ADVANCES IN DRUG RESEARCH

Volume 29

ACADEMIC PRESS

ADVANCES IN

DRUG RESEARCH

VOLUME 29

This Page Intentionally Left Blank

ADVANCES IN

DRUG RESEARCH

Edited by

BERNARD TESTA

School of Pharmacy, University of Lausanne, Lausanne, Switzerland

and

URS A. MEYER

Department of Pharmacology, Biocentre, University of Basel, Basel, Switzerland

VOLUME 29

ACADEMIC PRESS

Harcourt Brace & Company, Publishers

SAN DIEGO LONDON BOSTON NEW YORK
SYDNEY TOKYO TORONTO

This book is printed on acid-free paper.

Academic Press
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http:// www.apnet.com

Academic Press Limited
24–28 Oval Road, London NW1 7DX, UK
http:// www.hbuk.co.uk/ap/

ISBN 0-12-013329-6

A catalogue record for this book is available from the British Library

Typeset by Keyset Composition, Colchester, Essex
Printed in Great Britain by Hartnolls Ltd, Bodmin, Cornwall

97 98 99 00 01 02 EB 9 8 7 6 5 4 3 2 1

CONTENTS

Peroxidic Antimalarials

CHARLES W. JEFFORD

CONTRIBUTORS

G. M. J. BEIJERSBERGEN VAN HENEGOUWEN, *Department of Medicinal Photochemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, NL–2300 RA Leiden, The Netherlands*

I. DWIVEDY, *Medicinal Chemistry Division, Central Drug Research Institute, Lucknow 226 001, India*

A. GIANNIS, *Institut für Organische Chemie und Biochemie der Universität Bonn, D–53121 Bonn, Germany*

C. W. JEFFORD, *Department of Organic Chemistry, University of Geneva, CH–1211 Geneva 4, Switzerland*

S. RAY, *Medicinal Chemistry Division, Central Drug Research Institute, Lucknow 226 001, India*

F. RÜBSAM, *Institut für Organische Chemie und Biochemie der Universität Bonn, D–53121 Bonn, Germany*

PREFACE:
THE SEARCH FOR CONSENSUS

In his deep and thought-provoking book, the physicist Alan Cromer offers many insights on the historical developments and characteristics of science. One passage struck us as particularly relevant to the mission of book editors:

> Science is the search for consensus. But before there is sufficient evidence to form a consensus on a particular problem, scientists will pursue different approaches and suggest different solutions. (Cromer, 1995)

How do books fit into this view of science? Should they be repositories of the universal consensus, and thus serve mostly as slowly ageing teaching tools? Or should they review the evidence and solutions from which a consensus will emerge, serving as rapidly outdated sources for scientific debates? No doubt some editors have strong views on this question, but the faithful readers of *Advances in Drug Research* know that we have consistently pursued both views. In fact, this duality of approaches can be found in many chapters of the series, which present a consensus on a given subject, followed by recent evidence suggesting improved solutions and new interpretations. In this way, *Advances in Drug Research* serves as a medium for both education and debate, just as its editors are professional educators and researchers.

And how does the present volume serve the present and future consensus? The first chapter by Giannis and Rübsam is an extensive review of the role of peptidomimetics in drug design. Much has been written on this fast-evolving topic, but the authors have managed to bring forth principles of long-lasting value, simultaneously discussing recent advances and new concepts. We are convinced that their thorough and lucid treatment of the topic will be a source of clarity and inspiration to researchers and teachers in medicinal chemistry.

The second chapter by Beijersbergen van Henegouwen is a primer on medicinal photochemistry. It examines at length both the toxicological and therapeutic aspects of this largely ignored field, and does so in such a comprehensive and didactic manner that it should be an eye-opener to many readers.

The second part of the book contains two chapters on specific therapeutic classes. Here, the editors are pleased to welcome again distinguished scientists from the Central Drug Research Institute in Lucknow, whose chapter takes an original look at the many facets of estrogen antagonists. Ray and Dwivedy present the current consensus on these agents, but they also reveal some little-known aspects of their therapeutic potential. In so doing, they indeed contribute to enrich a lively debate.

The last chapter is devoted to the chemotherapy of malaria. This is a field of immense medical importance considering the appalling number of infected people and the yearly toll claimed by this disease. But malaria is more than the sum of its victims – it is one symptom among many of the incapacity of humankind to solve its most pressing social and medical problems. In their modest capacity, scientists can at least hope to make a contribution towards solving the medical problem. This is what this chapter, and one in the following volume, are about.

Jefford summarizes, with limpid elegance, his work and that of others to develop peroxidic antimalarials and to understand their mode of action. The story is a fascinating one, with nature providing a few lead compounds (e.g., artemisinin), and dedicated researchers improving remarkably on their pharmacodynamic and pharmacokinetic properties to create promising drug candidates.

Furthermore, this last chapter is noteworthy in that it is a kind of scientific testament written by a reportedly retired – yet still highly creative – scientist. Such chapters are of special value since elderly scientists are an often neglected yet inexhaustible source of guidance, having had ample time to make their own mistakes and to learn from them. As stated so aptly by Allen (1995)

> Remember, good judgment is the result of experience, and experience is the result of bad judgment.'

In passing, we note that this is a rule which greedy shareholders in general and pharmaceutical giants in particular have temporarily forgotten – but which may catch up with them.

BERNARD TESTA
URS A. MEYER

References

Allen, R. E. (1995). *Winnie-the-Pooh on Management*. p. 93. Methuen, London.
Cromer, A. (1995). *Uncommon Sense*, p. 151. Oxford University Press, New York.

This Page Intentionally Left Blank

Peptidomimetics in Drug Design

ATHANASSIOS GIANNIS* and FRANK RÜBSAM

Institut für Organische Chemie und Biochemie der Universität Bonn, D–53121 Bonn, Germany

'The investigation of the truth is in one way hard, in another easy. An indication of this is found in the fact that no one is able to attain the truth adequately, while, on the other hand, we do not collectively fail, but every one says something true about the nature of things, and while individually we contribute little or nothing to the truth, by the union of all a considerable amount is amassed.'

Aristotle

1 Introduction

A great diversity of peptides acting as neurotransmitters, neuromodulators, and hormones has been discovered and characterized during the last 30 years (Krieger, 1983; Schmidt, 1986; Schwarz, 1991). Many of these have been

ADVANCES IN DRUG RESEARCH, VOL. 29
ISBN 0-12-013329-6

found in both neuronal and in non-neuronal tissues. After binding to their membrane-bound receptors, which belong mainly to the category of G protein-coupled receptors, they influence cell–cell communication and adhesion, and control a series of vital functions such as cell proliferation, tissue development, metabolism, immune defence, digestion, perception of pain, reproduction, behaviour, and blood pressure (Savarese and Fraser, 1992; Berridge, 1993). Peptides are also involved in the pathogenesis and/or maintenance of several diseases. For these reasons, selective agonists and particularly antagonists are indispensable for the investigation of peptidergic systems and can also be potential therapeutic agents.

As a result of major advances in organic chemistry and in molecular biology (Jung and Beck-Sickinger, 1992) most bioactive peptides have been prepared in larger quantities and made available for pharmacological and clinical experiments. However, the use of peptides as drugs is limited by the following factors: (a) their low metabolic stability towards proteolysis in the gastrointestinal tract and in serum; (b) their poor transport from the gastrointestinal tract to the blood and also their poor penetration into the central nervous system, in particular, due to their relatively high molecular mass or the lack of specific transport systems, or both; (c) their rapid excretion through liver and kidneys; and (d) their side-effects caused by interaction of the conformationally flexible peptides with distinct receptors. In addition, a bioactive peptide can cause effects on several types of cells and organ systems, since peptide receptors and/or isoreceptors can be widely distributed in an organism. In recent years intensive efforts have been made to develop peptidomimetics (Veber and Freidinger, 1985; Rose *et al.*, 1985; De Grado, 1988; Freidinger, 1989; Morgan and Gainor, 1989; Hruby *et al.*, 1990; Griffith, 1991; Hirschmann, 1991; Hölzemann, 1991; Rizo and Gierasch, 1992; Giannis and Kolter, 1993; Gante, 1994; Liskamp, 1994) which display more favourable pharmacological properties than their endogenous prototypes. For the purpose of this review we define a peptidomimetic as a compound that, as the ligand of a receptor, can mimic or block the biological effects of a peptide (Morgan and Gainor, 1989; Veber, 1992). As the ligand of an enzyme it can serve as substrate or as inhibitor. Enzyme ligands will be considered here only in special cases. In contrast, we will discuss basic principles of peptidomimetic design, presenting selected examples of ligands developed for several G-protein-coupled receptors as well as ligands for proteins involved in cell adhesion. Emphasis will be given to rational approaches to small nonpeptide ligands. Physiological functions and mode of action of the endogenous peptides as well as possible uses of peptidomimetics in therapy will also be discussed. An exhaustive treatment is beyond the scope of this review.

2 Design of Peptidomimetics

2.1 INTRODUCTION

As for any drug, a peptidomimetic must fulfil the following requirements: (a) metabolic stability, (b) good bioavailability, (c) high receptor affinity and receptor selectivity, and (d) minimal side-effects. For the rational design of such compounds knowledge of the biosynthesis, transport, release, and inactivation of the peptide are extremely useful. Regardless of their mode of action (as neurotransmitters, neuromodulators or hormones), peptides show some common characteristics (van Nispen and Pinder, 1986; Brownstein, 1989). First, they are synthesized in the ribosomes as higher molecular forms (prepro forms). Thereafter the pro forms are formed by cleavage of an *N*-terminal signal peptide by peptidases. During the subsequent vesicular transport to the Golgi apparatus they are processed further to their active forms and finally transported to the intracellular storage pools. They are released from the latter after stimulation. The inactivation of the peptides and thus termination of their biological action result mainly from the action of proteolytic enzymes (endo- and exo-peptidases).

2.2 THE BIOACTIVE CONFORMATION

Of crucial importance for the rational design of peptidomimetics are insights into the three-dimensional structure of the peptide–receptor complex, and also the subsequent signal transduction and the coordination and interaction with other signal transduction systems and integration in the organism. Our understanding of the mechanisms of signal transduction of peptide receptors has increased greatly in recent years (Berridge, 1993). However, due to the hydrophobic nature and size of the G-protein-coupled receptors the detailed determination of their three-dimensional structure has not yet been possible. In the study of peptide–receptor interactions impressive progress has been made through site-directed mutagenesis, molecular modelling and analysis of structure–activity relationships.

For the development of peptidomimetics having a peptidic nature the endogenous ligand generally serves as lead structure. Peptides of small to medium size (<30–50 amino acid units) generally exist in dilute aqueous solution in a multitude of conformations in dynamic equilibrium (Fig. 1). If the ligand has the biologically active conformation (receptor-bound conformation), then an increased receptor affinity is expected, since the decrease in entropy on binding is less than that on the binding of a flexible ligand. In solution and in the absence of the receptor, the biologically active conformation may be poorly populated and is frequently quite different from the conformation obtained by, for example, X-ray or nuclear magnetic

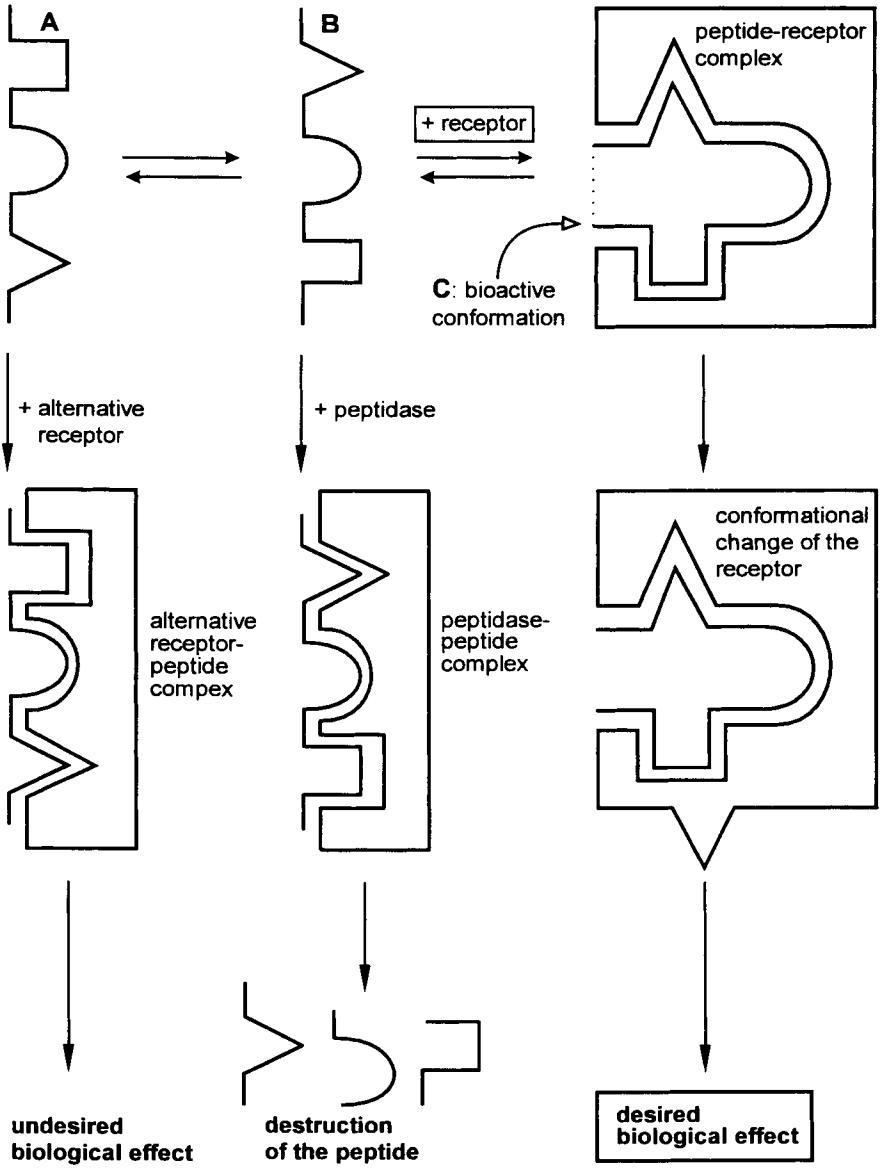


FIG. 1. In solution, peptides exist in a variety of conformations that are in dynamic equilibrium with each other. If a conformational constrain (broken line in C) is introduced in the bioactive conformation of the peptide, conformers A and B cannot exist. Thus, the interaction with alternative receptors and peptidases is suppressed or does not occur. In this way a desired biological effect can be obtained (modified from Veber and Freidinger, 1985).

resonance (NMR) methods (Kessler, 1982; Fesik, 1991; Jorgensen, 1991; Wüthrich *et al.*, 1991).

2.3 CONFORMATIONAL RESTRICTION

A successful method for the development of peptidomimetics involves synthesis of conformationally restricted compounds, i.e. locally or globally constrained peptide analogues that imitate the receptor-bound conformation of the endogenous ligands as closely as possible (Veber and Freidinger, 1985; Burt and Greer, 1988; Rizo and Gierasch, 1992). Investigations of these analogues show them to have increased metabolic stability, as well as increased receptor selectivity (Veber and Freidinger, 1985). The fact that, frequently, only a small number of three to eight amino acid side-chains in the peptide are responsible for the biological activity ('message') proves favourable for this approach (Kessler, 1982; Freidinger, 1989). In such cases the rest of the molecular framework may serve to present the pharmacophore in a specific conformation. In addition, in the part of the peptide not containing the message, additional binding affinity to various receptor types (an 'address') or to an interface (the plasma membrane) can be localized.

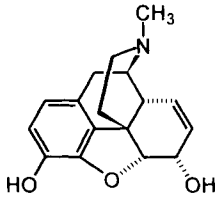
The metabolic stability of the peptide ligand can be controlled by the existence of cleavage sites for peptidases in this region of the molecule. Simplification of the structure of the ligand by removal or modification of this part leads to a simultaneous increase of the metabolic stability. Alternatively, the resistance toward amino- and carboxy-peptidases can be increased by acylation of the *N*-terminal group or by amidation of the *C*-terminus.

For the development of conformationally restricted peptide ligands the identification of the amino acids necessary for receptor recognition and activation is essential. For this reason shorter analogues are synthesized to identify the minimal sequence required for receptor binding and biological activity. Thereafter, the importance of parameters such as stereochemistry, charge, lipophilicity, and peptide backbone are examined by systematic changes in the individual amino acids. Compounds with rigid conformations are then produced, and the most active structures are selected by studying conformation–activity relationships. This mainly empirical procedure is time-consuming and costly. However, most of the peptidic drugs have been developed by this approach (Fauchère and Thurieau, 1992). Recently very promising new techniques for the controlled synthesis and the rapid examination of a great variety of peptides (peptide combinatorial libraries) have been established (Gallop *et al.*, 1994; Gordon *et al.*, 1994; Janda, 1994). Owing to the above-described disadvantages of drugs having peptidic nature and considering that their application can cause immunological reactions (Kaiser and Kezdy, 1984), the development of low-molecular-weight

nonpeptide ligands appears to be necessary. Another reason for the development of such compounds is that peptide analogues designed as antagonists often display agonist-like activity *in vivo*. Unfortunately, the agonist-like properties are usually not clearly revealed until the transition is made to *in vivo* assays (Rosenblatt, 1986).

2.4 LEAD STRUCTURES

For the development of nonpeptide peptidomimetics the discovery of a lead structure in large-scale screening is of crucial importance. Collections of substances (compound libraries) and single compounds, especially natural products, are examined for their biological activity. In most cases, lead compounds have some but not all of the desired pharmacological properties. They rarely become drugs as they may have low activity and selectivity, poor bioavailability and stability, and unwanted side-effects. For optimization many derivatives must be synthesized and evaluated to identify the compound with optimal properties. The opioid alkaloids are classic examples of nonpeptide ligands that were later shown to be mimetics of endogenous opioid peptides. Morphine (**1**), for example, imitates the pharmacological effects of β-endorphin (**2**), an endogenous opioid composed of 31 amino acids, and of the tetrapeptide Met-enkephalin (**3**). Conversely, the structurally related morphine derivative naloxone (**4**) represents a universal opioid receptor antagonist. These examples clearly demonstrate that it is possible in principle to find small nonpeptide compounds which act as agonists or antagonists for peptide receptors.



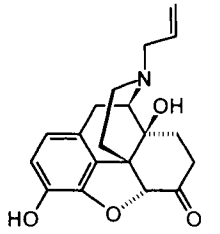
1

Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu

2

Tyr-Gly-Gly-Phe-Met

3



4

2.5 RATIONAL DESIGN

The rational design of low-molecular-weight nonpeptide ligands is still in its infancy. The transformation of a peptide into a nonpeptide ligand is one of the most challenging and exciting fields in medicinal chemistry. In this context it is noteworthy that (a) from the study of many peptide analogues it became apparent that for receptor recognition the side-chains of the amino acid residues are of crucial significance whereas for proteolytic enzymes the peptide backbone of the substrate participates considerably in binding affinity (Hirschmann, 1991; Marshall, 1992), and (b) conformation–activity studies of peptides suggested that their bioactive conformations are folded having molecular dimensions in the range of 10×15 Å (Freidinger, 1989). Furthermore through site-directed mutagenesis experiments the receptor residues essential for ligand recognition can be identified. Such knowledge, in combination with peptide synthesis, molecular modelling analysis and spectroscopic methods, helps to generate a pharmacophore concept and subsequently to select an appropriate scaffold or template that carries the functional groups necessary for receptor binding or receptor activation in their correct spatial arrangement. Subsequent optimization may provide compounds with improved bioavailability and pharmacodynamic. A general approach to peptidomimetics is shown in Fig. 2 (modified according to Marshall, 1993). Several examples demonstrating individual steps of Fig. 2 will be discussed later (see section 3) whereas examples of peptidomimetics (both peptide- and nonpeptide-based), including their possible use in therapy, are the topic of section 4. Biological, biochemical and medical aspects of endogenous peptides will also be discussed.

3 Strategies for the Development of Peptidomimetics

3.1 MODIFICATION OF AMINO ACIDS

Conformationally restricted nonproteinogenic (i.e. uncoded) amino acids have a great potential for elucidating the bioactive conformation of peptides. However, it must be kept in mind that there are only a few amino acid analogues that facilitate subtle and predictable restrictions of conformational flexibility without drastically changing the stereoelectronic properties of a peptide (Burgess *et al.*, 1995). In order to retain biological activity constraints must affect the conformation of the backbone but simultaneously retain crucial side-chain interactions with the receptor (Froimowitz and Hruby, 1989). For this purpose non-natural amino acids as well as chimeric amino acids (compounds which display structural features of two distinct amino acids; Marshall, 1993) have been developed (Fig. 3). They are obtained through α- or β-alkylation of the parent amino acid and/or

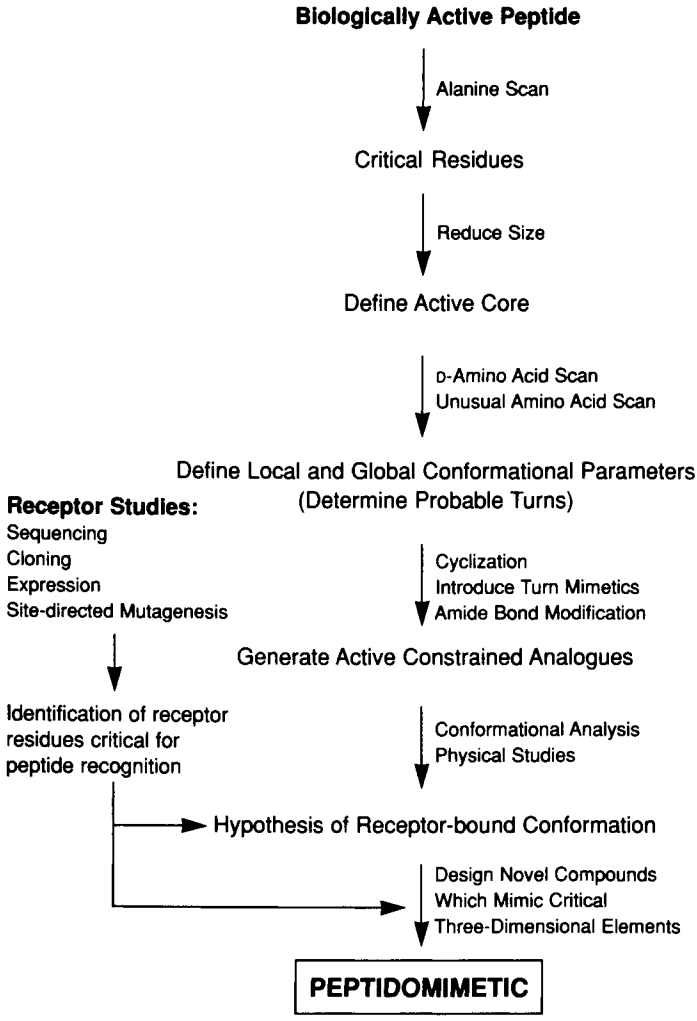


FIG. 2. Methods for the design of peptidomimetic drugs.

cyclization as well as introduction of an olefinic bond between the α- and the β-C-atom. In addition to these modifications, amino acids with sterically demanding side-chains and/or D-stereochemistry at the α-C atom can be used (Fig. 4).

The substitution of the α-hydrogen atom of an amino acid with a single methyl group (i.e. compounds of the general formula **5**) has profound (De Grado, 1988), although not always completely understood, effects on Φ, Ψ and χ angles (Fig. 5). Similarly, the structurally related cyclic amino

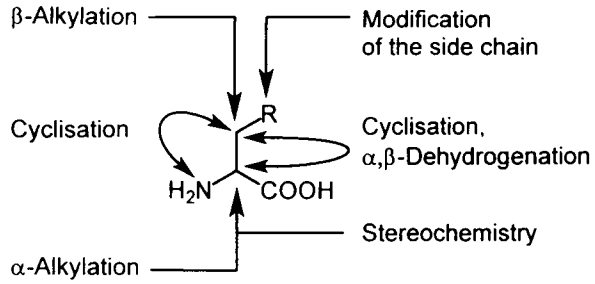


FIG. 3. Common amino acid modifications in peptidomimetics.

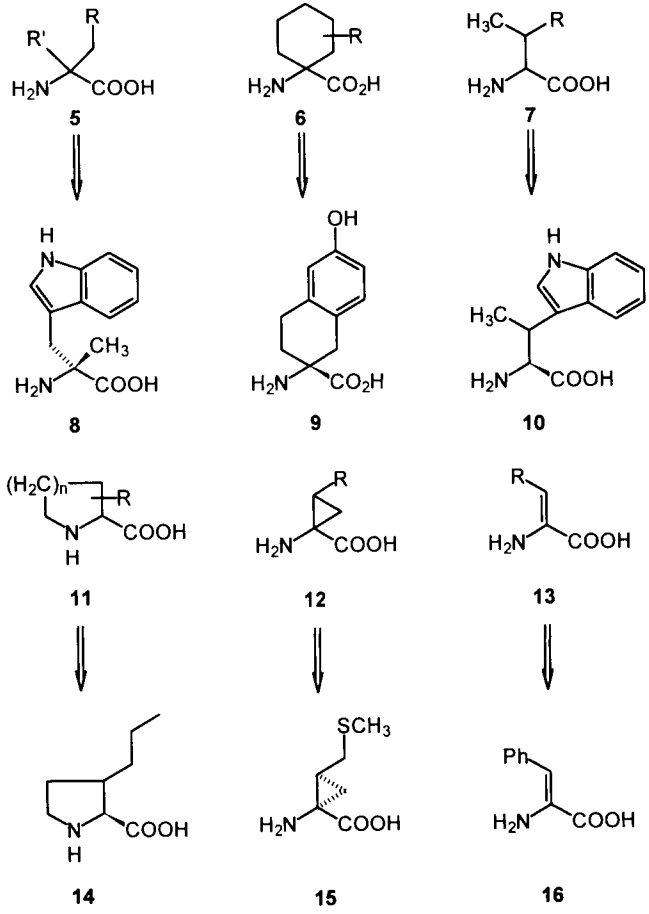


FIG. 4. Conformationally constrained amino acids.

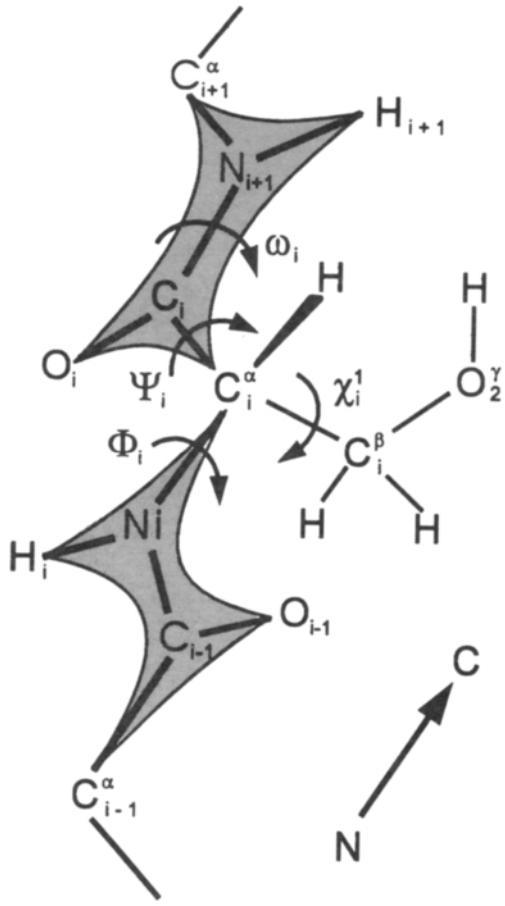


FIG. 5. Definition of the torsion angles Φ, Ψ, χ and ω of a peptide.

acids of type **6** can be used. The addition of one or several α-aminoisobutyric acid residues (α,α-dimethylglycine, **5**: $R' = CH_3$, $R = H$) in one peptide reduces the area of a Ramachandran plot (shows areas of sterically allowed Φ and Ψ angles for a peptide) on values that are associated with turn or helical conformations depending on the surrounding residues (Rose *et al.*, 1985). Interestingly, incorporation of the α,α-diethyl- or α,α-dibenzylglycines has been shown to favour full extended ($\Phi = \Psi = 180°$) conformations (Valle *et al.*, 1990). Introduction of a β-methyl group at the β-C atom of an amino acid yields compounds of the general formula **7**. Such a substitution has a profound effect on the χ torsion angle (Huang *et al.*, 1992). Several excellent methods for the synthesis of chiral C^{α}- and C^{β}-alkylated amino acids have been published during the last decade and have allowed the widespread use

of alkylamino acids as conformational constraints (Naef and Seebach, 1985; Seebach *et al.*, 1985; Fitzi and Seebach, 1988; Schöllkopf, 1983).

L-Proline is the only conformational constrained proteinogenic amino acid. Owing to the constraints of the pyrrolidine ring the Φ angle is fixed at approximately $-60°$ whereas the values for the corresponding Ψ angle are in the region between $-55°$ and $130°$. L-Proline cannot fit into regular, internal parts of either helix or β-sheets both because of its steric hindrance and the fact that, as part of a peptide, it does not have an NH for formation of an H-bond network. It is very common in the second position of β-turns (Richardson and Richardson, 1989). Another unique property of proline (and generally of *N*-alkyl amino acids) is that it can occur also with a *cis* configuration (Kessler, 1970; Stewart and Siddall, 1970). The energy difference between *cis* ($\omega = 0°$) and *trans* ($\omega = 180°$) configurated prolyl peptides is approximately 2 kcal mol^{-1} in favour of the *trans* (Schulz and Schirmer, 1979). Oligopeptides carrying one proline residue exist in dilute aqueous solutions as *cis/trans* mixtures containing up to 30% of the *cis* isomer (Davies and Thomas, 1978). The isomerization of the peptide bond is of particular interest in protein-folding processes (Fischer, 1994).

In order to extend the possibilities for design of constrained peptides using chimeric cyclic amino acids, many proline homologues of the type **11** containing more carbon atoms in the ring with or without additional substituents have been prepared and introduced into peptides (Hruby *et al.*, 1990; Toniolo, 1990; Liskamp, 1994).

3-Substituted 2,3-methane amino acids of the type **12** (cyclopropane amino acids, methanologues) have also been synthesized (Stammer, 1990; Es-Sayed *et al.*, 1995) and used for the same purpose (Burgess *et al.*, 1995). The cyclopropane ring introduces significant steric constraints into the amino acid residue. Because of the unsaturated character of the cyclopropane ring, latent chemical instability is incorporated into the peptide. Furthermore, because of the conjugation of the carbonyl group with the ring, small Ψ values are observed. The effects on the angles χ and Φ depend on the stereochemistry at the two stereogenic centres of the three-membered ring. Cyclopropane amino acids seem to favour turns in small linear peptides.

Another possibility for the synthesis of constrained peptides is the incorporation of α,β-dehydroamino acids **13**. Such amino acids have a rigidifying effect on the side-chain and on backbone conformations of flexible peptides (De Grado, 1988). Because of the conjugation of the carbonyl group with the olefinic bond small Ψ values are also observed here. In short linear or cyclic peptides dehydroamino acids stabilize β-turns (Bach and Gierasch, 1985).

Since the effect of the incorporation of the amino acids mentioned above on the conformational parameters Φ, Ψ, χ, and ω and also on the biological activity of the resulting peptides has been summarized in two excellent reviews (Hruby *et al.*, 1990; Toniolo, 1990), we will only refer here to a few

representative structures of individual compounds (Fig. 4) in order to indicate the use of non-ribosomal (i.e. nonproteinogenic) amino acids in peptidomimetic development.

- The β-methyl analogue of the amino acid tryptophane (**8**) is a component of a potent non-peptide cholecystokinin receptor antagonist (Horwell *et al.*, 1991) whereas the tyrosine analogue **9** is a component of a peptidic ligand with high selectivity for the δ opioid receptor (Toth *et al.*, 1992).
- The tryptophane derivative **10** was incorporated into cyclic somatostatin analogues in order to study their bioactive conformations (Huang *et al.*, 1992).
- The 3-propyl-substituted proline derivative **14** was introduced as a norleucine analogue in a *C*-terminal tetrapeptide of cholecystokinin. The resulting analogue showed high affinity for the CCK_B receptor (Holladay *et al.*, 1991).
- The 2*S*,3*S*-2,3-methanomethionine (2*S*,3*S*-cyclo-Met) (**15**) was introduced as a conformationally restricted substitute for methionine in the anti-opiate peptide Phe-Met-Arg-Phe-NH_2. NMR and quenched molecular dynamics studies indicated that this cyclopropane amino acid occupies the central position of a γ-turn in the tetrapeptide Phe-(2*S*,3*S*-cyclo-Met)-Arg-Phe-NH_2 (Burgess and Ho, 1994; Burgess *et al.*, 1995).
- Finally, dehydrophenylalanine (**16**) (Δ^z-Phe) was incorporated as a substitute of D-phenylalanine in the peptide cyclo(Gly^1-Pro^2-D-Phe^3-D-Ala^4-Pro^5). In this peptide the amino acid D-Phe occupies the i + 2 position of a type II β-turn. According to NMR solution studies the modified peptide cyclo(Gly^1–Pro^2–Δ^z–Phe^3–D–Ala^4–Pro^5) favours also a type II β-turn with dehydrophenylalanine in the i+2 position (Bach and Gierasch, 1985).

3.2 DIPEPTIDE AND OLIGOPEPTIDE ANALOGUES

Bridging between two consecutive amino acids in a peptide (Fig. 6) leads to a dipeptide mimetic, the flexibility of which is limited in comparison with

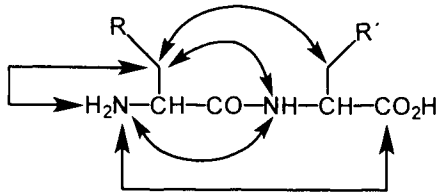


FIG. 6. Possibilities for bridging between two consecutive amino acids leading to dipeptide mimetics.

that of regular dipeptides. There are many such possibilities (Giannis and Kolter, 1993; Gante, 1994):

(1) Bridging the side-chain of one amino acid and the nitrogen of the peptide bond yields a lactam. The dipeptide analogue **17** has been used as substitute for the Gly^6–Leu^7 sequence in gonadotropin-releasing hormone (GnRH). Although the resulting modified GnRH showed an *in vivo* effect only 2.4 times greater than that of the native GnRH (Freidinger *et al.*, 1980), it provided an indication for the existence of a β-turn in the biologically active GnRH conformation. This observation is a landmark in the field of peptidomimetics and also initiated the application of lactams as turn inducing moieties and turn mimetics.

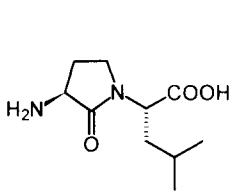
The derivative **18** represents a Leu-Ala analogue (Wolf and Rapoport, 1989) whereas the derivative **19** has been used as a substitute for Phe-His for the synthesis of a potent renin inhibitor (Kempf and Condon, 1990).

(2) Bridging between the C^{α} atom of one amino acid with the nitrogen atom of the peptide bond affords δ- and γ-lactams depending on the length of the spacer used for cyclization. Compound **20**, a Phe-His analogue, has been used for the synthesis of a chymotrypsin inhibitor whereas the tryptophyllysine substitute **21** has been utilized for the synthesis of somatostatin analogues (Freidinger, 1985).

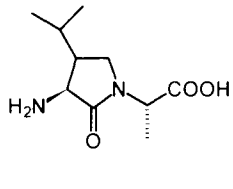
(3) Cyclization involving the side-chains of the two consecutive amino acids leads to mono- and bi-cyclic lactams. The derivative **22** represents an Ala-Ala analogue (Kemp and McNamara, 1984) whereas the bicyclic lactam **23** was used as an alternative for the Ala^7-D-Ala^8 sequence of Cyclosporin A (CsA). The resulting tricyclic CsA derivative (TCsA) was three times more potent than CsA as inhibitor of the rotamase activity of cyclophilin A (Alberg and Schreiber, 1993). Finally, the bicyclic lactam derivative **24** represents a surrogate for homoPhe-His-Leu and is one of the most potent angiotensin-converting enzyme (ACE) inhibitors (Flynn *et al.*, 1987).

(4) Bridging the two nitrogen atoms of a dipeptide moiety with a spacer containing two carbon atoms leads to piperazinones. Substituted piperazinones as for example the Asp-Phe analogue **25** are components of Leu-enkephalin analogues (DiMaio and Belleau, 1989) and cholecystokinin receptor antagonists (Kendrick *et al.*, 1992; Batt *et al.*, 1994). Chiral syntheses that enable an entry to all diastereomers of several 3,6-substituted piperazinones have recently been published (Kolter *et al.*, 1995).

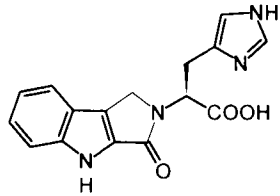
(5) End-to-end cyclization of a dipeptide yields diketopiperazines **26** which are the simplest cyclopeptides. They are among the most ubiquitous peptide derivatives found in nature and often display interesting pharmacological properties (Sammes, 1975). Recently, an interesting



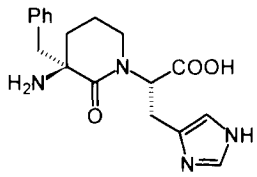
17 : Gly-Leu



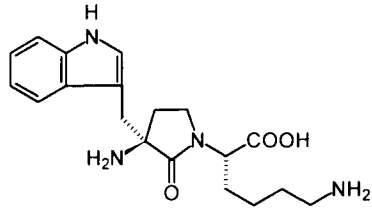
18 : Leu-Ala



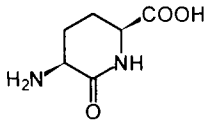
19 : Phe-His



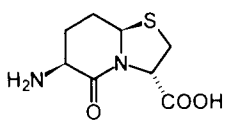
20 : Phe-His



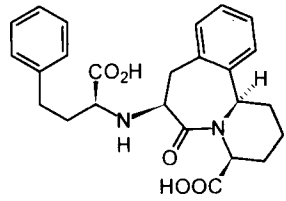
21 : Trp-Lys



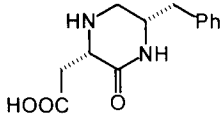
22 : Ala-Ala



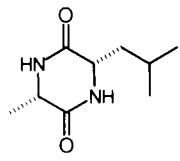
23 : Ala-D-Ala



24 : homo Phe-His-Leu



25 : Asp-Phe



26 : cyclo-Ala-Leu

and efficient combinatorial synthesis for *C*- and *N*-substituted diketopiperazines was reported (Gordon and Steele, 1995).

Several lactams of the types shown have been designed, to some extent, as turn mimetics. The structural variation of such compounds can be extended further by reducing or increasing the size of the lactam ring or by incorporation of heteroatoms (Yanagisawa *et al.*, 1987; Robl *et al.*, 1994; Cornille *et al.*, 1995).

In addition to the local conformational constraints described above, global restrictions of the conformational flexibility through bridging two distant

amino acids in a peptide can also be accomplished. The approach leads to cyclic peptide analogues and it is based on the general theory of peptide–receptor interactions that assumes a folded conformation of the peptide (Chipens, 1983; Rose *et al.*, 1985). Such a conformation may be stabilized by ionic interaction of the *C*-terminus with the positive charged *N*-terminus. This approach has been used extensively in the field of peptidomimetics (Hruby *et al.*, 1990; Toniolo, 1990; Rizo and Gierasch, 1992). Global constraints are usually achieved by linking two side-chains, or connecting one terminus with a side-chain or by end-to-end cyclization. For this purpose bifunctional spacers can be used. Because such cyclizations lead to an overall change of the peptide direction they offer a tool to investigate turns in peptides. Finally, cyclopeptides and generally constrained peptides are poor substrates for peptidases and this leads to their increased metabolic stability (Veber and Freidinger, 1985).

3.3 MODIFICATION OF THE PEPTIDE BACKBONE

The replacement of the peptide bond with suitable surrogates represents another possibility to influence the metabolic stability and lipophilicity of a peptide and to probe the importance of the peptide backbone in receptor recognition and biological activity. Furthermore, a different hydrogen-bonding pattern of the resulting peptide can be expected and this in turn may have profound effects on the secondary structure, on the mode of interaction with a receptor or a membrane and, consequently, on the biological activity. In addition it must be borne in mind that the peptide backbone can form complexes with for example Ca^{2+} which is present in extracellular fluids in millimolar concentrations. Complexation of the amide group of peptides with Ca^{2+} can lead to a dramatic change in peptide conformation (Zhorov and Ananthanarayanan, 1995).

The utilization of amide bond mimetics has been particularly important for the development of enzyme inhibitors (Spatola, 1983; Wiley and Rich, 1993; Gante, 1994). Popular amide group surrogates which have found broad application also in the development of receptor ligands include $-CH_2NH-$ (methylene amino) $-CH=CH-$ (*E*-alkene) and HN-CO (retro-inverso).

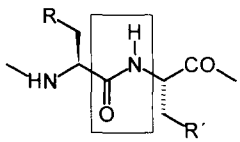
The exchange of the amide group for (CH_2NH) has led to the development, for example, of agonists of the cholecystokinin receptor (Rodriguez *et al.*, 1987), and the somatostatin receptor (Sasaki *et al.*, 1987) as well as antagonists of the bombesin receptor (Coy *et al.*, 1988). Peptides containing the isosteric CH_2NH group are often quite different, especially in their electronic properties, from their native prototypes. The methylene amino compounds may be obtained by reduction of the peptides with $LiBH_4/(CH_3)_3SiCl$ (Giannis and Sandhoff, 1989; Fincham *et al.*, 1992). Another preparative approach is the reductive amination of suitably protected α-aminoaldehydes

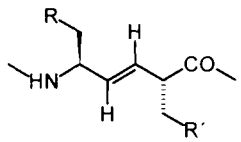
with amino acid derivatives (Curran *et al.*, 1992), during which racemization of the aminoaldehyde can occur. The racemization is overwhelmed by the use of configurationally stable α-aminoaldehydes or configurationally stable peptide aldehydes (Kolter *et al.*, 1992).

Another amide bond surrogate is the *E*-olefinic unit **27**, first introduced by Hann *et al.* (1980) and Cox *et al.* (1980a). Several analogues of Leu^5-enkephalin and Met^5-enkephalin containing the *E*-olefinic group were synthesized and their ability to serve as ligands for the opioid receptor was investigated (Cox *et al.*, 1980b). These studies suggested that the presence of a *trans*-amide bond between Tyr^1 and Gly^2 is not essential for activity, whereas that between Gly^2 and Gly^3 is important.

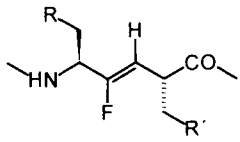
The fluoro-olefin unit **28** is an ever better amide bond substitute, mimicking both the steric and electronic properties of the peptide bond. The Phe-Gly moiety of a hexapeptide ligand for the substance P receptor was replaced by the Phe-CF=CH-CH_2CO unit leading to a peptide analogue with higher binding affinity to the substance P receptor (Allmendinger *et al.*, 1990a,b). In addition, these authors described an enantioselective synthesis of both antipodes of the Phe-Gly mimic. Stereoselective routes to several alkene isosteres were also recently published by Bol and Liskamp (1992), Yong and Lipton (1993) and also by Wipf and Fritsch (1994).

Various heterocycles such as **29–31** have been synthesized and used as amide bond mimics yielding peptide analogues showing interesting pharmacological activity (Gante, 1994). For example, potent neurokinin-1 (NK-1) antagonists containing the thiazole unit **29** were prepared (Gordon *et al.*, 1993), whereas ligands containing the imidazoline moiety **30** have been developed for opioid receptors (Jones and Ward, 1988). Tripeptide aldehydes bearing the chiral tetrahydropyrimidine unit **31** are potent cysteine protease inhibitors (Cheng *et al.*, 1994).

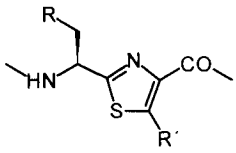




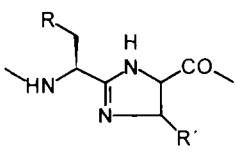
27



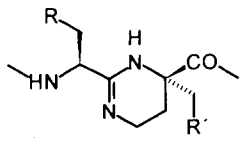
28



29



30



31

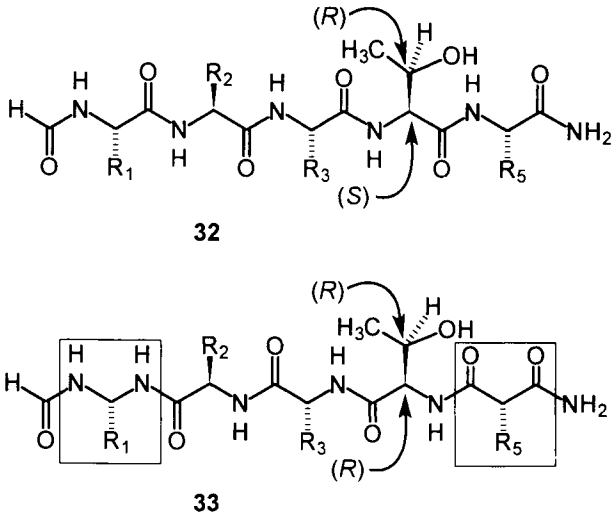


FIG. 7. Retro–Inverso modification of the parent pentapeptide **32** leads to the Retro–Inverso-Isomer **33** which is stable towards proteases.

A further approach for altering the peptide backbone is the use of retro–inverso modifications (Goodman and Chorev, 1979; Chorev and Goodman, 1993). In this method the sequence is reversed and the chirality at the α-C atom of each amino acid residue is inverted relative to the parent peptide **32**, whereas the chirality of the side-chains of L-threonine and L-isoleucine needs to be retained (Fig. 7). The resulting retro–inverso compounds **33** are no longer substrates for proteases. One problem is that for noncyclic peptides the end groups and, thereby, their charges are not complementary to those of the native peptide and must either be substituted by the introduction of 'false' ends or the retro-inverso structure must be incorporated into a larger peptide. An interesting synthesis of retro–inverso compounds was described recently by Dürr *et al.* (1992).

The effect of the retro–inverso modification on biological activity of peptides has been rather unpredictable and in many cases inactive compounds or analogues having low potencies were obtained (Cushman *et al.*, 1990). The difficulties in attaining successful applications of the retro–inverso isomers may be explained by the fact that these isomers are topologically nonidentical with their parent compounds (Freidinger and Veber, 1979). Interesting applications of this approach include a partially modified retro–inverso isomer of the immune-stimulating tetrapeptide tuftsin (Thr-Lys-Pro-Arg) which has been newly developed and displays remarkable resistance toward proteolytic degradation, combined with retention of high biological activity following oral administration (Verdini *et al.*, 1991).

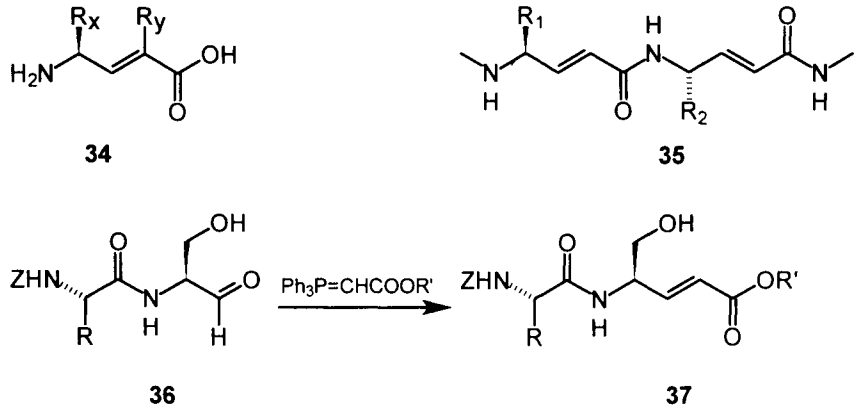


FIG. 8. Vinylogous amino acids and peptides.

Furthermore another promising retro–inverso derivative has been rationally developed as a mimetic of a hairpin loop of the CD4 receptor and applied successfully for the therapy of experimentally induced allergic encephalomyelitis in animals (Jameson *et al.*, 1994). These developments may be the driving force to continue the efforts on retro–inverso peptides.

Finally, we wish to bring attention to a recently published work in which the authors utilize vinylogous amino acids (**34**) in place of 'normal' amino acids (Hagihara *et al.*, 1992). Linear and cyclic peptides containing such amino acids are rich in secondary structure elements. Vinylogous peptides such as (**35**) (Fig. 8) are conceptually related to modified DNA containing a hexose as the next higher homologue of the deoxyribose, and could lead to the development of peptide analogues with unusual backbones. A new entry to peptides of type **37** (containing a 'normal' amino acid and vinylogous serin) via Wittig olefination of the configurationally stable dipeptide aldehydes **36** was published recently (Kolter *et al.*, 1992).

3.4 IMITATION OF SECONDARY STRUCTURES: MIMETICS OF β-TURNS

The β-turn is a structural motif common to many proteins (including the complementarity determining region of antibodies) as well as cyclic peptides and has been postulated in many cases for the bioactive conformation of linear peptides (Rose *et al.*, 1985). Because of the surface localization of turns in proteins they serve as sites for molecular recognition. For these reasons β-turns are the most frequently imitated secondary structures (Kitagawa *et al.*, 1995). β-Turn mimetics have also been designed, synthesized and incorporated in proteins in order to enforce defined secondary structures (Rose *et al.*, 1985; Hölzemann, 1992; Giannis and Kolter, 1993). Such

approaches as well as other secondary structure mimics (γ-turn, helix, β-sheet, hairpins) will not be discussed here. Rather, we will focus on β-turn mimetics which have been designed as receptor ligands *per se*. The requirements of such a conformation mimetic are the following: the desired conformation should be mimicked as exactly as possible, and the synthesis should permit the introduction of the desired side-chains in the correct stereochemistry. In addition, synthetic accessibility, stability under the conditions used for synthesis and removal of protecting groups must be considered.

In linear biologically active peptides, turns arise mainly due to the tendency to form intramolecular hydrogen bonds. They may be also induced by interaction with biological membranes and also by complexation with Ca^{2+} which is present in the serum. In order to emphasize these two important aspects we refer to two recent examples. First, Guba *et al.* (1994) have recently investigated, via NMR spectroscopy, the structure of the highly potent bradykinin antagonist Hoe 140 (D-Arg^0-Arg^1-Pro^2-Hip^3-Gly^4-Thi^5-Ser^6-D-Tic^7-Oic^8-Arg^9) in a membrane-like surrounding (SDS micelles). The conformation is characterized by a βII′-turn and a βII-turn comprising residues 6–9 and 2–5, respectively, with the three arginine side-chains anchored in the water phase and the rest of the molecule embedded in the hydrophobic phase. In contrast, the molecule has an unfolded conformation in pure H_2O. Second, the conformation of free and Ca^{2+}-bound forms of Met^5-enkephalin (a classical μ-opioid receptor ligand) were compared using a Monte Carlo method with energy minimization and found to be quite different. The Ca^{2+}-bound form contains a turn with a centrally located Gly-Gly moiety and the two aromatic groups in near proximity (Zhorov and Ananthanarayanan, 1995). Interestingly, the overall shape of the complex was found to have great similarity with the Ca^{2+}-bound form of morphine which represents a classical nonpeptide agonist of the μ-opioid receptor.

The selection criteria generally adopted for β-turns state that any tetrapeptide sequence in which the distance between the two stereogenic centres $\alpha C_{(1)}$ and $\alpha C_{(4)}$ is ≤ 7 Å and which occur in a non-helical region is a β-turn (Rose *et al.*, 1985). In many, but not all, cases a hydrogen bridge between the carbonyl group of the first amino acid residue of this sequence and the NH-function of the fourth amino acid is formed yielding a 10-membered ring (Fig. 9). The backbone conformation of the β-turn is highly variable. Such structures are classified according to the values of the torsion angles Φ_2/Ψ_2 and Φ_3/Ψ_3 (see Table 1; Ball *et al.*, 1990). However, a considerable number (up to 50%) of distorted β-turns with a single torsion angle differing by more than 45–50° from the ideal have been found (Ball *et al.*, 1993; Hutchinson and Thornton, 1994). As mentioned above, for peptide–peptide receptor interactions the three-dimensional arrangement of the side-chains of the amino acids are particularly important. Unfortunately, the relative disposition of the side-chains in β-turns (bonds 1–4, Fig. 9) is

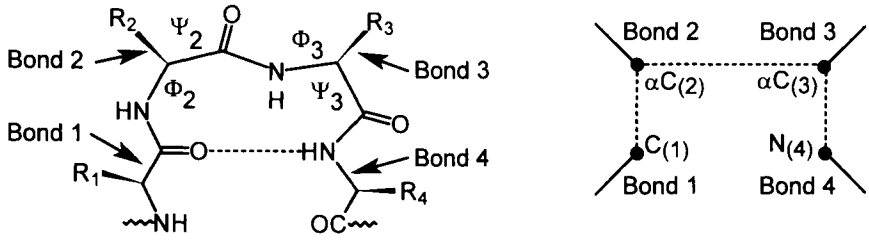


FIG. 9. β-Turns are commonly classified according to their torsion angles Φ_2/Ψ_2 and Φ_3/Ψ_3 but the relative disposition of the side-chains remains undefined by the current classification. In a more simple classification β-turns are described by a single dihedral angle $C_{(1)}$–$\alpha C_{(2)}$–$\alpha C_{(3)}$–N_4 which provides a complete description for any β-turn.

TABLE 1
Classification of β-turn types according to the mean dihedral angles of residues 2 and 3 in Figure 9

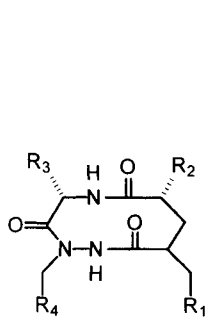
Turn Type	Φ_2 (deg.)	Ψ_2 (deg.)	Φ_3 (deg.)	Ψ_3 (deg.)
I	−60	−30	−90	0
I′	60	30	90	0
II	−60	120	80	0
II′	60	−120	−80	0
III	−60	−30	−60	−30
III′	60	30	60	30
IV	A turn with two or more angles differing by at least 40° from the above			
V	−80	80	80	−80
V′	80	−80	−80	80
VI	A *cis* Pro at position 3			
VII	A kink in the protein chain created by $\Psi_2 \approx 180°$ and $\lvert\Phi_3\rvert < 60°$ or $\lvert\Psi_3\rvert < 60°$ and $\Psi_3 \approx 180°$			

not clearly defined by the current classification and this is an obstacle to the construction of conformationally constrained molecules designed to be topographical mimics of the β-turn (Ball *et al.*, 1993). A classification based on the peptide backbone conformation may be entirely irrelevant for these molecules. In two interesting papers Ball *et al.* (1990, 1993) investigated a large number of β-turns reported in the literature and observed common topographical features across a variety of such secondary structure elements.

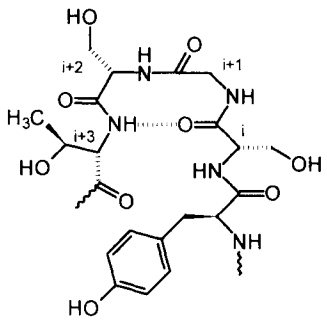
Based on these studies the authors proposed that β-turns could be described in terms of a single dihedral angle $C_{(1)}$-α$C_{(2)}$-α$C_{(3)}$-$N_{(4)}$. This angle was defined as β and provides a complete description of the spatial relationship between the entry and exit peptide bonds as well as the relative orientation of the intervening amino acid side-chains for any β-turn. This simplification was made possible by the reduction of the β-turn structure into two conformationally invariant groups namely the $C_{(1)}$-α$C_{(2)}$-α$C_{(3)}$ and α$C_{(2)}$-α$C_{(3)}$-$N_{(4)}$ units (Fig. 9). Despite large differences in the geometry of the peptide backbone the relative positions of bond 1, bond 2 and the α$C_{(2)}$ remain similar. This is because of the *trans* nature of the intervening peptide bond. The same is true for the relative positions of α$C_{(3)}$, bond 3 and bond 4. This simple description may be useful in the design of β-turn mimetics, which may be subsequently used as low-molecular receptor ligands.

Compounds of the general formula **38** containing a hydrazine amino acid (Saragovi *et al.*, 1991; Kahn, 1993) represent a landmark in the development of biologically active β-turn mimetics. Compound **39b** imitates a β-turn in the sequence Tyr-Ser-Gly-Ser-Thr **39a**, a component of the hypervariable region of a monoclonal antibody against the reovirus type-3 receptor and represents the first example of a low molecular mass immunoglobulin mimetic developed on the basis of an X-ray structure analysis of the antigen–antibody complex. Cyclopeptide **39b** is resistant towards proteases and imitates the binding and functional properties of the native antibody.

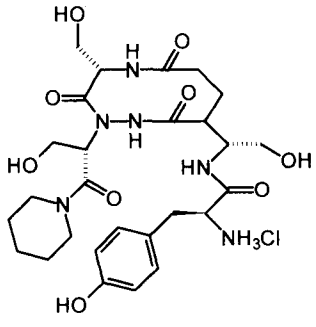
The macrocycles described above resemble compounds of type **40** and **41** originally developed by Olson *et al.* (1990) and Kemp and Stites (1988), respectively. A simple and efficient synthesis of a large library of chiral substituted heterocycles of the type **42** as β-turn mimetics containing the side-chains R_{i+1} and R_{i+2} of the parent peptide has been reported (Virgilio and Ellman, 1994). For the same purposes macrocycles of the general formula **43** were designed. A series of compounds containing the dipeptides Ala-Gly and cyclized with all stereoisomers of 6-amino-3,5-dimethylcaproic acid was



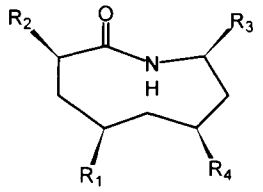
38



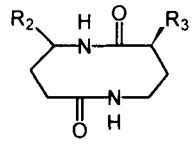
39a



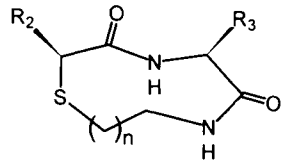
39b



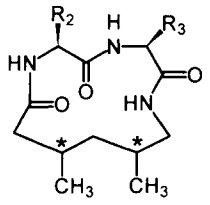
40



41



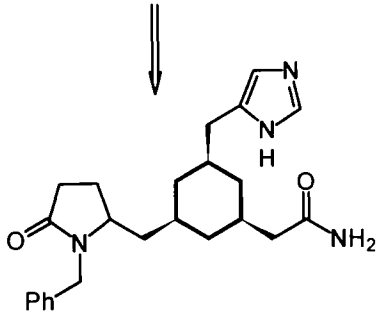
42



43

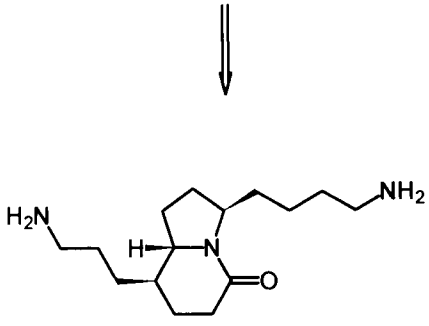
prepared by Kitagawa *et al.* (1995). A preliminary examination of these and other related compounds by NMR spectroscopy, circular dichroism and X-ray crystallography revealed that, depending on linker stereochemistry, different proportions of type II and type I exist in solution. Both type I and type II β-turn were observed also in the solid state. Although the use of synthetic linkers for the constraint of a dipeptide into various turns is not new, the ability of substituted linkers to affect the type of the turn is novel and may be useful in fine-tuning of biologically active peptidomimetics.

44: TRH
(pyroGlu-His-ProNH$_2$)



46

45: Lys-Pro-Arg



47

FIG. 10. Rational development of low-molecular-weight nonpeptide compounds as turn mimetics using cyclohexane or a bicyclic lactam moiety as scaffolds.

Olson *et al.* (1993) reported recently the design and synthesis of the 1,2,3-trisubstituted cyclohexane **46** as a TRH analogue with agonistic properties on the TRH receptor. On the basis of crystal and solution structures of TRH (**44**, Fig. 10) and TRH analogues they proposed a model for the pharmacophore which includes the lactam moiety of the pyroglutamyl group, the histidine imidazole ring, and the carboxamide function of the terminal prolineamide. For mimetic design they chose a starting conformation in which the peptide backbone approximates the Y-shaped X-ray structure of TRH. Subsequently, the cyclohexane ring has been used as a scaffold for placing the pharmacophoric groups in the correct spatial arrangement. The most active compound was found to be the *N*-benzyl derivative, **46**. The rational design of this derivative clearly demonstrates (a) the value of X-ray and NMR spectroscopical studies for generating a fruitful hypothesis concerning the bioactive conformation of a ligand, which is not always different from the solution or crystal structure, and (b) the possibility for the use of scaffolds for replacing the peptide backbone.

One of the earliest examples of rationally developed low-molecular non-peptide compounds as turn mimetics is the bicyclic lactam derivative **47** (Fig. 10; Kahn and Chen, 1987). This compound is a mimic of the immunosuppressing tripeptide Lys-Pro-Arg **45** which antagonizes the biological effects of the endogenous peptide tuftsin (Thr-Lys-Pro-Arg). *This example unequivocally demonstrates that rational development of nonpeptidal antagonists of peptide receptors is also possible using scaffolds carrying some critical side-chain groups of an endogenous peptide agonist.*

4 Examples of Peptidomimetics

4.1 OPIOIDS (ENDORPHINS, ENKEPHALINS, DYNORPHINS)

Morphine is the main constituent of opium. It has been used and misused for its pain-killing and euphoria-generating effects for over 2500 years (Brownstein, 1993).

Numerous morphine derivatives have been synthesized and examined in the search for morphine's mechanism of action and with the aim of developing a nonaddictive analgesic. Using these compounds it was possible to discover and investigate various opioid receptors in animal organisms. The discovery of such receptors (Pert and Snyder, 1973) suggested the existence of the corresponding endogenous ligands: the characterization of enkephalin in the mid-1970s (Hughes *et al.*, 1975) was followed by the discovery of endorphin (Nakanishi *et al.*, 1979) and dynorphin (Tachibama *et al.*, 1982). These endogenous opioids are biosynthesized by posttranscriptional processing (Lynch and Snyder, 1986) of the corresponding higher molecular mass precursors pro-opio-melanocortin, proenkephalin A, and proenkephalin B,

both in neurones and also in non-neuronal cells, for example in cells of the adrenal medulla and in leukocytes (Zurawski *et al.*, 1986; Smith *et al.*, 1986). Surprisingly, the presence of morphine and codeine in the brain of various animals was recently demonstrated (Weitz *et al.*, 1987a). To answer the question whether they are of exogenous origin or – as speculated at the beginning of this century (Mavrojannis, 1903) – of endogenous origin, cell culture experiments were conducted which showed that liver cells from mice possess the enzymes necessary for the decisive step of conversion of exogenously administered reticulin to salutaridine, which has the morphinan framework (Weitz *et al.*, 1987b). Adrenal and brain cells, however, are unable to convert reticulin into salutaridine. Thus, the question of the existence of a complete biosynthetic route for morphine in animal organisms remains unanswered.

From a pharmacological point of view, endogenous opioids are very similar to morphine (Simon and Hiller, 1989): they also have analgesic properties, lead to physical addiction and depression of respiration, depress the cough reflex, stimulate the release of vasopressin, and inhibit gastric fluid secretion and the motor activity of the intestine. In addition, they appear to act as tumour suppressants and as inhibitors of cell division (Maneckjee and Minna, 1992; Maneckjee and Minna, 1990; Zagon and McLaughlin, 1983).

The effects of opioids are transmitted by receptor-induced reduction of the intracellular levels of c-AMP and Ca^{2+}, apparently involving various G-proteins (Wollemann, 1990; Ginzler and Xu, 1991; Kiefer *et al.*, 1992). The overall effect of the opioid receptor activation is the inhibition of neural transmission.

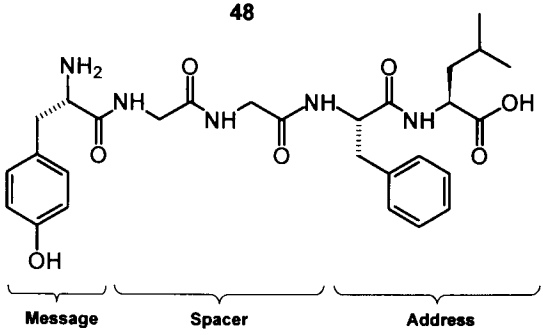
Opium alkaloids were subsequently shown to be mimetics of the endogenous opioids. Independent experiments on the interaction of opiate receptors with the corresponding agonists and antagonists demonstrated that there are at least three distinct types of opiate receptors with differing specificity, anatomic location (in both central and peripheral nervous systems; both pre- and post-synaptic in neurones), and physiological importance. These three types are designated μ, κ, and δ. The μ receptor mediates effects such as analgesia, respiratory depression, euphoria, miosis and gastrointestinal effects like nausea, vomiting and reduction of the gastrointestinal motility. The δ receptor mediates supraspinal analgesia whereas the κ receptor mediates analgesia at the spinal level. The latter receptor induces also dysphoria as well as weak respiratory depression (Brownstein, 1993).

The δ opioid receptor was recently cloned for the first time, expressed in COS cells, and characterized pharmacologically (Evans *et al.*, 1992; Kiefer *et al.*, 1992). Shortly thereafter the μ and κ receptors were also cloned (Chen *et al.*, 1993; Yasuda *et al.*, 1993). For detailed reviews on molecular biology and pharmacology of cloned opioid receptors the interested reader is referred to two recent articles (Uhl *et al.*, 1994; Knapp *et al.*, 1995).

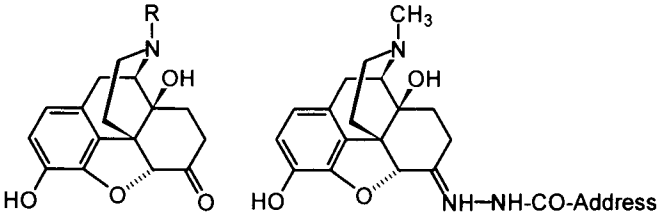
It is assumed that dynorphin (**48**) and Leu-enkephalin (**49**) are the

Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys

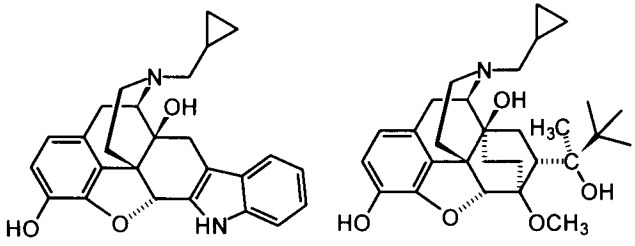
48



49

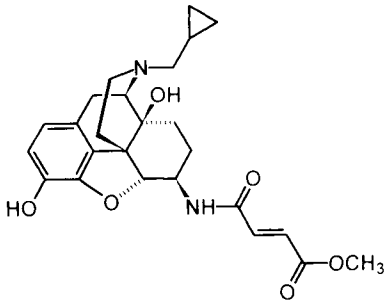


50 a,b **51**

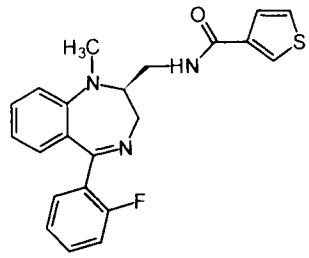


52 **53**

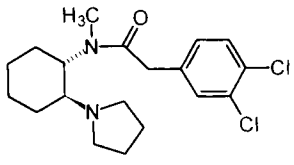
endogenous ligands for the κ and δ receptors, respectively, while an endogenous peptide with selectivity for the μ receptor has not yet been found (Portoghese, 1991). Comparison of the amino acid sequence of the opioid peptides shows that their *N*-terminals have the common sequence Tyr-Gly-Gly-Phe, regardless of the receptor specificity. This sequence, which is the same for all receptor types, is apparently essential for molecular recognition by the opiate receptor and is designated as 'message' (Schwyzer, 1982). A neighbouring sequence for the given receptor types, designated as 'address', is responsible for the selectivity. The message and address sequences may



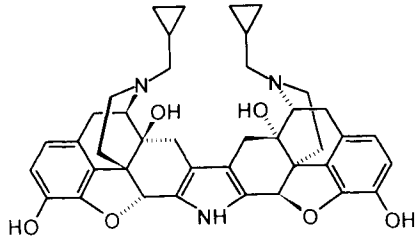
54



55



56



57

be different or slightly overlapping. According to a modified (Portoghese *et al.*, 1990; Portoghese, 1991, 1992) interpretation of this model, which also can be extended to nonpeptide ligands of opiate receptors, the message of opioids is located in the Tyr^1 residue and separated by two glycine residues that function as spacers from the address sequence, which begins with Phe^4. On the basis of this model it was possible to develop ligands of type **51** with selectivity for the δ and κ receptors from the μ-selective opioid antagonist oxymorphone (**50a**; R=methyl) by attaching the appropriate 'address' sequence. Naltrindole **52** was developed analogously as the first nonpeptide antagonist with selectivity for the δ opioid receptor (Portoghese *et al.*, 1990; Portoghese, 1991, 1992). The pyrrole ring of the indole residue of **52** serves as a rigid spacer, fixing the phenylene group in the conformation required for it to serve as a component of the δ address. Finally, a comparison of the structure of oxymorphone (**50a**) with that of naloxone (**50b** R=allyl) appears to be interesting: replacement of the *N*-methyl group of **50a** (agonist) with an *N*-allyl group leads to the universal opioid antagonist naloxone (**50b**). Other ligands for the various opioid receptors are the partial μ-receptor agonist buprenorphine (**53**), the selective and irreversible μ receptor antagonist β-funaltrexamine (**54**) (Portoghese *et al.*, 1980), as well as the κ-selective agonists tifluadom (**55**) (Romer *et al.*, 1982) and the disubstituted chiral cyclohexane derivative (**56**) (U-50488; Szmuszkovicz and von Voigtlander, 1982). The C_2-symmetric compound norbinaltorphimine

(NorBNI, **57**) (Portoghese *et al.*, 1987) is a highly potent opioid antagonist with good selectivity for the κ receptor.

A plethora of other peptide- and nonpeptide-based ligands of opioid receptors have been synthesized and investigated pharmacologically (Evans *et al.*, 1990; Portoghese *et al.*, 1990; Portoghese, 1991, 1992; Zimmerman and Leander, 1990). However, 'while our knowledge of the opioid receptor system has increased tremendously, this new information has not yet led to the development of a major new therapeutic agent' (Zimmerman and Leander, 1990). In particular, it has not yet been possible, despite initial hopes, to develop an opiate with strong analgesic potency that is not addictive and does not suppress respiration (a frequent cause of death following morphine overdose). Nevertheless, some successes and interesting new developments in the area have been achieved and they will be briefly discussed here.

Buprenorphine (**53**) is a partial agonist of the μ receptor. After sublingual application approximately 50% is absorbed (for comparison, only 5% of the morphine applied orally passes into the blood stream). After successful absorption buprenorphine binds slowly to the receptor and dissociates from it equally slowly. It is precisely this property that leads to the unusually low addiction potential of this drug. After termination of treatment only mild withdrawal symptoms are observed. This pharmacological profile, together with the absence of respiratory suppressant and psychotomimetic effects, has contributed to the clinical success of buprenorphine (Lewis, 1985).

Based on the knowledge that (a) nerve endings of peripheral sensory neurones possess opioid receptors and that (b) the leukocytes that migrate to inflamed tissues may help alleviate pain by virtue of their opioids (Stein *et al.*, 1990), a new approach for producing analgesia was recently reported: in arthroscopic knee operations morphine was applied intra-articularly. It has been shown that the postoperative analgesia is both stronger and longer-lasting than that obtained by intravenous application of morphine. The well-known side-effects of morphine do not appear with this type of application (Stein *et al.*, 1991). This is the first study in humans of the analgesic effects resulting from the administration of opioids in the vicinity of peripheral sensory-nerve terminals. These exciting results suggest that central opioid receptors are not the exclusive target of systemically administered morphine (Fig. 11) and confirm the necessity for the development of potent, orally active opioid analgesics that cannot pass the blood–brain barrier, thus precluding the serious side-effects triggered by their action in the central nervous system (Basbaum and Levine, 1991).

An interesting possibility for the treatment of pain is offered by the benzodiazepine derivative tifluadom (**55**), a κ receptor agonist. Although clinical studies are still not available, animal experiments show that tifluadom is an effective analgesic without undesired effects such as respiratory depression or addictive potential (Romer *et al.*, 1982).

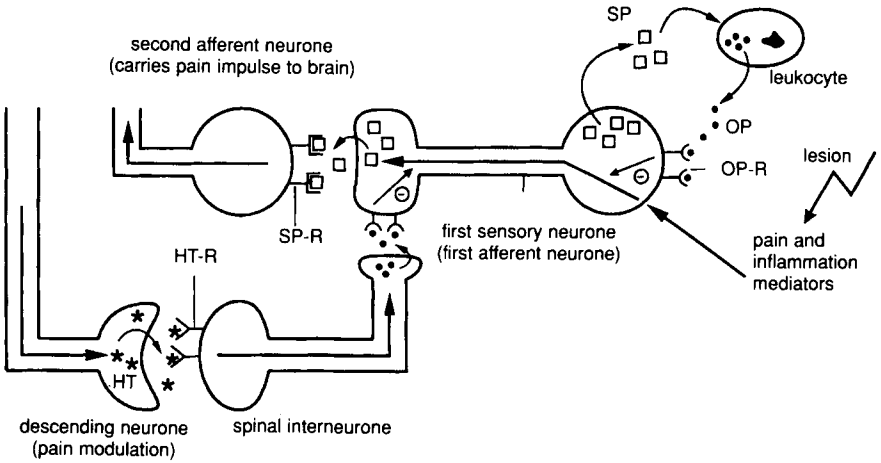


FIG. 11. Components of pain transmission. Specific interneurones of the spinal cord use opioids as neurotransmitters. The interneurones act directly on the centrally directed ends of the primary sensory neurones (first afferent neurone) and suppress the conduction of pain impulses from the periphery to the spinal cord by release of opioids. Substance P is an important neurotransmitter in the pain-transmitting synapses. The pain impulse is carried to the brain by the second afferent neurone. The neurone descending from the brain modulates the activity of the spinal interneurone and in this process uses serotonin, among others, as a neurotransmitter. The activation of the peripheral nerve endings of sensory neurones, for example, during inflammation, also leads to the release of substance P in the area of the lesion. This results in the migration of leukocytes in the area of inflammation. Endogenous opioids released from the leukocytes bind to the peripheral opioid receptors of the first afferent neurones, leading to a reduction in intensity of the pain impulses. Abbreviations: OP = opioid, OP-R = opioid receptor, 5HT = serotonin (5-hydroxytryptamine), 5HT-R = serotonin receptor, SP = substance P, SP-R = substance P receptor.

Considering the importance of substance P (Fig. 11) and cholecystokinin for the perception of pain and the fact that potent substance P and cholecystokinin receptor antagonists (see sections 4.2 and 4.5) are now available, the future of pain therapy can be viewed with optimism.

4.2 TACHYKININS (SUBSTANCE P, NEUROKININS A AND B)

Tachykinins substance P (SP, **58**), Neurokinin A (NKA, **59**) and Neurokinin B (NKB, **60**) are peptides with a length of 10 or 11 amino acids that act as neurotransmitters or neuromodulators in various parts of the central and peripheral nervous systems (Logan *et al.*, 1991; Mussap *et al.*, 1993). These

peptides have a common *C*-terminal sequence Phe-X-Gly-Leu-Met-NH_2. The best known and most investigated tachykinin, substance P (**58**), was discovered by von Euler and Gaddum (1931).

At least three receptors for the tachykinins designated as NKR 1–3 are known. The endogenous ligands are, corresponding to the different relative receptor affinities, SP for the NK1 receptor (NKR1), neurokinin A for NKR2, and neurokinin B for NKR3:

NKR1: SP > NKA > NKB
NKR2: NKA > NKB > SP
NKR3: NKB > NKA > SP.

NKR2 is found predominantly in the peripheral nervous system and NKR3 in the central nervous system, whereas NKR1 exists in both the central and peripheral nervous systems (Logan *et al.*, 1991). The receptors belong to the G-protein-coupled receptor superfamily with seven α-helical transmembrane domains as the common structural motif. Chimeric receptor studies revealed that the transmembrane domains (TM) V–VII are responsible for the recognition of the common tachykinin sequence (message sequence), whereas the segments I–IV and the *N*-terminal (extracellular) part of the receptors bind to the variable part of the tachykinin (address sequence) (Yokota *et al.*, 1992). Site-directed mutagenesis studies disclosed that three residues (Asn^{85}, Asn^{89}, and Tyr^{92}) in the second transmembrane domain of the NK1R and Tyr^{287} in the seventh transmembrane domain are involved in the binding of all tachykinin peptides (Strader *et al.*, 1994). It is worth noting that these four amino acid residues are conserved among the three tachykinin receptor subtypes. Asn^{85} has been proposed to interact with the *C*-terminal amide of SP. Tyr^{92} is involved in an aromatic interaction with residues on tachykinin peptides, whereas Tyr^{287} appears to be involved in hydrogen bonding and aromatic interactions with the peptide agonists. Moreover, Glu^{78} in the second transmembrane domain and Tyr^{205} in the fifth transmembrane domain appear to be involved in the receptor activation process (Huang *et al.*, 1994). Finally, using a photoreactive analogue of SP, Li *et al.* (1995) have shown that the *C*-terminal Gly-Leu-Met-NH_2 sequence of this neuropeptide inserts into a hydrophobic ligand-binding pocket formed by TM II and TM VII with contributions from other transmembrane domains. Position 8 of SP interacts with the second extracellular loop (NKR1 173–183) and position 3 of SP with the *N*-terminal extracellular tail of NKR1 (residues 1–21). These results suggest that peptide NK1R agonists interact with both the transmembrane and the extracellular domains of the receptor.

The individual NK receptors are quite homologous; after they bind their endogenous ligands they act by intracellular release of 1,4,5-inositoltrisphosphate, diacylglycerol, and cyclo-AMP (c-AMP), which act as second messengers.

The physiological function of tachykinins is still not completely understood but significant progress has been made in recent years. The biological effects of SP have been well examined. It triggers smooth muscles contraction of the respiratory, gastrointestinal and urogenital tract, and has diuretic and natriuretic properties. In addition, SP is a potent stimulator of the salivary and respiratory mucus secretion and the secretion of pancreatic juice and bile (Mussap *et al.*, 1993). The importance of tachykinins and particularly of SP is clearly demonstrated in pain and inflammation. This neuropeptide acts in the periphery, causing hypotension and symptoms characteristic of inflammation, including vasodilation and the extravasation of plasma. It induces the release of endogenous opioids from leukocytes and of interleukin-1, interleukin-6, tumour necrosis factor-α from monocytes (Lotz *et al.*, 1987; Harris, 1990; Stein *et al.*, 1990). Conversely, some cytokines appear to increase the rate of biosynthesis of SP (Freidin and Kessler, 1991). This mutual influence of SP and cytokines is important in neurogenic inflammation, i.e. in processes such as rheumatoid arthritis, Crohn's disease and colitis ulcerosa (Lotz *et al.*, 1987; Mantyh *et al.*, 1988; Harris, 1990; Payan, 1992). In general, the first afferent nociceptor neurone is unusual in that its centrally directed axon innervates the spinal dorsal horn, whereas its peripherally directed axon innervates tissues such as muscle, skin, and joints (Basbaum and Levine, 1991). Upon depolarization of these neurones, SP is released in the dorsal horn at pain-conveying synapses and also in the periphery where it maintains an inflammatory process. Furthermore, SP stimulates leukocytes within the area of an inflammatory event to release endogenous opioids, whose action on opioid receptors located on the peripheral end of the nociceptor neurone causes pain reduction (see Fig. 11). Neurokinin A has effects similar to those of SP, whereas neurokinin B operates as a pain reliever by the release of ligands of the opiate receptor (Logan *et al.*, 1991).

In the search for tachykinin antagonists many peptide analogues were prepared and pharmacologically tested (Logan *et al.*, 1991; Mussap *et al.*, 1993). The peptidic compounds initially developed showed, in most cases, low potency and allowed only an incomplete characterization of the tachykinin receptors, because of overlapping specificity. Other serious problems were the mixed agonist- and antagonist-like properties, the histamine-releasing effects and also the neurotoxicity of these tachykinin receptor ligands. The undecapeptide spantide I is an example for this. Folkers *et al.* (1990) have recently introduced spantide II, which has a better affinity for NKR1 and a diminished histamine-releasing effect. Another analogue is the antagonistic octapeptide (D-Pro^{4}-,D-Trp^{7,9,10}-Phe^{11})-SP(4-11) (Mizrahi *et al.*, 1984). In order to elucidate the essential domain of this antagonist the tripeptide derivative Boc-Gln-D-Trp(CHO)-Phe-OBzl was prepared and shown to exhibit potent inhibitory activity (IC_{50} 90 nM) in the binding assay using guinea pig lung membranes and tritium-labelled SP. It also antagonized SP-induced contraction of isolated guinea pig trachea strips. However, in *in*

vivo experiments it was inactive due to the rapid hydrolysis of the benzyl ester group at the *C*-terminus to produce Boc-Gln-D-Trp(CHO)-Phe-OH. The latter derivative is completely devoid of binding affinity (Hagiwara *et al.*, 1992). In order to overcome these problems the tripeptide lead was optimized to the metabolically stable amide Ac-Thr-D-Trp(CHO)-Phe-NMeBzl (**61**) (FR113680). *In vitro*, FR113680 acts as an SP-antagonist ($IC_{50} \approx 6$ nM) and inhibits SP-induced contraction of isolated guinea pig trachea strips (IC_{50} 2.3 μM). Furthermore, it was demonstrated *in vivo* that this antagonist suppressed the SP-induced bronchoconstriction and bronchial oedema in guinea pigs with ED_{50} of 0.42 mg kg^{-1} and 0.66 mg kg^{-1}, respectively, after intravenous administration. From these results FR113680 can be considered to be a potent and specific SP antagonist with no agonistic properties. However, its *in vivo* antagonistic activity was relatively weak.

A common structural feature of the above peptidic SP antagonists and of the potent and highly selective SP antagonist GR71251 (Ward *et al.*, 1990) is the existence of one or more aromatic and particularly tryptophane residues in their *C*-terminus. This could be the driving force to search for small nonpeptide ligands containing aromatic and amine residues. The first potent, competitive nonpeptide antagonist for the NK1 receptor is CP-96345 (**62**) ($K_i = 0.66$ nM in the binding assay using bovine caudate membranes and tritium-labelled SP) which emerged from a chemical file-screening process (Snider *et al.*, 1991; McLean *et al.*, 1991). Only the (2*S*,3*S*) enantiomer of **62** is effective; it shows no agonistic activity and has an affinity for the NK1 receptor comparable to that of its endogenous ligand, SP. The authors later optimized this initial lead and developed the (2*S*,3*S*) derivative **63** which is a more potent SP antagonist having a $K_i = 0.17$ nM in the binding assay using human IM-9 cells and tritium-labelled SP (Desai *et al.*, 1992). These results suggest that first, only the inner of the two phenyl ring of the benzhydryl group is critical for activity, and second that the 1-phenyl-2-benzylamine-1,2-diaminoethane group represents the pharmacophoric group (Desai *et al.*, 1994). The recently described tryptophane ester **64a** (X = O) and the metabolically stable ketone derivative **64b** ($X = CH_2$) also represent potent NK1R antagonists (MacLeod *et al.*, 1993; Merchant *et al.*, 1994). They display some structural features common to the above described chiral quinuclidine and piperidine derivatives, i.e. an indole side-chain and a bulky disubstituted aromatic group.

With the aid of site-directed mutagenesis the amino acid residues of the NK1R important for the recognition of these non-peptide antagonists were identified (Strader *et al.*, 1994; Cascieri *et al.*, 1994). The residues Gln^{165}, His^{197}, His^{265} and Tyr^{287} are critical sites of contact. His^{197} (TM V) interacts with the benzhydryl moiety of CP-96345 (**62**) possibly via amino–aromatic interactions. His^{265} (TM VI) appears to be in close proximity to the substituted benzyl moiety of the bound quinuclidine antagonists. Finally, the residue Gln^{165} (TM IV) of the NK1R seems to interact with the heteroatom

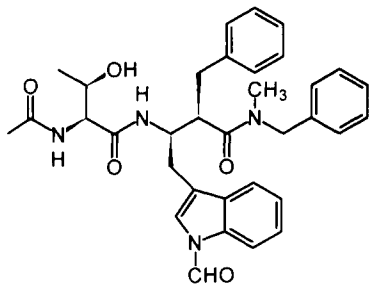
at the C3 position of the quinuclidine. Similar results were obtained for the tryptophane derivatives (**64**). In this case the indole moiety interacts with His^{197} and the bistrifluoromethyl side-chain with His^{265} (Cascieri *et al.*, 1994). The acylamino group appears to face the extracellular region of the receptor.

Taken together, these studies confirm that the extracellular domains of NKR1 play a minor role in nonpeptide antagonist binding and that nonpeptide antagonists use different receptor residues for binding than peptide agonists. In other words, pharmacologically competitive binding does not suggest that the two ligands utilize the same intermolecular interactions with the receptor.

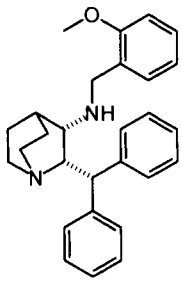
58: Arg-Pro-Lys-Pro-Gln-Gln-**Phe**-Phe-**Gly**-**Leu**-**Met**-NH_2

59: His-Lys-Thr-Asp-Ser-**Phe**-Val-**Gly**-**Leu**-**Met**-NH_2

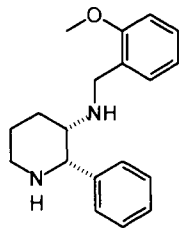
60: Asp-Met-His-Asp-Phe-**Phe**-Val-**Gly**-**Leu**-**Met**-NH_2



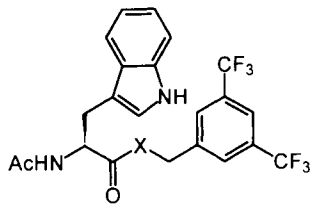
61



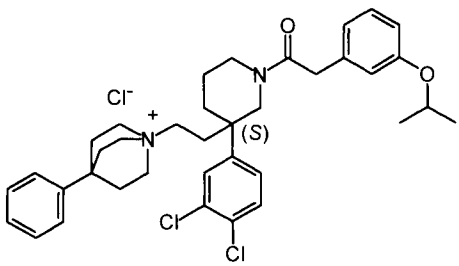
62



63



64a,b (X=O,CH_2)



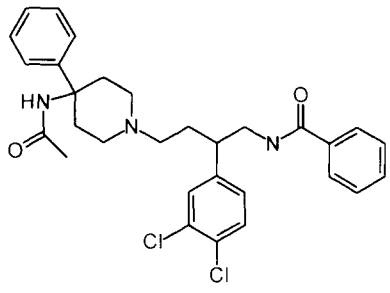
65

CP-96345 blocks SP-induced plasma extravasation and salivation in the rat following oral administration. In addition, intraperitoneal administration of CP-96345 inhibits *Clostridium difficile* toxin A-mediated enteritis in rats and dramatically reduces fluid secretion and mannitol permeability in rat ileal loops exposed to toxin A (Pothulakis *et al.*, 1994). Importantly, it has no inhibitory effect on the intestinal effects caused by administration of cholera toxin. However, CP-96345 has serious cardiovascular effects (in rats, guinea pigs and dogs). It causes hypotension and has negative inotropic, chronotropic and dromotropic actions on the heart. These effects are due to the 'verapamil-like' Ca^{2+} channel antagonism and are not related to blockade of the NK1 receptor (Constantine *et al.*, 1994).

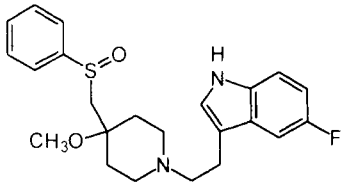
A newer NKR1 antagonist is **65** (SR 140333) discovered by a chemical file screening process (Emonds-Alt *et al.*, 1993; Oury-Donat *et al.*, 1994). This compound potently, selectively and competitively inhibits substance P binding to NK1 receptor. Using a human astrocytoma cell line and labelled SP, a K_i value of 0.74 nM was calculated. *In vitro* it is a potent antagonist in functional assays for NK1 receptors. *In vivo*, SR 140333 exerts, after intravenous administration, potent antagonism towards SP-induced hypotension in dogs, bronchoconstriction in guinea pigs and plasma extravasation in rats. The ED_{50} values range from 3 to 42 $\mu g\,kg^{-1}$ (i.v.). Contrary to CP-96345 this compound did not cause hypotension. Moreover, *in vivo* and at the level of the central nervous system it inhibits the NK1R-mediated release of acetylcholine and also the neuronal activities in the rat thalamus following nociceptive stimulation (ED_{50} 0.2 mg i.v.). These pharmacological properties make SR 140333 a promising drug candidate. Other potent nonpeptide antagonists are RP 67580 which is specific for the rat NK1 receptor (Garret *et al.*, 1991; Peyronel *et al.*, 1992) and a steroid derivative (Venepalli *et al.*, 1992).

Recently, compounds **66a** (SR 48968) (Advenier *et al.*, 1992) and **66b** (GR 159897) (Cooper *et al.*, 1994) were described as highly potent and selective competitive antagonists of the NK2 receptor of several species including humans. The common structural features of these compounds are the tertiary amine moiety of the piperidine substituted with a bulky arylalkyl group and the presence of aromatic moieties in position 4 of the piperidine. Derivatives SR 48968 and GR 159897 block, after oral administration, the agonist-induced smooth muscle constriction in several tissue preparations.

The dipeptide derivative Boc(S)Phe(S)αMePheNH$_2$ represents a rationally developed dipeptidic NK3 receptor antagonist (Boden *et al.*, 1994). Because several NKR3 receptor ligands (for example MePhe^7-NKB) have a Phe-Phe moiety as a common structural feature, the authors introduced an α-methyl group in the *C*-terminal Phe in order to restrict conformational flexibility and obtained Boc(S)PheαMe(S)PheNH$_2$. This derivative proved to be a low-affinity NK3R antagonist. By optimizing the *C*-terminus of this dipeptide lead compound and introducing a lipophilic alkyl group the urea derivative



66a



66b

Boc(S)Phe(R)αMePheNH$(CH_2)_7NH_2CONH_2$ was synthesized and proved to be a potent NK3R antagonist (IC_{50} = 16 nM).

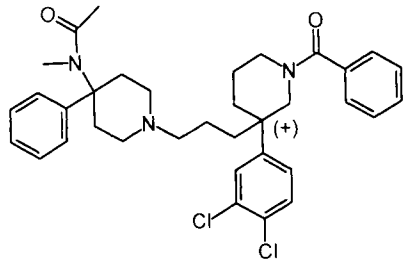
A piperidine-derived NK3R antagonist was described by Emonds-Alt *et al.* (1995). Compound **67** (SR 142801) potently inhibits *in vivo* the turning behaviour induced by intra-striatal injection of senctide (an NK3R agonist). SR 142801 also can cross the blood–brain barrier and is fully active after oral administration with a long-acting effect.

In summary, many selective and potent nonpeptide tachykinin antagonists have been developed in recent years. The study of the interaction of the quinuclidine and tryptophane derivatives with the NK1 receptor was studied in detail and gave important insights into interactions of these ligands with the NKR1. These results may be fruitful for the design of ligands for other G-protein-coupled receptors. Finally, comparing the structures of tachykinin receptor antagonists shown (**61–67**), it appears that the piperidine scaffold is well suited for the development of tachykinin antagonists. The availability of such compounds will help to further clarify the physiological and pathological role of tachykinins and may lead to new antiasthmatic and analgesic drugs.

4.3 SOMATOSTATIN (SOMATOTROPIN-RELEASE INHIBITING FACTOR, SRIF)

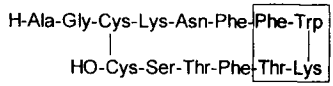
Somatostatin (SRIF) **68** (Scheme 1) is a cyclic tetradecapeptide synthesized as a preproprotein with a molecular mass of approximately 8 kDa (Reichlin, 1983a,b; Rens-Domiano and Reisine, 1992). Through alternative posttranscriptional processing it is transformed into the mature hormone and to SRIF-28, which is also biologically active. SRIF has been found in all regions of the brain and particularly in the hypothalamus. It inhibits the release of growth hormone (GH) by the pituitary gland. In other parts of the brain somatostatin acts as a neurotransmitter and as a neuromodulator.

SRIF has also been found in peripheral tissues such as the gastrointestinal

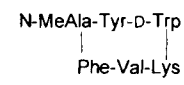


67

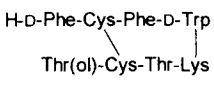
tract and secretory cells of the intestine and pancreas. It serves in these organs as an autocrine and paracrine regulator. SRIF formed in the δ cells of the pancreas inhibits the release of insulin and glucagon by the β and α cells of the pancreas, respectively. The motility of the digestive tract is inhibited



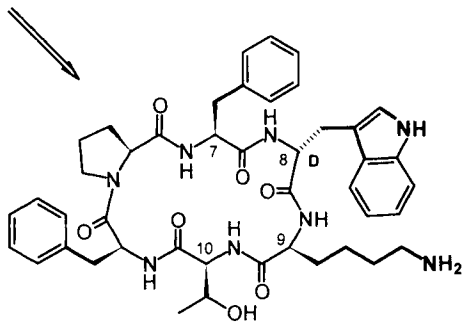
68: Somatostatin with a β-turn



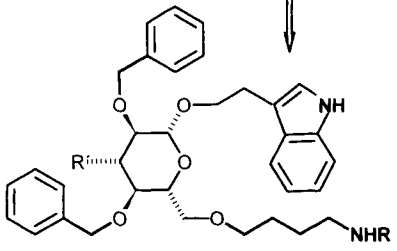
69: Seglitid



70: Octreotid



71



72: R=R′=H
73: R=CH_3CO, R′=OCH_2Ph

Scheme 1

by SRIF and the blood supply is restricted. The secretion of gastric acid and gastrin is also reduced. Another effect is the reduction in secretion of pancreatic enzymes and the absorption of carbohydrates, triglycerides and water.

SRIF has been also identified in the sensory *C*-fibres, in sympathetic and parasympathetic neurones in the peripheral nervous system (Payan, 1992). In addition, SRIF-mRNA has been identified in lymphocytes. In the immune system, SRIF has been shown to inhibit lymphocyte proliferation by blocking DNA and RNA synthesis at nanomolar concentrations (Payan, 1992). It inhibits also at subnanomolar concentrations the production of immunoglobulin A by cells isolated from secondary lymphatic organs such as spleen and intestinal Peyer's patches (van Hagen *et al.*, 1994). These findings indicate that SRIF plays an important role in the modulation of the immune response by the nervous system. It remains to be determined whether or not these biological effects on the immune system are mediated indirectly by a mechanism involving cytokines/growth factors or directly by interaction of SRIF with its receptor. For example, the cyclic SRIF analogue R-160 has been found to stimulate tyrosine phosphatase activity in cancers of many different organ sites. It is of interest to note that this analogue produces cytostatic effects and eliminates experimentally induced premalignant lesions in animals (Liebow *et al.*, 1993).

At present, five SRIF receptors denoted as SRIF-R 1–5 (Bell and Reisine, 1993; Ocarrol *et al.*, 1994) have been identified. They are located in normal tissues including neurones, secondary lymphatics and leukocytes as well as in malignant and granulomatous tissues. Most, but not all, biological effects of SRIF are mediated by the SRIF receptors which are coupled to pertussis-sensitive G-proteins.

The main therapeutic use of somatostatin is in the treatment of acute gastrointestinal diseases such as gastric ulcers and particularly in the treatment of life-threatening bleeding in the gastrointestinal tract (Reichlin, 1983a,b). It is also used in the treatment of pancreatitis and after surgery on the pancreas. Somatostatin has not been successful, however, in the treatment of diabetes mellitus. In patients with type I diabetes (juvenile form), a temporary reduction in blood glucose levels has been observed after infusion of somatostatin. The condition of patients with type II diabetes (adult diabetes) deteriorates after application of somatostatin because of inhibition of the remaining insulin secretion. Because SRIF is rapidly metabolized its pharmacological action is of short duration. In order to overcome these problems numerous peptide-based SRIF agonists have been synthesized and pharmacologically tested, especially by Veber; a critical and detailed discussion of this topic has been published recently (Veber, 1992). Seglitide (**69**) (MK 678, Scheme 1) (Veber *et al.*, 1984) is one cyclic analogue that is more potent than SRIF. Clinical trials with this compound were terminated because of serious side-effects such as steatorrhea. Another

somatostatin agonist is octreotide (SMS 201-995, **70**) (Bauer *et al.*, 1982). In the crystal structure of octreotide a β-II′-turn involving the (D)-Trp-Lys unit was identified (Pohl *et al.*, 1995). Octreotide is successfully used in the therapy of acromegalia (Lamberts *et al.*, 1985) and is the therapeutic principle of first choice in the symptomatic treatment of the carcinoid syndrome (Kvols *et al.*, 1986), Verner–Morisson syndrome and glucagonoma syndrome (Arnold *et al.*, 1994). Furthermore it has been used successfully in the treatment of intestinal scleroderma, which is otherwise very difficult to treat (Soudah *et al.*, 1991). ^{123}I-labelled octreotide has also been used as a diagnostic agent for the detection of various tumours bearing SRIF receptors (Lamberts *et al.*, 1990). The potential of SRIF and its analogues in the diagnosis of immune, granulomatous, and malignant diseases has been recently summarized (van Hagen *et al.*, 1994).

Seglitide, octreotide, and the early developed cyclopeptide **71** (Veber *et al.*, 1981) simulate a β-turn within the sequence Phe^7-Trp^8-Lys^9-Thr^{10} of SRIF (Scheme 1). This structural characteristic was postulated after extensive studies on structure–activity relationships. In agreement with the theory that a D-amino acid in the i+1 position stabilizes a β-turn (Chandrasekaran *et al.*, 1973), Trp^8 is replaced by D-Trp in these cyclopeptides. In order to define precisely the bioactive conformation of the derivative **71** Huang *et al.* (1992) have synthesized several analogues of **71** incorporating α- and β-methylated amino acid residues at positions 7, 8 and 11 (somatostatin numbering). Studies on the biological activity of these analogues accompanied by NMR investigations suggest a binding pocket for the SRIF analogues which consists of the side-chains of Trp^8 and Lys^9, the peptide backbone, and the side-chain of Phe^{11} in a folded conformation. The peptide backbone adopts a β–II′-turn about Trp^8-Lys^9 and a β–VI-turn about Phe^{11}-Pro^6. These results are similar to those proposed by Veber *et al.* (1981). A model for the SRIF pharmacophore is shown in Fig. 12 (Huang *et al.*, 1992).

The cyclopeptide **71** served as a lead compound not only for seglitide (**69**) but also for the first nonpeptide ligand **72** (Nicolaou *et al.*, 1990; Hirschmann *et al.*, 1992a). The dipeptide moiety Phe-Pro in **71** allows the side-chains of the four amino acids that comprise the β-turn to assume conformations which permit binding and agonist-like activity. The Phe group of the dipeptide sequence is thought to replace the hydrophobic region defined by Asn^5 and Thr^{12} of SRIF and provides a favourable hydrophobic interaction with the receptor. In agreement with this is the fact that a derivative of **71** in which the Phe-Pro moiety is replaced by Ala-Pro shows diminished affinity for the SRIF receptor (Veber *et al.*, 1981). Based on these studies Hirschmann and Nicolaou suggested that D-glucose could be a suitable scaffold for presenting the amino acid side-groups of the β-turn to the SRIF receptor. The initially synthesized compound **73** contains the side-chains of Phe^7, Trp^8, and Lys^9 at C-2, C-1, and C-6 of the D-glucose, respectively. The benzyl group at C-4 is thought to mimic the Phe-Pro moiety of the cyclopeptide **71**. The side-chain

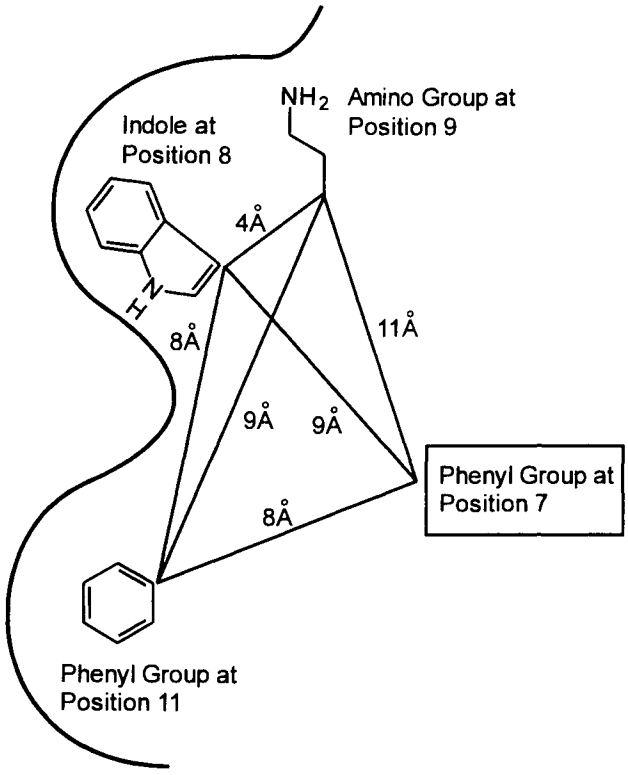


FIG. 12. Model for the somatostatin-release inhibiting factor (SRIF) pharmacophore.

of Thr^{10} was not incorporated because there was indication that this group is not critical for receptor binding. In the binding assay using radioactive-labelled SRIF the peptidomimetic had an IC_{50} value of 15 μM. However, the corresponding 3-deoxy-β-D-glucose derivative **72** displayed a higher affinity to the somatostatin receptor of the pituitary gland with an IC_{50} value of 1.3 μM. A compound that contains a 1,2,3,5-tetrasubstituted D-xylofuranose unit in place of the central deoxyglucose showed comparable activity (Papageorgiou *et al.*, 1992).

Investigations with **72** and **73** have shown the following interesting aspects: at low concentration **72** behaves as a somatostatin agonist, whereas at higher concentration it shows antagonistic properties. Thus, it is the first somatostatin *antagonist* ever reported. In addition, derivative **72** is also an antagonist of the G-protein-coupled β_2-adrenergic receptor (IC_{50} = 3 μM). Surprisingly, it displays a much higher affinity towards the NK1 receptor (agonistic effect, IC_{50} = 0.18 μM), whereas derivative **73** is a potent antagonist (IC_{50} = 60 nM) for the NK1 receptor. These results show again that a lipophilic ligand-

binding domain is a general feature of G-protein-coupled receptors and may be targeted for the design of specific nonpeptide ligands (agonists and antagonists) for this class of receptors.

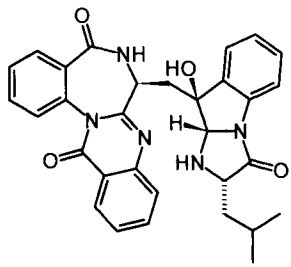
These results should be particularly valuable in the design and synthesis of specific and more potent ligands for the five currently known SRIF receptors. This is urgently necessary because at present no SRIF analogues or nonpeptide ligands have been identified that are truly monospecific. A number of SRIF agonists have been characterized with notably different affinities for the SRIF receptors. Seglitide is a potent ligand for both SRIF1 and SRIF2 receptors. It has been also used in order to obtain more insight into the selectivity determinants of these receptors. By employing chimeric receptors it was revealed that regions about the second and the third extracellular loops of these receptors contain the critical regions for seglitide affinity and selectivity (Fitzpatrick and Vandlen, 1994).

4.4 CHOLECYSTOKININ AND GASTRIN

Cholecystokinin (CCK) exists in numerous biologically active forms (CCK-58, CCK-39, CCK-33, CCK-8, CCK-4), having a common *C*-terminus which is essential for biological activity (Bradford, 1986; Nadzan and Kerwin, 1991). It exists in the nervous system both centrally and peripherally. CCK-8 (**74**) is the most common neuropeptide in the brain. CCK was originally demonstrated to be one of the hormones responsible for regulating the function of the digestive tract. Structurally it is related to the intestinal hormone gastrin (**75**). Peripherally, cholecystokinin is released from nerve endings in many regions of the body. It is also synthesized in neuroendocrine cells in the upper gastrointestinal tract; here it stimulates the contraction of

74: Asp-Tyr(SO_3H)-Met-Gly-**Trp-Met-Asp-Phe-NH_2 (CCK-8)**

75: pyroGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr(SO_3H)-Gly-Tyr-Gly-**Trp-Met-Asp-Phe-NH_2 (Gastrin)**



76

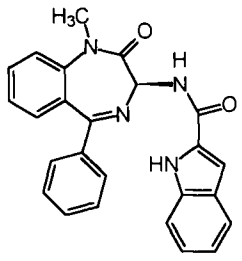
the gall bladder by simultaneous relaxation of the sphincter oddi, increases the release of insulin, enhances the secretion of enzymes from the pancreas, and inhibits the secretion of gastrin and gastric emptying.

In the central nervous system cholecystokinin acts as a neuromodulator/neurotransmitter, has an anxiogenic and appetite-suppressing effect, while at the spinal level it antagonizes the effect of opiates and thereby acts as an antianalgesic (Faris *et al.*, 1983).

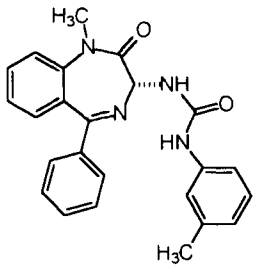
Two receptor subtypes have been well characterized: the predominantly peripheral CCK-A receptor and the largely centrally located CCK-B receptor. At the molecular level the binding of an agonist with the CCK-A receptor in acinus cells of the pancreas causes a G-protein-mediated release of inositol 1,4,5-trisphosphate and calcium ions, activation of the protein kinase C, and opening of calcium-dependent chloride channels. CCK-B has a similar mechanism of action (Silvene-Poirot *et al.*, 1993). Numerous CCK-A and CCK-B receptor ligands based on modification of the CCK peptides have been developed and investigated (Nadzan and Kerwin, 1991).

The area of indications for the CCK agonists appears to be limited, whereas the therapeutic potential of CCK antagonists lies in the treatment of diseases of the digestive tract (gastritis, pancreatitis, endocrinic tumours such as insulinomes and gastrinomes), neuropsychiatric disorders (anxiety, panic, cognitive dysfunction, schizophrenia), and pain.

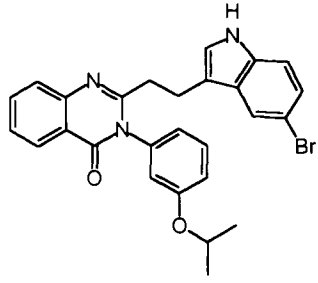
The discovery that the fungal metabolite asperlicin (**76**) (Chang *et al.*, 1985) is a weak CCK-A antagonist (IC_{50} = 1 μM) represents a major breakthrough in this area and opened the way for the development of the selective, orally active CCK-A antagonist Devazepide (**77**), (MK-329) with an IC_{50} value of 0.8 nM (Evans *et al.*, 1986), and also the CCK-B antagonist **78** (L-365260) with an IC_{50} value of 2 nM (Bock *et al.*, 1989). These two CCK antagonists differ only in the nature and stereochemistry of the side-chain. Recently, Beinborn *et al.* (1993) found that Val^{319} in the sixth transmembrane domain of the CCK-B receptor is critical for binding of L-365260. The structural



77



78

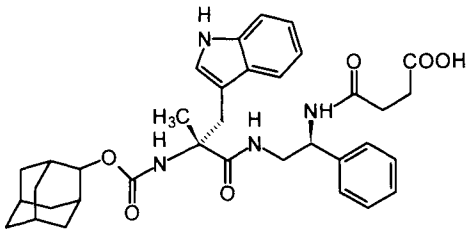


79

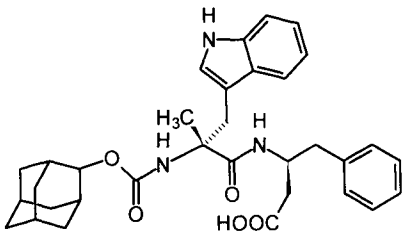
similarity of asperlicin with the clinically well-established benzodiazepines was the driving force for the synthesis of the compounds MK-329 and the structurally related derivative L-365260. It is worth noting here that some benzodiazepines such as diazepam act as weak CCK-A antagonists. In addition, the benzodiazepine derivative tifluadom (**55**) developed initially as a κ-opioid receptor agonist later proved to be a moderate CCK-A antagonist (Bock *et al.*, 1990).

Since the asperlicine structure is comprised of several heterocyclic systems, Yu *et al.* (1991) hypothesized that alternative substructures embedded within the molecular framework of this naturally occurring quinazolino-1,4-benzodiazepine derivative may provide a rational starting-point for the design of other nonpeptide CCK receptor ligands. Indeed, they synthesized several quinazolinone derivatives. One of these compounds, the derivative **79** subsequently proved to be a potent but nonselective CCK receptor antagonist.

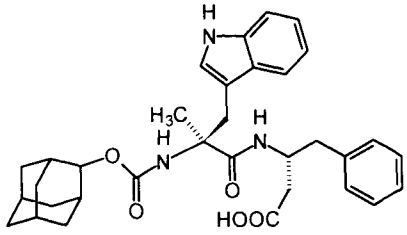
The observation that the *C*-terminal tetrapeptide sequence of CCK (Trp-Met-Asp-Phe-NH_2) is critical for biological activity at CCK receptors led to the synthesis of the dipeptide derivative Boc-Trp-Phe-NH_2 as a weak CCK-B agonist (Horwell *et al.*, 1987). The modification of this structure, supported by investigations of conformational energy (Pincus *et al.*, 1987) of the tetrapeptide Trp-Met-Asp-Phe-NH_2, culminated in the synthesis of the orally active derivative CI-988 (previously PD-134308, **80**), which proved to be a selective CCK-B antagonist (IC_{50} = 1.7 nM) (Horwell *et al.*, 1991).



80



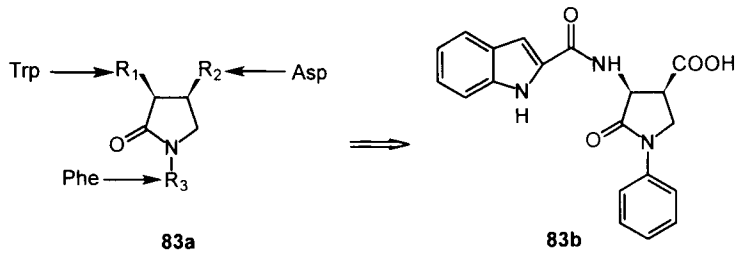
81



82

Compound CI-988 is the first example of a rationally developed nonpeptide ligand for a neuropeptide receptor. Another structurally related CCK-B receptor antagonist is compound **81** (PD-135666). Interestingly, inversion of the stereochemistry at the two stereogenic centres of **81** led to the corresponding enantiomeric derivative **82** which is a potent and selective CCK-A receptor antagonist (Higginbottom *et al.*, 1993).

Using the substituted pyrrolidinone of the general formula **83a** as scaffold and incorporating the side-chains of Trp, Asp, and Phe of the *C*-terminal region of the CCK peptides, Flynn *et al.* (1992) have developed the derivative **83b** (Scheme 2) which is a potent and orally active CCK-A antagonist (IC_{50} = 16 nM). For the antagonist activity the 3,4-*cis* stereochemical arrangement is necessary. Taken together, these results demonstrate again how an endogenous peptidal receptor ligand can be transformed to a nonpeptide receptor antagonist.



83a **83b**

Scheme 2

Compounds CI-988 (**80**) and L-365 260 (**78**) display interesting pharmacological properties in animal experiments. They have strong anxiolytic activity, but show no sedatory effects. No addiction-generating activity has been observed (Hughes *et al.*, 1990). In this respect they are far superior to the benzodiazepines. However, an appetite-increasing effect has been seen in animal experiments (Dourish *et al.*, 1989). Neither CI-988 nor L-365260 alone have analgesic properties; however, they can increase the analgesic effect of morphine and the duration of this effect (Wiesenfeld-Hallin *et al.*, 1990). This suggests that CCK is the physiological opioid antagonist (Wiertelak *et al.*, 1992).

4.5 GONADOTROPIN-RELEASING HORMONE (GnRH, LH-RH)

The gonadotropin-releasing hormone GnRH (**84**) is synthesized in the hypothalamus and then secreted directly into the hypophysioportal circulation (Conn and Crowley, 1991). In the anterior lobes of the pituitary gland it acts to release gonadotropins, i.e. luteinizing hormone (LH) and follicle-

stimulating hormone (FSH). After GnRH binds to its membrane-bound receptor, a G-protein-mediated activation of phospholipase C together with an increase in the intracellular concentration of free Ca^{2+} (Tse and Hille, 1992) occurs, and activation of calmodulin follows leading to the release of gonadotropins. Recent investigations (Tse and Hille, 1992) confirm the participation of leukotrienes as second messengers in these events.

The gonadotropins are heterodimeric glycoproteins with common α and different β subunits. They function as nonspecific superordinate sex hormones: FSH stimulates maturation of follicles and oestrogen biosynthesis in females, and the generation of spermatozoa in males; in females LH causes ovulation and formation of corpus luteum and oestrogen whereas in males the release of testosterone and biosynthesis of androgens is triggered.

Under physiological conditions GnRH is released in a pulsatile fashion (Fig. 13) (Conn and Crowley, 1991). Continuous application of GnRH (or a GnRH agonist) causes an initial increase in gonadotropin release by the pituitary gland, followed by reduction and finally cessation of gonadotropin release. The exact molecular mechanism responsible for this phenomenon has not yet been unequivocally clarified, but there is some evidence suggesting that it involves a down-regulation of the GnRH receptors in the pituitary gland or their decoupling from the intracellular effector system in the pituitary gland or both. Thus, the action of GnRH can be both imitated and antagonized by the choice of the method of application. In cases where the initial phase of increased secretion of LH, FSH, and sexual hormones should be avoided the application of GnRH antagonists is used.

GnRH and its analogues are used primarily in the following areas (Nestor and Vickery, 1988). (a) in the treatment of fertility disorders, for example in patients with a deficiency of GnRH. The use of an external pump, through which native GnRH is administered in a pulsatile manner, is necessary in order to simulate the physiological situation. (b) For the selective and reversible suppression of the pituitary gland–gonadal axis. The continuous application of GnRH is obligatory here. Alternatively, long-lasting GnRH agonists or antagonists can be used. Examples of diseases that can be treated with GnRH analogues include endometriosis, menometrorrhagia, polycystic ovarian disease, prostate carcinomas, uterine leiomyomas, and hyperandrogenism. GnRH agonists have been used hitherto for these purposes because they show fewer side-effects compared with the first-generation antagonists. For most of the antagonists tested to date, the side-effects are generally caused by the release of histamine due to mast cell degranulation. The areas of indication of GnRH analogues have been recently reviewed (Filicori, 1994).

Several peptidic GnRH analogues have been synthesized to date. The exchange of Gly^6 by lipophilic and/or D-amino acids as well as the replacement of Gly^{10} with *N*-ethylamide residue are very popular and have provided clinically useful agonists that are much more potent compared with

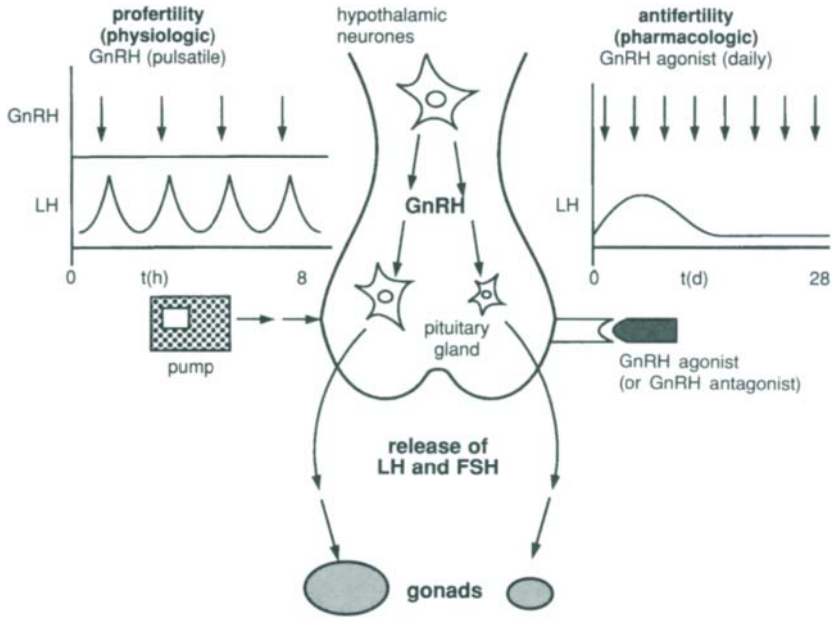


FIG. 13. Mechanism of action of gonadotropin-releasing hormone (GnRH) and GnRH agonists with long-lasting effects. For the treatment of fertility problems the pulsatile application of natural GnRH (physiological type of application, left) is necessary. This type of application imitates the physiological secretion of GnRH by the hypothalamus. For suppression of the pituitary gland–gonadal axis, long-lasting GnRH agonists (or antagonists) are administered in depot form or taken once daily (pharmacological application, right). After an agonistic initial phase, which can last several days to weeks, a long-lasting inhibition of the gonadotropin secretion results that is practically complete and selective. By terminating administration of the GnRH agonist the inhibition can be reversed (modified from Conn and Crowley, 1991).

GnRH. Among these are leuprolide (**85**), buserelin (**86**), and histrelin (**87**), which are 15, 20, and 210 times more active than native GnRH, respectively (Conn and Crowley, 1991). Another potent GnRH agonist is the azapeptide [D-Ser(tBu)^6,azaGly^{10}]-GnRH (goserelin) (Gante, 1989). The improved effect of these compounds can be traced back both to the blocking of catabolism at positions 6 and 10, and to their better receptor binding and increased lipophilicity, which leads to depot formation.

There are indications that the receptor-bound conformation of GnRH shows a Tyr^5-Gly^6-Leu^7-Arg^8-β-II′ turn (Momany, 1976). Incorporation of a β-turn mimetic in this position leads to peptide **89** in which the amino acids glycine and leucine in positions i + 1 and i + 2 of the postulated β-turn have been replaced by a γ-lactam. Although this compound shows an *in vivo* effect

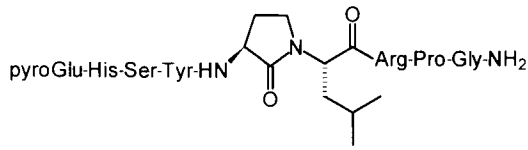
84: pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2

85: [D-Leu^6, Pro^9-NHEt]-GnRH

86: [D-Ser(tBu)^6, Pro^9-NHEt] -GnRH

87: [D-His(Bn)^6, Pro^9-NHEt] -GnRH

88: [D-3-(2-Naphtylalanin)^6] -GnRH



89

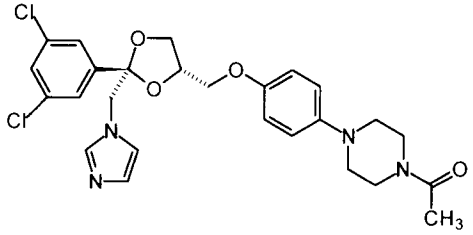
only 2.4 times greater than that of GnRH (Freidinger *et al.*, 1980), it provides an indication for the existence of a β-turn in the biologically active GnRH conformation. This observation was a turning-point in the field of peptidomimetics and initiated the application of lactams as turn mimetics.

GnRH agonists such as buserelin (Kuhn *et al.*, 1989) and leuprolide (Crawford *et al.*, 1989) find application in the treatment of metastasic prostate cancer, the growth of which is sex-hormone dependent. The simultaneous application of antiandrogens is necessary in order to forestall an androgen-induced initial intensification of the symptoms. Nafarelin has recently been used successfully in the therapy of the gynecological disease endometriosis, treatment of which is otherwise difficult (Henzl *et al.*, 1988). An increased oestrogen level seems to be responsible for the pathogenesis of this disease. In comparison with the steroid derivative danazol used also for this purpose, nafarelin possesses few side-effects and is well tolerated by patients (Barbieri, 1988). In this context it is worth noting that many endocrinologists believe that GnRH analogues are the most important advance since the discovery and application of synthetic oestrogens and progestagens in the treatment of gynecological diseases (Barbieri, 1988). These peptide analogues represent one of the most important new pharmaceutical contributions of the last two decades (Filicori, 1994).

Peptidal GnRH antagonists with negligible histamine-releasing activity were also developed. They contain several unusual amino acids. An example for such an antagonist is ganirelix (**90**), which contains several non-ribosomal amino acids with bulky and hydrophobic side-chains (Nestor *et al.*, 1992). Ganirelix is currently in phase II clinical trials and appears to be the most potent GnRH antagonist tested in humans. It has high potency for ovulation suppression ($ED_{50} = 0.29\ \mu g\ rat^{-1}$) and low histamine-releasing potency ($EC_{50} = 13\ mg\ ml^{-1}$).

[N-Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal(3)^3, D-hArg(Et)$_2^6$, hArg(Et)$_2^8$, D-Ala^{10}] - GnRH

90



91

The observation (Bhasin *et al.*, 1986) that high oral doses of the antimycotic drug ketoconazole (**91**) cause a reduction in serum testosterone levels and subsequent remission in prostate cancer led to the discovery that ketoconazole is a weakly competitive antagonist of the GnRH receptor (Biswanath *et al.*, 1989). Its affinity lies in the micromolar range. Thus, ketoconazol represents the first nonpeptide low-affinity ligand for the GnRH receptor.

4.6 ANGIOTENSIN II (A II)

The renin–angiotensin system plays an important role in the regulation of blood pressure. The enormous interest in this system has come about because of the high incidence of cardiovascular diseases, for example hypertension and heart failure. There are numerous possibilities for the pharmacological influence of the renin–angiotensin system, for example, by application of renin inhibitors, ACE inhibitors, or of antagonists of the A II receptor. ACE inhibitors have proven to be useful in the treatment of hypertension (Lawton *et al.*, 1992). In addition to the development of effective, orally active renin inhibitors (Kleinert *et al.*, 1992), dramatic activity has taken place in recent years in the development of angiotensin II analogues. While A II agonists are of interest for biochemical and pharmacological investigations, the A II antagonists are of enormous therapeutic potential and represent, at least, an alternative way for treating hypertension and cardiovascular diseases.

The biosynthesis of angiotensin II starts from angiotensinogen, a protein with a mass of about 60 kDa, which is largely synthesized in the liver and then passes into the circulation. In the kidneys it is cleaved by the aspartyl protease renin into the decapeptide angiotensin I, which is converted by the angiotensin-converting enzyme (ACE), a carboxypeptidase found primarily

in the lungs, into the biologically active angiotensin II (**92**). Its inactivation results from *C*-terminal cleavage of phenylalanine and leads to angiotensin III. Recent investigations suggest that angiotensin III is taken up into the cell and then inhibits cGMP-phosphodiesterase, resulting in opposite effects (relaxation of smooth muscle) to those of angiotensin II (Sharma *et al.*, 1991).

Angiotensin II is also synthesized in the brain. This octapeptide was the most potent substance known to increase blood pressure until the discovery of endothelin-1. It affects blood pressure directly by constriction of the blood vessels and indirectly by release of aldosterone through the cortex of the adrenal gland (Lawton *et al.*, 1992).

In addition to its action on blood pressure, angiotensin II transmits numerous other effects (Greenlee and Siegel, 1991), for example release of catecholamines from the adrenal medulla and stimulation of glycogenolysis in the liver. In the kidneys it stimulates the synthesis of prostaglandins and the resorption of sodium ions, and inhibits the release of renin and, thereby, its own biosynthesis. Angiotensin affects contraction of the uterus and stimulates ovulation, and in the brain it stimulates the secretion of vasopressin and adrenocorticotropic hormone (ACTH) by the pituitary gland and also functions as a neuromodulator.

Based on the differential selectivity of synthetic ligands, two receptor subtypes, AT1 and AT2, could be differentiated (Chiu *et al.*, 1989). Recently, an additional receptor subtype, AT-3, from the adrenal cortex of the rat, was cloned, sequenced, and expressed in COS cells and in *Xenopus* oocytes (Sandberg *et al.*, 1992).

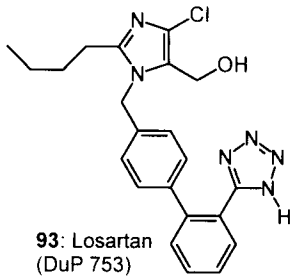
All known cardiovascular effects of angiotensin II have been attributed to the AT1 receptor, which has been demonstrated in several tissue types such as adrenal cortex, liver, kidney, brain, smooth muscles of blood vessels, bronchi, and the digestive tract. A G-protein-mediated action through various second-messenger systems, including the inhibition of adenylate cyclase activity, an increase in the intracellular calcium concentration by mobilization of intracellular reserves or by opening of calcium channels, and hydrolysis of phosphatidylinositol-4,5-bisphosphate could be demonstrated. The receptor subtype AT2 is found in fetal tissues including brain, in the adrenal cortex and in the uterus. Unlike AT1, AT2 is not deactivated by reaction with disulfide-reducing agents and is apparently not coupled to a G-protein. Its biological function is still unknown. A II binding to AT2 receptors has been shown to decrease intracellular cGMP levels (Sumners *et al.*, 1991). Recently, Buisson *et al.* (1995) using nondifferentiated NG108-15 cells (which exclusively express AT2 receptors) have found that A II decreases the T-type calcium current and that this effect is mediated by the involvement of a tyrosine phosphatase. Considering the abundance of T-type calcium channels in neurones it can be postulated that A II, via the AT2 receptor, modulates the Ca^{2+} channel and regulates the pacemaker

activity in neuronal cells. In addition, there are indications that angiotensin II plays a role in embryonic development. A growth-factor-like effect of angiotensin II is possibly mediated by the AT2 receptor (Feuillan *et al.*, 1991). A development-dependent differential expression of the two receptors AT1 and AT2 has been observed in the aorta (Viswanathan *et al.*, 1991) and the brain (Millan *et al.*, 1991) of the rat.

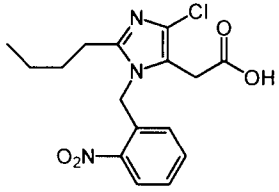
Peptidic A II antagonists such as saralasin ([Sar^1, Ala^8]A II) were developed in the 1970s. They display antihypertensive properties when administered intra-arterially but are not orally active, have short plasma half-lives, and often also possess agonist-like activity (Greenlee and Siegl, 1991). One of the newer peptide-based ligands of the A II receptor is [Sar^1,(2′,3′,4′,5′,6′,-Br_5)Phe^8]-A II, a strong noncompetitive antagonist with a long-lasting effect *in vivo* (Holk *et al.*, 1989). Derivatives of [Sar^1]-A II, in which Phe^8 is replaced by β,β'-diphenylalanine, are potent agonists of the A II receptor, whereas [Sar^1]-A II analogues with 2-naphthylalanine or 2-amino-2-indanecarboxylic acid in place of Phe^8 are antagonists. Global restriction of the conformational flexibility of A II through cyclization led to the agonist [$HCy^{3,5}$]-A II and to the agonist [Sar^1,$HCy^{3,5}$,Ile^8]-A II, both with higher affinity for the receptor (HCy = homocysteine) (Spear *et al.*, 1990). Model building suggests that the later ring structure is consistent with a receptor-bound conformation having any of a variety of three-residue turns, including a γ-turn. On the basis of physicochemical and spectroscopical investigations of A II and the superagonist [Sar^1]A II Matsoukas *et al.* (1994) have suggested a conformational model for A II characterized by clustering of the three aromatic rings and a charge relay system involving the triad Tyr hydroxyl–His imidazole–Phe carboxylate. According to these studies, the *N*-terminal domain of A II appears to play a crucial role in generating the biologically active charge relay conformation of the peptide hormone. In addition, these investigations confirmed that a Tyr-Ile-His bend is a predominant feature of the conformation of A II and [Sar^1]A II in the relatively nonpolar 'receptor simulating' environment provided by dimethyl sulfoxide.

The first potent, orally active nonpeptide antagonist of the AT1 receptor and prototype of a series of further compounds of similar structure (Greenlee and Siegel, 1991) is losartan (**93**) (DuP 753, MK-954) with an IC_{50} value of 19 nM (Carini *et al.*, 1991). Losartan was developed by overlaying the lead structure of *S*- 8308 (**94**), a weak but specific antagonist of the AT1 receptor discovered by screening (Furukawa *et al.*, 1982), with the structure of A II, using molecular modelling techniques. It was postulated that the imidazole moiety of **94** serves as a template to present mimetics of the Tyr^4 and Ile^5 side-chains as well as the *C*-terminal carboxyl group of A II. In clinical trials the blood pressure of volunteers could be reduced in a dose-dependent manner. These discoveries initiated intensive research in this area and culminated in the design of a great number of highly potent, orally active and selective antagonists of the AT1 receptor. A structural feature common

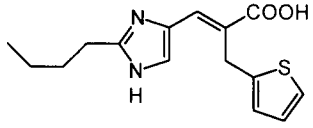
92: Asp-Arg-Val-**Tyr-Ile**-His-Pro-**Phe**



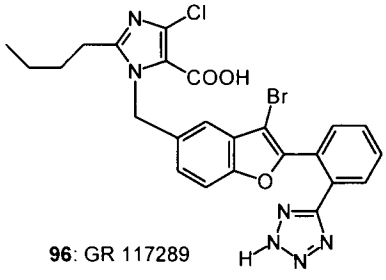
93: Losartan
(DuP 753)



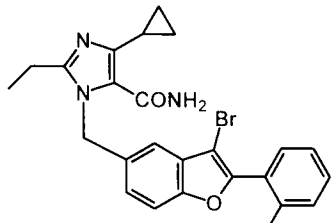
94: S-8308



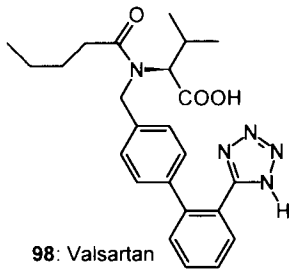
95: SK & F 108566



96: GR 117289



97: GR 138950 CF_3SO_2NH



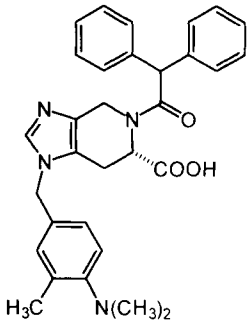
98: Valsartan

to most of the A II antagonists is an aromatic moiety derivatized with acidic groups such as carboxyl, tetrazole or sulfonamide. Examples are SK&F 108566 (**95**) (Weinstock *et al.*, 1991), GR 117289 (**96**) (Middlemmis and Watson, 1994) and GR 138950 (**97**) (Middlemmis and Watson, 1994) and valsartan (**98**) (Bühlmayer *et al.*, 1994). On the basis of comparative analysis of energy minimized conformations of losartan and the *C*-terminal pentapeptide Tyr-Ile-His-Pro-Ile of antagonist [Sar^1, Ile^8]A II it was speculated that the amide moiety of valsartan mimics the *C*-terminus whereas the phenyltetrazole part mimics the phenol group of Tyr^4 of A II.

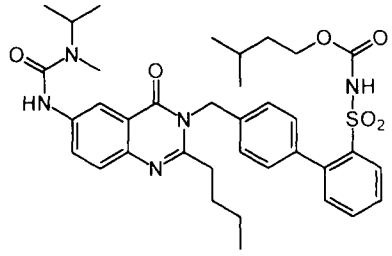
In an effort to characterize binding sites for nonpeptide A II antagonists on the human AT1 receptor Schambye *et al.* (1994) have systematically exchanged segments of the human A II receptor with corresponding segments

from an homologous *Xenopis laevis* receptor, which does not bind the nonpeptide antagonists. Substitution of the transmembrane domain VII of the human AT1 receptor dramatically reduced the binding affinity of, for example, losartan, SK&F 108566, GR 117289 and GR 138950. Conversely, this substitution has no effect on the binding of A II. Furthermore, it seems that the transmembrane domain VI and also the extracellular loop 3 of the AT1 receptor play an important role in the binding of the nonpeptide compounds. Exchanges of smaller segments and single residues in the transmembrane domains VI and VII revealed that the binding of the nonpeptide ligands was dependent on nonconserved residues located deep within these domains, in particular Asn-295 in transmembrane domain VII. From these studies it was concluded that the binding sites for peptide and nonpeptide ligands are rather different. In addition it was revealed that the binding sites for losartan and SK&F 108566 on one hand and for GR 117289 and GR 138950 on the other are overlapping but distinct. The last two compounds belong to the category of the so-called insurmountable antagonists, i.e. compounds that shift the dose–response curve to the right and simultaneously reduce the maximal response. Other studies showed that the binding of the tetrazole moiety of A II antagonists involves multiple contacts with residues such as Lys^{199} on TM V and His^{256} on TM VII (Noda *et al.*, 1995).

In recent years selective and potent antagonists of the AT2 receptor were also discovered: PD-123319 (**99**), a nonpeptide antagonist of the AT2 receptor (IC_{50} = 34 nM) is representative of other compounds with similar structures (Blankley *et al.*, 1991; Klutchko *et al.*, 1994). Recently, the quinazolinone L-162393 (**100**) was described as a potent antagonist of AT1 receptor (IC_{50} = 0.33 nM) and of the AT2 receptor (1.3 nM) (Glinka *et al.*, 1994). Compounds with antagonistic properties on both A II receptor subtypes have become of interest because AT1 antagonists lead to increased levels of A II which in turn could result, *in vivo*, in as-yet unidentified responses (Greenlee and Mantlo, 1994).



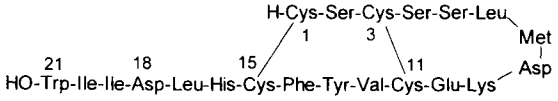
99: PD-123319



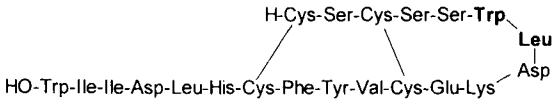
100: L-162393

4.7 ENDOTHELINS

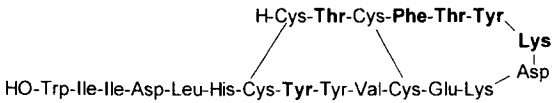
Endothelin-1 (ET-1, **101**), a peptide consisting of 21 amino acids which has a marked blood vessel contracting action (10 times stronger than angiotensin II), was first isolated in 1988 from cultivated endothelial cells (Yanagisawa *et al.*, 1988). Later, two other structurally related peptides were found, ET-2 (**102**) and ET-3 (**103**) (Inoue *et al.*, 1989; Matsumoto *et al.*, 1989). The endothelins are encoded by separate genes. The peptides and also their receptors are expressed in several vascular and nonvascular tissues such as lung, heart, kidney, eye, intestine, adrenal gland and brain (Simonson and Dunn, 1990; Rubanyi and Polokoff, 1994).



101



102



103

The biosynthesis of the most investigated member, endothelin-1, occurs by way of a preproendothelin (203 amino acids) and a proendothelin (39 amino acids). This peptide, also known as 'big endothelin', is cleaved by the endothelin-converting enzyme, which can be inhibited by the metalloprotease inhibitor phosphoramidon (McMahon *et al.*, 1991). To date, two mammalian endothelin (ET) receptor types have been isolated: ET_A with selectivity for endothelin-1 and ET_B without selectivity for the isopeptides. They are coupled by G-proteins to the phosphatidylinositol-4,5-bisphosphate second-messenger system and effect the mobilization of calcium ions from intracellular stores. Interestingly, after endothelin is bound to its receptor (Swiss 3T3 cells), a tyrosine kinase is also stimulated (Zachary *et al.*, 1991).

The endothelins which are structurally and pharmacologically related to the sarafotoxins have several biological actions (Simonson and Dunn, 1990; Doherty, 1991). They possess mitogenic effects, reduce the excretion of sodium ions by the kidneys, and mediate a series of other biological effects, for example contraction of smooth bronchial, intestinal, and uterine muscles.

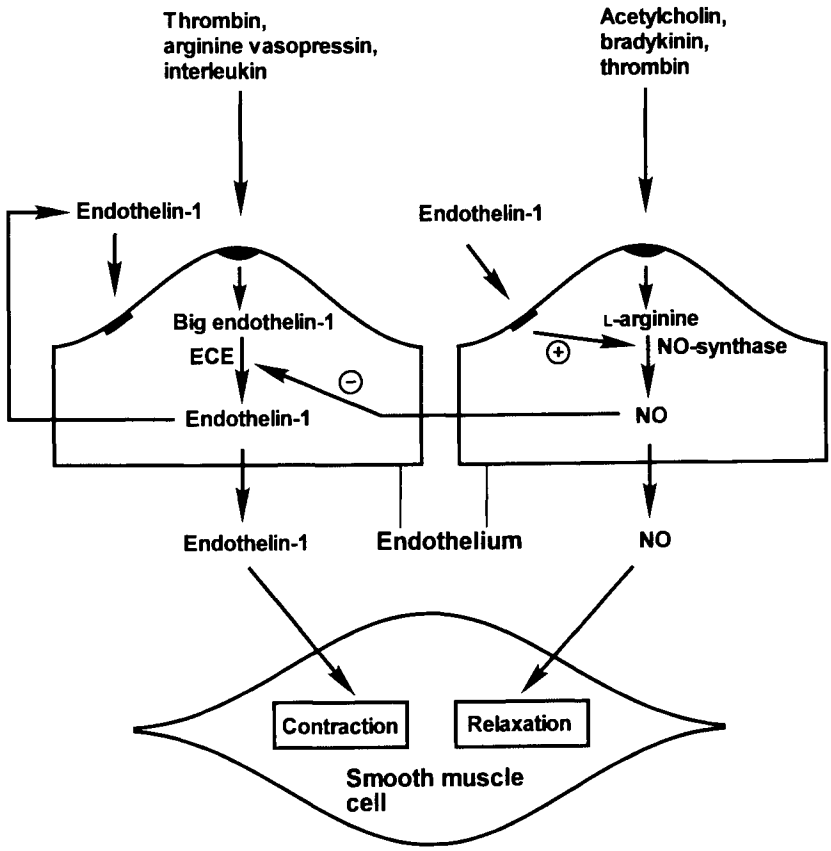


FIG. 14. Regulation of the vascular tone by endothelin-1 and NO.

Their haemodynamic effects are well studied. Intravenous administration of ET-1 leads, after an initial transient decrease of the arterial blood pressure, to a long-lasting increase. It is thought that the initial decrease in blood pressure is mediated through the ET_B receptor whereas the subsequent increase is mediated through the ET_A receptor (Takayanagi *et al.*, 1991; Clozel *et al.*, 1993a). Other studies suggest that the ET_B receptor mediates both vasodilation and vasoconstriction *in vivo* (Clozel *et al.*, 1992).

One important opponent of the effects of ET-1 on the blood pressure is the endothelium-derived relaxing factor nitric monoxide (NO). Activation of endothelial cells by several vasoactive hormones and neurotransmitters increases the production of the potent contractile peptide ET-1 and also the production of NO. In addition, the biosynthesis of NO is stimulated by binding of ET-1 to the ET_B receptor whereas nitric monoxide inhibits the formation of ET-1 from big-endothelin. This mutual influence of ET-1 and

NO is of key importance in the local regulation of vascular tone (Fig. 14, modified according to Vanhoutte, 1994).

Newer investigations challenge the opinion that endothelins are the 'bad guys of circulatory control' (Vanhoutte, 1994). The lower levels of ET-1 in mice heterozygous for the gene (ET-$1^{+/-}$) result in a modest increase in arterial blood pressure, rather than the expected reduction suggesting the existence of complex ET-1-mediated mechanisms that may function in peripheral tissues as well as in the central nervous system (Kurihara *et al.*, 1994). Furthermore ET-1 has profound effects on the heart. It evokes positive inotropic and chronotropic effects on myocardium. ET-1 inhibits the protein kinase A-dependent chloride current in ventricular myocytes (James *et al.*, 1994) whereas by inhibiting the L-type Ca^{2+} channel it hyperpolarizes the membrane and shortens the duration of the action potential in mammalian atrial myocytes, leading to suppression of electrical excitability of the heart. ET-1 strongly reduces the heart rate when it was increased by β-adrenoceptor stimulation (Ono *et al.*, 1994). By opposing the adverse effects of catecholamines on heart, ET-1 may reduce the risk of dangerous ventricular arrhythmias which occur in course of myocardial infarction (Haber and Lee, 1994). Recently, several investigations have also clarified the role of endothelins in embryonic development:

(1) ET-$1^{-/-}$ homozygous mice die of respiratory failure at birth and have abnormalities of the pharyngeal-arch-derived craniofacial tissues and organs (Kurihara *et al.*, 1994).
(2) The ET_B receptor plays a crucial role in the normal development of epidermal melanocytes and ganglion neurones in mice and humans (Puffenberger *et al.*, 1994).

Defects in the gene encoding the ET_B receptor leads to Hirschsprung's disease which is characterized by an absence of enteric ganglia in the distal colon and a failure of innervation in the gastrointestinal tract (Baynash *et al.*, 1994).

A linear ET-1, in which all four cysteine residues are replaced by alanine, acts as a selective agonist for the ET_B receptor (Saeki *et al.*, 1991). If the outer of the two disulfide bridges in ET-1 is exchanged for an amide bond between diaminopropionic acid and aspartic acid in position 15 (the position of the outer cysteine residue), a specific, potent antagonist for the ET_A receptor is obtained (Spinella *et al.*, 1991). The authors discuss a direct participation of the outer disulfide bridge in the recognition of ET-1 by ET_A, possibly by a disulfide exchange between ligand and receptor (Morrison *et al.*, 1988; Wilden *et al.*, 1989). Many other peptide analogues of the endothelins have been prepared and used for structure–activity relationships. It is clear that for the vasoconstrictor activity of ET-1 the sequence Asp^8-Lys^9-Glu^{10} as well as a free *N*-terminal amino group and the *C*-terminal

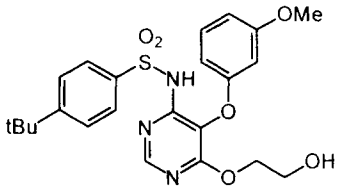
tryptophane residue are critical (Kimura *et al.*, 1988). Other studies suggested the importance of Leu^{17}, Asp^{18}, Ile^{20}, and Trp^{21} for receptor activation and implicated that the residues Asp^{8}, Tyr^{13}, Ile^{20}, and Trp^{21} are of significance for receptor binding (Hunt *et al.*, 1991).

Screening revealed the microbial cyclopeptide **104** as a selective ET_A receptor antagonist ($IC_{50} = 3\ \mu M$) (Ishikawa *et al.*, 1992). Optimization of this lead afforded the selective ET_A receptor antagonist BQ-123 (**105**) with an IC_{50} value of 22 nM. It was speculated that this cyclopeptide may be a mimic of the *C*-terminal residues 18–21 of ET-1 which are important for receptor binding.

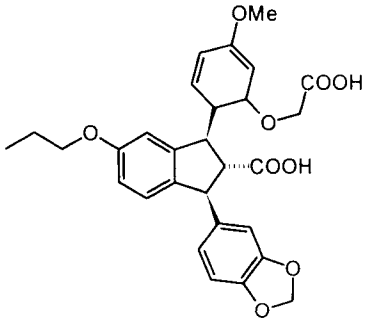
The first potent nonpeptide endothelin receptor antagonist was the sulfonamide Ro 46-2005 (**106**) discovered by a compound library screening (Clozel *et al.*, 1993b). This orally active compound inhibited the specific binding of radiolabelled ET-1 to human vascular smooth muscle cells (ET_A receptor) and rat aortic endothelial cells (ET_B receptor) with IC_{50} values of 0.22 μM and 1 μM, respectively. Low doses of Ro 46-2005 inhibited the depressor response to i.v. ET-1 but surprisingly potentiated its pressor

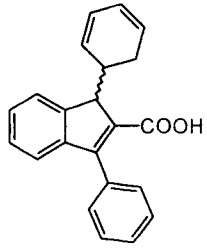
cyclo-(D-Trp-D-Glu-Ala-D-Val-Leu) cyclo-(D-Trp-D-Asp-Pro-D-Val-Leu)

104 **105**: BQ-123



106: Ro 46-2005





107: SB 209670 **108**: SK&F 66861

response. In contrast, the pressor effect of big ET-1 was inhibited by i.v. Ro 46-2005, even when it was given at low doses. On the basis of the observation that i.v. ET-1, not i.v. big ET-1, leads to an ET_B-dependent depressor response, the authors suggested that this depressor effect is solely a pharmacological observation and not a physiological phenomenon.

The first highly potent rationally developed nonpeptide ET_A receptor antagonist is the indane derivative (**107**) (SB 209670; Elliot *et al.*, 1994; Ohlstein *et al.*, 1994). The approach for the discovery of SB 209670 included the screening of compounds selected for their similarity to known antagonists of G-protein-coupled receptors and containing features of ET-1 known to be important to receptor binding. In this way the indene derivative SK&F 66861 (**108**) was identified (K_i: $ET_A = 7.3\,\mu M$, $ET_B > 30\,\mu M$). Comparison of a three-dimensional structure of SK&F 66861 with the low-energy conformation of ET-1 (according to NMR spectroscopic investigations in dodecylphosphocholine micelles) suggested that the 1- and 3-phenyl groups of SK&F 66861 may be mimics of a combination of two of the aromatic side-chains of Tyr^{13}, Phe^{14}, and Trp^{21}. In addition, the carboxyl group of SK&F 66861 was proposed to mimic either the Asp^{18} or the *C*-terminal carboxyl group in ET-1. The hypothesis that the carboxyl group in SK&F 66861 may mimic the Asp^{18} residue suggested the incorporation of a second acidic moiety into SK&F 66861 in order to mimic the important *C*-terminal carboxy function of Tyr^{21}. Furthermore, in view of the electron-rich characteristics of Tyr^{13} it was hypothesized that incorporation of electron-donating substituents onto the 1- or 3-phenyl groups of SK&F 66861 could have a favourable effect on receptor binding affinity. These efforts culminated in the synthesis of SB 209670 which is a potent competitive antagonist of radiolabelled ET-1 binding to cloned human ET_A ($K_i = 0.4$ nM) and ET_B receptors ($K_i = 14$ nM). SB 209670 produces dose-dependent inhibition of ET-1-mediated vasoconstriction in isolated vascular tissues and *in vivo* following intraduodenal administration. In hypertensive rats it produces dose-dependent reduction of the arterial blood pressure and also protects from ischaemia-induced neuronal degeneration in a gerbil stroke model.

Apart from pharmacological and pathophysiological aspects, the potential of ET_A-selective antagonists lies in the treatment of hypertension. Other possible applications for such antagonists include the treatment of asthma and also vasospasms in brain and heart. However, it must be kept in mind that ET_A blockers could have adverse effects on heart especially under pathological conditions such as myocardial ischaemia.

No concrete hypothesis concerning the potential of the ET_B receptor antagonists can be made at present, especially as the physiological significance of the ET_B receptor is still not completely clear. The accumulating data indicate the existence of multiple ET_B receptors subtypes, which may be classified as ET_{B1} (vasodilator) and ET_{B2} (vasoconstrictor) (Ohlstein *et al.*, 1994).

4.8 INTEGRIN LIGANDS AS MODULATORS OF CELL ADHESION

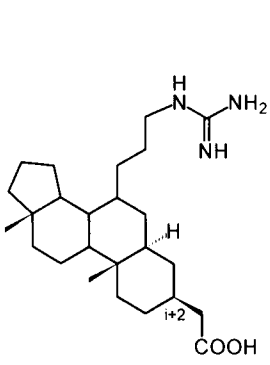
Integrins are noncovalently linked α/β heterodimeric proteins which play a critical role in cell-to-cell adhesion and in cell-to-extracellular matrix adhesion and interaction (Ruoslati and Pierschbacher, 1987; Hynes, 1992). Such interactions determine important biological phenomena such as cell morphology, differentiation and viability, cellular traffic and organogenesis, angiogenesis and blood clotting. To date 15 α subunits and 8 β subunits have been identified. Different $\alpha\beta$ complexes are expressed on different cells (Lucinscas and Lawler, 1994). For example the integrins $\alpha_L\beta_2$, $\alpha_M\beta_2$, and $\alpha_x\beta_2$ are expressed only on leukocytes whereas the integrins $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_v\beta_3$ are expressed on endothelial cells. In platelets, $\alpha_{IIb}\beta_3$ (known also as GPIIb/IIIa) is the major integrin. Furthermore tumour cells, for example human melanoma cells, express a_vb_3 (vitronectin receptor) which promotes a survival signal protecting these cells from apoptosis (programmed cell death) in the three-dimensional collagen matrix (Montgomery *et al.*, 1994).

The Arg-Gly-Asp (RGD) sequence on several proteins such as fibrinogen, fibronectin, vitronectin, laminin, osteopontin, and von Willebrand factor often serves as an endogenous ligand for the integrins. The binding is Ca^{2+}-dependent. A mechanism for binding of the RGD ligand by the platelet integrin $\alpha_{IIb}\beta_3$ was proposed recently (D'Souza *et al.*, 1994). According to these studies the $\beta 3$ residues 118–131 bind both ligand and cation. Formation of an unstable ternary intermediate complex between cation, ligand, and receptor with subsequent displacement of the Ca^{2+} from β_3 (118–131) seems to be central to the mechanism of ligand recognition by integrins.

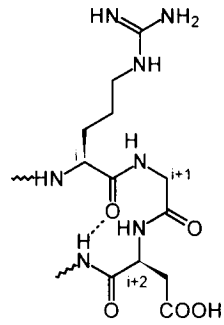
The specificity of the RGD-integrin interaction is generated by a combination of variations in the RGD conformation in different proteins and contributions of sequences near the RGD moiety (Ruoslati and Pierschbacher, 1987). Structure–activity investigations have revealed that in linear RGD peptides small structural modifications such as the exchange of alanine for glycine or glutamic acid for aspartic acid abolish the binding of the resulting peptides by integrins (Ruoslati *et al.*, 1994).

RGD analogues are potential candidates, for example by promoting wound healing, for treatment of thromboembolic diseases, and can also be considered as antimetastatic and anti-inflammatory drugs. A few selected examples discussed briefly in the following should highlight this potential:

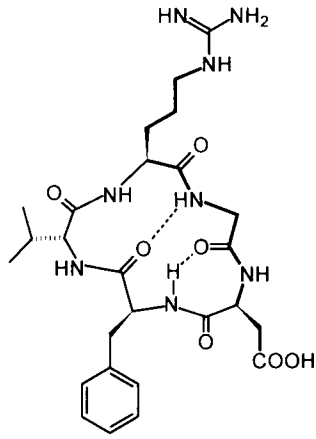
(1) A synthetic adhesive matrix containing the RGD moiety (Argidine) is in phase III clinical trials as wound-healing aid (Ruoslati *et al.*, 1994). A randomized double-blind, placebo-controlled trial with patients suffering from diabetic skin ulcers showed significant improvement of healing in the treatment group. The development of synthetic polymer materials containing the RGD moiety is a rapidly growing field in biomedicine with



109



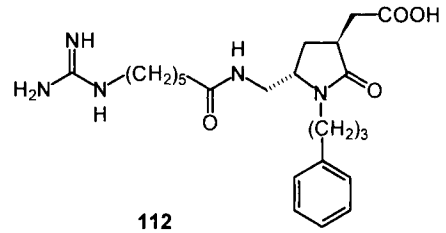
110



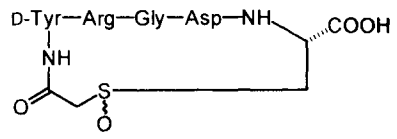
111: cyclo-RGDFv

many interesting and promising applications in tissue engineering (Ruoslati *et al.*, 1994; Hubbell and Langer, 1995).

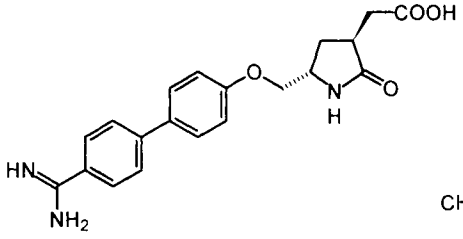
(2) The steroid derivative **109** was designed and synthesized as an analogue of a postulated type I β-turn **110** of the glycoprotein GpIIb/IIIa-bound sequence Arg-Gly-Asp of fibrinogen (Hirschmann *et al.*, 1992b). Compound **109** binds to the GpIIb/IIIa receptor and shows a moderate IC_{50} value of 100 μM when fibrinogen is used as ligand. In comparison the peptide cyclo(Arg-Gly-Asp-Phe-D-Val) (**111**), displays in IC_{50} value of 2 μM (Aumailley *et al.*, 1991; Müller *et al.*, 1992). The RGD conformation relevant for the binding by the glycoprotein GpIIb-IIIa is apparently imitated better by **111**. According to NMR spectroscopical investigations in dimethylsulfoxide solution, the Arg residue of the cyclopeptide lies in the i + 2 position of an extended βII'-turn, whereas the Asp residue lies in the central position of a γ-turn, so that the Arg and Asp side-chains are oriented nearly parallel (Müller *et al.*, 1992). However, it must be emphasized here that the receptor-bound conformation of RGD-containing peptides and RGD nonpeptide mimetics are still unknown. On the basis of the assumption that the GpIIb/IIIa-bound conformation of the lead peptide Arg-Gly-Asp-Phe includes either a β- or a γ-turn a γ-lactam was used as template. The binding groups were attached to the γ-lactam via flexible linkers, the length of which was optimized to yield a weakly active antagonist **112**. After further optimization of the very rigid lactam derivative **113** (BIBU 52) with an EC_{50} value of 80 nM was obtained (Austel *et al.*, 1994). This drug candidate possesses a negatively and a positively charged group at the appropriate distance, is a high-affinity ligand for GpIIb/IIIa and is also active *in vivo*. In an animal thrombosis model 1 mg kg^{-1} i.v. of BIBU 52 completely abolished thrombus formation for 1 h.



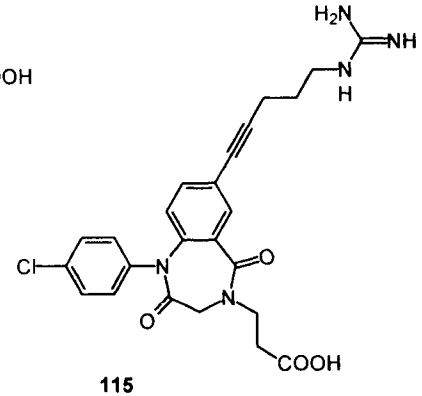
112



114 : G4120



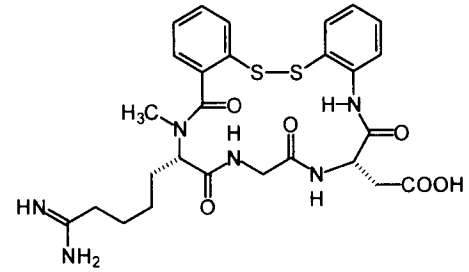
113: BIBU 52



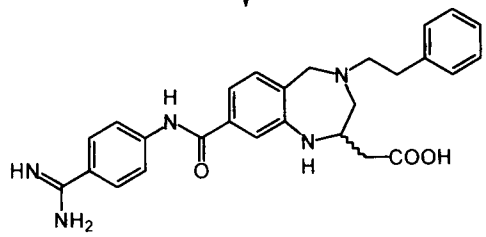
115

NMR studies of a rigid, potent RGD analogue **114** (G4120) and molecular dynamic simulations of flexible active analogues suggested a cupped (U-shaped) presentation of the RGD moiety of G4120 to the GpIIb/IIIa receptor (McDowel and Gadek, 1992). The D-Tyr side-chain of G4120 is spatially positioned nearly in the middle between the Arg and Asp side-chain. Subsequently the pyrrolo[1,4]benzodiazepine-2,5-dione was selected as a template to fit the contour and volume of the peptide backbone of G4120 (McDowel *et al.*, 1994). This template enabled the attachment of the critical Arg and Asp side-chains. The rational approach led to the benzodiazepine derivative **115** with an IC_{50} value of 9 nM using an ELISA essay.

Another rigid and potent GpIIb/IIIa ligand is the cyclopeptide **116** (Scheme 3). X-ray diffraction affirmed a 'turn-extended-turn' about the RGD moiety as described for both the major and the minor NMR derived conformers (Kopple *et al.*, 1992). These conformers differ mainly in the relative disposition of the guanidino group of Arg and the carboxyl group of Asp. The design process led to the benzodiazepine derivative **117** which mimics both compositional and conformational characteristics



116



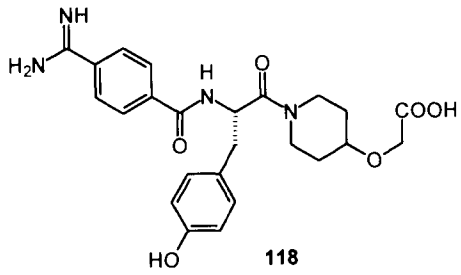
117

Scheme 3

of the cyclopeptide **116**. In competitive binding essays using biotinylated fibrinogen the benzodiazepine **117** displayed a K_i of 1.25 nM. It was also an inhibitor of platelet aggregation induced by ADP in human platelet-rich plasma with an IC_{50} value of 150 nM. In comparison, in both assays the cyclopeptide **116** displayed a K_i value of 0.62 nM and an IC_{50} of 57 nM, respectively (Ku *et al.*, 1993).

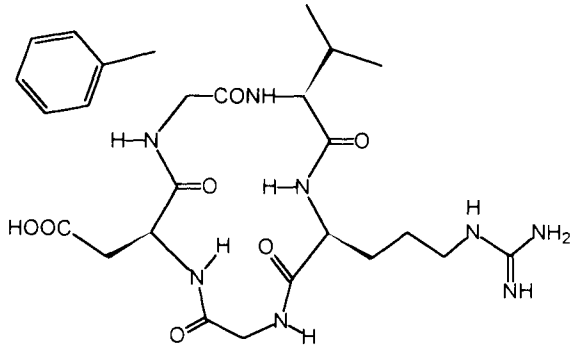
The above structural characteristics of G4120 suggest that an Arg-Tyr-Asp (RYD) group could be an alternative ligand for GpIIb/IIIa. Indeed, the RYD mimetic lamifiban (**118**; Ro 44-9883), which is currently in phase 3 clinical trials, is a potent inhibitor of the ADP-induced platelet aggregation determined in human platelet-rich plasma with an IC_{50} value of 30 nM (Alig *et al.*, 1992; Weller *et al.*, 1994). In addition, the OPG2 antibody binds also strongly to the GpIIb/IIIa receptor due to the presence of an Arg-Tyr-Asp moiety (Kodandapani *et al.*, 1995). Secondary structure analysis of the turn containing the RYD peptide indicated a 'β-type' turn with torsion angles that are not characteristic of tight β-turns.

(3) Malignant metastases are highly dependent on blood supply. Neovascularization is essential for their survival. Neovascularization begins with vasodilatation of the parent vessel (i.e. the vessel from which a new

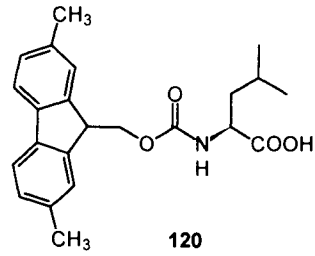


118

capillary sprout originates) followed by degradation of the basement membrane. Thereafter endothelial cells migrate through the degraded basement membrane toward the angiogenic stimulus (Folkman and Brem, 1992; Folkman, 1995). Tumour cells promote vascular endothelial cells entry into the cell cycle and expression of integrin a_vb_3 (Brooks *et al.*, 1994). After endothelial cells begin to move toward the angiogenic stimulus $\alpha_v\beta_3$ ligation provides a survival signal which finally results in differentiation and formation of mature blood vessel. A disruption of the $\alpha_v\beta_3$ ligation may lead to apoptosis with subsequent tumour regression because of shortage in blood supply. In order to test this hypothesis Brooks *et al.* (1994) used the cyclopeptide RGDfV (**119**) which was recently identified as a potent inhibitor of $\alpha_v\beta_3$-mediated cell adhesion with an IC_{50} of 100 nM (Aumailley *et al.*, 1991; Müller *et al.*, 1992; Pfaff *et al.*, 1994). A single intravascular injection of this peptide disrupts ongoing angiogenesis on the chick chorioallantoic membrane (Brooks *et al.*, 1994). This leads to the rapid regression of histologically distinct human tumours transplanted onto the chick chorioallantoic membrane. These results clearly demonstrate that $\alpha_v\beta_3$ antagonists may provide a new alternative approach for the treatment of malignancies or other



119 : cyclo RGDfV



120

diseases characterized by angiogenesis such as rheumatoid arthritis, psoriasis, haemangiomas and corneal neovascularization. Previously, it has been shown that antiangiogenic therapy with agents such as platelet factor 4 and the fumagilin derivative AGM 1470 generally has low toxicity and drug resistance does not develop (Folkman, 1995). In the future further $\alpha_v\beta_3$ antagonists (particularly nonpeptidal compounds) may be developed by optimization of known RGD analogues. For example, the toxin kistrin and also the cyclopeptide **114** are potent inhibitors of the $\alpha_v\beta_3$ binding to fibrinogen and vitronectin (McDowell and Gadek, 1992).

(4) Recently, the L-leucin derivative **120** (leumedin, NPC 15669) was identified in a screening process as an inhibitor of the function of the β_2-integrin Mac-1 (Burch *et al.*, 1991; Burch *et al.*, 1993). The precise mechanism of this inhibition is not clear. The derivative **120** inhibits leukocyte recruitment into inflammatory lesions in animals and inhibits endotoxin-induced neutropenia and lymphopenia in mice. It may be a valuable agent for blocking integrin-mediated leukocyte extravasation and can find application in the therapy of inflammatory diseases. Leukocyte recruitment into areas of inflammation is a complex, multistage event initiated through the interaction of the sialyl-Lewis-X oligosaccharide (SLe^x) group found on glycosphingolipids and particularly in glycoproteins on the extracellular face of the leukocyte membrane with the P- and L-selectin expressed on endothelial cells. (Springer, 1994; Giannis, 1994). For SLe^x-containing glycoproteins there are indications that the epitope recognized by the selectins consists of the sialyl-Lewis-X oligosaccharide group and an adjacent peptide sequence. Because of the above-described importance of RGD in cell adhesion events, Sprengard *et al.* (1995) suggested that a glycopeptide containing both the RGD group and the SLe^x moiety could be an alternative selectin ligand. Thereafter, compound Arg-Gly-Asp-Ala-NH-SLe^x was synthesized. Interestingly, this glycopeptide proved to be the most potent inhibitor of P-selectin today known in an assay employing P-selectin-IgG and HL-60 tumour cells (IC_{50} value of 26 μM). This work represents an important step toward the development of anti-inflammatory drugs.

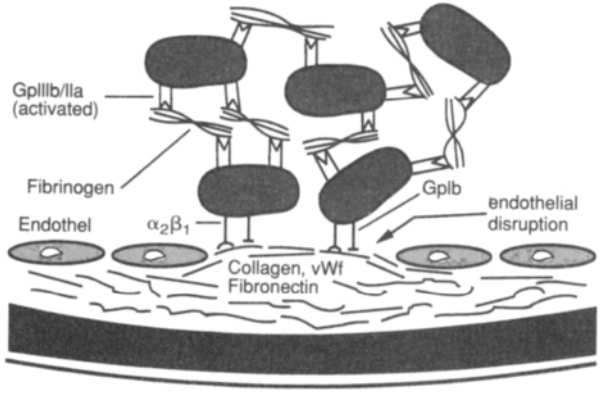


FIG. 15. Arterial thrombus formation. Endothelial disruption along the surface of an arterosclerotic plaque exposes tissue-bound von Willebrand factor (vWF), collagen, fibronectin and vitronectin from the subendothelial matrix. The platelet glycoprotein Ib (GpIb) binds vWF. In addition, the glycoprotein Ia/IIa (GpIa/IIa), an $\alpha_2\beta_1$ integrin, binds collagen. Adhesion of platelets to the subendothelial matrix leads to a conformational change and activation of integrin GpIIb/IIIa. This integrin can now bind the RGD sequence of the symmetrical molecule fibrinogen. Adherent, activated platelets degranulate and release a variety of other platelet activators such as adenosine diphosphate (ADP), serotonin and thromboxane metabolites. Adjacent platelets become secondarily activated by these soluble molecules. This adhesion cascade leads to thrombus formation. RGD mimetics block the binding of GpIIb/IIIa receptor to fibrinogen and prevent platelet aggregation.

In summary, after recognition of the importance of the RGD group for integrin-mediated cell adhesion several linear and cyclic RGD analogues were synthesized and their ability to act as alternative integrin ligands was investigated. On the basis of the subsequent pharmacological and physicochemical studies different hypotheses concerning the bioactive RGD conformation were generated, leading to numerous interesting and exciting new agents for the treatment of thromboembolic diseases. The design and synthesis of RGD mimetics were initiated after early studies in patients with the rare, inherited, autosomal bleeding disorder Glanzmann's thrombasthenia revealed the role of GpIIb/IIIa in platelet aggregation (Fig. 15). Subsequently, extensive clinical studies with the monoclonal anti-GpIIb/IIIa-antibody c7E3 (*abciximab*) were performed. This antibody potently inhibits platelet aggregation. In a double-blind trial of 2099 patients undergoing coronary angioplasty *abciximab* reduced the incidence of acute ischaemic events by 35% compared with placebo (Evaluation of c7E3 for the Prevention of Ischaemic Complications; EPIC Investigators, 1994). These results indicate that restenosis could be reduced by GpIIb/IIIa antagonists. A serious adverse

effect observed in patients receiving *abciximab* is bleeding. This problem should be addressed in future clinical evaluation of GpIIb/IIIa antagonists. These antagonists will be probably the first clinically useful 'anti-integrins' and should initiate the development of specific and potent low-molecular mimetics for the integrin ligands for use in anti-adhesion therapy (Lefkovits *et al.*, 1995). The potential of anti-integrins and RGD analogues as antimetastatic and anti-inflammatory drugs remains to be evaluated.

5 Summary and Outlook

Several native and modified peptides have been developed in the last years. The number of peptidic drugs is constantly increasing, and this trend will continue in the future (Williams and Nadzan, 1991; Fauchère and Thurieau, 1992). Many of these compounds are presently under clinical investigation whereas others were introduced for human therapeutic use. The GnRH and SRIF analogues belong to the last category. Despite the disadvantages referred to at the beginning of this article, the development of peptide-based receptor ligands will certainly remain of interest in the future (Griffith, 1991; Williams and Nadzan, 1991; Fauchère and Thurieau, 1992). The marked trend of recent years is the design and development of orally active nonpeptide ligands for peptide receptors. Of significance in this regard is the fact that it is possible to imitate or to block the pharmacological effect of a relatively large peptide at the receptor level with low molecular mass compounds. The opioid alkaloids are excellent examples for this fact. In the last years it was shown that several other clinically useful drugs have peptidomimetic properties:

(1) There are several lines of evidence that clinically useful benzodiazepines like diazepam are mimetics of endogenous peptides (Guidotti *et al.*, 1983; Alho *et al.*, 1985; Marquard *et al.*, 1986). In this connection it is important to note that benzodiazepines such as *N*-desmethyldiazepam were, surprisingly, found in the brains of people who had not been treated with benzodiazepines. Moreover, *N*-desmethyldiazepam could be detected in material obtained from human autopsies over 54 years ago, long before the first benzodiazepine was synthesized (Sangameswaran *et al.*, 1986; Stephenson, 1987; Rothstein *et al.*, 1992). This suggests that these compounds are of endogenous origin but the biosynthetic route is still not clear. Endogenous benzodiazepines have been implicated in the pathogenesis of hepatic encephalopathy (Basile *et al.*, 1991).
(2) Drugs based on sulfonylurea, long used for treatment of diabetes mellitus type II, are mimetics of the endosulfines, the endogenous peptide ligands of the sulfonylurea receptor (Virsolvy-Vergine *et al.*, 1992).
(3) The antibiotic erythromycin has been shown to be an agonist of the

polypeptide motilin, which is responsible for the coordination of the contraction of the gastrointestinal tract (Kondo *et al.*, 1988). This observation led to the successful application of erythromycin for the treatment of the delayed emptying of the stomach in diseases such as diabetes, thereby extending its indication area (Janssens *et al.*, 1990). So, it can be expected that 'as our knowledge base of receptors and enzymes expands, the number of known drugs classified as peptidomimetics will expand' (Portoghese, 1991).

Most of the membrane-bound peptide receptors known to date are coupled G-proteins in which the ligand-binding domain is in the hydrophobic core formed by the transmembrane α-helices. Several examples discussed above unequivocally show that nonpeptide antagonists use different receptor residues for binding to their G-protein-coupled receptors than the corresponding endogenous peptide agonists. In other words, pharmacologically competitive binding does not suggest that the two ligands utilize the same intermolecular interactions with the receptor. If one then looks at the structures of the above-mentioned nonpeptide receptor ligands, it is noticeable that they often contain hydrophobic and, in particular, aromatic groups which, in addition to their hydrophobic character groups, are also well suited for formation of hydrogen bonds with amino groups (Rodham *et al.*, 1993). Aromatic groups on the ligand generally appear to play a central role in recognition and in subsequent receptor activation. The removal of such groups often leads to an inversion of the action at the receptor (Marshall, 1992). This is shown impressively, for example, by a comparison of structures **72** (SP agonist) and **73** (SP antagonist).

A major issue in the design of ligands for peptide receptors with favourable pharmacological properties is the discovery of a lead structure. Most of the peptide receptor ligands described above were discovered by screening of natural products and compound libraries. However, several ligands were developed rationally with the aid of molecular modelling and physicochemical techniques, as demonstrated unequivocally by the examples of compounds **72**, **73**, **80–83b**, **107**, and **119**. In these cases the corresponding endogenous peptides served as the lead compound. The same applies also to the promising nonpeptidal RGD mimetics. The importance of the appropriate spatial arrangement was also demonstrated in studies of immunoglobulin mimetic **39b** and of the TRH analogue **46**, the development of which was based on X-ray crystallographic analyses. These mimetics, and also the somatostatin agonist octreotide (**70**) and the GnRH agonist **90**, demonstrate clearly the value of the concept of conformation restriction, the use of scaffolds, and the application of secondary structure mimetics in the development of receptor ligands that imitate bioactive peptide conformation. NMR spectroscopy under physiological conditions and/or in 'receptor simulating' environments has been shown to be increasingly useful in the investiga-

tion of bioactive peptide conformation as well as for generating fruitful hypotheses.

The significance of NMR spectroscopy in studies of peptide–receptor complexes (Fesik, 1991) was demonstrated recently for cyclosporin A and its interaction with the protein cyclophilin (Jorgensen, 1991; Wüthrich *et al.*, 1991). However, NMR studies are limited to small proteins (30–40 kDa) (Wüthrich, 1995). Large proteins and membrane proteins are generally not accessible with ^{1}H-NMR investigations. Recently, a new NMR technique, namely rotationally resonant magnetization exchange, was developed and used successfully in the investigation of the retinal–bacteriorhodopsin complex (Creuzet *et al.*, 1991). This technique may be useful for structural studies in membrane proteins.

Of great interest in peptidomimetic design is the fact that receptor proteins are becoming available in larger quantities with the help of molecular biology. In addition, through site-directed mutagenesis, receptor residues critical for ligand recognition can be identified. Such knowledge, in combination peptide synthesis, molecular modelling analysis and spectroscopic methods, helps to generate a pharmacophore concept and subsequently also in the design and optimization of lead compounds. However, a general method for the application of such knowledge to conformational design (Hoffmann, 1992) is not yet available. In conformational design the aim is often the conception of suitable scaffolds or templates that carry the functional groups required for binding to the receptor in a suitable spatial arrangement. Structure–activity investigations have shown that only a relatively small peptide sequence consisting of four to eight amino acids is responsible for biological activity, and that the amide backbone is not involved in occupation of the receptor and its activation (Hirschmann, 1991; Marshall, 1992). In addition, conformation–activity investigations show that these sequences of folded and compact bioactive conformations are generally 10×15 Å in size. These are the same dimensions of a benzodiazepine-like compound **77** or a cyclic hexapeptide such as **71** (Freidinger, 1989). The benzodiazepine template has proven to be versatile and leads not only to neuropeptide mimetics such as tifluadom (**55**), devazepide **77**, and L-365260 (**78**) but also for RGD mimetics such as **115** and **117**. Other pharmacologically important compounds which belong to the benzodiazepines are, for example:

- A new class of *ras*-farnesyltranferase inhibitors having a potential as cancer therapeutics (James *et al.*, 1993; Gibbs *et al.*, 1994). These peptidomimetics were developed rationally.
- Platelet-activating factor antagonists (Kornecki *et al.*, 1984).
- HIV protease inhibitors (HIV = human immunodeficiency virus) (Merluzzi *et al.*, 1990; Pauwels *et al.*, 1990; De Luca and Otto, 1992).
- Antitumour antibiotics such as anthramycin and tomaymycin (Kizu *et al.*, 1993).

Thus, it seems that benzodiazepines and related structures will continue to provide an interesting research field for both chemists and physicians.

The diverse pharmacological and biochemical properties of compounds **72** and **73** suggest the potential of the carbohydrate scaffold in the design of nonpeptide ligands. Carbohydrates offer advantages of structural diversity and the facile derivatization with a multitude of functional groups.

Finally, chiral substituted heterocycles like piperidine, pyrrolidine and piperazine derivatives, appear to be particularly attractive templates for the development of low-molecular receptor ligands: several orally active mimetics for CCK, tachykinins, angiotensin II and RGD belong to these categories of nitrogen-containing heterocycles. It is expected that in future rational design will become increasingly important for identification of lead structures as ligands for peptide receptors. However, because of dramatic developments of new receptor technologies and binding assays (Williams, 1991), screening of natural products, compound collections and particularly screening of low-molecular compounds generated by combinatorial synthesis will be valuable methods for the rapid progress in the field of peptidomimetics. There is currently a marked trend toward the synthesis of diverse compound libraries (diversomers) starting from readily available building blocks or templates. Although in the early phase of the combinatorial synthesis where polymeric structures predominate it is expected that in future rigid low-molecular (nonpolymeric) templates will receive more attention. Rigid templates resist hydrophobic collapse and prevent the formation of pharmacologically inactive conformations in aqueous media (Wiley and Rich, 1993).

References

Advenier, C., Rouissi, N., Nguyen, Q. T., Emonds-Alt, X., Breliere, J. C., Neliat, G., Naline, E. and Regoli, D. (1992). *Biochem. Biophys. Res. Commun.* **184**, 1418–1424.

Alberg, D. G. and Schreiber, S. L. (1993). *Science* **262**, 248–250.

Alho, H., Costa, E., Fujimoto, M., Cosenza-Murphy, D. and Guidotti, A. (1985). *Science* **229**, 179–182.

Alig, L., Edenhofer, A., Hadváry, P., Hürzeler, M., Knopp, D., Müller, M., Steiner, B., Trzeciak, A. and Weller, T. (1992). *J. Med. Chem.* **35**, 4393–4407.

Allmendinger, T., Furet, P. and Hungerbühler, E. (1990a). *Tetrahedron Lett.* **50**, 7297–7300.

Allmendinger, T., Felder, E. and Hungerbühler, E. (1990b). *Tetrahedron Lett.* **50**, 7301–7304.

Arnold, R., Frank, M. and Kajdan, U. (1994). *Digestion* **55** (Suppl. 3), 107–113.

Aumailley, M., Gurrath, M., Müller, G., Calvete, J., Timpl, R. and Kessler, H. (1991). *FEBS Lett.* **291**, 50–54.

Austel, V., Himmelsbach, F. and Müller, T. (1994). *Drugs Fut.* **19**, 757–764.

Bach, A. C. and Gierasch, L. M. (1985). *J. Am. Chem. Soc.* **107**, 3349–3350.

Ball, J. B., Andrews, P. R., Alewood, P. F. and Hughes, R. A. (1990). *FEBS Lett.* **273**, 15–18.

Ball, J. B., Hughes, R. A., Alewood, P. F. and Andrews, P. R. (1993). *Tetrahedron* **49**, 3467–3478.

Barbieri, R. L. (1988). *N. Engl. J. Med.* **318**, 512–514.

Basbaum, A. I. and Levine, J. D. (1991). *N. Engl. J. Med.* **325**, 1168–1169.

Basile, A. S., Hughes, R. D., Harrison, P. M., Murata, Y., Pannel, L., Jones, E. A., Williams, R. and Scolnick, P. (1991). *N. Engl. J. Med.* **325**, 473–478.

Batt, A. R., Kendrick, D. A., Mathews, E., Rooker, D. P., Ryder, H., Semple, G. and Szelke, M. (1994). *Bioorg. Med. Chem. Lett.* **4**, 867–872.

Bauer, W., Briner, U., Doepfner, W., Haller, R., Huguenin, R., Marbach, P., Petcher, T. J. and Pless, J. (1982). *Life Sci.* **31**, 1133–1140.

Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E. and Yanagisawa, M. (1994). *Cell* **79**, 1277–1285.

Beinborn, M., Lee, Y.-M., McBride, E. W., Quinn, S. M. and Kopin, A. S. (1993). *Nature* **362**, 348–350.

Bell, G. I., and Reisine, T. (1993). *Trends Neurosci.* **16**, 34–38.

Berridge, M. J. (1993). *Nature* **361**, 315–325.

Bhasin, S., Sikka, S., Fielder, T., Sod-Moriah, U., Levine, H. B., Swerdloff, T. S. and Rajfer, J. (1986). *J. Endocrinol.* **118**, 1229–1232.

Biswanath, D., Plattner, J. J., Bush, E. N., Jae, H.-S., Diaz, G., Johnson, E. S. and Perun, T. J. (1989). *J. Med. Chem.* **32**, 2036–2038.

Blankley, C. J., Hodges, J. C., Klutchko, S. R., Himmelsbach, R. J., Chucholowski, A., Conolly, C. J., Neergaard, S. J., van Nieuwenhze, M. S., Sebastian, A., Quin III, J., Essenburg, A. D. and Cohen, D. M. (1991). *J. Med. Chem.* **34**, 3248–3260.

Bock, M. G., Dipardo, R. M., Evans, B. E., Rittle, K. E., Whitter, W. L., Veber, D. F., Anderson, P. S. and Freidinger, R. M. (1989). *J. Med. Chem.* **32**, 13–16.

Bock, M. G., Dipardo, R. M., Evans, B. E., Rittle, K. E., Whitter, W. L., Veber, D. F., Freidinger, R. M., Chang, R. S. L., Chen, T. B. and Lotti, V. J. (1990). *J. Med. Chem.* **33**, 450–455.

Boden, P., Eden, E. J., Hodgson, J., Horwell, D. C., Howson, W., Hughes, J., McKnight, A. T., Meecham, K., Pritchard, M. C., Raphy, J., Ratcliffe, G. S., Suman-Chauhan, N. and Woodruff, G. N. (1994). *Bioorg. Med. Chem.* **4**, 1679–1684.

Bol, K. M. and Liskamp, R. M. J. (1992). *Tetrahedron* **48**, 6425–6438.

Bradford, H. F. (1986). 'Chemical Neurobiology', pp. 265–310. Freeman, New York.

Brooks, P. C., Clark, R. A. F. and Cheresh, D. A. (1994). *Science* **264**, 569–571.

Brownstein, M. J. (1989). *In* 'Basic Neurochemistry' (Siegel, G. J., Agranoff, B. W., Albers, R. W. and Molinoff, P. B., eds), pp. 287–309. Elsevier, Amsterdam.

Brownstein, M. J. (1993). *Proc. Natl Acad. Sci. USA* **90**, 5391–5393.

Bühlmayer, P., Furet, P., Criscione, L., de Gasparo, M., Whitebread, S., Schmidlin, T., Lattmann, R. and Wood, J. (1994). *Bioorg. Med. Chem. Lett.* **4**, 29–34.

Buisson, B., Laflamme, L., Bottari, S. P., de Gasparo, M., Gallo-Payet, N. and Payet, M. D. (1995). *J. Biol. Chem.* **270**, 1670–1674.

Burch, R. M., Weitzberg, M., Blok, N., Muhlhauser, R., Martin, D., Farmer, S. G., Bator, J. M., Connor, J. R., Ko, C., Kuhn, W., McMillan, B. A., Raynor, M., Shearer, B. G., Tiffany, C. and Wilkins, D. E. (1991). *Proc. Natl Acad. Sci. USA* **88**, 355–359.

Burch, R. M., Noronha-Blob, L., Bator, J. M., Lowe, V. C. and Sullivan, J. P. (1993). *J. Immunol.* **150**, 3397–3403.

Burgess, K. and Ho, K. (1994). *J. Am. Chem. Soc.* **116**, 799–800.

Burgess, K., Ho, K. and Pettit, B. M. (1995). *J. Am. Chem. Soc.* **117**, 54–65.

Burt, S. K. and Greer, J. (1988). *Annu. Rep. Med. Chem.* **23**, 285–294.

Carini, D. J., Duncia, J. V., Aldrich, P. E., Chiu, A. T., Johnson, A. L., Pierce, M. E., Price, W. A., Santella III, J. B., Wells, G. J., Wexler, R. R., Wong, P. C., Yoo, S. and Timmermans, P. B. M. W. M. (1991). *J. Med. Chem.* **34**, 2525–2547.

Cascieri, M. A., MacLeod, A. M., Underwood, D., Shiao, L.-L., Ber, E., Sadowski, S., Yu, H., Merchant, K. J., Swain, C. J., Strader, C. D. and Fong, T. M. (1994). *J. Biol. Chem.* **269**, 6587–6591.

Chandrasekaran, R., Lakshiminarayanan, A. V., Pandya, U. V. and Ramachandran, G. N. (1973). *Biochim. Biophys. Acta* **303**, 14–27.

Chang, R. S. L., Lotti, V. J., Monaghan, R. L., Birnbaum, J., Stapley, E. O., Goetz, M. A., Albers-Schonberg, G., Pachett, A. A., Liesch, J. M., Hensens, O. D. and Springer, J. P. (1985). *Science* **230**, 177–179.

Chen, Y., Mestek, A., Liu, J., Hurley, J. A., Yu, L. (1993). *Mol. Pharmacol.* **44**, 8–12.

Cheng, H., Keitz, P. and Jones, J. B. (1994). *J. Org. Chem.* **59**, 7671–7676.

Chipens, G. (1983). *In* 'Peptides. Structure and Function' (Hruby, V. J. and Rich, D. H., eds), pp. 865–868. Pearce Chem. Co., Rockford, Illinois.

Chiu, A. T., Herblin, W. F., McCall, D. E., Ardecky, R. J., Carini, D. J., Duncia, J. V., Pease, L. J., Wong, P. C., Wexler, R. R., Johnson, A. L. and Timmermans, P. B. M. W. M. (1989). *Biochem. Biophys. Res. Commun.* **165**, 196–203.

Chorev, M. and Goodman, M. (1993). *Acc. Chem. Res.* **26**, 266–273.

Clozel, M., Gray, G. A., Breu, V., Löffler, B.-M., Osterwaldet, R. (1992). *Biochem. Biophys. Res. Commun.* **186**, 867–873.

Clozel, M., Breu, V., Gray, G. A. and Löffler, B.-M. (1993a). *J. Cardiovasc. Pharmacol.* **22** (Suppl. 8), S377–S379.

Clozel, M., Breu, V., Burri, K., Cassal, J.-M., Fischli, W., Gray, G. A., Hirth, G., Löffler, B.-M., Müller, M., Neidhard, W. and Ramuz, H. (1993b). *Nature* **365**, 759–761.

Conn, P. M. and Crowley, W. F., Jr. (1991). *N. Engl. J. Med.* **324**, 93–103.

Constantine, J. W., Lebel, W. S., Woody, H. A., Benoit, P. A., Wolfgang, E. A. and Knight, D. R. (1994). *Eur. J. Pharmacol.* **252**, 275–282.

Cooper, A. W. J., Adams, H. S., Bell, R., Gore, P. M., McElroy, A. B., Pritchard, J. M., Smith, P. W. and Ward, P. (1994). *Bioorg. Med. Chem.* **4**, 1951–1956.

Cornille, F., Slomczynska, U., Smythe, M. L., Beusen, D. D., Moeller, K. D. and Marshall, G. R. (1995). *J. Am. Chem. Soc.* **117**, 909–917.

Cox, M. T., Heaton, D. W. and Horbury, J. (1980a). *J. Chem. Soc., Chem. Commun.* 799–800.

Cox, M. T., Gormley, J. J., Hayward, C. F. and Petter, N. N. (1980b). *J. Chem. Soc., Chem. Commun.* 800–802.

Coy, D. H., Heinz-Erian, P., Jiang, N.-Y., Sasaki, Y., Taylor, J., Moreau, J.-P., Wolfrey, W. T., Gardner, J. D. and Jensen, R. T. (1988). *J. Biol. Chem.* **263**, 5056–5060.

Crawford, E. D., Eisenberger, M. A., McLeod, D. G., Spaulding, J. T., Benson, R., Dorr, F. A., Blumenstein, B. A., Davies, M. A. and Goodman, P. J. (1989). *N. Engl. J. Med.* **321**, 419–424.

Creuzet, F., McDermott, A., Gebhard, R., van der Hoeff, K., Spijker-Assink, M. B., Herzfeld, J., Lugtenburg, J., Levitt, M. H. and Griffin, R. G. (1991). *Science* **251**, 783–786.

Cushman, M., Jurayj, J. and Moyer, J. D. (1990). *J. Org. Chem.* **55**, 3186–3194, cit. Lit.

Davies, J. S. and Thomas, W. A. (1978). *J. Chem. Soc. Perkin Trans.* **II**, 1157–1163.

De Grado, W. F. (1988). *Adv. Protein Chem.* **39**, 51–124.

De Luca, G. V. and Otto, M. J. (1992). *Bioorg. Med. Chem. Lett.* **2**, 1639–1644.

Desai, M. C., Lefkowitz, S. L., Thadeio, P. F., Longo, K. P. and Snider, M. R. (1992). *J. Med. Chem.* **35**, 4911–4913.

Desai, M. C., Lefkowitz, S. L., Bryce, D. K. and McLean, S. (1994). *Bioorg. Med. Chem. Lett.* **4**, 1865–1868.

DiMaio, J. and Belleau, B. (1989). *J. Chem. Soc. Perkin Trans.* **1**, 1687–1689, cit. Lit.

Doherty, A. M. (1991). *Annu. Rep. Med. Chem.* **26**, 83–92.

Dourish, C. T., Rycroft, W. and Iversen, S. D. (1989). *Science* **245**, 1509–1511.

D'Souza, S. E., Haas, T. A., Piotrowicz, R. S., Byers-Ward, V., McGrath, D. E., Soule, H.

R., Cierniewski, C., Plow, E. E., Smith, J. W. (1994). *Cell* **79**, 659–667.
Dürr, H., Goodman, M. and Jung, G. (1992). *Angew. Chem.* **104**, 773–774; (1992). *Angew. Chem. Int. Ed. Engl.* **31**, 785–787.
Elliot, J. D., Lago, M. A., Cousins, R. D., Gao, A., Leber, J. D., Erhard, K. F., Nambi, P., Elshourbagy, Kumar, C., Lee, J. A., Bean, J. W., DeBrosse, C. W., Eggleston, D. S., Brooks, D. P., Feuerstein, G., Ruffolo, R. R., Weinstock, J., Gleason, J. G., Peishoff, C. E. and Ohlstein, E. H. (1994). *J. Med. Chem.* **37**, 1553–1557.
Emonds-Alt, X., Doutremepuich, J.-D., Heaulme, M., Neliat, G., Santucci, V., Steinberg, R., Vilain, P., Bichon, D., Ducoux, J.-P., Proietto, V., van Broeck, D., Soubrié, P., Le Fur, G. and Brelière, J.-C. (1993). *Eur. J. Pharmacol.* **250**, 403–413.
Emonds-Alt, X., Bichon, D., Ducoux, J.-P., Heaulme, M., Miloux, B., Poncelet, M., Proietto, V., van Broeck, D., Vilain, P., Neliat, G., Soubrié, P., Le Fur, G. and Brelière, J.-C. (1995). *Life Sci.* **56**, 27–32.
EPIC Investigators (1994). *N. Engl. J. Med.* **330**, 956–961.
Es-Sayed, M., Devine, P., Burgess, L. E., de Meijere, A., Meyers, A. I. (1995). *J. Chem. Soc., Chem. Commun.* 141–142.
Evans, B. E., Bock, M. G., Rittle, K. E., Dipardo, R. M., Whitter, W. L., Veber, D. F., Anderson, P. S. and Freidinger, R. M. (1986). *Proc. Natl Acad. Sci. USA* **83**, 4918–4922.
Evans, S. M., Lenz, G. R. and Lessor, R. A. (1990). *Annu. Rep. Med. Chem.* **25**, 11–20.
Evans, C. J., Keith, D. E., Morrison, H., Magendzo, K. and Edwards, R. H. (1992). *Science* **258**, 1952–1955.
Faris, P. L., Komisaruk, B. R., Watkins, L. R. and Mayer, D. J. (1983). *Science* **219**, 310–312.
Fauchère, J.-L. and Thurieau, C. (1992). *In* 'Advances in Drug Research' (Testa, B., ed.), Vol. 23, pp. 127–159. Academic Press, London.
Fesik, S. W. (1991). *J. Med. Chem.* **34**, 2937–2945.
Feuillan, P., Millan, M. A. and Aguilera, G. (1991). *FASEB J.* **5**, A872.
Filicori, M. (1994). *Drugs* **48**, 41–58.
Fincham, C. I., Higginbottom, M., Hill, D. R., Horwell, D. C., O'Toole, J. C., Ratcliffe, G. S., Rees, D. C. and Roberts, E. (1992). *J. Med. Chem.* **35**, 1472–1484.
Fischer, G. (1994). *Angew. Chem. Int. Ed. Engl.* **33**, 1415–1436.
Fitzi, R., Seebach, D. (1988). *Tetrahedron* **44**, 5277–5292.
Fitzpatrick, V. D. and Vandlen, R. L. (1994). *J. Biol. Chem.* **269**, 24621–24626.
Flynn, D. L., Villamil, C. I., Becker, D. P., Gullikson, G. W., Moummi, C. and Yang, D.-C. (1992). *Bioorg. Med. Chem. Lett.* **2**, 1251–1256.
Flynn, G. A., Giroux, E. L. and Dage, R. C. (1987). *J. Am. Chem. Soc.*, **109**, 7914–7915.
Folkers, K., Feng, D.-M., Asano, N., Hakanson, R., Weisenfeld-Hallin, Z. and Leander, S. (1990). *Proc. Natl Acad. Sci. USA* **87**, 4833–4835.
Folkman, J. and Brem, H. (1992). *In* 'Basic Principles and Clinical Correlates', 2nd edition (Gallin, J. I., Goldstein, I. M. and Snyderman, R., eds), pp. 821–839. Raven Press, New York.
Folkman, J. (1995). *Nature Med.* **1**, 27–31.
Freidin, M. and Kessler, J. A. (1991). *Proc. Natl Acad. Sci. USA* **88**, 3200–3203.
Freidinger, R. M. (1985). *J. Org. Chem.* **50**, 3631–3633.
Freidinger, R. M. (1989). *Trends Pharmacol. Sci.* **10**, 270–274.
Freidinger, R. M. and Veber, D. F. (1979). *J. Am. Chem. Soc.* **101**, 6129–6131.
Freidinger, R. M., Veber, D. F., Perlow, D. S., Brooks, J. R. and Saperstein, R. (1980). *Science* **210**, 656–658.
Froimowitz, M. and Hruby, V. J. (1989). *Int. J. Pept. Prot. Res.* **34**, 88–96.
Furukawa, Y., Kishimoto, S. and Nishikawa, K. (1982). US-A 4 340 598 (patent).
Gallop, M. A., Barrett, R. W., Dower, W. J., Fodor, S. P. A. and Gordon, E. M. (1994). *J. Med. Chem.* **37**, 1233–1251.

Gante, J. (1989). *Synthesis* 405–413.

Gante, J. (1994). *Angew. Chem.* **106**, 1780–1802; (1994). *Angew. Chem. Int. Ed. Engl.* **33**, 1699–1720.

Garret, C., Carruette, A., Fardin, V., Moussaoui, S., Peyronel, J.-F., Blanchard, J.-C. and Laduron, P. M. (1991). *Proc. Natl Acad. Sci. USA* **88**, 10 208–10 212.

Giannis, A. (1994). *Angew. Chem. Int. Ed. Engl.* **33**, 178–180.

Giannis, A. and Kolter, T. (1993). *Angew. Chem.* **105**, 1303–1326; (1993). *Angew. Chem. Int. Ed. Engl.* **32**, 1244–1267.

Giannis, A. and Sandhoff, K. (1989). *Angew. Chem.* **101**, 220–222; (1989). *Angew. Chem. Int. Ed. Engl.* **28**, 218–220.

Gibbs, J. B., Oliff, A. and Kohl, N. E. (1994). *Cell* **77**, 175–178.

Ginzler, A. R. and Xu, H. (1991). *Proc. Natl Acad. Sci. USA* **88**, 4741–4745.

Glinka, T. W., de Laszlo, S. E., Siegl, K. S., Chang, R. S., Kivlighn, S. D., Schorn, T. S., Faust, K. A., Chen, T.-B., Zingaro, G. J., Lotti, V. J. and Greenlee, W. J. (1994). *Biorg. Med. Chem. Lett.* **4**, 81–86.

Goodman, M. and Chorev, M. (1979). *Acc. Chem. Res.* **12**, 1–7.

Gordon, D. W. and Steele, J. (1995). *Bioorg. Med. Chem. Lett.* **5**, 47–50.

Gordon, E. M., Barrett, R. W., Dower, W. J., Gallop, M. A., Fodor, S. P. A. and Gallop, M. A. (1994). *J. Med. Chem.* **37**, 1385–1401.

Gordon, T., Hansen, P., Morgan, B., Singh, J., Baizman, E. and Ward, S. (1993). *Bioorg. Med. Chem.* **3**, 915–920.

Greenlee, W. J. and Mantlo, N. B. (1994). *Bioorg. Med. Chem. Lett.* **4**, 15–16.

Greenlee, W. J. and Siegl, P. K. S. (1991). *Annu. Rep. Med. Chem.* **26**, 63–72.

Griffith, E. C. (1991). *In* 'A Textbook of Drug Design and Development' (Krogsgaard-Larsen, P. and Bundgaard, H., eds), pp. 486–528. Harwood Academic Publishers, Switzerland.

Guba, W., Haessner, R., Breipohl, G., Henke, S., Knolle, J., Santagada, V. and Kessler, H. (1994). *J. Am. Chem. Soc.* **116**, 7532–7540.

Guidotti, A., Forchetti, C. M., Corda, M. G., Konkel, D., Bennet, C. D. and Costa, E. (1983). *Proc. Natl Acad. Sci. USA* **80**, 3531–3535.

Haber, E. and Lee, M.-U. (1994). *Nature* **370**, 252–253.

Hagihara, M., Anthony, N. J., Stout, T. J., Clardy, J. and Schreiber, S. L. (1992). *J. Am. Chem. Soc.* **114**, 6568–6570.

Hagiwara, D., Miyake, H., Morimoto, H., Murai, M., Takashi, F. and Matsuo, M. (1992). *J. Med. Chem.* **35**, 3184–3191.

Hann, M. M., Sammes, P. G., Kennewell, P. D. and Taylor, J. B. (1980). *J. Chem Soc., Chem. Commun.* 234–235.

Harris, E. D., Jr (1990). *N. Engl. J. Med.* **322**, 1277–1289.

Henzl, M. R., Corson, S. L., Moghissi, K., Buttram, V. C., Berquist, C. and Jacobson, J. (1988). *N. Engl. J. Med.* **318**, 485–489.

Higginbottom, M., Horwell, D. C. and Roberts, E. (1993). *Bioorg. Med. Chem. Lett.* **3**, 881–884.

Hirschmann, R. (1991). *Angew. Chem.* **103**, 1305–1330; (1991). *Angew. Chem. Int. Ed. Engl.* **30**, 1278–1301.

Hirschmann, R., Nicolaou, K. C., Pietranico, S., Salvino, J., Leahy, E. M., Sprengler, P. A., Furst, G., Smith III, A. B., Strader, C. D., Cascieri, M. A., Candelore, M. R., Donaldson, C., Vale, W. and Maechler, L. (1992a). *J. Am. Chem. Soc.* **114**, 9217–9218.

Hirschmann, R., Sprengler, P. A., Kawasaki, T., Leahy, J. W., Shakespeare, W. C. and Smith III, A. B. (1992b). *J. Am. Chem. Soc.* **114**, 9699–9701.

Hoffmann, R. W. (1992). *Angew. Chem.* **104**, 1147–1157; (1992). *Angew. Chem. Int. Ed. Engl.* **31**, 1124–1134.

Holk, M., Bosse, R., Fischli, W., Gerold, H. and Escher, E. (1989). *Biochem. Biophys. Res. Commun.* **160**, 1350–1356.

Holladay, M. W., Lin, C. W., May, C. S., Garvey, D. S., Witte, D. G., Miller, T. R., Wolfram, C. A. W. and Nadzan, A. M. (1991). *J. Med. Chem.* **34**, 455–457.

Holzemann, G. (1991). *Kontakte (Darmstadt)* **1**, 3–12 (part 1).

Holzemann, G. (1991). *Kontakte (Darmstadt)* **2**, 55–63 (part 2).

Horwell, D. C., Beeby, A., Clarc, C. R. and Hughes, J. (1987). *J. Med. Chem.* **30**, 729–732.

Horwell, D. C., Hughes, J., Hunter, J. C., Pritchard, M. C., Richardson, R. S., Roberts, E. and Woodruff, G. N. (1991). *J. Med. Chem.* **34**, 404–414.

Hruby, V. J., Al-Obeidi, F. and Kazmierski, W. (1990). *Biochem. J.* **268**, 249–262.

Huang, R.-R. C., Yu, H., Strader, C. D. and Fong, T. M. (1994). *Biochemistry* **33**, 3007–3013.

Huang, Z., He, Y., Raynor, K., Tallent, M., Reisine, T. and Goodman, M. (1992). *J. Am. Chem. Soc.* **114**, 9390–9401.

Hubbell, J. A. and Langer, R. (1995). *Chem. Eng. News* **73**(11), 42–54.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A. and Morris, H. R. (1975). *Nature* **258**, 577–579.

Hughes, J., Boden, P., Costall, B., Domeney, A., Kelly, E., Horwell, D. C., Hunter, J. C., Pinnock, R. D. and Woodruff, G. N. (1990). *Proc. Natl Acad. Sci. USA* **87**, 6728–6732.

Hunt, J. T., Lee, V. G., Stein, P. D., Hedberg, A., Liu, E., McMullen, D. and Moreland, S. (1991). *Bioorg. Med. Chem. Lett.* **1**, 33–38.

Hutchinson, E. G. and Thornton, J. M. (1994). *Protein Sci.* **3**, 2207–2216.

Hynes, R. O. (1992). *Cell* **69**, 11–25.

Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 2863–2867.

Ishikawa, K., Furami, T., Nagase, T., Fujita, K., Hayama, T., Niiyama, K., Mase, T., Ihara, M. and Yano, M. (1992). *J. Med. Chem.* **35**, 2139–2142.

James, A. F., Xie, L.-H., Fujitani, Y., Hayashi, S. and Horie, M. (1994). *Nature* **307**, 297–300.

James, G. L., Goldstein, J. L., Brown, M. S., Rawson, T. E., Somers, T. C., McDowell, R. S., Crowley, C. W., Lucas, B. K., Levinson, A. D. and Marsters, J. C. (1993). *Science* **260**, 1937–1942.

Jameson, B. A., McDonnel, J. M., Marini, J. C. and Korngold, R. (1994). *Nature* **368**, 744–746.

Janda, K. D. (1994). *Proc. Natl Acad. Sci. USA* **91**, 10 779–10 785.

Janssens, J., Peters, T. J., Vantrappen, G., Tack, J., Urban, J. L., De Roo, M., Muls, E. and Bouillon, R. (1990). *N. Engl. J. Med.* **322**, 1028–1037.

Jones, R. C. F. and Ward, G. J. (1988). *Tetrahedron Lett.* **29**, 3853–3856.

Jorgensen, W. L. (1991). *Science* **254**, 954–955.

Jung, G. and Beck-Sickinger, A. G. (1992). *Angew. Chem.* **104**, 375–500; (1992). *Angew. Chem. Int. Ed. Engl.* **31**, 367–383.

Kahn, M. (1993). *Synlett.* 821–826.

Kahn, M. and Chen, B. (1987). *Tetrahedron Lett.* **28**, 1623–1626.

Kaiser, E. T. and Kezdy, F. J. (1984). *Science* **223**, 249–255.

Kemp, D. S. and McNamara, P. E. (1984). *J. Org. Chem.* **49**, 2286–2288.

Kemp, D. S. and Stites, W. E. (1988). *Tetrahedron Lett.* **29**, 5057–5060.

Kempf, D. J. and Condon, S. L. (1990). *J. Org. Chem.* **55**, 1390–1394.

Kendrick, D. A., Ryder, H., Semple, G. and Szelke, M. (1992). *Bioorg. Med. Chem. Lett.* **2**, 9–12.

Kessler, H. (1970). *Angew. Chem.* **82**, 237–253; (1970). *Angew. Chem. Int. Ed. Eng.* **9**, 219–235.

Kessler, H. (1982). *Angew. Chem.* **94**, 509–520; (1982). *Angew. Chem. Int. Ed. Engl.* **21**, 512–523.

Kiefer, B. L., Befort, K., Gaveriaux, C. and Hirth, C. G. (1992). *Proc. Natl Acad. Sci. USA* **89**, 12 048–12 052.

Kimura, S., Kasuya, Y., Sawamura, T., Shinmi, O., Sugita, Y., Yanagisawa, M., Goto, K. and Masaki, T. (1988). *Biochem. Biophys. Res. Commun.* **156**, 1182–1186.

Kitagawa, O., Velde, V. D., Dutta, D., Morton, M., Takusagawa, F. and Aubé, J. (1995). *J. Am. Chem. Soc.* **117**, 5169–5178.

Kizu, R., Draves, P. H. and Hurley, L. H. (1993). *Biochemistry* **32**, 8712–8722.

Kleinert, H. D., Rosenberg, S. H., Baker, W. R., Stein, H. H., Klinghofer, V., Barlow, J., Spina, K., Polakowski, J., Kovar, P., Cohen, J. and Denissen, J. (1992). *Science* **257**, 1940–1943.

Klutchko, S., Hamby, J. M. and Hodges, J. C. (1994). *Bioorg. Med. Chem. Lett.* **4**, 57–62.

Knapp, R. J., Malatynska, E., Collins, N., Fang, L., Wang, J. Y., Hruby, V., Roeske, W. R. and Yamamura, H. (1995). *FASEB J.* **9**, 516–525.

Kodandapani, R., Veerapandian, B., Kunicki, T. J. and Ely, K. R. (1995). *J. Biol. Chem.* **270**, 2268–2273.

Kolter, T., Klein, A. and Giannis, A. (1992). *Angew. Chem.* **104**, 1394–1395; (1992). *Angew. Chem. Int. Ed. Engl.* **31**, 1391–1392.

Kolter, T., Dahl, C. and Giannis, A. (1995). *Liebigs Ann.* 625–629.

Kondo, Y., Torii, K., Omura, S. and Ito, Z. (1988). *Biochem. Biophys. Res. Commun.* **150**, 877–882.

Kopple, K. D., Bauers, P. W., Bean, J. W., D'Ambrosio, C. A., Hughes, J. L., Peishoff, C. E. and Eggleston, D. S. (1992). *J. Am. Chem. Soc.* **114**, 9615–9623.

Kornecki, E., Ehrlich, Y. H. and Lenox, R. H. (1984). *Science* **226**, 1454–1456.

Krieger, D. T. (1983). *Science* **222**, 975–985.

Ku, T. W, Ali, F. E., Barton, L. S., Bean, J. W., Bondinell, W. E., Burgess, J. L., Callahan, J. F., Calvo, R. R., Chen, L., Eggleston, D. S., Gleason, J. G., Huffman, W. F., Hwang, S. M., Jakas, D. R., Karash, C. B., Keenan, R. M., Kopple, K. D., Miller, W. H., Newlander, K. A., Nichols, A., Parker, M. F., Peishoff, C. E., Samanen, J. M., Uzinskas, I. and Venslavsky, J. W. (1993). *J. Am. Chem. Soc.* **115**, 8861–8862.

Kuhn, J.-M., Billebaud, T., Navratil, H., Moulognuet, A., Fiet, J., Grise, P., Jouis, J.-F., Costa, P., Husson, J.-M., Dahan, R., Bertagna, C. and Edelstein, R. (1989). *N. Engl. J. Med.* **321**, 413–418.

Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W.-H., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, M. and Yazaki, Y. (1994). *Nature* **368**, 703–710.

Kvols, L. K., Moertel, C. G., O'Connell, M. J., Schutt, A. J., Rubin, J. and Hahn, R. G. (1986). *N. Engl. J. Med.* **315**, 663–666.

Lamberts, S. W. J., Uitterlinden, P., Verschoor, L., van Dongen, K. J. and del Pozo, E. (1985). *N. Engl. J. Med.* **313**, 1576–1580.

Lamberts, S. W. J., Bakker, W. H., Reubi, J.-C. and Krenning, E. P. (1990). *N. Engl. J. Med.* **323**, 1246–1249.

Lawton, G., Paciorek, P. M. and Waterfall, J. F. (1992). *In* 'Advances in Drug Research' (Testa, B., ed.), Vol. 23, pp. 162–220, Academic Press, London.

Lefkovits, J., Plow, E. F. and Topol, E. J. (1995). *N. Engl. J. Med.* **332**, 1553–1559.

Lewis, J. W. (1985). *In* 'Medicinal Chemistry – The Role of Organic Chemistry in Drug Research' (Roberts, S. M. and Price, B. J., eds), pp. 119–142. Academic Press, London.

Li, Y.-M., Marnerakis, M., Stimson, E. R. and Maggio, J. E. (1995). *J. Biol. Chem.* **270**, 1213–1220.

Liebow, C., Crean, D. H., Schally, A. V. and Mang, T. S. (1993). *Proc. Natl Acad. Sci.* **90**, 1897–1901.

Liskamp, R. M. J. (1994). *Recl. Trav. Chim. Pays-Bas* **113**, 1–19.

Logan, M. E., Goswami, R., Tomczuk, B. E. and Venepalli, B. R. (1991). *Annu. Rep. Med.*

Chem. **26**, 43–51.
Lotz, M., Carson, D. A. and Vaughan, J. H. (1987). *Science* **235**, 893–895.
Lucinscas, F. W. and Lawler, J. (1994). *FASEB J.* **8**, 929–938.
Lynch, D. R. and Snyder, S. H. (1986). *Annu. Rev. Biochem.* **55**, 773–799.
MacLeod, A. M., Merchant, K. J., Cascieri, M. A., Sadowski, S., Ber, E., Swain, C. J. and Baker, R. (1993). *J. Med. Chem.* **36**, 2044–2045.
Maneckjee, R. and Minna, J. D. (1990). *Proc. Natl Acad. Sci. USA* **87**, 3294–3298.
Maneckjee, R. and Minna, J. D. (1992). *Proc. Natl Acad. Sci. USA* **89**, 1169–1173.
Mantyh, C. R., Gates, T. S., Zimmerman, R. P., Welton, M. L., Passaro, E. P., Jr, Vigna, S. R., Maggio, J. E., Kruger, L. and Mantyh, P. W. (1988). *Proc. Natl Acad. Sci. USA* **85**, 3235–3239.
Marquard, H., Todaro, G. J. and Shoyab, M. (1986). *J. Biol. Chem.* **261**, 9727–9731.
Marshall, G. R. (1992). *Curr. Opin. Struct. Biol.* **2**, 904–919.
Marshall, G. R. (1993). *Tetrahedron* **49**, 3547–3558.
Matsoukas, J. M., Hondrelis, J., Keramida, M., Mavromoustakos, T., Makriyannis, Yamdagni, R., Wu, Q. and Moore, G. J. (1994). *J. Biol. Chem.* **269**, 5303–5312.
Matsumoto, H., Suzuki, N., Onda, H. and Fujino, M. (1989). *Biochem. Biophys. Res. Commun.* **164**, 74–80.
Mavrojannis, M. (1903). *Comptes Rend. Soc. Biol.* **55**, 1092–1094.
McDowel, R. S. and Gadek, T. R. (1992). *J. Am. Chem. Soc.* **114**, 9243–9253.
McDowel, R. S., Blackburn, B. K., Gadek, T. R., McGee, L. R., Rawson, T., Reynolds, M. E., Robarge, K. D., Somers, T. C., Thorsett, E. D., Tischler, M., Webb, R. R. and Venuti, M. C. (1994). *J. Am. Chem. Soc.* **116**, 5077–5083.
McLean, S., Ganong, A. H., Seeger, T. F., Bryce, D. K., Pratt, K. G., Reynolds, L. S., Siok, C. J., Lowe III, J. A. and Heym, J. (1991). *Science* **251**, 437–439.
McMahon, E. G., Palomo, M. A., Moore, W. M., McDonald, J. F. and Stern, M. K. (1991). *Proc. Natl Acad. Sci. USA* **88**, 703–707.
Merchant, K. J., Lewis, R. T. and MacLeod, A. M. (1994). *Tetrahedron Lett.* **35**, 4205–4208.
Merluzzi, V. J., Hargrave, K. D., Labadia, M., Grozinger, K., Skoog, M., Wu, J. C., Shih, C.-K., Eckner, K., Hattox, S., Adams, J., Rosenthal, A. S., Faanes, R., Eckner, R. J., Koup, R. A. and Sullivan, J. L. (1990). *Science* **250**, 1411–1413.
Middlemmis, D. and Watson, S. P. (1994). *Tetrahedron* **50**, 13 049–13 080.
Millan, M. A., Jacobowitz, D. M., Aguilera, G. and Catt, K. J. (1991). *Proc. Natl Acad. Sci. USA* **88**, 11 440–11 444.
Mizrahi, P., Escher, E., D'Orleans-Juste, P. and Regoli, D. (1984). *Eur. J. Pharmacol.* **99**, 193–202.
Momany, F. A. (1976). *J. Am. Chem. Soc.* **98**, 2990–2996.
Montgomery, A. M., Reisfeld, R. A. and Cheresh, D. A. (1994). *Proc. Natl Acad. Sci. USA* **91**, 8856–8860.
Morgan, B. A. and Gainor, J. A. (1989). *Annu. Rep. Med. Chem.* **24**, 243–252.
Morrison, D. B., Swanson, M. L., Swanson, L. J., Sweet, L. J. and Pessin, J. E. (1988). *J. Biol. Chem.* **263**, 7806–7813.
Müller, G., Gurrath, M., Kessler, H. and Timpl, R. (1992). *Angew. Chem.* **104**, 341–343; (1992). *Angew. Chem. Int. Ed. Engl.* **31**, 326–328.
Mussap, C. J., Geraghty, D. P. and Burcher, E. (1993). *J. Neurochem.* **60**, 1987–2009.
Nadzan, A. M. and Kerwin, J. F., Jr (1991). *Annu. Rep. Med. Chem.* **26**, 191–200.
Naef, R. and Seebach, D. (1985). *Helv. Chim. Acta* **68**, 135–143;
Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. J., Cohen, S. N. and Numa, S. (1979). *Nature* **278**, 423–427.
Nestor, J. J., Jr and Vickery, B. H. (1988). *Annu. Rep. Med. Chem.* **23**, 211–220.
Nestor, J. J., Tahilramani, R., Ho, T. L., Goodpasture, J. C., Vickery, B. H. and Ferrandon,

P. (1992). *J. Med. Chem.* **35**, 3942–3948.

Nicolaou, K. C., Salvino, J. M., Raynor, K., Pietranico, S., Reisine, T., Freidinger, R. M. and Hirschmann, R. (1990). *In* 'Peptides: Chemistry, Structure and Biology, Proceedings of the Eleventh American Peptide Symposium' (Rivier, J. E. and Marshall, G. R., eds), pp. 881–884. Escom, Leiden.

Noda, K., Saad, Y., Kinoshita, A., Boyle, T. P. and Graham, R. M. (1995). *J. Biol. Chem.* **270**, 2284–2289.

Ocarrol, A. M., Raynor, K., Lolait, S. J. and Reisine, T. (1994). *Mol. Pharmacol.* **46**, 291–298.

Ohlstein, E. H., Nambi, P., Douglas, S. A., Edwards, R. M., Gellai, M., Lago, A., Leber, Cousins, R. D., Gao, A., Frazee, J. S., Peishoff, C. E., Bean, J. W., Eggleston, D. S., Elshourbagy, Kumar, C., Lee, J. A., Yue, T.-L., Louden, C., Brooks, D. P., Weinstock, J., Feuerstein, G., Poste, G., Ruffolo, R. R., Gleason, J. G. and Elliot, J. D. (1994). *Proc. Natl Acad. Sci. USA* **91**, 8052–8056.

Olson, G. L., Voss, M. E., Hill, D. E., Kahn, M., Madison, V. S. and Cook, C. M. (1990). *J. Am. Chem. Soc.* **112**, 323–333.

Olson, G. L., Bolin, D. R., Bonner, M. P., Bös, M., Cook, C. M., Fry, D. C., Graves, B. J., Hatada, M., Hill, D. E., Kahn, M., Madison, V. S., Rusciecki, V. K., Sarabu, R., Sepinwall, Vincent, G. P. and Voss, M. E. (1993). *J. Med. Chem.* **36**, 3039–3049.

Ono, K., Tsujimoto, G., Sakamoto, A., Eto, K., Masaki, T., Ozaki, Y. and Satake, M. (1994). *Nature* **370**, 301–304.

Oury-Donat, F., Lefevre, I. A., Thurneyssen, O., Gauthier, T., Bordey, A., Feltz, P., Emonds-Alt, X., Le Fur, G. and Soubrie, P. (1994). *J. Neurochem.* **62**, 1399–1407.

Papageorgiou, C., Haltiner, R., Bruns, C. and Petcher, T. J. (1992). *Bioorg. Med. Chem. Lett.* **2**, 135–140.

Pauwels, R., Andries, K., Desmyter, J., Schols, D., Kukla, M., Breslin, H., Raeymaeckers, A., Van Gelder, J., Woestenborghs, R., Heykants, J., Schellekens, K., Janssen, M. A. C., De Clercq, E. and Janssen, P. (1990). *Nature* **343**, 470–474.

Payan, D. G. (1992). *In* 'Inflammation: Basic Principles and Clinical Correlates', Second Edition (Gallin, J. I., Goldstein, I. M. and Snyderman, R., eds), pp. 177–192. Raven Press, New York.

Pert, C. B. and Snyder, S. H. (1973). *Science* **179**, 1011–1014.

Peyronel, J.-F., Truchon, A., Mountonnier, C. and Garret, C. (1992). *Bioorg. Med. Chem. Lett.* **2**, 37–40.

Pfaff, M., Tangemann, K., Müller, B., Gurrath, M., Müller, G., Kessler, H., Timpl, R. and Engel, J. (1994). *J. Biol. Chem.* **269**, 20 233–20 238.

Pincus, M. R., Carty, R. P., Chen, J., Lubowsky, J., Avitable, M., Shah, D., Scheraga, H. A. and Murphy, R. B. (1987). *Proc. Natl Acad. Sci. USA* **84**, 4821–4825.

Pohl, E., Heine, A., Sheldrick, G. M., Dauter, Z., Wilson, K. S., Kallen, J., Huber, W. and Pfäffli, P. J. (1995). *Acta Cryst.* D48–59.

Portoghese, P. S. (1991). *J. Med. Chem.* **34**, 1757–1762.

Portoghese, P. S. (1992). *J. Med. Chem.* **35**, 1927–1937.

Portoghese, P. S., Larson, D. L., Sayre, L. M., Fries, D. S. and Takemori, A. E. (1980). *J. Med. Chem.* **23**, 233–234.

Portoghese, P. S., Lipkowski, A. W. and Takemori, A. E. (1987). *J. Med. Chem.* **30**, 238–239.

Portoghese, P. S., Sultana, M. S. and Takemori, A. E. (1990). *J. Med. Chem.* **33**, 1714–1720.

Pothulakis, C., Costagliuolo, I., LaMont, T., Jaffer, A., O'Keane, J. C., Snider, M. R. and Leeman, S. E. (1994). *Proc. Natl Acad. Sci. USA* **91**, 947–951.

Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., deWitt, D., Yanagisawa, M. (1994). *Cell* **79**, 1257–1266.

Reichlin, S. (1983a). *N. Engl. J. Med.* **309**, 1495–1501.
Reichlin, S. (1983b). *N. Engl. J. Med.* **309**, 1556–1563.
Rens-Domiano, S. and Reisine, T. (1992). *J. Neurochem.* **58**, 1987–1996.
Richardson, J. S. and Richardson, D. C. (1989). *In* 'Prediction of Protein Structure and the Principles of Protein Conformation' (Fassman, G. D., ed.), pp. 1–116. Plenum Press, New York and London.
Rizo, J. and Gierasch, L. M. (1992). *Annu. Rev. Biochem.* **61**, 387–418.
Robl, J. A., Cimarusti, M. P., Simpkins, L. M., Weller, H. N., Pan, Y. Y., Malley, M. and DiMarco, J. D. (1994). *J. Am. Chem. Soc.* **116**, 2348–2355.
Rodham, D. A., Suzuki, S., Suenram, R. D., Lovas, F. J., Dasgupta, S., Goddart, W. A. and Blakes, G. A. (1993). *Nature* **362**, 735–737.
Rodriguez, M., Lignon, M.-L., Galas, M.-C., Fulcrand, P., Mendre, C., Aumelas, A., Laur, J. and Martinez, J. (1987). *J. Med. Chem.* **30**, 1366–1373.
Romer, D., Buscher, H. H., Hill, R. C., Maurer, R., Petcher, T. J., Zeugner, H., Benson, W., Finner, E., Milkowski, W. and Thies, P. W. (1982). *Nature* **298**, 759–760.
Rose, G. D., Gierasch, L. M. and Smith, J. A. (1985). *Adv. Protein Chem.* **37**, 1–109.
Rosenblatt, M. (1986). *N. Engl. J. Med.* **315**, 1004–1013.
Rothstein, J. D., Garland, W., Puia, G., Guidotti, A., Weber, R. and Costa, E. (1992). *J. Neurochem.* **58**, 2102–2115.
Rubanyi, G. M. and Polokoff, M. A. (1994). *Pharmacol. Rev.* **46**, 325–415.
Ruoslati, E. and Pierschbacher, M. D. (1987). *Science* **238**, 491–497.
Ruoslati, E., Pierschbacher, M. D. and Border, W. A. (1994). *In* 'The Liver: Biology, and Pathobiology', 3rd edn (Arias, I. M., Boyer, J. L., Fausto, N., Jacoby, W. B., Schachter, D. A. and Shafritz, eds), pp. 889–906. Raven Press, New York.
Saeki, T., Ihara, M., Fukuroda, T., Yamagawa, M. and Yano, M. (1991). *Biochem. Biophys. Res. Commun.* **179**, 286–292.
Sammes, P. G. (1975). *In* 'Progress in the Chemistry of Natural Organic Compounds' (Herz, W., Griesebach, H. and Kirby, G. W., eds) **32**, 51–118, Springer, Vienna.
Sandberg, K., Ji, H., Clark, A. J. L., Shapira, H. and Catt, K. J. (1992). *J. Biol. Chem.* **267**, 9455–9458.
Sangameswaran, L., Fales, H. M., Friedrich, P. and De Blas, A. L. (1986). *Proc. Natl Acad. Sci. USA* **83**, 9236–9240.
Saragovi, H. U., Fitzpatrick, D., Raktabutr, A., Nakanishi, H., Kahn, M. and Greene, M. I. (1991). *Science* **253**, 792–795.
Sasaki, Y., Murphy, W. A., Heiman, M. L., Lance, V. A. and Coy, D. H. (1987). *J. Med. Chem.* **30**, 1162–1166.
Savarese, T. M. and Fraser, C. M. (1992). *Biochem. J.* **283**, 1–19.
Schambye, H. T., Hjorth, S. A., Bergsma, D. J., Sathe, G. and Schwartz, T. W. (1994). *Proc. Natl Acad. Sci. USA* **91**, 7046–7050.
Schmidt, G. (1986). *Top. Curr. Chem.* **136**, 109–159.
Schöllkopf, U. (1983). *Curr. Top. Chem.* **109**, 65–84.
Schulz, G. E. and Schirmer, R. H. (1979). *In* 'Principles of Protein Structure' (Cantor, C. R., ed.), pp. 17–26. Springer, New York.
Schwarz, J. H. (1991). *In* 'Principles of Neural Science' (Kandel, E. R., Schwarz, J. H. and Jessel, T. M., eds), pp. 213–224. Elsevier, New York.
Schwyzer, R. (1977). *Naturwissenschaften* **69**, 15–20.
Seebach, D., Aebi, J. D., Naef, R., Weber, T. (1985). *Helv. Chim. Acta* **68**, 144–154.
Sharma, R. K., Smith, J. R. and Moore, G. J. (1991). *Biochem. Biophys. Res. Commun.* **179**, 85–89.
Silvene-Poirot, S., Dufresne, M., Vayasse, N. and Fourmy, D. (1993). *Eur. J. Biochem.* **215**, 513–529.
Simon, E. J. and Hiller, J. M. (1989). *In* 'Basic Neurochemistry (Siegel, G. J., Agranoff, B.

W., Albers, R. W. and Molinoff, P. B., eds), pp. 271–285. Elsevier, Amsterdam.
Simonson, M. S. and Dunn, M. J. (1990). *FASEB J.* **4**, 2989–3000.
Smith, E. M., Morrill, A. C., Meyer III, W. J. and Blalock, J. E. (1986). *Nature* **321**, 881–882.
Snider, R. M., Constantine, J. W., Lowe III, J. A., Longo, K. P., Lebel, W. S., Woody, H. A., Drozda, S. E., Desai, M. C., Vinick, F. J., Spencer, R. W. and Hess, H.-J. (1991). *Science* **251**, 435–437.
Soudah, H. C., Hasler, W. L. and Owyang, C. (1991). *N. Engl. J. Med.* **325**, 1461–1467.
Spatola, A. F. (1983). *In* 'Chemistry and Biochemistry of Amino Acids, Peptides and Proteins' (Weinstein, B., ed.), pp. 267–357. New York, Dekker.
Spear, K. L., Brown, M. S., Reinhard, E. J., McMahon, E. G., Olins, G. M., Palomo, M. A. and Patton, D. R. (1990). *J. Med. Chem.* **33**, 1935–1940.
Spengard, U., Kretschmer, G., Bartnick, E., Hüls, C. and Kunz, H. (1995). *Angew. Chem.* **107**, 1104–1107; *Angew. Chem. Int. Ed. Engl.* **34**, 990–993.
Spinella, M. J., Malik, A. B., Everitt, J. and Andersen, T. T. (1991). *Proc. Natl Acad. Sci. USA* **88**, 7443–7446.
Springer, T. (1994). *Cell* **76**, 301–314.
Stammer, C. H. (1990). *Tetrahedron* **46**, 2231–2254.
Stein, C., Hassan, A. H. S., Przewlocki, R., Gramsch, C., Peter, K. and Herz, A. (1990). *Proc. Natl Acad. Sci. USA* **87**, 5935–5939.
Stein, C., Comisel, K., Haimerl, E., Yassouridis, A., Lehrberger, K., Herz, A. and Peter, K. (1991). *N. Engl. J. Med.* **325**, 1123–1126.
Stephenson, F. A. (1987). *Trends Neurosci.* **10**, 185–188.
Stewart, W. E. and Siddall, T. H. (1970). *Chem. Rev.* **70**, 517–551.
Strader, C. D., Fong, T. M., Tota, M. R. and Underwood, D. (1994). *Annu. Rev. Biochem.* **63**, 101–132.
Sumners, C., Tang, W., Zelezna, B. and Raizada, M. K. (1991). *Proc. Natl Acad. Sci. USA* **88**, 7567–7571.
Szmuszkovicz, J. and von Voigtlander, P. F. (1982). *J. Med. Chem.* **25**, 1125–1126.
Tachibama, S., Araki, K., Ohya, S. and Yoshida, S. (1982). *Nature* **295**, 339–340.
Takayanagi, R., Kitazumi, K., Tadasaki, C., Ohnaka, K., Aimoto, S., Tasaka, K., Ohashi, M. and Nawata, H. (1991). *FEBS Lett.* **282**, 103–106.
Toniolo, C. (1990). *Int. J. Peptide Protein Res.* **35**, 287–300.
Toth, G., Russel, K. C., Landis, G., Kramer, T. H., Fang, L., Knapp, R., Davis, P., Burks, T. F., Yamamura, H. I. and Hruby, V. J. (1992). *J. Med. Chem.* **35**, 2384–2391.
Tse, A. and Hille, B. (1992). *Science* **255**, 462–464.
Uhl, G. R., Childers, S. and Pasternak, G. (1994). *Trends Neurosci.* **17**, 89–93.
Valle, G., Crisma, M., Bonora, G., Toniolo, C., Lelj, F., Barone, V., Fraternali, F., Hardy, P. M., Langran-Goldsmith, A. and Maia, H. L. S. (1990). *J. Chem. Soc. Perkin Trans.* **2**, 1481–1487.
van Hagen, P. M., Krenning, E. P., Kwekkeboom, D. J., Reubi, J. C., Anker-Lugtenburg, vd P. J., Löwenberg, B. and Lamberts, S. W. J. (1994). *Eur. J. Clin. Invest.* **24**, 91–99.
Vanhoutte, P. M. (1994). *Nature* **368**, 693–694.
van Nispen, J. and Pinder, R. (1986). *Annu. Rep. Med. Chem.* **21**, 51–62.
Veber, D., Saperstein, R., Nutt, R., Freidinger, R., Brady, S., Curley, P., Perlow, D., Palveda, W., Colton, C., Zacchei, A., Tocco, D., Hoff, D., Vandlen, R., Gerich, J., Hall, L., Mandarino, L., Cordes, E., Anderson, P. and Hirschmann, R. (1984). *Life Sci.* **34**, 1371–1378.
Veber, D. F. (1992). *In* 'Peptides – Chemistry and Biology, Proceedings of the Twelfth American Peptide Symposium', (Smith, J. A. and Rivier, J. E., eds), pp. 3–14. Escom, Leiden.
Veber, D. F. and Freidinger, R. M. (1985). *Trends Neurosci.* **8**, 392–396.
Veber, D. F., Freidinger, R. M., Perlow, D. S., Paleveda, W. I., Jr, Holly, F. W., Strachan,

R. G., Nutt, R. F., Arrison, B. H., Homnick, C., Randall, W. C., Glitzer, M. S., Saperstein, R. and Hirschmann, R. (1981). *Nature* **292**, 55–58.

Venepalli, B. R., Aimone, L. D., Appel, K. C., Bell, M. R., Dority, J. A., Goswami, R., Hall, P. L., Kumar, V., Lawrence, K. B., Logan, M. E., Scensny, P. M., Seelye, J. A., Tomczuk, B. E. and Yanni, J. M. (1992). *J. Med. Chem.* **35**, 374–378.

Verdini, A. S., Silvestri, S., Becherini, C., Longobardi, M. G., Parente, L., Pepoloni, S., Perretti, M., Pileri, P., Pinori, M., Visconi, G. C. and Nencioni, L. (1991). *J. Med. Chem.* **34**, 3372–3379.

Virgilio, A. A. and Ellman, J. A. (1994). *J. Am. Chem. Soc.* **116**, 11 580–11 581.

Virsolvy-Vergine, A., Leray, H., Kuroki, S., Lupo, B., Dufour, M. and Bataille, D. (1992). *Proc. Natl Acad. Sci. USA* **89**, 6629–6633.

Viswanathan, M., Tsutsumi, K., Correa, F. M. A. and Saavedra, J. M. (1991). *Biochem. Biophys. Res. Commun.* **179**, 1361–1367.

von Euler, U. S. and Gaddum, J. H. (1931). *J. Physiol.* **72**, 74–87.

Ward, P., Ewan, G. B., Jordan, C. C., Ireland, S. J., Hagan, R. M. and Brown, J. R. (1990). *J. Med. Chem.* **33**, 1848–1851.

Weinstock, J., Keenan, R. M., Samanen, J., Hempel, J., Finkelstein, J. A., Franz, R. G., Gaitanopoulos, D. E., Girard, G. R., Gleason, J. G., Hill, D. T., Morgan, T. M., Peishoff, C. E., Aiyar, N., Brooks, D. P., Fredrickson, T. A., Ohlstein, E. H., Ruffolo, R. R., Jr, Stack, E. J., Sulpizio, A. C., Weidley, E. F. and Edwards, R. M. (1991). *J. Med. Chem.* **34**, 1514–1517.

Weitz, C. J., Lowney, L. I., Faull, K. F., Feistner, G. and Goldstein, A. (1987a). *Proc. Natl Acad. Sci. USA* **83**, 5335–5338.

Weitz, C. J., Faull, K. F. and Goldstein, A. (1987b). *Nature* **330**, 674–677.

Weller, T., Alig, L., Hürzeler-Müller, M., Kouns, W. C. and Steiner, B. (1994). *Drugs Fut.* **19**, 461–476.

Wiertelak, E. P., Maier, S. F. and Watkins, L. R. (1992). *Science* **256**, 830–833.

Wiesenfeld-Hallin, Z., Xu, X.-J., Hughes, J., Horwell, D. C. and Hockelt, T. (1990). *Proc. Natl Acad. Sci. USA* **87**, 7105–7109.

Wilden, P. A., Treadway, J. L., Morrison, D. B. and Pessin, J. E. (1989). *Biochemistry* **28**, 9734–9740.

Wiley, R. A. and Rich, D. H. (1993). *Med. Res. Rev.* **13**, 327–384.

Williams, M. (1991). *Med. Res. Rev.* **11**, 147–184.

Williams, M. and Nadzan, A. M. (1991). *In* 'A Textbook of Drug Design and Development' (Krogsgaard-Larsen, P. and Bundgaard, H., eds), pp. 1–25. Harwood Academic Publishers, Chur.

Wipf, P. and Fritsch, P. C. (1994). *J. Org. Chem.* **59**, 4875–4886.

Wolf, J.-P. and Rapoport, H. (1989). *J. Org. Chem.* **54**, 3161–3173.

Wollemann, M. (1990). *J. Neurochem.* **54**, 1095–1101.

Wüthrich, K. (1995). *Acta Cryst.* **D51**, 249–270.

Wüthrich, K., von Freiberg, B., Weber, C., Wider, G., Traber, R., Widmer, H. and Braun, W. (1991). *Science* **254**, 953–954.

Yanagisawa, H., Ishihara, S., Ando, A., Kanazaki, T., Miyamoto, S., Koike, H., Iijima, Y., Oizumi, K., Matsushita, Y. and Hata, T. (1987). *J. Med. Chem.* **30**, 1984–1991.

Yanagisawa, M., Kurihara, H., Kumura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988). *Nature* **332**, 411–415.

Yasuda, K., Raynor, K., Kong, H., Breder, C. D., Takeda, J., Reisine, T. and Bell, G. I. (1993). *Proc. Natl Acad. Sci. USA* **90**, 6736–6740.

Yokota, Y., Akazawa, C., Ohkubo, H. and Nakanishi, S. (1992). *EMBO J.* **11**, 3586–3591.

Yong, Y. F. and Lipton, M. A. (1993). *Bioorg. Med. Chem. Lett.* **3**, 2879–2882.

Yu, M. J., Thrasher, K. J., McCowan, J. R., Mason, N. R. and Mendelson, L. G. (1991). *J. Med. Chem.* **34**, 1508–1511.

Zachary, I., Gil, J., Lehmann, W., Sinnet-Smith, J. and Rozengurt, E. (1991). *Proc. Natl Acad. Sci. USA* **88**, 4577–4581.

Zagon, I. S. and McLaughlin, P. J. (1983). *Science* **221**, 671–673.

Zhorov, B. S. and Ananthanarayanan, V. S. (1995). *FEBS Lett.* **354**, 131–134.

Zimmerman, D. M. and Leander, J. D. (1990). *J. Med. Chem.* **33**, 895–902.

Zurawski, G., Benedik, M., Kamb, B. J., Abrams, J. S., Zurawski, S. M. and Lee, F. D. (1986). *Science* **232**, 772–775.

Medicinal Photochemistry: Phototoxic and Phototherapeutic Aspects of Drugs

GERARD M. J. BEIJERSBERGEN VAN HENEGOUWEN

Department of Medicinal Photochemistry, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, NL–2300 RA Leiden, The Netherlands

ADVANCES IN DRUG RESEARCH, VOL. 29
ISBN 0-12-013329-6

ABBREVIATIONS

A	acceptor
ALA	δ-aminolevulinic acid
BAL	bronchoalveolar lavage
BCA	bacteriochlorin *a*
BPD	benzoporphyrin derivative mono acid ring A
C	β-carotene
CAP	chloramphenicol
CARB	carbadox
CDZ	chlordiazepoxide
CHS	contact hypersensitivity
Co	coumarin
CPZ	chlorpromazine
CPZSO	chlorpromazinesulfoxide
CTCL	cutaneous T cell lymphoma
CYAD	cyadox
D	donor
desoxyOLAQ-4-M	desoxyolaquindox-4-monoxide
DLE	dialysable leukocyte extract
DNFB	2,4-dinitrofluorobenzene
EE_2	ethinylestradiol
GSH	glutathione
HMF	5-hydroxymethylene-2(5H)-furanone
HpD	haematoporphyrin derivative
IC	internal conversion
ISC	intersystem crossing
MB	methylene blue
MetHb	methaemoglobine
5-MOP	5-methoxypsoralen
8-MOP	8-methoxypsoralen
MRZ	mesoridazine
NE	norethisterone
NFA	nitrofurfural
NFT	nitrofurantoin
NIF	nifedipine
NONIF	nitrosoderivative of NIF
2-OH-PZ	2-hydroxypromazine
OLAQ	olaquindox
P	protoporphyrin
pABA	*p*-aminobenzoic acid
PBS	phosphate buffered saline
PDT	photodynamic therapy
pHABA	*p*-hydroxylaminobenzoic acid

pNB	*p*-nitrobenzaldehyde
pNBA	*p*-nitrobenzoic acid
pNBOH	*p*-nitrobenzylalcohol
pNOBA	*p*-nitrosobenzoic acid
pNOCAP	*p*-nitrosochloramphenicol
PpIX	protoporphyrin IX
PTR	protryptiline
PUVA	psoralen + ultraviolet A radiation
PZ	promazine
QUIN	quindoxin
R.CDZ	reduced CDZ
RG 777	1,4,6,8-tetramethyl-2H-furo[2,3-h]-quinolinone
S	singlet
SRZ	sulforidazine
T	triplet
TF	transfer factor
Th	T helper cells
TMA	4,6,4′-trimethylangelicin
TMP	4,8,5′-trimethylpsoralen
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNCB	2,4,6-trinitrochlorobenzene
TRZ	thioridazine
tum+	tumorigenic clones
tum−	non-tumorigenic clones
UVA	ultraviolet A radiation

1 Introduction

1.1 GENERAL REMARKS

Evolution of life is the consequence of continual responses to an ever-changing environment. In the evolution only those organisms survive which can respond adequately. Every living being is forced to respond to the pressure of its environment and, as a result of this, it is shaped by these responses. Thus, responses form an integrated part of an organism, or in other words each animal and plant is a reflection of its environment. A delicate balance exists between the organisms and their environment. Interfering in this balance poses new problems leading to new responses; the latter may be damaging or profitable for the functioning of an organism.

Light is an ubiquitous element of our environment with a tremendous impact on life. Although human photobiology is not yet studied very extensively some responses to light are known which indicate a subtle balance

between the human body and light (Davis, 1985; Holick, 1985; McDonagh, 1985; Morison, 1985; Czeisler *et al.*, 1989).

The interest in human photobiology is currently growing for a number of reasons. First, sunlight is beneficial to health but overexposure as a result of increased outdoor activities can cause skin cancer (Urbach, 1982) and accelerated aging of this vital organ (Kligman and Kligman, 1986). Further, artificial light sources, such as fluorescent tubes and the new and increasingly popular high-intensity lamps are now used world-wide for lighting of large public shopping areas, factory-buildings, offices and private houses. As a large part of the population spends much of its time in these artificially illuminated places, in which light intensity and spectral distribution differ considerably from those found outdoors, there is concern about the consequences for human health. Thus, it is not only because of lamps used for sun-bathing that the role of artificial light in human health and disease has begun to receive serious attention (Abramov, 1985). Second, spectral distribution and intensity of daylight will change if the ozone layer in the stratosphere, which filters out much of the short wavelength UV radiation (McElroy and Salwitch, 1989), is detoriorated by gases from, e.g., spray cans and supersonic aircraft. A change in the spectral distribution and intensity of daylight is expected to have serious consequences for human health. Third, drugs can also interfere in the balance between light and the human body. The effects may be beneficial and thus the combination of light and drug can be used as a therapeutic. Often however, the effects are unintended and uncontrolled and damage of organ functions occurs.

Phototoxicity, including photoallergy, as an unintended side-effect of the exposure of the body to (sun)light during drug therapy, will be dealt with in Part 2 of this chapter. In Part 3, attention is paid to therapeutic applications of the combination light plus drug (phototherapeutics).

Sunprotectors can prevent the acute and chronic effects of sunlight and enable people, who must work outside, to do their job all year round and others to have pleasure in outdoor activities. Sunprotectors, especially compounds which quench excited states or scavenge reactive intermediates such as free radicals formed on exposure to light, are currently the subject of extensive research. Although they are outside the scope of the series, some attention will be paid to them because they can be of help in diminishing phototoxic effects of drugs, including those of phototherapeutics. Some examples will be given in Part 3.

Rather than trying to be complete, emphasis will be placed on those drugs of which something is known about the molecular processes they undergo *in vivo* after absorption of light. Knowledge of these processes is important for identifying that part of the molecular structure that causes the (un)wanted photobiological effect. This makes it possible to alter the structure in such a way that the phototoxicity diminishes whereas the desired properties of the drug remain conserved. (Self-evidently with phototherapeutics too, struc-

ture–photoreactivity relationships are important for the optimization of the desired photobiological activity with respect to the side-effects.)

Photobiological effects of drugs often affect the light-exposed area of the body, namely the skin and eyes. However, normal photobiological processes in man, such as the light-induced vitamin D_3 production or the conversion of bilirubin in, e.g., premature babies, with visible light indicate that systemic effects can also occur as a result of the simultaneous exposure to (sun)light and a drug. In Parts 2 and 3 some examples will be given which support this last supposition.

Before so doing, however, some photochemical and photobiological facts will be dealt with on which Parts 2 and 3 are based. This will be done in a simplified and descriptive manner; for more detailed information see Anderson and Parrish (1982), Grossweiner (1989), Grossweiner and Smith (1989), Hawk and Parrish (1982) and Spikes (1989).

1.2 SOME PHOTOCHEMISTRY AND PHOTOBIOLOGY

1.2.1 Difference Between Thermochemistry and Photochemistry

Although the importance of photochemical reactions to mankind is tremendous, most people are familiar only with thermochemical reactions (reactions without light). They even refer to the latter as the normal reactions. This incorrect situation results from the fact that a standard course in chemistry only deals with thermochemical reactions.

Thermochemical reactions mostly proceed with molecules in their ground state. With regard to organic molecules, ground state normally means that each orbital is filled by one pair of two electrons and that the occupied orbitals are as close as possible to the nuclei; the molecule is in its lowest-energy electronic state. (The negatively charged electrons are as close as possible to the positively charged nuclei.)

Photochemistry is concerned predominantly with the processes molecules undergo after having absorbed ultraviolet radiation (200–380 nm) or visible light (380–800 nm). A photochemical reaction takes place with a molecule in an excited state, which is reached after absorption of a photon of UV or visible light. With this energy absorption by the molecule in its ground state, one of the two outer electrons moves to an orbital further away from the nuclei. (In general, radiation of longer wavelengths than 800 nm, such as infrared, is ineffective in causing a transition of an electron to another orbital. Radiation of shorter wavelengths than 200 nm, such as X- and γ-radiation, generally ionizes molecules; an outer electron is completely separated from the molecule.)

The important consequence of the fact that one of the electrons has moved to another orbital, is that the electron (or charge) distribution has become

FIG. 1. Evidently, excited and ground state have something in common: the skeleton.

quite different from that of the original ground-state molecule. In turn, this new charge distribution leads to a change in vibration and rotation of groups of atoms in the molecule. Because chemical reactivity is determined by the charge distribution and by the vibrations and rotations in a molecule, it is comprehensible that the reactivity of a given molecule in its ground state is quite different from that in its excited state. The excited molecule and its ground-state counterpart resemble each other, at the most, as far as their nuclear skeleton is concerned (Fig. 1). Thus, it is a common misconception that light would be a triggering force or accelerator of thermochemical reactions.

Although the same kind of reactions are found in photochemistry as in thermochemistry, the reactivity of a molecule in its ground state and in its excited state mostly differ from each other, not only quantitatively but also qualitatively. Some examples serve to illustrate the point:

(1) From resonance structures, A′ and D′, it is expected that if thermochemical (Δ) hydrolysis takes place the methoxy group at C_1 in A, and that at C_2 in D, are the ones that will be replaced by nucleophilic substitution (Fig. 2). This is indeed the case with A, where heating (Δ) at 80°C gives C, but not with D; even at 125°C product F is not formed. Obviously, in compound D, the distance between C_2 and C_6 is too large to make the $-OCH_3$ at C_2 susceptible to nucleophilic substitution as a result of the electron withdrawing activity of the $-NO_2$ at C_6.

In contrast, photohydrolysis ($h\nu$) takes place at room temperature in both cases. However, with A as well as with D, it is not the OCH_3 group *para* with respect to the $-NO_2$ which is substituted but the *meta*-

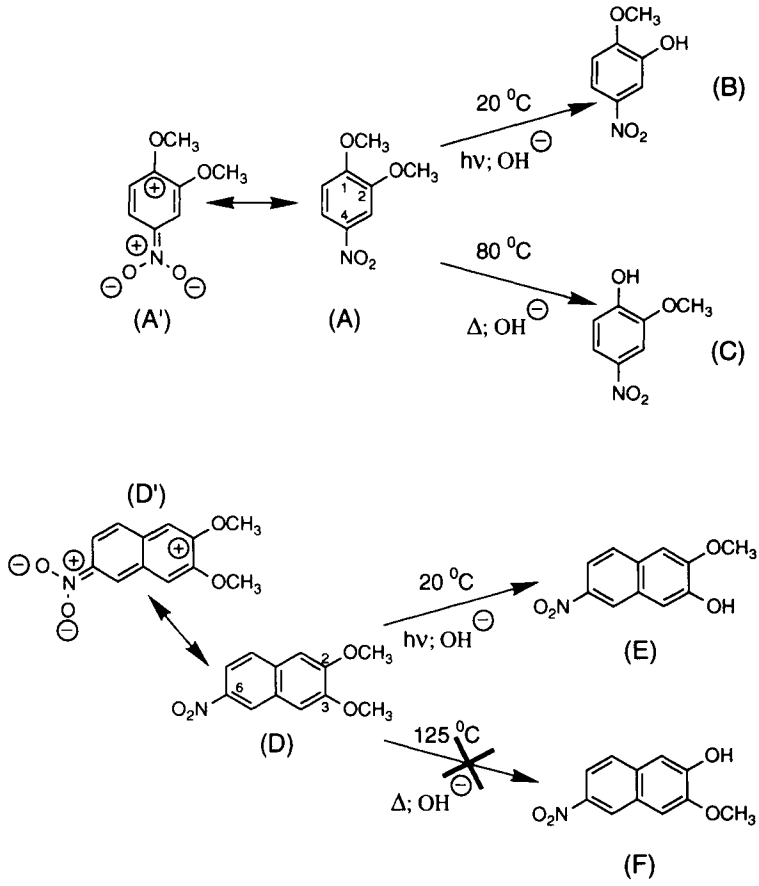


FIG. 2. Alkaline hydrolysis of 4-nitroveratrole (A) and 2,3-dimethoxy-6-nitronaphthalene (D) with (hν) and without (Δ) exposure to light.

oriented methoxyl; at C_2 in A and at C_3 in D, respectively (Havinga and Kronenberg, 1968; Beijersbergen van Henegouwen and Havinga, 1970).

Calculation of the charge distribution revealed that in the excited state involved the C-atoms *meta* with respect to the $-NO_2$ (C_2 in A; C_3 in D) are more positive than the *para* C-atoms (C_1 in A; C_2 in D). Unlike in the ground state of molecules A and D, the $-NO_2$ group proves to exert a *meta*-directing influence in the excited state.

(2) Thermochemically (Δ) the antibiotic chloramphenicol is very stable in aqueous solution; e.g., at 25°C and pH 7 it has a half-life of more than 2 years (Pandit, 1979). The major cause of chloramphenicol degradation

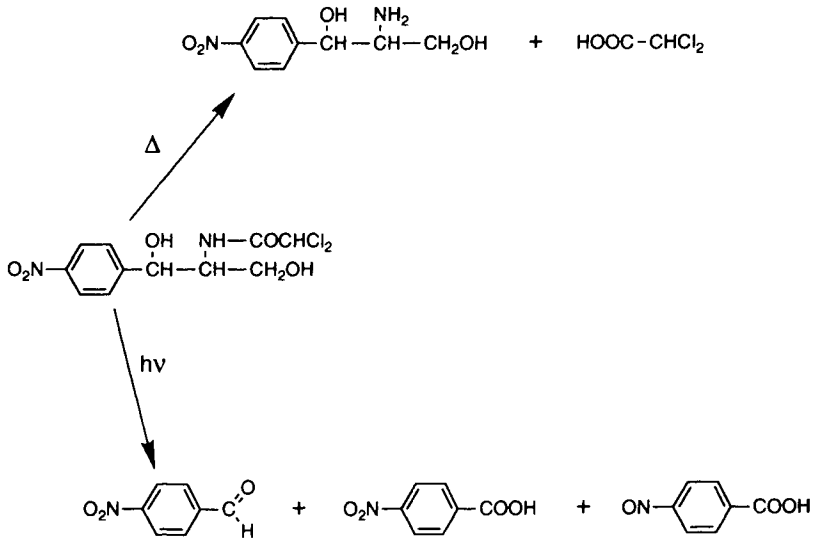


FIG. 3. Thermochemical (Δ) hydrolysis and photohomolysis (hν) of chloramphenicol in aqueous medium.

under these conditions can be attributed to the hydrolytic cleavage of the amide bond (Fig. 3).

In contrast, homolysis occurs photochemically (hν) at the carbon atom adjacent to the aromatic ring. During exposure to sunlight of moderate intensity ($I = 14\ W\ m^{-2}$ at 360 nm) for 45 min at room temperature, more than 80% of initial chloramphenicol ($10\ mg\ l^{-1}$ in 0.05 M phosphate, pH 7.0) proved to be decomposed. In addition to 20% unconverted material, 25% *p*-nitrobenzaldehyde, 15% *p*-nitrobenzoic acid and 36% *p*-nitrosobenzoic acid were found (De Vries *et al.*, 1984). Formation of these reactive photoproducts can have toxicological implications (see also section 2.2.5.3.), if only because the concentration in the aqueous humour of the eye after topical and systemic application of the drug is approximately 5–15 $mg\ l^{-1}$ and 5–30 $mg\ l^{-1}$ respectively (Bartlett, 1982) for some hours.

(3) The tranquillizer chlordiazepoxide ($1\ mg\ ml^{-1}$; pH 7) decomposes very slowly into demoxepam upon standing in the dark at room temperature ($t_{0.5} = 2$ h at 80°C; Dobrinska, 1979). Demoxepam is one of the main metabolites of chlordiazepoxide in man.

When the same solution is exposed to UV radiation ($\lambda_{max} = 350$ nm; $I = 20\ W\ m^{-2}$, intensity comparable to that on a sunny May day in Holland) the half-life is only 40 s. However, in contrast to the thermochemical (Δ) reaction where hydrolysis occurs, chlordiazepoxide isomerizes into an oxaziridine (Fig. 4). This photo-isomerization (hν) into

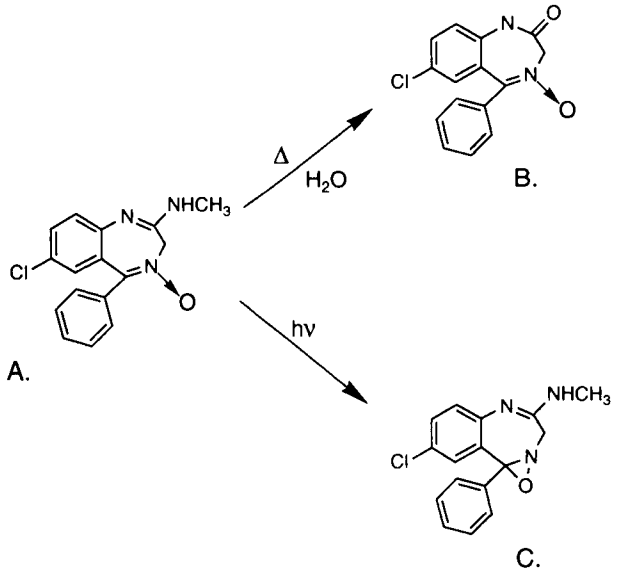


FIG. 4. Thermochemical (Δ) hydrolysis of chlordiazepoxide (A) into demoxepam (B) and photoisomerization (hν) into its oxaziridine (C).

a very reactive oxaziridine has been shown to be responsible for the phototoxic and photoallergic properties of chlordiazepoxide and other imino-*N*-oxides (see also section 2.2.6.).

Because a photochemical reaction only takes place with molecules in an excited state, it is important to pay attention to the following questions:

(1) What kinds of excited states exist?
(2) How are excited states formed and what processes can they undergo apart from chemical reactions?

1.2.2 What Kinds of Excited States Exist?

That there are singlet and triplet states has to do with two facts, namely that an electron turns around its own axis and that it has an electric charge on its 'surface'. Put together, this gives the electron an intrinsic magnetic field, identified with electron 'spin', of which the direction is found by the so-called cork-screw rule.

If there is only one electron, the 'spin' can be oriented in two directions relative to an external magnetic field H (Fig. 5a), 'spin up' (↑) and 'spin down' (↓). The energy difference between these two positions is determined by the strength of the external magnetic field H (the stronger the field the

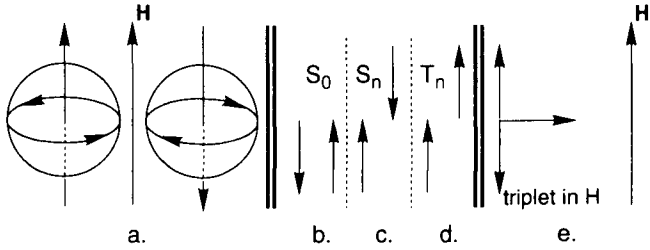


FIG. 5. a. The possible positions of an electron spin in an external magnetic field (H); b, c and d. the positions of the outer two electron spins in a normal organic molecule in its ground state (S_0), in an excited singlet (S_n) and in an excited triplet state (T_n); e. a triplet state split up by an external magnetic field H into three different states (parallel, opposite and perpendicular to H).

larger the energy difference). 'Spin up' (↑) has the lowest energy and 'spin down' (↓) the highest, because the orientation of the latter is opposite to H (Fig. 5a).

In the ground state, most organic molecules have each of their orbitals occupied by one pair of two electrons. Because, according to the Pauli exclusion principle, two electrons in the same orbital cannot be in an equal position, they have their spins opposite.

Such paired electrons with opposite spins are magnetically coupled; they neutralize each other's magnetism and, as a consequence of this, together they do not exert a magnetic field. Conversely, this means that an external magnetic field does not have any influence on either of these spins and thus, although they have opposite orientations, there is no energy difference between them. There remains only one single energy state when an external field (H) is applied on this electron pair; hence the name singlet state. (Fig. 5b; the subscript 0 indicates a ground-state molecule.)

The situation described also holds for most organic molecules in their ground state where, according to the Pauli exclusion principle, each orbital is filled with two electrons with their spin in opposite direction. Thus, in most ground-state molecules, there is one energy state only, called singlet state also.

In an excited state, with one electron promoted to an unoccupied orbital further away from the nuclei, there exist two possibilities:

(1) The excited electron did not change spin direction (Fig. 5c) and thus the total magnetic field of the molecule is still zero. Consequently, this kind of excited state is called singlet state.
(2) The excited electron did change its spin direction, resulting in a net magnetic field (Fig. 5d). From quantum mechanics it follows that this magnetic state can take up three positions in an external magnetic field (Fig. 5e): parallel, opposite and perpendicular to H. Because this entails

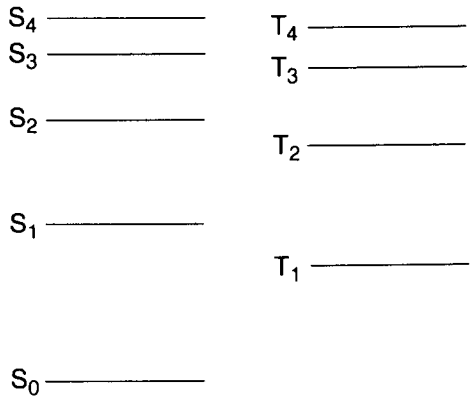


FIG. 6. Some electronic energy states of a normal organic molecule represented in a Jablonski diagram. Subscript 0 indicates the ground state, while the higher subscripts point to singlet (S) and triplet (T) excited states.

three different energy states one speaks of a triplet state. Normally, there is not an external magnetic field present and the state has only one energy content; still the name triplet state is in use for distinction from singlet state.

Both singlet and triplet states are distinguished from each other by their subscript n. This can have the values 0, 1, 2, 3, 4 etc. Zero indicates the ground state while the higher values point to one of the excited states. The higher the n value, the larger the distance between the nuclei and the orbital occupied by the promoted electron and the higher the energy content of the excited state (Fig. 6).

In Fig. 6 the usual situation is represented, namely that the ground state is a singlet (S_0). Because of its important role in photochemistry and photobiology, oxygen should be mentioned as an exception to this rule; its ground state is a triplet, T_0.

As can be seen in Fig. 6 the energy of each excited S_n is somewhat higher than that of its corresponding T_n. The reason is that in an excited S_n the promoted electron is still magnetically coupled to the one which stayed behind (Fig. 5c). This results in a smaller distance but also in a higher repulsive force between the two electrons because of their negative electric charges, and thus in a higher energy content. In an excited triplet T_n, there is no longer any magnetic attraction between the promoted electron and the one which stayed behind (Fig. 5d). The two electrons repel each other magnetically. As a consequence of this, the strong repulsive force between the two electric charges is lower than in the corresponding singlet state (S_n), where they still form a magnetic pair. As a result of this, the T_n state has a somewhat lower energy than the corresponding S_n state.

1.2.3 How are Excited States Formed and What Physical Deactivation Processes can they Undergo Apart from Reaction?

1.2.3.1 In the Absence of Surrounding Molecules. Absorption of energy, UV-radiation or visible light, by a molecule only occurs in discrete quantities, photons. If a photon is absorbed, then its energy corresponds to the difference in energy between the excited state involved and the ground state.

During excitation of the S_0 to one of the T_n states both the orbital and the spin of the electron must change. However, absorption takes place in only 10^{-15} s and this makes such a simultaneous change of both orbital and spin of the electron involved highly improbable. An important consequence of this is that with organic molecules, which normally have singlet ground state (S_0), absorption of light exclusively produces singlet excited states and not triplets.

With regard to absorption spectra the foregoing leads to the following results (see also Fig. 7):

(1) The absorption spectrum of a compound represents the transitions $S_0 \rightarrow S_n$ only;
(2) The shorter the wavelength the higher the energy of the transition ($E = hc/\lambda$). If there were no vibrations and rotations in a molecule the absorption spectrum would be more or less a line-spectrum (Fig. 7a). However, to each electronic state belongs a series of vibrational and rotational levels.

    Electronic transitions take place between S_0 mostly in the lowest vibrational level, and one of the vibrational levels of the S_1, S_2, S_3, etc. (see Fig. 7b; the rotational levels were not taken into account for the sake of clarity). This broadens the electronic transitions into bands (in the example in Fig. 7b, some transitions between the S_0 state and vibrational levels of the S_1 state can be seen in the long-wavelength band of the UV/visible absorption spectrum);
(3) ε, the molar extinction coefficient, is a measure of the probability that light of a given wavelength is absorbed or, in other words, the efficiency with which light of a given wavelength produces an excited state.

The lifetime of excited singlet states other than S_1, is usually smaller than 10^{-11} s; too short for a photochemical reaction. Within this short time, molecules in higher excited singlet states tumble down to the lowest vibrational level of their S_1 state. In fact, the energy gap between the higher excited states and the S_1 is much smaller than that between the S_1 and the ground state, S_0. This facilitates the rapid decay of highly excited states to the S_1 state. The larger energy gap between the S_1 and S_0 state results in a longer lifetime of the molecule in the S_1 state: 10^{-9}–10^{-6} s.

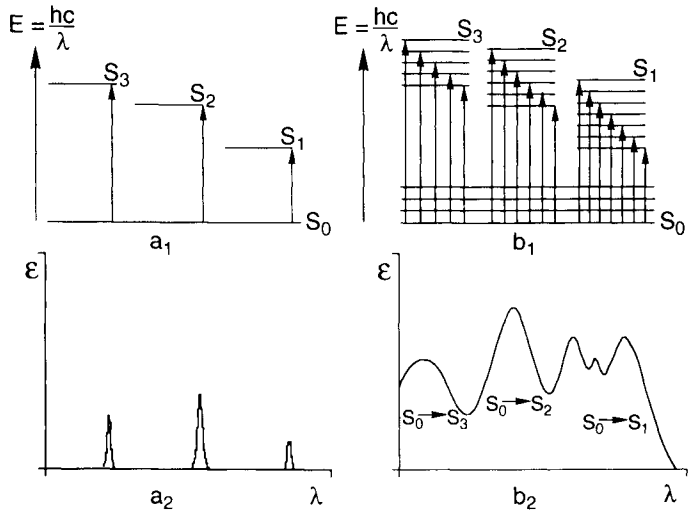


FIG. 7. Upper panels: Jablonski diagram for the electronic transitions between S_0 and S_1, S_0 and S_2 and S_0 and S_3 state: a_1 without and b_1 with vibrational sublevels. Lower panels a_2 and b_2: the corresponding UV/visible absorption spectra. Absorption of radiation is shown as straight arrows. E = energy; ε = molar extinction coefficient.

Important consequences of this are:

(1) Although absorption of light is an essential condition for photochemistry, the nature of the photochemical reaction is independent of the wavelength of the radiation used. This is due to the sequence:

$$h\nu\text{: } S_0 \rightarrow S_n$$

$$\Delta\text{: } S_n \rightarrow \rightarrow \rightarrow \rightarrow S_1 \rightarrow \text{reaction}$$

(2) The extinction coefficient (ε) is a measure of the efficiency with which light of a given wavelength ultimately produces the first excited singlet state. A consequence of (1) and (2) is that the rate of a photoreaction is a function of ε.

From the foregoing it follows that it is a misconception to believe that light of shorter wavelengths is more detrimental to chemicals than light of longer wavelengths. For instance, protoporphyrin is 50 times more photoreactive with visible light of 410 nm than with the same intensity of UV-radiation of 330 nm (see also Fig. 8). The reason for this is that $\varepsilon_{410} \approx 50 \times \varepsilon_{330}$.

As a result of photoreactivity of protoporphyrin, cells and tissues are damaged. Patients who suffer from impaired porphyrin metabolism have too

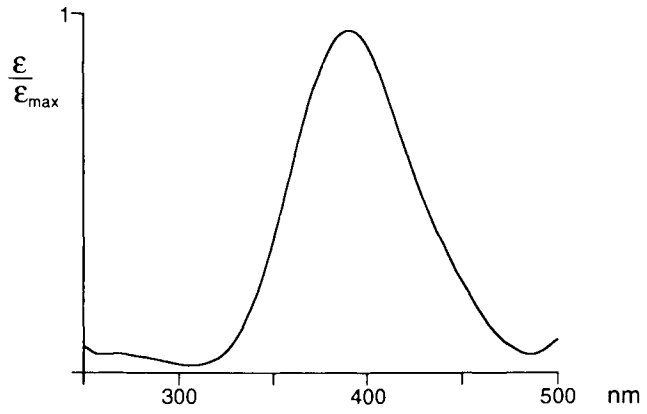


FIG. 8. Absorption spectrum of protoporphyrin.

much protoporphyrin in their blood and are very sensitive to visible light (Mathews-Roth, 1982).

Because, for the vast majority of organic molecules, light absorption ultimately leads to an S_1 state, it is important to know what kind of processes can take place from that excited state. In Fig. 9 the physical processes are represented without taking into account surrounding molecules, as for instance those of the solvent.

There are radiative and non-radiative ways to lose the excitation energy (Fig. 9). The non-radiative processes, a and b, initially proceed without loss of energy; a high vibrational level is reached of either the S_0 or the T_1 state, followed by a rapid cascade to the lowest vibrational level. Both processes are probable because only the orbital of the electron (a) or its spin (b) is changed. Process (a) is called internal conversion (IC) and (b) intersystem crossing (ISC).

The most important consequence of process (b) is that the triplet excited state, which is almost not formed upon light absorption, can often easily be obtained via the probable process $S_1 \rightarrow T_1$ (ISC). The other non-radiative process (Fig. 9e), from the triplet T_1 to a high vibrational level of the ground state S_0 is improbable because the orbital and the spin of the excited electron should be simultaneously changed.

Radiative processes are (c) from the S_1 and (d) from the T_1, called fluorescence and phosphorescence respectively. With (c) only the orbital and with (d) both the orbital and the spin should be changed, making (c) a probable and (d) an improbable process.

The important consequence of the fact that processes (a), (b) and (c) from the S_1 state are probable and (d) and (e), from the T_1 state, are improbable processes, is that the lifetime of the molecule in the triplet excited state is far longer than that in the singlet excited state. The intrinsic lifetime, without

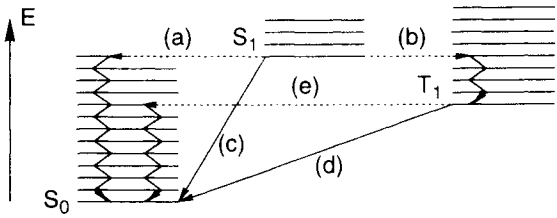


FIG. 9. Physical deactivation processes from the first excited singlet state without surrounding molecules being present (electronic and vibrational levels are represented). (a) $S_1 \rightarrow S_0$ + vibrational and rotational energy; (b) $S_1 \rightarrow T_1$ + vibrational and rotational energy; (c) $S_1 \rightarrow S_0 + h\nu$ (fluorescence); (d) $T_1 \rightarrow S_0 + h\nu$ (phosphorescence); (e) $T_1 \rightarrow S_0$ + vibrational and rotational energy.

surrounding molecules, is 10^{-3}–10 s for the T_1 state and 10^{-9}–10^{-6} s for the S_1 state. Because the intrinsic lifetime of a molecule in its T_1 state is much longer, the chance of colliding with a reaction partner is far greater than when it is in the S_1 state. This is the reason that the T_1 state is so important to photochemistry.

In addition to the physical relaxation processes mentioned (Fig. 9), competing photochemical reactions can take place in which surrounding molecules are not necessarily involved (unimolecular reaction). Thus, isomerization, rearrangement and radical formation are examples of reactions that a molecule in the excited S_1 or T_1 state can undergo. Because unimolecular reactions can proceed very rapidly, the chance that a molecule in its singlet excited state will undergo this kind of reaction should be taken into account.

1.2.3.2 In the Presence of Surrounding Molecules (in Particular Energy Transfer). In the presence of other molecules (O), formation of excited states and subsequent deactivation processes are essentially the same as described in section 1.2.3.1. A number of processes can occur, some of which are rather trivial:

$$S_1 \text{ (or } T_1) + O \rightarrow S_0 + O + \text{kinetic energy}$$

$$S_1 \text{ (or } T_1) + O \rightarrow \text{reaction products.}$$

The first process is called collisional deactivation to which, evidently, the T_1 state with its longer intrinsic lifetime is more susceptible. The same holds for the formation of reaction products. (However, one should be careful when considering the T_1 state as a prerequisite for photochemical reaction. Especially when reactants are very near, as for the complexation of drugs with biomolecules, the S_1 state can considerably contribute to product

formation. Thus, the photohydrolysis of 2,3-dimethoxy-6-nitronaphthalene proved to proceed from the S_1 state (Fig. 2).)

Apart from the trivial processes mentioned, the presence of other molecules can produce another effect, called *energy transfer*, which complicates the photochemical activity of drugs. This involves transfer of excitation energy from an electronically excited molecule (D = Donor) to the ground state of another molecule (A = Acceptor) resulting in deactivation of D and excitation of A. Excited D involves either the S_1 or the T_1 state. Light absorption, followed by radiationless deactivation of excited singlet states with $n > 1$, produces the S_1 state. The T_1 state results from intersystem crossing (ISC):

$$h\nu\text{: } D(S_0) \rightarrow D(S_n)$$

$$\Delta\text{: } D(S_n) \rightarrow \rightarrow \rightarrow \rightarrow D(S_1)$$

$$\text{ISC: } D(S_1) \rightarrow D(T_1)$$

Between organic molecules, of which the ground state usually is a singlet, two modes of energy transfer, (a) and (b), are possible (the arrows in parentheses represent the electron spins of the outer two electrons; subscript s or t means singlet or triplet):

$$\text{(a) } D_S(\uparrow\downarrow) + A_S(\uparrow\downarrow) \rightarrow D_S(\uparrow\downarrow) + A_S(\uparrow\downarrow)$$

$$\text{(b) } D_T(\uparrow\uparrow) + A_S(\uparrow\downarrow) \rightarrow D_S(\uparrow\downarrow) + A_T(\uparrow\uparrow)$$

Oxygen, which often plays a role in photochemical processes of biomolecules, is a triplet in its ground state. In connection with energy transfer, route (c), in which $A = O_2$ is also important:

$$\text{(c) } D_T(\uparrow\uparrow) + A_T(\downarrow\downarrow) \rightarrow D_S(\uparrow\downarrow) + A_S(\uparrow\downarrow)$$

(Note: singlet oxygen has a long lifetime. The reason is that the transition to the triplet ground state is improbable because the orbital and the spin should be simultaneously changed.)

In processes (a), (b) and (c), both D and A undergo an electronic transition. Consequently, this would mean that (a) would be the only probable process. The reason is that both with (b) and (c), a simultaneous change of the spin of the electrons, involved in the excitation of A and in the deactivation of D, also occurs. However, (b) and (c) can still occur by virtue of *exchange interaction* between D and A. This means that a molecular collision or near-collision between D and A should take place, such that the electron clouds of the donor and acceptor overlap. In the region of overlap, the excited electron of D and an outer electron of A are indistinguishable and can be exchanged without their spin being changed.

Although for route (a) to proceed a collision between D and A is not necessary and the distance between the two can be as much as 5 nm, the

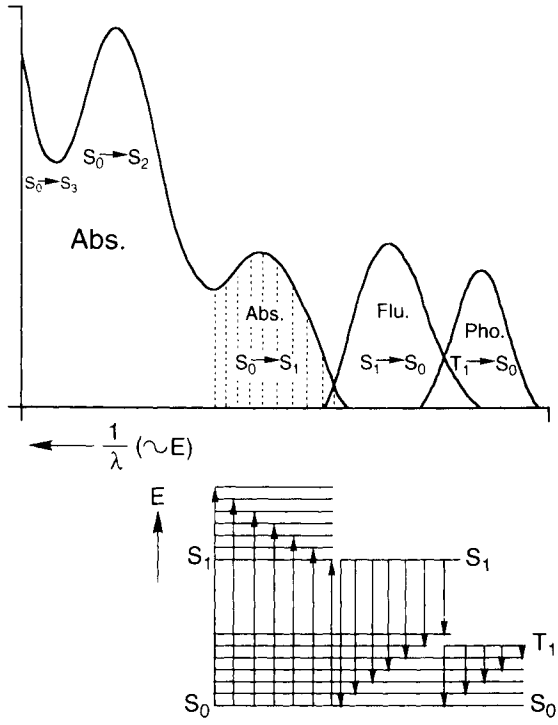


FIG. 10. Jablonski diagram of radiative transitions (lower panel) and corresponding electronic spectra (upper panel): $S_0 \rightarrow S_1$ (Abs. = absorption; $S_0 \rightarrow S_2$ and $S_0 \rightarrow S_3$ are not depicted in the diagram), $S_1 \rightarrow S_0$ (Flu. = fluorescence) and $T_1 \rightarrow S_0$ (Pho. = phosphorescence); vibrational levels included.

$S_0 \rightarrow S_1$ part of the absorption spectrum of the acceptor should partly overlap the fluorescence spectrum ($S_1 \rightarrow S_0$) of the donor: the energy difference between $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_0$ should not be too large. Because a (near) collision between donor and acceptor is a condition for the occurrence of both (b) and (c), it is understandable that a long lifetime of the excited D as a triplet will favour the occurrence of processes (b) and (c). Another condition for both (a), (b) and (c) to proceed, is that in each case the energy for deactivation for excited D exceeds that for excitation of ground state A; e.g., process (b): $E\ (D_T \rightarrow D_S) > E\ (A_S \rightarrow A_T)$.

Whether conditions of overlap of spectra, lifetime of excited state and difference in deactivation and excitation energy are fulfilled in a given case can be concluded from the study of the electronic spectra (absorption, fluorescence and phosphorescence) of D and A. (See also Fig. 10: phosphorescence can only be measured in rigid media; the relatively long intrinsic lifetime of the triplet ensures that, in solution, it is already

deactivated by collision with surrounding molecules, long before it can end its 'natural' life by emission of a photon of light.) Whereas absorption spectra mostly consist of more bands, the fluorescence and phosphorescence spectra have one band only. The reason is that the higher excited singlet states, formed upon absorption, live too briefly and tumble down in a non-radiative process to the lowest vibrational level of the S_1 state. From the latter energy level, fluorescence can take place which brings the molecule into one of the vibrational levels of its ground state, S_0. In the same way, phosphorescence occurs from the lowest vibrational level of the T_1 state, reached from the S_1 state by ISC. As a result of the latter, both fluorescence and phosphorescence bands of a given compound are at the long wavelength side of its absorption spectrum. Further, the fluorescence spectrum ($S_1 \rightarrow S_0$) is often a mirror-image of the long wavelength absorption band ($S_0 \rightarrow S_1$).

To summarize sections 1.2.3.1 and 1.2.3.2, one can state that:

- Independently of the wavelength, absorption of light ultimately brings a molecule from its ground state S_0 into its excited S_1 state. The higher the extinction coefficient the more efficiently light is used for the production of S_1 excited molecules.
- The molecule in the S_1 state has a short lifetime which is sufficient for unimolecular reactions, such as rearrangement and isomerization, and for bimolecular reactions if the reaction partner is very near (mean lifetime of the S_1 state is 1–20 ns in solution at room temperature.)
- The triplet excited molecule has more chance to react with other molecules because of its longer lifetime (mean lifetime in solution at room temperature can exceed 1 ms). Triplets are not directly formed from the ground state molecule by light absorption.
- Molecules in their T_1 state are obtained by intersystem crossing (ISC) from the excited S_1 state. Another important possibility of getting T_1 excited molecules is energy transfer, also called sensitization. In this process an acceptor (A) takes electronic energy over from an excited donor (D, also called sensitizer), resulting in excitation of A and deactivation of D. Excited oxygen is formed by energy transfer. However, instead of the T_1, the excited S_1 state is obtained. This singlet excited oxygen has a relatively long lifetime, in solution at room temperature 20 μs, because the transition to the triplet ground state is improbable.

1.2.3.3 Some Examples of Energy Transfer to Exemplify the Concepts Action Spectrum, Quencher *and* Quantum Yield.

(1) Energy transfer from an excited donor to a ground state acceptor mostly proceeds very efficiently. This holds for the processes (b) and (c), and not only for (a) in which a collision is not even necessary (distance effect). A frequently observed situation is that the donor itself does not react during the process of photosensitization. If this is the case, the donor can repeat

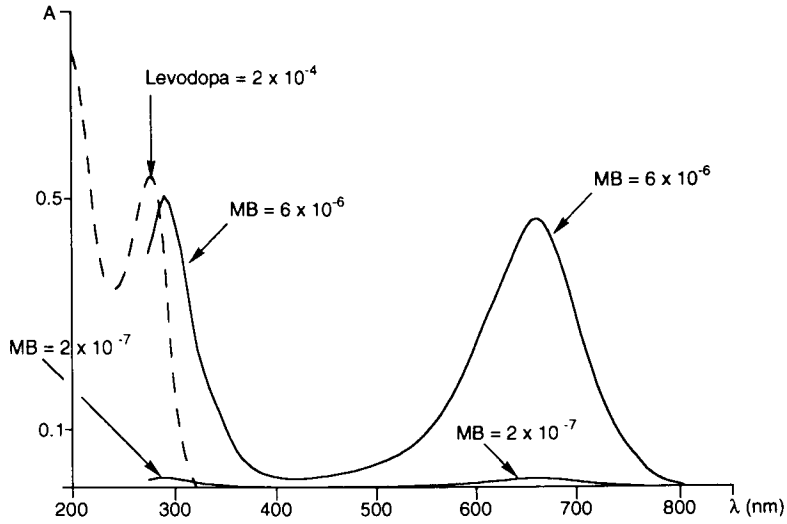


FIG. 11. UV/Visible absorption spectra of levodopa and methylene blue (MB). For comparison the spectrum of MB is shown for 6×10^{-6} M and for 2×10^{-7} M.

the transfer of absorbed energy numerous times per second and act as a catalyst. The consequence is that the donor, also called photosensitizer, can be present in a very low concentration.

The unexpected oxidation of catecholamines upon exposure to visible light can serve as an example to illustrate this and to introduce the concept of *action spectrum*. Catecholamines only absorb UV-radiation with $\lambda < 310$ nm (Fig. 11). It was therefore unexpected to find that a 2×10^{-4} M solution of levodopa in water was still sensitive to light while it was kept in colourless ordinary glass vials (less than 1% transmission below 320 nm). However, it is quite a common situation in photochemistry and photobiology that light is absorbed by a compound other than the one which decomposes. To identify the chromophore, which by absorption of light triggers the sequence of events eventually leading to the decomposition observed, an action spectrum can be helpful.

The action spectrum is a plot of the reaction rate against the wavelength of the light. Provided that at each wavelength the same light intensity (number of photons per second) is used the action spectrum resembles the absorption spectrum of the chromophore; they are identical if no other absorbing compounds are present. In the case of the levodopa solution a maximum in the action spectrum was found at 670 nm. This could be ascribed to methylene blue. High-performance liquid chromatography (HPLC) analysis gave further evidence and revealed that the concentration of this known photosensitizer was only 2×10^{-7} M. (The low absorption of

methylene blue at 670 nm is easily overlooked by superficial inspection of the UV/visible spectrum; see Fig. 11.)

The photo-oxidation of levodopa (De Mol and Beijersbergen van Henegouwen 1979; De Mol *et al.*, 1979a,b) by methylene blue (MB) under the influence of visible light ($\lambda = 670$ nm) can be summarized as follows ($MB_{S(0)}$ means MB in its S_0 state etc.):

$$h\nu + MB_{S(0)} \rightarrow MB_{S(1)}$$

$$MB_{S(1)} \rightarrow MB_{T(1)} \text{ (ISC)}$$

$$MB_{T(1)} + {}^3O_2 \rightarrow MB_{S(0)} + {}^1O_2 \text{ (energy transfer)}$$

$${}^1O_2 + \text{levodopa} \rightarrow \text{oxidation products}$$

$$\text{sum: } h\nu + {}^3O_2 + \text{levodopa} \rightarrow \text{oxidation products}$$

(2) An acceptor once excited can, in turn, transfer energy to another molecule also. An example of this is the β-carotene therapy of erythropoietic phorphyria (Mathews-Roth, 1982). A supposed mechanism underlying the protection of β-carotene (C) against the damaging effects of protoporphyrin (P) excited by visible light can be summarized as follows ($P_{S(0)}$ means P in its S_0 state etc.):

$$P_{S(0)} \rightarrow P_{S(1)}$$

$$P_{S(1)} \rightarrow P_{T(1)} \text{ (ISC)}$$

$$P_{T(1)} + {}^3O_2 \rightarrow P_{S(0)} + {}^1O_2 \text{ (energy transfer)}$$

$$C_{S(0)} + {}^1O_2 \rightarrow C_{T(1)} + {}^3O_2 \text{ (energy transfer)}$$

$$C_{T(1)} \rightarrow C_{S(0)} + \text{vibrational} + \text{rotational energy.}$$

Compared with 'normal' oxygen, ${}^3O_2$, singlet oxygen ${}^1O_2$ is very reactive with all kinds of biomacromolecules, DNA, proteins and lipids as constituents of cellular membranes. In fact, it is ${}^1O_2$ that damages tissue in patients suffering from erythropoietic porphyria. When such a patient is exposed to visible light, protoporphyrin (P) is photoexcited to its S_1 state, which reaches very efficiently its T_1 state by intersystem crossing (ISC). P in the T_1 state transfers its energy to ${}^3O_2$ with the damaging ${}^1O_2$ as the result.

β-Carotene can interfere in this process in two different ways (Krinsky, 1982): either by accepting energy from ${}^1O_2$, as depicted above, or directly from the triplet excited porphyrin. In both cases β-carotene reaches the triplet state which can be deactivated by internal conversion, followed by radiationless decay to the S_0 state, during which harmless heat is produced.

A compound such as β-carotene, which in the process of energy transfer plays the role of acceptor and which converts the excitation energy into heat without undergoing any reaction, is called a *quencher*. Quenchers can also be useful as protectors against the harmful effects of phototoxic drugs.

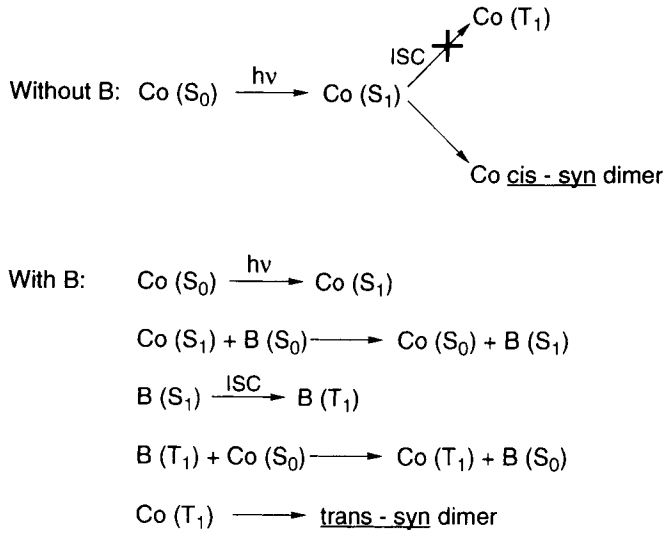


FIG. 12. Coumarin (Co) dimer formation in ethanol upon exposure to light: without and with benzophenone (B) present.

(3) Still further complicated by energy transfer is a reaction (e.g., Fig. 12) in which the same compound enters the process of sensitization both as donor and as acceptor (Calvert and Pitts, 1967). Coumarin (Co) in ethanol, brought into its S_1 state after light absorption, forms a *cis*-syn dimer with very low *quantum yield* (= mol dimer formed/number of photons absorbed by Co). Under the experimental conditions applied the quantum yield for ISC, $S_1 \rightarrow T_1$ is extremely low (quantum yield ISC = number of Co in T_1 state/number of photons absorbed by Co). The addition of a small amount of benzophenone (B) to the solution of Co in ethanol leads to the production of the *trans*-syn dimer of Co with high quantum yield. This is also the case when all of the exciting light is still absorbed by Co. Obviously the *trans*-syn dimer is formed from the T_1 state of coumarin.

The production of Co triplets proceeds through two energy transfer steps: Singlet excited Co molecules transfer energy to B, which undergoes ISC. Subsequently, triplet excited B transfers energy back to Co (see Fig. 12).

1.2.4 Photochemical Reactions

With respect to photochemical reactions taking place in biological systems, distinction is made between type I and type II reactions (Fig. 13). In type I reactions radicals are formed. In both the singlet excited and the triplet

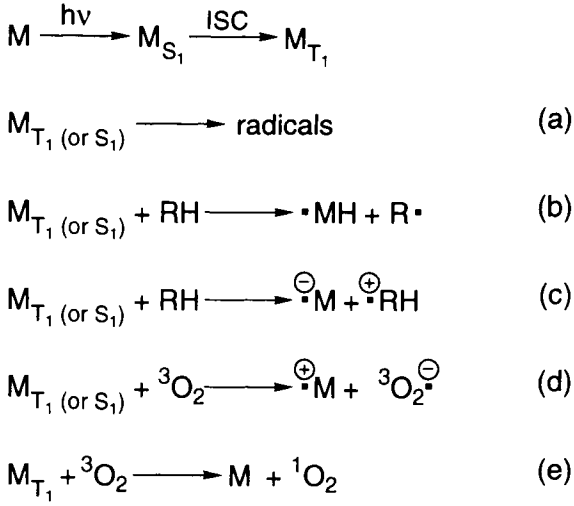


FIG. 13. Singlet or triplet excited molecule M starts a type I reaction (a), (b), (c) and (d). Triplet excited M transfers its energy to ground state oxygen (e): type II reaction. (RH = reactant).

excited molecule homolytic cleavage of a single bond can occur in a unimolecular process, resulting in the formation of two radicals (a). Radicals can also be produced in a bimolecular process when the molecule in the S_1 or T_1 state collides with a reactant (RH); in this case, however, the triplet excited state has more chance to react because of its longer lifetime (b, c and d). Oxygen often plays a role in type I reactions, not only directly as in (d), but also indirectly by participating in (a), (b) and (c). As a result of this, in addition to superoxide anion, peroxy radicals, hydrogen peroxide and hydroxyl radicals can be formed.

In a type II reaction the triplet excited molecule transfers its energy to oxygen resulting in singlet oxygen (e).

Photoreactions in which molecular oxygen plays a role (type II reactions and part of the type I reactions) are called photodynamic reactions. In recent literature the term type III reactions is found which means all other reactions in which both radicals and oxygen do not play a role (e.g., Figs 2, 4 and 12; the latter concerning cycloaddition).

1.2.5 Some Facts Pertinent to Human Photobiology

1.2.5.1 Spectral Composition of Sunlight; Subdivision of UV-radiation. Extraterrestrial solar radiation (200–800 nm) is essentially continuous, lacking only certain narrow wavelength bands because of absorption by the

sun's atmosphere. At midday, maximal intensity is in the region of 450–500 nm. Although there is little change in the spectral composition of sunlight as such, the intensity as a function of wavelength to which man is exposed can vary greatly depending, for example, on season, distance from the equator and height above sea level.

The subdivision of ultraviolet radiation (UVR) into three regions dates back to early observations by photodermatologists. UVA (320–400 nm) was considered as innoxious and responsible for pigmentation, UVB (290–320 nm) as a cause of sunburn and photocarcinogenicity and UVC (200–290 nm) as radiation, which, although photobiologically active because of its mutagenic and antimicrobial activity, is not relevant to human health provided that it is effectively absorbed by the ozone layer.

More recent research has modified this picture; e.g., both UVB and a part of UVA are carcinogenic. More photobiological effects have now been discovered which are important for the functioning of the human body and whose action spectra do not fit one of these UV-regions, e.g., influence of UV-radiation on the immune system (Morison, 1985). That UVC would not be relevant to human photobiology because of the above-mentioned reason is a conclusion often drawn but is incorrect because it is based upon a misconception of the process of light absorption. It follows from equations describing radiation absorption, that a certain portion is always transmitted. Whether the small amount of UVC transmitted by the ozone layer is important or not, should be assessed from its photobiological effects. In this connection, it is of interest to know that essential biomacromolecules have their maximal absorption in the UVC region, e.g., proteins at *c.* 280 nm and DNA at *c.* 260 nm (for comparison, current knowledge of human photobiology would not validate the conclusion that UVR is less important than visible light because UVR is only a few per cent of electromagnetic radiation of 200–800 nm).

Although, as shown above, the subdivision into UVA, UVB and UVC has limited value and is inevitably dated, it is frequently used in the literature. This is certainly useful in so far as it facilitates a preliminary discussion about observed phenomena. However, one can only obtain insight in the molecular processes underlying the occurrence of a photobiological effect by carefully defining the applied radiation in more rigorous spectrometric terms.

1.2.5.2 Optical Properties of the Skin and Eyes. Whether an interaction between light and a drug can take place in the eyes or the skin is dependent on pharmacokinetic parameters of the drug and its metabolites, but also on the extent to which light of different wavelengths penetrates the organ compartments.

Along the optic axis of the eye the following tissues absorb light: cornea, aqueous humour, lens, vitreous and retina. The cornea absorbs wavelengths below 295 nm and transmits all longer wavelength radiation to the aqueous

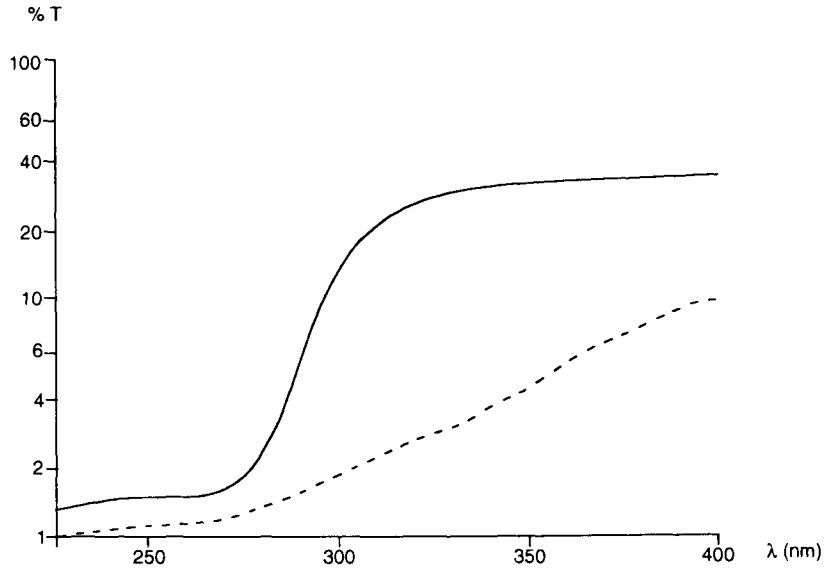


FIG. 14. The transmission (%) for the epidermis of black (------) and white (————) skin.

humour and lens. The lens contains chromophores which absorb wavelengths between 295–400 nm and transmit wavelengths longer than 400 nm to the retina (Andley, 1987).

Before reaching the dermis which contains the network of capillary blood vessels, light first passes the viable part of the epidermis. Skin transmission curves depend on numerous factors, of which skin pigmentation is one of the most important. In Fig. 14, a typical transmission curve is represented for the epidermis of black and white skin. The shoulder in the curves (at *c.* 280 nm) is caused by the absorption of light by proteins and nucleic acids. It proves that a considerable part of the incident UVR and visible light can penetrate the dermis of white skin: 1% at 250 nm, 5% at 290 nm, 20% at 300 nm, 35% at 320 nm and 50–60% from 350 nm upward (Anderson and Parrish, 1982; Everett *et al.*, 1966). These percentages are lower for black skin while *c.* 70% of the human population has a transmission curve between these two extremes.

Although only a small fraction of incident radiation of wavelengths less than 300 nm reaches the dermis, it cannot be assumed that these wavelengths exert no direct dermal effects. Delayed erythema induced by 254 nm radiation (transmission of Caucasion skin *c.* 1%) can involve direct dermal effects (van der Leun, 1966). Further, UVB plays a distinct role in photoaging of the dermis (Kligman and Kligman, 1986).

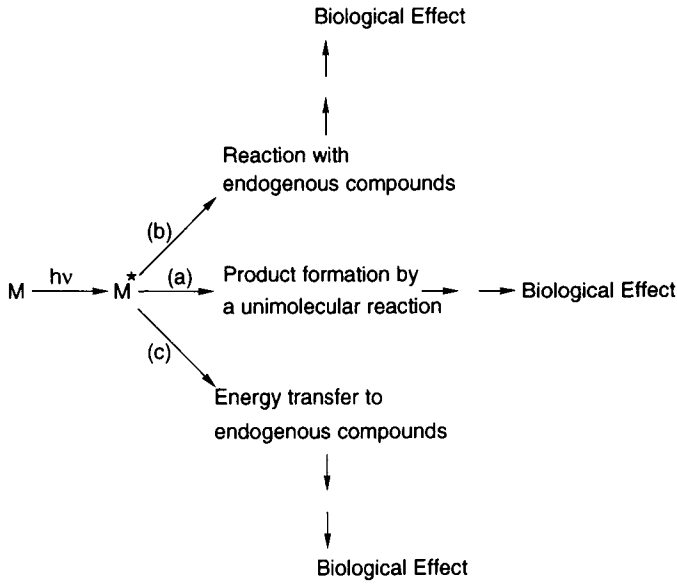


FIG. 15. Possible processes in a biological system initiated by a molecule (M) after absorption of a photon ($h\nu$).

1.2.5.3 Photoreactions Taking Place In Vivo. Absorption of light by an endogenous compound or a xenobiotic in the skin or eyes can provoke a biological effect as a result of the following molecular processes (see Fig. 15):

(a) After excitation, the compound under investigation can undergo a unimolecular reaction such as rearrangement, isomerization or decomposition. The resulting products or their metabolites can be biologically active compounds. The UVB induced conversion (maximum between 295 and 300 nm) of 7-dehydrocholesterol into previtamin D_3 is an example of this. By careful investigation, Holick (1985) unravelled much of this process and showed that previtamin D_3 in human skin participates in three different photochemical equilibrium reactions which control the amount of this very biologically active compound and prevents, for example, excessive exposure to sunlight resulting in vitamin D overproduction.

Vitamin D is essential for maintaining calcium and phosphorus homeostasis, which in turn, is responsible for maintaining a healthy skeleton. The vitamin D process is also important to research of the photobiological activity of drugs, because it demonstrates that in the light-exposed skin, compounds can be formed in an extremely low

concentration which exert their biological activity in parts of the body far away from the skin.

(b) After absorption of light, a compound in its excited state can react with endogenous molecules, e.g., proteins and DNA. An example of this is the irreversible photobinding of psoralens to DNA as a result of UVA absorption in the skin, which is considered to play an important role in the so-called PUVA(= *P*soralen + *UVA*)-therapy of psoriasis (see Part 3).

(c) Transfer of excitation energy to another molecule can occur, for instance to oxygen, leading to reactive singlet oxygen. This is supposed to play a role in the phototherapy of neonatal jaundice (Ennever, 1988). In neonatal jaundice and in the frequently occurring Gilberts syndrome, too much bilirubin is found in the blood. Bilirubin can cause brain damage. Upon exposure of the body to visible light, bilirubin undergoes a number of processes leading to products which are more easily excreted than the parent compound itself. In addition to structural and configurational isomerization, photooxidation is another of the processes taking place *in vivo*. During the latter process singlet oxygen, produced by energy transfer from the photoexcited bilirubin to oxygen, decomposes the parent molecule.

Because light-induced isomerization and oxidation of bilirubin prevents this biomolecule from exerting its brain-damaging effects, it can be considered as an example of a photobiological process with systemic effects. Of further interest is the fact that this process also takes place in healthy persons upon exposure to sunlight, demonstrating another beneficial effect of the latter (McDonagh, 1986).

1.2.5.4 Phototoxic Versus Phototherapeutic Xenobiotics (Drugs). As far as the molecular processes underlying the occurrence of photobiological effects are concerned, there is no essential difference between the phototherapeutics and the phototoxicons as a group; the same processes, a, b and c (Fig. 15), occur. One speaks of a phototherapeutic if the photoreaction is intentional and controlled. The latter concerns not only the site in the body where it occurs but also the doses of the drug and the light (wavelength region, intensity and exposure time) applied.

With both phototherapeutics and phototoxicons, the sequence of events which eventually leads to the biological effects starts with the absorption of a photon of UV-radiation or visible light. In a biological system a photoexcited drug or other xenobiotic can undergo a number of primary reactions (Fig. 15):

(a) Photochemical reaction of the compound as such, a unimolecular reaction, e.g., rearrangement, isomerization or decomposition. The reaction products or their metabolites can display their biological activity

by interaction with a receptor. At present there are no reports of phototherapeutic or phototoxic compounds acting in this way. Yet, this can be considered as a possibility; certainly if one takes into account that interaction of a photometabolite with a receptor is an essential part of a normal endogenous photobiological process in man. This process concerns the UVB induced rearrangement of 7-dehydrocholesterol into previtamin D_3 and subsequent steps.

Unstable photoproducts can react with endogenous molecules resulting in a biological effect. If the half-life of such a photoproduct is long, it can react in inner organs after having been formed in the skin. For example, phototoxic imino-*N*-oxides isomerize in the UVA exposed skin into an oxaziridine. The latter can irreversibly bind to biomacromolecules (see section 2.2.6).

Decomposition can also involve formation of radicals which, whether or not coupled to oxygen, can damage biomacromolecules. For instance, chlorpromazine photodecomposes with UVA into the phenothiazine radical which, *in vivo*, covalently binds to lipids and to proteins (see section 2.2.4).

(b) Photoreaction with endogenous molecules. An example of this is the irreversible binding of photoexcited psoralens to DNA as a result of UVA absorption in the skin, what is considered to play an important role in the so-called PUVA-therapy (see section 3.2.3).

Photoexcited M can also abstract an electron or an H-atom from, for example, endogenous glutathione and the so-formed M-derived radicals can damage biomacromolecules such as DNA, proteins and lipids as constituents of membranes.

(c) Energy transfer to endogenous compounds. This is exemplified by singlet oxygen formation from $^3O_2$, which is supposed to play an important role in the dye/visible light therapy of cancer (see section 3.2.1). Phototoxic compounds of quite different molecular structure can also produce singlet oxygen by energy transfer, e.g., phenylpropionic acid derivatives used as inflammatory drugs and tetracyclines.

Each of the essential bio(macro)molecules can be damaged by primary photoreactions (a), (b) and (c). Damage to DNA can lead to cell death but also to mutation. If DNA repair enzymes (proteins) are also inactivated, for instance by a simultaneously occurring primary photoreaction, mutation can be transferred and tumour formation can result. Photoreaction with proteins, or with lipids as constituents of membranes, can lead to cell death, but it can also trigger an immune response which eventually can lead to allergy. In addition to biomacromolecules such as DNA, proteins and membrane constituents, small endogenous molecules, such as glutathione which is important to the cellular defence, are targets of primary photoreactions.

The biological effects which can be observed with a given phototherapeutic or phototoxicon depend on a variety of factors, such as:

(1) The extent to which each of the primary photoreactions a, b and c occurs.
(2) The extent to which each of the essential bio(macro)molecules is damaged.
(3) The bioavailability of the given photoactive compound and its metabolites, not only in the organs exposed to light but also at cellular level.

For example, singlet molecular oxygen is a mutagenic species. However, although dyes are efficient singlet oxygen producers by energy transfer (c), mutagenesis is not an expected problem with the dye/visible light therapy (see section 3.2.1). The fact is that the dyes commonly used in this therapy, accumulate in the lipid material of the membranes and remain outside the cellular nucleus. Another example of bioavailability as a determining factor is chlorpromazine whose photoreactivity to DNA is known from many *in vitro* studies: irreversible binding is one of the possibilities. However, as a result of poor bioavailability in the cellular nucleus, this photoreaction was not found *in vivo*. Contrary to this, photobinding to proteins and lipids of epidermal cells was observed (see section 2.2.4). A consequence of the latter is that light-induced disorders of the immune system rather than photocarcinogenicity are to be expected.

A comparable situation was found with furocoumarins: photobinding to DNA got by far the most attention. However, it has been demonstrated that 8-methoxypsoralen, frequently used in PUVA therapy, photobinds *in vivo* not only to DNA but to a high extent also to proteins and lipid material of epidermal cells (Beijersbergen van Henegouwen *et al.*, 1989a; Schoonderwoerd *et al.*, 1991a). This can be an important finding with regard to e.g., immunological effects from PUVA (section 3.2.3).

2 Phototoxic Drugs and other Xenobiotics

2.1 INTRODUCTION

2.1.1 Phototoxic Versus *Photoallergic Xenobiotics*

Xenobiotics are extensively used in, for example, drugs, cosmetics, food and agriculture chemicals. Although very useful and almost indispensable they can produce adverse biological (toxic) effects.

Because of the different nature of these toxic effects, toxicology is subdivided. Subdivisions are, for example, genotoxicity, hepatotoxicity, immunotoxicity and neurotoxicity. Skin allergy is a part of immunotoxicity and thus belongs to a subdivision of toxicology.

Phototoxicology concerns the whole field of toxicology and thus includes all subdivisions. (The only condition is that light plays a role in the occurrence of the toxic effect.) The consequence of this is that if absorption of sunlight by a xenobiotic or its metabolites is an essential condition for the occurrence of toxic effects, the chemical is called phototoxic. Thus, it fits into the logic of toxicology and its subdivisions that photoallergic compounds are considered as a subdivision of phototoxic compounds.

2.1.2 Systemic Phototoxic Effects

It is commonly assumed that phototoxicity of drugs and other xenobiotics remains restricted to the organs exposed to light, namely the skin and eyes. However, there are natural photobiological processes in man by which the eventual effect occurs in a part of the body far from the sunlight-exposed organ in which photoexcitation of an endogenous compound took place.

The conversion of 7-dehydrocholesterol into vitamin D_3 and that of bilirubin into more water-soluble products, upon exposure of the body to UVB and to visible light respectively, are two examples of systemic photobiological effects of endogenous compounds. Vitamin D_3 is essential for proper bone calcification and the photoconversion of bilirubin, e.g., the visible light therapy of neonatal jaundice results in a decrease of brain-damaging effects.

Although the concept, sunlight + xenobiotic $\rightarrow$ systemic biological effects has not had much attention, some reports will be presented here, which address this question.

2.1.3 Aim of the Research of Phototoxic Xenobiotics

Insight into the molecular processes which a given phototoxicon undergoes *in vivo* after absorption of UV or visible light, is a prerequisite for the identification of that part of the molecular structure which causes the unwanted photobiological effect. Only after this identification will it be possible to alter the molecular structure of the xenobiotic in such a way that the phototoxicity diminishes whereas desired properties remain conserved. This research aim can be achieved by combining data from *in vitro* and *in vivo* investigations. Photoreactivity of the phototoxic xenobiotic and structural analogues should be studied:

(1) *In vitro*, whether or not in the presence of essential bio(macro)-molecules;
(2) In microbiological test systems (e.g., bacteria and yeast) and in human cells, whether or not in culture;
(3) In experimental animals or human volunteers.

Both with (1), (2) and (3), attention should be paid to the identification of the photoproducts and to the possibility that photobinding to and damage of biomacromolecules has occurred. These data can provide insight into the formation of reactive intermediates and into the reaction mechanism occurring *in vivo*.

With results from *in vitro* studies (1) and (2) only, it is almost impossible to predict which photo-induced reactions occur *in vivo*. This is caused by factors such as:

- Metabolism of the xenobiotic. Metabolites can differ in photoreactivity compared with the parent compound
- (Intracellular) distribution. The amount of the xenobiotic that reaches the site of exposure (e.g., skin or eyes) or specific sites within the cell (e.g., DNA or membranes of cell bodies)
- Absorption of radiation by skin components which determines the amount of light available to the drug
- Repair processes by which the organism can respond to photochemically induced damage.

On the other hand, the *in vivo* system is too complicated and without continuous help from *in vitro* research, the investigation of this cannot provide much insight. For this reason, the above mentioned research lines (1), and (2) and (3) should be performed in continuous interaction with each other.

The variety and number of phototoxic compounds are large. This, added to the fact that up to now there is only limited research effort devoted to this subject, means that for most phototoxic xenobiotics a relation between structure and *in vivo* photoreactivity is not available. Therefore, more attention will be paid in this chapter to xenobiotics of which something is known not only about the *in vitro* but also about the *in vivo* photochemistry. References in this review are mostly investigations which date from the last 10 years. For older literature or for additional information the reader is referred to other reviews (Johnson, 1984; Epstein and Wintroub, 1985; Kochevar, 1987; Kochevar, 1989; Greenhill and McLelland, 1990). Phototoxic xenobiotics not dealt with here are sufficiently covered in the reviews mentioned.

2.2 SPECIFIC EXAMPLES OF PHOTOTOXICONS

2.2.1 Tricyclic Dibenzocycloheptadienes

Although members of this group of antidepressants have been reported to cause photosensitization, research has been mainly limited to protriptyline (PTR, Fig. 16). The role of the stilbene-like double bond between C10 and

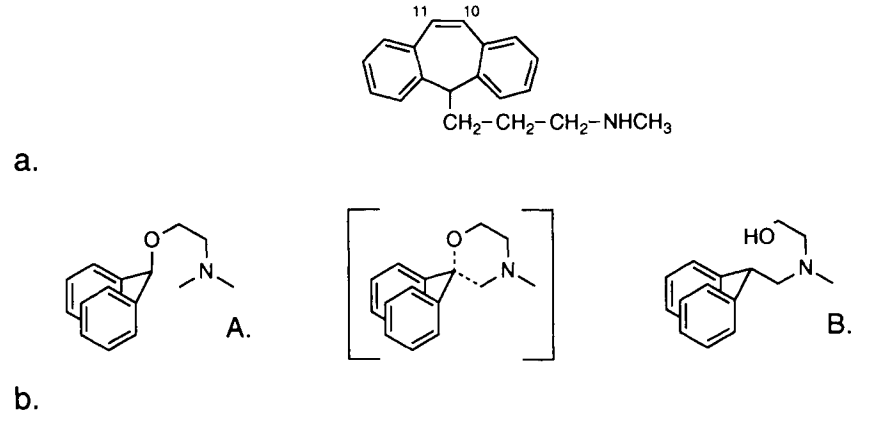


FIG. 16. a. Structure of protriptyline (PTR); b. Formation of photoproduct (B) by intramolecular rearrangement of diphenhydramine (A) via an exciplex.

C11 in the occurrence of phototoxicity, via for instance the formation of an epoxide, has been emphasized. Although this may be the case with PTR, it cannot be the cause of the phototoxicity of other tricyclic dibenzocycloheptadienes which do not have the C10–C11 double bond. In this connection, it is therefore of importance that Epling *et al.* (1988) compared the photoreactivity of members of this family. From the identification of the products of the photoreaction of PTR, amitriptyline, nortriptyline and imipramine, both in the presence and absence of oxygen, interesting conclusions could be drawn. Under all conditions photofragmentation of the parent compound is an important reaction – in some cases it is virtually the only photoreaction observed. The mechanism of photoreaction appears to involve excited state electron transfer from the side-chain nitrogen to the aromatic part of the molecule. Electron transfer, whether or not preceded by the formation of an intramolecular exciplex with a structure comparable to that represented for diphenhydramine (Fig. 16), can readily explain the occurrence of reactive intermediates such as radicals, radical anions and radical cations. These unstable intermediates can lead to cell damage by reaction with proteins, DNA/RNA or unsaturated lipids, eventually resulting in phototoxicity.

2.2.2 *Diphenhydramine*

Despite the fact that according to most pharmacopoeias, diphenhydramine should be protected from light and can cause photoallergic dermatitis (Emmett, 1974), no literature data were available on the photochemical

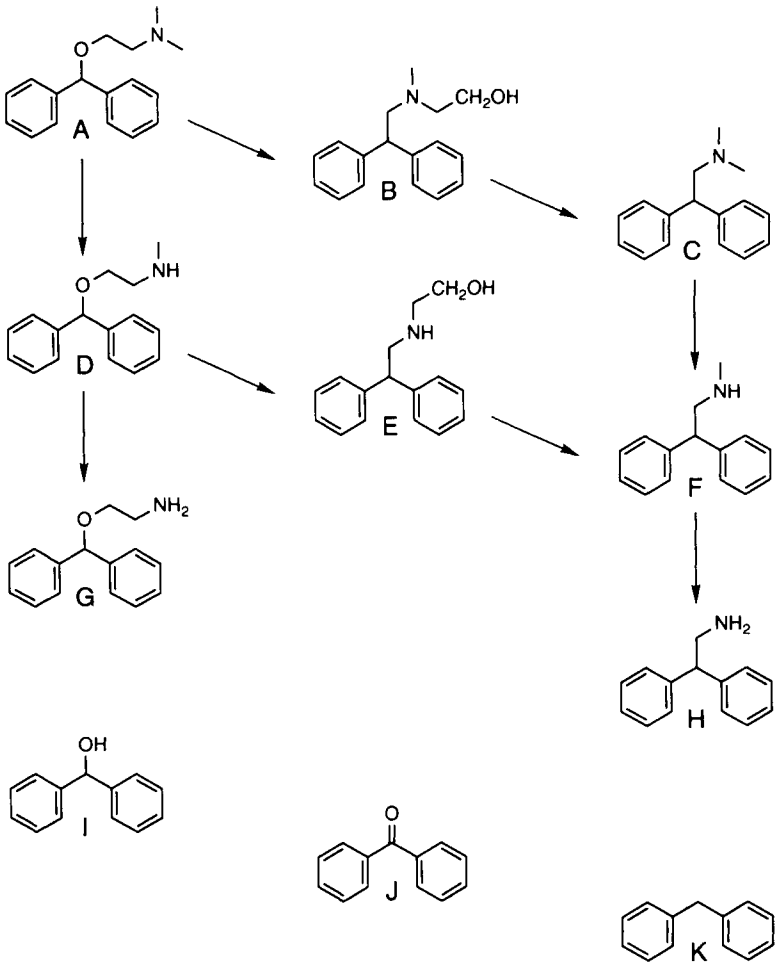


FIG. 17. Photodegradation of diphenhydramine (A) in water.

decomposition of this compound until some 10 years ago (Beijersbergen van Henegouwen *et al.*, 1987). Products isolated and identified are represented in Fig. 17. In addition to benzhydrol (I), benzophenone (J) and diphenylmethane (K), these involve products B to H which fit the reaction scheme. In addition to progressive *N*-demethylation, going from A to G and from C to H, intramolecular rearrangements occur with the formation of B from A (see also Fig. 16) and with that of E from D. By identification of photoproducts from the irradiation of pure B, C and D respectively, further evidence was obtained for the scheme represented in Fig. 17.

That upon irradiation of G no rearrangement product could be identified with a structure like E supports the mechanism according to which B (and

E) are formed via an intermediate, as is represented in Fig. 16b; this is not possible with G lacking a methyl group at the N-atom. Benzhydrol (I), benzophenone (J) and diphenylmethane (K) were found as a products of the irradiation of A, B, C, D and G.

If the HCl salt in water was irradiated instead of diphenhydramine base, the same products were formed but it took 15 times longer to reach the same degree of decomposition. This obviously reflects the importance of the N atom; thus, the formation of B or the demethylation of the dimethylamino group is facilitated if the electron pair on the N atom is available and not delocalized by proton binding.

The photodecomposition proceeds via the formation of unstable intermediates, probably radicals. Demethylation, loss of a CH_2OH fragment in a number of decomposition steps and the formation of polymeric material are an indication of this mechanism, which is further supported by the fact that product formation proceeds even if the irradiation is stopped.

With regard to photoallergy, the mechanism of the photoreaction may be of more importance: the formation of unstable intermediates facilitates a reaction with essential biomacromolecules, e.g., proteins, which is considered to be an important step for the onset of photoallergy.

The studies of diphenhydramine (Beijersbergen van Henegouwen *et al.*, 1987) and tricyclic antidepressants (Epling *et al.*, 1988) indicate that phototoxicity resulting from photofragmentation can be expected with drugs and xenobiotics having a side-chain nitrogen that can interact with the excited chromophore. The formation of intramolecular exciplexes from systems of the type $Ar\text{-}(CH_2)_n\text{-}NR_2$ has been reviewed (Kavarnos and Turro, 1986) and many examples of reactions such as fragmentation caused by formation of an exciplex (e.g., Fig. 16) have been given (Davidson, 1983; Kavarnos and Turro, 1986).

2.2.3 Contraceptive Steroids

In every pharmacopoeia it is stated that oral contraceptive steroids have to be protected from light. These compounds can also cause adverse photobiological effects which are mainly immunogenic in character. Despite the daily and prolonged use of these agents by a large part of the population, little was known about photoproducts at the beginning of the last decade. This situation mainly changed as a result of developments in NMR instrumentation, i.e., highly stable superconducting magnets interfaced to modern computer-controlled spectrometers, together with the rise of so-called two-dimensional (2D) nuclear magnetic resonance (NMR) techniques, making it possible to assign the ^1H and ^{13}C-NMR spectra of these very complex steroids (Sedee *et al.*, 1984a, 1985a,b) and to identify their photoproducts (Sedee *et al.*, 1985a,c,d).

Decomposition of a drug in the body into reactive intermediates or unstable products, can result from photoexcitation of the drug itself but can also be brought about by a photosensitizer. The latter case concerns not only exogenous chemicals, but also endogenous photosensitizers such as protoporphyrins, the quantity of which is raised in women taking oral contraceptive steroids – even to a level of provoking porphyria. For a number of steroids most frequently used in 'the pill' (norethisterone, norethynodrel, lynestrenol, ethynylestradiol and mestranol) these two methods of light-induced decomposition have been investigated. (Norethynodrel and lynestrenol are metabolized in the body into norethisterone, while ethynylestradiol is an important metabolite of mestranol. This is the reason that in the study described below, most attention is given to norethisterone and ethinylestradiol.)

With haematoporphyrin as a photosensitizer, all compounds except norethisterone decomposed when their solution in buffer pH 7.4 was exposed to visible light (Sedee and Beijersbergen van Henegouwen, 1985); hydroperoxides proved to be important intermediates (Sedee and Beijersbergen van Henegouwen, 1983, 1985; Sedee *et al.*, 1985a).

Mestranol showed to be more stable to photosensitization than EE_2 and because it is metabolized in the body into the latter, further research was done with EE_2, or with estrone if a radiolabelled compound was needed.

Both EE_2 and estrone are rapidly photooxidized and quantitatively form a 10β-hydroperoxide (Sedee and Beijersbergen van Henegouwen, 1983, 1985). This hydroperoxide easily decomposes into a *p*-quinol (Fig. 18).

After exposure to visible light for 15 min of a solution of [4-^{14}C]estrone, albumin and haematoporphyrin to visible light, 30% of the radioactivity was irreversibly bound to proteins. (It took 90 min exposure to daylight only – winter's day in Holland, sample at 1 m from the window – to get 8%

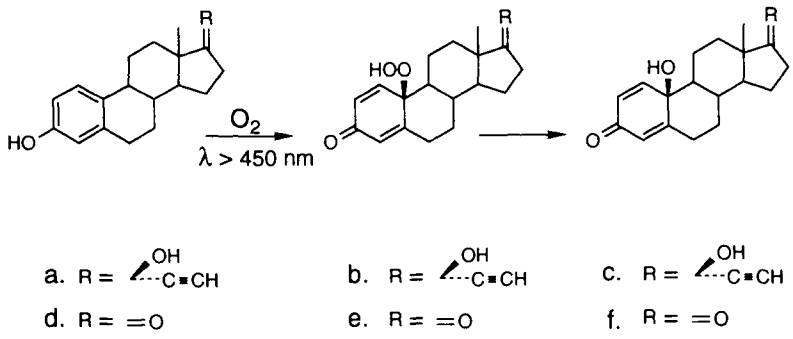


FIG. 18. Photosensitized decomposition of ethynylestradiol (a) and estrone (d) into a 10β-hydroperoxide (b, e) and decomposition of the latter into *p*-quinol (c, f).

irreversible binding to albumin.) The 10β-hydroperoxide and not the *p*-quinol proved to be the reactive intermediate (Sedee *et al.*, 1984b). Almost the same percentage was found when albumin was added after irradiation; this indicates that the lifetime of the hydroperoxide formed is long enough to find a target molecule.

Because a high level of endogenous photosensitizers is a common side-effect of contraceptive steroids, the photosensitized binding of estrogens can be pathologically relevant in allergic disorders (see references in Sedee *et al.*, 1984b) because phenolic steroids chemically bound to proteins have antigenic properties. Furthermore, EE_2 can induce the formation of anti-EE_2-antibodies in women who suffer from erythema caused by oral contraceptives. In lupus erythematosus, photosensitivity and other disorders with immunogenic features, the natural and synthetic estrogens have also been incriminated (see references in Sedee *et al.*, 1984b). That the skin is an important steroid-metabolizing organ, that subcutaneous adipose tissue is a site of steroid storage, that photosensitizers, whether or not endogenous, are present in the body and that 10–50% of visible light can reach the dermis, are facts which facilitate photosensitized irreversible binding of estrogens to proteins. It offers an explanation for allergic side-effects of estrogens.

The photosensitized reaction of estrone was similarly studied in the presence of DNA, because steroids produce their effect after the formation of a steroid–receptor complex via DNA. A very strong interaction was observed between DNA and the 10β-hydroperoxide compared with that between other steroids and DNA (Sedee *et al.*, 1984c).

If a photosensitizer is absent and the estrogen mestranol (or ethynylestradiol EE_2) itself absorbs the light, it is quite photostable.

Contrary to this, norethisterone decomposes by absorption of UVB but is stable towards photosensitization. With regard to the progestogens, norethisterone (NE) is the compound of choice in an investigation into the cause of light-induced side-effects of oral contraceptives; it is not only commonly applied but also is the principal metabolite of some of the most frequently used 19-nor-progestagens.

Norethisterone-4β,5β-epoxide is by far the main photoproduct of NE

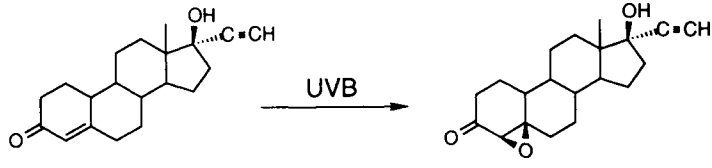
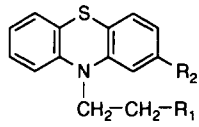


FIG. 19. Formation of the 4β, 5β-epoxide upon exposure of norethisterone to UVB.

(Fig. 19); 23% of the total amount of products after 30 min UVB-exposure (280–320 nm; $I = 12.5\ W\ m^{-2}$), which was raised after the irradiation to 34% by keeping the solution at 37°C for 4 h (Sedee *et al.*, 1983). NE is not prone to the skeletal rearrangement usually observed with steroids possessing a C10-methyl group. Under the reaction conditions applied (1.7×10^{-4} M NE in buffer pH 7.4 containing 10% alcohol) the *c.* 25 products identified were mainly formed by addition of solvent molecules, oxygen or a second steroid molecule (Sedee *et al.*, 1985c,d).

NE forms reversible complexes with serum proteins, receptor proteins of target tissue, and with steroid-metabolizing enzymes. The conjugated ketone function of NE is essential for this reversible binding; however, it is just this function which is also susceptible to photooxidation and photoaddition (Sedee *et al.*, 1983, 1985c,d). Thus, formation of an epoxide and photoaddition to other molecules suggested that NE could irreversibly bind to proteins under the influence of UVB. Human plasma (1.0 ml) or an albumin solution (38 mg ml^{-1}, 1.0 ml) was added to 4.0 ml [^{14}C]NE (5×10^{-7} M in 0.04 M phosphate buffer pH 7.4) and exposed to UVB (280–320 nm; $I = 12.5\ W\ m^{-2}$). Irreversible binding increased linearly with irradiation time and occurred very efficiently in both cases. After 40 min exposure to UVB, 33% of starting NE had photoreacted; half of this 33% proved to be photobound to proteins (Sedee *et al.*, 1984d). Interestingly, irreversible binding occurred even when proteins were added only several hours after the irradiation of NE (still 10% of decomposed NE after 22 h). Photoproducts incubated after the irradiation with a bacterial test system proved to be toxic and irreversible binding to bacterial macromolecules was also observed (Sedee *et al.*, 1985e).

Since contraceptive ingestion has been associated with allergic effects, irreversible binding of NE to proteins as a result of photoexcitation may be important. Irreversible binding occurring even several hours after irradiation implies that inner organs can also be a target of photoproducts (e.g., see sections 2.2.5.4 and 2.2.6.1). Therefore, some preliminary experiments were carried out with rats that received 100 μg [^{14}C]NE intraperitoneally followed by exposure to UVB for 3 h. The intensity of UVB used ($5\ W\ m^{-2}$) is quite normal in summer. With irradiated rats, irreversible binding was significantly higher to macromolecules of the skin but also to those of the liver than with control animals (Sedee *et al.*, 1985e). These preliminary findings deserve further investigation, especially because the amount of irreversible binding can increase considerably at higher ambient temperatures (see Fig. 25 and section 2.2.5.4). In this connection, it also is important to mention that NE-4β,5β-epoxide which is stable at 37°C and pH 7.4 for at least 1 hour, is suspected to be the cause of adverse effects on the liver (see references in Sedee *et al.*, 1985). A clinical trial has shown NE-4β,5β-epoxide to cause contact-allergy (H. van Weelden, personal communication, 1986).



R_1	R_2	COMPOUND
$-CH_2-N(CH_3)_2$	—Cl	CPZ
	—H	PZ
	—OH	2—OH-PZ
H_3C-N	$-S-CH_3$	TRZ
	$-\overset{\overset{O}{\|}}{S}-CH_3$	MRZ
	$-\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}-CH_3$	SRZ

FIG. 20. Structural formula of chlorpromazine (CPZ), promazine (PZ), 2-hydroxypromazine (2-OH-PZ), thioridazine (TRZ), mesoridazine (MRZ) and sulforidazine (SRZ).

2.2.4 Chlorpromazine and other Phenothiazines

Like many other compounds, such as polycyclic aromatic hydrocarbons, acridine derivatives or methylene blue, phenothiazine drugs are able to form molecular complexes with nucleic acids. Although these complexes, formed by partial intercalation between two adjacent base pairs, are reversible, they can cause adverse biological effects owing to interference with essential processes controlled by DNA. Therefore, much interest in the interaction between phenothiazine drugs and DNA remains justified; the situation can also be aggravated by irreversible binding to DNA/RNA, for instance by photoexcitation of the phenothiazine.

Most studies concern chlorpromazine (CPZ; Fig. 20), a prototype of this class of antipsychotic drugs. Thioridazine (TRZ; Fig. 20) not only resembles CPZ with regard to its pharmacological activity but also in adverse biological effects. As with CPZ, exposure to sunlight can cause a grey to purple skin pigmentation and ocular opacity in man. TRZ was found to be almost as phototoxic as CPZ in *Candida albicans* and in mice (see references in Schoonderwoerd *et al.*, 1990). Marko *et al.* (1985) found that phenothiazines, including TRZ, photochemically induce dynamic nuclear polarization in

nucleic acid bases and nucleotides. This polarization has been explained by electron transfer from photoexcited phenothiazine to the bases and seems to be correlated with the phototoxic potency of the drug. Results were recently obtained for TRZ and two of its major metabolites, mesoridazine (MRZ) and sulforidazine (SRZ), (Schoonderwoerd *et al.*, 1990) which correspond with this hypothesis:

In vitro, UVA-exposure (320–380 nm; max = 350 nm) of TRZ and MRZ in saline produced a transient colour (TRZ: blue; MRZ: purple). These coloured unstable intermediates are probably the result of photoionization (formation of a radical cation by abstraction of an electron from the sulfur atom). This was supported by the fact that TRZ and MRZ produced the same transient blue and purple colour, respectively, on treatment with peroxidase. (The results found with peroxidase correspond with those obtained by others; see referencs in Schoonderwoerd *et al.*, 1990). On UVA irradiation of SRZ, a coloured intermediate was not produced to a measurable extent, while only a slightly yellow colour was observed upon peroxidase treatment.

Ionization of the phenothiazines was accompanied by irreversible binding to DNA; with regard to the latter the same order was found for both peroxidase treatment and UVA exposure: TRZ > MRZ > SRZ. (Almost no photobinding with SRZ.) The rate of photodecomposition also decreased from TRZ, MRZ to SRZ. Oxidation of the sulfur which is substituted at the 2-position of the phenothiazine molecule thus makes this compound less susceptible to (photo-)ionization. The amount of phenothiazine which photoreacted divided by the amount irreversibly photobound to DNA gave the same result for TRZ, MRZ and SRZ. Therefore, although sulfoxidation diminishes the rate of phenothiazine photodecomposition, the *intrinsic* reactivity of the radical cation towards DNA seems the same for TRZ, MRZ and SRZ.

Photo-induced genetic effects, as investigated by means of a differential DNA repair test in *Escherichia coli*, also decreased from TRZ to MRZ to SRZ (Schoonderwoerd *et al.*, 1990). As MRZ has less genetic effects than TRZ and the metabolite SRZ did not induce any such effect at all, metabolism of the drug can be considered in this case as a detoxification process.

An interesting analogy exists between the above and the behaviour of CPZ. Peroxidase treatment of CPZ produced a red-coloured radical cation (De Mol and Busker, 1984) but, in contrast to TRZ and MRZ, UVA irradiation of CPZ did not yield this species. Photo-induced ionization of CPZ to this red-coloured intermediate occurs upon irradiation with UVC (254 nm; Gubitz *et al.*, 1973) but at longer wavelengths (> 270 nm) dechlorination was observed rather than ionization. Photohomolytic dechlorination into promazinyl radicals has been demonstrated *in vitro* by Motten *et al.* (1985). It is known that *in vitro* formation of the CPZ radical cation can lead to chlorpromazinesulfoxide (CPZSO), whereas dechlorination by

photohomolysis produces the promazinyl radical with promazine (PZ) and 2-hydroxypromazine (2-OH-PZ) as the stable products (see references in Schoonderwoerd *et al.*, 1989).

More recently (van den Broeke *et al.*, 1994) showed that irradiation of CPZ in buffered aqueous solution with UVB or UVA resulted in 65% and 90% 2-OH-PZ, 5% and 7% PZ and only 2% and 0% CPZSO under aerobic and anaerobic conditions, respectively. Thus, photodegradation of CPZ *in vitro* under conditions relevant to the *in vivo* situation proceeds almost entirely by dechlorination rather than by radical cation formation (the essential pathway of CPZSO production).

This *in vitro* study supported *in vivo* results earlier obtained by Schoonderwoerd *et al.* (1989). The latter investigators showed that *in vivo* (rats exposed to UVA (345–410 nm; maximum 370 nm; $I = 30\ W\ m^{-2}$) after i.p. administration of CPZ), photohomolysis is by far the dominant process as concluded from the photometabolites PZ and 2-OH-PZ. This *in vivo* finding is interesting with regard to the extent to which phototoxicity is to be expected with phenothiazines: the presence of an aromatic chlorine atom at the 2-position is important. This corresponds with the fact that PZ is much less phototoxic in mice than CPZ (Ljungren and Möller, 1977).

Many *in vitro* studies focus on the photo-induced addition of phenothiazines to DNA. CPZ has received most of the attention, not only in this respect, but also with regard to genetic effects resulting from UV exposure. However, side-effects of CPZ that can be connected with photomutagenic properties observed in cellular test systems, have not been reported. That *in vitro* data from research into photoreactivity of a drug should be verified *in vivo*, is demonstrated by results with CPZ:

Rats were exposed to UV-radiation with maximum intensity either at 310 nm ($I = 4\ W\ m^{-2}$), 370 nm ($I = 18\ W\ m^{-2}$) or at 420 nm ($I = 6.5\ W\ m^{-2}$) for 6 h after i.p. administration of [^{3}H]CPZ ($12\ mg\ kg^{-1}$). In each of the three cases, the treatment was repeated on each of 4 consecutive days. In the ears, eyes and skin of the back, irreversibly bound radioactivity was found after irradiation with 310 and 370 nm, but not with 420 nm light (Schoonderwoerd and Beijersbergen van Henegouwen, 1987). Irreversible binding was found to lipids and proteins only, but surprisingly not to DNA.

To investigate whether the bioavailability of CPZ is a causative factor of the latter finding, the drug was applied topically and a comparison (Schoonderwoerd *et al.*, 1991a) was made with 8-methoxypsoralen (8-MOP) whose *in vitro* photoreactivity to DNA has been the subject of many studies. After applying the same amount per cm^2 of either [^{3}H]-8-MOP or [^{3}H]-CPZ on the back skin, rats were exposed to UVA for 1 h (345–410 nm; maximum = 370 nm; $I = 30\ W\ m^{-2}$). Photobinding to epidermal biomacromolecules, determined immediately after the irradiation, was as follows: proteins, 8-MOP 0.25 ± 0.03 and CPZ $0.17 \pm 0.02\ nmol\ mg^{-1}$ protein; Lipids: 8-MOP 1.17 ± 0.21 and CPZ $0.81 \pm 0.08\ nmol\ mg^{-1}$ lipid;

DNA/RNA, 8-MOP 8.95 ± 0.5 and CPZ < 0.5 molecules per 10^4 nucleotides. With regard to photobinding to lipids and proteins, there was only a small difference between 8-MOP and CPZ. However, as far as DNA was concerned, the irreversible binding of CPZ was at least 16 times lower than that of 8-MOP. That this was caused by the low bioavailability of CPZ near the genetic material, was supported by *in vitro* data (Schoonderwoerd *et al.*, 1991a). With a fivefold lower light dose, but the same irradiation conditions as in the *in vivo* experiments, CPZ exceeded 8-MOP in photobinding to DNA ([8-MOP] = [CPZ] = 0.5 mM, [DNA] = 1 mg ml^{-1}; photobinding 8-MOP = 120 and CPZ = 260 molecules per 10^4 nucleotides). In this connection it is also important to mention that the photoproducts of CPZ *in vivo* (Schoonderwoerd *et al.*, 1989) correspond with those found under *in vitro* conditions (van den Broeke *et al.*, 1994). Thus, because of the low bioavailability near the genetic material, light-induced genotoxicity as a side-effect of CPZ is not to be expected.

When photobinding to epidermal biomacromolecules was investigated with rats killed several hours after UVA-exposure had been stopped, a remarkable difference between CPZ and 8-MOP was observed (Schoonderwoerd *et al.*, 1991a). For instance after 24 h, photobinding expressed as a percentage of that found immediately after the irradiation was: to proteins, CPZ 67%, 8-MOP 31%; and to lipids, CPZ 67%, 8-MOP 8%. After 5 days, photobinding of CPZ to lipids was still 24% (8-MOP 1%) while that of proteins was 41% and 24%, respectively. In connection with the fact that CPZ is known to cause photoallergy (Johnson, 1984; Epstein and Wintroub, 1985; Kochevar, 1987), it is interesting that the *in vivo* photobinding of CPZ is more persistent than that of 8-MOP. (Photobinding to proteins and membranes (lipids) is considered important in the sequence of events which eventually lead to photoallergy.)

It is noteworthy that photobinding of CPZ *in vivo* could be induced even with long-wave UVA (345–410 nm; maximum = 370 nm), whereas CPZ has its maximum in the UVB region, namely at 310 nm. This corresponds with detailed action spectrum studies showing that in general, patient CPZ-photosensitivity is enhanced by UVA rather than by UVB. Furthermore, CPZ-treated mice are also very sensitive to UVA (maximum between 330–380 nm; see references in Schoonderwoerd *et al.*, 1988).

A possible explanation for this maximum in the action spectrum at long wavelengths is that phototoxic effects can also be caused by CPZ metabolites. The possible role of CPZ sulfoxide (CPZSO) has been stressed in this connection (see references in Schoonderwoerd *et al.*, 1988). Sulfoxidation is a major metabolic route; more than half of a 50 mg oral dose of CPZ becomes available as the sulfoxide, therefore, a comparison between CPZ and CPZSO has been made (Schoonderwoerd *et al.*, 1988).

The bioavailability as a function of time in the skin, ears and eyes of rats after i.p. administration (13 mg kg^{-1}) was almost the same for CPZ

and CPZO. Despite this, UVA-exposure (345–410 nm; maximum 370 nm) resulted in less photobinding after CPZO than after CPZ administration (Schoonderwoerd *et al.*, 1988). Obviously, photobinding of CPZSO occurs less efficiently than that of CPZ, because CPZSO absorbed two times more photons emitted by the lamps than CPZ (Schoonderwoerd *et al.*, 1988). Because CPZSO binds to skin components upon UVA irradiation it can play a role in causing phototoxic side-effects, as observed after CPZ treatment. However, from *in vitro* investigations it was concluded that CPZSO binds considerably less to HSA upon irradiation with UVB (maximum = 310 nm) or with UVA (maximum = 370 nm), despite the fact that in both cases, it absorbed twice as much radiation than CPZ. The observed long wavelength maximum in the photosensitivity should therefore be attributed to CPZ rather than its sulfoxidated metabolites. As will be seen below, this hypothesis is also supported by the fact that CPZ, but not CPZSO, photobinds *in vitro* on exposure to 420 nm light:

In sharp contrast to CPZ, almost no photobinding was found in the eyes after CPZSO administration. An explanation for this was found by taking into account the fact that the optical properties of the eyes differ from those of the skin (most radiation with wavelengths shorter than 370 nm does not reach the vitreous body). Careful investigation of the absorption spectra of both CPZSO and CPZ revealed that CPZ still absorbs radiation with wavelengths longer than 380 nm but CPZSO does not. *In vitro*, it was demonstrated that CPZ but not CPZSO can photobind to HSA upon exposure to 380–480 nm light (maximum = 420 nm). Hence, CPZSO can photobind in a very small part of the eye only, thus providing an explanation for the *in vivo* results (Schoonderwoerd *et al.*, 1988).

The ratio of the bioavailability in the epidermis (Ep) and in the dermis (De) was the same for both compounds (0.15). Despite this, CPZSO photobound relatively more in the epidermis than in the dermis compared with CPZ (Ep_{bound}/De_{bound} 0.84 and 0.39, respectively). The difference was explained in a way comparable to that described for the eyes: CPZ photobinds more efficiently than CPZSO, especially with longer wavelength radiation and it is just this light which reaches the dermis. (percentage of incident light 0.27% at 297 nm; 9.5% at 313 nm; 19% at 365 nm; see references in Schoonderwoerd *et al.*, 1988).

2.2.5 Nitroarenes

2.2.5.1 Nifedipine. This drug belongs to an important group of calcium antagonists of which the prototypes had already been synthesized in the 1960s but of which new therapeutic possibilities were discovered in the early 1970s. They are used in the treatment of angina pectoris and arterial hypertension.

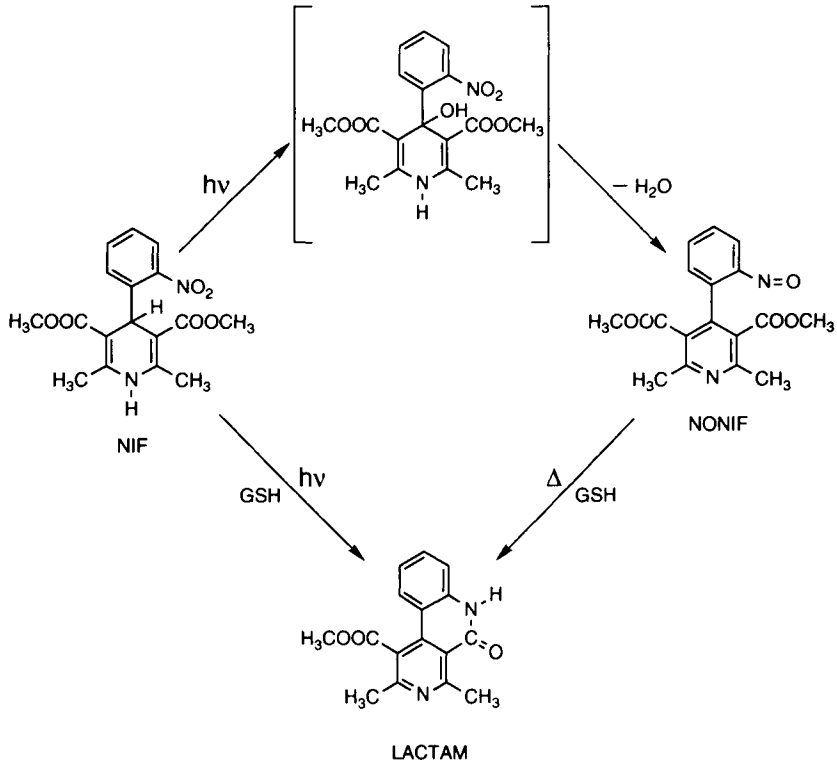


FIG. 21. Photodegradation (hν) of nifedipine (NIF) in 0.01 M phosphate buffer pH 7.4. Without glutathione (GSH) being present, the nitroso derivative (NONIF) is formed and with GSH the lactam derivative (both quantitatively). With GSH present, NONIF is an intermediate. This is demonstrated by the thermochemical reaction (Δ) of pure NONIF with GSH in the same medium, which also quantitatively produces the lactam.

Nifedipine (NIF) is extremely sensitive to ultraviolet radiation and to visible light up to 450 nm. The quantum yield for photodegradation is *c.* 0.5 (Thoma and Klimek, 1985a); statistically, this means that of every two photons absorbed, one causes decomposition of a NIF molecule.

The *ortho*-nitrophenyl derivatives, NIF (Fig. 21) and nisoldipine prove to be far more photolabile than corresponding 1,4-dihydro-4-(nitrophenyl)pyridines with a *meta*-nitro group, e.g., nicardipine, nitrendipine and nimodipine (De Vries and Beijersbergen van Henegouwen, unpublished results). An explanation for this can be an intramolecular redox reaction which simultaneously leads to reduction of the nitro group to nitroso and oxidation of the dihydropyridine ring to pyridine (Fig. 21). This redox

reaction proceeds more smoothly if both groups involved, nitro and dihydropyridine, are at a small distance from each other.

By far the main product of the photodecomposition is a nitroso compound (Thoma and Klimek, 1985a,b). De Vries and Beijersbergen van Henegouwen (1995) found that the UVA-induced conversion of NIF (1 μg ml^{-1} phosphate buffer pH 7.4) to its nitrosoderivative (NONIF) is even quantitative (more than 99%).

However when NIF is irradiated in the presence of glutathione (GSH), in plasma and in blood, NONIF is a short-lived intermediate only. With 10^{-3} M GSH as the only reactant present in phosphate buffer pH 7.4, a lactam is formed quantitatively. That NONIF is an instabile intermediate in the photoreaction with GSH was proved as follows. NIF was first converted into NONIF by UVA, without GSH being present. Subsequently, GSH was added and within 10 s of incubation in the dark, the same percentage of lactam (more than 99%) was formed as obtained during simultaneous irradiation of NIF with GSH.

As NIF is complexed with plasma proteins for *c.* 95%, its photolability and the high reactivity of its first and main photoproduct NONIF, implies the possibility of irreversible photobinding to proteins. This has been found by several investigators (Campbell *et al.*, 1984; Ichida *et al.*, 1989a,b). Irreversible photobinding to protein can provide an explanation for photosensitivity associated with NIF which has been reported several times (Thomas and Wood, 1986).

Preliminary results indicate that the photoreaction of NIF, with the lactam as an endproduct, also takes place *in vivo*. The clearing of NIF from the blood is also faster in the UVA-exposed rat than in the animal kept in the dark (De Vries and Beijersbergen van Henegouwen, unpublished results).

2.2.5.2 Artificial Musk Ambrette. Musk ambrette (Fig. 22), a widely applied perfume component, is a notorious photoallergen in man (Ford, 1984; Parker *et al.*, 1986). An important route to the occurrence of photoallergy is due to a reactive form of the photosensitizing agent which combines covalently with a protein to produce an allergen. Photobinding of musk ambrette to human serum albumin has been demonstrated by Barrett and Brown (1985).

From electron spin resonance (ESR) measurements it was concluded that the first step in the photodecomposition of musk ambrette with UVA is the production of two nitro anion radicals (Motten *et al.*, 1983). Photolysis of a 3% solution in CH_3OH/0.1 M NaOH gave an azoxy derivative (7%) besides an azo compound as the main product (42%).

These data have allowed us to postulate the mechanism of photodecomposition (Fig. 22). Disproportionation of a nitro anion radical upon light exposure forms a nitroso compound and the original musk ambrette. Reduction of the nitroso compound generates the corresponding

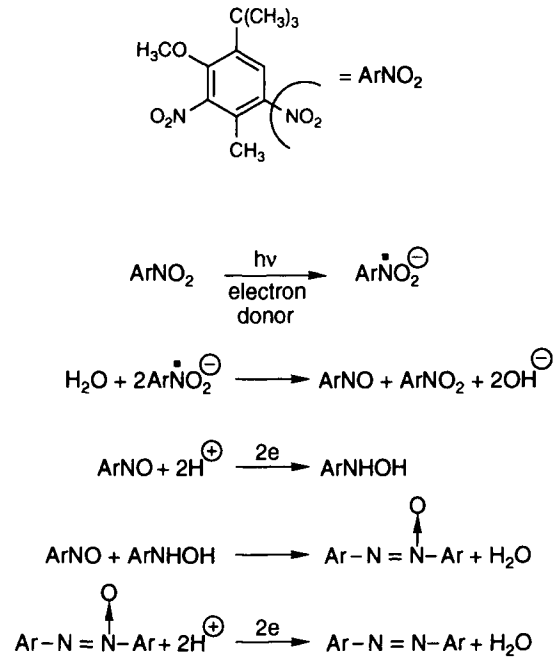


FIG. 22. Mechanism of the photodecomposition of musk ambrette ($ArNO_2$).

hydroxylamine derivative, which can condense with another nitroso molecule to yield the azoxy photoproduct. The condensation reaction and the subsequent reduction of the azoxy compound to an azo derivative occur only with relatively high concentrations of the reactants and are not relevant to the *in vivo* situation.

More important to the problem of photoallergy is the observation that in addition to nitro anion radicals, nitroso and hydroxylamine compounds are formed. The last two species are considered to be even more reactive with protein than the radical itself.

In addition to *in vitro* photochemical research, some photobiological experiments were performed with human lymphoid cells (Morison *et al.*, 1982); these showed that musk ambrette is phototoxic to immune cells, as measured by decreased incorporation of [^{3}H]thymidine into nuclear DNA.

Thus, it is supposed that *in vivo* in the light exposed skin haptens from musk ambrette (such as nitro anion radical, nitroso and hydroxylamine derivatives), trigger an immune response by reacting with plasma proteins and immune cells, leading to photoallergy.

2.2.5.3 Chloramphenicol. Chloramphenicol (CAP) is an antibiotic effective against a wide range of life-threatening bacteria. It is administered

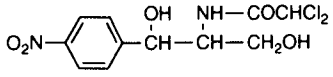
systemically, e.g., in the treatment of central nervous system infections, but also topically, e.g., against deep ocular infections, where it is a first choice drug. CAP is a bone marrow toxin affecting primarily the erythroid precursors. Although rare, anaemia as a side-effect of CAP can be fatal; this has also been observed after topical application (Frauenfelder *et al.*, 1993).

It is supposed that reactive intermediates damage the bone marrow cells. One of the routes, along which these reactive intermediates may occur, is metabolic reduction of the nitro group in the liver. *p*-Nitrosochloramphenicol (pNOCAP) has been extensively investigated in this regard; *in vitro* it proved to be very toxic towards bone marrow cells (Yunis *et al.*, 1980).

That reactive intermediates can also be formed as a result of exposure to sunlight was pointed out by De Vries *et al.* (1984). They showed that CAP *in vitro* was photodecomposed for *c.* 80% on exposure to sunlight for 45 min (UVA = 14 W m^{-2}). The initial concentration of CAP (10 mg l^{-1} in phosphate-buffered saline, PBS) was comparable with that found in blood after systemic administration or in the aqueous humour of the eye up to 1 h after topical application.

In addition to the 20% CAP remaining, 25% *p*-nitrobenzaldehyde (pNB), 36% *p*-nitrosobenzoic acid (pNOBA) and 15% *p*-nitrobenzoic acid (pNBA) were determined (Fig. 23).

The rapid photodecomposition of CAP under conditions relevant to the *in vivo* situation, and the reactivity of some of the photoproducts, necessitated further research (De Vries *et al.*, 1994). The toxicity of CAP and the photoproducts towards bone marrow cells *in vitro* was determined. For CAP, pNB and *p*-nitrobenzyl alcohol (pNBOH) the values found correspond quite well with those reported in the literature (Gross *et al.*, 1982) (Table 1). As can be seen pNB and pNOBA, with a similar value to pNOCAP (1.2), are far more toxic towards bone marrow cells than CAP.



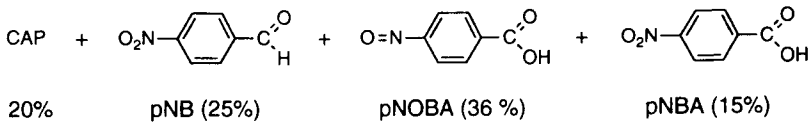


FIG. 23. Composition (Mol%) of a CAP solution (10 mg l^{-1} in PBS) after exposure to sunlight (UVA = 14 W m^{-2}) for 45 min. CAP = chloramphenicol, pNB = *p*-nitrobenzaldehyde, pNOBA = *p*-nitrosobenzoic acid, pNBA = *p*-nitrobenzoic acid.

TABLE 1

Toxicity towards bone marrow cells *in vitro* (De Vries *et al.*, 1994). Represented is %[^{3}H]thymidine incorporation; control without compound is put at 100%

Compound	%[^{3}H]Thymidine incorporated	%[^{3}H]Thymidine literature values
pNOCAP	n.d.	1.2
CAP	44	42.6
pNBA	57	n.a.
pNB	2	1.7
pNOBA	7	n.a.
pHABA	7	n.a.
pABA	73	n.a.
pNBOH	73	73

Abbreviations (also see Fig. 23): pHABA = *p*-hydroxylaminobenzoic acid, pABA = *p*-aminobenzoic acid, pNBOH = *p*-nitrobenzylalcohol; n.d., not determined; n.a., not available.

Literature values from Gross *et al.*, 1982 (CAP, pNB and pNBOH) and Yunis *et al.*, 1980 (pNOCAP).

To act as a bone marrow toxin, a reactive intermediate should be stable enough to be transported by the blood from its site of formation to the target tissue. Eyer *et al.* (1984) found that pNOCAP was rapidly eliminated from human blood (half life is less than 15 s). For this reason, they found it unlikely that pNOCAP, when enzymatically formed in the liver, could be the cause of bone marrow toxicity of CAP. In this regard, the photoproducts pNB and pNOBA seem to be more serious candidates; their half-life in human blood proved to be 1.7 min and 3.7 min, respectively. Thus, once formed photochemically in the skin they are stable enough to reach the bone marrow.

In the presence of GSH, endproducts of the photoreaction of CAP are *p*-nitrobenzoic acid (pNBA), *p*-nitrobenzyl alcohol (pNBOH) and *p*-aminobenzoic acid (pABA). pNB and pNOBA are intermediates only; when dissolved in PBS containing glutathione, pNB was converted to pNBA and pNBOH, while pNOBA gave pABA (De Vries *et al.*, 1994). It was proved, also by Ellis *et al.* (1992), that *p*-hydroxylaminobenzoic acid (pHABA) is an intermediate in the conversion of pNOBA to pABA. The *in vitro* bone marrow cell toxicity of pHABA is comparable to that of pNOBA (Table 1).

UVA-exposure of CAP in rat blood produced pNBA, pNBOH and pABA. Incubation of possible intermediates in the dark gave the same results as described for the photoreaction in PBS with GSH, indicating the same reaction mechanism (De Vries *et al.*, 1994).

TABLE 2

Generation of methaemoglobin (MetHb as a percentage of total Hb) in rat blood by CAP and photoproducts (0.1 mg ml^{-1}). UVA: 60 W m^{-2}; 345–410 nm; maximum = 370 nm; 30 min.

	% MetHb
Untreated blood	0.3
Blood + UVA	1.1
CAP	0.2
CAP + UVA	4.3
pNBA	0.2
pNOBA	30.9
pHABA	16.1
pABA	0.2
pNBOH	0.3

Abbreviations see Table 1.

With regard to the side-effects of CAP, irreversible binding to plasma proteins and cell constituents in the blood is considered important. Irreversible binding is likely to occur simultaneously with the formation of the reactive intermediates mentioned, pNB, pNOBA and pHABA. This indeed was found when [^{3}H]CAP in human blood was irradiated with UVA *in vitro*; irreversible binding was found to plasma proteins and also to cell constituents (De Vries *et al.*, 1994).

In various adverse effects from drugs, oxidative stress plays a key role. To investigate whether this is relevant to the occurrence of the side-effects of CAP, photoproducts were tested for their ability to generate oxidative stress (De Vries *et al.*, 1994). This was done by the determination of the amount of methaemoglobin (MetHb) formed during incubation of the photoproducts in blood (Table 2). Only the photoproducts pNOBA and pHABA, both without irradiation, proved to be able to generate considerable oxidative stress. This indicates that the photoproducts pNOBA and pHABA play an important role in the occurrence of oxidative stress resulting from CAP + UVA.

To determine whether oxidative stress also occurs *in vivo*, Wistar rats, whose backs were shaved, were given 100 mg CAP i.p. and exposed to UVA (45 W m^{-2}; 345–410 nm; maximum 370 nm) for 4 h. This resulted in 3.6 times the basic level of MetHb (De Vries *et al.*, 1994).

Further evidence that CAP photodecomposes *in vivo* was investigated as follows (De Vries *et al.*, 1994). Each of six rats (200 g) of which the back was shaved, was given 30 mg [^{3}H]CAP (i.p.). Three were exposed to UVA (50 W m^{-2}; 345–410 nm; maximum 370 nm) for 10 h and three kept in

light-poor environment. This was repeated the next day after which the rats were killed. After dialysis under non-equilibrium conditions, irreversible bound CAP was determined and expressed per mg protein (the latter is a measure for the amount of dorsal skin taken). Values found were 463 ± 13 and 51 ± 15 disintegrations per minute (d.p.m.) per mg protein for the UVA-irradiated and non-exposed rats respectively. Irreversible binding found *in vivo* corresponds with data from *in vitro* experiments with blood and evidences that the photodecomposition proceeds via the formation of reactive intermediates derived from CAP such as pNB, pNOBA and pHABA.

The results from the *in vitro* and *in vivo* photochemical research described above, justify the conclusion that the possibile relationship between sunlight exposure and anaemia, as a systemic side-effect of CAP, deserves further research.

2.2.5.4 Nitrofurantoin. The urinary tract disinfectant nitrofurantoin (NFT) is notorious for its high incidence of serious side-effects. Of these side-effects, 40% are thought to be allergic reactions affecting especially the lungs and the skin.

NFT is also known to cause a considerable number of haematological reactions; no less than 20% of the fatalities by NFT are caused by blood dyscrasia. Blood dyscrasia also include methaemoglobinemia, a clinical condition in which more than 1% of the blood haemoglobin has been oxidized to the ferric form.

Incomplete metabolic reduction of the nitro group of NFT in the liver resulting in reactive intermediates, has been proposed as the origin of most of the side-effects of this drug. However, the exact mechanism involved is not known.

In this context, and taking into consideration that NFT is photolabile on irradiation with UVA, the possible role of light in the activation of NFT was investigated.

On UVA-exposure of a NFT solution in PBS (1 mM, pH = 7), 60% of the photodecomposed material proved to be converted into 5-nitrofurfural (NFA).

NFA is also photolabile. Under the same conditions, up to 70% of NFA loses nitrite with formation of 5-hydroxymethylene-2(5H)-furanone (HMF). Extensive research of product formation and reaction kinetics revealed that HMF is a tautomer of 5-hydroxyfurfural and that the conversion of NFA into the latter aldehyde probably proceeds via nucleophilic substitution with a sigma-complex as an intermediate (Fig. 24) (Busker and Beijersbergen van Henegouwen, 1987).

Since nitrite is a major photoproduct of NFA and is a well-known inducer of methaemoglobin (MetHb), it was investigated whether photodecomposition of NFT in human blood *in vitro* results in increased MetHb and nitrite

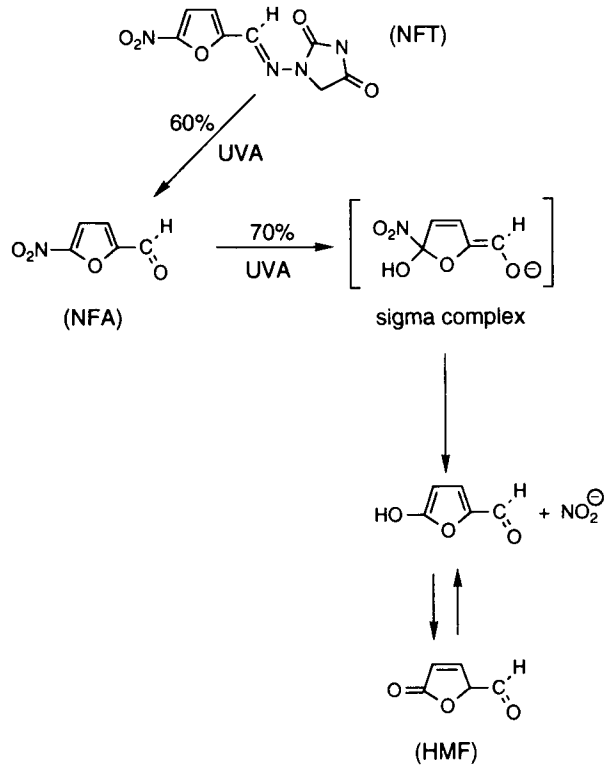


FIG. 24. Photodecomposition of nitrofurantoin (NFT) into 5-nitrofurfural (NFA) and 5-hydroxymethylene-2(5H)-furanone (HMF).

levels. A considerable increase in both MetHb and nitrite concentration was observed only after simultaneous exposure to NFT and UVA. The first photoproduct of NFT, NFA, was found to play an important role in the formation of MetHb (Busker *et al.*, 1988a).

To investigate a possible relation between exposure to (sun)light and methaemoglobinaemia as a side-effect of NFT, *in vivo* experiments were performed with rats (Busker *et al.*, 1988a). The experimental conditions and results are summarized in Table 3. NFA was given intraperitoneally (i.p.) instead of orally (p.o.) and in lower doses per day because it is a (photo)metabolite of NFT. Exposure to UVA alone (b) or administration of NFT or NFA, both without exposure to UVA (c and e respectively) gave a MetHb% which did not differ significantly from the normal value (a) (for more details see Busker *et al.*, 1988a).

The rat can nullify an increased MetHb level very efficiently and is not the most suited species for this experiment. Nevertheless, the increase of the

TABLE 3

Methaemoglobin (MetHb) level of rats (measured in triplicate) after the treatments mentioned in text (a–f). Normal MetHb = 0.5%. UVA exposure (25 $W m^{-2}$; 320–410 nm; maximum = 370 nm) for 12 h day^{-1}; c and d (and also e and f) differ significantly ($P < 0.01$; Student *t*-test; Busker *et al.*, 1988a)

	Compound	Days	Doses (mg day^{-1})	UVA	MetHb%	*n*
a	–	4	–	No	0.5	10
b	–	4	–	Yes	0.6	10
c	NFT	4	12 (p.o.)	No	0.5	8
d	NFT	4	12 (p.o.)	Yes	1.0	12
e	NFA	2	3 (i.p.)	No	0.4	5
f	NFA	2	3 (i.p.)	Yes	1.3	6

MetHb% is clear, it occurs only as a result of simultaneous exposure to NFT (or NFA) and UVA.

Together with the *in vitro* photochemical data mentioned above, these *in vivo* experimental results strongly point to a relation between (sun)light exposure and methaemoglobinaemia as a systemic side-effect of NFT.

Allergic drug reactions are considered to arise from an immune response against adducts between the drug and biomacromolecules, e.g., plasma proteins. The formation of adducts may result from activation by biotransformation. However, because of the photolability of NFT, irreversible binding to biomacromolecules can also be caused by UVA radiation.

In vitro, efficient photobinding to human serum albumin was demonstrated for both NFT (up to 50 nmol mg^{-1} protein) and its primary photoproduct NFA. Incubation in the dark of end-products of the photodecomposition of NFT and NFA, such as HMF, with plasma proteins also resulted in irreversible binding. Protein amino groups, and to a lesser extent thiol groups, proved to be targets for binding. Furthermore, a significant decrease in isoelectric point of albumin was observed (Busker *et al.*, 1989).

Whether adducts are also formed *in vivo* was investigated in rats (Busker *et al.*, 1988b). Each of eight rats (150 g) of which the back was shaved, was given 80 mg kg^{-1} [^{14}C]NFT (p.o.). Four were exposed to 50 $W m^{-2}$ UVA (345–410 nm; maximum = 370 nm) for 10 h day^{-1} and four were kept in a dimly-lit environment. This was repeated the next day, after which the rats were killed. Irreversible bound NFT was determined and expressed per mg protein as a measure for the amount of organ material taken. The results are represented in Fig. 25.

More irreversible binding was found in the UVA exposed animals than in those kept in a dimly lit environment, this involved the skin of the back (both epidermis and dermis), ears, plasma protein and spleen and also (not

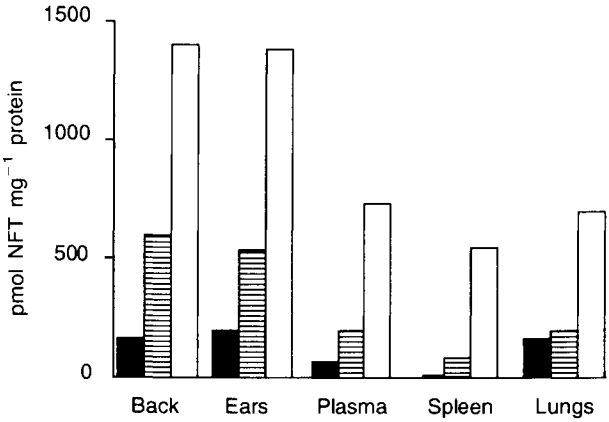


FIG. 25. Irreversible photobinding of NFT to biomacromolecules in various organs (NFT 80 mg kg^{-1} plus UVA for 2 days). Except for the lungs, UVA 22°C and Dark differ significantly ($P < 0.0001$, Student t-test). □ UVA 32°C; ▤ UVA 22°C; ■ Dark.

represented in Fig. 25) the tail and eyes. When rats were kept at 32°C instead of 22°C during NFT/UVA treatment, even more irreversible binding was observed (Fig. 25). This was probably caused by an increased dermal blood flow. Under these conditions, irreversibly bound radioactivity was found even in the lungs and (not represented) in kidneys and liver (Busker *et al.*, 1988b). Other experiments showed that the photobinding increased with dose and light intensity. Furthermore, the same amount of irreversible binding was found in rats killed immediately after the last NFT/UVA treatment and in those sacrificed 5 days later. The latter observation gave additional evidence that photobinding was irreversible.

The irreversible binding found in inner organs can be explained by systemic distribution of plasma proteins to which NFT has been photobound in the skin. In addition, reactive photoproducts of NFT, such as NFA and HMF, can be transported and eventually covalently bound to tissue biomacromolecules far away from their site of formation.

The immunogenic properties of photoadducts between NFT and plasma proteins have also been demonstrated (Busker, 1989). Photoadducts produced *in vitro* with plasma proteins of rabbits, were injected into the same animals from which blood was taken. It was demonstrated that photoadducts can indeed induce formation of antibodies (titres ranging from 150 to 1500 were found; controls were < 60).

The wide variety of dermatological reactions in NFT-therapy are frequently of the allergic type. This may be caused by covalent binding after incomplete enzymatic reduction of NFT in the liver. However, we found significantly more covalent binding in rats exposed to UVA than in those kept in the dark.

Therefore, the photoactivation of NFT in the skin should be considered as a route to (immunologically mediated) skin damage or rash.

Furthermore, the presence of irreversible bound NFT throughout the system (blood and organs, especially the spleen) indicates that light-induced adverse effects are not restricted to light-exposed skin. Photoreactions of a drug in the skin can also result in systemic allergic effects.

2.2.6 *Imino*-N-*oxides*

2.2.6.1 Chlordiazepoxide. Extensive research into the *in vitro* photochemistry of phototoxic chlordiazepoxide (CDZ) showed that an oxaziridine is the first and main product. This reactive intermediate was also found on UVA exposure of other imino-*N*-oxides such as the major metabolites of CDZ (Fig. 26) (Cornelissen *et al.*, 1980).

In vitro, imino-*N*-oxides bind irreversibly to plasma proteins upon UVA exposure, probably via an oxaziridine as an intermediate (Bakri *et al.*, 1988). Analogues of CDZ without an oxygen attached to the nitrogen are photostable and photobinding to proteins does not occur (Bakri *et al.*, 1988).

Based on these *in vitro* results, it was expected that the *N*-oxide group, in both CDZ and its metabolites, was responsible for phototoxicity. This was confirmed with a microbiological test system; survival of *Salmonella typhimurium* TA100 was plotted against concentration of the compound investigated. Compounds lacking an oxygen to the nitrogen of the C=N group were not phototoxic. Furthermore, phototoxicity curves of CDZ and some other imino-*N*-oxides proved to correspond quite well with curves for the toxicity (= without UVA exposure) of their oxaziridines (Cornelissen *et al.*, 1980; De Vries *et al.*, 1983).

UVA-induced effects were also extensively investigated *in vivo* with rats (Bakri *et al.*, 1983, 1985; Beijersbergen van Henegouwen, 1988). Some typical results are summarized here (see also Fig. 27). As can be seen, UVA has a considerable influence on CDZ metabolism. The percentage of CDZ and *N*-oxide metabolites is only about half of that found without UVA irradiation. In contrast, the percentage of metabolites lacking the oxygen attached to nitrogen, including R.CDZ, is about five times higher in UVA exposed rats.

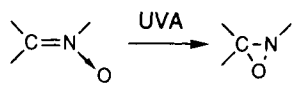
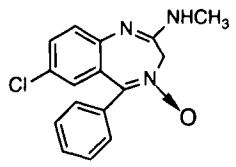
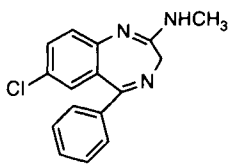


FIG. 26. Photoisomerization of an imino-*N*-oxide into an oxaziridine.



Chlordiazepoxide (CDZ)



Reduced CDZ (R.CDZ)

	UVA	Dark
CDZ+	20%	37%
R.CDZ+	14%	3%
Unknown	11%	5%

FIG. 27. Urinary excretion (45% of dose) of CDZ metabolites with rats either UVA-exposed or kept in dimly-lit environment (Dark). CDZ+ are imino-*N*-oxides, including CDZ. Metabolites lacking the oxygen attached to the nitrogen are indicated as R.CDZ+ and include R.CDZ.

An explanation can be proposed in the light of the reaction of oxaziridines, formed by photoisomerization in the UVA-exposed skin, with thiol group containing compounds. In the presence of GSH, which is abundantly present in the body, CDZ on UVA irradiation and its oxaziridine without light proved to react spontaneously with the GSH giving R.CDZ as the ultimate product (Beijersbergen van Henegouwen, 1988). These reactions with the GSH occurring in the UVA-exposed rats will be the cause of a lower percentage of *N*-oxides (e.g., CDZ) and a higher percentage of reduced metabolites (e.g., R.CDZ).

Another observation with regard to the metabolism of CDZ concerns the extent of glucuroconjugation, which was two times lower in UVA exposed rats (Bakri *et al.*, 1983).

As was expected from *in vitro* photochemical data, irreversible binding to organ material was also found with rats exposed to the combination CDZ/UVA. In a typical experiment (Bakri *et al.*, 1983) each of four rats (200 g) was given one single dose only of [^{14}C]CDZ (25 mg kg^{-1}, i.p.). On 18 consecutive days (on which no CDZ was administered), two of them were exposed to UVA (14 W m^{-2}; 320–380 nm; maximum = 350 nm) for 10 h day^{-1} whereas the two others were kept in a dimly-lighted environment. Thereafter, the rats were killed and organ material was submitted to dialysis for 26 days under non-equilibrium conditions. Irreversibly bound radioactivity was found only in UVA exposed rats (e.g., c.p.m. $\times 10^{-2}$ mg^{-1} tissue

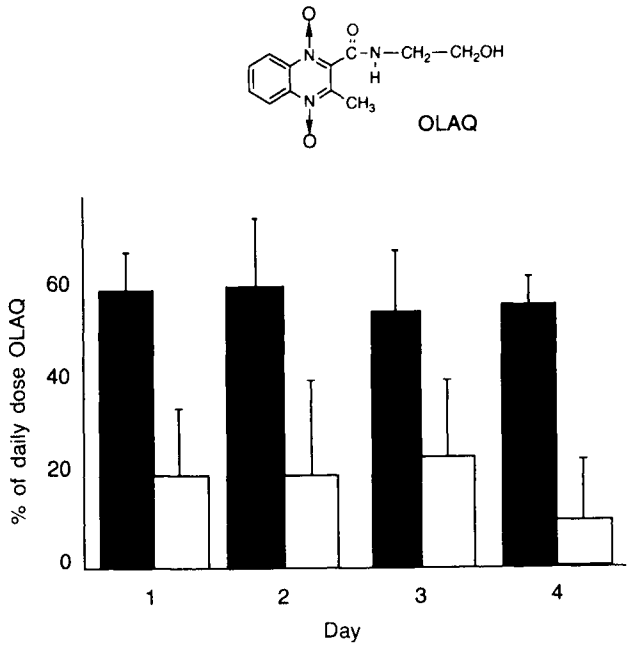


FIG. 28. Daily urinary excretion (mol% of dose) of unconverted olaquindox (OLAQ) with rats exposed to UVA (□) or kept in dimly-lit environment (Dark ■). UVA and Dark differ significantly ($P < 0.001$, Student t-test).

from skin of the back 2440 ± 30; from skin of the abdomen 910 ± 20; ears 1940 ± 80 and liver 1030 ± 90).

The considerable change in metabolism and the irreversible binding to biomacromolecules in the liver show that the phototoxicity of CDZ is not restricted to the UVA-exposed area but can also involve inner organs.

Further confirmation of the responsibility of the *N*-oxide function for the phototoxic effects was obtained by comparing CDZ with R.CDZ *in vivo*. These investigations with R.CDZ were performed under the same conditions as with CDZ. (For instance, with a dose of R.CDZ 1.5 times that of CDZ, both compounds had the same UVA absorption and bioavailability in the skin as a function of time.) UVA exposure of rats did not have any influence on metabolism of R.CDZ and no irreversible binding to biomacromolecules was observed (Bakri *et al.*, 1985). Similar differences have been found between diazepam-*N*-oxide and diazepam (Beijersbergen van Henegouwen, 1988). In contrast to diazepam-*N*-oxide, diazepam proved to be non-phototoxic.

2.2.6.2 Olaquindox. Olaquindox (OLAQ) belongs to a group of quindoxin (QUIN) derived compounds (Fig. 28). OLAQ finds extensive applica-

tion in cattle breeding, pig husbandry and poultry farming, as a feed additive with antimicrobial and growth-promoting effects. QUIN has also been used as such but was withdrawn from the market due to persistent photocontact dermatitis in man. In quite a number of cases, light sensitivity continued for more than 4 years (Zaynoun *et al.*, 1976).

OLAQ has also been reported to cause photoallergy in man (Francalanci *et al.*, 1986). Symptoms of OLAQ-induced photoallergy resemble those of QUIN. Furthermore, severe phototoxicity was observed with pigs.

QUIN-derived compounds belong to the group of imino-*N*-oxides and proved to form an oxaziridine upon irradiation *in vitro* (see also Fig. 26). Furthermore, they are highly photoreactive with proteins, indicating photoallergic properties (De Vries *et al.*, 1990a).

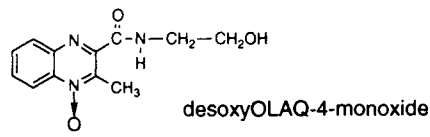
Rats exposed to the combination of OLAQ and UVA, but not to either OLAQ or UVA alone, suffered from severe erythema of the skin, oedema of the feet and necrosis of the ears (De Vries *et al.*, 1990b). The same experiments were performed with QUIN and some analogues of OLAQ, carbadox (CARB) and cyadox (CYAD), which are also extensively used as a feed additive with antimicrobial and growth-promoting effects. The results show that CARB is the most phototoxic followed by QUIN and OLAQ. CYAD, although very reactive *in vitro* (De Vries *et al.*, 1990a), was not taken up from the intestine into the bloodstream like the other compounds and thus could not be reached by the UVA. Because of this, a photochemical reaction could not take place, which provides an explanation for the fact that this compound was not phototoxic after oral administration.

In addition to skin phototoxicity, a profound influence on metabolism was found. Each of eight rats (140 g) of which the back was shaved, was given OLAQ (60 mg kg^{-1}, p.o.). Four of them were exposed to UVA (60 W m^{-2}; 345–420 nm; maximum = 370 nm) for 12 h day^{-1} and the other four were kept in poor-light environment. Urine was collected every 24 h. The procedure was repeated on each of four consecutive days (intervals 24 h).

With rats kept in a dimly-lighted environment, OLAQ was excreted for about 60% of the daily dose. However, with UVA irradiation unconverted OLAQ found in urine was only about 20% of the dose (see also Fig. 28).

Without UVA-exposure, reduction of the parent imino-*N*-oxide is only a minor metabolic route. This situation changed drastically as a result of UVA-irradiation; instead of about 2% desoxyolaquindox-4-monoxide (desoxyOLAQ-4-M), about 30% of this compound was found (see also Fig. 29).

This decrease of imino-*N*-oxide in favour of a reduced metabolite has been mentioned above for chlordiazepoxide (Fig. 27) and also for diazepam-*N*-oxide. Together with *in vitro* photochemical data (occurrence of an oxaziridine and photoreactivity with proteins) the formation of desoxyOLAQ-4-M *in vivo* is an important indication that photoisomerization



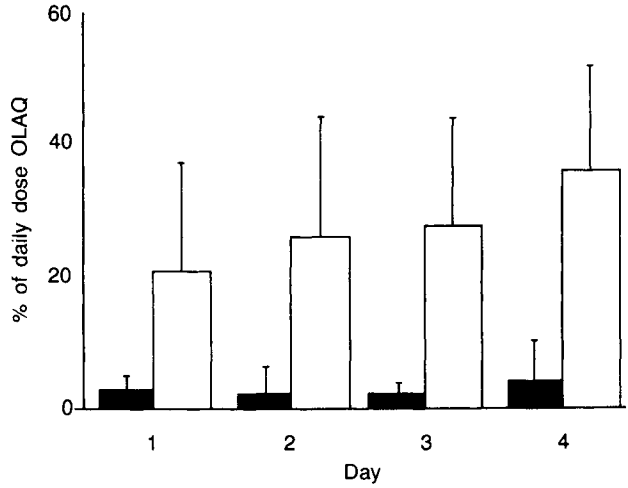


FIG. 29. Daily urinary excretion (mol% of dose olaquindox = OLAQ) of the metabolite desoxyolaquindox-4-monoxide with rats exposed to UVA (□) or kept in dimly-lit environment (Dark ■). UVA and Dark differ significantly ($P < 0.001$, Student *t*-test).

of OLAQ to an oxaziridine is responsible for the phototoxic/photoallergic effects.

2.2.6.3 Methaqualone-N-oxide. The foregoing results with imino-*N*-oxides were confirmed by *in vitro* and *in vivo* investigations of methaqualone-*N*-oxide, a major metabolite of the drug methaqualone (Pöhlmann *et al.*, 1986). In this case too, a reactive oxaziridine proved to be involved.

2.3 CONCLUSION

Only knowledge of the part of the functional group responsible for phototoxicity can provide the opportunity to alter a phototoxicon in such a way that the adverse photobiological effects diminish while the desired ones remain conserved (e.g., research on chlordiazepoxide and derivatives). This aim can be reached more efficiently when combining photoreactivity data from both *in vitro* and *in vivo* investigations.

More attention should be paid to the possibility that phototoxic effects can affect also inner organs and hence be systemic (e.g., research on nitrofurantoin).

3 Phototherapeutics

3.1 INTRODUCTION

There is a growing interest in drugs having photobiological effects because of their possible application as phototherapeutics.

With both phototoxic drugs and phototherapeutics, the eventual biological effect is the result of photochemical reactions which occur in the body. (The reader is referred to section 1.2.5.4 for more detailed information, e.g., about the sequence of events which starts with the absorption of a photon of UV-radiation or visible light and eventually leads to a biological effect.) One speaks of a phototherapeutic if the photoreactions are intended and controlled. The latter can involve the selective destruction of the affected part of a tissue, for example with the photodynamic therapy of tumours (see section 3.2.1). However, the control of the photoreactions can be even more refined in that photomodification, but certainly not killing of leukocytes takes place; this is the case with a recent development of PUVA: photopheresis (see section 3.2.4).

Potentially, phototherapeutics have a considerable advantage over conventional drugs because side-effects are negligible. On one hand, a phototherapeutic without light does not have biological activity. On the other hand the radiation used, visible light or UVA, is harmless without the phototherapeutic drug being present. Only as a result of simultaneous exposure to the phototherapeutic and light, can a biological effect occur. The consequence of the latter is that the treatment can be very specific. In addition to pharmacokinetic parameters, as with conventional drugs, the properties of light contribute to a high extent to the specificity of a phototherapeutic. (Light can be directed very precisely to the treated tissue or cells and its wavelength region, intensity and exposure time can be controlled.)

3.2 SPECIFIC EXAMPLES OF PHOTOTHERAPEUTICS

3.2.1 Dyes for Photodynamic Therapy (Abnormal Proliferating Tissues)

3.2.1.1 General Description. During the past 20 years there has been increasing interest in the use of porphyrins and other tetrapyrroles in combination with visible light for the treatment of tumours and other abnormal proliferating tissues (for detailed reviews with many references see:

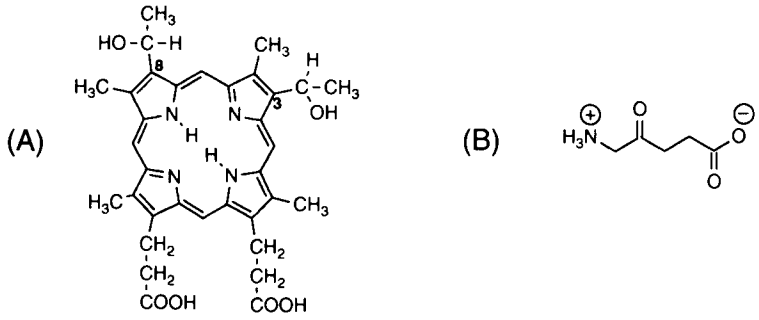


FIG. 30. A. Haematoporphyrin (Hp). Haematoporphyrin derivative (HpD) may contain several Hp units linked, e.g. at position 3 or 8 via ether bonds, $-CH(CH_3)-O-CH(CH_3)-$, with the porphyrin rings folded over each other. B. δ-Aminolevulinic acid (ALA), precursor of the endogenous porphyrin protoporpyrin IX (PpIX), represented as zwitterion.

Jori, 1985; Moan, 1986; Stables and Ash, 1995). This therapeutic modality requires the presence of oxygen and is therefore called photodynamic therapy (PDT).

In the predominant mechanism, a porphyrin (e.g., Fig. 30) excited by visible light transfers its excess of energy to oxygen resulting in the formation of singlet oxygen which is very damaging to biological systems. Simultaneously, the porphyrin falls back to its ground state, ready to repeat this energy transfer numerous times.

The treatment is selective because only the combination of visible light and porphyrin is tissue-damaging and the porphyrin is preferentially taken up in significant amounts by hyperproliferating cells and is retained there much longer than in normal tissues. The short lifetime of singlet oxygen results in a very small range of action (0.1 μm) and limits the destruction of cells and tissues to its site of formation.

Although some reports on the use of PDT for the clinical treatment of atheromatous plaques and psoriasis have appeared, until now the therapy has been most frequently applied against solid tumours.

Clinical protocols generally involve intravenous administration (2.5 or 5 mg kg^{-1}) of so-called haematoporphyrin derivative (HpD or Photofrin, a complex mixture of porphyrins). This is followed by irradiation between 48 and 72 h after administration, when most of the drug is cleared from normal tissue. Irradiation is performed with a laser or an adequately filtered non-coherent light source emitting at 620–630 nm, which is directed towards the affected tissue through optical fibres.

As a result of irradiation, destruction of vessels which provide the tumour with blood, takes place. In addition, tumour cells are killed, mainly by photo-oxidative modification of mitochondrial and plasma membranes.

(Because of their lipophilicity the porphyrin derivatives are more membrane-bound and their concentration close to DNA is relatively low. As a consequence of this, damage to DNA appears to be a minor result of HpD photosensitization).

During the past years some thousands of patients have been subjected to PDT and most of them have benefited from the treatment. Curative effects are most frequently observed with both superficial and deep-sited primary tumours of relatively small dimensions. To date, early cancers of the skin, bladder, eyes, vagina and oral cavity appear most suitable for this kind of treatment. Complete remission has also been obtained in early stages of lung cancer.

The only documented side-effect of PDT is skin photosensitivity, which may persist for some weeks.

3.2.1.2 Improvement of Efficacy of PDT (less Photosensitivity as a Side-effect).

(1) From a pharmaceutical point of view, one of the main disadvantages of haematoporphyrin derivative HpD and other related commercialized products such as Photofrin is that they are mixtures the composition of which is not constant. Not only the identity but also the desired photobiological activity and dark toxicity of the components is unknown. This was an important reason for the synthesis of pure compounds, but not the only one.

(2) Newer photosensitizers, such as chlorins, bacteriochlorins and phthalocyanins have also been synthesized because of their high extinction coefficient at the long wavelength end of the visible spectrum. This is especially important in view of the treatment of tumours which, because of their size, are less accessible for optical radiation. (Photodynamic treatment of superficial lesions can be very effective with light of about 400 nm, where compounds such as haematoporphyrin have a far higher extinction coefficient than in the red part of the light.)

(3) The search for photosensitizers other than HpD also concerns the pharmacokinetic behaviour. This should be such that the uptake in the tumour is higher than in normal tissue and that the excretion from the latter is faster. Improvement with regard to this and thus also to lower photosensitivity as a side-effect of PDT, has been obtained with, e.g., the benzoporphyrin derivative, meso-tetra-(hydroxyphenyl)-chlorin, and with mono-L-aspartyl chlorin e6.

(4) Other possibilities which deserve attention regarding improvement of the efficacy of PDT with concomitant less skin photosensitivity is drug targeting. For example, the use of antibody-haematoporphyrin (or other photosensitizer) conjugates as was shown by experiments with mice. The latter were injected subcutaneously with myosarcoma cells and subsequently with haematoporphyrin linked to antibodies against tumour

cells bearing myosarcoma associated antigens. The survival of those animals, which were exposed to whole-body irradiation with visible light, appeared to be increased.

More efficient targeting of the tumour with a porphyrin can also be achieved by delivering the phototoxic drug incorporated in liposomes.

(5) Improvement of PDT and less skin photosensitivity can also be achieved by the use of a precursor which is enzymatically converted in the body into a porphyrin. δ-Aminolevulinic acid (ALA) is such a precursor; although it is not a sensitizer itself, it has rather recently been introduced for PDT (Kennedy *et al.*, 1990). Upon administration, ALA is enzymatically converted into protoporphyrin IX (PpIX), an intermediate product in the cellular haem synthesis. Clinically, PpIX shows to be a useful PDT photosensitizer (Stables and Ash, 1995).

The most important advantage of treating diseased tissues with ALA instead of exogenously applied photosensitizers, is the fact that photosensitizing effects do not remain for more than 24 h. This property is explained by the relatively high susceptibility of PpIX for enzymatic degradation, as well as by its photo-instability.

However, the fact that ALA is a zwitterion (Fig. 30) at physiological pH – and therefore has low lipid-solubility – causes problems for the treatment of diseases. Because of its low lipid solubility, ALA poorly passes biological barriers such as the walls of stomach and intestine, the stratum corneum of the skin and cellular membranes. In order to reach levels of PpIX that are clinically relevant, it is necessary to administer fairly high doses (Kennedy *et al.*, 1990).

A solution to the poor bioavailability of ALA can be provided by the prodrug concept. A prodrug is a pharmacologically inactive derivative of a parent drug molecule that requires spontaneous or enzymatic transformation within the body in order to release the active drug. It has improved delivery properties over the parent molecule. Being more lipophilic, ALA-prodrugs are expected to pass through cellular membranes more easily than ALA. After having entered the site of action, the prodrug is converted enzymatically in order to release ALA, which is in turn converted into PpIX. Recently, it was shown *in vitro* and *in vivo* that derivatives of ALA (e.g., esters) do indeed enhance the amount of accumulated PpIX considerably compared with ALA (Kloek and Beijersbergen van Henegouwen, 1996).

(6) Skin photosensitivity as a side-effect can also be controlled in another way, namely by protection of the skin. Photoprotectors which absorb the visible light and are also acceptable for cosmetic reasons are not available. However two alternatives deserve attention. The first one is β-carotene. This compound is used as a drug against photosensitivity in patients with the genetic disorder erythropoietic protoporphyrin (EPP). In EPP the combination of visible light and endogenous porphyrins also

damage skin cells according to the mechanism described above: formation of singlet oxygen by energy transfer. β-Carotene is a known quencher of singlet oxygen (see section 1.2.3.3) and it is plausible that this drug may be useful also in suppressing the side-effects of PDT.

The second alternative is the application of antioxidants on the skin. During the reaction of singlet oxygen formed as a result of photoexcitation of a PDT dye in the skin, a variety of reactive intermediates such as superoxide anion, hydroxyl radicals and peroxyl radicals are produced. This will exhaust the natural defence system of the skin, which is based on amongst others water-soluble radical scavengers such as vitamin C and glutathione, and vitamin E as lipophilic antioxidant. Support of the natural defence system during the period after PDT is a solution which deserves attention. Compounds which can be considered as potentially useful are, for example, vitamin E acetate and derivatives of cysteine. These can act as prodrugs which are enzymatically converted into vitamin E and cysteine (glutathione); their protective activity against reactive intermediates remains for hours after their application on the skin (van den Broeke and Beijersbergen van Henegouwen, 1994; Beijersbergen van Henegouwen *et al.*, 1995; Steenvoorden and Beijersbergen van Henegouwen, 1997, in press).

3.2.2 Dyes for Photodynamic Therapy (Infections)

Inactivation of viruses, bacteria and fungi by the photosensitizing action of visible light absorbing compounds is a well-documented phenomenon (Jarrat *et al.*, 1982; Melnick and Wallis, 1982; Rapp and Li, 1982). So far, however, because of controversial risk/benefit ratios this potent way of killing microorganisms has not yet found its place in clinical photomedicine. This is unfortunate, since only a few photosensitizers have been tried. Much attention has been paid to heterotricyclic acridine-like dyes, which, however, seem less favourable because they are suspected to be (photo)mutagenic.

Non-toxic dyes, e.g., those already used for centuries as natural food constituents, deserve more attention in this respect. This has been demonstrated with curcumin, which was found to have a much higher antimicrobial activity in combination with light than without irradiation (Tønnesen *et al.*, 1987).

3.2.3 PUVA Treatment of the Skin

3.2.3.1 Clinical Aspects. Currently, the administration of psoralens (P) and long-wave ultraviolet radiation (UVA) is the most widely used form of a drug-plus-light therapy and is commonly referred to as 'PUVA' (detailed reviews with many references are: Parrish *et al.*, 1982; Dall'Acqua and Rodighiero, 1985). Psoralens are tricyclic aromatic compounds in which the

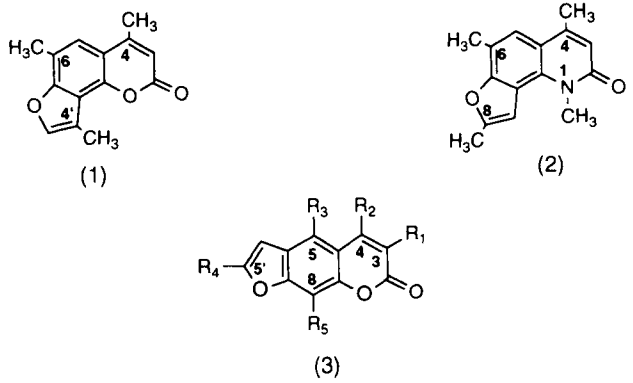


FIG. 31. (1): 4,6,4′-Trimethylangelicin (TMA); (2): 1,4,6,8-Tetramethyl-2H-furo[2,3]-quinolinone (RG 777) (3): 8-Methoxypsoralen (8-MOP, $R_1=R_2=R_3=R_4=H$; $R_5=OCH_3$); 5-Methoxypsoralen (5-MOP, $R_1=R_2=R_4=R_5=H$; $R_3=OCH_3$); Psoralen (Ps, $R_1=R_2=R_3=R_4=R_5=H$); 3-Carbethoxypsoralen (3-CPs, $R_1=CO_2C_2H_5$; $R_2=R_3=R_4=R_5=H$); 4,8,5′-Trimethylpsoralen (TMP, $R_1=R_3=H$; $R_2=R_4=R_5=CH_3$).

furan ring is fused with a coumarin moiety, hence the name furocoumarin (Gasparro, 1994a). A number of linear and angular furocoumarins are in use for PUVA (Fig. 31); some are commercialized, e.g., 8-methoxypsoralen (8-MOP) and 4,8,5′-trimethylpsoralen (TMP) while others, e.g., 3-carbethoxypsoralen, 5-methoxypsoralen (5-MOP) and 6-methylangelicins such as 4,6,4′-trimethylangelicin (TMA) are applied in clinical trials.

Originally, PUVA was used to treat psoriasis, a hyperproliferative skin disease and vitiligo, an acquired pigmentary alteration in the skin (Parrish *et al.*, 1974). PUVA is now used for a wide variety of skin diseases, such as psoriasis, vitiligo, lichen planus, persistent palmoplantar pustulosis and solar urticaria (Dall'Acqua and Rodighiero, 1985; Parrish *et al.*, 1982; Honig *et al.*, 1994).

Two psoralens are presently established as standard photosensitizers in PUVA of psoriasis. These are 8-MOP, administered orally for systemic treatment and TMP, which is used topically in the form of PUVA-baths as a whole-body treatment.

From clinical trials it was concluded that, with regard to the overall high tolerability and good therapeutic efficacy, high-dose 5-MOP can be considered as a valuable alternative drug for oral photochemotherapy of psoriasis. Because of its increased benefit/side-effect ratio, 5-MOP seems to be particularly advantageous for the treatment of light-sensitive subjects and patients with 8-MOP intolerance (Tanew *et al.*, 1988).

Vitiligo is a relatively common condition characterized by the progressive loss of normal skin coloration in certain skin areas. Although a minority of

patients may develop repigmentation when exposed to sunlight or artificial UV radiation, only PUVA is effective in inducing a permanent cosmetically acceptable result (Hönigsmann, 1988). Several psoralens are in use or have been tested for PUVA of vitiligo. Psoralen, 8-MOP, 5-MOP and TMP have proved effective in reconstituting normal skin coloration. Controlled studies in generalized vitiligo with PUVA using 8-MOP and/or TMP have yielded success rates ranging from 25 to 40%. Repigmentation of more than 70% usually is considered an excellent treatment response.

Interestingly, repopulation with melanocytes of vitiliginous skin occurs independently of the drug's ability to induce pigmentation in normal skin during PUVA. This indicates that the stimulation of melanogenesis and the induction of repopulation with melanocytes may be mediated by different mechanisms (Hönigsmann, 1988).

3.2.3.2 Side-effects. There is no controversy that PUVA with 8-MOP can promote human non-melanoma cancers (Chang *et al.*, 1992; Stern and Laird, 1994). What is under discussion is the magnitude of the problem and whether or not PUVA with 8-MOP is only a promotor of cancer in previously compromised skin (e.g., with arsenicals or with ionizing radiation) or a full carcinogen.

There is no evidence that accelerated cataract formation occurs or that the risk of melanoma is increased. These are two potential toxicities of greater clinical importance (Parrish *et al.*, 1982). However, with respect to these possible long-term side-effects more research is necessary for a correct evaluation of the benefit/risk ratio.

Among known side-effects which result from the combination of 8-MOP and UVA, erythema and hyperpigmentation, a temporary failure to elicit an immune response to new antigens (Friedman, 1986) is important to mention.

3.2.3.3 Mechanistic Aspects. The diversity of photobiological (side-) effects of furocoumarins is reflected in the different reactions which can occur as a result of their photo-excitation by UVA; all have been observed *in vitro* (Dall'Acqua *et al.*, 1985; De Mol *et al.*, 1986):

(1) Cycloaddition, to the pyrimidine bases of DNA with the 3,4- and/or 4',5'-double bond. Because of stereochemical reasons 3CPs and the angelicins give mono-adducts only, whereas the linear furocoumarins, such as 8-MOP and TMP can also form cross-links.
(2) Covalent binding to proteins (and phospholipids) via, among others, free radical formation.
(3) Energy transfer which produces reactive forms of oxygen such as singlet oxygen, resulting in oxidation of guanine residues in DNA of amino acids in proteins (a.o. enzymes) and of unsaturated fatty acids in phospholipids.

Despite the fact that there was no evidence for that from *in vivo* data, cycloaddition to DNA has been considered as essential for the phototherapeutic effect for many years. The reason for this was that the antiproliferative activity, important for the treatment of psoriasis, can be explained in this way.

As a consequence of the emphasis put on photocycloaddition, the photoreactivity of furocoumarins was considered as an oxygen-independent process. However, by extensive analysis of the photoreactivity of several furocoumarins in the presence or absence of oxygen the following results were obtained:

- Furocoumarins can produce singlet oxygen (De Mol and Beijersbergen van Henegouwen, 1979; De Mol and Beijersbergen van Henegouwen, 1981). This was concluded from the observation that in the presence of 2-methyl-2-pentene, a known chemical quencher of $^1O_2$, the characteristic products were formed. Furthermore, this was proved by kinetic analysis of the photoreaction in D_2O and H_2O (because of the lifetime of singlet oxygen in these two solvents being different), and also by the determination of the β-value, an important kinetic parameter in relation to singlet oxygen production.
- Furocoumarins produce singlet oxygen even when complexed with or covalently bound to DNA (De Mol *et al.*, 1981a). It was also proved that singlet oxygen plays a role in the mutation induction by 8-MOP and at least in a part of its photobinding to DNA (Beijersbergen van Henegouwen *et al.*, 1989b; De Mol *et al.*, 1981b, 1986).
- Photoexcited furocoumarins, including 8-MOP can be oxidized by ground-state oxygen; the oxidized species O_2-8-MOP proved to be stable in organic solvents but rapidly reacts with unsaturated fatty acids in liposome membranes (Potapenko *et al.*, 1982).
- Oxygen proved to play a distinct role in the photobinding of furocoumarins to proteins (Yoshikawa *et al.*, 1979). The reactive intermediates, which eventually covalently bound to protein, were assumed to be formed by the action on the furocoumarin of singlet oxygen generated by the furocoumarin itself (Veronese *et al.*, 1981).

Emphasis on photoreactivity with DNA is gradually decreasing, not only because of these *in vitro* investigations of the role of oxygen in the photoreaction of furocoumarins, whether or not in the presence of biomacromolecules. This also is the case since substantial evidence has been obtained that the immune system also plays a role with a number of diseases which are treated with PUVA (Morison and Parrish, 1982). The latter was an indication that photobinding to proteins and cellular membranes also plays a role.

As photobinding to DNA, and later also to other biomacromolecules as well, was supposed to be an important event underlying the occurrence of photobiological effects from furocoumarins, the question arose whether this would take place *in vivo*. To that aim a method has been developed (Beijersbergen van Henegouwen *et al.*, 1989a). In particular, the question was addressed to what extent each of the different kinds of biomacromolecules was a target for photobinding. In a typical experiment, we compared 8-MOP with TMA (Schoonderwoerd *et al.*, 1991a). In contrast to findings from *in vitro* experiments, we found that 8-MOP photobinds far more to epidermal DNA and proteins than TMA, but less to lipids.

Removal of photobound 8-MOP and TMA from epidermal proteins and lipids *in vivo*, occurred with the same rate. This proved not to be the case with DNA; even after 1 week there was still a considerable amount of 8-MOP irreversibly bound to this biomacromolecule. These *in vivo* data fit with the observation that the carcinogenic potential of TMA in experimental animals is less than that of 8-MOP plus UVA. The data also correspond with results from *in vitro* research, which prove that TMA forms monoadducts with DNA and photoaddition of the linear furocoumarin 8-MOP produced crosslinks. (Crosslinks are more difficult to repair and proved to be more mutagenic.)

In vivo, photobinding to proteins, DNA and to the lipid part of membranes also proceeds to a great extent via radicals whether or not coupled to oxygen. This was demonstrated by the influence of antioxidants which were topically applied on the skin before PUVA treatment (Schoonderwoerd *et al.*, 1991b; Beijersbergen van Henegouwen *et al.*, 1995); e.g., a decrease of photobinding 60–80% was found with cysteine derivatives (van den Broeke and Beijersbergen van Henegouwen, 1994). This decrease was due to the antioxidant activity and not to absorption of UVA.

3.2.4 PUVA Treatment of Buffy Coat (Photopheresis)

3.2.4.1 Clinical Aspects. A recent development of PUVA is an extracorporeal form of photochemotherapy called photopheresis or extracorporeal photochemotherapy (Moor and Gasparro, 1996). After oral administration of 8-MOP, 1.5 l of the patients blood is collected and separated into three fractions: erythrocytes, leukocyte-enriched blood (the buffy coat) and plasma. The erythrocyte fraction is immediately returned to the patient. The leukocyte/plasma fraction is exposed to UVA within an irradiation chamber (Edelson *et al.*, 1987; Heald and Edelson, 1989; Edelson, 1991) and thereafter returned to the patient.

Photopheresis is also based on simultaneous exposure to 8-MOP and UVA. However, regarding the immune system, the main difference with classical PUVA is that with photopheresis the immune-competent skin cells are not exposed to photoactivated 8-MOP.

The eventual result of the treatment is a specific suppressive response against the pathogenic clone of T cells (Edelson, 1991). Of interest with regard to the immunomodulatory response is the fact that from the leukocytes that pass through the photopheresis apparatus only a part will actually be exposed to photoactivated 8-MOP. Furthermore, one has to take into account that only a small percentage of all the leukocytes treated are malignant.

The only side-effect noted in the 13 years that photopheresis has been used in the clinic is nausea (Armus *et al.*, 1990; Heald, 1991; Oziemski *et al.*, 1991; Heald *et al.*, 1992; Prinz *et al.*, 1995).

Photopheresis for the treatment of cutaneous T cell lymphoma: In 1982 photopheresis was first used for the treatment of cutaneous T cell lymphoma (CTCL), a $CD4^+$ T cell malignancy. The FDA approved the use of photopheresis for CTCL. From the initial study and several follow-up studies it became clear that in about 25% of the patients a complete response occurs, whereas about 50% of the patients showed a partial response (Edelson *et al.*, 1987; Heald and Edelson, 1989; Armus *et al.*, 1990; Edelson, 1991; Heald, 1991; Oziemski *et al.*, 1991; Heald *et al.*, 1992; Prinz *et al.*, 1995).

The positive results obtained with patients suffering from CTCL and promising results in animal experiments prompted clinicians to use photopheresis for other T-cell mediated diseases.

Photopheresis for systemic sclerosis: Rook *et al.*, 1992 reported the results of a multicentre trial on the use of photopheresis in the treatment of systemic sclerosis. Systemic sclerosis is an autoimmune disorder characterized by excessive deposition of collagen within the skin and frequently within visceral organs, including the heart, lungs, kidney and gastrointestinal tract (Rook *et al.*, 1992). In the trial reported, 97 patients with systemic sclerosis of recent onset (mean symptom duration: 1.83 years, median 1.51 years) and progressive skin involvement entered a randomized, parallel-group, single-blinded clinical trial comparing photopheresis with treatment with D-penicillamine. (From a previous study by Steen *et al.* (1987), indications were obtained that D-penicillamine is therapeutically active in systemic sclerosis.) Assessment of disease activity included skin severity (skin thickness), area of skin involvement and oral aperture measurement. After 6 months of therapy, it was found that improvement of the skin severity score occurred in about 70% of the patients in the photopheresis group and in about 30% of the patients receiving D-penicillamine. Adverse effects of photopheresis were restricted to nausea, whereas six patients permanently discontinued the use of D-penicillamine due to adverse effects. The conclusion of this multicentre trial is that for patients with systemic sclerosis of recent onset, photopheresis is a well-tolerated treatment that may partially reverse the process that results in cutaneous sclerosis (Rook *et al.*, 1992).

The results of this study were confirmed by (Di Spaltro *et al.*, 1993). They treated nine patients with early systemic sclerosis. The results

showed a significant improvement of patients and their conclusion was that photopheresis may be beneficial in selected early cases of progressive systemic sclerosis.

Preliminary studies of photopheresis for other (autoimmune) diseases: Four patients with pemphigus vulgaris who were resistant to corticosteroids and immunosuppressive drugs responded to photopheresis (Rook *et al.*, 1990). Three of the patients eventually experienced long-term remissions.

Photophoresis proved to be effective in rheumatoid arthritis (Malawista *et al.*, 1991); four out of seven patients treated showed a significant improvement in joint score.

Knobler *et al.* (1992) showed in an open clinical trial on patients with systemic lupus erythematosus that photopheresis was able to suppress this disease. Eight out of the 10 treated patients completed the trial. In seven out of eight, there was a significant response to the treatment, with no or minor side-effects. The authors concluded that photopheresis leads to clinical improvement of systemic lupus erythematosus, but that further research in controlled clinical trials is needed.

Interesting results were also obtained in the treatment of patients with late-stage HIV disease (Bisaccia *et al.*, 1993; Cotrill *et al.*, 1994). It was shown, amongst others, that the majority of patients maintained persistently elevated HIV-specific antibodies to p24, gp120, gp66/31, gp55 and gp41. This increase in antibody titres was accompanied by a stable clinical course characterized by stable or increased body weight, resolution of adenopathy and absence of opportunistic infections.

In a limited clinical trial (three patients) photopheresis proved its effectiveness in severe atopic dermatitis (Prinz *et al.*, 1994). The therapeutic effectiveness was reflected by a marked reduction of IgE serum levels (one of the clinical manifestations of atopic dermatitis is an excessive overproduction of IgE antibodies) in all patients. The serum levels of IgG, IgM and IgA as well as the profile of circulating lymphocytes remained unchanged.

Photopheresis for allograft rejection: Promising results with photopheresis were obtained with prevention of allograft rejection.

Several studies showed that photopheresis is a successful adjuvant therapy to prevent rejection of heart transplants (Constanzo-Nordin *et al.*, 1992, 1993; Meiser *et al.*, 1994; Wieland *et al.*, 1994; Barr *et al.*, 1995). Barr *et al.* (1995) concluded that photopheresis induced a significant reduction in the intimal hyperplasia seen in the coronary arteries of the transplanted heart.

There is some controversy over the use of photopheresis to prevent the rejection of renal transplants. Horina *et al.* (1995) found no effect of photopheresis on the incidence of rejection in two patients receiving a renal graft, although the T cell count was reduced by photopheresis with 30–40%. In contrast to these findings is a study performed by Sünder-Plassman *et al.*

(1995); they observed a positive response in three renal graft receiving patients using a higher frequency of treatment cycles. More studies with larger groups of patients will provide answers concerning optimal regimen for successful interference of renal graft rejection (Moor and Gasparro, 1996).

That lung allograft rejection can also be controlled by photopheresis was concluded from a study of three patients with chronic rejection effects after single-lung transplantation. Their pulmonary function stabilized following the initiation of photopheresis (Slovis *et al.*, 1995).

In addition to these reports concerning allograft rejection, photopheresis appears to be effective in controlling graft-versus-host disease, as can be concluded from the work of Owsianowski *et al.* (1994) and Rossetti *et al.* (1995).

These clinical experiments show that a broad spectrum of immunological disorders can be treated with photopheresis. To date the mechanism underlying photopheresis has not been elucidated. Interleukin determination and cell type detection in the clinic proved that not all diseases and not all patients respond in the same manner. Some clinical reports mention an increase in natural killer (NK) cells (Prinz *et al.*, 1995) and in monocyte/macrophage activity (Vowels *et al.*, 1992). The latter increase was concluded from an increase of the production of tumour necrosis factor-α, interleukin (IL)-1 and IL-6 after photopheresis.

That there is need to know more about the photochemical and photoimmunological reactions occurring during photopheresis is illustrated by the debate that is going on concerning the use of photopheresis for the treatment of systemic sclerosis (Fries *et al.*, 1992; Rook *et al.*, 1992).

For ethical reasons clinical studies are not suitable to investigate the mechanistic aspects of photopheresis; *in vitro* and *in vivo* (animal) models are indispensable for this purpose.

Therapeutic aspects as studied with animal models:

Presence of blood components other than leukocytes As already stated above, photopheresis is based on the exposure of a mixture of 8-MOP rich buffy coat, plasma and saline to UVA in a special irradiation chamber. If photomodification of biomacromolecules (of leukocytes) is needed to induce specific immune suppression, this may be hindered by the presence of plasma in the irradiation chamber. Much photoactivated 8-MOP will be scavenged by plasma proteins. This is supported by the well-established fact that 8-MOP can photomodify various biomacromolecules *in vivo* including proteins (Schoonderwoerd *et al.*, 1991a,b; van den Broeke and Beijersbergen van Henegouwen, 1994). Photoreactivity with plasma proteins can pose a problem other than loss of the desired biological effect; it may be the cause of photoallergy (Kochevar, 1979).

Photoallergy resembles contact hypersensitivity (CHS): the only difference

is that chemicals producing photoallergic reactions require activation by light in order to provoke the immune response (Gerberick and Ryan, 1990). Irreversible binding of the photoexcited compound to proteins resulting in the formation of an antigen, is supposed to be the initial step in photoallergy (Barratt *et al.*, 1987). The antigen triggers immunologic reactions which eventually result in the symptoms of photoallergy (Kochevar, 1979; Morison and Kochevar, 1983).

Although 8-MOP is considered to be a moderate photoallergic compound, there are some reports of 8-MOP-induced photoallergy (Fulton and Willis, 1968; Ljundgren, 1977; Plewig *et al.*, 1978; Sidi and Bourgeois-Gavardin, 1953). Induction of photoallergy may have serious implications for patients whose immune system is already derailed. (As is the case with patients treated with photopheresis.)

The possibility that the presence of proteins during photopheresis can result in photoallergy was investigated with 8-MOP and with chlorpromazine (CPZ). CPZ was chosen as a model compound because it is a well-established photoallergen (Harber *et al.*, 1982; Horio, 1984; Giudici and Maguire, 1985; Gerberick and Ryan, 1990). In addition, much is known about the photochemical behaviour of CPZ, both *in vitro* and *in vivo* (Schoonderwoerd *et al.*, 1989, 1991b; van den Broeke and Beijersbergen van Henegouwen, 1993a,b, 1994; van den Broeke *et al.*, 1994).

To investigate the possible risk of photoallergy, the rat was used as a model. The experimental conditions regarding, e.g., concentration of the drug and the dose of UVA, were relevant for photopheresis treatment and comparable to those applied in the *in vivo* studies by our group with 8-MOP and CPZ.

UVA exposure of either 8-MOP or CPZ in the presence of plasma, proved to induce photoallergy (van Iperen and Beijersbergen van Henegouwen, 1996a); although the concentration 8-MOP needed to install photoallergy was 4.5 times higher than that for CPZ and higher than the therapeutic level of 8-MOP during clinical photopheresis. Photoallergy did not occur if the irradiation of 8-MOP or CPZ was performed in the presence of lymphocytes only. Irradiation of either plasma, or lymphocytes followed by incubation with one of the photosensitizers in the dark, did not lead to a photoallergic response either. Although 8-MOP did not induce photoallergy at therapeutic concentration, the risk of inducing photoallergy with clinical photopheresis is still present. One reason is that there is repeated exposure of the patients' plasma to the combination of 8-MOP and UVA, as they receive two photopheresis treatments a month for a longer period of time (Heald and Edelson, 1989). Repeated exposure to an antigen can induce an allergic response (Ishida *et al.*, 1994). Another reason is that patients receiving photopheresis treatment already have an immune system that is compromised and might therefore be susceptible to a photoallergen.

We therefore concluded that the presence of plasma during irradiation

should be avoided in order to prevent the risk of induction of photoallergy (van Iperen and Beijersbergen van Henegouwen, 1996a).

Not only photoallergy can be the result of the presence of plasma during photopheresis. The therapeutic result of photopheresis can also be negatively influenced because suppression of the immune response which is the cause of the disease does not take place. The latter conclusion was drawn from an animal study in which we looked at the effect of different bloodcomponents (van Iperen and Beijersbergen van Henegouwen, 1996c). This study was performed in our animal model for photopheresis. This model used the Wistar-derived rat with contact hypersensitivity (CHS) for 2,4-dinitrofluorobenzene (DNFB), or 2,4,6-trinitrochlorobenzene (TNCB). In fact, CHS is the immune response in this model of which the possibility to suppress it specifically is studied with photopheresis. For a more detailed description of this model see section 3.2.4.2.

In our study on the effect of the different blood components, it was found that plasma, upon exposure to 8-MOP and UVA, causes an increase of the CHS immune response. The same held true for animals treated with erythrocytes that were exposed to both 8-MOP and UVA, regardless whether the incubation with 8-MOP preceded the UVA irradiation or not. This was probably caused by the presence of lysed erythrocytes, as the reinfusion of lysed erythrocytes induced the same increase of the immune response. Leukocytes that were lysed before intravenous injection into the CHS animals proved to serve as an adjuvant as well: increase of the CHS response (also see Berger *et al.*, 1990).

From the foregoing, it can be concluded that the efficacy of the treatment in the clinic can benefit from these data. Avoidance of plasma is certainly possible. (However, the same holds for the remaining erythrocytes if one could use far less blood than the 1.5 l in the current treatment regime; see below.)

In connection with this, future studies will certainly aim at knowledge about the leukocytes involved in the occurrence of specific immunosuppression by photopheresis.

Amount of blood used In a study closely related to the one in which we studied the effect of the different blood components, we found that photopheresis with the buffy coat from 0.2% of the total amount of blood is enough to establish specific immune suppression (van Iperen and Beijersbergen van Henegouwen, 1996d). Potentially, this would mean for the clinical situation that 15 ml blood instead of 1.5 l is sufficient to induce the desired specific suppression.

Down-scaling of the amount of blood used for photopheresis will lead to an even more patient-friendly therapy. It will also simplify the procedure; the exposure of buffy coat from 15 ml blood is easier and does not require complicated apparatus. (In this study we were interested in the treatment of an amount of blood that would be easy to handle and could easily be

obtained, even from children; 0.2% of total blood is such an amount. Whether this is the lowest amount of buffy coat that is sufficient to obtain specific immune suppression, is not yet known.)

Diseases other than those already treated with clinical photopheresis In a recent study with our animal model (for a more detailed description see section 3.2.4.2), we found that photopheresis is active in suppressing an asthmatic response (van Iperen and Beijersbergen van Henegouwen, 1997a, in press).

Although asthma can result from an extremely heterogeneous group of disorders, there is evidence that T cells play an important role in the pathogenesis of asthma. Recent research has shown that asthma is a special type of airway inflammation orchestrated by T lymphocytes (Garssen *et al.*, 1991; Van Loveren *et al.*, 1991; Holgate, 1993).

The fact that the immune system plays such an important role in the pathogenesis of asthma indicates that therapeutic strategies should be based on modification of the immune system. For this reason we investigated the effect photopheresis can have in this disease. We used an existing murine model for asthma (Garssen *et al.*, 1991; Van Loveren *et al.*, 1991). Briefly, mice were sensitized by the application of 2,4,6-trinitrochlorobenzene (TNCB) on the shaved dorsal skin. Five days later, the immune system of the animals was challenged by intratracheal injection of 2,4,6-trinitrobenzenesulfonic acid (TNBS, the water soluble form of TNCB). This resulted in sensitized animals. The resulting lung inflammation was assessed by a bronchoalveolar lavage (BAL) cell count. The number of leukocytes present in the BAL is a measure for the asthmatic response (Durham and Kay, 1985; Blythe *et al.*, 1986).

Sensitized animals were treated with photopheresis. Briefly, leukocytes (isolated from TNCB sensitized animals) were simultaneously exposed to 8-MOP and UVA. The treated cells were then reinfused intravenously. Five days later, the immune system of the animals was challenged by applying the hapten TNBS intratracheally. Forty-eight hours later bronchoalveolar lavage (BAL) fluid was collected and the number of leukocytes and macrophages present in the BAL was determined. Photophoresis treatment led to 80% suppression of the influx of cells. Furthermore, the suppression generated proved to be transferable with spleen cells from photopheresis treated animals (van Iperen and Beijersbergen van Henegouwen, 1997a, in press).

More research should be done to elucidate the role that photopheresis can play in the treatment of asthma.

3.2.4.2 Mechanistic Aspects

Mechanistic aspects: models for investigation:

Animal model for systemic lupus erythematosus Berger *et al.* (1990) were the first with a report of an animal model for photopheresis. This concerns

the MRL/l mouse. This animal develops a progressive, virulent autoimmune disease that has many of the features of systemic lupus erythematosus. Photophoresis was performed prior to the onset of overt disease when a relatively intact immune system was still present. Monitoring of the mice was done by evaluation of disease parameters such as lymphoid organomegaly, survival, lymphocyte phenotype and anti-DNA antibody production.

It was found that the weight and size of the lymph nodes and spleen was significantly reduced in animals treated with photopheresis (compared with control animals). The survival of mice that received photopheresis treatment was significantly prolonged compared with the control animals. The anti-doublestranded DNA antibody titre was in the background range for treated animals (at 19 weeks of age), whereas the control animals had high levels of this antibody. In addition, lymph node cells from treated mice retained the possibility to respond to mitogens such as LPS and Con A, thus demonstrating that photopheresis induces specific immune suppression (Berger 1989; Berger *et al.*, 1990).

Animal models for allograft and xenograft rejection In a primate model of cardiac allograft and xenograft rejection (Pepino *et al.*, 1989), it was found that photopheresis increased graft survival, specifically suppressed the mixed leukocyte culture response to the donor and suppressed the formation of anti-donor antibodies (Berger, 1989; Pepino *et al.*, 1989). In addition, biopsy-proven rejection episodes were reversed in two photochemotherapy animals, while cyclosporin-treated animals demonstrated pathology consistent with progressive rejection.

These studies demonstrate that the vigorous immune response to xenoantigens can be suppressed with photopheresis.

Another animal model used to study photopheresis is a murine model in which skin allograft rejection is studied (Perez *et al.*, 1989, 1992) Briefly, in this model BALB/c mice received CBA/j skin grafts. Their splenocytes were treated with 8-MOP/UVA and reinfused in naive BALB/c mice. Recipient mice were tested for tolerance to alloantigens in mixed leukocyte culture, cytotoxicity, delayed type hypersensitivity assays and challenge with fresh CBA/j graft. It was found that the response of splenocytes of recipients to CBA/j alloantigens in the mixed leukocyte test and cytotoxicity test was low. In addition, *in vivo*, the delayed type hypersensitivity response was specifically suppressed to the relevant alloantigen compared with controls. Moreover, BALB/c mice treated in this way retained a CBA/j skin graft for up to 42 days post-transplantation without visual evidence of rejection (control BALB/s mice receiving a CBA/j skin graft reject this graft after 7 days). Control photopheresis-treated BALB/c mice receiving instead of CBA/j, B10 skin graft rejected this skin within 7 days (Perez *et al.*, 1989).

In a follow-up study, it was found that DNA located on the surface of T cells (the so-called cmDNA) plays a role in the induction of the suppression

immune obtained. It was found that leukocytes pretreated with DNAse (cmDNA removed) were unable to induce suppression after treatment with 8-MOP/UVA (Perez *et al.*, 1992). A refinement of the skin graft model showed that photopheresis is able to induce specific suppression of the immune response, even if there is only a difference in a locus of the MHC molecule (Perez *et al.*, 1991).

Animal model based on contact hypersensitivity We developed an easy-to-handle animal model for photopheresis (van Iperen and Beijersbergen van Henegouwen, 1992, 1993). The animal model uses the male Wistar-derived rat and the immune response to be studied with photopheresis is CHS. CHS was induced by the application of 2,4-dinitrofluorobenzene (DNFB), a commonly used hapten, to the shaved dorsal skin of male rats. Photophoresis was carried out and, as a measure of the effect of the treatment, ear swelling was determined after challenge with DNFB. The transfer and specificity of the suppression was investigated by transferring splenocytes from photopheresis-treated animals (with the CHS response against DNFB suppressed) to naive syngeneic animals. Immediately after receiving these cells intravenously, the animals were sensitized with DNFB (to investigate the transfer of the suppression), or with 2,4,6-trinitrochlorobenzene (TNCB; to determine the specificity of the suppression).

With both DNFB and TNCB, antigens are formed by nucleophilic substitution of the fluoro- and chloro atom, respectively. In the case of DNFB, this results in an antigen consisting of an endogenous biomacromolecule to which a 2,4-dinitrophenyl fragment is attached. With TNCB, the antigen only differs from that of DNFB in that the phenyl fragment attached to the biomacromolecule has three nitro groups instead of two. The fact that these two haptens (and thus the resulting antigens) are chemically so closely related makes them particularly suitable for the investigation of the specificity of the immune suppression.

8-MOP proved to induce cell-mediated specific immune suppression just as with clinical photopheresis. (After injection of splenocytes from photopheresis treated animals in naive rats, CHS induced by DNFB was suppressed, whereas CHS induced by TNCB was normal. In addition, it was found that water-soluble mediators released by the cells after UVA exposure and before injection into the animals, are not responsible for the induction of the suppression observed.)

With this animal model, we determined a dose-response curve for UVA/8-MOP induced immune suppression. It showed that there is a correlation of $r = 0.921$ on a log scale between the dose of UVA and the immune suppressive response (van Iperen *et al.*, 1996a).

Mechanistic aspects: emphasis on cellular biomacromolecules: As stated above, the combination of 8-MOP and UVA leads to the induction of DNA damage (Gasparro, 1994a). This DNA damage leads to a proliferative arrest

of treated cells (Song and Tapley, 1979). However, this arrest cannot explain the immune suppression observed with photopheresis, as only about 10% of the cells are treated. That destruction of the malignant cells is not the major mode of action is endorsed by the results obtained with leukapheresis treatment for patients with CTCL. The removal of malignant T cells from patients with CTCL by leukapheresis at the same or even greater frequency than photopheresis, often have little effect on disease progression (Wolfe *et al.*, 1994).

The damage of DNA might also lead to the induction of apoptosis (Raf, 1992). Apoptosis is a process in which enzymes are produced by the cell that lead to chromatin condensation, the inhibition of cell–cell interactions and cytoskeleton disruption. Apoptosis culminates in the phagocytosis of the apoptotic cells by neighbouring cells. Recently, Marks and Fox (1991) suggested that the treatment of cells with 8-MOP and UVA could induce apoptosis. This induced apoptosis could lead to the death of a selected group of cells which in the process of disassembling could release partially degraded (and perhaps photomodified) proteins. These proteins could be the source of new oligopeptide fragments which may be displayed in surface MHC molecules of surviving cells, resulting in a higher level of antigenicity of these cells (Gasparro, 1994b).

That 8-MOP/UVA have an effect on the antigenicity of cells was demonstrated by Gasparro *et al.* (1993). They show that a highly tumourigenic P815 mouse mastocytoma cell line (tum+) with repeated cycles of 8-MOP/UVA led to the production of non-tumourigenic clones (tum−) (Moor and Gasparro, 1996). The inoculation in mice of the tum− clones led to a longer survival of these mice as compared with a group of mice that were inoculated with the original tum+ clone. In immuno-compromised mice the tum− clones were able to induce a tumour, but this induction was prevented by adoptive transfer of immune cells from tum− immunized mice. In addition, immunization of mice with tum− clones offered immune-mediated protection when mice were challenged with the original P815 (tum+) clone (Gasparro *et al.*, 1993; Moor *et al.*, 1995; Schmitt *et al.*, 1995). The increase in antigenicity was confirmed in two follow-up studies. In these studies, it was shown that the treatment of cells (P815 mastocytoma and RMA) with 8-MOP/UVA leads to the upregulation of MHC class I synthesis (Moor *et al.*, 1995; Schmitt *et al.*, 1995).

In addition, cytofluorometric analysis of monocytes isolated from CTCL patients and treated *in vitro* with UVA/8-MOP show upregulation of MHC class II (HLA class II) expression (Felli *et al.*, 1995). However, it must be stressed that this finding conflicts with two reports: one in which it was shown that in psoriatic patients undergoing PUVA no change in MHC expression could be observed (Gilmour *et al.*, 1993) and another in which PUVA on murine skin proved that MHC class II-positive Langerhans cells, upon exposure to 8-MOP/UVA, lost the expression of this surface molecule; the

latter effect was completely reversible (Aberer *et al.*, 1986). Further research in this field is needed to clarify the effect of 8-MOP/UVA on the expression of MHC class II.

The increase observed in expression of MHC class I and II can lead to an enhanced presentation of (photomodified) oligopeptides (see also above).

The effect of 8-MOP/UVA on other surface molecules has also been studied. It was found that the combination of 8-MOP and UVA leads to a decrease of expression of the adhesion molecule ICAM-1 in murine epidermal cells (Tang and Udey, 1992). Not only was the expression of ICAM-1 decreased, the expression of the epithelial growth factor (EGF) receptor (involved in the tyrosine kinase signalling pathway) was also decreased. This was found both in a human epithelial cell line and in human melanocytes (Kao and Yu, 1992; Laskin and Lee, 1991).

T-helper cells can be divided in different subsets; TH1 and TH2 cells. These cell clones differ in cytokine synthesis pattern. TH1 cells produce among others IL-2, IFN-γ, IFN-β and leucotriene, whereas TH2 cells secrete mediators such as IL-4, IL-5, IL-6 and IL-10 (Mosmann, 1991; Gasparro, 1994c). There is also a difference in function. TH1 cytokines augment cell-mediated (CD8+) immunity via T cell proliferation and macrophage activation, while the TH2 cytokines augment humoral responses and suppress cell-mediated immunity (Gasparro, 1994c; Saed and Fiveson, 1994a). Recently, Saed and Fiveson (1994b) showed that 8-MOP/UVA was able to induce a switch in the cytokine profile of Sézary syndrome cells from TH2 to TH1. This observed switch in TH subset will have a dramatic effect on the course of the disease, as it is known that the cytokines excreted from both TH1 and TH2 exert opposing regulatory effects (Kimber, 1994).

Mechanistic aspects: emphasis on photosensitizers (drugs): One of the ways that may lead to greater insight into the mechanism underlying photopheresis is the use of photosensitizers other than 8-MOP.

For this reason, we investigated the effect singlet oxygen producing photosensitizers have on the induction of immune suppression (van Iperen *et al.*, 1995; Van Iperen and Beijersbergen van Henegouwen, 1997b, in press). These dyes are used in the photodynamic therapy of cancer (PDT) (Henderson and Dougherty, 1992; see section 3.2.1). The dye in its excited triplet state, formed after absorption of light, transfers energy to oxygen. As a result of this, singlet oxygen is produced which causes damage to various biological structures, especially lipid-rich membranes (Henderson and Dougherty, 1992; see section 3.2.1.). The dyes we investigated were bacteriochlorin *a* (BCA), benzoporphyrin derivative mono acid ring A (BPD) and Photofrin®. With all three dyes we were able to induce immune suppression, but it was found that the suppression generated was antigen non-specific. Not only was the CHS response directed towards DNFB

suppressed, but the response to CHS induced by TNCB was also suppressed (van Iperen *et al.*, 1995; Van Iperen and Beijersbergen van Henegouwen, 1997b, in press).

The difference in activity in our animal model for photopheresis (specific suppression versus non-specific suppression) between 8-MOP and the PDT sensitizers, implies that photobinding to biomacromolecules may provide the clue to the underlying mechanism of photopheresis. This supposition is supported by Perez *et al.* (1992).

Up to the present, much emphasis has been put on the photobinding of 8-MOP to DNA as the crucial step in the sequence of events leading to the beneficial effect of photopheresis. However, as mentioned above, it is known from *in vivo* investigations, that 8-MOP not only photobinds to DNA, but also to lipids, as constituents of membranes and to proteins.This prompted us to investigate drugs that can photobind upon irradiation; considered especially of interest were drugs of which it is known that the photobinding is quite different from that of 8-MOP.

With this in mind, we looked at the effect chlordiazepoxide (CDZ) nitrofurantoin (NFT) and chlorpromazine (CPZ) have in our animal model for photopheresis. The photochemical reactivity of these compounds has extensively been investigated, both *in vitro* and *in vivo* (see sections 2.2.4, 2.2.5.4 and 2.2.6.1). It proved that all three drugs were capable of inducing suppression of the CHS response. CDZ and NFT were responsible for a partial immune suppression, whereas CPZ induced complete suppression of the DNFB-induced CHS (van Iperen and Beijersbergen van Henegouwen, 1992).

In a follow-up study, we showed that the suppression induced by CPZ is antigen specific just as with 8-MOP (van Iperen and Beijersbergen van Henegouwen, 1996a). In this study, we also compared the photobinding of CPZ and 8-MOP, which took place under the conditions as applied for photopheresis with this animal model. We used both human and rat lymphocytes for this investigation. It is known that *in vivo* the CPZ photobinding differs from that of 8-MOP (e.g., section 2.2.4). Under the same experimental conditions, CPZ does not photobind to DNA in the rat skin *in vivo*, whereas 8-MOP does. Poor bioavailability of CPZ near the genetic material proved to be the cause of this. We found that, under the conditions we used in our animal model for photopheresis to expose lymphocytes to the combination photosensitizer and UVA, the photobinding of 8-MOP to DNA was 22 times that of CPZ. In contrast, photobinding of CPZ to proteins proved to be more than 20 times that of 8-MOP. (Data found with rat lymphocytes were almost the same as those with human leukocytes.)

This difference between 8-MOP and CPZ is interesting with regard to the mechanism underlying photopheresis. The results indicate that not only the photobinding to DNA is crucial, but that the binding to proteins is also

important. In addition, we concluded that CPZ is an interesting candidate drug for photopheresis (van Iperen and Beijersbergen van Henegouwen, 1996b).

In a recent study, we compared the effect of 4,6,4′-trimethylangelicin (TMA) with that of 8-MOP (van Iperen *et al.*, 1996a). Both 8-MOP and TMA are furocoumarins (for the chemical structure of TMA see Fig. 31). The photodegradation of the two compounds (in the presence and absence of rat lymphocytes) was followed with HPLC and the damaging effects on rat lymphocytes was monitored with the MTT-assay. In the MTT test, viable cells convert a soluble tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble formazan precipitate (Mosmann, 1983). This conversion is mediated by the mitochondrial enzyme, succinate dehydrogenase. The amount of formazan produced is proportional to the number of viable cells present (Mosmann, 1983). It proved that, although TMA was completely photodegraded and its effect on lymphocytes was in the same range as 8-MOP, TMA was unable to induce immune suppression in this photopheresis setting.

One explanation for these results is found in a study performed by Bordin *et al.* (1993). They showed that 8-MOP, unlike TMA was capable of inducing DNA-protein cross-linking. This cross-linking of DNA and proteins can lead to the induction of p53, a tumour suppressor protein; p53 will prevent the progression through the cell cycle until the DNA damage is repaired (Zhan *et al.*, 1993). In the cytoplasm, NF-κB (a transcription factor) may be released. NF-κB translocates to the nucleus and may gain access to specific binding sites and leads to the upregulation of several genes. After repair of the damage, the molecules (such as p53 and NF-κB) the cells have orchestrated in their response are no longer needed and will be degraded by cellular enzymes. These degradation products can be presented by MHC class I, that has been up-regulated during the 8-MOP/UVA exposure (Moor *et al.*, 1995; Schmitt *et al.*, 1995) . As a result of these induced events, these cells have altered immunogenic properties and may stimulate the immune system in a way as to induce specific suppression.

Another way to explain the inability of TMA to induce immune suppression is found in a study by Schoonderwoerd *et al.* (1991a). This study showed that the photobinding of 8-MOP and TMA to biomacromolecules *in vivo* is different. TMA has a preference for lipids; its photobinding to lipids is two times higher than that of 8-MOP. This is probably due to the more hydrophobic character of TMA compared with 8-MOP: the partition coefficient *n*-octanol/water is about 20 times higher for TMA than for 8-MOP. The more hydrophobic nature of TMA probably also leads to a lower bioavailability in the nucleus *in vivo*. Whereas the *in vitro* DNA-photobinding is higher for TMA than for 8-MOP (Guitto *et al.*, 1984), it was shown (Schoonderwoerd *et al.*, 1991a) that the *in vivo* photobinding to DNA is for TMA eight times less than that of 8-MOP.

Due to its hydrophobic nature, TMA might be incapable of binding to DNA in a sufficient amount. (The results of the MTT-test appear to conflict with the conclusion that the bioavailability of TMA in the nucleus is too low to induce a sufficient amount of DNA-photobinding. However, the MTT test only provides information concerning the status of the mitochondria and therefore not necessarily on the extent of photobinding to DNA in the nucleus).

To determine whether the hydrophobic nature of TMA was responsible for its poor performance in photopheresis, we examined the activity of 4,8,5′-trimethylpsoralen (TMP), 1,4,6,8-tetramethyl-2H-furo[2,3-h]-quinolinone (RG 777) and psoralen (for the chemical structures see Fig. 31). TMP and psoralen are (just as 8-MOP and TMA) furocoumarins. The lipophilicity of TMP is in the same range as that of TMA. RG 777 is also a lipophilic compound. Both TMP and RG 777 can photobind to biomacromolecules, DNA, proteins and lipids (Dall'Acqua *et al.*, 1985). The last also holds for psoralen, however, its lipophilicity resembles that of 8-MOP.

It was found that even when TMP and RG 777 were completely photodegraded, they were not, or hardly, able to induce immunosuppression. This in contrast with psoralen. Psoralen induced, just as 8-MOP, a complete suppression of the CHS response (van Iperen *et al.*, 1996b). This study supports the idea that lipophilicity plays an important role in the mechanism underlying photopheresis. A compound with a pronounced lipophilic character is unable to induce suppression. This implies that hydrophobic parts of the leukocytes do not play an important role in the cascade of events leading to the induction of specific immune suppression.

An interesting target for photobinding and possibly important for the mechanism for photopheresis are immune transfer factors. In 1955; Lawrence showed that dialysable leukocyte extract (DLE) can transfer cutaneous hypersensitivity response from a skin test positive individual to a normal skin test negative individual. He called the factor responsible for this phenomenon transfer factor (TF). Since then, it was shown that with DLE it is possible to transfer specific immune responses from one individual to another (Fudenberg and Pizza, 1993). This transfer is even possible between species (Steele *et al.*, 1976; Burger *et al.*, 1979; Klesius *et al.*, 1981). The structure of these factors is not yet completely elucidated. It appears that transfer factors are a family of highly polar, hydrophilic molecules of low molecular mass (approximately 3.5 to 6 kDa) and that they have an oligoribonucleotide–peptide structure (Wilson *et al.*, 1982; Kirkpatrick, 1992; Fudenberg and Pizza, 1993). Because TF can transfer specific immune responses, it is likely that the individual TFs differ structurally in a manner similar to the subtle variations in antigen-binding sites at the hypervariable region of immunoglobulins, or in T cell receptors for antigens (Fudenberg and Pizza, 1993).

The fact that both DLE and photopheresis do have a specific mode of action prompted us to investigate the role of TFs in photopheresis (van Iperen *et al.*, 1996c). In this study we found that DLE from 14×10^6 leukocytes of CHS rats obtained immediately after exposure to the combination of 8-MOP, or CPZ and UVA was able to suppress the CHS response for 50% and 40%, respectively. Curiously, we found the same percentage if we used 60×10^6 leukocytes (van Iperen *et al.*, 1996c). These findings lead to the conclusion that TFs may play a role in the cascade of events leading to suppression after photopheresis treatment. Further research is needed to elucidate the exact role TFs play in the mechanism underlying photopheresis.

3.2.4.3 Conclusion. Although photopheresis therapy is a fresh shoot on the tree of phototherapies, the clinical results are such that one can expect that this therapy will become a standard treatment modality in many hospitals.

There is still a lot of research needed to elucidate the mechanism of action underlying photopheresis. Such mechanistic knowledge will lead to optimization and broadening of the scope of this therapy.

4 Conclusions

The variety in molecular structure of phototoxic drugs is immense. This implies that almost all classes of compounds contain members with adverse photobiological effects.

Before the introduction of a particular drug, careful *in vitro* and *in vivo* investigations can help to estimate the risk that phototoxicity will show to be a side-effect. This kind of research is all the more needed before commercialization, because photoreactivity of drugs in the body can be the cause of side-effects of both topical and systemic nature.

Medicinal photochemistry can provide the knowledge of the part of the molecular structure which is responsible for the phototoxicity of a particular drug. With this insight, analogues can be found which still have the desired pharmacological activity, but which are deprived of phototoxicity. (Self-evidently, medicinal photochemists play a comparable role in the optimization of a potential phototherapeutic drug.)

Because of their specificity and lack of side-effects, the search for new phototherapeutics deserves further attention.

References

Aberer, W., Romani, N., Elbe, A. and Stingl, G. (1986). Effects of physiochemical agents on murine langerhans cells and Thy-1 positive dendritic epidermal cells. *J. Immunol.* **136**, 1210–1216.

Abramov, I. (1985). Health effects of interior lighting: discussion. *In* 'The Medical and Biological Effects of Light' (Wurtman, R. J., Baum, M. J. and Potts, J. T., Jr, eds) Annals of the New York Academy of Sciences, Vol. 453, pp. 365–370. The New York Academy of Sciences, New York.

Anderson, R. R. and Parrish, J. A. (1982). Optical properties of human skin. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp. 147–194. Plenum Press, New York.

Andley, U. P. (1987). Photodamage to the eye. *Photochem. Photobiol.* **46**, 1057–1066.

Armus, S., Keyes, B., Cathll, C., Berger, C., Crater, D., Scarborough, D., Klainer, A. and Bisaccia, E., (1990). Photopheresis for the treatment of cutaneous T cell lymphoma. *J. Am. Acad. Dermatol.* **23**, 898–902.

Bakri, A., Beijersbergen van Henegouwen, G. M. J. and Chanal, J. L. (1983). The photopharmacology of the tranquilizer chlordiazepoxide in relation to its phototoxicity. *Photochem. Photobiol.* **38**, 177–183.

Bakri, A., Beijersbergen van Henegouwen, G. M. J. and Chanal, J. L. (1985). Involvement of the N_4-oxide group in the phototoxicity of chlordiazepoxide in the rat. *Photodermatol.* **2**, 205–212.

Bakri, A., Beijersbergen van Henegouwen, G. M. J. and de Vries, H. (1988). Photobinding of some 7-chloro-1,4-benzodiazepines to human plasma protein *in vitro* and photopharmacology of diazepam in the rat. *Pharm. Weekbl. Sci.* **10**, 122–129.

Barr, M. L., McLaughlin, S. N., Murphy, M. P., Stouch, B. C., Wiedermann, J. G., Marboe, C. C., Schenkel, F. A. Berger, C. L. and Rose. E. A. (1995). Prophylactic photopheresis and effect on graft atherosclerosis in cardiac transplantation. *Transplant Proc.* **27**, 1993–1994.

Barratt, M. D. and Brown, K. R. (1985). Photochemical binding of photoallergens to human serum albumin: a simple *in vitro* method for screening potential photoallergens. *Toxicol. Lett.* **24**, 1–6.

Barratt, M. D., Goodwin, B. F. J. and Lovell, W. W. (1987). Induction of photoallergy in Guinea-pigs by injection of photoallergen–protein conjugates. *Int. Arch. Aller. appl. Immunol.* **84**, 385–389.

Bartlett, J. G. (1982). Chloramphenicol. *Med. Clin. North Am.* **66**, 91–102.

Beijersbergen van Henegouwen, G. M. J. (1988). *In vitro* and *in vivo* research on phototoxic xenobiotics: structure-reactivity relationships. *Arch. Toxicol. Suppl.* **12**, 3–9.

Beijersbergen van Henegouwen, G. M. J. and Havinga, E. (1970). Photoreactions of aromatic compounds. XX. Photosubstitution of nitronaphthalenes in alkaline media. *Rec. Trav. Chim.* **89**, 907–912.

Beijersbergen van Henegouwen, G. M. J., van de Zijde, H. J., van de Griend, J. and de Vries, H. (1987). Photochemical decomposition of diphenhydramine in water. *Int. J. Pharm.* **35**, 259–262.

Beijersbergen van Henegouwen, G. M. J., Wijn, E. T., Schoonderwoerd, S. A. and Dall'Acqua, F. (1989a). A method for the determination of PUVA-induced *in vivo* irreversible binding of 8-methoxypsoralen (8-MOP) to epidermal lipids, proteins and DNA/RNA. *J. Photochem. Photobiol. B: Biol.* **3**, 631–635.

Beijersbergen van Henegouwen, G. M. J., Wijn, E. T., Schoonderwoerd, S. A., de Vries, H. and Dall'Acqua, F. (1989b). Singlet oxygen mediated photobinding of 8-methoxypsoralen to DNA and genotoxicity in *E. coli*. *Z. Naturforsch.* **44c**, 819– 823.

Beijersbergen van Henegouwen, G. M. J., Junginger, H. E. and de Vries, H. (1995). Hydrolysis of RRR-α-tocopheryl acetate (vitamin E acetate) in the skin and UV protecting activity (an *in vivo* study with the rat). *J. Photochem. Photobiol. B: Biol.* **29**, 45–51.

Berger, C. L. (1989). Experimental murine and primate models for dissection of the immunosuppressive potential of photochemotherapy in autoimmune disease and transplantation. *Yale J. Biol. Med.* **62**, 611–620.

Berger, C. L., Perez, M., Laroche, L. and Edelson, R. L. (1990). Inhibition of autoimmune

disease in a murine model of systemic lupus erythematosus induced by exposure to syngeneic photoinactivated lymphocytes. *J. Invest. Dermatol.* **94**, 52–57.

Bisaccia, E., Berger, C., DiSpalto, F. X. and Klainer, A. S. (1993). Extracorporeal photopheresis in AIDS-related complex: extended trial. *J. Acquir. Immune Defic. Syndr.* **6**, 386–392.

Blythe, S., England, D., Esser, B., Junk, P. and Lemanske, R. F., Jr (1986). IgE antibody mediated inflammation of rat lung: histologic and bronchoalveolar lavage assessment. *Am. Rev. Respir. Dis.* **134**, 1246–1251.

Bordin, F., Carlassare, F., Busulini, L. and Baccichetti, F. (1993). Furocoumarin sensitization induces DNA-protein cross-links. *Photochem. Photobiol.* **58**, 133–136.

Burger, D. R., Klesius, P. H., Vandenbrak, A. A., Vandenbark, R. M., Vetto, R. M. and Swann, A. I. (1979). Transfer of keyhole limpet haemocyanin dermal reactivity to man with bovine TF. *Cell. Immunol.* **43**, 192–196.

Busker, R. W. (1989). Photoreactivity of nitrofurantoin as a possible cause of some of its side-effects. Ph.D.-thesis, Leiden University.

Busker, R. W. and Beijersbergen van Henegouwen, G. M. J. (1987). The photolysis of 5-nitrofurfural in aqueous solutions: nucleophilic substitution of the nitro-group. *Photochem. Photobiol.* **45**, 331–335.

Busker, R. W., Beijersbergen van Henegouwen, G. M. J., Menke, R. F. and Vasbinder, G. (1988a). Formation of methemoglobin by photoactivation of nitrofurantoin and of 5-nitrofurfural in rats exposed to UVA light. *Toxicology* **51**, 255–266.

Busker, R. W., Beijersbergen van Henegouwen, G. M. J., Van Ballegooie, E. P. and Vasbinder, G. (1988b). Irreversible binding of nitrofurantoin to skin and to plasma proteins of UV-A exposed rats. *Photochem. Photobiol.* **48**, 683–688.

Busker, R. W., Beijersbergen van Henegouwen, G. M. J., Vaassen, A. J. H. and Menke, R. F. (1989). Irreversible photobinding of nitrofurantoin and of nitrofurfural to plasma proteins *in vitro*. *J. Photochem. Photobiol. B: Biol.* **4**, 207–219.

Calvert, J. G. and Pitts, J. N., Jr (1967). Photocyclodimerization. *In* 'Photochemistry' (Calvert, J. G. and Pitts, J. N., Jr, eds) pp. 536–537. John Wiley and Sons, Inc., New York, London and Sydney.

Campbell, K. P., Lipshutz, G. M. and Denney, G. H. (1984). Direct photoaffinity labeling of the high affinity nitrendipine-binding site in subcellular membrane fractions isolated from canine myocardium. *J. Biol. Chem.* **259**, 5384–5387.

Chang, T-Y., Heinrich, L. A. Schultz, M. D., Reizner, G. T., Kumm, R. C. and Cripps, D. J. (1992). PUVA and skin cancer. A historical cohort study on 492 patients. *J. Am. Acad. Dermatol.* **26**, 173–177.

Constanzo-Nordin, M. R., Hubbell, E. A., O'Sullivan, J., Johnson, M. R., Mullen, G. M., Heroux, A. L., Kao, W. G., McManus, B. M., Pifarre, R. and Robinson, J. A. (1992). Photophoresis versus corticosteroids in the therapy of heart transplant rejection. Preliminary clinical report. *Circulation*, **86** supII., 242–250.

Constanzo-Nordin, M. R., MacManus, B. M., Wilson, J. E., O'Sullivan, E. J., Hubbell, E. A. and Robinson, J. A. (1993). Efficacy of photopheresis in the rescue therapy of acute cellular rejection in human heart allografts: A preliminary clinical and immunopathologic report. *Transplant. Proc.* **25**, 881–883.

Cornelissen, P. J. G., Beijersbergen van Henegouwen, G. M. J. and Mohn, G. R. (1980). Structure and photobiological activity of 7-chloro-1,4-benzodiazepines. Studies on the phototoxic effects of chlordiazepoxide, desmethylchlordiazepoxide and demoxepam using a bacterial indicator system. *Photochem. Photobiol.* **32**, 653–661.

Cottrill, C. M., Klainer, A. S. and Bissacia, E. (1994). The role of extracorporeal photochemotherapy in the treatment of AIDS. *In* 'Extracorporeal Photochemotherapy: Clinical Aspects and the Molecular Basis for Efficacy' (Gasparro, F. P., ed.) pp. 81–100. CRC Press, Boca Raton.

Czeisler, C. A., Kronauer, R. E., Allan, J. S., Duffy, J. F., Jewett, M. E., Brown, E. N. and Ronda, J. M. (1989). Bright light induction of strong type (Type 0) resetting of the human circadian pacemaker. *Science* **244**, 1328–1333.

Dall'Acqua, F. and Rodighiero, G. (1985). Biological and medicinal aspects of furocoumarins (psoralens and angelicins). *In* 'Primary Photo-Processes in Biology and Medicine' (Bensasson, R. V., Jori, G., Land, E. J. and Truscott, T. G., eds.), pp. 277–295. Plenum Press, New York.

Dall'Acqua, F., Caffieri, S. and Rodighiero, G. (1985). Photoreactions of furocoumarins (psoralens and angelicins). *In* 'Primary Photo-Processes in Biology and Medicine' (Bensasson, R. V., Jori, G., Land, E. J. and Truscott, T. G., eds.), pp. 259–272. Plenum Press, New York.

Davidson, R. S. (1983). The chemistry of excited complexes: a survey of reactions. *Adv. Phys. Org. Chem.* **19**, 1–130.

Davis, D. M. (1985). Calcium metabolism in healthy men deprived of sunlight. *In* 'The Medical and Biological Effects of Light' (Wurtman, R. J., Baum, M. J. and Potts, J. T., Jr, eds), Annals of the New York Academy of Sciences, Vol. 453, pp. 21–27. The New York Academy of Sciences, New York.

De Mol, N. J. and Beijersbergen van Henegouwen, G. M. J. (1979). Formation of singlet molecular oxygen by 8-methoxypsoralen. *Photochem. Photobiol.* **30**, 331–335.

De Mol, N. J. and Beijersbergen van Henegouwen, G. M. J. (1981). Relation between some photobiological properties of furocoumarins and their extent of singlet oxygen production. *Photochem. Photobiol.* **33**, 815–819.

De Mol, N. J. and Busker, R. W. (1984). Irreversible binding of the chlorpromazine radical cation and of photoactivated chlorpromazine to biological macromolecules. *Chem.-Biol. Interact.* **52**, 79–92.

De Mol, N. J., Beijersbergen van Henegouwen, G. M. J. and Gerritsma, K. W. (1979a). Photodecomposition of catecholamines. Photoproducts, quantum yield and action spectrum of adrenaline. *Photochem. Photobiol.* **29**, 7–12.

De Mol, N. J., Beijersbergen van Henegouwen, G. M. J. and Gerritsma, K. W. (1979b). Photochemical decomposition of catecholamines-II. The extent of aminochrome formation from adrenaline, isoprenaline and noradrenaline induced by ultraviolet light. *Photochem. Photobiol.* **29**, 479–482.

De Mol, N. J., Beijersbergen van Henegouwen, G. M. J. and van Beele, B. (1981a). Singlet oxygen formation by sensitization of furocourmarins complexed with, or covalently bound to DNA. *Photochem. Photobiol.* **34**, 661–666.

De Mol, N. J., Beijersbergen van Henegouwen, G. M. J., Mohn, G. R., Glickman, B. W. and van Kleef, P. M. (1981b). On the involvement of singlet oxygen in mutation induction by 8-methoxypsoralen and UVA irradiation in *Escherichia coli* K-12. *Mutation Res.* **82**, 23–30.

De Mol N. J., Beijersbergen van Henegouwen, G. M. J., Weeda, B., Knox, C. N. and Truscott, G. (1986). Photobinding of psoralens to bacterial macromolecules *in situ* and induction of genetic effect in a bacterial test system. Effects of singlet oxygen diagnostic aids D_2O and DABCO. *Photochem. Photobiol.* **44**, 747–755.

De Vries, H. and Beijersbergen van Henegouwen, G. M. J. (1995). Photodegradation of nifedipine under *in vivo*-related circumstances. *Photochem. Photobiol.* **62**, 959–963.

De Vries, H., Beijersbergen van Henegouwen, G. M. J. and Wouters, R. (1983). Correlation between phototoxicity of some 7-chloro-1,4-benzodiazepines and their (photo)chemical properties. *Pharm. Weekbl. Sci.* **5**, 302–308.

De Vries, H., Beijersbergen van Henegouwen, G. M. J. and Huf, F. A. (1984). Photochemical decomposition of chloramphenicol in a 0.25% eyedrop and in a therapeutical intraocular concentration. *Int. J. Pharm.* **20**, 265–271.

De Vries, H., Bojarski, J., Donker, A. A., Bakri, A. and Beijersbergen van Henegouwen,

G. M. J. (1990a). Photochemical reactions of quindoxin, olaquindox, carbadox and cyadox with protein, indicating photoallergic properties. *Toxicology* **63**, 85–95.

De Vries, H., Beijersbergen van Henegouwen, G. M. J., Kalloe, F. and Berkhuysen, M. H. J. (1990b). Phototoxicity of olaquindox in the rat. *Res. Vet. Sci.* **48**, 240–244.

De Vries H., Hemelaar, P. J., Gevers, A. C. M. and Beijersbergen van Henegouwen, G. M. J. (1994). Photoreactivity of chloramphenicol *in vitro* and *in vivo*. *Photochem. Photobiol.* **60**, 249–252.

Di Spaltro, F. X., Cottrill, C., Cahill, CX., Degnan, E., Mulford, G. J., Scarborough, D., Franks A. J., Jr, Klainer, A. S. and Bisaccia, E. (1993). Extracorporeal photochemotherapy in progressive systemic sclerosis. *Int. J. Dermatol.* **32**, 417– 420.

Dobrinska, M. R. (1979). *In* 'Chemical Stability of Pharmaceuticals' (Connors, K. L., Amidon, G. L. and Kennon, L., eds.), pp. 208–217. John Wiley and Sons, New York.

Durham, S. R. and Kay, A. B. (1985). Eosinophils, bronchial hyperreactivity and late-phase asthmatic reactions. *Clin. Allergy* **15**, 411–418.

Edelson, R., Berger, C., Gasparro, F., Jegasothy, B., Heald, P., Wintroub, B., Vonderheid, E., Knobler, R., Wolff, K., Plewig, G., McKiernan, G., Christiansen, I., Oster, M., Hönigsmann, H., Wilford, H., Kokoschka, E., Rehle, T., Perez, M., Stingl, G. and Laroche, L. (1987). Treatment of cutaneous T-cell lymphoma by extracorporeal photochemotherapy. *N. Engl. J. Med.* **316**, 297–303.

Edelson, R. L. (1991). Photopheresis: present and future aspects. *J. Photochem. Photobiol. B: Biol* **10**, 165–174.

Ellis, M. K., Hill, S. and Foster, P. M. D. (1992). Reactions of nitrosonitrobenzenes with biological thiols: identification and reactivity of glutathion-*S*-yl conjugates. *Chem. Biol. Interact.* **82**, 151–163.

Emmett, E. A. (1974). A case of photoallergic dermatitis to a topically applied diphenhydramine preparation was observed, and the diagnosis was confirmed by photopatch testing. *Arch. Dermatol.* **110**, 249–252.

Ennever, J. F. (1988). Phototherapy for neonatal jaundice. *Photochem. Photobiol.* **47**, 871–876.

Epling, G. A., Sibley, M. T., Chou, T. T. and Kumar, A. (1988). Photofragmentation of phototoxic dibenzocycloheptadiene antidepressants. *Photochem. Photobiol.* **47**, 491–495.

Epstein, J. H. and Wintroub, B. U. (1985). Photosensitivity due to drugs. *Drugs* **30**, 42–57.

Everett, M. A., Yeargers, E., Sayre, R. M. and Olson, R. L. (1966). Penetration of epidermis by ultraviolet rays. *Photochem. Photobiol.* **5**, 533–542.

Eyer, P., Lierheimer, E. and Schneller, M. (1984). Reactions of nitrosochloramphenicol in blood. *Biochem. Pharmacol.* **33**, 2299–2308.

Felli, A., Chimenti, S., Smitt, I. M., Christensen, I., Berger, C. L. and Edelson, R. (1995). Treatment with 8-Methoxypsoralen (8-MOP)/UVA increases MHC class II expression on human monocytes. *J. Invest. Dermatol.* **104**, 647.

Ford, R. A. (1984). Photoallergenicity testing of fragrance material. A review on photoallergy by musk ambrette in male human patients. *Nippon Koshohin Kagakkaishi* **8**, 301–304.

Francalanci, S., Gola, M., Giorgini, S., Muccinelli, A. and Sertoli, A. (1986). Occupational photocontact dermatitis from olaquindox. *Contact Dermatitis* **15**, 112–114.

Frauenfelder, F. T., Morgan, R. L. and Yunis. A. A. (1993). Blood dyscrasias and topical chloramphenicol. *Am. J. Ophthalmol.* **115**(6), 812–813.

Friedman, P. S. (1986). Effects of ultraviolet radiation on immune responses of skin. *Abstracts Book of First European Congress of Photobiology*, S37.

Fries, J. F., Seibold, J. R. and Medsger. T. A. (1992). Photophoresis for scleroderma? No! (editorial). *J. Rheumatol.* **19**, 1011–1013.

Fudenberg, H. H. and Pizza. G. (1993). Transfer factors 1993: new frontiers. *In* 'Progress in drug research, 42' (Jucker, E. ed.), pp. 309–400. Birkhäuser Verlag, Basel.

Fulton, J. E. and Willis, I. (1968). Photoallergy to methoxsalen. *Arch. Dermatol.* **98**, 445–450.

Garssen, J., Nijkamp, F. P., van der Vliet, H. and van Loveren, H. (1991). T-cell-mediated induction of airway hyperreactivity in mice. *Am. Rev. Respir. Dis.* **144**, 931–938.

Gasparro, F. P. (1994a). Psoralen Photochemistry. *In* 'Extracorporeal Photochemotherapy: Clinical Aspects and the Molecular Basis for Efficacy (Gasparro, F. P., ed.)' pp. 13–36. CRC Press, Boca Raton.

Gasparro, F. P. (1994b). The impact of psoralen photoadducts on cells. *In* 'Extracorporeal Photochemotherapy: Clinical Aspects and the Molecular Basis for Efficacy' (Gasparro, F. P., ed.), pp. 37–58. CRC Press, Boca Raton.

Gasparro, F. P. (1994c). Mechanistic events underlying the response of CTCL patients to photopheresis. *In* 'Extracorporeal Photochemotherapy: Clinical Aspects and the Molecular Basis for Efficacy (Gasparro, F. P. ed.)' pp. 101–120. CRC Press, Boca Raton.

Gasparro, R. P., Malane, M. S., Maxwell, V. M. and Tigelaar, R. E. (1993). The treatment of masocytoma cells with 8-methoxypsoralen and long wavelength ultraviolet radiation enhances cellular immunogenicity. *Photochem. Photobiol.* **58**, 682–688.

Gerberick, G. F. and Ryan, C. A. (1990). Predictive mouse ear-swelling model for investigating topical photoallergy. *Food Chem. Toxicol.* **28**, 361–368.

Gilmour, J. W., Vestey, J. P. and Norval, M. (1993). The effect of UV therapy on immune function in patients with psoriasis. *Br. J. Dermatol.* **129**, 28–38.

Giudici, P. A. and Maguire, H. C. (1985). Experimental photoallergy to systemic drugs. *J. Invest. Dermatol.* **85**, 207–211.

Greenhill, J. V. and McLelland, M. A. (1990). Photodecomposition of drugs. *In* 'Progress in Medicinal Chemistry' Vol. 27 (Ellis, G. P. and West, G. B., eds.), pp. 51– 121. Elsevier, Amsterdam.

Gross, B. J., Blanchflower, R. V., Burke, T. R., Lees, D. E. and Pohl, L. R. (1982). Bone marrow toxicity *in vitro* of chloramphenicol and its metabolites. *Toxicol. and Appl. Pharmacol.* **64**, 557–565.

Grossweiner, L. I. (1989). Photophysics. *In* 'The Science of Photobiology' 2nd edn, (Smith, K. C., ed.), pp. 1–45. Plenum Press, New York.

Grossweiner, L. I. and Smith, K. C. (1989). Photochemistry. *In* 'The Science of Photobiology' 2nd edn (Smith, K. C., ed.), pp. 47–78. Plenum Press, New York.

Gubitz, R. H., Akera, T. and Brody, T. M. (1973). Comparative effects of substituted phenothiazines and their free radicals on (Na^+, K^+)-activated adenosine triphosphatase. *Biochem. Pharmacol.* **22**, 1229–1235.

Guiotto, A., Rodighiero, P., Manzini, P., Pastorini, G., Bordin, F., Baccichetti, F., Carlassare, F., Vedaldi, D., Dall'Acqua, F., Tamaro, M., Recchia, G. and Cristofolini, M. (1984). 6-Methylangelicins: A new series of potential photochemotherapeutic agents for the treatment of psoriasis. *J. Med. Chem.* **27**, 959–967.

Harber, L. C., Armstrong, R. B. and Ichikawa, H. (1982). Current status of predictive animal models for drug photoallergy and their correlation with drug photoallergy in humans. *J. Natl. Cancer Inst.* **69**, 237–241.

Havinga, E. and Kronenberg, M. E. (1968). Some problems in aromatic photosubstitution. *Pure Appl. Chem.* **16**, 137–152.

Hawk, J. L. M. and Parrish, J. A. (1982). Responses of normal skin to ultraviolet radiation. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp.219–260. Plenum Press, New York.

Heald, P. W. (1991). Photopheresis for cutaneous T cell lymphoma *Ann. N.Y. Acad. Sci.* **636**, 171–177.

Heald, P. W. and Edelson, R. L. (1989). Photophoresis for T cell mediated diseases. *Adv. Dermatol.* **3**, 25–40.

Heald, P., Rook, A., Perez, M., Wintroub, B., Knobler, R., Jegasothy, B., Gasparro, F.,

Berger, C. and Edelson, R. (1992). Treatment of erythrodermic cutaneous T cell lymphoma with extracorporeal photochemotherapy. *J. Am. Acad. Dermatol.* **27**, 427–433.

Henderson, B. W. and Dougherty, T. J. (1992). How does photodynamic therapy work? *Photochem. Photobiol.* **55**, 145–157.

Holgate, S. T. (1993). Asthma: past present and future. *Eur. Respir. J.* **6**, 1507–1520.

Holick, M. F. (1985). The photobiology of vitamin D and its consequences for humans. *In* 'The Medical and Biological Effects of Light' (Wurtman, R. J., Baum, M. J. and Potts, J. T., Jr, eds), Annals of the New York Academy of Sciences, Vol. 453, pp. 1–13. The New York Academy of Sciences, New York.

Honig, B., Morison, W. L. and Karp, D. (1994). Photochemotherapy beyond psoriasis. *J. Am. Acad. Dermatol.* **31**, 775–790.

Hönigsmann, H. (1988). Photoreactivity of different psoralens in the treatment of vitiligo. *In* 'Light in biology and medicine, Volume 1' (Douglas, R. H., Moan, J. and Dall'Acqua, F., eds.), pp. 315–319. Plenum Press, New York.

Horina, J. H., Müllegger, R. R., Horn, S., Holzer, H., Halwachs, G., Kerl, H. and Wolf, P. (1995). Photophoresis for renal allograft rejection. *Lancet* **346**, 61.

Horio, T. (1984). Photoallergic reaction classification and pathogenesis. *Int. J. Dermatol.* **23**, 376–382.

Ichida, S., Fujisue, T. and Masada, A. (1989a). Characteristics of specific binding of nitrendipine and PN 200–110 to various crude membranes: induction of irreversible binding by UV irradiation. *J. Biochem. (Tokyo)* **105**, 760–766.

Ichida, S., Fujisue, T. and Masada, A. (1989b). Photoaffinity labeling with dihydropyridine derivatives of crude membranes from rat skeletal, cardiac, ileal and uterine muscles and whole brain. *J. Biochem. (Tokyo)* **105**, 767–774.

Ishida, M., Amesara, R., Ukai, K. and Sakakura, Y. (1994). Antigen (DNP-As)-induced allergic rhinitis model in guinea pigs. *Ann. Allergy* **72**, 240–244.

Jarrat, M., Hubler, W., Jr and Panek, W. (1982). Dye-light phototherapy of viral, bacterial and fungal infections. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A. eds.), pp. 561–570. Plenum Press, New York.

Johnson, B. E. (1984). Light sensitivity associated with drugs and chemicals. *Physiol. Pathophysiol. Skin* **8**, 2541– 2606.

Jori, G. (1985). Molecular and cellular mechanisms in photomedicine: porphyrins in cancer treatment. *In* 'Primary Photo-Processes in Biology and Medicine' (Bensasson, R. V., Jori, G., Land, E. J. and Truscott, T. G., eds.), pp. 381–392. Plenum Press, New York.

Kao, C. and Yu, H. (1992). Comparison of the effect of 8-methoxypsoralen (8-MOP) plus UVA (PUVA) on human melanocytes in vitiligo vulgaris and *in vitro*. *J. Invest. Dermatol.* **98**, 734–740.

Kavarnos, G. J. and Turro, N. J. (1986). Photosensitization by reversible electron transfer: theories, experimental evidence and examples. *Chem. Rev.* **86**, 401–449.

Kennedy, J. C., Pottier, R. H. and Pross, D. C. (1990). Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience. *J. Photochem. Photobiol. B: Biol.* **6**, 143–148.

Kimber, I. (1994). Cytokines and regulation of allergic sensitization to chemicals. *Toxicology* **93**, 1–11.

Kirkpatrick, C. H. (1992). Purification and composition of transfer factors. *Molec. Immunol.* **29**, 167.

Klesius, P. H., Qualls, D. F., Elston, A. L. and Fudenberger, H. H. (1981). Effects of bovine transfer factor (TFd) in mouse coccidiosis (*Eimeria ferrisi*). *Clin. Immunol. Immunopathol.* **10**, 247–260.

Kligman, L. H. and Kligman, A. M. (1986). *Photodermatol.* **3**, 215–227.

Kloek, J. and Beijersbergen van Henegouwen, G. M. J. (1996). Prodrugs of 5-aminolevulinic acid for photodynamic therapy. *Photochem. Photobiol.* **64**, 994–1000.

Knobler, R. M., Graninger, W., Lindmaie, A., Trautinger, F. and Smolen, J. S. (1992). Extracorporeal photochemotherapy for the treatment of lupus erythematosus. *Arthritis Rheum.* **35**, 319–324.

Kochevar, I. E. (1979). Photoallergic responses to chemicals. *Photochem. Photobiol.* **30**, 437–442.

Kochevar, I. E. (1987). Mechanisms of drug photosensitisation. *Photochem. Photobiol.* **45**, 891–895.

Kochevar, I. E. (1989). Phototoxicity of nonsteroidal inflammatory drugs. *Arch. Dermatol.* **125**, 824–826.

Krinsky, N. I. (1982). Photobiology of carotenoid protection. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp. 397–407. Plenum Press, New York.

Laskin, J. D. and Lee, E. (1991). Psoralen binding and inhibition of epidermal growth factor binding by psoralen/ultraviolet light (PUVA) in human epithelial cells. *Biochem. Pharmacol.* **41**, 125–132.

Lawrence, H. S. (1955). The transfer in humans of delayed skin sensitivity to streptococcal M substance and to tuberculin with disrupted leukocytes. *J. Clin. Invest.* **34**, 219.

Ljundgren, B. (1977). Psoralen photoallergy caused by plant contact. *Contact Dermatol.* **3**, 85–90.

Ljunggren, B. and Möller, H. (1977). Phenothiazine phototoxicity: an experimental study on CPZ and related tricyclic drugs. *Acta Dermatovener.* **57**, 325–329.

Malawista, S. E., Trock, D. H. and Edelson, R. L. (1991). Treatment of rheumatoid arthritis by extracorporeal photochemotherapy. *Arthritis Rheum.* **34**, 646–654.

Marko, J., Vermeersch, G., Febvay-Garot, N. and Lablanche-Combier, A. (1985). Photo-CIDNP in nucleic acid bases and their nucleotides induced by chlorpromazine and analogues. *Photochem. Photobiol.* **42**, 213–221.

Marks, D. I. and Fox, R. M. (1991). Mechanism of photochemotherapy-induced apoptotic cell death in lymphoid cells. *Biochem. Cell. Biol.* **69**, 754–760.

Mathews-Roth, M. M. (1982). Beta-carotene therapy for erythropoietic protoporphyria and other photosensitivity diseases. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp. 409–440. Plenum Press, New York.

McDonagh, A. F. (1985). Light effects on transport and excretion of bilirubin in newborns. *In* 'The Medical and Biological Effects of Light' (Wurtman, R. J., Baum, M. J. and Potts, J. T., Jr, eds), Annals of the New York Academy of Sciences, Vol. 453, pp. 65–72. The New York Academy of Sciences, New York.

McDonagh, A. F. (1986). Sunlight-induced mutation of bilirubin in a long-distance runner. *N. Engl. J. Med.* **314**, 121.

McElroy, M. B. and Salawitch, R. J. (1989). Changing composition of the global stratosphere. *Science* **243**, 763– 770.

Meiser, B. M., Kur, F., Reichenspurner, H., Wagner, F., Boos, K. S., Vielhaufer, S., Weiss, M., Rohrbach, H., Kreuzer, E., Überfuhr, P. and Reichart, B. (1994). Reduction of the incidence of rejection by adjunct immunosuppression with photochemotherapy after heart transplantation. *Transplantation* **57**, 563–568.

Melnick, J. L. and Wallis, C. (1982). Photodynamic inactivation of herpus virus. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp. 545– 559. Plenum Press, New York.

Moan, J. (1986). Porphyrin photosensitization and phototherapy. *Photochem. Photobiol.* **43**, 681–690.

Moor, A. C. E. and Gasparro, F. P. (1996). Biochemical aspects of psoralen photochemotherapy. *Clin. Dermatol.* **14**, 353–365.

Moor, A. C. E., Schmitt, I. M., Beijersbergen van Henegouwen, G. M. J., Chimenti, S., Edelson, R. L. and Gasparro, F. P. (1995). Treatment of 8-MOP and UVA enhances MHC

class I synthesis in RMA cells: preliminary results. *J. Photochem. Photobiol. B: Biol.* **29**, 193–198.

Morison, W. L. (1985). Photoimmunology: study of the effects of nonionizing radiation on the immune system. *In* 'The Medical and Biological Effects of Light' (Wurtman, R. J., Baum, M. J. and Potts, J. T., Jr, eds), Annals of the New York Academy of Sciences, Vol. 453, pp. 105–113. The New York Academy of Sciences, New York.

Morison, W. L. and Kochevar, I. E. (1983). Photoallergy. *In* 'Photoimmunology' (Parrish, J. A., Kripke, M. L. and Morison, W. L., eds.), pp. 227–253. Plenum Publishing Corporation, New York.

Morison, W. L. and Parrish, J. A. (1982). Photo-immunology. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp. 293–320. Plenum Press, New York.

Morison, W. L., McAuliffe, D. J., Parrish, J. A. and Bloch, K. J. (1982). *In vitro* assay for phototoxic chemicals. *J. Invest. Dermatol.* **78**, 460–463.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63.

Mosmann, T. R. (1991). Cytokine secretion patterns and cross-regulation of T cell subsets. *Immunol. Res.* **10**, 183–188.

Motten, A. G., Chignell, C. F. and Mason, R. P. (1983). Spectroscopic studies of cutaneous photosensitizing agents-VI. Identification of the free radicals generated during the photolysis of musk ambrette, musk xylene and musk ketone. *Photochem. Photobiol.* **38**, 671–678.

Motten, A., Buettner, G. R. and Chignell, C. F. (1985). Spectroscopic studies of cutaneous photosensitizing agents-VIII. A spin trapping study of light induced free radicals from chlorpromazine and promazine. *Photochem. Photobiol.* **42**, 9–16.

Owsianowski, M., Gollnick, H., Siegert, W., Schwerdtfeger, R. and Orfanos, C. E. (1994). Succesful treatment of chronic graft-versus-host disease with extracorporeal photopheresis. *Bone Marrow. Transplant.* **14**, 845–848.

Oziemski, M. A., Rockman, S. P., Marks, D. I., Green, M. D., Varigos, G. A. and Fox, R. M. (1991). Extracorporeal photochemotherapy in the treatment of cutaneous T cell lymphoma. Complexity of objective evaluation. *Int. J. Dermatol.* **30**, 814–817.

Pandit, N. (1979). *In* 'Chemical Stability of Pharmaceuticals' (Connors, K. A., Amidon, G. L. and Kennon, L., eds.), pp.201–207. John Wiley and Sons, New York.

Parker, R. D., Buehler, E. V. and Newmann, E. A. (1986). Phototoxicity, photoallergy and contact photosensitization of nitro musk perfume raw materials. *Contact Dermatitis* **14**, 103–109.

Parrish, J. A., Fitzpatrick, T. B. and Tanenbaum, L. (1974). Photochemotherapy of psoriasis with oral methoxalen and longwave ultraviolet light. *N. Engl. J. Med.* **291**, 1207–1211.

Parrish, J. A., Stern, R. S., Pathak, M. A. and Fitzpatrick, T. B. (1982). Photochemotherapy of skin diseases. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A. eds.), pp. 595–625. Plenum Press, New York.

Pepino, P., Berger, C.L., Fuzesi, L., Panza, A., Pierson, R. N., Gutierrez, C., Marboe, C. C., Smith, C. R., Reemtsma, K. and Rose, E. A. (1989). Primate cardiac allo- and xenotransplantation: Modulation of the immune response with photochemotherapy. *Eur. Surg. Res.* **21**, 105–113.

Perez, M., Edelson, R., Laroche, L. and Berger, C. (1989). Inhibition of antiskin allograft immunity by infusions with syngeneic photoinactivated effector lymphocytes. *J. Invest. Dermatol.* **92**, 669–676.

Perez, M. I., Berger, C. L., Yamane, Y., John, L., Laroche, L. and Edelson, R. L. (1991). Inhibition of anti-skin allograft immunity induced by infusions with photoinactivated effector T lymphocytes – The congenenic model. *Transplantation* **51**, 1283–1289.

Perez, M. I., Yamane, Y., John, L., Gasparro, F. P. and Edelson, R. L. (1992). DNA associated with the cell membrane is involved in the inhibition of the skin rejection response induced

by infusions of photodamaged alloreactive cells that mediate rejection of skin allograft. *Photochem. Photobiol.* **55**, 839–849.

Plewig, G., Hofmann, C. and Braun-Falco, O. (1978). Photoallergic dermatitis from 8-methoxypsoralen. *Arch. Dermatol. Res.* **261**, 201–211.

Pöhlmann, H., Theil, F. P., Franke, P. and Pfeifer, S. (1986). *In vitro-* und *in vivo-*Untersuchungen zur fotochemischen Reaktivität von Methaqualon-1-oxid. *Pharmazie* **41**, 856–858.

Potapenko, A. Ya, Moshin, M. V., Krasnovski, A. A. and Sukurov, V. L. (1982), Dark oxidation of unsaturated lipids by the photooxidized 8-methoxypsoralen. *Z. Naturforsch.* **37c**, 70–74.

Prinz, B., Nachbar, F. and Plewig, G. (1994). Treatment of severe atopic dermatitis with extracorporeal photopheresis. *Arch. Dermatol. Res.* **287**, 48–52.

Prinz, B., Behrens, W., Hölzle, E. and Plewig, G. J. (1995). Extracorporeal photopheresis for the treatment of cutaneous T cell lymphoma – the Düsseldorf and München experience. *Arch. Dermatol. Res.*, **287**, 621–626.

Raf, M. C. (1992). Social controls on cell survival and cell death. *Nature* **356**, 397–400.

Rapp, F. and Li, J.-J. H. (1982). Transforming activity of viruses after dye-light inactivation. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds.), pp. 571–594. Plenum Press, New York.

Rook, A. H., Heald, P., Nahass, G., Ditre, C., Witmer, W., Lazarus, G. and Edelson, R. L. (1990). Extracorporeal photochemotherapy for drug-resistant pemphigus vulgaris. *Ann. Intern. Med.* **112**, 303–305.

Rook, A. H., Freundlich, B., Jegasothy, B. V., Perez, M. I., Barr, W. G., Jimenez, S. A., Rietschel, R. L., Wintroub, B., Kahaleh, M. B., Varga, J., Heald, P. W., Steen, V., Massa, M. C., Murphy, G. F., Perniciaro, C., Istfan, M., Ballas, S. K. and Edelson, R. L. (1992). Treatment of systemic sclerosis with extracorporeal photochemotherapy: results of a multicenter trial. *Arch. Dermatol.* **128**, 337–346.

Rossetti, F., Zulian, F., Dall'Amico, R., Messina, C., Montini, G. and Zacchello, F. (1995). Extracorporeal photochemotherapy as single therapy for extensive, cutaneous, chronic graft-versus-host disease. *Transplant* **15**, 149–151.

Saed, G. and Fiveson, D. P. (1994a). Augmentation of Th-1 cytokines in the peripheral blood of Sézary syndrome patients after treatment with extracorporeal photopheresis. *J. Invest. Dermatol.* **103**, 29–33.

Saed, G. and Fiveson, D. P. (1994b). Quantitation of interferom gamma mRNA levels in psoralen/UVA-treated HUT-78 cells by competitive PCR. *Biochem. Biophys. Res. Com.* **203**, 935–942.

Schmitt, I. M., Moor, A. C. E., Patrignelli, R., Chimenti, S., Beijersbergen van Henegouwen, G. M. J., Edelson, R. L. and Gasparro, F. P. (1995). Increased surface expression of class I MHC molecules on immunogenic cells derived from the xenogenization of P815 mastocytoma cells with 8-methoxypsoralen and long-wavelength ultraviolet radiation. *Tissue Antigens* **46**, 45–49.

Schoonderwoerd, S. A. and Beijersbergen van Henegouwen, G. M. J. (1987). Irreversible photobinding to skin constituents after systemic administration of chlorpromazine to Wistar rats. *Photochem. Photobiol.* **46**, 501–505.

Schoonderwoerd, S. A., Beijersbergen van Henegouwen, G. M. J. and Luijendijk, H. J. (1988). Photobinding of chlorpromazine and its sulfoxide *in vitro* and *in vivo*. *Photochem. Photobiol.* **48**, 621–626.

Schoonderwoerd, S. A., Beijersbergen van Henegouwen, G. M. J. and van Belkum, S. (1989). *In vivo* photodegradation of chlorpromazine. *Photochem. Photobiol.* **50**, 659–664.

Schoonderwoerd, S. A., Beijersbergen van Henegouwen, G. M. J. and Panday, I. R. (1990). UVA-induced genetic effects of thioridazine, mesoridazine and sulforidazine: an *in vitro* study. *Z. Naturforsch.* **45c**, 638–644.

Schoonderwoerd, S. A., Beijersbergen van Henegouwen, G. M. J., Persons, C. C. M., Caffieri, S. and Dall'Acqua, F. (1991a). Photobinding of 8-methoxypsoralen, 4,6,4′-trimethylangelicin and chlorpromazine to Wistar rat epidermal biomacromolecules *in vivo*. *J. Photochem. Photobiol. B: Biol.* **10**, 257–268.

Schoonderwoerd, S. A., Beijersbergen van Henegouwen, G. M. J. and Persons, C. C. M. (1991b). Effect of alpha-tocopherol and di-butyl-hydroxytoluene (BHT) on UV-A-induced photobinding of 8-methoxypsoralen to Wistar rat epidermal biomacromolecules *in vivo*. *Arch. Toxicol.* **65**, 490–494.

Sedee, A. G. J. and Beijersbergen van Henegouwen, G. M. J. (1983). A synthesis for *p*-quinol compounds from phenols fused with other saturated rings. *Tetrahedron Lett.* **24**, 5779–5780.

Sedee, A. G. J. and Beijersbergen van Henegouwen, G. M. J. (1985). Photosensitized decomposition of contraceptive steroids: a possible explanation for the observed (photo)allergy of the oral contraceptive pill. *Arch. Pharm.* **318**, 111–119.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J. and Blaauwgeers, H. J. A. (1983). Isolation, identification and desitometric determination of norethisterone-4β,5β-epoxide after photochemical decomposition of norethisterone. *Int. J. Pharm.* **15**, 149–158.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., Guijt, W. and Haasnoot, C. A. G. (1984a). Assignment of the ^{1}H- and ^{13}C-NMR spectra of norethisterone using two dimensional NMR spectroscopy. *J. Chem. Soc. Perkin Trans II*, 1755–1759.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., Lusthof, K. J. and Lodder, G. (1984b). Photosensitized irreversible binding to protein via a hydroperoxide intermediate: an explanation of (photo-)allergic side-effects of estrogens. *Biochem. Biophys. Res. Commun.* **125**, 675–681.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., de Mol, N. J. and Lodder, G. (1984c). Interaction of the photosensitization products of oestrone with DNA: comparison with the horseradish peroxidase catalyzed reaction. *Chem.-Biol. Interactions* **51**, 357–363.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., Lusthof, K. and Lodder, G. (1984d). Irreversible binding of norethisterone to human serum protein induced by UV-B light. *Pharm. Res.* **1**, 126–129.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., de Vries, M. E. and Erkelens, C. (1985a). Mestranol: the assignment of its ^{1}H- and ^{13}C-NMR spectra by means of two-dimensional NMR spectroscopy, and its photochemical decomposition. *Steroids* **45**, 101–118.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., Guijt, W. and Haasnoot, C. A. G. (1985b). Conformational analysis of steroids in solution: 17β-Hydroxy-19-nor-5α, 17α-pregn-20-yn-3-one and its 5β-isomer studied by nuclear magnetic resonance. *J. Org. Chem.* **50**, 4182–4187.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., Guijt, W. and Haasnoot, C. A. G. (1985c). Structure elucidation of two isomeric steroids: photolytical and thermal reaction products from norethisterone studied by two-dimensional nuclear magnetic resonance. *Pharm. Weekbl. Sci. Ed.* **7**, 202–207.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J., de Vries, H., Guijt, W. and Haasnoot, C. A. G. (1985d). The photochemical decomposition of the progestogenic 19-norsteroid, norethisterone, in aqueous medium. *Pharm. Weekbl. Sci. Ed.* **7**, 194–201.

Sedee, A. G. J., Beijersbergen van Henegouwen, G. M. J. and Bakri, A. (1985e). Investigation into the effects of the photodecomposition of norethisterone by UV-B light on *Salmonella typhimurium* TA98 and the rat. *Contraception* **32**, 207–216.

Sidi, E. and Bourgeois-Gavardin, J. (1953). Mise au point du traitement par 'l'Ammi Majus'. *Presse Medicine* **61**, 436–440.

Slovis, B. S., Loyd, J. E. and King, L. E., Jr (1995). Photophoresis for chronic rejection of lung allografts. *N. Engl. J. Med.* **332**, 962.

Song, P. S. and Tapley, K. J. (1979). Photochemistry and photobiology of psoralens. *Photochem. Photobiol.* **29**, 1177–1197.

Spikes, J. D. (1989). Photosensitization. *In* 'The Science of Photobiology' 2nd edn, (Smith, K. C., ed.), pp. 79–110. Plenum Press, New York.

Stables, G. I. and Ash, D. V. (1995). Antitumour treatment: photodynamic therapy. *Cancer Treatment Reviews* **21**, 311–323.

Steele, R. W., Eichberg, J. W., Heberling, R. L., Eller, J. J., Kalter, S. S. and Kniker, W. T. (1976). *In vivo* transfer of cellular immunity to primates with transfer factor prepared from human or primate leukocytes. *Cell. Immunol.* **22**, 110–120.

Steen, V. D., Medsger T. A., Jr and Rodnan, G. P. (1987). D-Penicillamine therapy in progressive systemic sclerosis (scleroderma). *Ann. Intern. Med.* **97**, 367–369.

Steenvoorden, D. P. T. and Beijersbergen van Henegouwen, G. M. J. (1997). The use of endogenous antioxidants to improve photoprotection. *J. Photochem. Photobiol. B: Biol.* in press.

Stern, R. S. and Laird, S. (1994). The carcinogenic risk of treatments for severe psoriasis. *Cancer* **73**, 2759–2764.

Sünder-Plassman, G., Druml, W., Steininger, R., Hönigsmann, H. and Knobler, R. (1995). Renal allograft rejection controlled by photopheresis. *Lancet* **346**, 506.

Tanew, A., Ortel, B. and Hönigsmann, H. (1988). 5-Methoxypsoralen and other new furocoumarins in the treatment of psoriasis. *In* 'Light in biology and medicine, Volume 1' (Douglas, R. H., Moan, J. and Dall'Acqua, F., eds.), pp. 183–188. Plenum Press, New York.

Tang, A. and Udey, M. C. (1992). Doses of ultraviolet radiation that modulate accessory cell activity and ICAM-1 expression are ultimately cytotoxic for murine epidermal Langerhans cells. *J. Invest. Dermatol.* **99**, 71S–75S.

Thoma, K. and Klimek, R. (1985a). Untersuchungen zur Photostabilität von Nifedipin, 1. Mitt.: Zersetzungskinetik und Reaktionsmechanismus. *Pharm. Ind.* **47**, 207–215.

Thoma, K. and Klimek, R. (1985b). Untersuchungen zur Photostabilität von Nifedipin, 2. Mitt.: Einflusz von Milieubedingungen. *Pharm. Ind.* **47**, 319–327.

Thomas, S. E. and Wood, M. L. (1986). Photosensitivity reactions associated with nifedipine. *Br. Med. J.* **292**, 992.

Tønnesen, H. H., de Vries, H., Karlsen, J. and Beijersbergen van Henegouwen, G. M. J. (1987). Studies on curcumin IX (Investigation of the photobiological activity of curcumine using bacterial indicator systems). *J. Pharm. Sci.* **76**, 371–373.

Urbach, F. (1982). Photocarcinogenesis. *In* 'The Science of Photomedicine' (Regan, J. D. and Parrish, J. A., eds), pp. 261–292. Plenum Press, New York.

van den Broeke, L. T. and Beijersbergen van Henegouwen, G. M. J. (1993a). Thiols as potential UV radiation protectors: an *in vitro* study. *J. Photochem. Photobiol. B: Biol.* **17**, 279–286.

van den Broeke, L. T. and Beijersbergen van Henegouwen, G. M. J. (1993b). UV-radiation protecting efficacy of thiols, studied with UVA-induced binding of 8-MOP and CPZ to rat epidermal biomacromolecules *in vivo*. *Int. J. Radiat. Biol.* **63**, 493–500.

van den Broeke, L. T. and Beijersbergen van Henegouwen, G. M. J. (1994). UV-radiation protecting efficacy of cysteine derivatives, studied with UVA-induced binding of 8-MOP and CPZ to rat epidermal biomacromolecules *in vivo*. *Int. J. Radiat. Biol.* **67**, 411–420.

van den Broeke, L. T., Ouijja, H., Bojarski, J. and Beijersbergen van Henegouwen, G. M. J. (1994). *In vitro* photodegradation of chlorpromazine. *Photochem. Photobiol.* **59**, 140–144.

van der Leun, J. C. (1966). Ultraviolet erythema: a study on diffusion processes in human skin. Ph.D. Thesis, Utrecht, The Netherlands.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1992). An animal model for extracorporeal photochemotherapy based on contact hypersensitivity. *J. Photochem. Photobiol. B: Biol.* **15**, 361–366.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1993). An animal model and new photosensitizers for photopheresis. *Photochem. Photobiol.* **58**, 571–575.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1996a). Photophoresis: the risk of photoallergy. *J. Photochem. Photobiol. B: Biol.* **34**, 225–228.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1996b). Chlorpromazine; a candidate drug for photopheresis. *J. Photochem. Photobiol. B: Biol.* **34**, 217–224.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1996c). Photophoresis: The effect of the different bloodcomponents on the immune suppressive effect of photopheresis. *In* 'Clinical and Mechanistic Aspects of Photophoresis', pp. 59–66. Ph.D thesis, Leiden University, Leiden.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1996d). Photophoresis: the amount of irradiated buffy coat (an animal study). *In* 'Clinical and Mechanistic Aspects of Photophoresis', pp. 69–76. Ph.D thesis, Leiden University, Leiden.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1997a). Photophoresis, a possible therapy for airway hypersensitivity. *J. Photochem. Photobiol. B: Biol.* In press.

van Iperen, H. P. and Beijersbergen van Henegouwen, G. M. J. (1997b). Singlet oxygen producing photosensitizers in photopheresis. *J. Photochem. Photobiol. B: Biol.* in press.

van Iperen, H. P., Schuitmaker, H. J. and Beijersbergen van Henegouwen, G. M. J. (1995). Non-specific systemic immune suppression induced by photodynamic treatment of lymph node cells with bacteriochlorin *a*. *J. Photochem. Photobiol. B: Biol.* **28**, 197–202.

van Iperen, H. P., Brun, B. M., Caffieri, S., Dall'Acqua, F., Gasparro, F. P. and Beijersbergen van Henegouwen, G. M. J. (1996a). The lack of efficacy of 4,6,4′-Trimethylangelicin to induce immune suppression in an animal model for photopheresis (a comparison with 8-MOP) *Photochem. Photobiol.* **63**, 577–582.

van Iperen, H. P., Sbrogiò, E., Burriesci, A., Steenvoorden, D. P. T., Caffieri, S., Dall'Acqua, F. and Beijersbergen van Henegouwen, G. M. J. (1996b). Furocoumarins for photopheresis: the effect of lipophylicity on the immune suppressive ability. *In* 'Clinical and Mechanistic Aspects of Photophoresis', pp. 131–141. Ph.D thesis, Leiden University, Leiden.

van Iperen, H. P., IJland, S. A. J. and Beijersbergen van Henegouwen, G. M. J. (1996c). The role of transfer factors. *In* 'Clinical and Mechanistic Aspects of Photophoresis', pp. 121–128. Ph.D thesis, Leiden University, Leiden.

van Loveren, H., Garssen, J. and Nijkamp, F. P. (1991). T cell-mediated airway hyperreactivity in mice. *Eur. Respir. J. 4 suppl. 13*, 16s–26s.

van Weelden, H. (1986). Personal Communication, Department of Dermatology, University of Utrecht, The Netherlands.

Veronese, F. M., Schiavon, O., Bevilacqua, R., Bordin, F. and Rodighiero, G. (1981). The effect of psoralens and angelicins on proteins in the presence of UVA-irradiation. *Photochem. Photobiol.* **31**, 351–354.

Vowels, B. R., Cassin, M., Boufal, M. H., Walsh, L. J. and Rook, A. H. (1992). Extracorporeal photochemotherapy induces the production of tumor necrosis factor-α by monocytes: implications fro the treatment of cutaneous T cell lymphoma and systemc sclerosis. *J. Invest. Dermatol.* **98**, 686–692.

Wieland, M., Thiede, V. L., Strauss, R. G., Piette, W. W., Kapelanski, D. P., Landas, S. K., Hunsicker, L. G., Vance, S. J. and Randels, M. J. (1994). Treatment of severe cardiac allograft rejection with extracorporeal photochemotherapy. *J. Clin. Apheresis* **9**, 171–175.

Wilson, G. B., Paddock, G. V. and Fudenberg, H. H. (1982). Bovine 'transfer factor': an oligoribonucleotide which initiates antigen-specific lymphocyte responsiveness. *Thymus* **4**, 335.

Wolfe, J. T., Lessin, S. R., Singh, A. H. and Rook, A. H. (1994). Review of immunomodulation by photopheresis: treatment of cutaneous T cell lymphoma, autoimmune disease, and allograft rejection. *Artif. Organs* **18**, 888–897.

Yoshikawa, K., Mori, N., Sakakibara, S., Mizuno, N. and Song, P-S. (1979). Photo-conjugation

of 8-methoxypsoralen with proteins. *Photochem. Photobiol.* **29**, 1127–1133.

Yunis, A. A., Miller, A. M., Salem, Z., Corbett, M. D. and Arimura, G. K. (1980). Nitroso-chloramphenicol: possible mediator in chloramphenicol-induced aplastic anemia. *J. Lab. Clin. Med.* **96**, 36–46.

Zaynoun, S., Johnson, B. E. and Frain-Bell, W. (1976). The investigation of quindoxin photosensitivity. *Contact Dermatitis* **2**, 343–352.

Zhan, O., Carrier, F. and Fornace, A. J. (1993). Induction of cellular p53 activity by DNA-damaging agents and growth arrest. *Mol. Cell. Biol.* **13**, 4242–4250.

Development of Estrogen Antagonists as Pharmaceutical Agents

SUPRABHAT RAY and INDRA DWIVEDY

Medicinal Chemistry Division, Central Drug Research Institute, Lucknow 226001, India

ADVANCES IN DRUG RESEARCH, VOL. 29
ISBN 0-12-013329-6

LIST OF ABBREVIATIONS

ADD	Androstene-3,17-dione
DMBA	7,12-Dimethyl benzanthracene
E_1	Estrone
E_2	Estradiol
E_3	Estriol
ED	Effective dose
ELA	Estrogenicity lowering agents
ER	Estrogen receptor
ERE	Estrogen response element
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
GnRF	Gonadotrophin-releasing factor
HDL	High density lipoproteins
LD	Lethal dose
LH	Luteinizing hormone
LHRH	LH-releasing hormone
MED	Minimum effective dose
P	Progesterone
PTH	Parathyroid hormone
SAR	Structure activity relationship
TAE	Triaryl ethylenes
TAP	Triaryl propenones
TGF	Transforming growth factor
TRH	Thiroxiphin-releasing hormone
VLDL	Very low density lipoproteins

1 **Introduction**

Estrogen antagonists are agents which diminish or nullify the effect of estrogens, creating a situation akin to ovariectomization. However, unlike ovariectomy, these agents are, at times, target specific. A lowered level of estrogen can be attained by (i) estrogen synthesis inhibitors (compounds that interfere with aromatase, the enzyme responsible in the final step conversion of cholesterol to estradiol in the multistep biosynthetic pathway), or (ii) estrogen action inhibitors (agents that compete with the uptake of endogenous estradiol by estrogen receptors for estrogenic response at the target tissue). We may coin the expression Estrogenicity Lowering Agents (ELA) for these two categories grouped together.

Interference with estrogen level may also occur owing to compounds such as progesterone through indirect or some unknown mechanism. However,

in this review, the main emphasis has been given on estrogen action inhibitors, generally known as estrogen antagonists.

Whereas estrogen synthesis inhibitors do not possess estrogen agonistic effect, action inhibitors usually show both estrogenic and antiestrogenic properties (partial agonist or partial antagonists) in different ratios. The variance in the ratio of the two activities (estrogenic/antiestrogenic) differentiates them and identifies them for a typical pharmaceutical use.

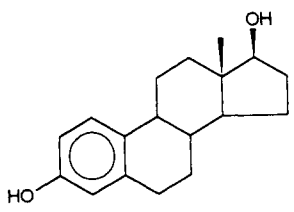
2 Role of Estrogens in Nature

The development of ELA as pharmaceuticals can be best appreciated in the light of beneficial and detrimental effects of estradiol in the body, both under physiological and non-physiological situations. Discussions on biological effects are given under respective sections.

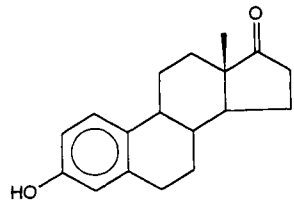
Estrogens were first identified with female reproduction and were classified along with progesterone as female sex hormones. The feminine character and the reproductive cycle of females are mainly controlled by these hormones. Estradiol (**1**) and its metabolites estrone (**2**) and estriol (**3**), are the major endogenous estrogens.

A logistic play with the estrogen level has resulted in the introduction of agents for fertility regulation as well as for infertility. Subsequently, characterization of various other estrogen dependent developments has opened up the scope for the use of ELA in many other areas shown in Fig. 1.

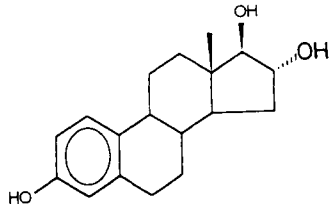
Unfortunately, screening of antiestrogens has been mostly reported in one



1



2



3

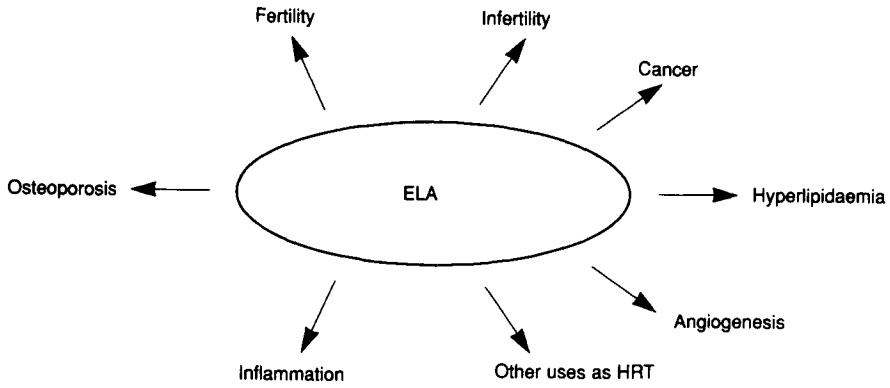


FIG. 1. Various uses of estrogenicity lowering agents.

specific area. Their scope in other fields has either not been evaluated due to lack of facility, toxicity or some such reason or is not worth reporting. In fertility regulation, the designing of a large number of non-steroidal agents is antiestrogen-related. However, in many cases data on their antiestrogenic potential is not mentioned. Based on available literature reports, a study of antiestrogens in some of the important areas will be discussed.

3 Mechanisms of Action of Estrogen Antagonists

3.1 AROMATASE INHIBITORS

In the biosynthetic pathway, cholesterol is converted in a stepwise manner to estradiol. The enzyme responsible for the production of estrogens from androgens is aromatase or estrogen synthetase, a cytochrome P450 enzyme. Aromatization is the last step in this synthetic sequence.

At the initial step of aromatization of the androgen (see Fig. 2) two stereospecific hydroxylations at C-19 produce the 19-aldehyde (Akhtar and Skinner, 1968). The third hydroxylation probably takes place at C-2 giving rise to the 2β-hydroxy-19-aldehyde intermediate which is rapidly converted into estrogen (Cole and Robinson, 1988). In this process, 1β- and 2β-hydrogens are stereospecifically eliminated as water (Fishman *et al.*, 1968) and the C-19 fragment is eliminated as formic acid (Skinner and Akhtar, 1969). The inhibition of the enzyme aromatase selectively reduces production of estrogen without interfering with the formation of other steroidal hormones. Such compounds lower plasma estrogen level and may be more effective and safer than hysterectomy/ovariectomy. Aromatase inhibitors are showing much promise in the treatment of estrogen-dependent cancer (discussed in Section 5).

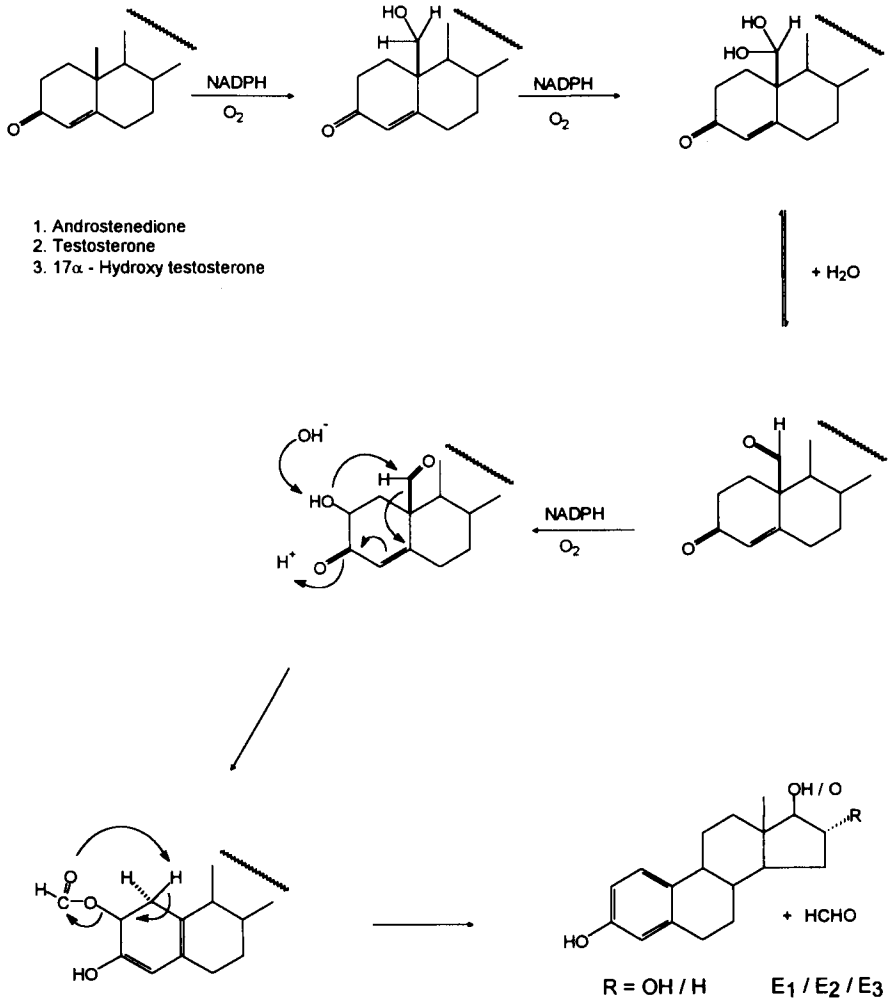


FIG. 2. Enzymatic conversion of androgens to estrogens.

3.2 ESTROGEN ACTION INHIBITORS

The mechanism of action of competitive inhibitors of estrogens involves their initial interaction with the estrogen receptors. The reason for their antagonistic effect is their inability to interact in subsequent steps to produce hormonal response – a phenomenon that is not fully understood.

The estrogen receptor (ER) is an intracellular protein which mediates estrogen action in target cells such as those of uterus, mammary glands and pituitary. Activated by the hormonal ligands, it regulates gene expression

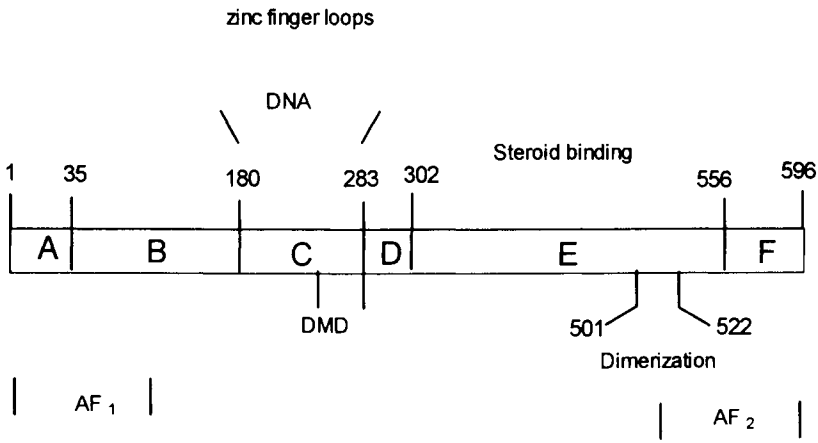


FIG. 3. Estrogen receptor protein.

and biological response (Katzenellenbogen *et al.*, 1985; Evans, 1988; Korach *et al.*, 1994).

The ER is a nuclear receptor and a member of a superfamily of zinc-containing receptors which includes receptors for other steroid hormones, thyroid hormones, vitamin D and retinoic acid as well as orphan receptors with undiscovered ligands (Evans, 1988). Sequence analysis of these receptors has revealed two regions of high sequence homology, which suggests that the receptors are organized into distinct domains (Green *et al.*, 1986; Greene *et al.*, 1986; Krust *et al.*, 1986; Kumar *et al.*, 1986). The ER protein is composed of six functional domains designated A–F (Green *et al.*, 1986) shown in Fig. 3. The E region is for binding of the estrogenic ligand. That both estrogens and antiestrogens bind to the same site cystein 530 of the ER from MCF-7 human breast cancer cells of the hormone binding domain, has been shown by covalent attachment of an estrogen affinity label, ketononestrol aziridine, and an antiestrogenic affinity label tamoxifen aziridine (Harlow *et al.*, 1989).

The DNA-binding domain, located near the mid portion of the receptor, has a zinc finger motif and recognizes DNA sequences called 'estrogen response elements' (ERE) housed within estrogen-regulated genes. The ERE sequence is most likely an inverted repeat 5′-GGTCA-NNN-TGACC-3′ (Klock *et al.*, 1987; Klein-Hitpass *et al.*, 1988). It is a perfect palindrome, composed of two half-sites spaced by three nucleotides. This spacing is important for receptor–DNA interaction. The ERE half sites may contain one or two base modifications but still retain ER binding. Binding of the ER to an ERE is not enough for transactivation function (Green, 1990; Gronemeyer, 1991; King, 1992; Power, 1992; Beniahmad and Tsai, 1993; Truss and Beato, 1993). The ER binds to the response element possibly as

a dimer, with one ER molecule occupying each half-site. The dimerization of the ER can occur prior to binding to DNA or the ligand (Sabbah *et al.*, 1989). The ligand is not essential for binding of the ER to an ERE (Bocquel *et al.*, 1989; Curtis and Korach, 1990; Fawell and Lees, 1990). However, in the absence of the hormone (ligand), the ER is unable to activate transcription. The mechanism by which this activation occurs is not clearly understood.

Domains A, B and F are involved in transcriptional activation through activator function regions AF-1 and AF-2. Domain D is a hinge region. Its functional part is not very clear.

In the process of hormone action the estrogenic ligand diffuses through cellular and nuclear membranes to bind to the nuclear receptor of the estrogen which results in 'activation' of the receptor. This activated form of the ER interacts with the ERE of the DNA sequence and stimulates transcription of estrogen-responsive genes which can lead to a physiological response (Fig. 4). This entire process is highly complex and is not fully understood.

There are some specific growth factors which may act in an agonistic or antagonistic manner in producing estrogenic response (Aronica and Katzenellenbogen, 1993; Smith *et al.*, 1993; Migliaccio *et al.*, 1993; Ignar-Trowbridge *et al.*, 1993). Antiestrogenic compounds e.g. 4-OH-tamoxifen have been found to inhibit tumour growth not only by blocking ER-mediated estrogen action of antagonist ligand but also impeding growth factor activity by decreasing receptor concentration or altering their functionalities (Freiss *et al.*, 1990).

Recent studies by Katzenellenbogen *et al.* (1995) indicated that some of the contact sites of estrogens and antiestrogens are possibly different. It has been suggested that estrogen agonists, antagonists, protein kinase activators, and growth factors alter the biological effectiveness of the ER through changes in phosphorylation state of the ER and/or proteins involved in the ER-specific response pathway.

Compounds which inhibit estrogen action are competitive inhibitors of estrogen. Such agents compete with endogenous estrogen for binding to the estrogen receptor. However, their failure in one or more of the subsequent steps may result in their inability to arouse estrogenic response either completely or partially (Jordan, 1984; Parker, 1993).

A study of these antiestrogenic molecules would suggest the presence of an estrogenic molecular subunit resembling the estradiol and diethylstilbestrol framework, responsible for an effective binding of the compound to the ER at the estradiol-binding site and a subunit, usually carrying a basic nitrogen-containing residue, which causes an estrogen antagonizing effect. This latter subunit is possibly located in space in a definite region which leads to its binding to the receptor on a site other than the estradiol-binding site. This additional binding also contributes towards the binding affinity of the

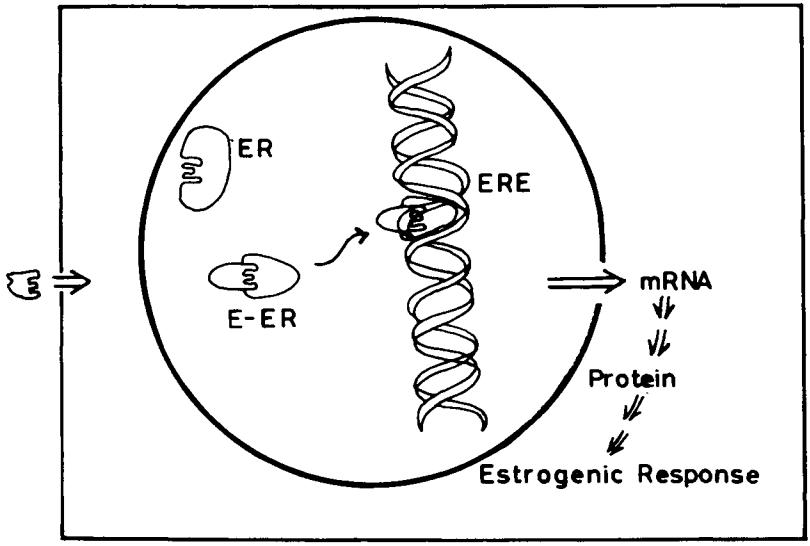


FIG. 4. Mechanism of estrogen action.

molecule and interferes with initiation of hormonal response by some mechanism which is not fully understood. Based on this study and assuming a structural complementarity between the ligand and the estrogen binding site on the receptor, we have earlier proposed a model (Durani *et al.*, 1979) which suggests the topography of the estrogen/antiestrogen binding pockets of the ER.

Since all antiestrogens carry the ER binding core, they may also possess intrinsic estrogenic activity. The subunit initiating antiestrogenic response, when separated from the above core, does not bind to the ER. It is therefore apparent that, whereas structure–activity relationship (SAR) studies of estrogen may lead to more potent estrogens, it is not possible at this state of knowledge to develop pure antiestrogens based on ligand–receptor interaction studies. The design of antiestrogens on the basis of DNA binding is another approach towards their development (Hendry *et al.*, 1994).

4 ELA in Fertility Regulation

An appreciation of the role of ELA in fertility regulation needs an understanding of the female reproductive physiology. In the female, after attaining puberty, a reproductive cycle is set in operation involving the hypothalamus–pituitary–gonad axis, (Fig. 5), in which a synchronized balance of the two hormones estrogen (E_2) and progesterone (P) (Fig. 6)

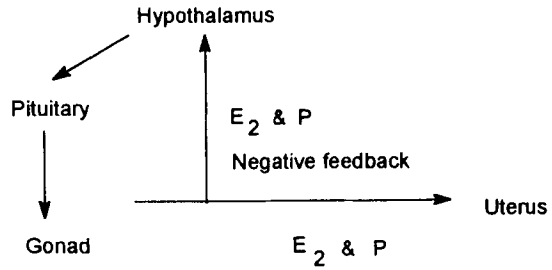


FIG. 5. Hormonal regulation of the reproductive cycle.

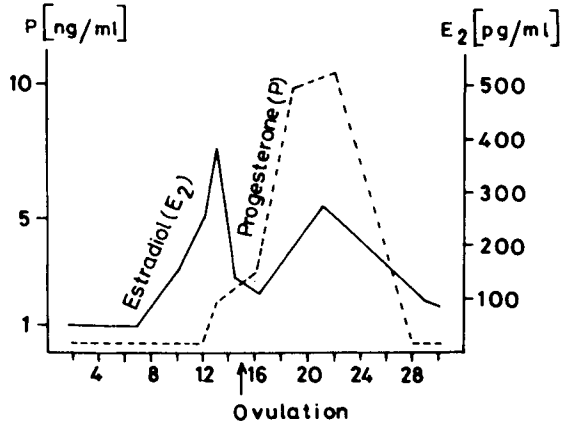


FIG. 6. Changes in plasma hormone levels during the menstrual cycle in women; —— estradiol (E_2); ---, progesterone (P).

plays a pivotal role. Under the influence of E_2 and P (low levels), release of the gonadotrophin-releasing factor (GnRF) takes place from the hypothalamus which acts on the pituitary, triggering production of gonadotrophins (FSH and LHRH). The effect of gonadotrophins causes development of follicle and subsequent release of ova (ovulation) from it and is associated with production of E_2 and P. A negative feedback mechanism operates by which higher levels of E_2 and P suppress gonadotrophin release and ovulation is inhibited. In a successful reproductive cycle the ova released are fertilized in the oviduct and travel down to settle in the uterus where the growth of endometrium caused by the estrogen release from the ovary is synchronized to be just ripe to receive it. At this stage, under the influence of a low surge of estrogen, decidualization takes place. Progesterone released initially from the ovary and then from the placenta, maintains the endometrium lining for a proper nurture of the implanted blastocyst to term.

Fall in the P level causes degeneration of the endometrium lining – the initiation of luteolysis.

Estrogen plays a vital role in this reproductive process (Mueller *et al.*, 1972). It causes suppression of gonadotrophin production and its secretion via the hypothalamus–pituitary–gonad axis (Flerko, 1959; Da Lage, 1966; Schwartz, 1969) discussed above. Estrogen is involved in growth and differentiation of mammary tissues (Lyons *et al.*, 1958), accelerated growth and cornification of vaginal epithelium (Bigger and Claringbold, 1954; Ladinsky *et al.*, 1968), growth and differentiation of uterine tissue (Mueller *et al.*, 1958; Oka and Shimke, 1969; O'Malley *et al.*, 1969) and synthesis and accumulation of fat in adipose cells (Gassner, 1958). Transportation of the ova or the fertilized egg in the oviduct is also greatly influenced by estrogen (Harper, 1965; Humphrey and Martin, 1968; Aref *et al.*, 1974). A low estrogen surge in the post-ovulatory period possibly (Ghosh and Sengupta, 1995) helps in the decidualization process.

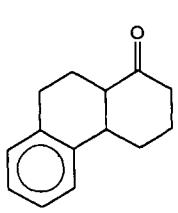
Utilizing the negative feedback effect of estrogen in suppressing ovulation, pharmacological doses of estrogen, preferably in combination with progesterone, were developed for achieving contraception. However, higher doses of estrogen under normal situations or even physiological levels under abnormal conditions were found to cause harmful side-effects which included induction and proliferation of cancer, and hypertension. Attempts to suppress the harmful effects while retaining the beneficial ones led to the discovery of estrogen antagonists discussed below.

4.1 FERTILITY INHIBITORS

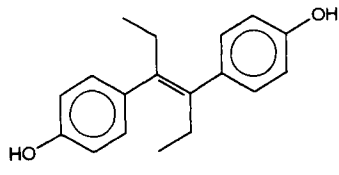
Fertility inhibitors may be classified into two groups (i) non-steroidal and (ii) steroidal inhibitors. These are discussed below.

4.1.1 Non-steroidal Estrogen Antagonists

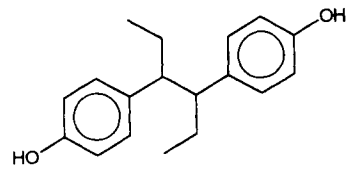
Beginning with the structure elucidation of the natural estrogen estradiol (**1**) attempts were made to synthesize compounds with structural similarity to estradiol as estrogens. This led to the phenanthrene derivative **4** (Cook *et al.*, 1933) as the first synthetic estrogen, followed by potent estrogens diethylstilbestrol (**5**) (Dodds *et al.*,1938a), hexestrol (**6**) (Campbell *et al.*, 1938), dienestrol (**7**) (Dodds *et al.*, 1938b), a large number of other stilbene derivatives (Solmssen, 1945) and simultaneously of triarylethylenes (**8**) (Grundy, 1957). Estrogenic activity was found in many other vastly different structural prototypes and non-steroidal compounds isolated from plants (Farnsworth *et al.*, 1975a,b).



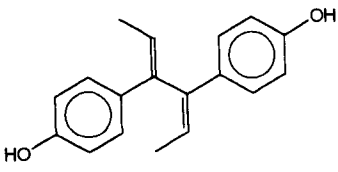
4



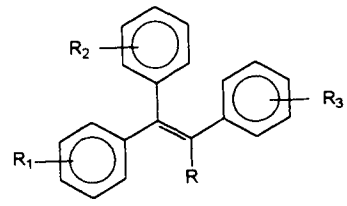
5



6



7



8

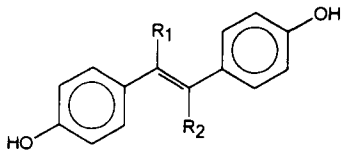
It was interesting to note that the structural variation was at times associated with qualitative and quantitative differences in their estrogenic activity. With the realization of beneficial and detrimental properties of estrogens, structural modifications of estrogens for achieving compounds with better pharmaceutical profiles formed a challenging area which is described in the following sections.

4.1.1.1 Diaryl Alkanes/alkenes and Related Compounds. Emmens *et al.* (1958) observed antiestrogenic activity in dimethylstilbestrol (**9**), ethylstilbestrol (**10**) and propylstilbestrol (**11**) in preventing cornification but not in methylethyl- (**12**), methylpropyl- (**13**) or dipropylstilbestrol derivatives (**14**). Of these, dimethylstilbestrol was most potent. It inhibited the increase in vaginal mitosis and epithelial thickness following estrogenic stimulation.

The antifertility effect of dimethylstilbestrol in mouse was, however, assigned to its estrogenic activity (Emmens, 1965).

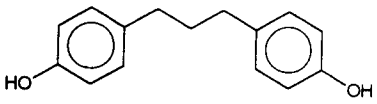
In *in vitro* assays, competitive inhibition of estradiol-17β activation of placental isoelectric dehydrogenase was shown by stilbestrol (**15**) and 1,3-bis (*p*-hydroxyphenyl)propane (**16**) (Villee and Hagerman, 1957).

The cyclic structure related to diethylstilbestrol, 3,9-dihydroxy-5,6,6aα,6bβ,11,12,12aβ,12bα-octahydrodibenzo[a,g]biphenylene (**17**) was found to be 100% active as a post-coital antifertility agent in rats at 100 mg kg^{-1} day^{-1} oral doses with only 16% uterotrophic activity compared with diethylstilbestrol which showed ED_{100} of 36 μg kg^{-1} day^{-1} (Ruehle *et al.*, 1980). This partial dissociation of antifertility and estrogenic activities of **17** could be due to its antiestrogenic effect.

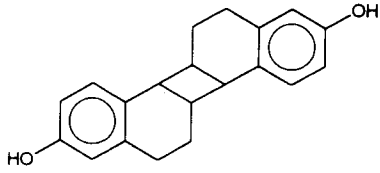


9–15

9: $R_1 = R_2 = Me$
10: R_1–H,R_2 = Et
11: R_1 = H,R_2 = Pr
12: R_1 = Me,R_2 = Et
13: R_1 = Me,R_2 = Pr
14: $R_1 = R_2$ = Pr
15: $R_1 = R_2$ = H



16

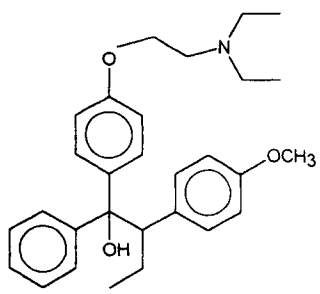


17

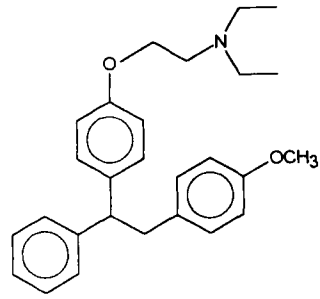
4.1.1.2 Triaryl Alkanes/alkenes

Triaryl Ethylenes/Ethanes. The discovery of triarylethylene (TAE) molecules as estrogens was reported shortly after that of stilbestrol. A number of TAEs elicited more prolonged hormonal effects which diminished on hydroxylation and had a limited estrogenic profile. Thus, some triarylethylene derivatives blocked vaginal cornification while others were antiuterotrophic. Unlike estradiol, these synthetic estrogens were found to be orally active. However, none of these compounds could be developed into a pharmaceutical agent with a distinct advantage.

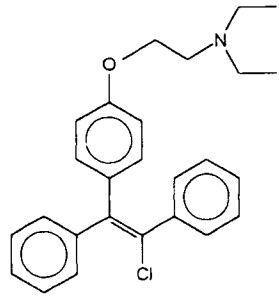
The most important landmark in the development of antiestrogens was the finding that the introduction of a basic (tertiary aminoalkoxy) side-chain onto the triaryl ethylene/ethane molecules at an appropriate position imparted estrogen antagonistic activity to the molecule. The first of these agents, MER-25 (**18**) (Lerner *et al.*, 1958), was only weakly estrogenic and counteracted biological effects of estrogens when administered concurrently. MER-25 was found to have no other apparent hormonal or antihormonal activity in mice, rats, chicks, rabbits and monkeys. Interestingly, MER-25 exhibited antifertility activity in rats (Segal and Nelson, 1958; Chang, 1959). This observation generated interest in the development of antiestrogens for fertility regulation. Preliminary clinical trials with MER-25 (Kistner and



18



19



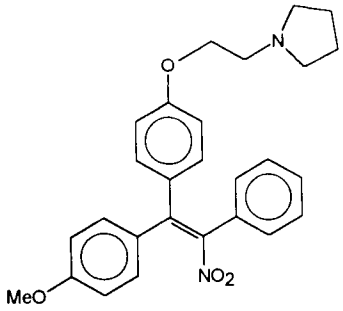
20

Smith, 1959; Smith and Kistner, 1963), showed it to be of low efficacy and to have toxic effects, for which trials were discontinued.

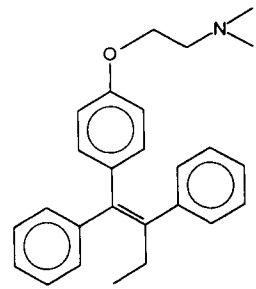
Simultaneously, Allen *et al.* (1960) reported a number of amino derivatives of triphenylethanol, triphenylethylene and triphenylethane which showed antiestrogenic, antigonadotrophic, anti-inflammatory, and cholesterol lowering activities. Antiestrogenic activity of 1-(*p*-(β-diethylaminoethoxy)phenyl)-1-phenyl-2-(*p*-methoxyphenyl)ethane (MRL-37, **19**) was reported by Greslin *et al.* (1959).

Out of the aminoalkoxytriphenylethylene derivatives prepared during this period, 1-(*p*-(β-diethylaminoethoxy)phenyl)-2-chloro-1,2-diphenylethylene dihydrogen citrate (clomiphene, MRL-41, **20**) was of particular interest. It was found to possess antigonadotrophic, estrogenic and antiestrogenic properties in rats (Holtkamp *et al.*, 1960). It blocked the action of endogenous and exogenous steroidal or non-steroidal estrogens with remarkable effectiveness in rats, mice, rabbits and monkeys (Gaunt *et al.*, 1961). Clomiphene was found to induce ovulation in women. This profertility property of the compound is discussed in section 4.2.1.

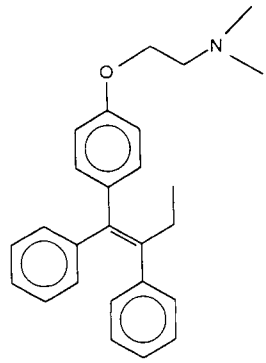
All the three compounds MER-25 (Segal and Nelson, 1958), MRL-37, and MRL-41(Segal and Nelson, 1961) elicited antifertility activity in rats.



21



22



23

Dewald and coworkers (1966) have reported 1-(*p*-methoxy)phenyl-1(*p*-(β-pyrrolid-inoethoxy)phenyl-2-nitro-2-phenylethylene, CN-55945-27, **21**) to be antizygotic. It provided full protection against pregnancy in mice (50 μg), rats (25 $\mu g\,kg^{-1}$) and in dogs (250–500 $\mu g\,kg^{-1}$) when fed continuously in diet. It has weak uterotrophic, antiestrogenic and antideciduogenic activities. It was observed that this compound stimulated pituitary LH secretion, but had no effect on FSH. Its anti-implantation effect is possibly owing to its antiestrogenicity and/or luteolytic action via LH (Callantine *et al.*, 1966).

The *trans*-isomer (tamoxifen, ICI 46474, **22**) of 1-(*p*-(β-dimethylamino ethoxyphenyl)-1,2-diphenylbut-1-ene was investigated by Harper and Walpole (1966, 1967a,b) and found to be a potent antiestrogen, weakly estrogenic and mildly antigonadotrophic. However, its *cis*-isomer (ICI 47699, **23**) was a potent estrogen in rat and mouse tests, antigonadotrophic and preventing implantation when given on days 1–4 of pregnancy at doses needed to induce vaginal cornification.

Tamoxifen prevented pregnancy in rats at an oral dose of 30 $\mu g\,kg^{-1}$ given on days 2–4 post coitum. A single oral dose (120 $\mu g\,kg^{-1}$) on day 4 of pregnancy was found to be most effective.

In clinical studies the effect of tamoxifen was found to be dependent on the dose, duration of treatment, age and sex of patients.

In premenopausal women tamoxifen produced no significant change in gonadotrophin secretion (Groom and Griffiths, 1976; Tanaka *et al.*, 1978; Manni *et al.*, 1979; Sherman *et al.*, 1979). Tamoxifen administration caused an increase in serum estradiol and progesterone concentrations (Boyns and Groom, 1972; Groom and Griffiths, 1976), suggesting stimulation of follicular activity. An increase in estradiol concentration level indicated the antiestrogenic nature of tamoxifen. Administration of tamoxifen to postmenopausal women caused a reduction of the elevated basal level of LH and FSH (Golder *et al.*, 1975; Willis *et al.*, 1976).

Tamoxifen treatment decreased prolactin release induced by TRH (Golder *et al.*, 1975; Willis *et al.*, 1976; Tanaka *et al.*, 1978). It caused a significant rise in circulating cortisol levels (Markham *et al.*, 1980; Levin *et al.*, 1981) and no change in serum androgen or progesterone levels (Willis *et al.*, 1976; Daxenbichler *et al.*, 1979; Coombes *et al.*, 1982). There is possibly no change in the estrogen level on tamoxifen treatment (Golder *et al.*, 1975; Kiang and Kennedy, 1977; Coombes *et al.*, 1982). However, a rise in the estrogen level has also been reported (Szamel *et al.*, 1979).

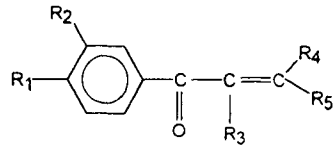
At the uterine level, tamoxifen acted as an antiestrogen at the endometrium (El-Sheikha *et al.*, 1972; Masson and Klopper, 1972).

Administration of tamoxifen at clinical doses did not consistently block either ovulation or menstruation. Continuous therapy with tamoxifen prevented the appearance of a secretory endometrium (Sherman *et al.*, 1979); this could be due to its blocking effect on progesterone receptor synthesis. However, a short-term treatment with tamoxifen has been found to increase progesterone receptor synthesis (Baulieu *et al.*, 1981b).

Because of the potent antiestrogenic property of tamoxifen, it has found use in the treatment of estrogen dependent cancer (discussed in section 5.2.1.1).

1,2,3-Triaryl-2-propen-1-ones (TAPs). In an attempt to prepare antiestrogens for fertility regulation, a number of TAPs (2,3-diarylacrylophenones) of molecular prototype **24** were synthesized (Iyer *et al.*, 1969) as a structural modulation of triarylethylenes.

Since the main aim of this study was to develop antifertility agents, these compounds were screened primarily for anti-implantation activity. The phenolic compounds were found to be inactive. Compounds having a basic ether residue at R_1, a phenyl group at R_3 and an aryl group at R_4 substituted with 2- or 4-halogen, 3-methoxy, 3,4-dichloro, 3,4-dimethoxy or 3,4-methylenedioxy showed significant activity. Of these, 1-(4-(β-pyr-



24

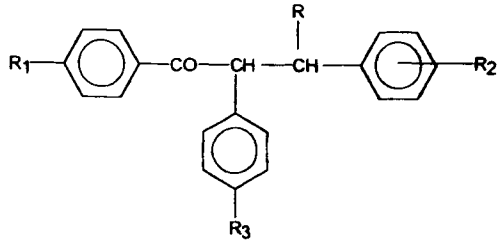
rolidinoethoxyphenyl)-2,3-diphenyl-3-(4-chlorophenyl)-2-propen-1-one (**24**, R_1 = β-pyrrolidinoethoxy; R_2 = H, R_3 = R_4 = C_6H_5, R_5 = 4-Cl-C_6H_5) was fully effective at 1 mg kg^{-1} dose in inhibiting implantation in rats. Shifting of the basic chain or the halogen atom to other parts of the molecule had a detrimental effect on activity. In this study, the Z-isomers, usually present as impurity, were found to be more potent anti-implantation agents. In another study of E and Z isomers of TAPs (Mittal *et al.*, 1985), their relative binding affinity (RBA) for estrogen receptors, uterotrophic and anti-uterotrophic activities were determined. It was found that whereas both E and Z isomers bind to the receptor and possess both estrogenic and antiestrogenic activities, the E-isomers were more potent. The triphenol (**24**, R_1 = OH, R_2 = R_5 = H, R_3 = R_4 = p-OHC_6H_4) was a potent antiestrogen, causing 70% decrease in E_2-stimulated uterine weight. Hormonal activity of basic ethers, which are likely to be more antiestrogenic, has not been reported.

1,2,3-Triarylpropan-1-ones and 2,3,3-triarylpropanols: Erythro-1,2,3-triaryl-2-propan-1-ones (2,3-diarylpropiophenones, **25**) (Gopalchari *et al.*, 1970), carrying a basic ether chain at the para position of 1-phenyl and an alkyl substituent (methyl (Me) or ethyl (Et)) at C-3 showed ten-fold more anti-implantation activity compared with the corresponding *threo* isomer. The presence of an Me or Et substituent at C-3 provided a bulk giving a staggered conformation, possibly desired for the observed activity of the *erythro* isomers (**25**, R = Et). Transfer of the basic ether chain to the phenyl ring at C-2 reduced activity.

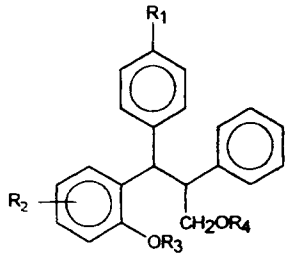
All the compounds showing anti-implantation activity were found to be antiestrogenic at their minimum effective dose (MED) (Mehrotra and Karkun, 1975).

In a study of *erythro*- and *threo*-2,3,-triarylpropanols (Kole, 1977) (**26**) the basic ether of the *erythro* isomer (**26**, R_1 = OMe, R_2 = R_4 = H; R_3 = $CH_2CH_2N(CH_2)_4$) inhibited pregnancy at 20 mg kg^{-1} dose in rats. Whether this was due to its estrogenic or antiestrogenic effect has not been studied.

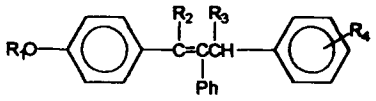
1,2,3-Triarylpropenes: As a logical extension of the work described in the above two sections, synthesis and biological evalua-



25



26

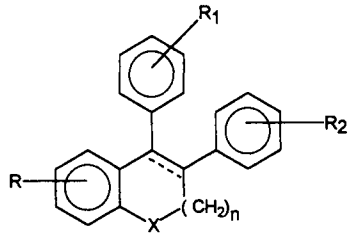


27

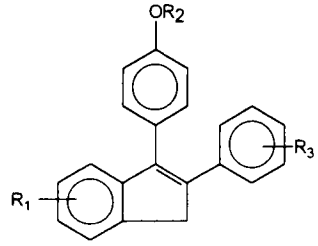
tion of 1,2,3-triarylpropenes (**27**) was also carried out by Gopalchari *et al.* (1971). Significant anti-implantation activity was reported in a number of compounds of which 3-(*m*-methoxphenyl)-2-phenyl-1-(*p*(β-pyrrolidinoethoxyphenyl)propene (**27**, $R_1 = CH_2CH_2N(CH_2)_4$), $R_2 = R_3 = H$, R_4 = 3-OMe) active at 0.5 mg kg^{-1} dose in rats was most potent. However, no study on their hormonal activity was reported.

Cyclized triarylethylenes and related compounds: Various anti-estrogenic structural prototypes having the triarylalkyl unit in cyclized form (**28**) were evaluated for fertility regulating activity. These may be subclassified as follows:

Carbocyclic compounds A series of basic ethers of 2,3-diarylindenes were prepared by Lednicer *et al.* (1961, 1965) as the first group of cyclized triarylethylenes. Compounds lacking basic ether chain did not show



28 X= O,S,NH,CH_2; n= 0-2

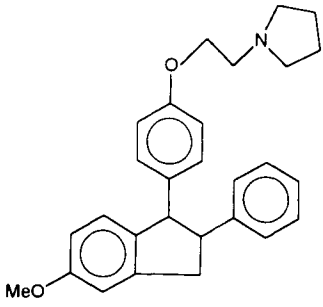


29

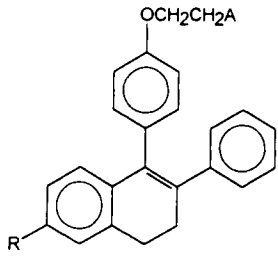
antifertility activity. Structure–activity relationship studies further showed that a 6-methoxy substituent significantly enhanced antifertility activity. A two-carbon ether chain was found to produce optimum activity. Among the basic amino moieties studied, the activity was observed in the order pyrrolidine > diethylamine > morpholine. Of the 2,3-diphenylindenes, U-11555A (**29**, R = 6-OMe; $R_2 = CH_2CH_2N(C_2H_5)_2$; R_3 = H) has been investigated extensively in rodents (Duncan *et al.*, 1962; Chang, 1964; Chang and Yanagimachi, 1965; Morris *et al.*, 1967). This compound possessed weak uterotrophic antideciduogenic and antiestrogenic activities and was found to be devoid of progestational and antigonadotrophic effects. It prevented implantation in rabbits at 15 mg kg^{-1} (Morris *et al.*, 1967) and in rats at 0.1 mg kg^{-1} dose (Lednicer *et al.*, 1965). The corresponding pyrrolidino compound (**29**, R_1 = 6-OMe, $R_2 = CH_2CH_2$ $(CH_2)4$; R_3 = H) was found to be most active (MED_{100} in rats: 0.025 mg kg^{-1}).

In the indane series, both 1,2-*cis*- and *trans*-isomers of 5-methoxyindane (**30**) showed anti-implantation activity at 0.5 mg kg^{-1} and 0.25 mg kg^{-1} dose respectively. The *cis*- and *trans*-indanes (**30**) were 152 and 108 times less estrogenic compared with ethynylestradiol. Both isomers showed antiestrogenic property and were devoid of progestational and antiprogestational activities (Malik and Rastogi, 1981; Malik *et al.*, 1989).

The next higher homologue of indenes 1,2-diphenyl-3,4-dihydronaph-



30



31

thalenes (**31**) was also synthesized by Lednicer *et al.* (1963). Structure–activity relationships for 1,2-diphenyl-3,4-dihydronaphthalenes were similar to that of 2,3-diphenylindenes. Potentiation of antifertility activity was observed with the introduction of a 6-methoxy group, and a pyrrolidinoethoxy substituent imparted highest activity (MED_{100} in rats 0.025 mg kg^{-1}).

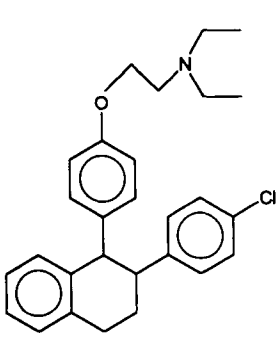
Two compounds of the above series, U-11100A (**31**, R = OMe, A = $N(CH_2)4$, nafoxidene) and U-10520A (**31**, R = OMe, A = $N(C_2H_5)_2$) were investigated by Duncan *et al.* (1963), for oral antifertility activity in rodents (rats, rabbits and guinea pigs). The compounds caused full inhibition of pregnancy in rats at 0.025 mg kg^{-1} and 0.25 mg kg^{-1} doses (1–5 days) or at 0.25 mg kg^{-1} and 2.5 mg kg^{-1} doses respectively on a single dose schedule. The compounds possessed mild uterotrophic, antiestrogenic and anti-deciduogenic activities and were devoid of antigonadrotrophic property.

In rhesus monkeys oral administration of U-11100A caused only a partial inhibition of pregnancy even at a high dose of 250 mg day^{-1}. No fetal abnormalities were observed (Morris *et al.*, 1967). In rabbits, U-11100A did not show blastotoxic effect and the viability of blastocysts remained unaffected (Morris *et al.*, 1967).

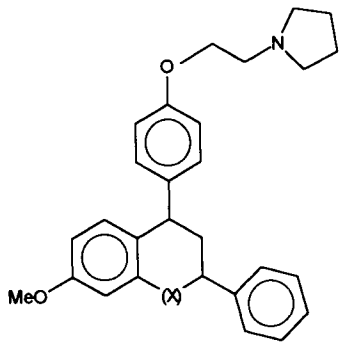
In a later study of the mechanism of contraceptive action of U-11555A and U-11100A by Siddiqui *et al.* (1976), it was found that inhibition was not due to their interference with ova transport or delayed implantation but could be due to their effect on ovarian hormone.

Lednicer *et al.* (1967) synthesized a large number of 3,4-dihydronaphthalenes and 1,2,3,4-tetrahydro-1-naphthols. Many of the compounds possessed significant antifertility activity. It was concluded that the presence of a basic ether chain at a particular position in space is required for estrogen antagonistic activity. The oxygen atom in the basic chain can be replaced by a methylene group without any significant loss in biological activity.

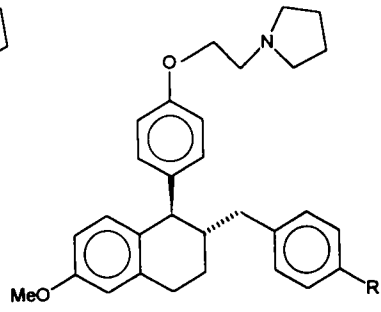
Of the *cis*- and *trans*-tetrahydro-1,2-diphenylnaphthalenes reported by Bencze *et al.* (1965, 1967), the most active compound **32** prevented implantation in rats at 20 $\mu g\, kg^{-1}\, day^{-1}$. It was weakly uterotrophic and antiestrogenic. In an SAR study, it was interesting to note that shifting of



32



33



34: R=H
35: R=OMe
36: R=Cl

the phenyl moiety of 1,2-diaryltetrahydronaphthalene, to position 3 (compound **33**, $X = CH_2$), led to abolition of antifertility activity (Malik and Rastogi, 1984). Similar effects were also observed in 2,4-diarylchromans (**33**) (X = O) (Srivastava *et al.*, 1993).

Antifertility activity was partially retained in the 2-benzyl derivatives **34** (R = H), **35** ($R = OCH_3$), **36** (R = Cl), which were active at 1,5 and 1 mg kg^{-1} dose, respectively (Tewari and Rastogi, 1980).

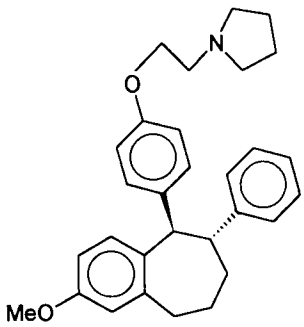
Anti-implantation activity of 1,2-*trans*-1-*p*-(β-pyrrolidinoethoxyphenyl)-2-phenyl-7-methoxybenzosuberan (**37**) was found to be at 0.2 mg kg^{-1} (1–5 day) and 1.5 mg kg^{-1} single oral (day 1) doses. It was 1000-fold less estrogenic compared with ethynylestradiol (Sangwan *et al.*, 1986) at 0.2 mg kg^{-1} dose and antiestrogenic at a single-day contraceptive dose. It was free from progestational and antiprogestational activities.

Trans-1-(*p*-(β-pyrrolidinoethoxyphenyl)-2-benzyl-7-methoxybenzosuberan (**38**) was active at 1 mg kg^{-1} and at 5 mg kg^{-1} single oral doses (Sangwan and Rastogi, 1981).

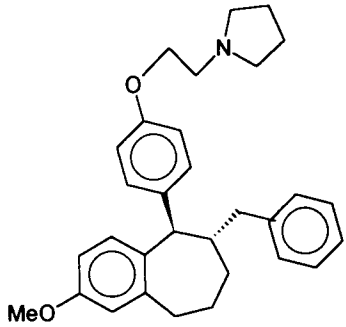
Diphenylbenzocycloheptene derivatives (**39**) prepared by McCagne *et al.*, (1988) were reported to be full estrogens in rats.

Taking a clue from the reported antiestrogenic activity of acrylophenone derivatives (Iyer and Gopalchari, 1969), molecular structures with a carbonyl group inserted into the triarylethylene nucleus were investigated by Jones *et al.* (1979). This led to the development of a potent antiestrogen trioxifene (**40**). It was equally potent when given orally or subcutaneously. In mice, it significantly inhibited uterotrophic response to estrone at 1 μg per animal per day dose at which it was only weakly uterotrophic. It fully prevented fertility in rats at 5 μg per animal per day.

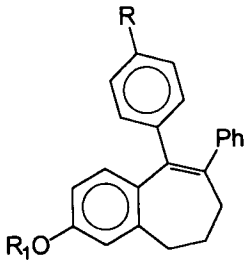
Trioxifen in *in vitro* binding studies with rat uterine cytosol estrogen receptors showed very high binding affinity (1.7 × estradiol) (Jones *et al.*, 1979).



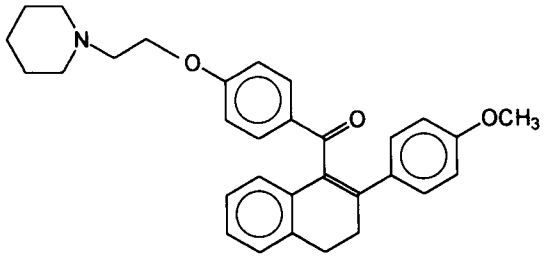
37



38



39



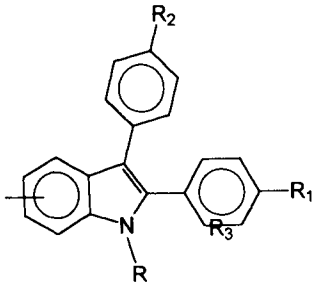
40

Nitrogen heterocycles Preparation of the 2,3-diarylindole derivative **41** was reported by Iyer and Gopalchari (1966) and simultaneously by Landquist and Marsden (1966). Two of the basic ether derivatives (**41**: R = R_2 = R_3 = H, R_1 = diethylamino-/pyrrolidino-ethoxy) were found to be active at 5 mg kg^{-1} dose in rats. It was found that the activity of the compounds was not due to their estrogenic effect.

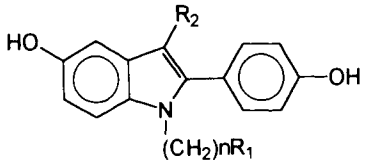
2-Phenylindoles (**42**) with amino, pyrrolidino, piperidino and morpholino groups as amino function (R_1), completely suppressed estrone-stimulated uterine growth. Highest binding affinity (20–30%) was found in compounds having a methyl group in position 3 (R_2 = Me) and a hexamethylene chain (n = 6) (VonAngerer *et al.*, 1990).

Paul *et al.* (1972) have reported preparation and antifertility activity of 1-phenyl-2-phenethyl-1,2,3,4-tetrahydroisoquinolines (**43**). Most of the active compounds elicited frank estrogenic property. The most active compound (+,+)-1-(*p*-(2-(1-pyrrolidinyl)ethoxy)phenyl)-2-(β-methylphenethyl)-1,2,3,4-tetrahydroisoquinoline, showed impeded estrogenic property.

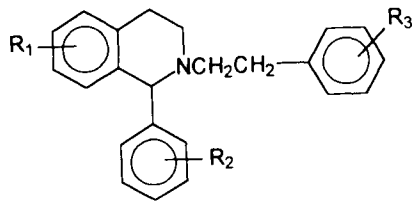
Sulfur heterocycles Crenshaw and Witt (1967) prepared substituted 3,4-dihydro-3-phenyl-4-hydroxy-4-(aminoalkoxy/alkylthiophenyl)



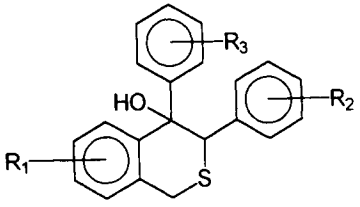
41



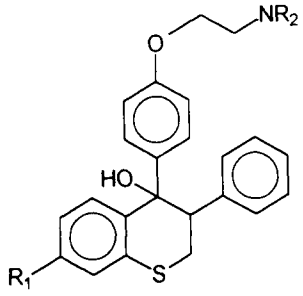
42



43



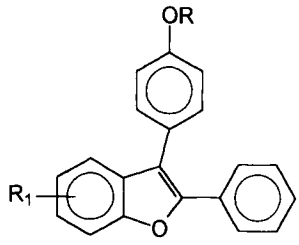
44



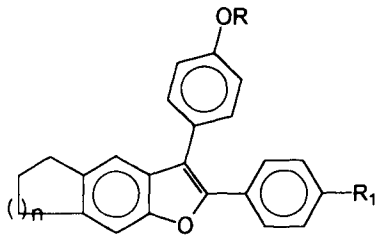
45

isothiochromans (**44**) as antifertility agents. The preferred compound 3,4-dihydro-7-methoxy-3-phenyl-4-hydroxy-4-(*p*-(2-(1-pyrrolidinyl)ethoxy)-phenyl)-1H-2-benzothiopyran (**44**, R_1 = 7-OMe,R_2 = H,R_3 = pyrrolidino-ethoxy) inhibited pregnancy in mice when administered orally a dose of 0.1 mg kg^{-1} day^{-1}. The antifertility activity of these compounds was parallel to their estrogenicity. Pruitt *et al.* (1990) reported preparation of thiochroman derivatives (**45**) as potential antiestrogens. No biological data were given.

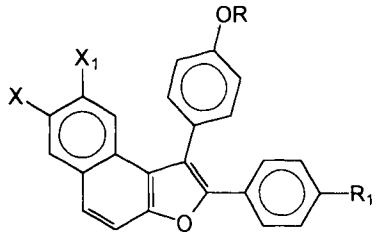
Oxygen heterocycles The preparation of a series of basic ethers of 1,2-diphenylbenzofurans (**46**) was reported by Grover *et al.* (1965).



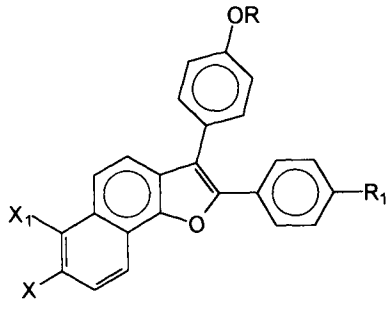
46



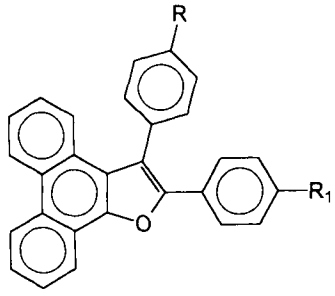
47



48



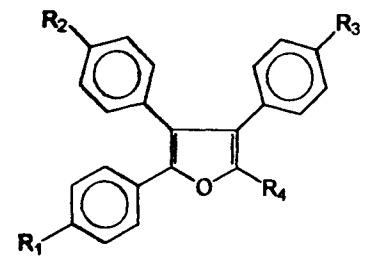
49



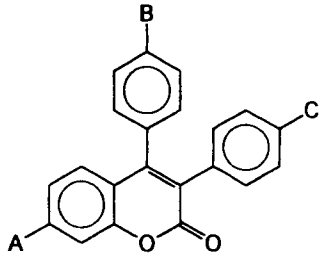
50

Structure–activity relationships were similar to indenes described earlier. Compound **46** (R = $CH_2CH_2N(CH_2)4$, R_1 = 6-OMe) was found to be most active (MED_{100}: 4 mg kg^{-1} in rats). 5,6-Polymethylene derivatives **47** showed significant activity. The tetramethylene compound (**47**, $n = 2$, R = pyrrolidinoethyl, R_1 = H) was active at 2 mg kg^{-1}.

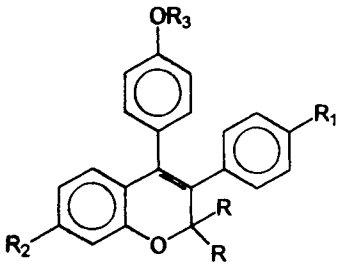
A significant difference in the antifertility activity of naphtho[2,1-b]furan



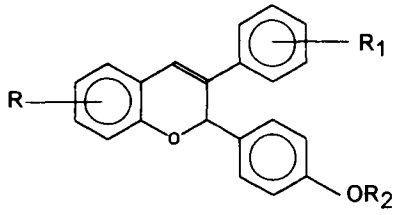
51



52



53



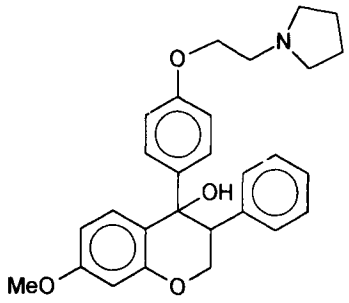
54

(**48**, $X = X_1 = R_1 = H$, R = pyrrolidinoethoxy) and naphtho[1,2-d]furan (**49**, $X = X_1 = H$, R = pyrrolidinoethoxy, $R_1 = H$) active at 2 and 20 mg kg^{-1} doses, respectively, suggested that increase in planar area of the heterocyclic residue along a particular orientation is important for increased antifertility activity (Chawla *et al.*, 1970). 2,3-Diarylphenanthrofuran (**50**) did not show any significant activity (Gupta *et al.*, 1977).

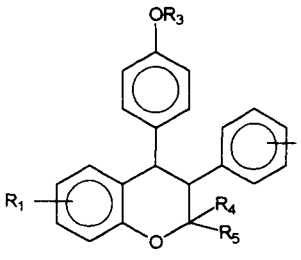
Two of the promising compounds of the furan series, compounds **46** (R = pyrrolidinoethyl, R_1 = 6-OMe) and **48** ($X = X_1 = R_1 = H$, R = pyrrolidinoethyl), were evaluated in greater detail. Both were weak estrogens and weak antiestrogens. They were devoid of other hormonal properties, with virtually inert endocrine and gross pharmacological properties (Chawla *et al.*, 1970). The compounds caused some fetal resorption in rats but did not produce genital abnormalities or teratogenicity. Post-natal sexual development and subsequent fertility remained normal.

A number of 2,3,4-triarylfurans showed moderate antifertility activity. Compounds carrying substituents at 3 and 4 phenyl rings showed antifertility activity at relatively lower doses (**51**: $R_1 = R_2 = R_4 = H$, R_3 = OH (2 mg kg^{-1}); $R_1 = R_3 = R_4 = H$, R_2 = pyrrolidinoethoxy (1 mg kg^{-1}); R_1 = pyrrolidinoethoxy, $R_2 = R_3 = R_4 = H$ (5 mg kg^{-1})) (Dikshit *et al.*, 1974; Munshi *et al.*, 1974).

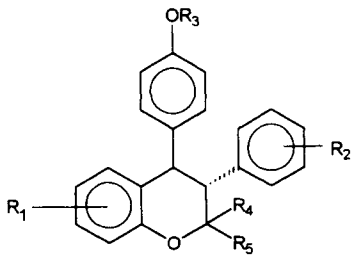
The next higher homologues of furans, benzopyrans, were studied



55



56



57

extensively. Lednicer *et al.* (1965) synthesized and evaluated a series of 3,4-diphenylcoumarins (**52**) for antifertility activity. Among basic ethers, the most potent compound **52** (A = $OCH_2CH_2N(C_2H_5)_2$, B = H, C = H) showed full inhibition of pregnancy on oral administration to rats only at 50 mg kg^{-1} dose and was weakly uterotrophic at this contraceptive dose.

3,4-Diphenylcoumarin with a pyrrolidinoethoxy substituent (**52**, A = OCH_3, B = β-pyrrolidinoethoxy, C = H) was reported to be active at 20 mg kg^{-1} (Ray *et al.*, 1970). A fivefold increase in antifertility activity was observed when the pyrrolidinoethyl basic ether chain was replaced by a 3-*n*-butylamino-2-hydroxy-propyl unit (**52**, A = OCH_3, B = $OCH_2CH(OH)CH_2HN$-*n*-butyl, C = H) (Ray *et al.*, 1987).

Study of 3,4-diphenylchromene derivatives (**53**) as implantation inhibitors has been reported by many groups (Carney *et al.*, 1966; Ray *et al.*, 1976). Compound **53** (R = R_1 = H, R_2 = OCH_3, R_3 = pyrrolidinoethyl), prevented implantation in rats at 0.1 mg kg^{-1} dose. It was weakly antiestrogenic and possessed considerable uterotrophic activity (Durani *et al.*, 1979). Introduction of an alkyl group at C-2 (R = alkyl) had a detrimental effect on the antifertility activity of the compound which increased with the size of the bulk (Ray *et al.*, 1976).

Highly significant antiestrogenic activity was observed in

2,3-diarylchromenes (**54**) substituted with a basic moiety on the 2-phenyl ring. Compound 85/287 (**54**, R = R_1 = H, R_2 = piperidinoethyl) elicited potent estrogen antagonistic activity in rats without agonistic activity. It inhibited pregnancy in rats at an oral dose of 0.25 mg kg^{-1}, administered on days 1–5. The monohydroxy compound **54** (R = H, R_1 = *p*-OH, R_2 = piperidinoethyl) was found to be more potent compared to 85/287. Both compounds were found to be superior antiestrogens compared with tamoxifen, trioxifene and LY117018 (Saeed *et al.*, 1990; Sharma *et al.*, 1990a,b).

7-Methoxy-4-(*p*-(β-pyrrolidinoethoxy)phenyl-isoflavan-4-ol (**55**) was found to possess antifertility activity in rats at a 5 mg kg^{-1} oral dose (Gopalchari and Iyer, 1966).

The 3,4-diarylchromans with two asymmetric carbon atoms C-3 and C-4, existed as two racemic pairs *cis* (**56**) and *trans* (**57**). The most active compound prepared by Carney *et al.* (1966) (**57**, R_1 = R_4 = R_5 = H, R_2 = *p*-Cl, R_3 = diethylaminoethyl) prevented pregnancy in rats at a dose of 1 mg kg^{-1}. Enhancement in antifertility activity was observed on incorporation of a 7-OMe substituent and replacement of the basic chain by a pyrrolidinoethyl group. Thus, the *cis*-isomer **56** (R_1 = 7-OMe, R_2 = R_4 = R_5 = H, R_3 = pyrrolidinoethyl) was active at a 1 mg kg^{-1} dose while the corresponding *trans* isomer **57** (R_1 = 7-OMe, R_2 = R_4 = R_5 = H, R_3 = pyrrolidinoethyl) inhibited pregnancy at a 0.25 mg kg^{-1} dose (Ray *et al.*, 1971).

On incorporation of a methyl group at C-2, the 2,3-*cis*-3,4-*cis*-isomer **56** (R_4 = R_2 = H, R_5 = CH_3, R_1 = 7-OMe, R_1 = 7-OMe, R_3 = pyrrolidinoethyl) was active at 2.0 mg kg^{-1} while the 2,3-*trans*-3,4-*trans*-isomer **57** (R_1 = 7-OMe,R_2 = R_4 = H,R_5 = CH_3,R_3 = pyrrolidino ethyl) was fully active at a 1 mg kg^{-1} dose) (Kole *et al.*, 1978).

The gem-dimethyl *trans*-chroman **57** (R_1 = 7-OMe,R_2 = H, R_4 = R_5 = CH_3, R_3 = pyrrolidinoethyl) (Ray *et al.*, 1976) was also found to inhibit implantation in rats at 0.25 mg kg^{-1} dose administered on days 1–5. This compound (generic name centchroman) was also effective in inhibiting pregnancy in mouse, dog and rhesus monkey. It prevented pregnancy in women and was marketed as the first oral non-steroidal contraceptive. Centchroman is discussed in detail in section 4.1.2.

4.1.2 Centchroman – A Non-steroidal Contraceptive

4.1.2.1 Chemistry. Centchroman (*trans*-1-(2-(4-(7-methoxy-2,2-dimethyl-3-phenyl-3,4-dihydro-2H-1-benzopyran-4-yl)-phenoxy)ethyl)-pyrrolidine hydrochloride (**57**, R_1 = 7-OMe, R_2 = H, R_3 = pyrrolidinoethyl, R_4 = R_5 = Me) is the first non-steroidal oral contraceptive introduced to the market. It was synthesized (Ray *et al.*, 1976) and developed by the Central Drug Research Institute, Lucknow, India (Anand and Ray, 1977; Kamboj *et al.*, 1992).

Centchroman is a racemate mixture and is marketed under the trade names Saheli and Centron. Its X-ray crystallography (Ray *et al.*, 1994) showed its molecular similarity to antiestrogenic compounds tamoxifen and nafoxidine. Centchroman has been resolved into its (+)- and (-)-antipodes.

4.1.2.2 Antifertility Action. Centchroman is an estrogen antagonist with weak estrogenic activity and no other hormonal property (Kamboj *et al.*, 1992). It binds to the estrogen receptor with an affinity of 5.4% that of estradiol (Durani *et al.*, 1979).

l-Centchroman displayed a sevenfold higher receptor affinity for estrogen receptors compared with the (+)-form, and a corresponding difference in stimulation of the uterine growth and anti-implantation activity (Salman *et al.*, 1986).

Centchroman prevented pregnancy in rat, mouse, dog and rhesus monkey when administered in a single dose within 24 h of coitus. The antifertility effect was readily reversible (Kamboj *et al.*, 1977). It did not interfere with endocrine functions and had no gross pharmacological effects except for anorexigenic activity at about 40 times and anti-inflammatory activity (equivalent to phenylbutazone) at about 64 times the contraceptive dose (Dhawan and Srimal, 1973; Chak *et al.*, 1977; Kamboj *et al.*, 1977).

Administration of centchroman to rhesus monkeys at two and five times the human contraceptive dose (on kg body weight basis) for 1 year, did not affect the basal and peak levels of peripheral plasma estradiol, progesterone, FSH and LH. This showed that the compound did not disturb the hypothalamus–pituitary–ovarian axis (Nigam *et al.*, 1985). When administered to immature and mated female rats up to 10 times and 50 times the contraceptive doses, respectively, it had no effect on the ovaries (Singh *et al.*, 1982). Weekly doses up to 120 mg given to women did not suppress ovulation and the hormone cycle (Vaidya *et al.*, 1977).

Centchroman given post-coitally to rats slightly accelerated ovum transport, stimulated blastocyst formation and delayed zona pellucida shedding (Singh *et al.*, 1986) but had no effect on the viability of embryos (Kamboj *et al.*, 1977; Singh and Kamboj, 1981; Singh *et al.*, 1983; Singh *et al.*, 1986).

Centchroman appears to manifest its contraceptive action primarily by causing asynchrony between ovum transport and endometrium development for nidation.

4.1.2.3 Metabolism. In an *in vitro* metabolic study using rat liver homogenate eight metabolites of centchroman were identified (Ratna *et al.*, 1986). 7-Desmethyl centchroman (**57**: R_1 = 7-OH, R_2 = H, R_3 = pyrrolidinoethyl, $R_4 = R_5$ = Me) was found to be the major metabolite. This metabolite elicited binding affinity for estrogen receptors which was comparable to estradiol (112%) (Durani *et al.*, 1979) and antifertility activity similar to centchroman. It appears to be the major active metabolite of centchroman.

4.1.2.4 Pharmacokinetics. Tissue distribution and excretion profile of radiolabelled centchroman was studied in rats (Ratna *et al.*, 1994) and rhesus monkeys (Mishra *et al.*, 1992). After oral administration, well-perfused organs (liver, heart, lung, kidney, intestine, etc.) retained more radiolabel than the less perfused organs (such as genital organs, thyroid, fat, adrenal, etc.). Its main route of excretion was through faeces. In women, the terminal disposition half-life of the drug was found to be about 170 h (Paliwal *et al.*, 1989).

4.1.2.5 Toxicity. The LD_{50} of centchroman i.p. in mice and rats is 400 mg kg^{-1}. It was found to be well tolerated in young adult albino rats in chronic (210 days) and prolonged toxicity studies. The haematological, biochemical and histopathological findings showed no evidence of toxicity. In adult female rhesus monkeys daily administration of centchroman for 3 months did not cause any adverse effect on gross behaviour, food consumption, blood haemogram, kidney and liver function, except for anorexia at 64 times the contraceptive dose (Mukherjee *et al.*, 1977).

Centchroman was found to be devoid of any mutagenic and carcinogenic activities.

4.1.2.6 Clinical Evaluation. Centchroman was evaluated as a contraceptive in a post-coital schedule, once weekly schedule, and a twice weekly followed by weekly schedule. Post-coital use of centchroman at 60 mg dose provided acceptable pregnancy protection. In a study of 103 parous women for 650 months' duration of use, only one pregnancy was reported. There were no side-effects except prolongation of menstrual cycles in 12% of cases beyond 45 days (Puri *et al.*, 1988).

In a once weekly schedule, a dose titration study at doses ranging from 10 to 120 mg $week^{-1}$ in 579 women volunteers for 3891 months of use, 30 mg doses gave the best ratio between acceptable pregnancy protection and menstrual delay. Prolongation of menstrual cycles occurred more at higher doses. Most of the cases showing delayed cycles, resumed cyclicity while on the drug and some resumed cycles within 40 days after withdrawal of the drug (Puri *et al.*, 1988).

In the contraceptive efficacy trials of centchroman at a 30 mg weekly schedule consisting of 992 subjects for a total of 13,965 months of use, a Pearl Index (Pearl Index = no. of pregnancies × 1200/total no. of exposures, expressed as per 100 women year) of 2.84 was observed. A significant improvement in the Pearl Index to 1.83 was recorded in 377 subjects for 3932 months of use when the dosing schedule was 30 mg twice weekly for 3 months followed by 30 mg once weekly (Kamboj *et al.*, 1992). This latter combination has been recommended for use of centchroman as a contraceptive.

No side-effects, except prolongation of menstrual cycles to more than 45 days in 8% of cases, were observed. The duration and flow of menstrual blood were normal or less but not more in prolonged cycles.

Centchroman administration was found to be quite safe. Haematology, biochemical tests, laparoscopy and ultrasonographic examinations showed no adverse effect of the drug. Generally observed side-effects of steroidal contraceptives such as nausea, vomiting, dizziness and breakthrough bleeding, effect on lipid profiles, HDL cholesterol and platelet functions were not found with centchroman. Babies born to subjects who failed to take the drug correctly were found to be normal.

The contraceptive effect of centchroman was readily reversible and subsequent pregnancies were normal (Kamboj *et al.*, 1992).

In a recent study it was found that centchroman passes into the breast milk. However, the amount of the drug to which the infant is exposed is about 2.5% of the maternal dose (30 mg once a week) which is unlikely to be of any physiological consequence to suckling babies (Paliwal *et al.*, 1994).

4.2 FERTILITY INDUCERS

Childbirth is an important aspect of married life. Infertility often leads to a strained relationship and at times mental and physical problems. In a study of 6306 married women in the UK, it was found (Cooke, 1976) that 22% of women in the age group 16–44 years had involuntary impairment of reproductive potential. Impairment of fertility in women shows an upward trend with increasing age above 20 (Lamb, 1972). Other factors such as social environment also have an influence.

The major causes of infertility in women could be ovulatory disorders, tubal disorders (Behrman and Kistner, 1975; Cox, 1975), cervical factors (Horne *et al.*, 1974; Behrman and Kistner, 1975; Mathews *et al.*, 1975), and uterine and endometrial factors (Raymont *et al.*, 1969). Of these disorders, ovulatory failure constitutes about 42.9% (Cox, 1975). The incidences, however, are likely to differ in different countries.

Therapeutic use of ovarian steroids to induce uterine bleeding in amenorrhoeic women was first reported by Kaufmann (1933), who used sequential injections of estradiol benzoate and progesterone for this purpose. However, no consistent beneficial effects could be found on application of ovarian steroids for follicular maturation or ovulation (Tsai and Yen, 1971; Craft *et al.*, 1975).

In a subsequent development, clomiphene citrate, which inhibits gonadotrophin release and ovulation in rats (Holtkamp, 1960), was found to be an effective ovulation inducer in humans (Greenblatt, 1966) and eventually became a drug for ovulation inductions (see section 4.2.1).

Two other antiestrogens, centchroman (see section 4.1.2) and tamoxifen (see section 5.2.1.1), which are drugs for fertility inhibition and breast cancer respectively, have also been tried for ovulation induction (Roy *et al.*, 1979).

In a preliminary study (Roy *et al.*, 1976), in four out of eight anovulatory women receiving centchroman, there was evidence of ovulation in 11 of 19 cycles. Basal body temperature, levels of urinary total estrogen and/or pregnanediol, blood progesterone, vaginal cytology, cervical mucus, and uterine bleeding were parameters observed for induction of ovulation.

Tamoxifen is also used for induction of ovulation in patients with anovulatory infertility but less widely compared with clomiphene. It is given at a daily dose of 20–40 mg for 4 or 5 days shortly after menstruation or with amenorrhea. In about 70% of cases, ovulation is achieved (Klopper and Hall, 1971; Ruiz-Velasco *et al.*, 1979).

Patients who do not respond to clomiphene may respond to tamoxifen treatment. However, spontaneous abortion rates were found to be higher with tamoxifen (Ruiz-Velasco *et al.*, 1979). Side-effects were found to be less with tamoxifen treatment (Senior *et al.*, 1978).

4.2.1 Clomiphene: A Drug for Fertility Induction

Clomiphene (**20**) exhibited estrogenic and antiestrogenic effects in mice and rats and effectively inhibited fertilization in these species by its gonadotrophin-suppressing, ovulation-inhibiting and blastotoxic effects (Holtkamp *et al.*, 1960; Segal and Nelson, 1961). However, in women, it did not suppress ovulation. Conversely, it was found to induce ovulation in anovulating women (Greenblatt *et al.*, 1961) and is now extensively used for this purpose.

4.2.1.1 Chemistry. Clomiphene citrate (**20**) or 2-(4-(2-chloro-1,2-diphenylethenyl)phenoxy)-*N*,*N*-diethylethamine, 2-hydroxy-1,2,3-propanetricarboxylate (1:1) MRL-41 (clomid) is a racemic mixture of *cis*- and *trans*-isomers. The original assignment of *cis*- and *trans*-geometry was later found to be incorrect as shown by crystallographic studies (Ernst *et al.*, 1976). The corrected *cis*-isomer now called zuclomiphene, is more estrogenic and the presently assigned *trans*-isomer, called enclomiphene, is relatively more antiestrogenic. The racemic mixture used for induction of ovulation is generally composed of 38% zuclomiphene and 62% enclomiphene.

4.2.1.2 Fertility-inducing Action. Clomiphene decreased uterine weight in intact and unilaterally ovariectomized young adult female rats. It also decreased the weight of the uterus of gonadotrophin treated intact and hypophysectomized rats. This suggested anti-uterotrophic activity of the compound. However, when administered to hypophysectomized rats, it caused a uterotrophic effect. These findings showed estrogenic and anti-estrogenic effects of clomiphene (Holtkamp *et al.*, 1960; Roy *et al.*, 1964c; Karkun and Mehrotra, 1973).

Clomiphene is primarily estrogenic in adult mice (Emmens, 1965; Clitheroe and Bonny Castle, 1966; Pollard and Martin, 1968; Terenius, 1971).

Uptake of radioactive estradiol intravenously administered to immature rats by the uterus, pituitary and hypothalamus was inhibited in the case of animals pretreated with clomiphene (Roy *et al.*, 1964a).

Clomiphene in lower doses stimulated secretion of gonadotrophins, particularly LH (Roy *et al.*, 1964b). It was found to stimulate ovulation in pseudo-pregnant rats when given in low doses (Watnick and Neri, 1968; Taubert *et al.*, 1969).

The precise mechanism by which clomiphene causes induction of ovulation is not clearly understood. Involvement of both hypothalamus (Igarashi *et al.*, 1967) and pituitary (Docke, 1971; Hsueh *et al.*, 1978) in clomiphene action has been reported. It is generally believed that its antiestrogenic property inhibits the negative feedback effect of estrogens causing gonadotrophin secretion and ovulation. However, agonistic property of clomiphene should show up at higher doses. It is also reported that clomiphene suppresses gonadotrophin secretion in ovariectomized or post-menopausal women (Czygan and Schulz, 1972; Hashimoto *et al.*, 1976). Interestingly, it was found that clomiphene and its isomers were agonistic and antagonistic over an identical dose range (Roy *et al.*, 1964a,b). Further, it was observed that ethynylestradiol, which is known to cause negative feedback effect in women, failed to interfere with the ovulation inducing effect of clomiphene even at high doses. These observations suggested that with clomiphene a differential cell stimulation may occur at the hypothalamic–pituitary level resulting in negative and positive feedback effects (Clark and Markaverich, 1982).

4.2.1.3 Pharmacokinetics and Metabolism. A comparative study of the metabolism and disposition of clomiphene and tamoxifen in immature rats was carried out by Ruenitz and Bagley (1985). After i.p. administration of the drug to immature female rats, the recovery of clomiphene from faeces over a period of 24 h was 57% compared with 19% of tamoxifen. Most of the clomiphene was eliminated unchanged whereas most of the tamoxifen was eliminated as metabolites, mostly as 4-hydroxytamoxifen. It was concluded that the effects of tamoxifen are due in part to its metabolites whereas the effects of clomiphene are mainly due to the unchanged compound. Some of the metabolites of clomiphene produced upon incubation of the drug with rat liver microsomes are the 4-hydroxy-, *N*-desethyl- and *N*-oxide compounds.

On oral administration of clomiphene to rats, no detectable urinary elimination of the drug or its metabolites was seen. 4-Hydroxy-clomiphene was the only detectable metabolite in faecal extracts.

4-Hydroxy-clomiphene had no uterotrophic effect at doses up to 50 mg day^{-1} but effectively inhibited uterotrophic effect of estradiol (0.5 mg day^{-1}) at doses of 2 μg day^{-1} (Ruemotz *et al.*, 1983).

In women receiving clomiphene citrate, the presence of unchanged clomiphene citrate and lesser amounts of a number of unidentified metabolites was seen after 2–3 h of drug administration (Geier *et al.*, 1987).

4.2.1.4 Toxicology. LD_{50} values of clomiphene in rats by i.p. and oral routes were found to be 530 and 5750 mg kg^{-1}, respectively. Chronic exposure to clomiphene in food at doses of 5–40 mg kg^{-1} day^{-1} resulted in decreased body weight gain and food intake in both male and female rats. Loss of hair was also observed in the mid-dorsal line (Newberne *et al.*, 1966).

Haematological values in clomiphene-treated rats and dogs were found to be normal. Cataract formation was observed in the general toxicity studies by Newberne *et al.* (1966). This could be due to inhibition of cholesterol metabolism which results in the build up of desmosterol.

Exposure of female rats to daily treatment of clomiphene (1–54 mg kg^{-1}, days 6–20) resulted in high fetal mortality (Diener and Hsu, 1967). Reduced ossification causing skeletal deformation and/or retardation of fetuses was observed at high doses (Diener and Hsu, 1967; Souma *et al.*, 1972). Abnormal bone development was also observed in rabbits (Morris, 1970).

Chronic administration of clomiphene (Newberne *et al.*, 1966) caused squamous metaplasia of the uterine epithelium, ovarian atrophy and occasional ovarian cysts. On neonatal exposure it caused premature vaginal opening, persistent vaginal estrus, decreased ovarian weight, inflammation in the oviduct and enlarged cleft clitoris (Leavitt and Meismer, 1968; Gellert *et al.*, 1971; Morishita *et al.*, 1979).

Administration of clomiphene to pregnant rats resulted in various abnormalities in the mother and the pups (McCormack and Clark, 1979) such as extreme stromal and glandular development with hyalinization and small angular nuclei, severely metaplastic and disorganized luminal epithelium, follicular cyst formation, etc. The female pups born appeared normal at 3 weeks of age but by 7 weeks, areas of luminal epithelial metaplasia could be seen in the uterus.

The above observed reproductive teratology is also seen with estrogens (Hale, 1944; Kincl *et al.*, 1965) and hence could be due to the estrogenic property of clomiphene.

Clomiphene administration to male rats resulted in decreased weight of testes, epididymis and other sex accessory structures. It inhibited most testicular functions (Newberne *et al.*, 1966; Flickinger, 1977). These toxic effects are possibly due to estrogenic effect of clomiphene.

Clomiphene treatment given to adult dogs resulted in ovarian atrophy with many atretic follicles. Dilation of endometrial glands was observed in the uterus. In the males, there was inhibition of spermatogenesis.

No general teratogenic effect of clomiphene was observed in monkeys (Courtney and Valerio, 1968).

4.2.1.5 Clinical Studies. Based on studies in rodents, clomiphene was first tested in humans for inhibition of fertility and was found to be ineffective in suppressing gonadotrophin release. Greenblatt *et al.* (1961) observed that clomiphene induced ovulation in anovulatory women and had no suppressive effects on ovulation in normal women.

Clomiphene is administered beginning on day 5 of a cycle following either spontaneous or induced menstruation. Initially, a 50 mg day^{-1} dose is given for 5 days. If no indications of pregnancy occur during the next 40 days, the treatment is continued, usually with a daily dose of 100 mg or higher (the manufacturer, Merrel & Co., does not recommend doses higher than 100 mg) (Adams *et al.*, 1971; Drake *et al.*, 1978; Gorlitsky *et al.*, 1978). If the pregnancy is not achieved in the first two trials, treatment may be continued for more cycles.

Induction of ovulation in clomiphene-treated women is from 60 to 70%, whereas the pregnancy rate is 35%. This lower pregnancy rate could be due to (i) changes in cervical mucus making it hostile to sperm penetration, (ii) follicular luteinization or (iii) inadequate luteal phase (Asch and Greenblatt, 1976).

Generally, the blood levels of LH increase during the period of clomiphene treatment (Jacobsen *et al.*, 1968; Vandenberg and Yen, 1973). A slight rise in FSH level may also occur. This increase in gonadotrophin levels stimulates follicular growth and increase in ovarian estrogen secretion. Ovulation occurs approximately 7 days after clomiphene treatment.

Clomiphene is also used in combination with other drugs such as estrogens, human chorionic gonadotrophin (hCG), bromocryptine, dexamethasone, human menopausal gonadotrophin (HMG) and gonadotrophin releasing hormone (GnRH). hCG is injected after clomiphene administration for improving the luteal phase and pinpointing the day of ovulation (O'Herlihy *et al.*, 1982).

In patients with galactorrhoea and hyperprolactinaemia, a combination of bromocryptine and clomiphene is used (Hirvonen *et al.*, 1976).

In patients with polycystic ovarian disease causing abnormal secretion of steroidal hormones from adrenal glands a combination of dexamethasone with clomiphene has been found to give better results (Lobo *et al.*, 1982).

In some patients showing resistance to clomiphene treatment, addition of HMG (Kistner, 1976) or GnRH (Phansey *et al.*, 1980) is beneficial.

4.2.1.6 Toxicity Studies in Humans. No abnormalities were reported in the reproductive tract of clomiphene-treated women or their children. Increased incidences of trisomy and other chromosomal abnormalities were found in the offspring of clomiphene-treated mothers (Oakley and Flynt, 1972; Boue and Boue, 1973). Endometrial tissue from women receiving clomiphene also showed some chromosomal abnormalities (Charles *et al.*, 1973).

The most common side-effect of clomiphene was ovarian enlargement caused by cyst formation in the follicle, corpus luteum or in luteinized follicles (Kistner, 1975; Natrajan and Greenblatt, 1979). The second most common side-effect of clomiphene was hot flushes. This was most likely due to the antiestrogenic property of the drug. Other side-effects observed in some subjects under clomiphene treatment were abdominal pelvic discomfort (5.5%), nausea and/or vomiting (2.2%), and breast discomfort (2%) (Clark and Markaverich, 1982).

5 Antiestrogens for the Treatment of Cancer

Estrogens play a complex and critical role in the promotion and growth of breast cancer. A correlation between tumour growth and the menstrual cycle was first observed by Cooper in 1836 and Beatson (1896) showed regression of metastatic lesions following oophorectomy in premenopausal women with advanced breast cancer. The era of endocrine therapy for mammary cancer began in 1952 when Huggins and Bergenstal effected remission of advanced breast cancer in postmenopausal women on bilateral adrenalectomy. Following significant success by surgical ablation of endocrine glands causing hormone deprivation, attempts were made to achieve similar hormone deficiency by chemical means. This led to the study of antiestrogens for the treatment of hormone-dependent cancer. This included study of non-steroidal and steroidal estrogenicity lowering agents described in the following sections.

5.1 ESTROGEN SYNTHESIS INHIBITORS: AROMATASE INHIBITORS

Aromatase inhibitors are gaining increasing importance particularly in the treatment of estrogen-dependent cancer. From the discussion on the mechanism of action of aromatase inhibitors given in section 3.1, it would be clear that aromatase inhibitors interfere with the production of endogenous estrogens and do not possess inherent estrogenic activity. A large number of non-steroidal and steroidal compounds show an aromatase-inhibiting property. An attempt is made here to cover important compounds belonging to both categories; however, it is not comprehensive.

5.1.1 Non-steroidal Aromatase Inhibitors

A large number of heterocyclic compounds with potent aromatase-inhibiting activity have been reported in the last 15 years. The heterocyclic portion is usually pyridine, pyrimidine, imidazole, and triazole, some in their cyclized forms. These are discussed below.

5.1.1.1 Pyridines: Aminoglutethimide and Related Compounds. Aminoglutethimide (3-(aminophenyl)-3-ethyl piperidine-2,6-dione, AG, **58**) is the first aromatase inhibitor to be used clinically in the treatment of postmenopausal breast cancer (Santen *et al.*, 1978; Harris, 1985; Sutherland *et al.*, 1985). Aminoglutethimide inhibits tumour growth by lowering estrogen biosynthesis through its effect on the enzyme complex, aromatase, which converts androsten-3,17-dione and testosterone into estrone and estradiol. However, its action is not selective; it also inhibits other enzyme systems involved in steroid biosynthesis and has other side-effects (Santen *et al.*, 1991). Its major action is against the initial step in estrogen biosynthesis which is the conversion of cholesterol into pregnenolone by the enzyme complex, desmolase (Camacho *et al.*, 1967). Inhibition of desmolase decreases corticosteroid production, requiring hydrocortisone as replacement therapy to prevent a reflux rise in adrenocorticotropic hormones (ACTH) for patients receiving aminoglutethimide. AG was approved in Switzerland for breast cancer in April 1991 and in USA for the treatment of Cushing's syndrome. Its side-effects include lethargy, dizziness, ataxia, skin rash, occasional hypothyroidism and rare blood dyscrasia (Santen *et al.*, 1991).

It has been found that the (+)-enantiomer of aminoglutethimide is more selective for aromatase versus sterol side-chain cleavage enzymes (Graves and Salhanick, 1979).

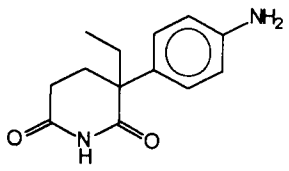
A number of analogues of aminoglutethimide (**59–65**) have been reported to possess more selectivity against aromatase (Foster *et al.*, 1985). Of these, the 4-pyridyl analogue **62** showed strong inhibition of aromatase (85% at 20 $\mu g\ ml^{-1}$) without having any inhibitory effect on the enzyme desmolase.

A structurally simple analog of aminoglutethimide, 4-cyclohexylaniline (**66**), is an effective inhibitor of human placental aromatase enzyme. It was found to be a more potent aromatase inhibitor than (+)-aminoglutethimide. Both **58** and **66** lose their enzyme inhibitory activity on acetylation suggesting a role for the arylamine moiety in these compounds (Kellis and Vickery, 1984).

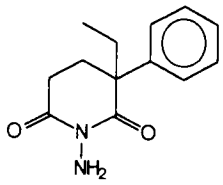
The pyrrolidine analogue, 3-(4-aminophenyl)pyrrolidine-2, 5-dione (**67**) was found to be a potent aromatase inhibitor (Jones *et al.*, 1986).

Hartmann *et al.* (1994) reported the synthesis and aromatase inhibitory activity of pyridyl-substituted indanones (**68**), indanes (**69**) and tetralins (**70**). Compounds **69** (X = 5-OCH_3, Y = H) and **70** (X = 6-OCH_3) were found to be most active in *in vitro* tests, their relative potencies being 154 and 163 (related to aminoglutethimide). However, all compounds showed poor inhibition in *in vivo* tests.

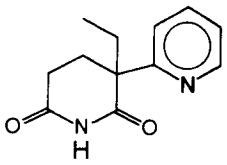
5.1.1.2 Pyrimidine Derivatives. Fenarimol (α-(2-chlorophenyl)-α-(4-chlorophenyl)-5-pyrimidine methanol, **71**) which is used as a fungicide, caused a decrease in fertility in male rats. It was found to inhibit estrogen biosynthesis (Kenneth *et al.*, 1987).



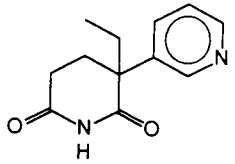
58



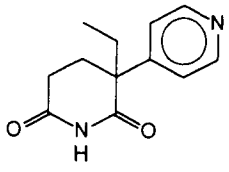
59



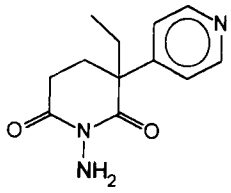
60



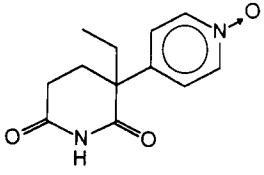
61



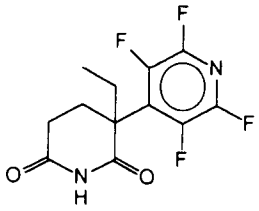
62



63

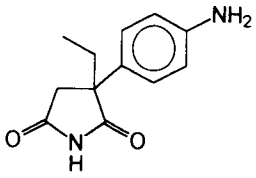


64



65

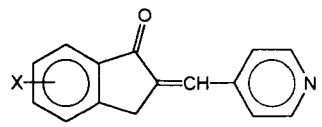
66



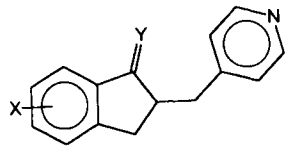
67

LY 56110 (α,α-bis(4-chlorophenyl)-5-methylpyrimidine, **72**) structurally similar to fenarimol, was found to be a significantly more potent inhibitor of rat ovarian microsome aromatase activity, with an IC_{50} of 29 nM (Kenneth *et al.*, 1987).

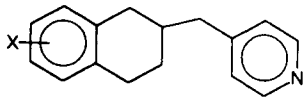
LY 56110 at oral doses of 10–30 mg kg^{-1} day^{-1} for 5 weeks, caused regression of established tumours in rats. However, this compound caused



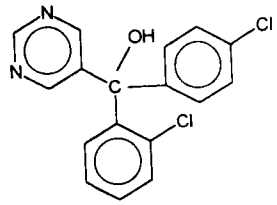
68



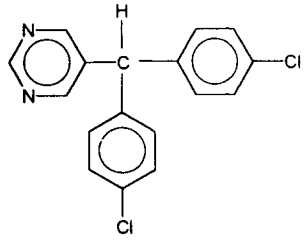
69



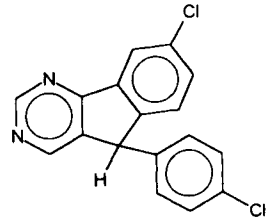
70



71



72

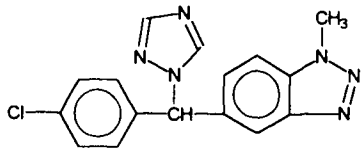


73

a moderate increase in liver weight and had an unacceptably long biological half-life (Kenneth *et al.*, 1987).

The cyclized compound LY 113174 (8-chloro-5-(4-chlorophenyl)-5H-indeno[1,2-d]pyrimidine, **73**) exhibited inhibition of aromatase activity and tumour growth similar to LY 56110 ($IC_{50} = 24$ nM). At a dose of 30 mg kg^{-1} day^{-1}, 75% of the rats appeared to be tumour-free (Kenneth *et al.*, 1987).

5.1.1.3 Imidazole Derivatives. Vorazole (6-(4-chlorophenyl)(1H-1,2,4-triazole-1-yl)methyl)-1-methyl-1H-benzotriazole, R 76713, **74**) in *in vitro* studies showed a very potent inhibition of the aromatase enzyme in rat granulosa cells exhibiting an IC_{50} value of 3.0 ± 0.2 nM. It is 1000 times more effective than aminoglutethimide in the same system and is a very highly selective aromatase inhibitor.



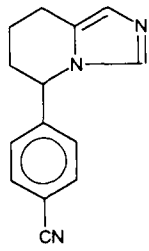
74

A single oral dose of R 76713 (0.05 mg kg^{-1}) effectively lowered plasma estrogen level of PMSG-primed female rats by more than 90%. At a 1 mg kg^{-1} single oral dose, the plasma estradiol level was suppressed completely for 24 h (Wouters *et al.*, 1989).

R 76713 has been found to be highly effective in patients and is well tolerated (Brodie, 1994). The inhibitory activity of R 76713 resides in its dextro isomer R 83842 (Krekels *et al.*, 1992). On oral administration of R 83842 (5 mg kg^{-1}), for five days, the uterine weight of tumour-bearing animals was reduced. *Ex vivo* aromatase inhibition in JE G-3 tumours from these animals was found to be 93%.

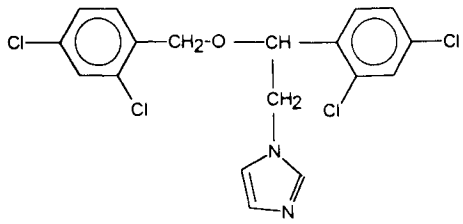
The (−)-enantiomer R 83839 is many-fold less active than R 83842. In PMSG-primed rats, R 83842 reduced plasma estradiol by 50% 2 h after oral administration of 0.0034 mg kg^{-1}, whereas 0.011 mg kg^{-1} of R 76713 and 0.25 mg kg^{-1} of R 83839 were required for the same effect (Wouters *et al.*, 1990).

Fadrozole (4-(5,6,7,8-tetrahydro-imidazo[1,5a]pyridin-5-yl)benzonitrile, CGS 16949A, **75**) is a potent, specific aromatase inhibitor without intrinsic androgenic or estrogenic properties (Ronald *et al.*, 1987; Schieweek *et al.*, 1988).

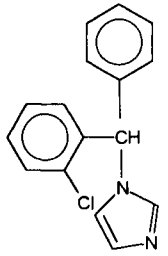


75

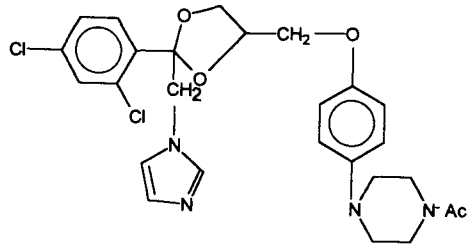
In a clinical trial study of 12 postmenopausal women with breast cancer, a daily dose of 0.6–16 mg for 2 weeks caused lowering of estrogen levels in plasma and urine, as obtained with aminoglutethimide, but complete suppression was not achieved. CGS 16949A was well tolerated and the treatment was associated with little side-effects (Santen *et al.*, 1989). Similar



76



77



78

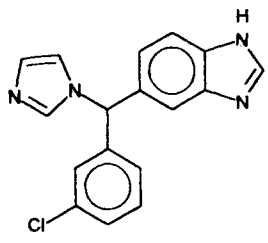
results were reported of a phase I clinical trial of CGS 16949A (Lipton *et al.*, 1990).

In vitro, fadrazole is 300- to 1000-fold more active as an aromatase inhibitor than aminoglutethimide (Lamberts *et al.*, 1989). Potent aromatase-inhibiting activity of human placental microsomes was found in imidazole derivatives miconazole (**76**), clotrimazole (**77**) and ketoconazole (**78**) which showed broad spectrum antimycotic activity and were effective against a wide range of fungal pathogens (Mason *et al.*, 1985; Jones *et al.*, 1990).

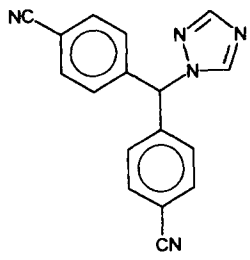
These compounds showed inhibition selectivity for the enzyme aromatase and their action was reversible (Covey *et al.*, 1981; Robert *et al.*, 1978; Osawa *et al.*, 1982).

Liarozole fumarate ((+)-5-(3-chlorophenyl)(1H-imidazol-1-yl)methyl)-1H-benzimidazole (E)-2-butenedioate (2:3), R-85246, **79**) is an aromatase inhibitor which also acts on other cytochrome P450-dependent enzymes (Mahler *et al.*, 1994). It inhibited retinoic acid metabolism in MCF-7 human breast cancer cells and inhibited tumour growth (Wouters *et al.*, 1992). Liarozole has been found to be effective in the treatment of prostatic cancer (Mahler *et al.*, 1993) and is presently in phase III trials.

5.1.1.4 Triazoles. Letrozole (4-(1-cyclophenyl)-1-(1,2-4-triazolyl)methyl) benzonitrile, CGS20267, **80**) is a non-steroidal aromatase inhibitor



79



80

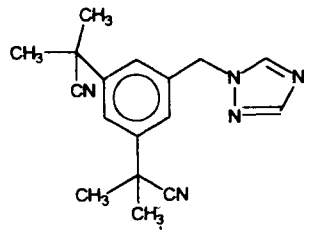
(Bhatnagar *et al.*, 1990; Di Salle *et al.*, 1994). It is a highly selective inhibitor of the enzyme aromatase (Brodie, 1994).

Letrozole administered orally to patients (0.1 and 0.25 mg day^{-1}) for 12 weeks, caused suppression of plasma E_2, E_1 and estrone sulfate levels within 24 h. No change in the levels of cortisol or aldosterone was observed even after provocative stimulation with ACTH. Urinary sodium and potassium levels remained unchanged. The compound has been found to be more potent than aminogluthimide (Bhatnagar *et al.*, 1990).

Arimidex (2,2′-(5-(1H-1,2,4-triazole-1-yl-methyl)-1,3-phenylene)bis(2-methylpropio-nitrile), ICI-ZD1033, **81**) (Prous *et al.*, 1995) was found to exhibit potent and selective aromatase inhibition. In animals, arimidex elicits maximum aromatase suppressive activity at a dose of *c.* 0.1 mg kg^{-1}.

In humans, daily doses of 1–10 mg of **81** suppressed estradiol levels. Its absorption was rapid. In a clinical trial on 17 postmenopausal women with advanced breast cancer, a dose of 10 mg kg^{-1} of ZD-1033 showed stabilization of the disease in four patients for 15–20 months (Plourde *et al.*, 1994).

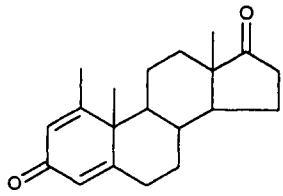
Phase III studies are in progress to assess the efficacy and safety of arimidex in the treatment of advanced breast cancer (Plourde *et al.*, 1995).



81

5.1.2 Steroidal Aromatase Inhibitors

As discussed in section 3.1, the enzyme aromatase is capable of converting 4-androstene-3,17-dione or testosterone into estrone or estradiol. The activity of this enzyme is dependent on two proteins (1) a flavoprotein,



82

NADPH-dependent cytochrome P450 reductase, and (2) an aromatase-specific cytochrome P450 (Thompson and Siiteri, 1974; Osawa *et al.*, 1982a). Many derivatives of androstenedione are not substrates of the enzyme, but bind to its active site, thereby inhibiting estrogen biosynthesis (Brueggemeier *et al.*, 1978; Covey and Hood, 1981; Metcalf *et al.*, 1981). A large number of such androstene-3,17-dione (ADD) derivatives have been found to show an aromatase-inhibiting property. Some of the important compounds are discussed below.

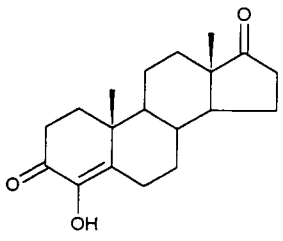
5.1.2.1 1-Substituted ADD Derivatives. Atamestane (1-methyl-1,4-androstadiene-3,17-dione, SH 4839, **82**) was designed as an irreversible inhibitor of estrogen biosynthesis (Henderson *et al.*, 1986). It was conceived that introduction of a methyl group at C-1 would prevent facile aromatization of the 1,4-androstadiene-3,17-dione system to estrone.

SH-4839 showed high affinity for aromatase. It was found to be a more potent inhibitor of human placental aromatase (K_i of 2.5×10^{-7} mol l^{-1}), compared to 4-hydroxy-4-androstene-3,17-dione (**83**) ($K_i = 2.5 \times 10^{-7}$ M). Its irreversible nature of inhibition and lack of other endocrinological effects make it a promising drug for the treatment of estrogen-dependent disease states.

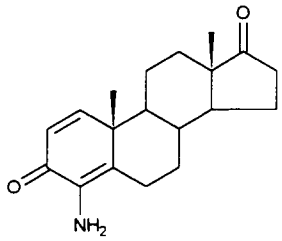
5.1.2.2 4-Substituted ADD Derivatives. Formestane (4-hydroxy-androst-4-ene-3,17-dione, 4-OHA, **83**) is the most studied steroidal aromatase inhibitor introduced into the market as an intramuscular formulation for the treatment of advanced breast cancer in postmenopausal women (Stein *et al.*, 1990). Its efficacy is comparable to aminogluthimide and it is devoid of side-effects (Brodie *et al.*, 1987). Its LD_{50} and LD_{10} values are 4.325 g kg^{-1} and 2.9 g kg^{-1}, respectively (Coombes *et al.*, 1984).

4-OHA is a strong inhibitor of aromatase but not of desmolase (Brodie *et al.*, 1977). In contrast to aminoglutethimide, 4-OHA binding to aromatase is irreversible (Brodie *et al.*, 1981).

In a clinical study, intramuscular injection of 4-OHA (500 mg) to 11 postmenopausal women with advanced breast cancer, caused reduction of serum estradiol for at least one week in all patients. The only side-effects observed were pain at the injection site and hot flushes (Coombes *et al.*, 1984).



83



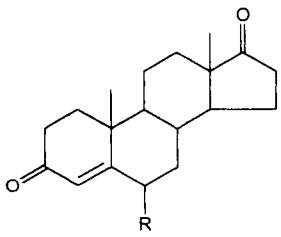
84

The acetyl derivative of 4-OHA has also been found to be a potent inhibitor of human placental aromatase and caused regression of 7,12-dimethylbenzanthracene (DMBA) induced tumours in rats (Brodie *et al.*, 1979, 1982).

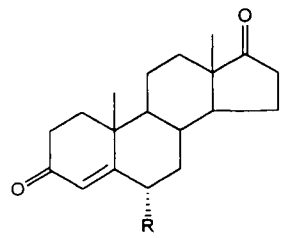
Minamistane (4-aminoandrosta-1,4,6-triene-3,17-dione, FCE 24928, **84**) was selected from a series of 4-amino-androstene-dione derivatives as a novel irreversible aromatase inhibitor (Di Salle *et al.*, 1990). It caused time-dependent inhibition of human placental aromatase with K_i of 50 nM. This compound was found to be more potent than 4-OHA in rats for microsomal ovarian aromatase activity. In immature rats FCE 24928 did not show any intrinsic androgenic activity unto 100 mg kg^{-1} day^{-1} s.c.

5.1.2.3 6-Substituted ADD Derivatives. A number of 6-substituted ADD derivatives (**85** and **86**) have been evaluated for aromatase inhibition activity (Numazawa and Oshibe, 1994). Alkyl derivatives **85a–d** and **86a–d** and also 6α-benzyl **86g** and 6β-vinyl **85h** showed potent competitive inhibitory activity in human placental microsomes with apparent K_i values ranging from 1.4 to 12 nM. Ethyl derivatives **85b** and **86b** were found to be most potent (K_i 1.4 nM and 4.7 nM, respectively).

6β-Bromoandrostenedione (**85j**) is a mechanism-based irreversible inhibitor of human placental aromatase (K_i 0.8 μM) while the corresponding 6α-epimer (**86j**) is a competitive inhibitor (K_i 3.4 nM) (Osawa *et al.*, 1987). A single *in vivo* administration of **85j** caused significant suppression of estrogen secretion from ovaries while **86j** was ineffective at the same dose.



85



86

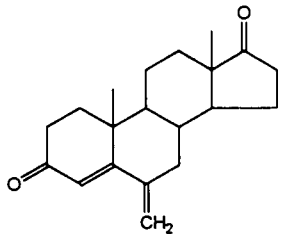
a: CH_3 f: C_6H_5
b: C_2H_5 g: $CH_2C_6H_5$
c: C_3H_7 h: $CH{=}CH_2$
d: C_4H_9 i: $C{\equiv}CH$
e: $CH(CH_3)_2$ j: Br

Both 6-methylenandrost-4-ene-3,17-dione (6-MAD, **87**) and exemestane (6-methylenandrosta-1,4-diene-3,17-dione, FCE 24304, **88**) (Buzzetti *et al.*, 1993) are potent inhibitors of human placental aromatase. Exemestane is an orally active irreversible inhibitor (Giudici *et al.*, 1988) effective against DMBA-induced mammary tumours in rats (Zaccheo *et al.*, 1989).

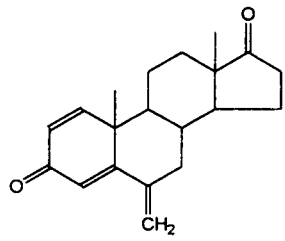
Derivatives of 3β-hydroxy-androst-4-en-6-one (**89**, R = OCHO, $OCOCH_3$ or H) were found to possess potent human placental microsomal aromatase inhibition activity with IC_{50} values in the range 1.7–3.3 μM, using 4-OHA as a substrate (Numazawa *et al.*, 1989a,b).

5.1.2.4 7-Substituted ADD Derivatives. A number of 7-substituted 4,6-androstadiene-3,17-diones (**90**) have been found to show potent aromatase inhibitory activity. 7-Benzyl and 7-phenethyl substituted compounds showed better inhibition compared with 7-phenyl, as in the first two cases the phenyl group could possibly protrude into the 7α-pocket (Li and Brueggemeier, 1990).

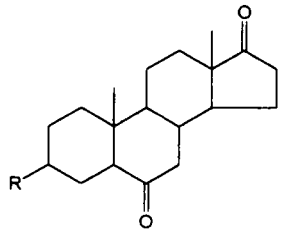
7α-Thiophenyl-substituted derivatives of androstenedione (**91**) and 2,4-androstadienedione (**92**) derivatives to possess potent aromatase inhibitory activity. Introduction of electron donating and withdrawing groups on the phenyl ring of **91** showed no linear correlation with the inhibitory potential. The *p*-iodophenyl compound appeared to be quite potent (Darby *et al.*, 1985). The most potent inhibitor, 7α-(4′-(aminophenyl)thio)-4-androstene-3,17-dione (7α-APTA, **91**), showed an apparent K_i of 18 nM (Li and Brueggemeier, 1990). These aromatase inhibitors inhibited aromatase activity in MCF-7 cells and caused reduction of tumour volumes in DMBA-induced rat mammary tumour model. 7α-(4′-Amino)phenylthio-1,4-androsta-1-diene-3,17-dione,(7α-APTADD, **92**) was found to be an irreversible inhibitor.



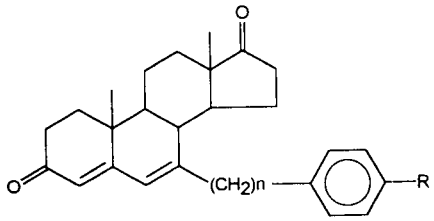
87



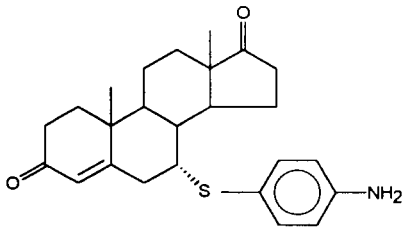
88



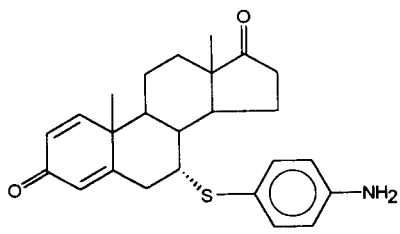
89



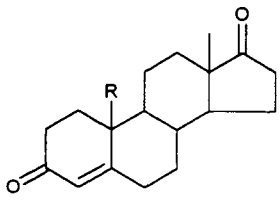
90



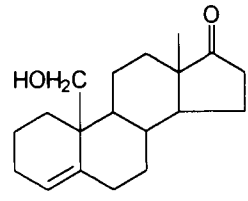
91



92



93



94

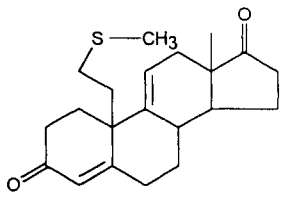
5.1.2.5 10-Substituted ADD Derivatives. Involvement of a C-10 methyl group during the aromatization process formed the basis for development of 10-substituted ADD derivatives as irreversible aromatase inhibitors. Among various compounds studied, allelic and acetylenic substitution led to potent aromatase inhibitors (**93**: R = CH_3, CH_2OH, CHO, CH_2—C≡CH, CHOH—C≡CH, —CH=C=CH_2).

MDL-18, 962 (10-(2-propynyl)estr-4-ene-3,17-dione (**93**: R = $CH_2C{\equiv}CH$) showed specific inhibition property (Johnston *et al.*, 1984; Johnston, 1987). It was found to be a highly potent irreversible inhibitor of human placental aromatase (apparent $K_i = 4.5 \mp 1.3$ nM). *In vivo* evaluation showed that MDL-18, 962 could inactivate aromatase and cause decrease in the weight of an estrogen target tissue (Metcalf *et al.*, 1981). ED_{50} for inhibition of peripheral aromatization of androgen by MDL 18,962 in female baboons was 0.01 mg kg^{-1} i.v. and 4 mg kg^{-1} p.o. (Longcope *et al.*, 1988).

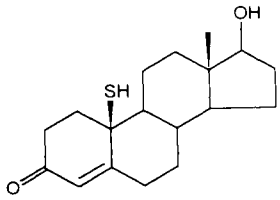
19-Hydroxy-3-deoxy-androst-4-en-17-one (**94**) was found to be a more potent inhibitor of human placental aromatase ($IC_{50} = 0.27$ nM) compared with 19-hydroxy-androsten-3,17-dione ($IC_{50} = 4.7$ mM) (Numazawa *et al.*, 1989).

RU 54115 (**95**) was reported to be a very potent inhibitor of human placental aromatase (K_i of 0.5 nM). *In vivo* it lowered serum estrogen level in pregnant mare serum gonadotrophin (PMSG) primed female rats with an ED_{50} of 0.4 mg kg^{-1} when given s.c. and 4 mg kg^{-1} administered orally.

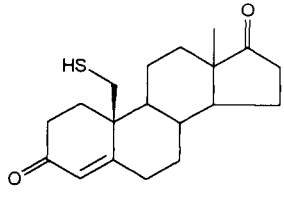
RU 54115 prevented growth of DMBA induced mammary tumours in rats



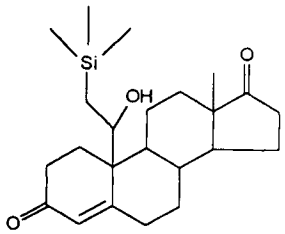
95



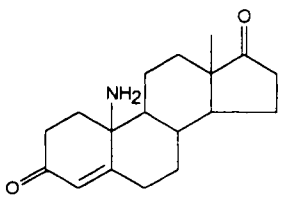
96



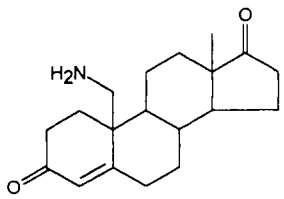
97



98



99



100

at an oral dose of 25 mg kg^{-1} day^{-1} (Delaisi *et al.*, 1992). 17β-Hydroxy-10β-mercapto-estr-4-en-3-one (96) and 19-mercapto-androst-4-en-3,17-dione (**97**) also inhibited human placental aromatase activity (Bednarski *et al.*, 1985).

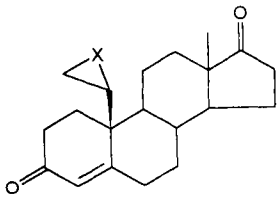
The silyl derivative, 10-(1-hydroxy-2-(trimethyl silyl)ethyl)estr-4-ene-3,17-dione (**98**) exhibited ability to inhibit human placental aromatase with an apparent K_i of 562 ∓ 12 nM (Burkhart *et al.*, 1985).

Aromatase inhibitory activity was also observed with the amino derivatives, 19-amino-estr-4-en-3,17-dione (**99**) and 19-aminoandrost-4-en-3,17-dione (**100**) (Lovett *et al.*, 1984). However, these compounds were inferior to **91** or 4-OHA.

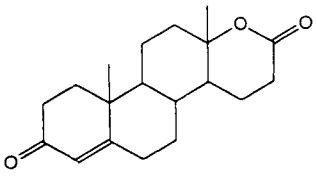
Introduction of an oxirane (**101**, X = O) or a thiirane (**101**, X = S) ring at C-10 led to compounds with significant human placental aromatase inhibiting activity. These acted in a competitive manner. 19*R*-Isomers were more potent inhibitors and exhibited affinities 36-fold (10-oxirane) and 80-fold (thiirane) greater than the corresponding 19*S*-isomers (Kellis *et al.*, 1987). Thiirane compounds (**101**, X = S) were more potent than corresponding oxiranes (**101**, X = O) (Childers *et al.*, 1991).

A D-ring modified analogue of androstenedione, Δ^1-testolactone (D-homo-17α-oxaandrosta-1,4-diene-3,17-dione, **102**) (Serwin *et al.*, 1989) showed inhibition of peripheral aromatization of androstenedione in nine postmenopausal women with metastatic breast cancer. Oral administration of 250 mg of testolactone given every 6 h. reduced E_1 level significantly; it is a potent aromatase inhibitor (Barone *et al.*, 1979).

Administration of Δ^1-testolactone (**102**), 500 mg dose twice daily for 4 weeks in nine patients with idiopathic oligospermia, showed inhibition of



101



102

estradiol levels by about 30%, and enhanced the secretion of FSH (30%), 17-hydroxyprogesterone (17-OHP) (40%) and testosterone (30%) (Dony *et al.*, 1985).

5.2 COMPETITIVE INHIBITORS OF ESTROGENS

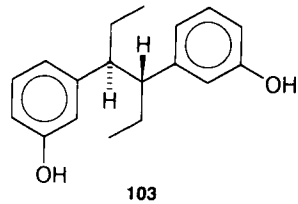
A large number of non-steroidal and steroidal compounds have been found to compete with endogenous estradiol for ER binding and also for direct interaction with growth factors, thereby inhibiting estrogen action. Use of such compounds is described under appropriate sections. A more generalized discussion on development of these compounds is given under section 4.1.1. In this section only compounds and groups of compounds which are of particular relevance to inhibition of cancer will be described.

5.2.1 Non-steroidal Antiestrogens

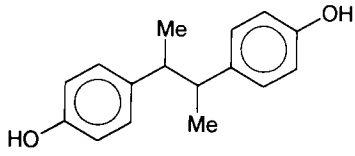
5.2.1.1 Aryl Alkanes and Alkenes

1,2-Diarylethane derivatives: Hexestrol (**6**) is a frank estrogen. Structural modification of the hexestrol molecule has led to compounds which elicit antiestrogenic activity. Variation of the alkyl chains in the 1,2-position (Hartman *et al.*, 1981) and tetralkylation in the 1,1,2,2-position of the 1,2-diphenylethane skeleton (Hartmann *et al.*, 1980), led to a number of active compounds. Another effective structural variation was the shifting of the hydroxyl groups from the *para* to the *meta* position. Compounds **103–111** are some of the active antiestrogens belonging to this class. Compound **103** (*meta*-hexestrol) and its higher homologues **108** and **109** reduced estrone-stimulated mouse uterine growth; their inhibition was 53, 50 and 45%, respectively (Hartmann *et al.*, 1981); all three compounds showed weak estrogenic activity in the mouse weight gain test and caused vaginal cornification. Compounds **105** (*meta*-butestrol), **108** and **109** exhibited a dose-dependent growth inhibition on the MCF-7 human breast cancer cell line (IC_{50} 10^{-6}–10^{-9} M) and also showed a marked dose dependent inhibition on the DMBA-induced hormone dependent mammary carcinoma of the Sprague–Dawley rat (Hartmann *et al.*, 1981; Engel *et al.*, 1983). *Meta*-hexestrol is also active on the hormone-independent human MDA-MB 231 breast cancer cell line (IC_{50} 2.5×10^{-6}M). Its antiestrogenicity is comparable to nafoxidine (Hartmann *et al.*, 1980; Kranzfelder *et al.*, 1982).

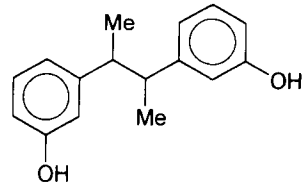
The tetramethyl-1,2-diphenylethanes (**106**,**107**) exhibited strongest anti-estrogenic activity. In contrast to *meta*-hexestrol, these compounds showed only slight or no estrogenic properties, depending on the test system (Hartmann *et al.*, 1980).



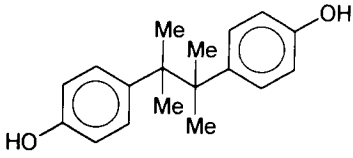
103



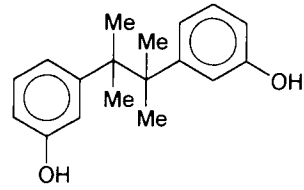
104



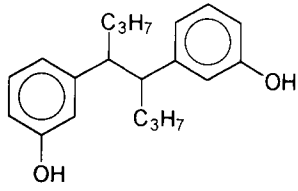
105



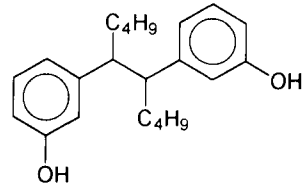
106



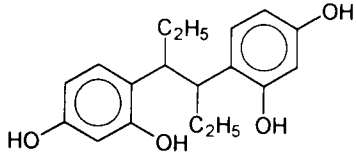
107



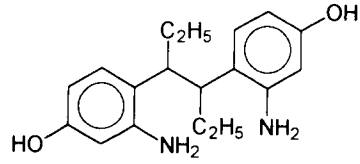
108



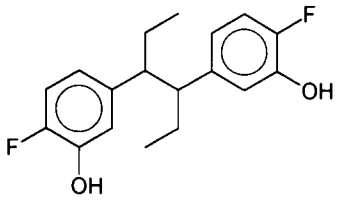
109



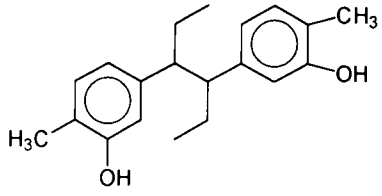
110



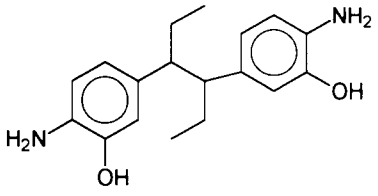
111



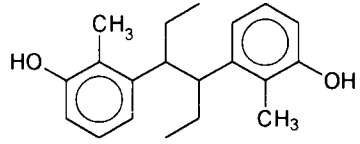
112



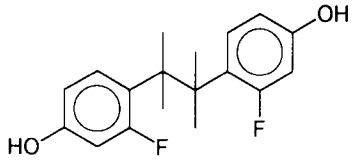
113



114



115



116

2,2′-Dihydroxyhexestrol (**110**) and 2,2′-diaminohexestrol (**111**) elicited full uterotrophic effects in high doses (25 μg day^{-1}) and inhibited estrone-stimulated uterine growth in small doses (59% and 78%, respectively, at 5 μg day^{-1}) (Hartmann *et al.*, 1983).

Compounds **112–115** are some other derivatives of metahexestrol which possessed antiestrogenic property (Hartmann *et al.*, 1984). The fluorinated derivative **116** caused strong dose-dependent inhibition of the DMBA-induced mammary tumour in rats. It reduced total tumour area by 47% and affected a complete remission in 74% of the tumours at a dose of 5 mg kg^{-1} day^{-1} (Hartman *et al.*, 1985). Recently, a number of thioether derivatives of 1,2-diarylethane (117) have been reported to possess significant antiestrogenic property with weak antiproliferative activity. The ether chain is very similar to that present in some of the steroidal antiestrogens discussed in section 5.2.2.1.

Two of the active compounds belonging to thioether class are **117** (R = H or Et). Compound **117** (R = Et) was found to be ten-fold less antiestrogenic

compared with EM-139 (see section 5.2.2.1) and similar RBA for ER. This derivative is devoid of estrogenic activity (Auger *et al.*, 1995). Compound **117** (R = H) is 100-fold less estrogenic in ER+ZR-75-1 cells than EM-139 (Parer *et al.*, 1994).

Triaryl alkanes and alkenes: One of the first non-steroidal antiestrogens discovered, MER-25 (**18**) (see section 4.1.1.2) (Lerner, 1981) was shown to cause 50% inhibition of 18-54-5F (derived from human pituitary tumour tissue) cell growth at concentration of $2–3 \times 10^{-7}$ M (Wyche and Noteboom, 1979). Since then a number of triaryl ethylenes and ethanes have been found to possess antiestrogenic activity and were evaluated for antitumour activity.

Clomiphene citrate (**20**) was the first antiestrogen to show activity in breast cancer patients (Herbst *et al.*, 1964). A clinical trial with clomiphene was reported by Hecker *et al.* (1974). At 200–300 mg daily dose to 51 cases of advanced breast cancer, 20 patients (39%) showed objective tumour remissions lasting between 70 and 660 days with a mean duration of 365 days. Only mild side-effects were reported; thus, clomiphene appeared to be effective in the treatment of breast cancer. However, it is not currently approved for this purpose.

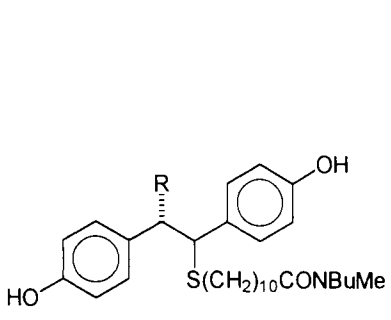
A series of triaryl ethylene compounds related to 4-hydroxy-clomiphene (**118**) has been reported as novel class of antiestrogens (Sutherland *et al.*, 1986) in which the vinyl chloro substituent was replaced by ethyl (X = Et), Br (X = Br), H (X = H), CN (X = CN), or NO_2 (X = NO_2). These derivatives possess antiproliferative activity in MCF-7 human mammary carcinoma cells. In the concentration range 10^{-10}–10^{-8} M, cell proliferation was inhibited by 60–70%. The inhibitory effect was estrogen reversible and was in the order Cl > Et > Br > NO_2 > H which was parallel to their RBA values for estrogen receptors.

The most noteworthy compound which has evolved out of the work on triaryl ethylenes and ethanes is tamoxifen (**22**) which is discussed in section 5.2.1.5.

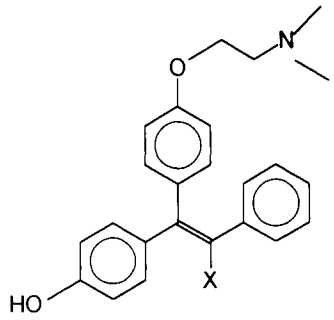
Droloxifen (E)-3-(1-(4-(2-(dimethylamino)ethoxy)phenyl)-2-phenyl-1-butenyl) phenol, EN 090075, **119**), a 3-hydroxyphenyl derivative of tamoxifen, is an estrogen antagonist. Compared to tamoxifen, it has a 10–60-fold higher RBA for ER and is less estrogenic in rats. Droloxifene inhibited different ER-positive breast cancer cells more effectively than tamoxifen (Tominaga *et al.*, 1993; Hasmann *et al.*, 1994).

In vivo, droloxifene displayed increased growth inhibition of different tumours of animal (R 3230Ac and 13762) and human origin (T61). Droloxifene was devoid of any *in vivo* or *in vitro* carcinogenic or mutagenic effects, whereas tamoxifen caused liver tumours and induced DNA-adduct formation in rats.

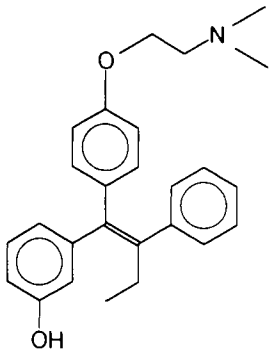
The pharmacokinetic studies of droloxifene in animals and pre- and



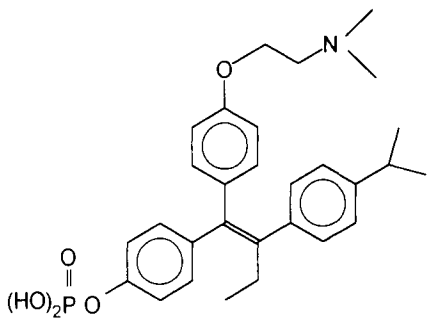
117



118



119



120

post-menopausal patients showed that the compound was rapidly and almost completely absorbed when given orally (Tanaka *et al.*, 1994).

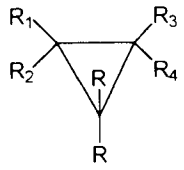
In a phase I trial in 30 patients with advanced refractory metastatic breast cancer, administration of droloxifene (20, 40, 100, 200 or 300 mg daily) caused adverse reactions such as hot flushes, nausea, and fatigue which were not dose related (Buzdar *et al.*, 1994). In a phase II trial in 268 postmenopausal patients with metastatic breast cancer, droloxifene at 20, 40 or 100 mg daily dose produced response rates of 30, 47 and 44%, respectively, with mild toxicity (Rauschning and Pritchard 1994). Similar results were reported by Serin (1993) for a multicentre, double-blind randomized trial in 369 postmenopausal patients with advanced breast cancer.

A phosphate derivative of tamoxifen, TAT 59 (E-4-(1-(4-(2-(dimethylamino) ethoxy)phenyl)-2-(4-(1-methylethyl)phenyl)-1-butenyl)phenol) dihydrogen phosphate ester, **120**) at 10^{-6} M concentration, reduced the levels of 10^{-8} M estradiol.

TAT 59 inhibited growth of MCF-7 cells in a dose-dependent manner. Activity was found in the order TAT > droloxifene > tamoxifen (Tominaga *et al.*, 1993).

5.2.1.2 Cyclized Di- and Triaryl-alkanes / alkenes. The following cyclized aryl-alkane and alkene derivatives have shown promising anticancer activity.

Diarylcyclopropyl and triarylcyclopropyl derivatives: Some gem-dichlorocyclopropyl and cyclopropyl analogues (R_1,R_2,R_3 = H or Ar; R = H or Cl, **121**) of stilbene were found to possess antiestrogenic activity (Magarian



121

and Benzamin, 1975; Stobaugh *et al.*, 1982). 1,1-Dichloro-2,3-diphenylcyclopropane (**121**, $R_1 = R_3$ = Ph; $R_2 = R_4$ = H, R = Cl) showed antiestrogenic properties without agonistic activity in the mouse and was comparable to tamoxifen against hormone-dependent DMBA-induced rat mammary tumour model (Pento *et al.*, 1982, King *et al.*, 1985).

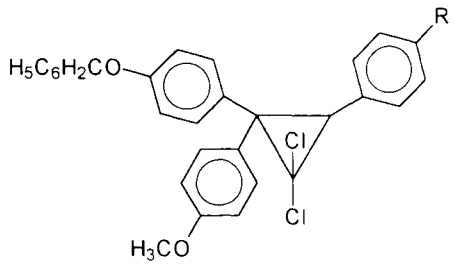
Compounds **122–124** are other antiestrogenic compounds of the triaryl-cyclopropyl series which inhibit growth of estrogen-receptor-positive MCF 7 human breast cancer cells in culture (Day *et al.*, 1991; Hossain *et al.*, 1994).

It is interesting to note that in this series, the presence of a ω-substituted aminoalkoxy group is not essential for antiestrogenic activity. Removal of the benzyl group resulted in complete loss of antiestrogenic activity. Phenolic derivatives were earlier reported to be estrogenic (Iyer and Gopalchari, 1976).

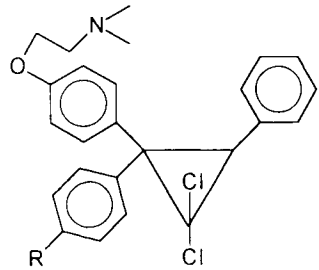
Nafoxidine: Nafoxidine (1-(2-(4-(3,4-dihydro-6-methoxy-2-phenyl-naphthyl)phenoxy) ethylpyrrolidine, **31**) is a non-steroidal antiestrogen prepared by the Upjohn company (Lednicer *et al.*, 1963) (see section 4.1.1.2.5). In 1967, Bloom *et al.* suggested the use of nafoxidine in the treatment of breast cancer. Nafoxidine has been investigated extensively in Europe and limited studies were reported from the United States (European Breast Cancer Group, EORTC, 1972; Legha *et al.*, 1978).

Nafoxidine prevented estradiol induced growth *in vitro* of DMBA-induced hormone-dependent rat mammary carcinoma cells (Jensen *et al.*, 1967). It had antitumour effects on the rat mammary carcinoma and prevented tumour induction and inhibited tumour growth (Terenius, 1971).

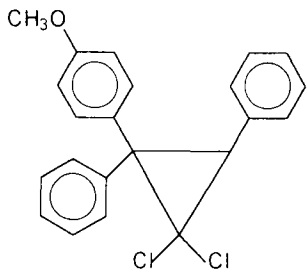
In a comparative study of nafoxidine and ethynylestradiol in postmenopausal women with advanced breast cancer, nafoxidine showed a twofold higher objective remission (31%) (Heuson *et al.*, 1975).



122



123



124

In a study of 52 women (all but one was postmenopausal) with advanced breast cancer, 85% of whom were resistant to or had relapsed after previous endocrine therapy, oral administration of nafoxidine (60–90 mg) three times a day for 1–36 months resulted in an objective response rate of 37%.

Trioxifene and related compounds. Trioxifene (**40**) developed by Jones *et al.* (1979) is a potent antiestrogen similar to tamoxifen, with very weak agonistic activity. However, it was found to be less effective in rats compared with tamoxifen in inhibiting DMBA-induced mammary carcinoma (Wakeling and Valcaccia, 1983). *In vitro*, it inhibited growth of human breast cancer cells (MCF-7) more efficiently compared with tamoxifen (Wakeling *et al.*, 1984).

The hydroxy derivatives of trioxifene (**125**, R = H or OH) suppressed growth of MCF-7 human breast cancer cells causing 36% and 50% inhibition at 30 nM and 4.5 nM concentration, respectively (Jones *et al.*, 1992).

Cyclized trioxifene derivatives, benzo[a]fluorene counterparts (**126**, R = H or OH) were also found to be more potent antiestrogens and inhibited growth of MCF-7 breast cancer cell proliferation more effectively than tamoxifen (Jones *et al.*, 1992).

Thiophene analogues LY 117018 (**127**) and LY 139481 (Raloxifine, **128**) (Black *et al.*, 1983) had much reduced partial agonistic activity in rat uterus

compared to tamoxifen (Black and Goode, 1980; Wakeling and Valcaccia, 1983) but were less effective antitumour agents than tamoxifen. In *in vitro* studies of growth inhibition of human breast cancer cells (MCF-7), the order of potency was 4-hydroxy-tamoxifen > LY139481 = Ly117018 > trioxifene > tamoxifen. However, because of a short biological half-life, these hydroxylated trioxifene-related compounds elicited inferior *in vivo* activity compared with tamoxifen.

6-Hydroxy-2-(4-hydroxyphenyl)benzo[b]thien-3-yl)-(4-(2-(1-piperidinyl)ethoxy) phenyl methanone hydrochloride (LY 156758, hydrochloride salt of LY 139481) was evaluated for antitumour effects in the DMBA-induced rat mammary tumour at $1\,mg\,kg^{-1}$ and $10\,mg\,kg^{-1}$ doses. It significantly inhibited tumour growth (Jones *et al.*, 1984).

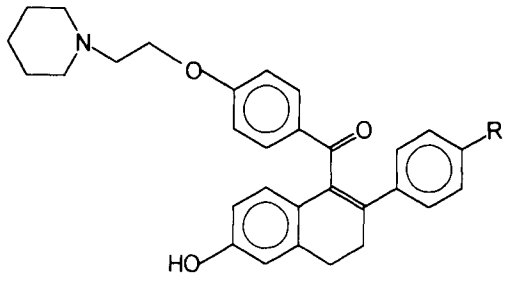
Centchroman. Centchroman (**57**), a potent antiestrogen with weak estrogenic activity, is an oral contraceptive agent (see section 4.1.2). Its antiestrogenic property and near inert pharmacological profile led to its evaluation in the advanced stages III and IV of breast cancer (Mishra *et al.*, 1989). To date, 158 patients of advanced breast cancer have been treated with centchroman. Various dosage schedules of centchroman, i.e. 60 mg AD/OD/BD and 120 mg AD for 6–52 weeks, were evaluated. An overall positive response rate of 58% was observed. Centchroman was found to be well tolerated and was devoid of distressing side-effects (Asthana *et al.*, 1994).

The anti-inflammatory activity of centchroman (Dhawan and Srimal, 1973) possibly helps in giving partial relief to the patients.

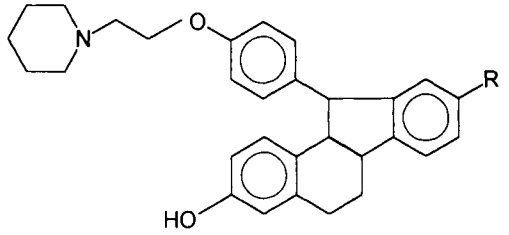
5.2.1.3 Indole Derivatives. Recently, 2-arylindole derivatives (**129**) have been reported as pure antiestrogens (VonAngerer *et al.*, 1990). These compounds possess an aminoalkyl chain at the indole nitrogen (R_1 = H, CH_3; R_2R_3 = H_2, $(CH_3)_2$, $(C_2H_5)_2$, $(CH_2C_6H_5)_2$, (CH_2)x: x = 4,5). In this series, the most active compound was found to be ZK 119010 (2-(4-hydroxyphenyl)-3-methyl-1-(6-(1-pyrrolidinyl)-hexyl)-indol-5-ol). In immature mice, it prevented uterotrophic and vaginotrophic effect of E_2 in a dose-dependent manner and was more potent than tamoxifen or ICI 164,384 in exerting antiestrogenic effect (Nishino *et al.*, 1991).

Two of the 2-phenylindole derivatives (**130**) (n = 10 or n = 11) with carbamoylalkyl chain as present in 7-substituted steroidal antiestrogens (see section 5.2.2.1) proved to be pure antiestrogens both in *in vitro* and *in vivo* assays. In estrogen-sensitive MCF-7 breast cancer cells, these two strongly inhibited cellular growth (VonAngerer *et al.*, 1994). 2-Phenyl indole derivatives have been found to inhibit DMBA-induced mammary carcinoma of Sprague–Dawley rats (VonAngerer *et al.*, 1984).

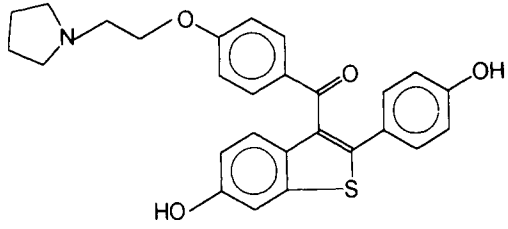
The (4-cyanobenzyl)indole derivative **131** with its high ER-binding affinity and strong antiestrogenicity, strongly inhibited the growth of DMBA-induced



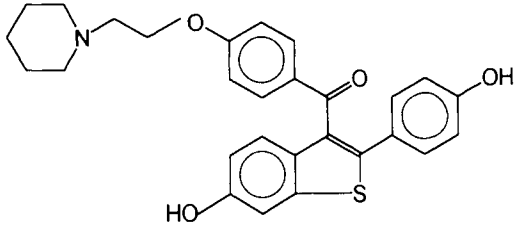
125



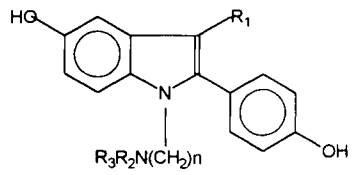
126



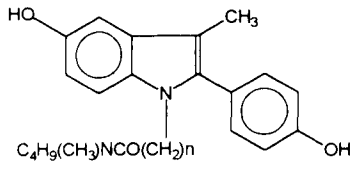
127



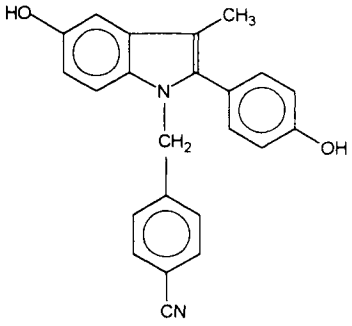
128



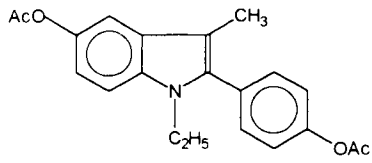
129



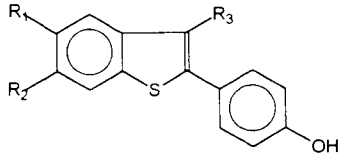
130



131



132



133

tumours at 12 mg kg^{-1} dose in Sprague–Dawley rats (VonAngerer and Stohmeier, 1987).

Zindoxifene (1H-indol-5-ol-2-(4-(acetoxy)phenyl)-1-ethyl-3-methyl acetate, D 16726, **132**) is an antiestrogen very active against experimental hormone-dependent mammary tumours (VonAngerer *et al.*, 1985). In rat prostate carcinoma model, zindoxifene in combination with a low dose of cisplatinum, inhibited tumours by 91% (VonAngerer *et al.*, 1992). It is presently undergoing phase I clinical trials.

Structure–activity relationship studies of 2-phenylindoles suggest the need for an alkyl substituent on the indole nitrogen for receptor binding (Fritsche and Bunsenges, 1964). The appropriate positions for the hydroxyl groups appear to be the *para* position in the 2-phenyl ring and the 5 or 6 position in the indole nucleus.

2-Arylbenzothiophene derivatives (**133**) with a hydroxyl group in position 5 or 6 and a short alkyl group at C-3 showed high RBA for ER

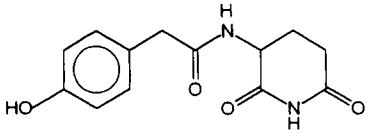
and tumour-inhibiting activity. Highest RBA (60%) was observed for 2-(4-hydroxyphenyl-3-ethyl-5-hydroxy benzo[b]thiophene (**133**; $R_1 = OH$, $R_2 = H$, $R_3 = Et$). Its administration ($3 \times 4.2\ mg\ kg^{-1}$) caused 83% reduction of tumour weight versus control (tamoxifen at the same dose caused 74% reduction) in mammary tumours in mice (VonAngerer and Erber, 1992).

5.2.1.4 Piperidinedione Derivatives. A recent approach in designing antiestrogens is based on computer graphics and energy calculations of DNA–ligand complexes (Hendry *et al.*, 1994). The rationale behind this approach is the finding that genes encode the information for both enzymes and receptors which, in turn, govern the initiation of biological activity. The degree of fit of a certain molecule into specific sites in DNA correlates with biological activity (Uberoi *et al.*, 1985; Hendry *et al.*, 1986, 1992; Hendry, 1988). Hormone agonists fit into a specific site in DNA in a similar manner. In the case of hormone antagonists, certain parts of the molecule generally fit certain portions of the site occupied by agonists, but possess additional features which may interact differently with DNA.

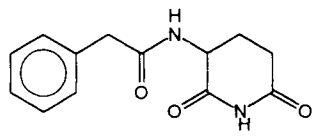
By utilizing computer modelling on the basis of various physical models, estradiol has been shown to fit on unbound DNA at the site 5′-d Td G-3′,5′-dCd A-3′ (Lehner *et al.*, 1987; Hendry, 1988). Stereospecific hydrogen bond formation between hydroxyl groups of estradiol and other estrogenic ligands with phosphate oxygen on adjacent DNA strands, forming a bridge between both sugar–phosphate backbones, correlated well with the estrogenic response of the ligand. Antagonists tamoxifen, 4-hydroxytamoxifen and LY-117018 bind to DNA in a different manner: a portion of the structural framework extended out of the site in DNA into the major or minor grooves (Hendry, 1988).

It is known that the ligand–receptor complex interacts with the DNA component. Estrogen receptors alone may reside in the ERE portions of DNA but the initiation of hormonal activity occurs only when ligand is coupled to ER. It has thus been speculated that in this ligand–ER interaction with ERE, the receptor protein upon binding to DNA in concert with other transcription factors may cause a specific conformational change in DNA, giving rise to the site into which ligand can fit.

In this computer-based study, *p*-hydroxyphenylacetylamino-2,6-piperidinedione (**134**) has been found to fit better into the DNA complex than phenyl acetylamino-2,6-piperidinedione (**135**). Similarly, 4-hydroxytamoxifen



134



135

fitted better than tamoxifen and this correlated well with their inhibitory effect on MCF-7 (E3) human breast cancer cell growth. However, piperidinediones also decrease protein kinase activity (Copland *et al.*, 1993) which would cause a dose-dependent decline in ER level and subsequently of progesterone receptors, which would suppress growth of MCF-7 cells. More data are required to substantiate the validity of this approach for the evaluation of antiestrogenic compounds.

5.2.1.5 Tamoxifen: A Drug for the Treatment of Breast Cancer.

Chemistry: Tamoxifen [(*Z*)-2-(4-(1,2-diphenylbut-1-enyl)phenoxy)-*N*,*N*-dimethylethanamine (ICI 46,474, **22**) was synthesized (Bedford and Richardson, 1966) and developed (Harper and Walpole, 1966) by Imperial Chemical Industries (UK patent, 1,013,907; 1,064,629). It is sold as a monocitrate salt for endocrine therapy of breast cancer. In contrast to the estrogen antagonistic property of tamoxifen, the E-isomer (ICI 47,699, **23**) is a conventional estrogen (Harper and Walpole, 1966). The stereochemistry of tamoxifen was determined by dipole moment measurements and NMR studies (Bedford and Richardson, 1966) and confirmed by X-ray crystallography (Kilbourn and Owston, 1970).

Biological Actions: Tamoxifen is a competitive inhibitor of estrogen. It inhibits uptake of [^{3}H]estradiol into the target tissue. This inhibition is dose-related. Administration of tamoxifen (5 mg on 2 consecutive days) to mature rats prevented accumulation of [^{3}H]estradiol in target tissues (Jordan, 1976a). Similarly large doses of tamoxifen (1.5 mg on two consecutive days) administered to ovariectomized mice produced long-term vaginal refractoriness to estradiol stimulation (Emmens, 1971; Jordan, 1975b), and decreased ability of [^{3}H]estradiol to bind to uterus and vaginal tissue (Jordan, 1975b).

In women, pretreatment with tamoxifen (either 30 mg more than 36 h or 40 mg more than 48 h) before hysterectomy, and subsequent injection of [^{3}H]estradiol showed lower concentration of [^{3}H]estradiol in the uterine endometrium than those who did not receive tamoxifen pretreatment (Lunan and Green, 1974).

Tamoxifen (*Z*-isomer) binds to the estrogen receptor with a relative binding affinity of 2.0, whereas the isomer ICI 47,699 binds with ten-fold less affinity (RBA = 0.2) (Robertson *et al.*, 1982). 4′-OH-tamoxifen has a binding affinity similar to that of estradiol (Binart *et al.*, 1979; Wakeling and Slater, 1980; Coezy *et al.*, 1982). Robertson *et al.* (1982) observed an RBA of 285 for 4-OH-tamoxifen.

A study of receptor–ligand interaction kinetics for tamoxifen showed its association rate to be four times slower than estradiol whereas the dissociation rate was about 100 times faster (Borgna and Rochefort, 1979). However, this faster dissociation rate may not be the sole reason for its antiestrogenicity

since the more potent 4-OH-tamoxifen does not show this type of behaviour (Rochefort, 1979).

Tamoxifen behaves as a full estrogen agonist, a partial agonist or as an antagonist, depending upon the species studied, the tissue under investigation and the end-point measured. Tamoxifen generally acts as agonist in mice (Harper and Walpole, 1966, 1967a; Terenius, 1971; Black and Goode, 1980). However, prolonged administration to ovariectomized mice or large doses produced an antiestrogenic effect (Emmens, 1971; Lee, 1971; Jordan, 1975b). Agonistic activity of tamoxifen was also observed in guinea pigs (Furr *et al.*, 1979).

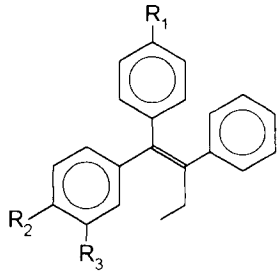
Tamoxifen behaves as partial agonist in rats (Harper and Walpole, 1967a; Marois and Marois, 1977), rabbits (Labhsetwar, 1971; Koseki *et al.*, 1977) and hamster (Furr *et al.*, 1979). Tamoxifen (Sutherland *et al.*, 1977; Baulieu *et al.*, 1981a) and 4'-OH-tamoxifen (Binart *et al.*, 1979) inhibited estrogen-stimulated growth of the oviduct in the chick and thus acted as pure antagonists. There are some controversial reports regarding action of tamoxifen on the pituitary gland of the hen, suggesting it to be estrogenic (Sommerville *et al.*, 1980) or antiestrogenic (Wilson and Cunningham, 1981), on the basis of its effect on LH release.

Tamoxifen antagonizes the effect of estrogen in amphibians (Rastogi, 1972; Rastogi and Chieffi, 1975), reptiles (Rastogi and Chieffi, 1975) and fish (Billard and Peter, 1977). In the pigtailed monkey it did not induce perineal swelling in the ovariectomized animal and also caused inhibition of swelling induced by estradiol (Furr *et al.*, 1979). Administration of tamoxifen to intact animals during the first 15–20 days of the cycle caused partial suppression of perineal swelling. This suggested antiestrogenic effect of tamoxifen in monkeys.

Metabolism: The metabolism of tamoxifen has been described in mice (Fromson *et al.*, 1973a; Wilking *et al.*, 1981, 1982), rat, dog, rhesus monkey (Fromson *et al.*, 1973a) and man (Fromson *et al.*, 1973b; Adam *et al.*, 1979, 1980; Kemp *et al.*, 1983). The metabolites characterized in these studies are shown in Table 1.

Effect of Tamoxifen on Tumour: Animal Tumour Model. Tamoxifen inhibited tumour growth in animals, particularly in estrogen-receptor-positive models. It acted directly through interaction with estrogen receptors thereby inhibiting estradiol binding (Nicholson and Golder, 1975; Powell-Jones *et al.*, 1975a,b; Jordan and Dowse, 1976; Nicholson *et al.*, 1978). It also acted on the pituitary and inhibited prolactin release. Role of prolactin in tumour growth has been identified (Pearson *et al.*, 1969; Kelly *et al.*, 1974; Leung *et al.*, 1975; Bradley *et al.*, 1976; Costlow *et al.*, 1976; Jordan *et al.*, 1980). Prolactin also increased estrogen receptor concentration (Sasaki and Leung, 1975; Vignon and Rochefort, 1976; Hawkins *et al.*, 1977).

TABLE 1
Tamoxifen metabolites



Compound	R_1	R_2	R_3
Tamoxifen	$OCH_2CH_2N(CH_3)_2$	H	H
Metabolite A[a]	$OCH_2CH_2N(CH_3)_2$	H	H
Metabolite B	$OCH_2CH_2N(CH_3)_2$	OH	H
Metabolite C	$OCH_2CH_2N(CH_3)_2$	OH	OCH_3
Metabolite D	$OCH_2CH_2N(CH_3)_2$	OH	OH
Metabolite E	OH	H	H
Metabolite X	$OCH_2CH_2NHCH_3$	H	H
Metabolite Y	OCH_2CH_2OH	H	H
Metabolite Z	$OCH_2CH_2NH_2$	H	H
Tamoxifen N-oxide	$OCH_2CH_2NO(CH_3)_2$	H	H

[a]In metabolite A water is added across the ethylenic double bond.

Tamoxifen may also act on the ovary and cause inhibition of estradiol synthesis. Thus, tamoxifen was found to be effective in carcinogen-induced (Nicholson and Golder, 1975; Manni *et al.*, 1977; Levy *et al.*, 1981) and radiation-induced (Welsch *et al.*, 1981) rat mammary tumours, mouse mammary cancer (Nohno *et al.*, 1981), a carcinogen induced rat endometrial cancer (Sekiya and Takamizawa, 1976), rat prostate cancers (Ip *et al.*, 1980; Noble, 1980a,b) and renal cancer in hamsters (Dodge, 1977).

Effect of Tamoxifen on Human Tumour Models: Tamoxifen caused inhibition of growth and cell numbers (Lippman *et al.*, 1976; Coezy *et al.*, 1982) in estrogen-responsive human breast cancer cell lines MCF-7 and CG-5. Thus, its inhibitory effect was observed in [^{3}H]thymidine incorporation (Lippman *et al.*, 1976; Lippman and Aitken, 1980), DNA polymerase activity (Edwards *et al.*, 1980) and in causing a reduction in DNA content of cultures (Horwitz and McGuire, 1978). The effect of tamoxifen produced in these cell lines is possibly through estrogen receptors present in them. Tamoxifen is ineffective in three cell lines (G.11, MDA-231 and HT-39) which do not have

estrogen receptors. However, Green *et al.* (1981) have shown inhibitory effect of tamoxifen in estrogen-receptor-negative cell line BT-20. The precise mechanism of inhibition caused by tamoxifen, therefore, is not fully understood.

Pharmacokinetic studies: On i.v. administration of tamoxifen to spayed female mice, its concentration was found to be higher in lung, liver, adrenal, kidney, pancreas, uterus, salivary glands and mammary tissue than in the blood (Wilking *et al.*, 1981, 1982). On oral administration to patients, serum tamoxifen concentration was found to be maximum after 3 h and steady serum concentration was achieved after 4 weeks (Patterson *et al.*, 1980). Half-lives of tamoxifen and a major metabolite *N*-demethylated tamoxifen, in healthy males, have been reported to be 4 and 7 days, respectively (Adam, 1981).

Toxicology:

- Acute toxicity: Tamoxifen was found to have very low acute toxicity. On oral administration LD_{50} values in mice range from 3 to 6 g kg^{-1} and in rats from 1.12 to 2.5 g kg^{-1} (Furr *et al.*, 1979; Watanabe *et al.*, 1980). LD_{50} values by i.p. route, in mice and rats, were 200 mg kg^{-1} and 600 mg kg^{-1} respectively and by i.v. route around 62.5 mg kg^{-1} in both the species.
- Chronic toxicity: In rats, at daily oral doses 2, 20 or 100 mg kg^{-1} no haematological or biological changes were observed; however, rate of growth of animals was depressed (Furr *et al.*, 1979). Histopathological changes were observed in the reproductive tract with loss around the neck in highest dose groups. The antiestrogenic effects of tamoxifen included reduction of ovarian weights, disappearance of endometrial glands in the uterus, and reduction in the number of corpora lutea. Reduction in weights of testis and accessory sex organs was observed in male rats. Histopathological changes produced on daily oral doses of 0.5 and 20.0 mg kg^{-1} for 3 months were found to be reversible.

  In mice and dogs the effects produced were those of estrogen, which is different from in humans where it acts predominantly as an antiestrogen. In the marmoset oral doses of 0.8, 4 and 8 mg kg^{-1} for 6 months produced no significant clinical or histopathological changes (Furr *et al.*, 1979).
- Teratology: Tamoxifen did not show any teratogenic effects in rats (Furr *et al.*, 1979) and rabbits (Furr *et al.*, 1979; Esaki and Sakai, 1980a,b) when administered maximum doses which did not terminate pregnancy.

Clinical Studies for Treatment of Tumours: Tamoxifen is the main drug for endocrine therapy for advanced breast cancer. Its clinical efficacy in breast cancer treatment was first demonstrated by Cole *et al.* (1971) and since then several studies have been published on its use (Patterson *et al.*, 1981; Noguchi *et al.*, 1990). In Patterson's review (Patterson *et al.*, 1981)

of 50 major studies, it was shown that tamoxifen treatment of advanced breast cancer resulted in an objective remission of disease in 33% of subjects and clinical benefit to 51% of cases. A daily dose of 20–40 mg is effective for treatment. Ward (1973) has suggested that 40 mg daily is an optimal dose. Activity of tamoxifen is mediated mainly through estrogen receptors. Accordingly, response rates increase from around 33% from an unselected group of patients to 48% for those with tumours containing estrogen receptors. However, in approximately 50% of patients with ER-positive tumours, tamoxifen treatment was found to be ineffective (Noguchi *et al.*, 1990). Many patients with ER-negative tumours showed objective remission of the disease after tamoxifen treatment. Better response rates were reported in patients with progesterone-receptor-positive tumours (Horwitz *et al.*, 1975).

Difference in effectiveness was observed between a dose of 30 mg day^{-1} and 90 mg day^{-1} (Rose *et al.*, 1982). Tamoxifen administration to premenopausal women suffering from breast cancer, produced a response rate of 31%. In postmenopausal patients the higher age group (age > 50 years) showed a better response. Response rates increase with the age of the patient (Patterson *et al.*, 1981). Patients with soft tissue disease were better responders (56%) compared with those with dominant metastases in bone (33%) and viscera (35%).

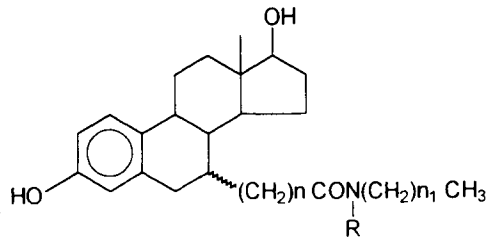
A number of combinations of tamoxifen with other endocrine therapies have been reported. These are combinations with (1) diethylstilbestrol, (2) oxyprogesterone acetate, (3) fluoxymesterone, (4) nandrolone, (5) megestrol acetate, (6) aminoglutethimide, (7) aminoglutethimide and danazol, (8) bromocriptine, and (9) prednisone (Furr and Jordan, 1984). No significant advantage was gained from these combinations.

A combination of tamoxifen with cytotoxic chemotherapy produced better results compared with tamoxifen treatment alone (Fisher *et al.*, 1981; Furr and Jordan, 1984). The combination is particularly useful when the cytotoxic chemotherapy alone fails to yield high response. An approach often practised is the initial use of tamoxifen, because of its lack of side-effects, followed by cytotoxic therapy on failure or relapse.

Tamoxifen has been used successfully in adjuvant therapy. After masectomy and radiotherapy, tamoxifen treatment given to postmenopausal women checks or delays recurrence of the disease (Palshof, 1981; Baum *et al.*, 1983; Rose *et al.*, 1983).

5.2.2 *Steroidal Antiestrogens*

Non-steroidal estrogen antagonists generally possess some agonistic activity. For this reason attempts were made to develop steroidal estrogen antagonists. Although substitutions at various carbon atoms of estradiol have been tried



136

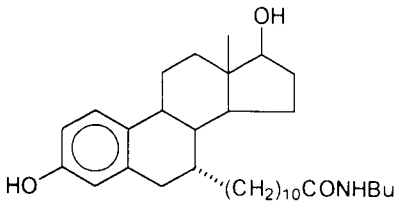
for this purpose, success was achieved in two classes of compounds namely (i) 7α-substituted and (ii) 11β-substituted estradiol derivatives, as discussed below.

5.2.2.1 7α-Substituted Estradiol Derivatives. Truong *et al.* (1973), while developing affinity chromatography agents for purification of estrogen receptors, identified that compounds with long alkyl substituents at 7α-position, retained high binding affinity to estradiol receptors.

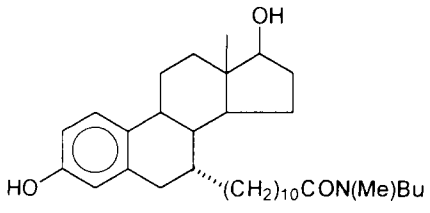
Based on the above observation, the possibility of developing estrogen antagonists by introducing appropriate groups at C-7 of estradiol was investigated by Wakeling and Bowler (1987). A number of 7-alkylamide derivatives of estradiol (**136**) were synthesized by Bowler *et al.* (1989) and evaluated for agonistic, antagonistic and tumour inhibiting activities. Structure–activity relationship studies revealed that the anti-uterotrophic activity resides exclusively in the 7α-isomer. Only a limited length of carbon chain ($n_1 = 4$–6) was required for pure antiestrogenic property. It was also observed that the amide group could be moved along the chain within certain limits ($n = 4$–6) provided that the total length was 16–18 atoms. Increases in length and bulk of *N*-substituent resulted in decreased antiestrogenicity. Branching of the butyl chain did not help in increasing activity. Introduction of an aryl substituent in the chain led to increased agonistic activity. Compounds **137–141** are some of the active members of this series.

Subcutaneous administration of ICI 163,964 (**137**) to rats produced 100% antagonistic effect at a $10\,mg\,kg^{-1}$ dose. However, oral administration showed partial agonistic effect. This was assigned to its likely metabolism to the acid. The *N*-methyl analogue ICI 164,384 (**138**) was found to be three times more potent as an estrogen antagonist when given either orally or subcutaneously.

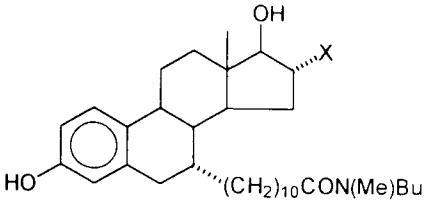
ICI 164,384 did not promote premature vaginal opening as was observed with tamoxifen. It was a more potent inhibitor of cell growth than tamoxifen in MCF-7 and ZR-75-1 breast cancer cells in tissue culture. It also inhibited growth of DMBA-induced mammary tumour (Wakeling and Bowler, 1987). Upon oral administration, ICI 164,384 was found to be less potent than tamoxifen. Ovariectomy along with ICI 164,384 treatments produced better



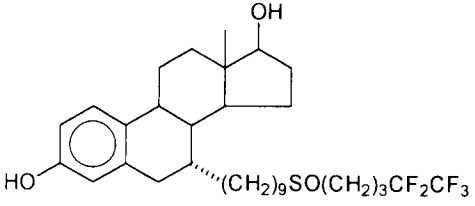
137



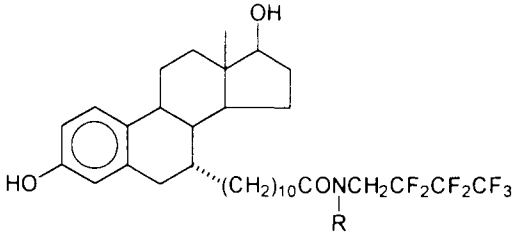
138



139



140



141

effect than tamoxifen. This could be due to absence of central antiestrogenic activity which would suppress secretion of prolactin, the hormone which controls growth of these tumours (Arafah *et al.*, 1980). However, it may be noted that prolactin does not play a major role in growth of human breast cancer (Jordan and Koerner, 1976).

The heptafluoro analogue (**141**, R = H) showed pure antagonistic activity when administered subcutaneously. The tertiary amide (**141**, R = CH_3) (ED_{50} 2 mg kg^{-1}) was three times more potent than the corresponding non-fluorinated compound (ED_{50} 6 mg kg^{-1}).

A series of 16α-halogenated compounds of 7α-undecanamide group (**139**) have been reported as pure antiestrogens (Levesquie *et al.*, 1991). At a low 3 μg dose (twice daily), the 16α-chloro compound (**139**, X = Cl) showed 74 $\pm$ 7% reversal of the stimulatory effect of estradiol-17β (0.01 μg, twice

daily) on mouse uterine weight while a complete reversal was achieved at 20 μg dose (twice daily).

The antiuterotrophic activity of the sulfoxide derivative ICI 182,780 (**140**) was found to be ten-fold greater than ICI 164,384. It had no agonistic effect and was peripherally selective in its action. It was a more effective inhibitor of MCF-7 breast cancer cell growth than 4-hydroxytamoxifen. *In vivo*, the antitumour activity of **140** was shown by xerografts of MCF-7 and Br10 human breast cancers in athymic mice. A single injection of **140** (5 mg in oil suspension) to mice inhibited tumour growth for 1 month (Wakeling and Bowler, 1992).

In a clinical trial study a long-acting formulation of **140** in castor oil was intramuscularly administered (250 mg month^{-1}) to 19 postmenopausal women with advanced breast cancer, resistant to tamoxifen therapy. Of these, seven patients responded partially, while six patients progressed in less than 8 weeks and the remaining six did not respond. No effects on the liver or the hypothalamic–pituitary axis of the patients were observed (Howell *et al.*, 1995).

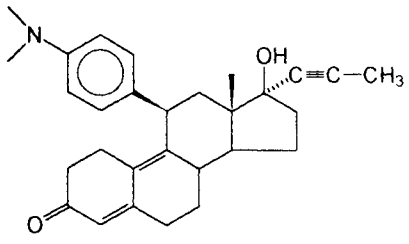
A likely mechanism of action of this alkylamide substituted estradiol derivative could be prevention of conformation change of the ligand–ER complex associated with receptor activation (Jordan, 1984).

5.2.2.2 11β-Substituted Estradiol Derivatives. The concept of developing 11β-substituted estradiol derivatives as antiestrogens by Roussel Uclaf is possibly an offshoot of the work which led to the development of RU 38,486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)estra-4,5-dien-3-one, **142**), an active antiprogestational agent (Ray and Sharma, 1987). Compounds **143–146** prepared by Roussel Uclaf are some of the potent antiestrogenic molecules.

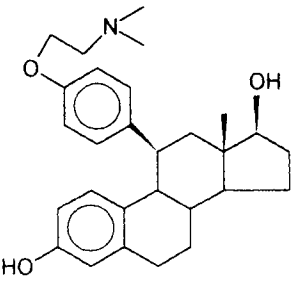
RU 3941 (3,17β-dihydroxy-11β-(4-dimethylaminoethoxyphenyl)-estra-1,3,5,(10)-triene, **143**) is a potent antiestrogenic and antiproliferative compound. However, it also displayed partial agonistic activity (Claussner *et al.*, 1992).

N-Methyl-*N*-isopropyl-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)-undecan amide (RU 51625) (**144**; R = H) and its 17α-ethynyl derivative (RU 53637) (**144**; R=C≡CH) showed significant RBA for ER and a stronger antiproliferative effect than tamoxifen *in vitro*. Both these compounds showed antitumour activity in nude mice by the percutaneous route. RU 53637 was significantly more potent than RU 51625 when given orally (Claussner *et al.*, 1992).

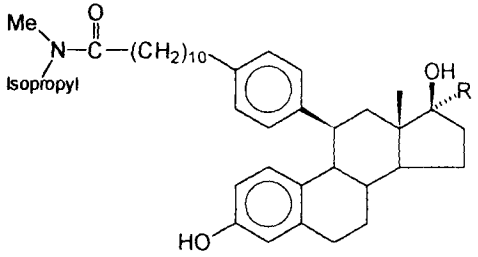
The sulfide derivative **145** exhibited potent antiproliferative effect on estrogen-positive MCF-7 cell line in different culture conditions and was of particular interest when cells were stimulated by growth factors. It inhibited E_2-induced growth of MCF-7 tumours implanted in nude mice (Nique *et al.*, 1994).



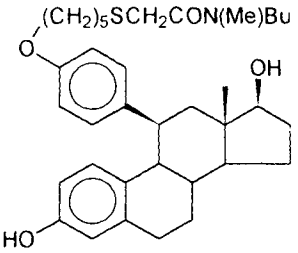
142



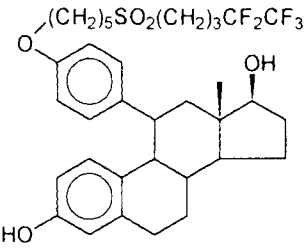
143



144



145



146

RU 58668 (11β-(4-(5-(4,4,5,5,5-pentafluoropentylsufonyl)pentaloxy)-phenyl) estra-1,3,5(10)triene-3,17β-diol), **146**) elicited pure antiuterotrophic effect in mice and rats without any agonistic activity. *In vitro*, it showed affinity for ER equivalent to that of 4-OH-tamoxifen (van de Velde *et al.*, 1994).

RU 58668 displayed potent antiproliferative activity on the MCF-7 human mammary cancer cell line stimulated either by exogenous growth factors or by paracrine interaction with non-tumoural cells, models in which tamoxifen showed poor activity. It inhibited the growth of the progesterone receptor-rich T47D cell line. *In vivo*, RU 58668 induced long-term regression of human

mammary tumours implanted in nude mice suggesting its potential use for the treatment of advanced breast cancer. It not only induced the regression of estradiol-stimulated or tamoxifen-stimulated MCF-7 tumours, but was also able to induce the regression of such tumours whose growth was not sustained by any exogenous stimulation.

A short-term toxicity study (15 days) was conducted on rats. RU 58668 was administered daily by subcutaneous route in aqueous methylcellulose suspension at doses of 20, 60 and 200 mg kg^{-1}. No serious side-effects were observed, except for nodule formation at the injection site, which could be due to poor resorption of the compound. Blood analysis showed no significant modifications of haematological parameters (Nique and Van de Velde, 1995).

6 Estrogen Antagonists in the Management of Osteoporosis

6.1 INTRODUCTION

Osteoporosis is another area where antiestrogens are emerging as very promising pharmaceuticals for the treatment of this disease. Osteoporosis is a disease characterized by low bone mass (Kanis *et al.*, 1994), micro-architectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in risk of fracture (Bouillon, 1991).

The two most common forms of osteoporosis which are associated with advancing age are (i) postmenopausal osteoporosis in women and (ii) senile osteoporosis in both men and women. The former, also called type I or high turnover osteoporosis, usually affects women after a menopause and the latter, known as type II or low turnover osteoporosis, is seen in men over 70 years of age and in women after 20 years of menopause. In both the cases, the skeletal mass shrinks, relatively faster in type I osteoporosis. A further subdivision is seen in type I group, some (35%) lose bone faster, 'fast losers' (3% per year in the first two following years) and the rest (65%) are 'normal losers' who lose only a minor amount of bone minerals (Riis, 1995).

Changes in bone metabolism during estrus cycle of rats (Whitson *et al.*, 1978) and menstrual cycle in women (Nielsen *et al.*, 1990) have been reported. Cessation of estrogen release after a menopause or on oophorectomy results in increased bone turnover. The rapid bone loss can be restored by estrogen administration (Horsman *et al.*, 1977; Lindsay *et al.*, 1988; Wronski *et al.*, 1985, 1986; Turner *et al.*, 1987; Christiansen *et al.*, 1990; Seibel *et al.*, 1993).

In contrast, in female rats, decreases in duodenal calcium transport and skeletal growth at puberty have been related to increase in circulating estrogen level (Thomas *et al.*, 1987).

The above findings would suggest a correlation between skeletal responsiveness and estrogen status. Further, the dependency of an onset of both

forms of osteoporosis on fall in levels of sex hormones, which occurs in advanced age, has been well established (Slemenda *et al.*, 1987). However, it has been found that 17β-estradiol is effective on the skeleton only in pharmacological doses and not in physiological doses, suggesting the possibility of the role of other ovarian hormones as well in bone metabolism (Turner *et al.*, 1988).

In the present context we limit our discussion to bone remodelling and osteoporosis related to estrogen status and treatment.

6.2 BONE REMODELLING

Bone is a tissue that is continuously remodelling. It is composed of both mineral (70%) and organic components (30%). The mineral, calcium, is present as hydroxyapatite crystals ($Ca_{10}(PO_4)_6(OH)_2$) and the organic material is mainly type I collagen (Ross, *et al.*, 1989).

A major part of the bone forming the human skeleton (80%) is of compact form, called cortical bones which form the shell of the bones or shaft of long bones. The other type of bone mass (20%) is spongy or cancellous, called trabecular bone, and found in vertebra, distal ends, long and flat bones. This inner mesh-work of bones is made up of thin plate-like structures. Trabecular bone has a larger surface area and is more prone to bone loss (Cummings *et al.*, 1985). Women may lose about 35% of their cortical bone and 50% of trabecular bone over their lifetimes (Riggs *et al.*, 1981; Mazess, 1982).

There are three types of bone cells involved in the remodelling process:

(1) Osteoprogenitor cells or bone lining cells: these are resting cells located on internal bone surfaces and are about to be used to produce bone.
(2) Osteoclasts: these are large, multinucleated bone-resorbing cells, present in relatively fewer numbers.
(3) Osteoblasts: these are bone-forming cells, present on the surface of the developing bones.

Osteoclasts and osteoblasts are derived from different stem cells. However, under the influence of possibly local coupling factors, their activity is highly synchronized. Bone formation takes place by the bone remodelling unit in a highly organized manner. The initiation of the bone resorption begins with an activation process in which, under the influence of bone-resorbing hormones, the lining cells which form the inactive bone cover release proteolytic enzymes such as a collagenase and plasminogen activator (Hamilton *et al.*, 1985). These enzymes expose the bone surface to osteoclastic resorption (Chambers *et al.*, 1985). Osteoclasts may also find access to bone surface through contraction of the lining cells (Shen *et al.*,

1986). Subsequently, replication of the osteoclast progenitors and their differentiation, migration and fusion under the control of local and systemic hormones (Baron *et al.*, 1986) lead to mature osteoclasts. Osteoclasts have a vacuolar-type proton pump (Blair *et al.*, 1989) which is similar to kidney H^+-ATPase. With the help of the ruffled surface it creates an acidic microenvironment which dissolves the alkaline bone mineral. Lysosomal cysteine proteinases (Ohsawa *et al.*, 1993; Tagami *et al.*, 1994) in the process, degrade bone matrix, including collagen, at a low pH (Baron *et al.*, 1985) resulting in bone resorption and formation of a cavity. It has also been shown that osteoblast-like cells, in the presence of a parathyroid hormone (PTH), release a soluble factor that stimulates osteoclastic bone resorption (McSheehy and Chambers, 1986).

After the end of the resorption process, the reversal phase starts. Osteoblast precursors are attracted to the resorbed site and, as they mature, they secrete collagen and matrix constituents known as osteoid which fill in the cavity to form new bones (Marcus, 1989). The process of new bone formation is completed by mineralization of the newly formed bone. The total time taken in this complex process is about 3–4 months and involves many local and systemic factors (Raisz, 1988) including estrogens and is not fully understood.

During growth, under normal condition, bone formation is faster than bone resorption and results in a net gain in bone mass. In a normal early adult life, the two processes are rightly balanced with bone loss fully compensated by bone gain. In the third situation when the bone formation fails to keep up with the bone loss, it leads to net loss of bone mass, known as osteoporosis or osteopenia.

In both type I and type II osteoporosis, the bone loss could be due to two different anomalies (Parfitt *et al.*, 1983): higher osteoclastic activity due to larger number of osteoclast cells with normal bone formation (Frost, 1969), or to impaired bone formation with normal or even decreased osteoclastic activity (Cummings *et al.*, 1985; Erickson *et al.*, 1985). Either mechanism may be operative, depending upon individual cases.

Generally, in osteoporotic patients there is no imbalance in the proportion of a mineral to an organic phase or any significant abnormality in the structure of either the organic matrix or the mineral phase (Robbins and Cotran, 1979).

6.3 ROLE OF ESTROGENS IN CALCIUM REGULATION AND BONE REMODELLING

The bone remodelling process largely depends on calcium regulation in the body. Calcium is one of the main constituents of the bone. Formation of the bone and its quality depend upon the supply, absorption, incorporation and withdrawal of calcium (Morris, 1993). Calcium has a protective effect on

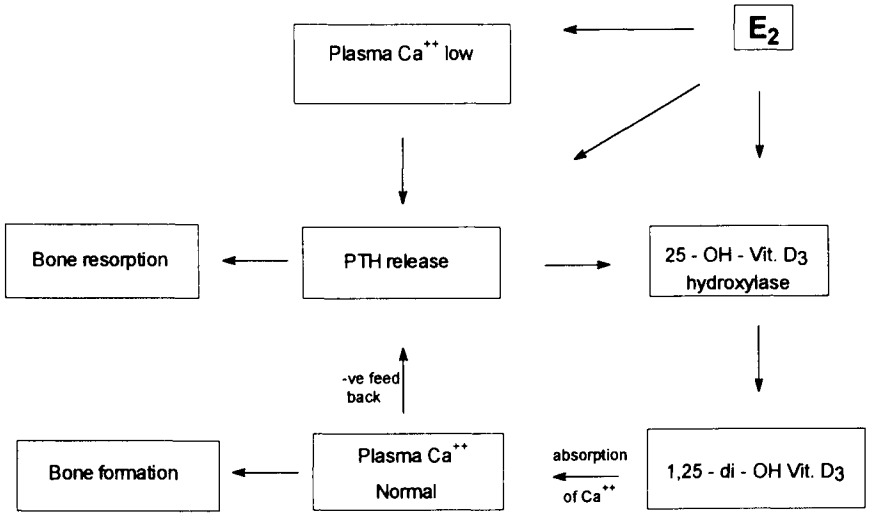


FIG. 7. Calcium regulation by estrogens.

bone. Subjects under calcium treatment have shown lower risk of hip fracture (Holbrook *et al.*, 1988); calcium inhibits bone resorption. Clinical trials with calcium supplementation have shown reduced rates of bone loss (Heaney, 1987). Calcium is more effective in senile osteoporosis, particularly in inhibiting cortical bone resorption (Rubin, 1991). It stabilizes bone loss and may improve bone mineralization and rectifies malfunctioning bone metabolism.

The regulation of calcium is governed mainly by the parathyroid hormone (PTH) and 1,25-di-OH-vitamin D_3. In the regulation process, described below (see Fig. 7), estrogen plays a significant role.

The deficiency of plasma calcium is sensed by the thyroid gland which secretes PTH, responsible for promoting bone resorption. A parathyroid hormone thus produced, simultaneously stimulates 25-OH-vitamin D_3 1-hydroxylase in the kidney to convert 25-OH-vitamin D_3 to the active metabolite 1,25-di-OH-Vitamin D_3 which helps in the absorption of calcium enteral. Elevation of plasma calcium level helps in the formation of bone and also prevents the release of PTH via a negative feedback mechanism.

In the normal course, 1,25-di-OH-vitamin D_3 is formed in the body and helps to maintain plasma calcium levels and bone formation. A decline in its level is found in old age (Omdahl *et al.*, 1982), which results in poor absorption of calcium. Calcium requirement of postmenopausal women (1400 mg day^{-1}) was found to be increased compared with premenopausal women (1000 mg d) (Heaney *et al.*, 1982). This excess requirement was due to lowered levels of 1,25-di-OH-vitamin D_3 in postmenopausal women and

consequently inefficient calcium absorption. Supplementation of 1,25-di-OH-vitamin D_3 helped in the absorption of calcium.

In the osteoporotic patients it was found that plasma levels of 1,25-di-OH-vitamin D_3 was significantly low, resulting in poor absorption of calcium. In such cases, possibly, plasma calcium concentrations were maintained through its supply from the skeleton under the influence of PTH.

Estrogens were found to stimulate formation of 1,25-di-OH-vitamin D_3 in the kidney (Castillo *et al.*, 1977) and to reduce urinary calcium excretion in postmenopausal women (Albright *et al.*, 1941), thereby maintaining calcium levels and supporting bone formation. Estrogens also influence PTH release. In the normal postmenopausal women, estrogens were shown to depress the set point of PTH stimulation by lowering of plasma calcium (Boucher, 1989) and thereby preventing bone resorption.

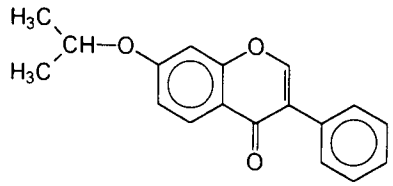
Estrogens may also have a direct effect on a bone metabolism. Estrogen receptors have been found in osteoblasts (Turner *et al.*, 1993), in some osteoblast cell lines (Eriksen *et al.*, 1988; Benz *et al.*, 1991), and in osteoclasts (Oursler *et al.*, 1991) which respond to estrogen challenge. It has also been found that avian osteoclasts produce estrogen receptor mRNA and exhibit estrogen dose-dependent inhibition of bone resorption (Eriksen *et al.*, 1987; Oursler *et al.*, 1991).

Estrogens may also interfere with bone resorption by increasing serum calcitonin concentrations (Brown *et al.*, 1980; Whitchead *et al.*, 1982; Stevenson *et al.*, 1983; Greenberg *et al.*, 1986; Gennari *et al.*, 1990). Calcitonin in turn inhibits osteoclast formation (Feldman *et al.*, 1980) and its action by moving it away from the bone surface (Kallio *et al.*, 1972) or changing its ruffled structure (Chambers and Dunn, 1983).

Whatever their mechanism, estrogens decrease bone resorption and provide long-term protection against bone loss in ovariectomized rats (Wronski *et al.*, 1991). A daily administration of 17β-estradiol (2 mg), norethisterone (1 mg) and calcium (500 mg) to postmenopausal women, led to a significant increase in bone mass of the spine and forearm (Christiansen and Riis, 1990).

6.4 ESTROGEN ANTAGONISTS

The use of estrogens for the treatment of osteoporosis is associated with a number of potential hazards. Estrogen therapy given to postmenopausal women could lead to endometrial cancer (Judd *et al.*, 1983). Hypertension, venous thrombosis and gallstone are some other important side-effects observed in patients who are on estrogen treatment (Stampfer *et al.*, 1985; Wilson *et al.*, 1985). In the recent past some antiestrogenic agents, discussed below, devoid of the above mentioned major side effects, are showing much promise in the chemotherapy of osteoporosis.



147

6.4.1 *Ipriflavone*

Ipriflavone (7-isopropoxy-3-phenyl-4H-1-benzopyran-4-one, **147**) is a derivative of the naturally occurring class of isoflavones. In postmenopausal women it did not induce LH secretion and is therefore devoid of estrogenicity (Melis *et al.*, 1992).

Ipriflavone when administered orally to rats, sheep and chickens (Yamazaki *et al.*, 1986a,b) was found to increase calcium concentrations in the bone. A decrease in the bone remodelling and prevention in bone loss was observed when ipriflavone was given to women after ovariectomy or to postmenopausal osteoporotic patients (Agnusdei *et al.*, 1989, 1992; Passeri *et al.*, 1992; Gambacciani *et al.*, 1993). Ipriflavone also protected bone loss in senile osteoporosis (Nakamura *et al.*, 1992) and reduced bone pain in osteoporotic patients (Scali *et al.*, 1991).

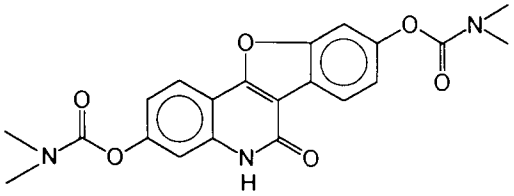
In combination with low doses of estrogens, ipriflavone effectively improved bone density which appeared to be a very satisfactory approach for treatment (Melis *et al.*, 1992).

Ipriflavone inhibits bone resorption in *in vivo* studies (Yamazaki *et al.*, 1986a,b). *In vitro* studies indicated that this inhibition could be due to direct suppression of both basal (Tsuda *et al.*, 1986) and PTH induced osteoclastic activity (Bonucci *et al.*, 1992). It has also been suggested that ipriflavone may interfere with osteoclast formation (Benvenuti *et al.*, 1991; Notoya *et al.*, 1992).

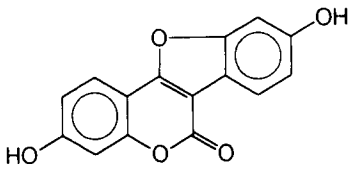
Ipriflavone in appropriate doses has been found to be safe and effective in preventing bone loss in postmenopausal osteoporosis and in acute ovarian deficient states (Brandi, 1993).

6.4.2 *KCA-098*

KCA-098 (3,9-bis (*N*,*N*-dimethyl-carbamoyloxy)benzofuro[3,2-c]quinoline-6(5H)-one, **148**) is structurally related to coumestrol (**149**), a weak estrogen derived from plant (Rabasseda. 1995). Similar to ipriflavone, KCA-098 was



148



149

found to be devoid of uterotrophic effects in rats (Tsutsumi *et al.*, 1994). It inhibits bone resorption in chicks and also increased bone formation.

Administration of KCA-098 to ovariectomized rats (3.0 mg kg^{-1} day^{-1}, p.o.) for 16 weeks caused partial reversal of the decrease in calcium and phosphorus content of femora, and complete normalization of the breaking force (Kojima *et al.*, 1994a). In rats, KCA-098 stimulated calcitonin secretion and inhibited PTH secretion stimulated by a calcium-deficient diet (Kojima *et al.*, 1994b).

6.4.3 Clomiphene

Clomiphene (2-(4-(2-chloro-1,2-diphenylethenyl)phenoxy)-*N*,*N*-diethylethanamine, **20**) is one of the very early discoveries in the area of antiestrogens. It is a mixture of two isomeric forms. Its *trans*-isomer enclomiphene shows partial estrogen agonist/antagonistic activities while the *cis*-form, zuclomiphene, is an estrogen agonist (Clark and Markaverich, 1982). It has been developed as a drug for fertility induction (see section 4.2.1).

Beall *et al.* (1984) showed that chronic administration of clomiphene citrate to ovariectomized rats prevented deterioration of femur structure and maintained total body calcium. This potential to reduce bone mineral loss without causing undesirable side-effects such as uterine hyperstimulation, has also been reported by other workers (Jordan *et al.*, 1987; Chakraborty *et al.*, 1991).

Clomiphene prevented estrogen-deficiency osteopenia and allowed lowering of blood estrogen levels and uterine shrinkage in buserelin-treated rats (Goulding *et al.*, 1992). At higher concentration it inhibited PTH-stimulated bone resorption *in vitro* (Stewart and Stern, 1986).

The mechanism of action of clomiphene is not clearly understood. Whether the estrogenic isomer mainly contributes toward its positive effect on bone is not certain.

6.4.4 Tamoxifen

Tamoxifen (2-(4-(1,2-diphenyl-1-butenyl)phenoxy)-*N*,*N*-dimethyl ethanamine, **22**) is an antiestrogen. Its effect has been found to be

species-dependent. It is an estrogen agonist in the mouse uterus (Terenius, 1971), a pure antagonist in a chicken oviduct (Sutherland *et al.*, 1977) and a mixed agonist–antagonist in the rat uterus (Harper and Walpole, 1967a; Clark *et al.*, 1973; Ferguson and Katzenellenbogen, 1977; Jordan *et al.*, 1978; Katzenellenbogen *et al.*, 1979).

The effect of tamoxifen on bone is also species-dependent. In birds, tamoxifen caused bone resorption and antagonized the inhibition of radial bone growth by estrogens and also estrogen-induced medullary bone formation. It inhibited osteoblastic activity and induced osteoclastic activity (Kusuhara and Ishida, 1986; Turner *et al.*, 1987a). It prevented bone loss in ovariectomized mice (Bronlik, 1991) and rats (Jordan *et al.*, 1987; Turner *et al.*, 1987a, 1988; Goulding and Gold, 1989, 1990; Moon *et al.*, 1991). Tamoxifen acted as a potent estrogen agonist on a rat skeleton. It prevented the increase in bone formation which results from ovarian deficiency and also prevented bone resorption at the medullary surface (Turner *et al.*, 1987b).

Similar to clomiphene, tamoxifen prevented bone loss caused by estrogen deficiency created with the LHRH agonist buserelin, used in the treatment of endometriosis, without inducing a uterotrophic effect (Goulding *et al.*, 1992). It slowed down bone loss in postmenopausal women (Love *et al.*, 1988; Turken *et al.*, 1989; Mazess *et al.*, 1990).

The mechanism of action of tamoxifen in a bone metabolism may involve a direct interaction with estrogen receptor present in bone cells (Paassen *et al.*, 1978; Gray *et al.*, 1987; Eriksen *et al.*, 1988; Komm *et al.*, 1988).

At high doses, tamoxifen inhibited PTH-stimulated bone resorption of fetal bones in culture (Stewart and Stern, 1986) and was also found to be cytotoxic to osteoclast cells (Arnett *et al.*, 1986).

A partial isomerization of the *trans*-tamoxifen to the estrogenic *cis*-form in the biophase cannot be completely ruled out, which might also explain part of its agonistic character in bone tissue.

6.4.5 Raloxifene/Keoxifene

Raloxifene (6-hydroxy-2-(4-hydroxyphenyl)benzo(b)thien-3-yl)(4-(2-(1-piperidinyl) ethoxy)phenylmethane, LY 139481, **128**) is a potent antiestrogen developed by Eli Lilly, USA. It showed high affinity for estrogen receptors (Jones *et al.*, 1979) and remarkable tissue specificity. It has been found to behave like an estrogen agonist, comparable to ethinylestradiol, in the skeleton and in lowering serum cholesterol when given orally to ovariectomized rats. Bone mineral density in the distal femur and proximal tibia was significantly greater than observed in ovariectomized control rats. However, in uterine tissue its effect was like a pure antiestrogen, more potent than tamoxifen with insignificant uterotrophic activity (Black *et al.*, 1994).

Raloxifene showed estrogen-like activity on bone measurements in growing rats. It prevented radial and longitudinal bone growth and cancellous osteopenia after ovariectomy. It did not prevent cancellous bone formation in ovariectomized rats (Evans *et al.*, 1994). Its administration effectively prevented body weight gain of female rats after ovariectomy (Jordan *et al.*, 1987). Because of its target specific activity, this compound appears to hold much promise in the treatment of postmenopausal osteoporosis.

6.4.6 Centchroman

Centchroman (*trans*-1-(2-(4-(7-methoxy-2,2-dimethyl-3-phenyl-3,4-dihydro-2H-1-benzopyran-4-yl)-phenoxy)ethyl)-pyrrolidine hydrochloride, **57**), a partial estrogen antagonist, has been introduced as a weekly oral pill for fertility regulation (see section 4.1.3) (Kamboj *et al.*, 1992). A recent finding with this drug is its potential to prevent bone loss in the ovariectomized mice (Bain *et al.*, 1993; Labroo *et al.*, 1994). Administration of centchroman to ovariectomized mice prevented decreases in cancellous bone area of the distal femur, without causing hypertrophy. Use of centchroman in osteoporosis is under evaluation.

7 **Miscellaneous**

In addition to the use of ELA mentioned under sections 2 to 6, there are many other areas where the use of ELA as a pharmaceutical may be of advantage or indicated. Some of these areas are briefly discussed below.

7.1 ANGIOGENESIS INHIBITORS

The term angiogenesis was coined by Hertig (1935). It is the generation of new blood vessels, which, in the adult, is restricted to situations including wound healing, and the formation of corpus luteum, endometrium and placenta. These conditions of normal angiogenesis are regulated in an orderly manner by angiogenesis inhibitors and stimulators. In normal angiogenesis, only 0.1% of vascular endothelial cells present in the organism proliferate (Denekamp, 1990).

Under certain pathological conditions, the process of angiogenesis is no longer orderly and controlled and results in proliferation of 10% or more of vascular endothelium cells (Denekamp, 1990). In these situations disorders may result in occurrence of diabetic retinopathy, atherosclerosis, psoriasis and growth of solid malignant tumours. Pathological angiogenesis is also seen during the rejection of the organ transplant. The aspect of pathological angiogenesis of most concern is possibly induction of solid tumours.

TABLE 2
Pathological angiogenesis

a) Overshooting angiogenesis (angiogenic diseases)	b) Insufficient angiogenesis (angiostatic diseases)
Atherosclerosis	Delayed wound healing
Solid cancers	Nonhealing fractures
Diabetic retinopathy	Hemifacial microsomia
Psoriasis	
Rheumatoid arthritis	
Retrolental fibroplasia	
Neovascular glaucoma	
Haemangioma	
Angiofibroma	

Angiogenesis is a complex multistep process which is not fully understood. Both in physiological and pathological angiogenesis, sprouting from pre-existing capillaries and venules in the endometrium takes place. The sprouts merge to form a capillary network. Differentiation of some newly formed capillaries into arterioles and venules forms a microcirculation and a three-dimensional differentiation into glandular and subepithelial capillary formation and causes vascular stroma in the tissue. Whereas the physiological angiogenesis is an orderly process which progresses to a set time schedule, pathological angiogenesis is continuous and less organized.

Folkman and Klagsbrun (1987) have classified pathological angiogenesis into two classes, as shown in Table 2. Pathological angiogenesis may result from an imbalance between angiogenic factors (Folkman and Klagsbrun, 1987) and inhibitors (Blood and Zetter, 1990; Bouck, 1990; Schweigerer and Fotis, 1992).

A number of agents (Table 3) have been identified as inhibitors of angiogenesis (Bicknell and Harris, 1991) and many more are likely to be added up.

A large number of non-steroidal and steroidal antiestrogens are effective in inhibiting growth of hormone-dependent cancer (see section 5). Whether their effectiveness is due solely to competitive blocking of estrogen receptor, has been questioned (Rochefort, 1987; Jordan and Murphy, 1990). An alternative mechanism operating at least partly could be their effect on a number of growth factors responsible for cellular proliferation (Freiss *et al.*, 1990).

In relatively recent studies (Jordan, 1990; Knabbe *et al.*, 1991) tamoxifen (**22**) was found to be effective even in estrogen-receptor-negative breast cancer. Knabbe *et al.* (1991) observed enhancement of transforming growth

TABLE 3
Inhibitors of angiogenesis

Inhibitors	References
Inferferons α and β	Sidky and Borden (1989)
Platelet factor-4	Taylor and Folkman (1982)
TIMP-1 and TIMP-2	Carmichael *et al.* (1986); Stetler-Stevenson *et al.* (1989)
Thrombospondin	Rastinejad *et al.* (1989)
Steroid/β-cyclodextrin tetradecasulphate	Folkman *et al.* (1989)
Pencillamine	Matsubara *et al.* (1989)
Vitamin D_3 analogues	Oikawa *et al.* (1990)
Herbimycin A	Oikawa *et al.* (1989)
Minocycline	Tamargo *et al.* (1991)
Fumigallin and angioinhibins	Ingber *et al.* (1990)
15-Deoxy spergualin	Oikawa *et al.* (1991)
Sulfated chitin derivatives	Murata *et al.* (1991)
Somatostatin analogues	Woltering *et al.* (1991)
Gly-Arg-Gly-Asp-Ser	Nicosia and Bonanno (1991)
Genistein	Fotsis *et al.* (1993)
Antiestrogens	Gagliardi and Collins (1993)

factor-β (TGFβ) upon tamoxifen treatment. It was suggested that TGFβ causes inhibition of tumourigenesis. TGFβ peptides were found to inhibit angiogenesis by reducing endothelial cell mitosis (Schultz and Grant, 1991). Non-steroidal estrogens were also found to inhibit protein kinase C, which also causes angiogenesis (Morris *et al.*, 1988).

Gagliardi and Collins (1993) have shown that partial estrogen antagonists, clomiphene (**20**), tamoxifen (**22**), and nafoxidine (**31**) and the pure estrogen antagonist ICI 164,384 (**138**) and ICI 182,780 (**140**) exhibit inhibition of angiogenesis in the chick egg chorioallantoic membrane. They elicit significant angiostatic activity in a dose-related manner. The addition of up to fivefold of 17β-estradiol to the disks containing clomiphene, tamoxifen or ICI 182,780 did not alter their angiostatic effect. This would suggest that their antitumour activity is exerted via a mechanism other than direct inhibition of estrogen action.

Interestingly genistein (**150**), an estrogenic compound derived from plants, was found to inhibit endothelial cell proliferation *in vitro* (Fotsis *et al.*, 1993). Genistein possibly inhibits basic fibroblast growth factor (FGF) or tyrosine kinase activities of epidermal growth factor receptor (Akiyama *et al.*, 1987). There is a possibility of involvement of other growth factors (Chodak *et al.*, 1988; Proctor *et al.*, 1991).

The fate of a large number of antiestrogenic compounds, known today as

angiogenesis inhibitors, is yet to be revealed. A structure–activity (angiogenesis inhibition) data of antiestrogens, if generated, would be of much help in designing antiestrogens for more effective treatment of tumours.

7.2 ANTI-INFLAMMATORY AGENTS

Uterotrophic effect caused by estrogens and inflammation have certain common biological aspects. In both cases, increased fluid inhibition and blood flow results in swelling of the tissue. In both of these instances, release of prostaglandins may be a common factor (Willis, 1970; Caldwell *et al.*, 1972; Saksena and Harper, 1972; Ryan *et al.*, 1974). It has been shown that a number of anti-inflammatory compounds effectively inhibit implantation (Rankin, *et al.*, 1979; Mehrotra, 1982). Similarly, anti-inflammatory activity has also been found to be associated with some potent antiestrogens which were developed as anti-implantation agents and a possible link between the two was studied (Verma *et al.*, 1989).

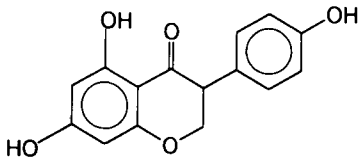
Allen and co-workers (1959) synthesized a number of amino-alkoxyphenylmethane derivatives. Of these, 9-(*p*-(β-diethylaminoethoxy)benzal)thioxanthine (**151**) and 9-(*p*-(β-diethylaminoethoxy)benzal fluorene, HCl (**152**) showed antiestrogenic, antigonadotrophic, anti-inflammatory and blood cholesterol lowering activities. Similar activities were reported by this group of 1,1,2-triphenyl-ethanol, ethylene and ethanes (**153**) in which one of the phenyl group was substituted with a basic ether moiety (Allen *et al.*, 1960).

Lerner *et al.* (1975) observed that diethylstilbestrol (**5**), clomiphene (**20**), ethamoxytriphetol (**18**) and triparamol (MER-29, **154**) were 0.18, 1.0, 0.02 and 0.01 times more potent in the *in vitro* inhibition of prostaglandin synthetase, respectively, than indomethacin (**155**). However, in *in vivo* carrageenan-induced rat paw oedema, the non-steroidal estrogen diethylstilbestrol was found to be more potent than the antiestrogenic compounds cloimiphene, MER-25 and MER-29.

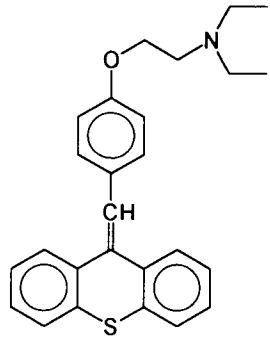
Anti-inflammatory activity was observed in some 3,4-diarylchromene and chroman derivatives substituted with a basic side-chain which imparted antiestrogenic activity to such compounds. In rats, at a dose of 80 mg kg^{-1} p.o., the chroman derivative centchroman (**57**), and two other antiestrogens, tamoxifen (**22**) and nafoxidine (**31**), showed 88%, 36% and 11% inhibition, respectively (Verma *et al.*, 1989).

A detailed study of anti-inflammatory activity of centchroman was reported by Dhawan and Srimal (1973).

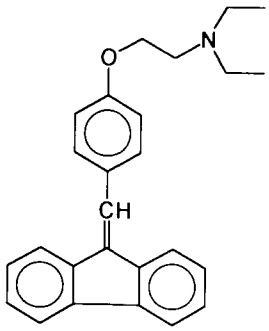
Centchroman inhibited carrageenan-induced oedema in mice with ED_{50} 96.0 mg kg^{-1} compared with 78.0 mg kg^{-1} for cortisone. In rats, the ED_{50} of centchroman was 36.0 mg kg^{-1} while the ED_{50} of cortisone was 45.0 mg kg^{-1}.



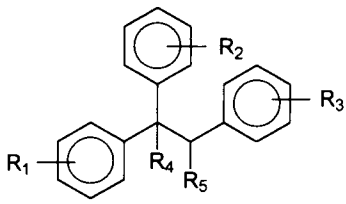
150



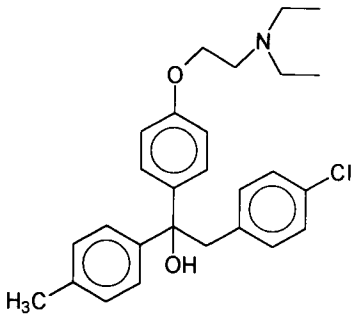
151



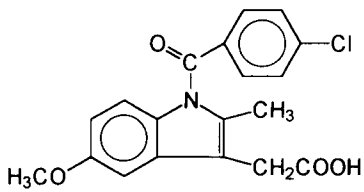
152



153



154



155

Centchroman was found to possess significant anti-inflammatory activity in acute and chronic models of inflammation. The specific inhibition by centchroman of bradykinin-induced spasm of the guinea-pig bronchial tree was similar to that of phenylbutazone. Centchroman had a lower ulcerogenic index (0.8) than phenylbutazone, hence fewer chances of gastric irritation. It did not cause leucopenia and eosinopenia as produced by phenylbutazone.

The anti-inflammatory activity of centchroman is not mediated via the pituitary–adrenal axis but results possibly from a direct effect on the inflamed tissue. This anti-inflammatory property of centchroman, which is in phase III clinical trials for the treatment of advanced breast cancer, possibly produces a partial relief to the patient.

7.3 ENDOMETRIOSIS

Endometriosis is a disorder that leads to the presence of functioning endometrial tissue in various sites throughout the pelvis or in the abdominal wall. In the treatment of endometriosis, LHRH agonists are administered. This leads to hypoestrogenism which results in osteoporosis (Devogelaer *et al.*, 1987; Matta *et al.*, 1987; Steingold *et al.*, 1987; Dowsett *et al.*, 1988; Franssen *et al.*, 1989; Scharla *et al.*, 1990). Estrogen administration to such cases causes excessive endometrial growth. This could be prevented by giving LHRH agonist buserelin along with tamoxifen, which mimics the actions of estradiol on the skeleton without producing uterotrophic effect (Jordan *et al.*, 1987; Turner *et al.*, 1987a, 1988).

Other antiestrogens which would prevent the onset of osteoporosis without causing uterotrophic effect hold much promise in this area.

7.4 HYPOLIPIDAEMIC AGENTS

Susceptibility to premature coronary heart disease has a direct relationship to increased serum levels of cholesterol and low-density and very-low-density lipoproteins (VLDL). The role of estrogens in inhibiting hyperlipidaemia has been studied by various groups. In pharmacological doses, estrogen administration to rats affected marked hypocholesterolemia (Fewster *et al.*, 1967; Aftergood *et al.*, 1968). Different results have been reported on use of estrogens administered in various doses on serum triglyceride concentrations. Some studies reported increases in concentrations (Kim and Kalkhoff, 1975; Weinstein *et al.*, 1975), whereas others reported no change (Aftergood *et al.*, 1968). Davis and Roheim (1978) showed that estrogen-treated rats have severe hypolipidaemia characterized by nearly complete absence of cholesterol and triglyceride as well as apolipoproteins.

A number of antiestrogens possess hypolipidemic activity. Aminoalkoxyphenyl methane derivatives (Allen *et al.*, 1959) such as 9-(*p*-(β-diethylaminoethoxy) benzal)thioxanthine (**151**) and 9-(*p*-(β-diethylaminoethoxy)benzal fluorene, HCl, **152**) elicited blood cholesterol lowering potential along with other activities. Antiestrogenic 1,1,2-triarylethanol, ethylene and ethane derivatives (**153**) also possessed cholesterol lowering activity.

Recently the antiestrogenic compound droloxifene (**119**) and its derivatives have been found to be useful in lowering elevated plasma triglycerides, cholesterol and lipoproteins for the treatment of atherosclerosis and as cardioprotectants (Denecke, 1994).

The mechanism of action of estrogens in causing hypolipidaemia includes decreased hepatic secretion of VLDL (Kim and Kalkhoff, 1975; Weinstein *et al.*, 1975a). It was found that estradiol inhibited the specific activity of a hepatic glyceride transacylase involved in triglyceride synthesis. Effectiveness of antiestrogens as hypolipidemic agents could be due to their agonistic component.

The use of estrogens in hyperlipidaemia produces unwanted side-effects such as thromboembolic disorders, etc. An antiestrogenic compound which would specifically inhibit hyperlipidaemia, without the side-effects of estrogens, would be of interest.

8 Conclusion

Estrogenicity lowering agents are gaining increasing importance in various pharmaceutical areas. Their use is indicated where the presence of estrogens is the cause of the ailment or their selective biological manifestation is needed. Compounds may be developed that inhibit estrogen synthesis. However, since estrogen is involved in many ways with normal human physiology, inhibition of estrogen synthesis may lead to complications and its use may be restricted to extreme diseases such as cancer.

Estrogen antagonists, or competitive inhibitors of estrogens, generally have both agonistic and antagonistic properties. The ratio of the two activities is crucial for their different requirements as pharmaceuticals. Based on ligand–receptor interaction studies, structural requirements for estrogen agonist activity have been worked out. Compounds resembling estradiol with appropriately positioned receptor-binding parameters can be designed to have potent estrogenic activity.

The antiestrogenic property of a compound arises when an additional group, usually a ω-substituted aminoalkoxy unit, is attached onto an estrogenic ligand at an appropriate place. These antiestrogenic units do not have an independent identity as antiestrogens. Therefore, it is not possible to develop a pure antiestrogen using such basic units alone. Their binding to the estrogen receptor is primarily due to the presence of the estrogenic subunit; because of this part of the molecular component, such compounds may elicit partly agonistic activity which the antiestrogenic subunit is unable to completely inhibit. Thus, with the present knowledge it remains a matter of speculation and trial to design molecules with the desired estrogenic and antiestrogenic profile for a particular end use.

The list of estrogenicity lowering agents, presently in the clinic for various

pharmaceutical uses, is rather short. However, a large number of them are under different developmental stages. It is conceivable that in the near future ELA will gain pharmaceutical significance.

Acknowledgement

We are thankful to Dr A. P. Bhaduri for initiating this report.

References

Adam, H. K. (1981). *In* 'Chemotherapeutic effects of "Nolvadex" (Tamoxifen Citrate)', pp. 29–34. Custom Commun. Postgrad. Med. McGraw Hill, New York.

Adam, H. K., Douglas, E. J. and Kemp, J. V. (1979). *Biochem. Pharmacol.* **27**, 145–147.

Adam, H. K., Patterson, J. S. and Kemp, J. V. (1980). *Cancer Treat. Rep.* **64**, 761–764.

Adams, R., Mishell, D. R., Jr and Israel, R. (1971). *Obstet. Gynecol.* **39**, 562–566.

Aftergood, L., Hernandez, H. J. and Alfin-Slater, R. B. (1968). *J. Lipid Res.* **9**, 447–452.

Agnusdei, D., Zacchei, F., Bigazzi, S., Cepollar, C., Nardi, P., Montagna, M. and Gennarie, C. (1989). *Drugs Expt. Clin. Res.* **XV**, 97–104.

Agnusdei, D., Camporeale, A., Zacchei, F., Gennari, C., Baroni, M. C., Costi, D., Biondi, M., Passeri, A. and Sbrenndi, C. (1992). *Curr. Ther. Res.* **51**, 82–91.

Akhtar, M. and Skinner, S. J. M. (1968). *Biochem. J.* **109**, 318–321.

Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. (1987). *J. Biol. Chem.* **262**, 5592–5595.

Albright, F., Smith, P. H. and Richardson, A. M. (1941). *JAMA* **116**, 2465–2474.

Allen, R. E., Palopoli, F. P., Schumann, E. L., Day, W. C. and Feil, V. J. (1959). US Pat. No. 2,891,957, June 23, 1959.

Allen, R. E., Palopoli, F. P., Schumann, E. L. and van Campen, M. G., Jr (1960). US Pat. No. 2,914,561,4 Nov. 24, 1959. *Chem. Abstr.* **54**, 5581 e.

Anand, N. and Ray, S. (1977). *Ind. J. Exp. Biol.* **15**, 1142–1143.

Arafah, B. M., Manni, A. and Pearson, O. H. (1980). *Endocrinology* **107**, 1364–1369.

Aref, I., Gottschewski, G. H. M. and Hafez, E. S. E. (1974). *Contraception* **10**, 291–298.

Arnett, R., Lindsay, R. and Dempster, D. W. (1986). *J. Bone Mineral Res.* **1**(Suppl. 1), 99 (Abstract).

Aronica, S. M. and Katzenellenbogen, B. S. (1993). *Mol. Endocrinol.* **7**, 743–752.

Asch, R. H. and Greenblatt, R. B. (1976). *J. Reprod. Med.* **17**, 175–180.

Asthana, O. P., Gupta, S. N., Kapur, B. M. L., Khanna, N. N., Lalitha, N., Mittal, V. K., Mishra, N. C., Krishnan Nair, Mj. and Kamboj, V. P. (1994). Cancer Congr. Abstr. No. PSBR. New Delhi, India. Oct. 30–Nov. 5, pp. 3–13.

Auger, S., Merand, Y., Pelletier, J. D., Poirier, D. and Labrie, F. (1995). *J. Steroid Biochem. Molec. Biol.* **52**, 547–565.

Bain, S. D., Celino, D. L., Lantry, M., Strachan, M. I., Edwards, M. W., Piggott, J. R. and Labroo, V. M. (1993). Ann. Meeting Am. Soc. Bone & Mineral Society Sept. 18–21, Tampa, FL.

Baron, R., Neff, L., Louvard, D. and Courtoy, P. J. (1985). *J. Cell Biol.* **101**, 2210–2222.

Baron, R., Neff, L., Van, P. T., Nefussi, J. R. and Vignery, A. (1986). *Am. J. Pathol.* **122**, 363–378.

Barone, R. M., Shamohki, I. M., Siiteri, P. K. and Judd, H. L. (1979). *J. Clin. Endocr. Metab.* **49**, 672–676.

Baulieu, E. E., Binart, N., Calelli, M. G., Geynet, C., Lebeau, M. C., Mester, J. and Sutherland, R. (1981a). *Rev. Endocrine-related Cancer Suppl.* **9**, 185–195.

Baulieu, E. E., Mortel, R., Levy, C., Namer, M. and Robel, P. (1981b). *Excerpta Med.* **551**, 234–249.

Baum, M., the Nolvadex Adjuvant Trial Organisation (1983). *Lancet* 257–261.

Beall, P. T., Misra, L. K., Young, R. L., Spjut, H. J., Evans, H. J. and LeBlanc A. (1984). *Calcif. Tissue Int.* **36**, 123–125.

Beatson, G. T. (1896). *Lancet* **ii**, 104–162.

Bedford, G. R. and Richardson, D. N. (1966). *Nature* **212**, 733–734.

Bednarski, P. J., Porubek, D. J. and Nelson, S. D. (1985). *J. Med. Chem.* **28**, 775–779.

Behrman, S. J. and Kistner, R. W. (1975). *In* 'Progress in Infertility', 2nd edn (Behrman, S. J. and Kistner, R. W., eds). Little, Brown & Co., Boston, MA.

Bencze, W. L., Carney, R. W. J., Barsky, L. I., Renzi, A. A., Dorfman, L. and DeStevens, G. (1965). *Experientia* **21**, 261.

Bencze, W. L., Barsky, L. I., Carney, R. W. J., Renzi, A. A. and DeStevens, G. (1967). *J. Med. Chem.* **10**, 138–144.

Beniahmad, A. and Tsai, M. J. (1993). *J. Cell Biochem.* **51**, 151–156.

Benvenuti, S., Tanini, A., Frediani, U., Bianchi, S., Masi, L., Casano, R., Bufaalino, L., Serio, M. and Brandi, M. L. (1991). *J. Bone Min. Res.* **6**, 987–996.

Benz, D. J., Haussler, M. R. and Komm, B. S. (1991). *J. Bone Min. Res.* **6**, 531–541.

Bhatnagar, A. S., Hausler, A., Schieweck, K., Lang, M. and Bowman, R. (1990). *J. Steroid Biochem. Molec. Bio.* **37**, 1021–1027.

Bicknell, R. and Harris, A. L. (1991). *Eur. J. Cancer* **27**, 781–785.

Biggers, J. D. and Claringbold, P. J. (1954). *J. Endocrinol.* **11**, 277–284.

Billard, R. and Peter, R. E. (1977). *Gen. Comp. Endocr.* **32**, 213–220.

Binart, N., Catelli, M. H., Greynot, C., Puri, V., Hahnel, R., Mester, J. and Baulieu, E. E. (1979). *Biochem. Biophys. Res. Commun.* **91**, 812–818.

Black, L. J. and Goode, R. L. (1980). *Life Sci.* **26**, 1453–1458.

Black, L. J., Jones, C. D. and Falcone, J. F. (1983). *Life Sci.* **32**, 1031–1036.

Black, L. J., Sato, M., Rowley, E. R., Magee, D. E., Bekele, A., Williams, D. C., Cullinan, G. J., Bendele, R., Kauffman, R. F., Bensch, W. R., Frolik, C. A., Termine, J. D. and Bryant, H. U. (1994). *J. Clin. Invest.* **93**, 63–69.

Blair, H. C., Teitelbaum, S. L., Ghiselli, R. and Gluck, S. (1989). *Science* **245**, 855–857.

Blood, C. H. and Zetter, B. R. (1990). *Biochim. Biophys. Acta Rev. Cancer* **1032**, 89–118.

Bloom, H. J. C., Roe, F. J. C. and Mitchley, B. C. V. (1967). *Cancer* **20**, 2118–2124.

Bocquel, M. T., Kumar, V., Stricker, C., Chambon, P. and Gronemeyer, H. (1989). *Nucl. Acids Res.* **17**, 2581–2595.

Boncher, A., D'Amour, P., Hamel, L., Fugere, P., Gascon-Barre, M., Lepage, R. and Ste-Marie, L. G. (1989). *J. Clin. Endocrinol. Metab.* **68**, 831–836.

Bonucci, I., Ballanti, P., Martelli, A., Mereto, E., Brambilla, G., Bianco, P. and Bufalino, L. (1992). *Calcif. Tissue Int.* **50**, 314–319.

Borgna, J. L. and Rochefort, H. (1979). *Cr. hebd. Seanc. Acad. Sci. Paris* **287**, 1141–1144.

Bouck, N. P. (1990). *Cancer Cells* **2**, 179–185.

Boue, J. G. and Boue, A. (1973). *Lancet* **i**, 679–680.

Bouillon, R. J., Burckhardt, P., Christiansen, C., Fleisch, H. A., Fujita, T., Gennari, C., Martin, T. J., Mazzuoli, G., Melton, L. J., Ringe, J. D., Riis, P., Peck, W. A., Samsioe, G. and Shulman, L. E. (1991). *Am. J. Med.* **90**, 107–110.

Bowler, J., Lilley, T. J., Pittam, J. D. and Wakeling, A. E. (1989). *Steroids* **54**, 71–99.

Boyns, A. R. and Groom, G. V. (1972). *In* Proc. of a Workshop ICI-46474-Work in Progress, pp. 35–39. ICI Pharmaceuticals Division plc, Macclesfield.

Bradley, C. J., Kledzik, G. S. and Meites, J. (1976). *Cancer Res.* **36**, 319–324.

Brandi, M. L. (1993). *Amer. J. Med.* **95**, 5A-69S.

Brodie, A. M. H. (1994). *J. Steroid Biochem. Molec. Biol.* **49**, 281–287.
Brodie, A. M. H., William, C. S., Abubakar, A. S. and Harry, J. B. (1977). *Endocrinology* **100**, 1684–1695.
Brodie, A. M. H., Marsh, D. and Brodie, H. J. (1979). *J. Steroid Biochem.* **10**, 423–429.
Brodie, A. M. H., Wesley, M. G., James, R. H., Chon-Hwa Tsai-Morris, Patrick, A. M. and Cecil, H. R. (1981). *Steroids* **38**, 693–702.
Brodie, A. M. H., Brodie, H. J., Garrett, W. M., Hendrickson, J. R., Marsh, D. A. and Tsai-Morris, C. H. (1982). *Biochem. Pharmacol.* **31**, 2017–2023.
Brodie, A. M. H. Coombes, R. C. and Dowsett, M. (1987). *J. Steroid Biochem.* **27**, 899–903.
Bronlik, P. D. (1991). *Endocrine Regul.* **25**, 217–219.
Brown, E. M., Gardner, D. G., Aurbach, G. D. (1980). *Endocrinology* **106**, 133–138.
Brueggemeier, R. W., Floyd, E. E. and Counsell, R. E. (1978). *J. Med. Chem.* **21**, 1007–1011.
Brueggemeier, R. W., Li, P. K., Chen, H. H., Moh, P. P. and Katlic, N. E. (1990). *J. Steroid. Biochem. Molec. Biol.* **37**, 379–385.
Burkhart, J. P., Weintraub, P. M., Wright, C. L. and Johnston, J. O. (1985). *Steroids* **45**, 357–374.
Buzdar, A. U., Kau, S., Hortobagyi, G. N., Theriault, R. L., Booser, D., Holmes, F. A., Walters, R., Krakoff, I. H. (1994). *Cancer Chemother. Pharmacol.* **33**, 313.
Buzzetti, F., Di Salle, E., Longo, A., Briatico, G. (1993). *Steroids* **58**, 527–532.
Caldwell, B. V., Tillson, S. A., Brock, W. A. and Speroff, L. (1972). *Prostaglandins* **1**, 217–228.
Callantine, M. R., Humphrey, R. R., Lee, S. L., Windsor, B. L., Schottin, N. H., O'Brien, O. P. (1966). *Endocrinology* **79**, 153–167.
Camacho, A. M., Cash, R., Brough, A. J., Wilroy, R. S. (1967). *J. Am. Med. Assoc.* **202**, 20–26.
Campbell, N. R., Dodds, E. C., Lawson, W. (1938). *Nature* **142**, 1121.
Carmichael, D. F., Sommer, A., Thompson, R. C. *et al.* (1986). *Proc. Natl Acad. Sci. USA* **83**, 2407–2411.
Carney, R. W. J., Bencze, W. L., Wojtkunski, J., Renzi, A. A., Dorfman, L. and DeStevens, G. (1966). *J. Med. Chem.* **9**, 516–520.
Castillo, L., Tanaka, Y., Deluca, H. F., Sunde, M. L. (1977) *Arch. Biochem. Biophys.* **179**, 211–217.
Chak, I. M., Dura, P. R., Kar, K., Srimal, R. C., Dhawan, B. N. (1977). *Ind. J. Exp. Biol.* **15**, 1159–1167.
Chakraborti, P. K., Brown, J. L., Ruff, C. B., Nelson, M. F. and Mitchell, A. S. (1991). *J. Steroid Biochem. Molec. Biol.* **40**, 725–729.
Chamber, T. J. and Dunn, C. J. (1983). *Calcif. Tissue Int.* **35**, 566–579.
Chambers, T. J., Darby, J. A., Fuller, K. (1985). *Cell Tissue Res.* **241**, 671–675.
Chang, M. C. (1959). *Endocrinology* **65**, 339–342.
Chang, M. C. (1964) *Fert. Steril.* **15**, 97–106.
Chang, M. C. and Yanagimachi, R. (1965). *Fert. Steril.* **16**, 281–291.
Charles, D., Turner, J. H. and Redmond, C. (1973). *J. Obstet. Gynaec. Br. Commonw.* **80**, 264–270.
Chawla, H. P. S., Grover, P. K., Anand, N., Kamboj, V. P. and Kar, A. B. (1970). *J. Med. Chem.* **13**, 54–59.
Childers, W. E., Silverton, J. V., Kellis, J. T., Jr, Vickery, L. E. and Robinson, C. H. (1991). *J. Med. Chem.* **34**, 1344–1349.
Chodak, G. W., Hospelhorn, V., Judge, S. M., Mayforth, R., Koeppen, H. and Sasse, J. (1988). *Cancer Res.* **48**, 2083–2088.
Christiansen, C., Riis, B. J. (1990). *J. Clin. Endocrinol. Metab.* **71**, 836–841.

Christiansen, C., Christiansen, M. S., McNair, P., Hagen K-Estocklund, C. and Transbol, I. (1990). *Eur. J. Clin. Invest.* **10**, 273–279.
Clark, J. H. and Markaverich, B. M. (1982). *Pharmac. Ther.* **15**, 467–519.
Clark, J. H., Anderson, J. N., Peck, E. J., Jr (1973). *Steroids* **22**, 707–718.
Claussner, A., Nedelec, L., Nique, F., Philibert, D., Teutsch, G. and Vande Velde, P. (1992). *J. Steroid Biochem. Molec. Biol.* **41**, 609–614.
Clitheroe, J. H. and Bonny Castle, D. D. (1966). *Fed. Proc.* **25**, 252.
Coezy, E., Borgna, J. L. and Rochefort, H. (1982). *Cancer Res.* **42**, 317–323.
Cole, M. P., Jones, C. T. A. and Todd, I. D. H. (1971). *Br. J. Cancer* **25**, 270–275.
Cole, P. A. and Robinson, C. H. (1988). *J. Am. Chem. Soc.* **110**, 1284–1285.
Cook, J. W., Dodds, E. C., Hewett, C. L. (1933). *Nature* **131**, 56–57.
Cooke, I. D. (1976). WHO Symposium on Advances in Fertility Regulation, 16–19 Nov., Moscow, USSR, pp. 88–110.
Coombes, R. C., Powles, T. J., Rees, L. H., Ratchiffe, W. A., Nash, A. G., Henk, M., Ford, H. T., Gazet, J. C. and Neville, A. M. (1982). *Br. J. Cancer* **46**, 30–34.
Coombes, R. C., Gross, P., Dowsett, M., Gazet, J. C., Brodie, A. (1984). *Lancet* **ii**, 1237–1239.
Cooper, A. P. (1836) *In* 'The Principles and Practice of Surgery' Vol. 1, pp. 333–335. E. Cox, London.
Copland, J. A., Hendry, L. B., Chu, C. K., Wood, J. C., Wrenn, R. W., Pantazis, C. G. and Mahesh, V. B. (1993). *J. Steroid Biochem. Molec. Biol.* **46**, 451–462.
Costlow, M. L., Buschow, R. A. and McGuire, W. L. (1976). *Cancer Res.* **36**, 3324–3329.
Courtney, K. D. and Valerio, D. A. (1968). *Teratology* **1**, 163–172.
Covey, D. F. and Hood, W. F. (1981). *Endocrinology* **108**, 1597–1599.
Covey, D. F., Hood, W. F. and Parikh, V. D. (1981). *J. Biol. Chem.* **256**, 1076–1079.
Cox, L. W. (1975). *Br. J. Obstet. Gynaec.* **82**, 2–6.
Craft, I. L., Foss, G. L., Warren, R. J. and Fotherby, K. (1975). *Contraception* **12**, 589–598.
Crenshaw, R. R. and Witt, D. (1967). U.S. Pat. No. 3,332,956, patented July 25, 1967.
Cummings, S. R., Kelsey, J. C., Neviti, M. C. and O'Dowd, K. J. (1985). *Epidemiology Rev.* **7**, 178–208.
Curtis, S. W. and Korach, K. S. (1990). *Mol. Endocrinol.* **4**, 276–286.
Czygan, P. J. and Schulz, K. D. S. (1972). *Gynec. Invest.* **3**, 126–134.
Da Lage, C. (1966). *In* 'European Review of Endocrinology' (Jayler, M. F., ed.) Suppl. 2 Part 1, p. 79. Pergamon, Oxford.
Darby, M. V., Lovett, J. A., Brueggemeier, R. W., Groziak, M. P. and Counsell, R. E. (1985). *J. Med. Chem.* **28**, 803–807.
Davis, R. A. and Roheim, P. S. (1978). *Atherosclerosis (Shannon)* **30**, 293–299.
Daxenbichler, G., Margreiter, R. and Dapunt, O. (1979). *Cancer Treat. Rep.* **63**, 1213.
Day, B. W., Magarian, R. A., Jain, P. T., Pento, J. T., Mousissian, G. K. and Meyer, K. L. (1991). *J. Med. Chem.* **34**, 842–851.
Delaisi, C., Doucet, B., Hartmann, C., Tric B., Gourvest J. F. and Lesuisse, D. (1992). *J. Steroid Biochem. Molec. Biol.* **41**, 773–777.
Denecke, R. (1994). Ger. Offen DE 4,401,554, Aug. 18, 1994; *Chem. Abstr.* **121**, 222020 n.
Denekamp, J. (1990). *Cancer Metas. Rev.* **9**, 267–282.
Devogelaer, J. P., Dedeuxch, C. N., Donnez, J. and Thomas, K. (1987). *Lancet* **i**, 1498–1499.
DeWald, H. A., Bird, O. D., Rodney, G., Kaump, D. H. and Black, M. L. (1966). *Nature* **211**, 538–539.
Dhawan, B. N. and Srimal, R. C. (1973). *Brit. J. Pharmacol.* **49**, 64–73.
Di Salle, E., Giudici, D., Ornati, G., Briatico, G., D'Alessio, R., Villa, V. and Lombardi, P. (1990). *J. Steroid Biochem. Molec. Biol.* **37**, 369–374.

Di Salle, E., Briatico, G., Gindici, D., Ornati, G., Zaccheu, T., Buzzetti, F., Nesi, M. and Panzeri, A. (1994). *J. Steroid Biochem. Mol. Biol.* **49**, 289–294.
Diener, R. M. and Hsu, B. Y. D. (1967). *Toxic Appl. Pharmacol.* **10**, 565–576.
Dikshit, D. K., Munshi, K. L., Kapil, R. S., Kamboj, V. P. and Anand, N. (1974). *Ind. J. Chem.* **12**, 1144–1146.
Docke, F. (1971). *J. Reprod. Fert.* **24**, 45–54.
Dodds, E. C., Goldberg, L., Lawson, W. and Robinson, R. (1938a). *Nature* **142**, 34.
Dodds, E. C., Goldberg, L., Lawson, W. and Robinson, R. (1938b). *Nature* **141**, 247–248.
Dodge, A. H. (1977). *Eur. J. Cancer Clin. Oncol.* **13**, 1377–1387.
Dony, J. M., Smalb, A. G., Rolland, R., Fauser, B. C. and Thomas, C. M. (1985). *Fertil. Steril.* **43**, 787–792.
Dowsett, M., Cantwell, B., Lal, A., Jeffcoate, S. L. and Harris, A. L. (1988). *J. Clin. Endocrinol. Metab.* **66**, 672–677.
Drake, I. S., Tredway, D. R., Buchanan, G. C. and Grunert, G. H. (1978). *Fertil. Steril.* **30**, 274–277.
Duncan, G. W., Stucki, J. C., Lyster, S. C. and Lednicer, D. (1962). *Proc. Soc. Exp. Biol. Med.* **109**, 163–166.
Duncan, G. W., Lyster, S. C., Clark, J. J. and Lednicer, D. (1963). *Proc. Soc. Exp. Biol. Med.* **112**, 439–442.
Durani, S., Agarwal, A., Saxena, R., Setty, B. S., Gupta, R. C., Kole, P. L., Ray, S. and Anand, N. (1979). *J. Steroid Biochem.* **11**, 67–77.
Edwards, D. P., Murthy, S. R. and McGuire, W. L. (1979). *Biochem. Biophys. Acta* **560**, 457–486.
Edwards, D. P., Murthy, S. R. and McGuire, W. L. (1980). *Cancer Res.* **40**, 1722–1726.
El-Sheikha, Z., Klopper, A. and Beck, J. S. (1972). *Clin. Endocrol.* **1**, 275–282.
Emmens, C. W. and Cox, R. I. (1958). *J. Endocrinol.* **17**, 265–271.
Emmens, C. W. (1965). *Acta Endocrinol.* **49**, 83–89.
Emmens, C. W. (1971). *J. Reprod. Fertil.* **26**, 176–182.
Engel, J., Hartman, R. W., Schonenberger, H. (1983). *Drugs Future* **8**, 413–419.
Eriksen, E. F., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C. and Riggs, B. L. (1987). *J. Bone Min. Res.* **2**(Suppl. 1), 238.
Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C. and Riggs, B. L. (1988). *Science* **241**, 84–86.
Erikson, E. F., Mosekil de, L. and Melsen, F. (1985). *Bone* **6**, 141–146.
Ernst, S., Hite, G., Cantrell, J. S., Richardson, A., Jr and Benson, H. D. (1976). *J. Pharm. Sci.* **65**, 148–150.
Esaki, K. and Sakai, Y. (1980a, 1980b). *Preclin. Rep. Cent. Inst. Exp. Anim.* **6**, 217–231, 233–238.
European Breast Cancer Group, EORTC (1972). *Eur. J. Cancer* **8**, 387–389.
Evans, G., Bryant, H. U., Magee, D., Sato, M. and Turner, R. T. (1994). *Endocrinology* **134**, 2283–2288.
Evans, R. M. (1988). *Science* **240**, 889–895.
Fawel, S. E. and Lees, J. A. (1990). *Cell* **60**, 953–962.
Feldman, R. S., Krieger, N. S. and Tashjian, A. J., Jr (1980). *Endocrinology* **107**, 1137–43.
Ferguson, E. R. and Katzenellenbogen, B. S. (1977). *Endocrinology* **100**, 1242–1251.
Fewster, M. E., Pirrie, R. E. and Turner, D. A. (1967). *Endocrinology* **80**, 263–271.
Fisher, B., Redmond, C., Brown, A., Wolmark, N., Wittliff, J., Fisher, E. R., Plotkin, D., Bowman, D., Sachs, S., Wolter, J., Frelick, R. and Desser, R. (1981). *N. Engl. J. Med.* **305**, 1–6.
Fishman, J., Guzik, H. and Dixon, D. (1968). *Biochem.* **8**, 4304–4309.
Flerko, B. (1957). *Arch. Anal. Microse. Morphol. Exp.* **46**, 159.
Flickinger, C. J. (1977). *Am. J. Anat.* **149**, 533–562.

Folkman, J. and Klagsbrun, M. (1987). *Science* **235**, 442–447.
Folkman, J., Weisz, P. B., Joullie, M. M., Li, W. and Ewing, W R. (1989). *Science* **243**, 1490–1493.
Foster, A. B., Jarman, M., Leung, C. H., Rowlands, M. G., Taylor, G. N., Plevey, R. G. and Sampson, P. (1985). *J. Med. Chem.* **28**, 200–204.
Fotsis, T., Pepper, M., Adlercruetz, H., Fleischmann, G., Hase, T., Montesano, R. and Schweigerera, L. (1993). *Proc. Natl Acad. Sci. USA* **90**, 2690–2694.
Franssen, A. M. H. W., Kauer, F. M., Chadha, D. R., Zijlstra, J. A., Rolland, R. (1989). *Fertil. Steril.* **51**, 401–408.
Fransworth, N. R., Bingel, A. S., Cordell, G. A., Grane, F. A. and Fong, H. H. S. (1975a). *J. Pharma. Sci.* **64**, 535–598.
Fransworth, N. R., Bingel, A. S., Cordell, G. A., Crane, F. A. and Fong, H. H. S. (1975b). *J. Pharma. Sci.* **64**, 717–754.
Freiss, G., Prebois, C., Rochefort, H. and Vignon, F. (1990). *J. Steroid Biochem. Mol. Biol.* **37**, 777–781.
Fritsche, H. and Bunsenges, B. (1964). *Phys. Chem.* **68**, 459.
Fromson, J. M., Pearson, S. and Bramah, S. (1973a). *Xenobiotica* **3**, 693–709.
Fromson, J. M., Pearson, S. and Bramah, S. (1973b). *Xenobiotica* **3**, 711–714.
Frost, H. M. (1969). *Calcif. Tiss. Res.* **3**, 211.
Furr, B. J. A. and Jordan, V. C. (1984). *Pharmac. Ther.* **25**, 127–205.
Furr, B. J. A. and Nicholson, R. I. (1982). *J. Reprod. Fert.* **64**, 529–539.
Furr, B. J. A., Patterson, J. S., Richardson, D. N., Slater, S. R. and Wakeling, A. E. (1979). Tamoxifen. *In* 'Pharmacological and Biochemical Properties of Drug Substances', (Goldberg, M. E., ed.) Vol. 2, pp. 355–399. American Pharmaceutical Association, Washington, D.C.
Gagliardi, A. and Collins, D. C. (1993). *Cancer Res.* **53**, 533–535.
Gambacciani, M., Spinetti, A., Cappagli, B., Taponeco, F., Felipetto, R., Parrini, D., Cappelli, N. and Fioretti, E. (1993). *J. Clin. Invest.* **16**, 333–337.
Gassner, F. X., Reifenstein, E. C., Algeo, J. W. and Mettox, W. E. (1958). *Recent Prog. Horm. Res.* **14**, 183–217.
Gaunt, R., Chart, J. J. and Renzi, A. A. (1961). *Science* **133**, 613–621.
Geier, A., Lunenfeld, B., Pariente, C., Koter Emeth, S., Shadmi, A., Kokia, E. and Blankstein, J. (1987). *Fertil. Steril.* **47**, 778–784.
Gellert, R. J., Bakke, J. L. and Lawrence, N. L. (1971). *Fertil. Steril.* **22**, 244–250.
Gennari, C. and Agnusdei, D. (1990). *J. Steroid Biochem. Mol. Biol.* **37**, 451–455.
Giudici, D., Ornati, G., Briatico, G., Buzzetti, F., Lombardi, P. and Di Salle, E. (1988). *J. Steroid Biochem.* **30**, 391–394.
Golder, M. P., Phillips, M. E. A., Baum, M., Griffiths, K., Fahmy, D. R., Henk, J. M., Jones, J. and Preece, P. E. (1975). *Br. J. Cancer* **32**, 246–247.
Gopalchari, R. and Iyer, R. N. (1966). *Ind. J. Chem.* **4**, 331–332.
Gopalchari, R., Iyer, R. N., Kamboj, V. P. and Kar, A. B. (1970). *Contraception* **2**, 199–205.
Gopalchari, R., Iyer, R. N., Kamboj, V. P. and Kar, A. B. (1971). *Ind. J. Exp. Biol.* **9**, 104–105.
Gorlitsky, G. A., Kase, N. G. and Speroff, L. (1978). *Obstet. Gynecol.* **51**, 265–269.
Goronemeyer, H. (1991). *Annu. Rev. Genet.* **25**, 89–123.
Goulding, A. and Gold, E. (1990). *Calcif. Tissue Int.* **46**, 14–19.
Goulding, A. and Gold, E. (1989). *J. Endocrinol.* **121**, 293–298.
Goulding, A., Gold, E. and Feng, W. (1992). *Bone Mineral* **18**, 143–152.
Graves, P. E. and Salhanick, H. A. (1979). *Endocrinology* **105**, 52–57.
Gray, T. K., Flynn, T. C., Gray, K. M. and Nabell, L. M. (1987). *Proc. Natl Acad. Sci. USA* **84**, 267–6271.

Green, M. M., Whybourne, A. M., Taylor, I. W. and Sutherland, R. L. (1981). *In* 'Non-Steroidal Antiestrogens' (Sutherland, R. L. and Jordan, V. C., eds) pp. 397–412. Academic Press, Sydney.

Green, S. (1990). *J. Steroid Biochem. Molec. Biol.* **37**, 747–751.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P. and Chambon, P. (1986). *Nature* **320**, 134–139.

Greenberg, C., Kukreja, S. C., Bowser, E. N., Hargis, G. K., Henderson, W. J. and Williams, G. A. (1986). *Endocrinology* **118**, 2594–2598.

Greenblatt, R. B. (1966). *In* 'Ovulation' (Greenblatt, R. B., ed.), p. 134. Lippincott Co., Philadelphia, Toronto.

Greenblatt, R. B., Barfield, W. E., Jungck, E. C. and Ray, A. W. (1961). *J. Am. Med. Ass.* **178**, 101–104.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. and Shine, J. (1986). *Science* **231**, 1150–1154.

Greslin, J. G., Van Maanen, E. F., Davis, R. H. and Holtkamp. D. E. (1959). Programme of 41st Meeting Endocrine Soc., p. 82.

Groom, G. V. and Griffiths, K. (1976). *J. Endocrinol.* **70**, 421–428.

Grover, P. K., Chawla, H. P. S., Anand, N., Kamboj, V. P. and Kar, A. B. (1965). *J. Med. Chem.* **8**, 720–721.

Grundy, J. (1957). *Chem. Rev.* **57**, 281–416.

Gupta, R. C., Srivastava, S. C. and Anand, N. (1977). *Ind. J. Chem.* **15B**, 91–92.

Hale, H. B. (1944). *Endocrinology* **35**, 499–506.

Hamilton, J. A., Lingelbach, S., Partridge, N. C. and Martin, T. J. (1985). *Endocrinology* **116**, 2186–2191.

Harlow, K. W., Smith, D. N., Katzenellenbogen, J. A., Greene, G. L. and Katzenellenbogen, B. S. (1989). *J. Biol. Chem.* **264**, 17,476–17,485.

Harper, M. J. K. (1965). *Endocrinology* **77**, 114–123.

Harper, M. J. K. and Walpole, A. L. (1966). *Nature* **212**, 87.

Harper, M. J. K. and Walpole, A. L. (1967a). *J. Endocrinol.* **37**, 83–92.

Harper, M. J. K. and Walpole, A. L. (1967b). *J. Reprod. Fert.* **13**, 101–119.

Harris, A. L. (1985). *Exp. Cell Biol.* **53**, 1–8.

Hartmann, R. W., Krazfelder, G., Angerea, E. V. and Schoneuberger, H. (1980). *J. Med. Chem.* **23**, 841–848.

Hartmann, R. W., Buchborn, H., Krazfelder, G. and Schonenberger, H. (1981). *J. Med. Chem.* **24**, 1192–1197.

Hartmann, R. W., Schwarz, W. and Schonenberger, H. (1983). *J. Med. Chem.* **26**, 1137–1144.

Hartmann, R. W., Heindle, A. and Schonenberger, H. (1984). *J. Med. Chem.* **27**, 577–585.

Hartmann, R. W., Schwarz, W., Heindle, A. and Schonenberger, H. (1985). *J. Med. Chem.* **28**, 1295–1301.

Hartmann, R. W., Bayer, H. and Grun, G. (1994). *J. Med. Chem.* **37**, 1275–1281.

Hashimoto, T., Miyai, K., Izumi, K. and Kumahara, Y. (1976). *J. Clin. Endocr. Metab.* **42**, 593–594.

Hasmann, M., Rattel, B. and Loeser, R. (1994). *Cancer Letter* **84**, 101–116.

Hawkins, R. A., Hill, A., Freedman, B., Killen, E., Buchan, P., Miller, W. R. and Forrest, A. P. M. (1977). *Eur. J. Cancer Clin. Oncol.* **13**, 223–228.

Heaney, R. P. (1987). *Clinical Obstet. Gynecol.* **30**, 833–846.

Heaney, R. P., Gallagher, J. C., Johnston, C. C., Neer, R., Parfitt, A. M. and Whedon, G. D. (1982). *Am. J. Clin. Nutr.* **36**(Suppl. 5), 986–1013.

Hecker, E., Begh, I., Levy, C. M., Magin, C. A., Martinez, J. C., Lowreiro, J. and Garola, R. E. (1974). *Eur. J. Cancer* **10**, 747–749.

Henderson, D., Norbisrath, G. and Kerb, U. (1986). *J. Steroid Biochem.* **24**, 303–306.

Hendry, L. B. (1988). *J. Steroid Biochem.* **31**, 493–523.
Hendry, L. B., Bransome, E. D., Jr, Lehner, A. F., Muldoon, T. G., Hutson, M. S. and Mahesh, V. B. (1986). *J. Steroid Biochem.* **24**, 843–852.
Hendry, L. B., Muldoon, T. G. and Mahesh, V. B. (1992). *J. Steroid Biochem. Molec. Biol.* **42**, 659–670.
Hendry, L. B., Chu, C. K., Copland, J. A. and Mahesh, V. B. (1994). *J. Steroid Biochem. Molec. Biol.* **48**, 495–505.
Herbst, A. L., Griffiths, C. T. and Kistner, R. W. (1964). *Cancer Chemother. Rep.* **43**, 39–41.
Hertig, A. T. (1935). *Carnegie Contrib. Embryol.* **25**, 37–81.
Heuson, J. C., Engelsman, E., Blonk-van Der Wijst, J., Maas, H., Droghmans, A., Michel, J., Nowakowski, H. and Gorins, A. (1975). *Brit. Med J.* **2**, 711–713.
Hirvonen, E., Ranta, T. and Seppala, M. (1976). *Int. J. Fertil.* **21**, 255.
Holbrook, T. L., Barrett-Connor, E. and Wingard, D. L. (1988). *Lancet* **ii**, 1046–1049.
Holtkamp, D. E., Greslin, J. G., Root, C. A. and Lerner, L. J. (1960). *Proc. Soc. Exp. Biol. Med.* **105**, 197–200.
Horne, H. W., Jr, Kundsin, R. B. and Kosasa, T. S. (1974). *Fertil. Steril.* **25**, 380–389.
Horsman, A., Simpson, M., Kirby, P. A. and Nordin, B. E. C. (1977). *Br. J. Radiol.* **50**, 504–507.
Horwitz, K. B. and McGuire, W. L. (1978). *J. Biol. Chem.* **253**, 8185–8191.
Horwitz, K. B., McGuire, W. L., Pearson, O. H. and Segaloff (1975). *Science, N.Y.* **189**, 726–727.
Hossain, M. B., Helm, D. V. D., Schmitz, F. J., Pordesimo, E. O., Magarian, R. A., Meyer, K. L., Overacre, L. B. and Day, B. W. (1994). *J. Med. Chem.* **37**, 1670–1683.
Howell, A., Defriend, D., Robertson, J., Blamey, R. and Walton, P. (1995). *Lancet* **345**, 29–30.
Hsueh, A. J. W., Erickson, G. F. and Yen, S. S. C. (1978). *Nature* **273**, 57–59.
Huggins, C. and Bergenstal, D. M. (1952). *Cancer Res.* **12**, 134–141.
Huggins, C., Briziarelli, G. and Sulton, H. (1959). *J. Exp. Med.* **104**, 25–41.
Humphrey, K. W. and Martin, L. (1968). *Aust. J. Biol. Sci.* **21**, 1239–1248.
Igarashi, M., Ibuki, Y., Kubo, H., Kamioka, J., Yokota, N., Ebara, Y. and Matsumoto, S. (1967). *Am. J. Obstet. Gynecol.* **97**, 120–123.
Ignar-Trowbridge, D. M., Teng, C. T., Ross, K. A., Parker, M. G., Korach, K. S. and McLachlan, J. A. (1993). *Mol. Endocrinol.* **7**, 992–998.
Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H. and Folkman, J. (1990). *Nature* **348**, 555–557.
Ip, M. M., Milholland, R. J. and Rosen, F. (1980). *Cancer Res.* **40**, 2188–2193.
Iyer, R. N. and Gopalchari, R. (1966). *Ind. J. Chem.* **4**, 520–523.
Iyer, R. N. and Gopalchari, R. (1976). *Ind. J. Chem.* **14B**, 194–198.
Iyer, R. N., Gopalchari, R., Kamboj, V. P. and Kar, A. B. (1969). *Ind. J. Pharm.* **31**, 49–54.
Jacobsen, A., Marshall, J. R. and Ross, G. T. (1968). *Am. J. Obstet. Gynec.* **101**, 1025–1031.
Jensen, E. V., DeSombre, E. R. and Jungblut, P. W. (1967). *In* 'International Symposium on Endogenous Factors Influencing Host-tumor Balance' (Wissler, R. W., Dao, T. L. and Wood, S., eds), p. 15. Chicago University Press, Chicago.
Johnston, J. O. (1987). *Steroids* **50**, 105–120.
Johnston, J. O., Wright, C. L. and Metcalf, B. W. (1984). *Endocrinology* **115**, 776–785.
Jones, C. D., Suarez, T., Nassey, E. H., Black, L. J. and Tinsley, F. C. (1979). *J. Med. Chem.* **22**, 962–966.
Jones, C. D., Jevnikar, M. G., Pike, A. J., Peters, M. K., Black, L. J., Thompson, A. R., Falcone, J. F. and Clemens, J. A. (1984). *J. Med. Chem.* **27**, 1057–1066.

Jones, C. D., Winter, M. A., Hirsch, K. S., Stamm, N., Taylor, H. M., Holden, H. E., Davenport, J. D., Krumkalns, E. V. and Suhr, R. (1990). *J. Med. Chem.* **33**, 416–429.
Jones, C. D., Blaszczak, Goettel, M. E., Suarez, T., Crowell, T. A., Mabry, T. E., Ruenitz, P. C. and Srivatsan, V. (1992). *J. Med. Chem.* **35**, 931–938.
Jones, G. W., Nazareth, W. M., Daly, M. J., James, M. I., Nicholls, P. J. and Smith, H. J. (1986). *Anticancer Res.* **6**, 393.
Jordan, V. C. (1975). *J. Reprod. Fert.* **52**, 251–258.
Jordan, V. C. (1976a). *Eur. J. Cancer Clin. Oncol.* **12**, 419–424.
Jordan, V. C (1976). *Cancer Treat Rep.* **60**, 1409–1419.
Jordan, V. C. (1984). *Pharmacol. Rev.* **36**, 245–276.
Jordan, V. C. (1990). *Breast Cancer Res. Treat.* **15**, 125–136.
Jordan, V. C. and Dowse, L. J. (1976). *J. Endocrinol.* **68**, 297–303.
Jordan, V. C. and Koerner, S. (1976). *J. Endocr.* **68**, 305–311.
Jordan, V. C. and Murphy, C. S. (1990). *Endocrine Rev.* **11**, 578–610.
Jordan, V. C., Dix, C. J., Naylor, K. E., Prestwich, G. and Rowsby, L. (1978). *J. Toxicol. Environ. Health* **4**, 363.
Jordan, V. C., Allen, K. E. and Dix, C. J. (1980). *Cancer Treat. Rep.* **64**, 745–759.
Jordan, V. C., Phelps, E. and Lindgren, J. U. (1987). *Breast Cancer Res. Treat.* **10**, 31–35.
Judd, H. L., Medrum, D. R., Degtos, L. J. and Henderson, B. E. (1983). *Ann. Inter. Med.* **98**, 195–205.
Kallio, D. M., Garant, P. R. and Minkin, C. (1972). *J. Ultrastructure Res.* **39**, 205–216.
Kamboj, V. P., Kar, A. B., Ray, S., Grover, P. K. and Anand, N. (1971). *Ind. J. Exp. Biol.* **9**, 103–104.
Kamboj, V. P., Setty, B. S., Chandra, H., Roy, S. K. and Kar, A. B. (1977). *Ind. J. Exp. Biol.* **15**, 1144–1150.
Kamboj, V. P., Ray, S. and Dhawan, B. N. (1992). *Drugs Today* **28**, 227–232.
Kanis, J. A., Melton, L. J., Christiansen, C., Johnston, C. C. and Khaltaev, N. (1994). *J. Bone Min. Res.* **9**, 1137–1141.
Karkun, J. N. and Mehrotra, P. K. (1973). *Ind. J. Exp. Biol.* **11**, 7–14.
Katzenellenbogen, B. S., Bhakoo, H. S., Ferguson, E. R., Lan, N. C., Tatee, T., Tsai, T. S. and Katzenellenbogen, J. A. (1979). *Recent Progr. Horm. Res.* **35**, 259–300.
Katzenellenbogen, B. S., Miller, M. A., Mullick, A. and Sheen, Y. Y. (1985). *Breast Cancer Res. Treat.* **5**, 231–243.
Katzenellenbogen, B. S., Montano, M. M., Goff, R. L., Schodin, D. J., Kraus, W. L., Bhardwaj, B. and Fujimoto, N. (1995). *J. Steroid Biochem. Molec. Biol.* **53**, 387–393.
Kaufman, C. (1933). *Klin. Wochschr.* **12**, 1557–1562; *Chem. Abstr.* **28**, 8123.
Kellis, J. T. and Vickery, L. E. (1984). *Endocrinology* **114**, 2128–2137.
Kellis, J. T., Jr, Childers, W. E., Robinson, C. H. and Vickery, L. E. (1987). *J. Biol. Chem.* **262**, 4421–4426.
Kelly, P. A., Bradley, C., Shiu, R. P. C., Meites, J. and Friesen, H. G. (1974). *Proc. Soc. Exp. Biol. Med.* **146**, 816–819.
Kemp, J. V., Adam, H. K., Wakeling, A. E. and Slater, R. (1983). *Biochem. Pharmacol.* **32**, 2045–2052.
Kenneth, S. H., Jones, C. D., Terry, D. L., Nancy, B. S., Gregory, P. S., Harold, M. T. and Dix, E. W. (1987). *Steroids* **50**, 201–217.
Kiang, D. T. and Kennedy, B. J. (1977). *Ann. Intern. Med.* **87**, 687–690.
Kilbourn, B. T. and Owston, P. G. (1970). *J. Chem. Soc.* B. 1–5.
Kim Hak-Joong and Kalkhoff, R. K. (1975). *J. Clin. Invest.* **56**, 888–896.
Kincl, F. A., Pi, A. F., Magueo, M., Lasso, L., Oriol, A. and Dorfman, R. I. (1965). *Acta Endocrinol.* **49**, 193–206.
King, M. M., Magarian, R. A., Terao, J. and Brueggemann, G. L. (1985). *J. Natl Cancer Inst.* **74**, 447–451.

King, R. J. B. (1992). *Clin. Endocrinol. (Oxf)* **36**, 1–14.
Kistner, R. W. (1975). *In* 'Progress in Infertility', 2nd edn (Behrman, S. J. and Kistner, R. W., eds), pp. 509–536. Little, Brown & Co., Boston.
Kistner, R. W. (1965). *Am. J. Obstet. Gynecol.* **92**, 380–411.
Kistner, R. W. (1976). *Fertil. Steril.* **27**, 72–82.
Kistner, R. W. and Smith, O. W. (1959). *Surg. Forum* **10**, 725–729.
Klein-Hitpass, L., Ryffel, G. U., Heittinger, E. and Cato, A. C. B. (1988). *Nucl. Acids Res.* **16**, 647–663.
Klock, G., Strahle, U. and Schutz, G. (1987). *Nature* **329**, 734–736.
Klopper, A. and Hall, M. (1971). *Br. Med. J.* **1**, 152–154.
Knabbe, C., Zugmaier, G., Schmahl, M., Dietel, M. and Lippman, M. E. (1991). *Am. J. Clin. Oncol.* **14**(Suppl. 2), 515–520.
Kojima, M., Tsutsumi, N., Nagata, H., Itoh, F., Ujii, A., Kawashima, K., Endo, H. and Okazaki, M. (1994a). *Biol. Pharm. Bull.* **17**, 504–508.
Kojima, M., Tsutsumi, N., Arai, N., Itoh, F., Ujiie, A., Kawashima, K., Endoh, H. and Okazaki, M. (1994b). *Iyakuhin Kenkyu* **25**, 453–458.
Kole, P. L., Ray, S. and Anand, N. (1977). *Ind. J. Chem.* **15B**, 835–838.
Kole, P. L., Ray, S. and Anand, N. (1978). *Ind. J. Chem.* **16B**, 722–723.
Komm, B. S., Terpening, C. M., Benz, D. J., Graeme, K. A., Gallegos, A., Kore, M., Greene, G. L., O' Malley, B. W. and Hausser, M. R. (1988). *Science* **241**, 81–84.
Korach, K. S., Migliaccio, S. and Davis, V. L. (1994). *In* 'Principles of Pharmacology: Basic Concepts and Clinical Applications' (Munson, P. L., Mueller, R. A. and Breese, G. R., eds), pp. 809–825. Chapman and Hall, New York.
Koseki, Y., Zava, D. T., Chamness, G. C. and McGuire, W. L. (1977). *Steroids* **30**, 169–177.
Kranzfelder, G., Hartmann, R. W., Angerer, E. V., Schonenberger, H. and Bogden, A. (1982). *J. Cancer Res. Clin. Oncol.* **103**, 165–180.
Krekels, M. D. W. G., Wouters, W., Vanginckel, R., Jansseus, B., Callens, M. and De Coster, D. (1992). *J. Steroid Biochem. Mol. Biol.* **41**, 761–764.
Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J. M. and Chambon, P. (1986). *EMBO J.* **5**, 891–897.
Kumar, V., Green, S., Staub, A. and Chambon, P. (1986). *EMBO J.* **5**, 2231–2236.
Kusuhara, S. and Ishida, K. (1986). *Jpn J. Zootech. Sci.* **57**, 726.
Labhsetwar, A. P. (1971). *J. Reprod. Fert.* **25**, 295–297.
Labroo, V. M., Piggott, J. R., Bain, S. D., U.S. Pat. No. 5,280,040 dt. Jan. 18, 1994.
Ladinsky, J. L., Gruchow, H. W. and Peckhman, B. M. (1968). *J. Endocrinol.* **41**, 161–169.
Lamb, E. J. (1972). *Fertil. Steril.* **23**, 320–325.
Lamberts, S. W., Bruining, H. A., Marzouk, H., Zuiderwijk, J., Uitterlinden, P., Blijd, J. J., Hackeng, W. H. and De Jong, F. H. (1989). *J. Clin. Endocrinol. Metab.* **69**, 896–901.
Landquist, J. K. and Marsden, C. J. (1966). *Chem. Ind.* 1032–1033.
Leavitt, W. W. and Meismer, D. M. (1968). *Nature* **218** (5137), 181–182.
Lednicer, D., Babcock, J. C., Lyster, S. C., Stucki, J. C. and Duncan, G. W. (1961) *Chem. Ind.* 2098–2099.
Lednicer, D., Lyster, S. C. and Duncan, G. W. (1963a). *J. Med. Chem.* **8**, 725–726.
Lednicer, D., Babcock, J. C., Lyster, S. C. and Duncan, G. W. (1963b). *Chem. Ind.* 408–409.
Lednicer, D., Babcock, J. C., Marlatt, P. E., Lyster, S. C. and Duncan, G. W. (1965). *J. Med. Chem.* **8**, 52–57.
Lednicer, D., Lyster, S. C. and Duncan, G. W. (1967). *J. Med. Chem.* **10**, 78–84.
Lee, A. E. (1971). *Acta Endocrinol.* **67**, 345–352.
Legha, S. S., Davis, H. L. and Muggia, F. M. (1978). *Ann. Int. Med.* **88**, 69–77.

Lehner, A. F., Muldoon, T. G., Mahesh, V. B., Bransome, E. D., Jr and Hendry, L. B. (1987). *Molec. Endocrinol.* **1**, 377–387.

Lerner, L. J. (1981). *In* 'Non-steroidal antiestrogens: Mol. Pharmacol. Antitumour Act', (Sutherland, R. L. and Jordan, V. C., eds), pp. 1–16. Academic Sydney, Australia.

Lerner, L. J., Holthaus, F. J., Jr, and Thompson, C. R. (1958). *Endocrinology* **63**, 295–318.

Lerner, L. J., Carminati, P. and Schialti, P. (1975). *Proc. Soc. Exp. Biol. Med.* **148**, 329–332.

Leung, B. S., Sasaki, G. H. and Leung, J. S. (1975). *Cancer Res.* **35**, 621–627.

Levesquie, C., Merand, Y., Dufour, J., Labrie, C. and Labrie, F. (1991). *J. Med. Chem.* **34**, 1624–1630.

Levin, J., Markhzm, M. J., Greenwald, E. S., Wollner, D., Kream, J., Zumoff, B. and Fukushima, D. K. (1981). *Cancer* **47**, 1394–1397.

Levy, J., Liel, Y., Feldman, B., Afllallo, L. and Glick, J. M. (1981). *Eur. J. Cancer Clin. Oncol.* **17**, 1023–1026.

Li, Pui-kai and Brueggemeier, R. W. (1990). *J. Med. Chem.* **33**, 101–105.

Lindsay, R., Hart, D. M., Forrest, C. and Baird, C. (1980). *Lancet* **ii**, 1151–1153.

Lindsay, R. (1988). *In* 'Sex Steroids in the Pathogenesis and Prevention of Osteoporosis'. (Riggs, B. L., ed.), p. 337. Raven Press, New York.

Lippman, M. E. and Aitken, S. C. (1980). *In* 'Hormones and Cancer' (Iacobelli, S., Linder, H. R., King, R. J. B. and Lippman, M. E., eds), pp. 3–19. Raven Press, New York.

Lippman, M. E., Bolan, G. and Huff, K. (1976). *Cancer Res.* **36**, 4595–4601.

Lipton, A., Harvey, H. A., Demers, L. M., Hangan, J. R., Mulagha, M., Kochak, G. M., Fitzsimmons, S., Snaders, S. I. and Santen, R. J. (1990). *Cancer* **65**, 1279–1286.

Lobo, R. A., Paul, W., March, C. M., Granger, L. and Kletz, O, A. (1982). *Obstet. Gynecol.* **60**, 497–501.

Longcope, C., Femino, A. and Johnston, J. O. (1988). *Endocrinology* **122**, 2007–2011.

Love, R. R., Mazess, R. B., Tormey, D. C., Barden, H. S., Newcomb, P. A. and Jordan, V. C. (1988). *Bone Cancer Res. Treat.* **12**, 297–302.

Lovett, J. A., Darby, M. V. and Counsell, R. E. (1984). *J. Med. Chem.* **27**, 734–740.

Lunan, C. B. and Green, B. (1974). *Clin. Endocr.* **3**, 465–480.

Lyons, W. R., Li, C. H. and Johnson, R. E. (1958). *Recent Prog. Horm. Res.* **14**, 219–254.

Magarian, R. A. and Benzamin, E. J. (1975). *J. Pharm. Sci.* **64**, 1626–1632.

Mahler, C., Verhelst, J. and Denis, L. (1993). *Cancer* **71**(Suppl. 3), 1068–1073.

Mahler, C., Verheist, J., Denis, L., de Porre, P., De Coster, R., Freyne, E. and Bruynseeis, J. (1994). *Drugs of the Future* **19**, 552–556.

Malik, M. S. and Rastogi, S. N. (1981). *Ind. J. Chem.* **20B**, 174–175.

Malik, M. S. and Rastogi, S. N. (1984). *Ind. J. Chem.* **23B**, 834–838.

Malik, M. S., Rastogi, S. N., Singh, M. M., Sreenivasulu, S., Agnihotri, A., Kalra, V. and Kamboj, V. P. (1989). *Eur. J. Med. Chem.* **24**, 193–197.

Manni, A., Trujillo, J. E., Marshall, J. S., Brodkey, J. and Pearson, O. H. (1979). *Cancer* **43**, 444–450.

Manni, A., Trujillo, J. E. and Pearson, O. H. (1977). *Cancer Res.* **37**, 1216–1219.

Marcus, R. (1989). *Hosp. Prac.* **24**, 189–218.

Markham, M. J., Levin, J., Greenwald, E. S., Wollner, D., Kream, J., Zumoff, B. and Fukushima, D. K. (1980). *Proc. Am. Ass. Cancer Res.*, Abst. 678.

Marois, M. and Marois, G. (1977). *Cr. Soc. Biol.* **171**, 280–286.

Mason, J. I., Murry, B. A., Olcott, M. and Sheets, J. J. (1985). *Biochem. Pharmacol.* **34**, 1087–1092.

Masson, G. M. and Klopper, A. (1972). *In* 'Proc. Workshop ICI 46474-Work in Progress'. pp. 40–43. ICI Pharmaceuticals Division plc, Macclesfield.

Matsubara, T., Saura, R., Hirohata, K. and Ziff, M. (1989). *J. Clin. Invest.* **83**, 158–167.

Matta, W. H., Shaw, R. W., Hesp, R. and Katz, D. (1987). *Br. Med. J.* **294**, 1523–1524.

Matthews, C. D., Elmslie, R. G., Clapp, K. H. and Svigos, J. M. (1975). *Fertil. Steril.* **26**, 988–990.

Mazess, R. B. (1982). *Clin. Orthop.* **165**, 239–252.

Mazess, R. B., Barden, H. S. and Love, R. R. (1990). *Bone Min. Res.* **5**(Suppl. 2), Abst. 425.

McCagne, R., Leclercq, G. and Jordan, V. C. (1988). *J. Med. Chem.* **31**, 1285–1290.

McCormack, S. and Clark, J. H. (1979). *Science* **204**, 629–631.

McSheehy, P. M. J. and Chambers, T. J. (1986). *Endocrinology* **119**, 1654–1659.

Mehrotra, P. K. and Karkun, J. N. (1975). *Ind. J. Exp. Biol.* **13**, 69–70.

Mehrotra, P. K. (1982). *Ind. J. Exp. Biol.* **20**, 719–720.

Melis, G. B., Paoletti, A. M., Cagnacci, A., Bufalino, L., Spinetti, A., Gambacciani, M. and Fioretti, P. (1992). *J. Endocrinol. Invest.* **15**, 755–761.

Metcalf, B. W., Wright, C. L., Burkhart, J. P. and Johnston, J. O. (1981). *J. Am. Chem. Soc.* **103**, 3221–3222.

Migliaccio, S., Wetsel, W. C., Fox, W. M., Washburn, T. F. and Korach, K. S. (1993). *Mol. Endocrinol.* **7**, 1133–1143.

Mishra, N. C., Nigam, P. K., Gupta, R., Agarwal, A. K. and Kamboj, V. P. (1989). *Int. J. Cancer* **43**, 781–783.

Mishra, N. C., Ratna, S., Ray, S. and Roy, S. K. (1992). *Med. Sci. Res.* **20**, 259–260.

Mittal, S., Durani, S. and Kapil, R. S. (1985). *J. Med. Chem.* **28**, 492–497.

Moon, L. Y., Wakley, G. K. and Turner, R. T. (1991). *Endocrinology* **129**, 1568–1574.

Morishita, H., Nakago, K., Ii, K., Hashimoto, T., Kawamoto, M., Tanaka, T., Higuchi, K., Miyauchi, Y. and Ozasa, T. (1979). *Acta Endocrinol.* **92**, 577–584.

Morris, J. M. (1970). *Contraception* **2**, 85–97.

Morris, J. M., van Wagenen, G., McCann, T. and Jacob, D. (1967). *Fert. Steril.* **18**, 18–34.

Morris, N. (1993). *Fert. Steril.* **59**, 707–725.

Morris, P. B., Hida, T., Blackshear, P. J., Klintworth, G. K. and Swain, J. L. (1988). *Am. J. Physiol.* **254**, C318–C322.

Mueller, G. C., Herranen, A. M. and Jervell, K. F. (1958). *Recent Prog. Horm. Res.* **14**, 95–139.

Mueller, G. C., Vonderhaar, B., Kim, U. H. and Mahieu, M. E. (1972). *Recent Prog. Horm. Res.* **28**, 1–44.

Mukherjee, S. S., Sethi, N., Srivastava, G. N., Roy, A. K., Nityanand, S. and Mukherjee, S. K. (1977). *Ind. J. Exp. Biol.* **15**, 1162–1166.

Munshi, K. L., Dikshit, D. K., Kapil, R. S. and Anand, N. (1974). *Ind. J. Chem.* **12**, 336–343.

Murata, J., Saiki, I., Makabe, T., Tsuta, Y., Tokura, S. and Ayuma, I. (1991). *Cancer Res.* **51**, 22–26.

Nakamura, S., Morimoto, S., Takamoto, T., Onishi, T., Fukuo, K., Koh, E., Kitano, S., Miyashita, Y., Yasuda, O., Tamatani, M., Nakahashi, T. and Ogihara, T. (1992). *Calcif. Tissue Int.* **51**, 530–534.

Natrajan, P. K. and Greenblatt, R. B. (1979). *In* 'Clomiphene Citrate: Induction of Ovulation', (Greenblatt, R. B., ed.), pp. 35–76. Lea & Febiger, Philadelphia, PA.

Newberne, J. W., Kuhn, W. L. and Elsea, J. R. (1966). *Toxicol. Appl. Pharmacol.* **9**, 44–55.

Nicholson, R. I. and Golder, M. P. (1975). *Eur. J. Cancer Clin. Oncol.* **11**, 571–579.

Nicholson, R. I., Davies, P. and Griffiths, K. (1978). *Revs Endocrine-related Cancer*, April Suppl. 306–321.

Nicosia, R. F. and Bonanno, E. (1991). *Am. J. Pathol.* **138**, 829–833.

Nielsen, H. K., Brixen, K., Bouillon, R. and Mosekilde, L. (1990). *J. Clin. Endocrinol. Metab.* **70**, 1431–1437.

Nigam, P. K., Malaviya, B., Chowdhury, S. R., Kamboj, V. P. and Chandra, H. (1985).

Contraception **32**, 283–300.
Nique, F. and Van de Velde, P. (1995). *Drugs of the Future* **20**, 362–366.
Nique, F., van de Velde, P., Bremaud, J., Hardy, M., Philbert, D. and Teutsch, G. (1994). *J. Steroid Biochem. Molec. Biol.* **50**, 21–29.
Nishino, Y., Schneider, H., Michna and von Angerer, E. (1991). *J. Endocrinol* **130**, 409–414.
Noble, R. L. (1980a). *Cancer Res.* **40**, 3547–3550.
Noble, R. L. (1980b). *Cancer Res.* **40**, 3551–3554.
Noguchi, M., Tajiri, K., Taniya, T., Kumaki, T., Ashikari, A. and Miyazaki, I. (1990). *Oncology* **47**, 19–24.
Nohno, T., Omukai, Y., Watanabe, S., Saito, T. and Senoo, T. (1981). *Proc. 8th Int. Congr. Pharmac.*, Abst. 646.
Notoya, K., Yoshida, K., Taketomi, S., Yamazaki, I. and Kumegawa, M. (1992). *Calcif. Tissue Int.*, **51**(Suppl.), 63–66.
Numazawa, M. and Oshibe, M. (1994). *J. Med. Chem.* **37**, 1312–1319.
Numazawa, M., Mutsumi, A., Hoshi, K. and Koike, R. (1989a). *Biochem. Biophys. Res. Comm.* **160**, 1009–1014.
Numazawa, M., Mutsumi, A. and Tsuji, M. (1989b). *Steroids* **54**, 299–311.
O'Herlihy, C., Pepperell, R. J. and Robinson, H. P. (1982). *Obstet. Gynecol.* **59**, 40.
O'Malley, B. W., McGuire, W. L., Kohler, P. O. and Korenman, S. G. (1969). *Recent Prog. Horm. Res.* **25**, 105–160.
O'Neal, J. (1987), *Steroids* **50**, 105–120.
Oakley, G. P. and Flynt, J. W. (1972). *Teratology* **5**, 264.
Ohsawa, Y., Nitatori, T., Higuchi, S., Kominani, E. and Uchiyama, Y. (1993). *J. Histochem. Cytochem.* **41**, 1075–1083.
Oikawa, T., Hirotani, K., Shimamura, M., Ashinofu, H. and Iwaguchi, T. (1989). *J. Antibiotics* **42**, 1202–1204.
Oikawa, T., Hirotani, K., Ogasawara, H., Katayama, T., Nakamura, O., Iwaguchi, T. and Hiragun, A. (1990). *Eur. J. Pharmacol.* **178**, 247–250.
Oikawa, T., Shimamura, M., Ashino-Fuse, H., Iwaguchi, T., Ishizuka, M. and Takeuchi, T. (1991). *J. Antibiotics* **44**, 1033–1035.
Oka, T. and Schimke, R. T. (1969). *J. Cell. Biol.* **43**, 123–137.
Omdahl, J. L., Garry, P. J., Hunsaker, L. A., Hunt, W. C. and Goodwin, J. S. (1982). *Am. J. Clin. Nutr.* **36**, 1225–1233.
Osawa, Y., Tochigi, B., Hiashiyama, T., Yarborough, C., Nakamura, T. and Yamamoto, T. (1982a). *Cancer Res. Suppl.* **42**, 3299s–3306s.
Osawa, Y., Yarborough, C. and Osawa Y. (1982b). *Science* **215**, 1249–1251.
Osawa, Y., Osawa, Y. and Coon, M. J. (1987). *Endocrinology* **121**, 1010–1016.
Oursler, M. J., Osdoby, P., Pyfferoen, J., Riggs, B. L. and Spelsberg, T. C. (1991). *Proc. Natl Acad. Sci. USA* **88**, 6613–6617.
Paassen, H. C., Poortman, J., Borgart-Creutzburg, I. H. C., Thijssen, J. H. H. and Duursman, S. A. (1978). *Calcif. Tissue Res.* **25**, 249.
Paliwal, J. K., Gupta, R. C., Grover, P. K., Asthana, O. P. and Srivastava, J. S. (1989). *Pharmac. Res.* **6**, 1048–1051.
Paliwal, J. K., Grover, P. K., Asthana, O. P., Nityanand, S. and Gupta, R. C. (1994) *Br. J. Clin. Pharmac.* **38**, 485–486.
Palshof, T. (1981). *Revs Endocrine-related Cancer Suppl. 7*, 65–75.
Parfitt, A. M., Mathews, C. H. E., Villanueva, A. R., Kleerekoper, M., Frame, B. and Rao, D. S. (1983). *J. Clin. Invest.* **72**, 1396–1409.
Parker, M. G. (1993). *J. Neuro. Endocrinol.* **5**, 223–228.
Passeri, M., Biondi, M., Costi, D., Bufalino, L. Castigli, G. N., Dipeppe, C. and Abate, G. (1992). *Bone Mineral* (Suppl. 1), 557–562.

Patterson, J. S., Settatree, R. S., Adam, H. K. and Kemp, J. V. (1980). *In* 'Breast Cancer – Experimental and Clinial Aspects' (Mouridsen, H. T. and Palshof, T., eds), pp. 89–92. Pergamon Press, Oxford.

Patterson, J. S., Edwards, D. G. and Battersby, L. A. (1981). *Jap. J. Cancer Clins* Suppl. (November) 157–183.

Paul, R., Coppola, J. A. and Cohen, E. (1972). *J. Med. Chem.* **15**, 720–726.

Pearson, O. H., Llerena, O., Llerena, L., Molina, A. and Butler, T. (1969). *Trans. Ass. Am. Phys.* **82**, 225–238.

Pento, J. T., Magarian, R. A. and King, M. M. (1982). *Cancer Lett.* **15**, 261–269.

Phansey, S. A., Barnes, M. A., Williamson, H. O., Sagel, J. and Nair, R. M. G. (1980). *Fert. Steril.* **34**, 448–451.

Plourde, P. V., Dyroff, M. and Dukes, M. (1994). *Breast Cancer Res. Treat.* **30**, 103–111.

Plourde, P. V., Dyroff, M., Dowsett, M., Demers, L., Yates, R. and Webster, A. (1995). *J. Steroid Biochem. Molec. Biol.* **53**, 175–179.

Poirier, D., Anger, S., Merand, Y. and Labrie, F. (1994). *J. Med. Chem.* **37**, 1115–1125.

Pollard, I. and Martin, L. (1968). *Steroids* **11**, 897–907.

Powell-Jones, W., Davies, P. and Griffiths, K. (1975a). *J. Endocrinol.* **66**, 437–438.

Powell-Jones, W., Jenner, D. A., Blamey, R. W., Davies, P. and Griffiths, K. (1975b). *Biochem. J.* **150**, 71–75.

Power, R. F., Conneely, O. M. and O'Malley, B. W. (1992). *Trends Pharmacol.* **13**, 318–323.

Proctor, A. J., Coombs, L. M., Cairns, J. P. and Knowles, M. A. (1991). *Oncogene* **6**, 789–795.

Prous, J. Mealy, N. and Castaner, J. (1995). *Drugs of the Future* **20**, 30–32.

Pruitt, V. L., Berlin, K. D. and Hirsch, K. S. (1990). *Org. Prep. Proced. Int.* **22**, 235–244.

Puri, V., Kamboj, V. P., Chandra, H., Ray, S., Kole, P. L., Dhawan, B. N. and Anand, N. (1988). Results of multicentric trial of centchroman. *In* 'Pharmacology for Health in Asia' (Dhawan, B. N., Agrawal, K. K., Arora, R. B., Parmar, S. S., eds), pp. 439–447. Allied Publishers, New Delhi.

Rabasseda, X., Mealy, N. and Castaner, J. (1995). *Drugs of the Future*, **20**, 235–237.

Raisz, L. G. (1988). *New Engl. J. Med.* **318**, 818–828.

Rankin, J. C., Ledford, B. E. and Baggett, B. (1979). *Biol. Reprod.* **20**, 399–404.

Rastinejad, F., Polverini, P. J. and Bouck, N. P. (1989). *Cell* **56**, 345–355.

Rastogi, R. K. (1972). *Steroid Lipids Res.* **3**, 59–64.

Rastogi, R. K and Chieffi, G. (1975). *Gen. Comp. Endocr.* **26**, 79–91.

Ratna, S., Roy, S. K., Ray, S., Kole, P. L., Salman, M., Madhusudanan, K. P., Sircar, K. P. and Anand, N. (1986). *Drug Development Res.* **7**, 173–178.

Ratna, S., Mishra, N. C., Ray, S. and Roy, S. K. (1994). *J. Basic Applied Biomed.* **2**, 31–36.

Rauschning, W. and Pritchared, K. I. (1994). *Breast Cancer Res. Treat.* **31**, 83.

Ray, S. and Sharma, I. (1987). *Pharmazie* **42**, 656–661.

Ray, S., Grover, P. K. and Anand, N. (1970). *Ind. J. Chem.* **8**, 961–963.

Ray, S., Grover, P. K. and Anand, N. (1971). *Ind. J. Chem.* **9**, 727–728.

Ray, S., Grover, P. K., Kamboj, V. P., Setty, B. S., Kar, A. B. and Anand, N. (1976). *J. Med. Chem.* **19**, 276–279.

Ray, S., Singh, M. M., Agarwal, A. K. and Kamboj, V. P. (1987). *Contraception* **35**, 283–287.

Ray, S., Tandon, A., Dwivedy, I., Wilson, S. R., O Neil, J. P. and Katzenellenbogen, J. A. (1994). *J. Med. Chem.* **37**, 696–700.

Raymont, A., Arronet, G. H. and Arrata, W. S. M (1969). *Int. J. Fertil.* **4**, 141.

Riggs, B. L., Wahner, H. W., Dunn, W. L., Mazess, R. B., Offord, K. P. and Melton, L. J. (1981). *J. Clin. Invest.* **67**, 328–335.

Riis, B. J. (1995). *The Amer. J. Med.* **98**(Suppl. 2A). 2A-29S.

Robbins, S. L. and Cotran, R. S. (1979). *In* 'Pathologic Basis of Disease'. (Robbins, Cotran and Kumar, V., eds), W. B. Saunders Company, Philadelphia, PA.

Robert, W. B., Floyd, E. E. and Counsell, R. E. (1978). *J. Med. Chem.* **212**, 1007.

Robertson, D. W., Katzenellenbogen, J. A., Long, D. J., Rorke, E. A. and Katzenellenbogen, B. S. (1982). *J. Steroid Biochem.* **16**, 1–13.

Rochefort, H. (1987). *Trends Pharmacol.* **8**(4), 126–128.

Rochefort, H., Garcia, M. and Borgna, J. L. (1979). *Biochem. Biophys. Res. Commun.* **88**, 351–357.

Ronald, E. S., Lynn, B. M., Wilbur, K. S., Jong, M. W. and Leslie, J. B. (1987). *Steroids* **50**, 147–161.

Rose, C., Theilade, K., Boesen, E., Salimtschik, M., Dombernowsky, P., Brunner, N., Kjaer, M. and Mowidsen, H. T. (1982). *Breast Cancer Res. Treat.* **2**, 395–400.

Rose, C., Thorpe, S. M., Mouridsen, H. T., Anderson, J. A., Brincker, H. and Andersen, K. W. (1983). *Breast Cancer Res. Treat.* **3**, 77–84.

Ross, M. H., Reith, E. J. and Romrell, L. J. (1989). 'Histology a Text and Atlas' (Kimberly Kist), Second edn. Williams & Wilkins. Baltimore, MD.

Roy, S., Greenblatt, R. B. and Mahesh, V. B. (1964c). *Acta Endocrinol.* **47**, 657–668.

Roy, S., Mahesh, V. B. and Greenblatt, R. B. (1964a). *Acta Endocrinol.* **47**, 669–675.

Roy, S., Mahesh, V. B. and Greenblatt, R. B. (1964b). *Acta Endocrinol.* **47**, 645–656.

Roy, S., Kumari, G. L., Madoiya, K., Prakash, V. and Ray, S. (1976). *Fert. Steril.* **27**, 1108–1110.

Roy, S., Dutta, J. K. and Kumari, G. L. (1979). *In* 'Recent Adv. Reprod. and Fertil.' (Talwar, G. P., ed.) p. 359. Elsevier, North-Holland.

Rubin, R. D. (1991). *Am. J. Med. Sci.* **301**, 281–298.

Ruehle, P. H., Browne, C. E., Vickery, E. H., Beller, N. R., Eisenbraun, E. J., Loghry, R. A. and van der Helm, D. (1980). *J. Med. Chem.* **23**, 1410–1414.

Ruemotz, P. C., Bagley, J. R. and Mokler, C. M. (1983). *Biochem. Pharmacol.* **32**, 2941–2947.

Ruenitz, P. C. and Bagley, J. R. (1985). *Drug Metab. Dispos.* **13**, 582–586.

Ruiz-Velasco, V., Rosas-Arceo, J. and Matute, M. M. (1979). *Int. J. Fert.* **24**, 61–64.

Ryan, M. J., Clark, K. E., VanOrden, D. E., Farley, D., Edvinsson, L., Sjoberg, N. O., vanOrden, III, L. S. and Brody, M. J. (1974). *Prostaglandins* **5**, 257–268.

Sabbah, M., Redeuilh, G. and Baulieu, E. E. (1989). *J. Biol. Chem.* **264**, 2397–2400.

Saeed, A., Sharma, A. P., Durani, N., Jain, R., Durani, S. and Kapil, R. S. (1990). *J. Med. Chem.* **33**, 3210–3216.

Saksena, S. K. and Harper, M. J. K. (1972). *Prostaglandins* **2**, 511.

Salman, M., Ray, S., Anand, N., Agarwal, A. K., Singh, M. M., Setty, B. S. and Kamboj, V. P. (1986). *J. Med. Chem.* **29**, 1801–1803.

Sangwan, N. K. and Rastogi, S. N. (1981). *Ind. J. Chem.* **20B**, 140–143.

Sangwan, N. K., Prasad, M., Rastogi, S. N., Jehan, Q. and Setty, B. S. (1986). *Ind. J. Chem.* **25B**, 832–837.

Santen, R. J., Santner, S., Davis, S., Veldhuis, J., Samojlik, E. and Ruby, E. (1978). *J. Clin. Endocr. Metabol.* **47**, 1257–1265.

Santen, R. J., Demers, L. M., Adlercreutz, H., Harvey, H., Santner, S., Sanders, S. and Lipton, A. (1989). *J. Clin. Endocrinol. Metab.* **68**, 99–106.

Santen, R. J., Samojlik, E. and Worgnl, T. J. (1991). Aminoglutethimide, product profile. *In* 'Parmanual, a New Comprehensive Guide to the Therapeutic Use of Aminoglutethimide', (Santen, R. J. and Henderson, I. C., eds), pp. 101–160. Karger, Basel.

Sasaki, G. H. and Leung, B. S. (1975). *Cancer* **35**, 645–651.

Scali, G., Mansanti, P., Zurlo, A., Miola, F., Parti, M., Nardelli, A., Magnani, D., Zagathi, A. M., Kambanis, J. and Bufalino, L. (1991). *Curr. Ther. Res.* **49**, 1004–1010.

Scharla, S. H., Minne, H. W., Waibel-Treber, S., Schaible, A., Lempert, U. G., Wuster, C., Leyendecker, G. and Ziegler, R. (1990). *J. Clin. Endocrinol. Metab.* **70**, 1055–1061.
Schieweek, K., Bhatnagar, A. S. and Matter, A. (1988). *Cancer Res.* **48**, 834–838.
Schultz, G. S. and Grant, M. B. (1991). *Eye* **5**, 178–180.
Schwartz, N. B. (1969). *Recent Prog. Horm. Res.* **25**, 1–55.
Schweigerer, L. and Fotsis, T. (1992). *Eur. J. Pediatr.* **151**, 472–476.
Segal, J. S. and Nelson, W. O. (1958). *Proc. Soc. Exp. Biol. Med.* **98**, 431–436.
Segal, S. J. and Nelson, W. O. (1961). *Anat. Rec.* **139**, 273.
Seibel, M. J., Cosman, F., Shen, V., Gordon, S., Dempster, D. W., Ratchiff, A. and Lindsay, R. (1993). *J. Bone Mineral. Res.* **8**, 881–889.
Sekiya, S. and Takamizawa, H. (1976). *J. Obstet. Gynaec. Br. Commonw.* **83**, 183–186.
Senior, B. E., Cawood, M. L., Oakey, R. E., McKiddie, J. M. and Siddle, D. R. (1978). *Clin. Endocr.* **8**, 381–389.
Serin, D. (1993). *Breast Cancer Res. Treat.* **28**, Abst. 20.
Serwin, P. F., McMullan, P. C. and Covey, D. F. (1989). *J. Med. Chem.* **32**, 651–658.
Sharma, A. P., Saeed, A., Durani, S. and Kapil, R. S. (1990a). *J. Med. Chem.* **33**, 3222–3229.
Sharma, A. P., Saeed, A., Durani, S. and Kapil, R. S. (1990b). *J. Med. Chem.* **33**, 3216–3222.
Shen, V., Rifas, L., Kohler, G. and Peck, W. A. (1986). *J. Bone Miner. Res.* **1**, 243–249.
Sherman, B. M., Chapler, F. K., Crickard, K. and Wycoff, D. (1979). *J. Clin. Invest.* **64**, 398–404.
Siddiqui, U., Heald, P. J. and Watson, J. (1976). *J. Endocrinol.* **69**, 39.
Sidky, Y. A. and Borden, E. C. (1989). *Cancer Res.* **47**, 5155–5161.
Singh, M. M. and Kamboj, V. P. (1981). *Biol. Reprod.* **25**, 171–180.
Singh, M. M., Wadhwa, V. and Kamboj, V. P. (1982). *Ind. J. Exp. Biol.* **20**, 448–451.
Singh, M. M., Wadhwa, V., Sethi, N. and Kamboj, V. P. (1983). *J. Reprod. Fert.* **68**, 165–170.
Singh, M. M., Bhalla, V., Wadhwa, V. and Kamboj, V. P. (1986). *J. Reprod. Fert.* **76**, 317–324.
Skinner, S. J. M. and Akhtar, M. (1969). *Biochem. J.* **114**, 75–81.
Slemenda, C., Hui, S. L., Longoope, C. and Johnston, C. C. (1987). *J. Clin. Invest.* **80**, 1261–1269.
Smith, C. L., Conneely, O. M. and O'Malley, B. W. (1993). *Proc. Natl Acad. Sci. USA* **90**, 6120–6124.
Smith, O. W. and Kistner, R. W. (1963). *J. Am. Med. Assoc.* **184**, 122–130.
Solmssen, U. V. (1945). *Chem. Rev.* **37**, 481–598.
Sommerville, B. A., Luck, M., Scanes, C. G., Harvey, S. and Chadwick, A. (1980). *IRCS Med. Sci. Libr. Compend.* **8**, 864–865.
Souma, J. A., Marshall, L. D. and Abdul Karim, R. W. (1972). *Toxic Appl. Pharmacol.* **23**, 339–343.
Srivastava, V., Ray, S. and Singh, M. M. (1993). *Ind. J. Chem.* **32B**, 569–571.
Stampfer, M. J., Willett, W. C., Coltiz, G. A., Bosner, B., Speizer, F. E. and Henneknes, C. H. (1985). *N. Engl. J. Med.* **313**, 1044–1049.
Stanek, J., Alder, A., Bellus, D., Bhatnagar, A. S., Hausler, A. and Schieweck, K. (1991). *J. Med. Chem.* **34**, 1329–1334.
Stein, R. C., Dowsett, M., Hedbey, A., Davenport, J., Gazet, J. C., Ford, H. T. and Coombes, R. C. (1990). *Cancer Chemother. Pharmacol.* **26**, 75–78.
Steingold, K. A., Cedars, M., Lu, J. K. H., Randle, D., Judd, H. L. and Meldrum, D. R. (1987). *Obstet. Gynecol.* **69**, 403–411.
Stetler-Stevenson, W. G., Krutzsch, H. C. and Liotta, L. A. (1989). *J. Biol. Chem.* **264**, 17,374–17,378.

Stevenson, J. C., Abeyasek, G., Hillyard, C. J., Phang, K. G., Macintyr, I., Campbell, S., Lane, G., Townssend, P. T., Young, O. and Whitehea, M. I. (1983). *Eur. J. Clin. Invest.* **13**, 481–487.

Stewart, P. J. and Stern, P. H. (1986). *Endocrinology* **118**, 125–131.

Stobaugh, J. F., Magarian, R. A., Pento, J. T. (1982). *J. Pharm. Sci.* **71**, 1126–1128.

Sutherland, C. M., Muchmore, J. H., Carter, R. D. (1985). *South Med. J.* **78**, 987–989.

Sutherland, R. L., Walts, C. K. W., Ruenitz, P. C. (1986). *Biochem. Biophys. Res. Commun.* **140**, 523–529.

Sutherland, R., Mester, J., Baulieu, E. E. (1977), *Nature* **267**, 434–435.

Szamel, I., Hermann, I., Borvendeg, J., Vincze, B., Hindy, I., Kerpel-Fronius, S. and Eckhardt, S. (1979). *Acta Med. Hung.* **36**, 341.

Tagami, K., Kamioka, H., Sumitani, K., Kawata, T., Lenarcic, B., Turk, V., Kakegawa, H. and Katunuma, N. (1994). *FEBS Lett.* **342**, 308–312.

Tamargo, R. J., Bok, R. A., Brem, H. (1991). *Cancer Res.* **51**, 672–675.

Tanaka, M., Abe, K., Ohnami, S., Adachi, I., Yamaguchi, K. and Miyakawa, S. (1978). *Jap. J. Clin. Oncol.* **8**, 141–148.

Tanaka, Y., Sekiguchi, M., Sawamoto, T., Hata, T., Esumi, Y., Sugai, S., Ninomiya, S. (1994). *Eur. J. Drug Metab. Pharmacol.* **19**, 47–58.

Taubert, H. D., Schwantje, R. and May, E. (1969). *Acta Endocrinol. Suppl.* **138**, 202.

Taylor, S. and Folkman, J. (1982). *Nature* **297**, 307–312.

Terenius, L. (1971). *Acta Endocrinol.* **66**, 431–447.

Tewari, S. C. and Rastogi, S. N. (1980). *Ind. J. Chem.* **19B**, 1060–1064.

Thomas, M. L., Hope, W. G. and Ibarra, M. J. (1987). *S. Int. Congr. Excerpta Med.* 735 (*Calcium Regul. Bone Metab.*) 260–265.

Thompson, E. A. and Siiteri, P. K. (1974). *J. Biol. Chem.* **249**, 5364–5372.

Tominaga, T., Yoshida, Y., Matsumoto, A., Hayashi, K., Kosaki, G. (1993). *Anticancer Res.* **13**, 661–665.

Truong, H., Geynet, C., Millet, C., Soulignac, O., Bucourt, R., Vignau, M., Torelli, V. and Baulieu, E. E. (1973). *FEBS Lett.* **35**, 289–294.

Truss, M. and Beato, M. (1993). *Endocrinol. Rev.* **14**, 459–479.

Tsai, C. C. and Yen, S. S. C. (1971). *J. Clin. Endocr. Metab.* **33**, 917–923.

Tsuda, M., Kitazaki, T., Ito, T. and Fujita, T. (1986). *J. Bone Min. Res.* **1**, 207–211.

Tsutsumi, N., Kawashima, K., Arai, N., Nagata, H., Kojima, N., Ujiie, A. and Enmdo, H. (1994). *Bone Mineral* **24**, 201–209.

Turken, S., Siris, E., Seldin, D., Flaster, E., Hyman, G. and Lindsay, R. (1989). *J. Natl. Cancer Inst.* **81**, 1086–1088.

Turner, R. T., Vandersteenhoven, J. J. and Bell, N. H. (1987a). *J. Bone Miner. Res.* **2**, 115.

Turner, R. T., Wakley, G. K., Hannon, K. S. and Bell, N. H. (1987b). *J. Bone Miner. Res.* **2**, 449–456.

Turner, R. T., Wakley, G. K., Hannon, K. S. and Bell, N. H. (1988). *Endocrinology* **122**, 1146–1150.

Turner, R. T., Bell, N. H. and Gay, C. V. (1993). *Poultry Sci.* **72**, 728–740.

Uberoid, N. K., Hendry, L. B., Muldoon, T. G., Myers, R. B., Segaloff, A., Bransome, E. D. and Mahesh, V. B. (1985). *Steroids* **45**, 325–340.

Vaidya, R., Joshi, U., Maherji, P., Rege, N., Betrabet, S., Joshi, L., Seth, A. and Devi, P. K. (1977). *Ind. J. Exp. Biol.* **15**, 1173–1176.

van de Velde, P., Nique, F., Bouchoux, F., Bremaud, J., Hameau, M.-C., Lucas, D., Moratille, C., Viet, S., Philibert, D. and Teutsch. (1994). *J. Steroid Biochem. Molec. Biol.* **48**, 187–196.

Vandenberg, G. and Yen, S. S. C. (1973). *J. Clin. Endocrinol.* **37**, 356–365.

Verma, P., Arora, P. K., Salman, M., Ray, S. and Srimal, R. C. (1989). *Ind. J. Pharm. Sci.* **51**, 48–50.
Vignon, F. and Rochefort, H. (1976). *Endocrinology* **98**, 722–729.
Villee, C. A. and Hagerman, D. D. (1957). *Endocrinology* **60**, 552–558.
VonAngerer, E. and Erber, S. (1992). *J. Steroid Biochem. Molec. Biol.* **41**, 557–562.
VonAngerer, E. and Strohmeier, J. (1987). *J. Med. Chem.* **30**, 131–136.
VonAngerer, E., Prekajac, J. and Strohmeier, J. (1984). *J. Med. Chem.* **27**, 1439–1447.
VonAngerer, E., Prekajac, J. and Berger, M. R. (1985). *Eur. J. Cancer Clin. Oncol.* **21**, 531–537.
VonAngerer, E., Knebel, N., Kager, M. and Ganss, B. (1990). *J. Med. Chem.* **33**, 2635–2640.
VonAngerer, E., Birnboeck, H., Kager, M. and Mancher, A. (1992). *J. Cancer Res. Clin. Oncol.* **118**, 339–343.
VonAngerer, E., Biberger, C., Holler, E., Koop, R. and Leichtl, S. (1994). *J. Steroid Biochem. Molec. Biol.* **49**, 51–62.
Wakeling, A. E. and Bowler, J. (1987a). *J. Endocrinol.* **112**, R7-R10.
Wakeling, A. E. and Bowler, J. (1987b). *J. Steroid Biochem.* **31**, 645–653.
Wakeling, A. E. and Bowler, J. (1992). *J. Steroid Biochem. Molec. Res.* **43**, 173–177.
Wakeling, A. E. and Slater, S. R. (1980). *Cancer Treat. Rep.* **64**, 741–744.
Wakeling, A. E. and Valcaccia, B. (1983). *J. Endocrinol.* **99**, 455–465.
Wakeling, A. E., Valcaccia, B., Newboult, E. and Green, L. R. (1984). *J. Steroid Biochem.* **21**, 111–120.
Ward, H. W. C. (1973). *Br. Med. J.* **1**, 13–14.
Watanabe, M., Tanaka, H., Koizumi, H., Tanimoto, Y., Toni, R. and Yanagita, T. (1980). *Jitchuken Zenrinsho Kenkyicho* **6**, 1–36.
Watnick, A. S. and Neri, R. O. (1968). *Acta Endocrinol. Kbh.* **59**, 611–621.
Weinstein, I., Seedman, S. and Veldhuis, M. (1975a). *Proc. Soc. Exp. Biol. Med.* **149**, 181–184.
Weinstein, I., Belitsky, R., Seedman, S., Fregly, M. J. and Thrasher, T. N. (1975b). *Biochem. Pharmacol.* **24**, 1583–1588.
Welsch, C. W., Goodrich-Smith, M., Brown, C. K., Miglorie, N. and Clifton, K. H. (1981). *Eur. J. Cancer Clin. Oncol.* **17**, 1255–1258.
Whitead, M. I., Lane, G., Townsend, P. T., Abeyasekera, G., Hillyard, C. J. and Stevenson, J. C. (1982). *Acta Obstet. Scand.* **106**(Suppl.), 27–32.
Whitson, S. W., Dawson, L. R., Jee, W. S. S. (1978). *Endocrinology* **103**, 2006–2010.
Wilking, N., Applegren, L. E., Carlstrom, K., Nordgren, T. and Theve, N. O. (1981). *Revs Endocrine-related Cancer* **9**(Suppl.), 223–239.
Wilking, N., Applegren, L. E., Carlstrom, K., Pousette, A. and Theve, N. O. (1982). *Acta Pharmac. Tox.* **50**, 161–168.
Willis, A. L. (1970). *Pharmac Res. Commun.* **2**, 297–304.
Willis, K. J., London, D. R. and Butt, W. R. (1976). *J. Endocrinol.* **69**, P51.
Wilson, P. W. F., Garrison, R. J. and Castelli, W. P. (1985). *N. Engl. J. Med.* **313**, 1038–1043.
Wilson, S. C. and Cunningham, F. J. (1981). *J. Endocr.* **88**, 309–316.
Woltering, E. A., Barrie, R., O'Dorisio, T. M., Arce, D., Ure, T., Cramer, A., Holmes, D., Robertson, J. and Fassler, J. (1991). *J. Surg. Res.* **50**, 245–251.
Wouters, W., De Coster, R., Van Dun, J., Krekels, M. D. W. G., Gelder, J., Sanz, G., Venet, M. and Jannen, M. (1990). *J. Steroid Biochem. Molec. Biol.* **37**, 1049–1054.
Wouters, W., Van Dun, J., Dillen, A., Coene, M. C., Cools, W. and De Coster, R. (1992). *Cancer Res.* **52**, 2841–2846. 324–328.
Wronski, T. J., Yen, C. F. and Scott, K. S. (1991). *J. Bone Mineral. Res.* **6**, 387–394.
Wyche, J. H. and Noteboom, W. D. (1979). *Endocrinology* **104**, 1765–1773.

Yamazaki, I., Shino, A., Shimizu, Y., Shirakawa, Y. and Kinoshita, M. (1986a). *Life Sci.* **8**, 951–958.

Yamazaki, I., Shino, A. and Tsukuda, R. (1986b). *J. Bone Mineral Metab.* **3**, 205–210.

Zaccheo, T., Giudici, D., Lombardi, P. and Di Salle, E. (1989). *Cancer Chemother. Pharmacol.* **23**, 47–50.

Peroxidic Antimalarials

CHARLES W. JEFFORD

Department of Organic Chemistry, University of Geneva, CH–1211 Geneva 4, Switzerland

1 Introduction

Malaria has been known since ancient times and today it is one of the major causes of morbidity and death in the world. It is estimated that some 2.5 billion people are at risk. The areas most affected are the northern part of South America, Central America, sub-Saharan Africa, the Indian subcontinent, South-East Asia, Vietnam, Indonesia, and the southern rim of the

ADVANCES IN DRUG RESEARCH, VOL. 29
ISBN 0-12-013329-6

Pacific basin. Every year some 300 million new clinical cases arise, killing between 2 and 3 million, most of whom are young African children (Anonymous, 1994).

The disease is transmitted by the bite of the female *Anopheles* mosquito which infects humans with one of four species of protozoa, *Plasmodium malariae*, *vivax*, *ovale* or *falciparum*. The parasites invade red blood cells and multiply causing intermittent fever, anaemia, and debilitation. The gravity of the infection depends on the species. *P. vivax* and *P. ovale* are usually non-lethal, but pass through a dormant liver stage which brings about relapses long after the original infection. *P. falciparum* is the most dangerous because the level of parasitaemia is the highest. Moreover, the parasitized erythrocytes tend to block the microvasculature of vital organs, such as the brain, frequently killing the host in a matter of hours.

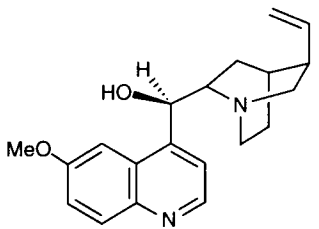
1.1 CHEMOTHERAPY BEFORE 1960

Before embarking on the theme of the chapter, it is instructive to briefly review the drugs that are available for treating malaria. Surprisingly, they are remarkably limited in number and structural variety. Apart from the traditional remedy, quinine (**1**), an alkaloid extracted from the bark of the cinchona tree (Duran-Reynal, 1946), the chief synthetic drugs are quinoline derivatives, such as primaquine (**2**), pamaquine (**3**), amodiaquine (**4**), chloroquine (**5**) and an acridine derivative, mepacrine (**6**). They were developed as replacements for **1**, the production of which was a Dutch monopoly until 1930 (Sweeney and Strube, 1979; Black *et al.*, 1981).

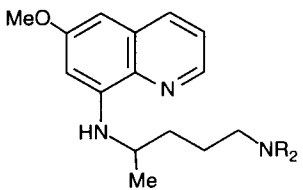
In the period 1939–1945, proguanil or paludrin (**7**), its metabolite, cycloguanil (**8**), pyrimethamine (**9**), and trimethoprim (**10**) were rationally designed as inhibitors of dihydrofolate reductase. They act selectively by blocking the synthesis of DNA by the parasite at the schizont stage without affecting the analogous enzymes in the host (Hesp and Willard, 1984).

1.2 CHEMOTHERAPY AFTER 1960

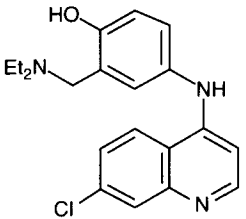
With this stock of drugs in hand for prophylaxis and cure, malaria might have been regarded as a vanquished or at least a manageable disease (Russell, 1955). Unfortunately, this was not the case. In 1960, or thereabouts, several disturbing trends appeared. Malaria began increasing in most endemic countries, with the possible exception of China (Brown, 1992). The incidence of the malignant parasite *P. falciparum* became more marked, notably in India and Brazil. In addition, new strains resistant to chloroquine emerged (Peters, 1987; Wernsdorfer, 1991, 1994). Accordingly, certain measures were taken to remedy the situation.



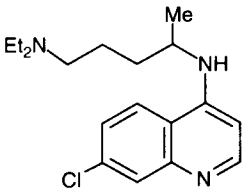
1



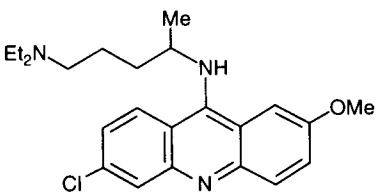
2 R = H
3 R = Et



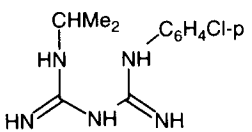
4



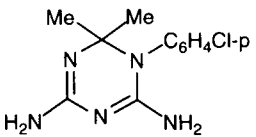
5



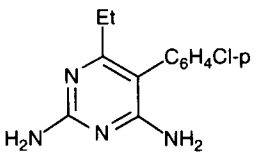
6



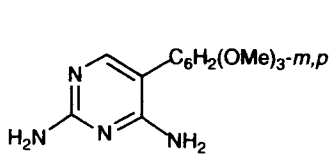
7



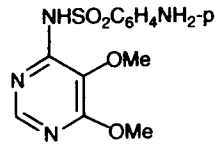
8



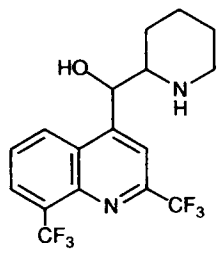
9



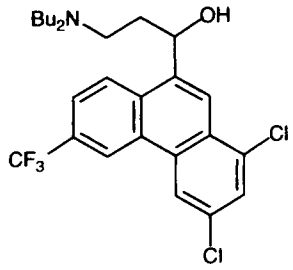
10



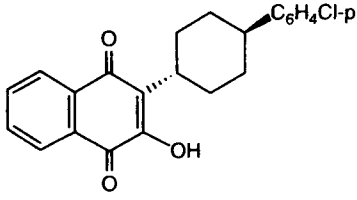
11



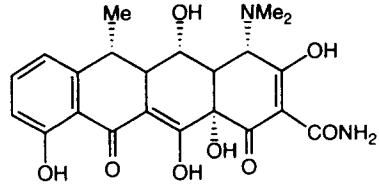
12



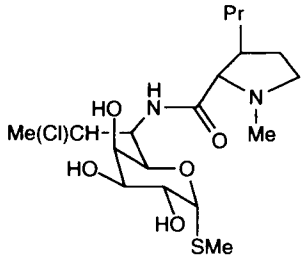
13



14



15



16

Sulfonamides, already known as inhibitors of dihydrofolate synthase, were brought to the fore, especially sulfadoxine (**11**). Its synergistic combination with **9**, known as Fansidar®, was deployed as a second-line treatment when chloroquine failed. Yet another quinoline derivative, mefloquine (**12**), was developed under the aegis of the World Health Organization. For prophylaxis, **12** was used in conjunction with **9** and **11** (Fansimef®), the idea being that the triple combination would foil the development of resistance

to **12** by the parasite. Other drugs were salvaged from earlier discovery programs, such as halofantrine (**13**) and atovaquone (**14**), a powerful antagonist of plasmodial ubiquinone. Certain antibiotics, doxycycline (**15**) and clindamycin (**16**), have been enlisted as adjuncts to quinine for treatment of chloroquine-resistant falciparum malaria (Rieckmann, 1984; Clara, 1990; Kremsner, 1990).

1.3 DISADVANTAGES OF TRADITIONAL ANTIMALARIALS

In the interim, many of the older drugs (**2–4**, **6**) were found to be too toxic and were either abandoned or only employed in a specific context (e.g. **2**). The hopes raised by the new and resurrected drugs were short-lived. Most turned out to be palliatives at best, while others proved unacceptable due to toxic side-effects (Oaks *et al.*, 1991). The protection supposedly conferred by Fansidar on mefloquine against it inducing resistance in the parasite has been questioned on the basis of the different pharmacokinetics of the three constituents (White, 1987). In any event, the risk of Stevens–Johnson syndrome and mortality on taking **11** renders it unsafe for prophylaxis (Miller *et al.*, 1986).

Mefloquine, like chloroquine, soon encountered resistance by new strains of *P. falciparum* in parts of Africa and in Thailand. Furthermore, it is neurotoxic, causing stupor and psychiatric disorders. Halofantrine, an expensive drug like mefloquine, is now deemed unsuitable for prophylaxis because of erratic absorption and recent instances of cardiotoxicity. The utility of doxycycline may be vitiated by the long treatment times needed for cure and the attendant risk of gastrointestinal toxicity and photosensitivity. Quinine itself is never totally effective. Its administration is often accompanied by notoriously toxic side-effects, collectively termed cinchonism. Consequently, only chloroquine is left, but of diminished effectiveness, and presently restricted to use in the Middle East, Mexico and Central America above Panama.

Originally, only *P. falciparum* became resistant to chloroquine, but recently cases of resistance by *P. vivax* have been reported in Papua New Guinea (Schuurkamp *et al.*, 1992). Such resistance is not specific to the drug in question, chloroquine, but usually embraces the other quinoline antimalarials.

Other ineluctable trends are global warming, which will enlarge the malarial domain, and the alarming expansion of the world population. There is no doubt that the incidence of *P. falciparum* and multi-drug-resistant strains will continue to rise and that malaria will encroach into economically prosperous areas in South-East Asia. Therefore, the search for new drug candidates is a matter of utmost urgency. Experience has shown that variations and reformulations of existing drug types (*vide supra*) are not going

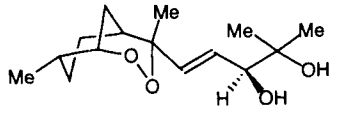
to provide an adequate response to the growing menace. Instead, studies will need to focus on compounds belonging to entirely new chemical classes. Other burning questions such as the development of vaccines, antivector measures, and the human aspects of malaria are beyond the scope of this chapter and have received attention elsewhere (Peters, 1994).

2 The Advent of Peroxidic Antimalarials

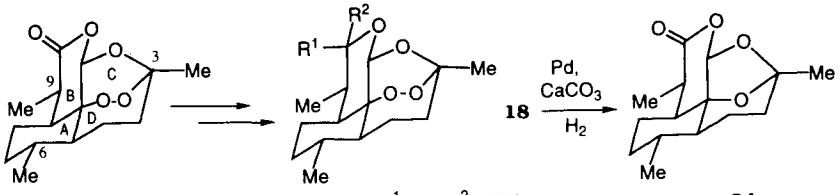
The discovery in 1979 that two sesquiterpenes derived from Chinese medicinal plants, yingzhaosu A (**17**) and qinghaosu or artemisinin (**18**), display powerful antimalarial activity was not only opportune, but fulfilled the aforementioned criteria. Yingzhaosu A (**17**) is obtained from the roots of a perennial vine, *Artabotrys uncinatus* (Lam.) Merr., which grows sparsely in the coastal regions of Guandong and Hainan island (Liang, *et al.*, 1979). It is a bridged bicyclic peroxide, which is not in itself a rarity for a natural product, but uncommon as a pharmacological entity.

Conversely, **18** is more common in occurrence, but almost unique in structure (Liu *et al.*, 1979). Artemisinin, the preferred name, is the active principle of *Artemisia annua* Linn., otherwise known as sweet or annual wormwood, a shrub that grows wild nearly anywhere and which is widespread in China. Infusions of the leaves of the plant have been dispensed by Chinese traditional herbalists as an antipyretic for more than 1000 years (Klayman, 1985; Luo and Shen, 1987; Butler and Wu, 1992).

The structure of **18** proved difficult to elucidate at first, although it was known to possess the cadinane or amorphane skeleton (Stefanovic *et al.*,



17



18

19 $R^1 = H, R^2 = OH$
20 $R^1 = OH, R^2 = H$
21 $R^1 = OMe, R^2 = H$
22 $R^1 = OEt, R^2 = H$
23 $R^1 = H, R^2 = O_2C(CH_2)_2CO_2Na$

24

1972). X-ray analysis revealed that **18** was a tetracyclic molecule enclosing a 1,2,4-trioxane ring locked in a boat conformation (Qinghaosu Research Group, 1980).

An important difference between **17** and **18**, which had a bearing on drug development, is their relative natural abundance. Apparently, **17** is not a constituent of the plant. It is formed after the roots have been collected and stored in the dark for 2 months or more (Liang, 1985). In other words, securing **17** in useful quantities is difficult. In contrast, *A. annua* is easy to cultivate and harvest. Artemisinin (**18**) is obtained by extracting the leaves and flowering tops of the plant. The yields vary from 0.01 to 0.50% depending on the plant variety, habitat, and growing conditions. Consequently, from the start, kilogram quantities of **18**, as a colourless, crystalline solid, melting point (m.p.) 159°C, were available for chemical studies.

A particular reaction of **18**, which has been greatly exploited, is reduction by sodium borohydride to the α- and β-lactols **19** and **20**. Acid-catalysed alkylation of the epimeric lactols or dihydroartemisinins gives mainly the β-ethers, whereas the α-esters preponderate on base-catalysed acylation (Li *et al.*, 1981). In addition, several carbonates and sulfonates were also prepared. Some 20 derivatives exhibited antimalarial activity 10 times greater than that of **18**. Finally, the choice of drug candidates among these, so-called, first-generation artemisinin derivatives was winnowed down to β-artemether (**21**), β-arteether (**22**), and sodium artesunate (**23**) (Luo and Shen, 1987). The peroxide link was shown to be part of the pharmacophore, since deoxyartemisinin (**24**), obtained by catalytic hydrogenation of **18**, was devoid of activity (China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials, 1982a,b). In China, the efficacy of **18**, **21** and **23** in the clinic was demonstrated early on by the successful treatment of millions of patients (Hien and White, 1993).

The preceding results, in addition to their intrinsic worth, greatly stimulated new research with far-reaching repercussions. The most obvious extension was the elaboration of improved, first-generation derivatives of **18**. Next, total syntheses of **17** and **18** were undertaken. The biogenetic precursor to **18**, artemisinic acid, which occurs more abundantly in the plant, has been turned to advantage and converted to **18** by simple procedures. Other naturally occurring peroxides were sought out and screened for activity. More importantly, new methods have been devised for the synthesis and design of structurally simpler peroxides, 1,2,4-trioxanes, and 1,2,4,5-tetroxanes displaying high artemisinin-like activity. Investigations into the nature of the pharmacophore and the mode of action responsible for killing the parasite have enabled the design of peroxidic antimalarials to be refined still further. In summary, the discovery of **17** and **18** has begun a new chapter in the chemotherapy of malaria.

It should be stated that very little has been reported on **17** while the literature dealing with **18** and its first-generation derivatives is abundant.

Three useful reviews covering practically all aspects of artemisinin (**18**) have recently appeared (Woerdenbag *et al.*, 1990, 1994; Meshnick *et al.*, 1996).

3 Assays for Antimalarial Activity

An integral part of drug discovery is the assay used to gauge activity. In the past, various tests have been employed, the discussion of which is beyond the scope of this chapter (Sweeney and Strube, 1979; Peters, 1980; Howells, 1982). For the purposes of understanding the data, which will be subsequently presented, the main *in vitro* and *in vivo* methods used since 1982 are briefly outlined.

3.1 *IN VITRO* ACTIVITY. THE DESJARDINS TEST

In vitro activity is usually assayed as a primary screen. The test sample is compared with a reference compound (artemisinin, chloroquine, etc.) with respect to its ability to inhibit the uptake of [^{3}H] hypoxanthine by *P. falciparum* clones in the culture medium (Desjardins *et al.*, 1979; Milhous *et al.*, 1985). The purine base is required for the synthesis of DNA by the growing parasite. The radioactivity so incorporated into the parasites is measured in a scintillation counter enabling the effect of the added sample to be determined. Concentrations of sample inhibiting the growth by 50% and 90% (IC_{50} and IC_{90}) are usually expressed in ng ml^{-1}. Two clones are used for estimating the effectiveness of the sample towards resistance. The Sierra Leone D-6 clone is sensitive to chloroquine, pyrimethamine and sulfadoxine (**5**, **9** and **11**), but resistant to mefloquine **12**. Conversely, the Indochina W-2 clone is resistant to **5**, **9** and **11**, but susceptible to (**12**).

3.2 *IN VIVO* ACTIVITY. THE PETERS TEST

On the basis of results obtained from *in vitro* testing, selected samples can then be evaluated by the '4-day' test (Peters *et al.*, 1975). The test sample is compared with a reference compound (artemisinin, chloroquine, etc.) with respect to its ability to suppress parasitaemia in the mouse. Male, Swiss albino mice (20 ± 2 g each) in groups of five are inoculated intravenously with about 10^7 infected red cells of *P. berghei* N or *P. yoelii* NS, which are sensitive and resistant to chloroquine, respectively. The samples to be tested are dissolved or suspended with sonication in a 0.2% solution of Tween 80 or 0.5% carboxymethyl cellulose and made up to yield doses such as 3, 10 and 30 mg kg^{-1}. The resulting sets of doses are administered to three groups of

mice once a day for four successive days either subcutaneously (s.c.) or orally (p.o.) following inoculation. On the fifth day, thin blood films are taken from each mouse, stained, examined, and the percentage of parasitized red cells noted. Effective dose levels for 50% and 90% suppression of parasitaemia (ED_{50} and ED_{90}) are obtained from the dose–activity curve and expressed in $mg\,kg^{-1}$.

3.3 *IN VIVO* ACTIVITY. THE RANE TEST

Another *in vivo* test, devised as a primary screen, measures the mortality of mice on receiving an inoculum of 10^6 infected red cells of *P. berghei* N (Osdene *et al.*, 1967; Ager, 1984). Normally, all mice die within 6 days or so. Extension of the life span of infected mice by s.c. administration of a single dose of sample to more than twice that of untreated mice indicates that the drug is active at the dose chosen. Survival beyond 60 days is regarded as a cure. Samples are usually made up in arachis oil by sonication into doses of 640, 320, 160, 80 and 40 $mg\,kg^{-1}$. The minimum effective dose (MED) is compared with the maximum tolerated dose (MTD), the dose that produces no more than one in five deaths due to toxicity.

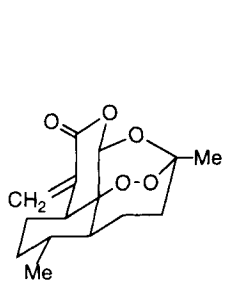
Other tests in monkeys are used at a later stage in drug development and will not be discussed here.

4 Naturally Occurring Antimalarial Trioxanes and Peroxides

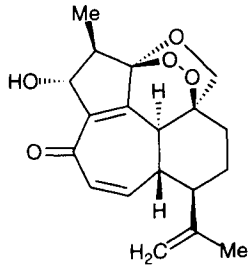
4.1 OCCURRENCE

At the time of its isolation, **18** was unprecedented in being the sole 1,2,4-trioxane occurring in Nature. Even today only three others are known. Artemisitene (**25**) has also been isolated from *A. annua* (Acton and Klayman, 1985). The other two are the diterpenes, caniojane (**26**) and its 1,11-epimer, isolated from the roots of *Jatropha grossidenta*, a plant growing in central Paraguay (Jakupovic *et al.*, 1988). It is likely that **26** would be active, but nothing has been reported about it to date. Artemisitene is about five times less active than **18** *in vitro* (Acton and Klayman, 1987).

Peroxides are quite common as genuine plant constituents, but only about a dozen have significant activity (Rücker *et al.*, 1991; Rücker, 1995). The cyclic peroxides **27–29** are representative and are reported to have IC_{50} values of about $1\,\mu g\,ml^{-1}$ compared with $0.01\,\mu g\,ml^{-1}$ found for artemisinin (**18**) under the test conditions. The most noteworthy are ascaridol derivatives, exemplified by sesquiascaridol **30**, which are more effective being about 10 times less active *in vitro* than **18**.



25



26

Further examination of the roots of *A. uncinatus* revealed a second potent antimalarial artefact, yingzhaosu C (**31**). Another active peroxide has been recently isolated from a species of Thai cardamom (*Amomum krevanh* Pierre) (Kamchonwongpaisan *et al.*, 1995). The molecule possesses an unusual dimeric pinane structure (**32**) and exhibits an IC_{50} value of 0.17 μM or 59 ng ml^{-1}.

4.2 TOTAL SYNTHESIS

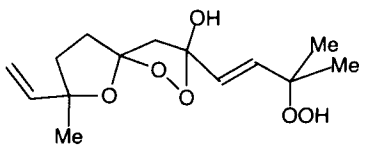
Just one synthesis of yingzhaosu A (**17**) has appeared so far, while many of artemisinin (**18**) have been reported. As several of these have been reviewed (Zaman and Sharma, 1991; Zhou and Xu, 1994), only the salient points will be presented. However, more recent results will be discussed and the merits of the various methods will be compared.

4.2.1 Synthesis of Yingzhaosu A

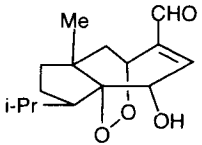
R-(−)-carvone (**33**) provides the desired chiral centres of **17** (Xu *et al.*, 1991). Epoxidation of **33**, rearrangement to the aldehyde followed by addition of methyl Grignard reagent, and dehydration, yield the methyl derivative **34**. Dye-sensitized photo-oxygenation of **34** in the presence of acid gives the hydroperoxide **35** which instantly undergoes Michael addition to create the bicyclic core structure **36**. Removal of the carbonyl group, extension of the vinyl chain and formal bis-hydroxylation produces **17**.

4.2.2 Syntheses of Artemisinin

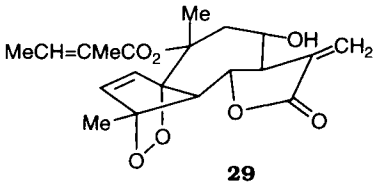
Many different chiral starting materials have been exploited to install a suitably functionalized A ring for elaboration into **18**. However, in each case, the trioxane part, ring C, is subsequently formed in much the same way. In



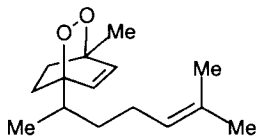
27



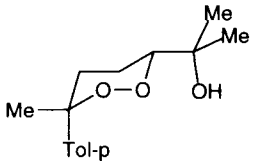
28



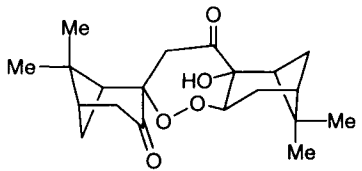
29



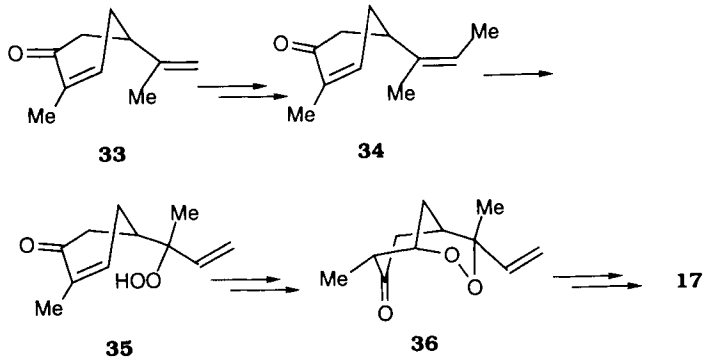
30



31

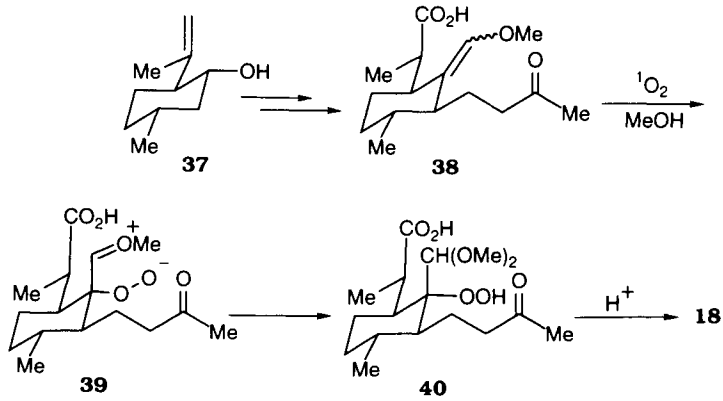


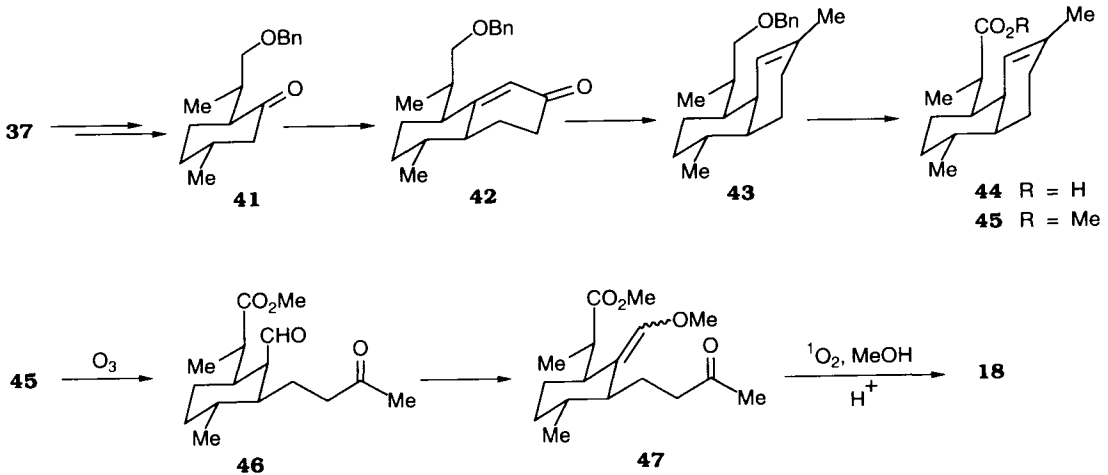
32

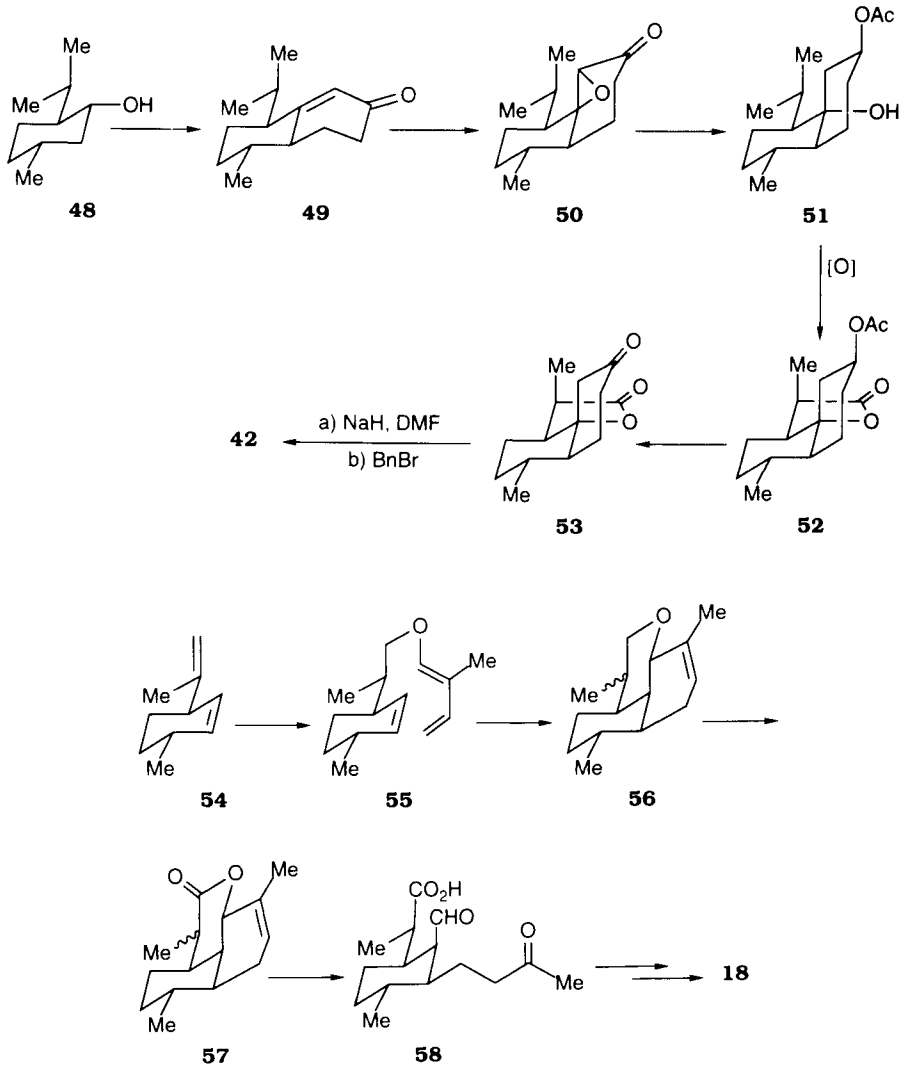


the first reported synthesis (Schmid and Hofheinz, 1983), (−)-isopulegol (**37**) is transformed into the E-methoxymethylidene derivative, **38**. Photo-oxygenation of its sodium salt in methanol is presumed to pass through the zwitterionic peroxide **39** which on quenching with methanol produces the hydroperoxy acetal **40**. Acidification of **40** with formic acid gives **18** in low (30%) yield. Nucleophilic attack by the hydroperoxy substituent on the carbonyl group creates the D-ring, while rings B and C form by acid-catalysed attachment of the carboxylic group and the erstwhile carbonyl oxygen atom to the acetal function.

The next synthesis also starts with **37**, which is procured from *R*-(+)-citronellal by Lewis acid catalysis (Xu *et al.*, 1986). Conversion to the cyclohexanone **41**, annelation to the enone **42**, methylation to the *cis*-fused decalin **43**, deprotection and oxidation of the alcohol affords the key intermediates, dihydroartemisinic acid (**44**) and its methyl ester **45**. Ozonolysis of **45** cleaves the ring to the ketone-aldehyde **46**, which after selective protection can be enolized to the methyl ether **47**. As before, methanol-mediated photo-oxygenation yields a hydroperoxy acetal which on acidification condenses to **18**.

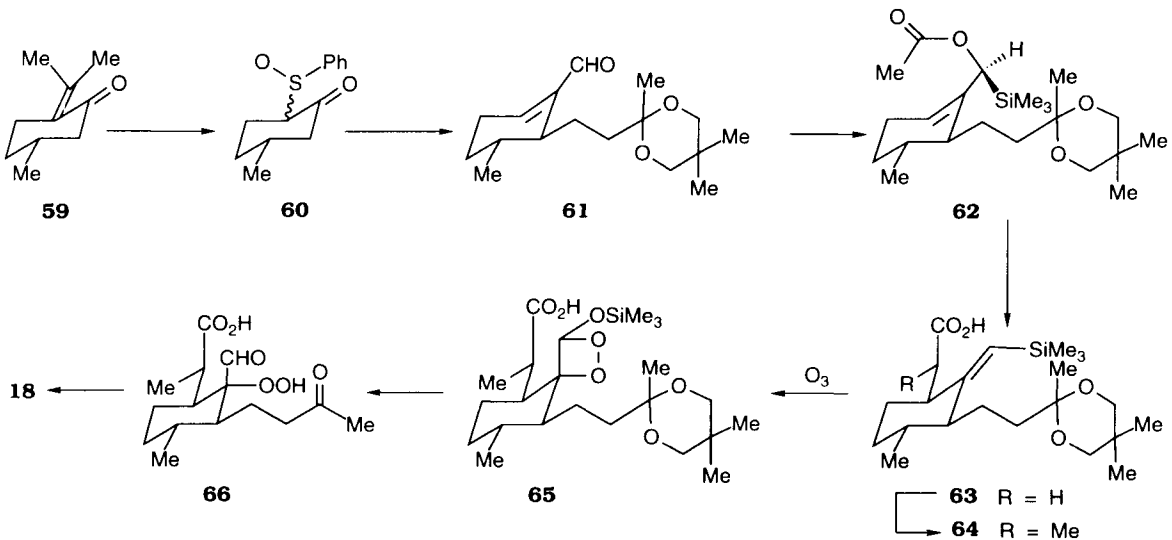



37
OBn
Me
O
Me
41
OBn
Me
O
Me
42
OBn
Me
Me
Me
43
CO2R
Me
Me
Me
44 R = H
45 R = Me
45
O3
CO2Me
CHO
Me
O
Me
Me
46
CO2Me
OMe
Me
O
Me
Me
47
1O2, MeOH
H+
18



The last part of the previous route has been grafted onto a circuitous synthesis of **42** from (−)-menthol (**48**) (Bhonsle *et al.*, 1994). The structurally similar enone **49**, secured from **48**, is converted through its epoxide **50** to the acetate **51** by reduction with lithium aluminium hydride. Treatment of **51** with lead tetracetate and iodine brings about oxidation of the contiguous methyl group with closure to the tricyclic lactone **52** as the main product. Deacetylation and oxidation yield the corresponding cyclohexanone **53**, the reduction of which with sodium hydride in dimethylformamide, followed by benzylation, furnishes the requisite key intermediate **42**.

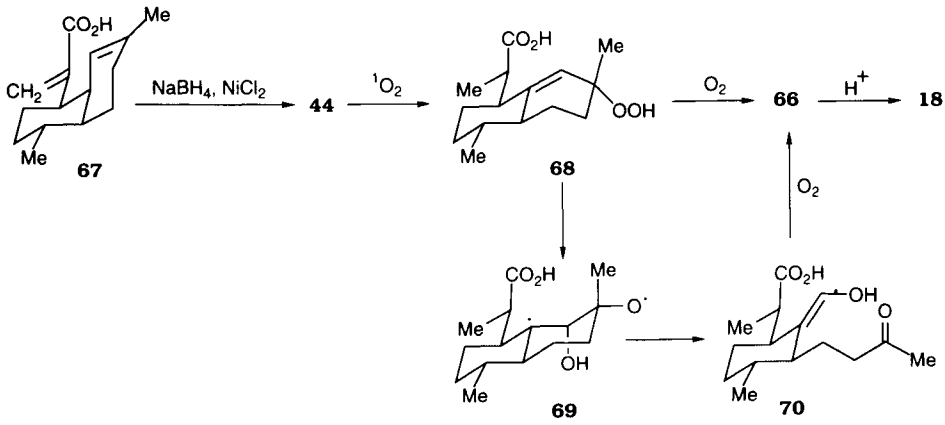
Another approach to the trioxane precursor **46** or rather its acid derivative


59
60
61
62
63 R = H
64 R = Me
65
66
18
O3

(**58**) has been achieved from (+)-isolimonene (**54**) (Ravindranathan *et al.*, 1990). Conversion of **54** to the diastereomeric alcohols by regioselective hydroboration and oxidation, and thence to the dienol ethers **55**, sets up the required framework. On heating, the tethered diene undergoes [4 + 2] addition to give the epimeric tricyclic ethers **56**. Epoxidation, reduction with LAH, and oxidation with $RuCl_3$ and $NaIO_4$ provide the epimeric lactones **57**. Separation of the α-epimer and oxidative cleavage give **58** and then artemisinin (**18**) in the usual way.

Yet another route to the hydroperoxy derivative of **58** makes use of *R*-(+)-pulegone (**59**) as the source of chirality (Avery *et al.*, 1992). Conversion to the known sulfoxide **60**, attachment of the protected 2-oxobutyl side-chain and eliminative formylation furnish the cyclohexenyl carbaldehyde **61** as a suitably functionalized, future ring *A*. Sequential treatment of **61** with tris-(trimethylsilyl)aluminium etherate and acetic anhydride gives solely acetate **62**, which on treatment with lithium diethylamide rearranges to the acid **63**. Attachment of the missing methyl group and ozonation of the homologue **64** generate the frangible siloxydioxetane **65**. The action of trifluoroacetic acid on **65** first deprotects it giving the tetra-functionalized intermediate **66** and then artemisinin (**18**) by acid-catalysed condensation.

The transient acid **66** can also be obtained in just a few steps from artemisinic acid **67**, the biogenetic precursor to **18**, which is about 10 times more abundant than **18** in the plant. A chemical mimic of the natural process entails the reduction and oxygenation of **67**. Treatment with excess sodium borohydride and nickel chloride yields dihydroartemisinic acid **44** which, by methylene-blue sensitized photo-oxygenation, gives the allylic hydroperoxide **68**. Solution of **44** in hexane in the air and addition of a little trifluoroacetic acid produces the labile hydroperoxy acid **68** and thereafter **18** in yields of no better than 30% (Roth and Acton, 1989; Acton and Roth, 1992).



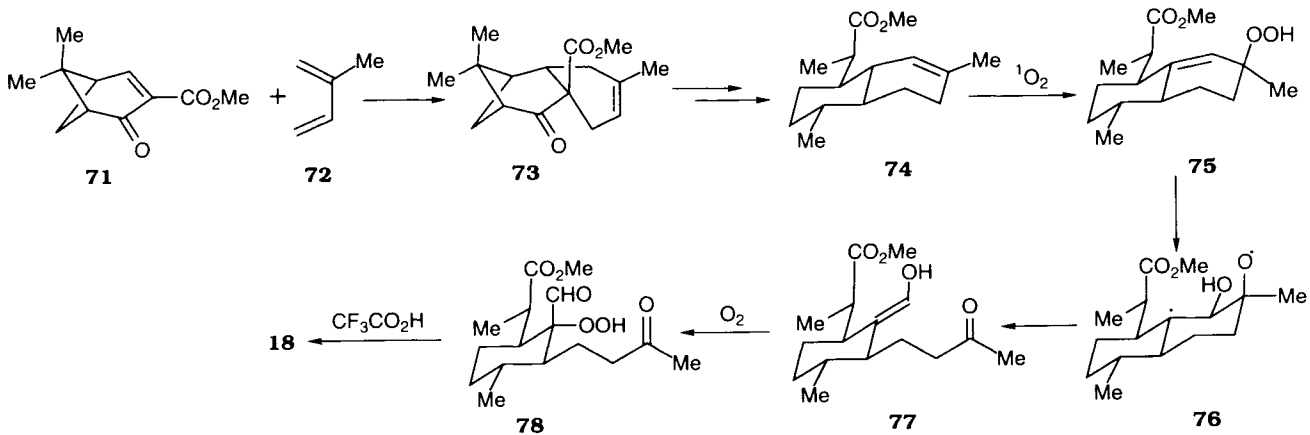
A variant of the previous procedure makes use of cupric trifluoromethanesulfonate as catalyst with or without $Fe(phenanthroline)_3(PF_6)$ in acetonitrile as solvent (Haynes and Vonwiller, 1990). However, no particular advantage is gained as yields are still less than 30%. From experiments conducted with **67**, it is now deduced that the rearrangement of **68** to **66** involves the intermediate E-enol **70** (Vonwiller *et al.*, 1995). This hitherto puzzling transformation of **68** may be rationalized in terms of homolysis of the O–O bond and 1,2-rearrangement of the severed hydroxyl radical to give the 1,4-diradical **69**. Subsequent β-cleavage of the C–C bond then occurs readily thanks to the greater thermodynamic stability of the resulting keto-enol **69**. Autoxidation by air gives the hydroperoxide **66**. Finally, catalysis by added trifluoroacetic acid or by vestiges of trifluoromethanesulfonic acid in the copper salt brings about closure to **18**.

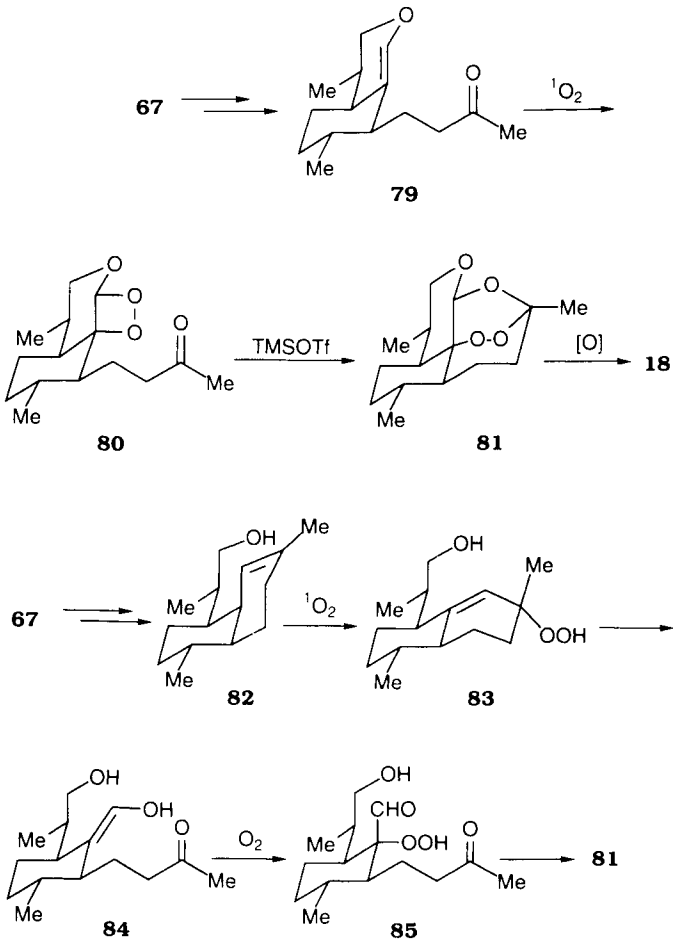
Whether copper has a role to play is problematical. The original suggestion that cupric ion converts **68** into its peroxy radical which then cyclizes to a dioxetanyl radical is not supported by precedent (Courtneidge, 1992). Moreover, such a peroxy radical, if formed, would most probably lose oxygen to give the stable allylic radical (Dussault *et al.*, 1996).

Of mechanistic relevance to the foregoing discussion is the behaviour of non-natural or *trans*-fused methyl dihydroartemisinate (**74**) which is also convertible to **18** when subjected to the same sequence of photo-oxygenation, autoxidation and acid-catalysis (Liu *et al.*, 1993). The synthesis starts with the Diels–Alder addition of isoprene (**72**) to the pinenone ester **71**. The adduct **73** is then transformed in some 20 steps into **74**. Although the actual intermediates are unknown, methylene-blue sensitized photo-oxygenation of **74** must give the epimeric hydroperoxide **75**. Homolysis of the O–O bond and rearrangement to the 1,4-diradical **76** set up the carbon skeleton for easy β-scission to the Z-enol **77**. Autoxidation in the presence of acid slowly generates the hydroperoxide **78**, which condenses to artemisinin (**18**) in the customary 30% yield.

A conventional and more efficient usage of artemisinic acid (**67**) for preparing **18** begins with its initial conversion in three standard steps into the dihydropyran **79** (Ye and Wu, 1990). Photo-oxygenation of **79** in dichloromethane solution with methylene blue as sensitizer produces the 1,2-dioxetane (**80**) *in situ* which, on treatment with trimethylsilyl trifluoromethanesulfonate (TMSOTf), cyclizes mainly to deoxoartemisinin (**81**). Oxidation of the tetrahydropyran ring with $RuCl_3$-$NaIO_4$ affords **18** in an overall yield of 37% from **67**.

Interestingly, the precursor to dihydropyran **79**, the alcohol **82** prepared from **67** by reduction, provides an alternative route to deoxoartemisinin (**81**) (Jung *et al.*, 1989). Methylene blue-sensitized photo-oxygenation of **82** for 2 h leads to the hydroperoxy-aldehyde **85**. The latter, on direct treatment with Dowex-resin under oxygen, yields **81**, undoubtedly, in keeping with the preceding mechanistic rationale, through the intermediacy of the allylic

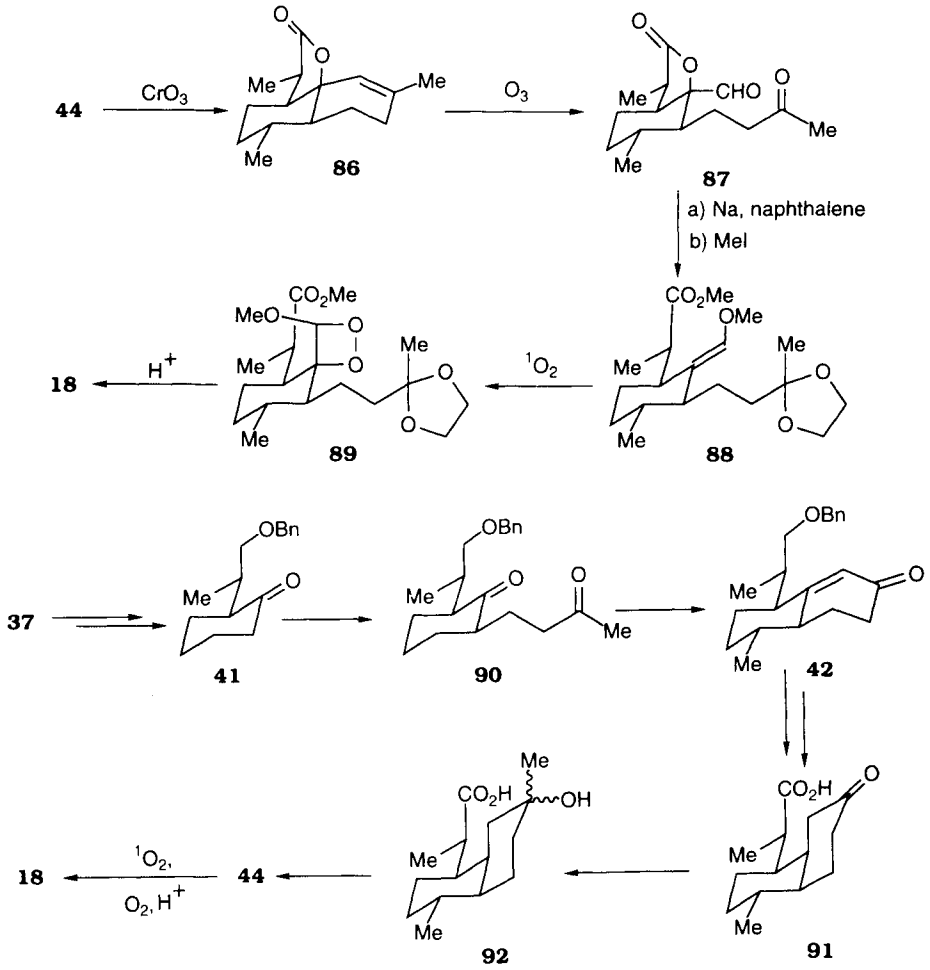

Me
Me
CO2Me
O
71
+
Me
72
Me
Me
CO2Me
Me
O
73
CO2Me
Me
Me
Me
74
1O2
CO2Me
OOH
Me
Me
Me
75
CO2Me
O
HO
Me
Me
Me
76
CO2Me
OH
Me
O
Me
Me
77
O2
CO2Me
CHO
Me
OOH
O
Me
Me
78
CF3CO2H
18



hydroperoxide **83** and the product of its rearrangement, the enol **84**. It is no surprise that the yield for these two steps is only 18%.

An attempt to make better use of dihydroartemisinic acid (**44**) has been reported (Lansbury and Nowak, 1992). Oxidation with chromic oxide and 3,5-dimethylpyrazole to the lactone **86** and ozonolysis to the formyl-ketone **87** provides the proto-artemisinin skeleton. Selective protection of the ketone group and reductive cleavage of **87** with sodium naphthalenide in the presence of methyl iodide followed by protection set up the required methyl enol ether **88** for the cyclo-addition of singlet oxygen. The 1,2-dioxetane **89** so obtained, in the presence of camphorsulfonic acid, opens and combines with the concomitantly liberated carboxylic and ketone functions so forming **18** in overall yields of 30–35%.

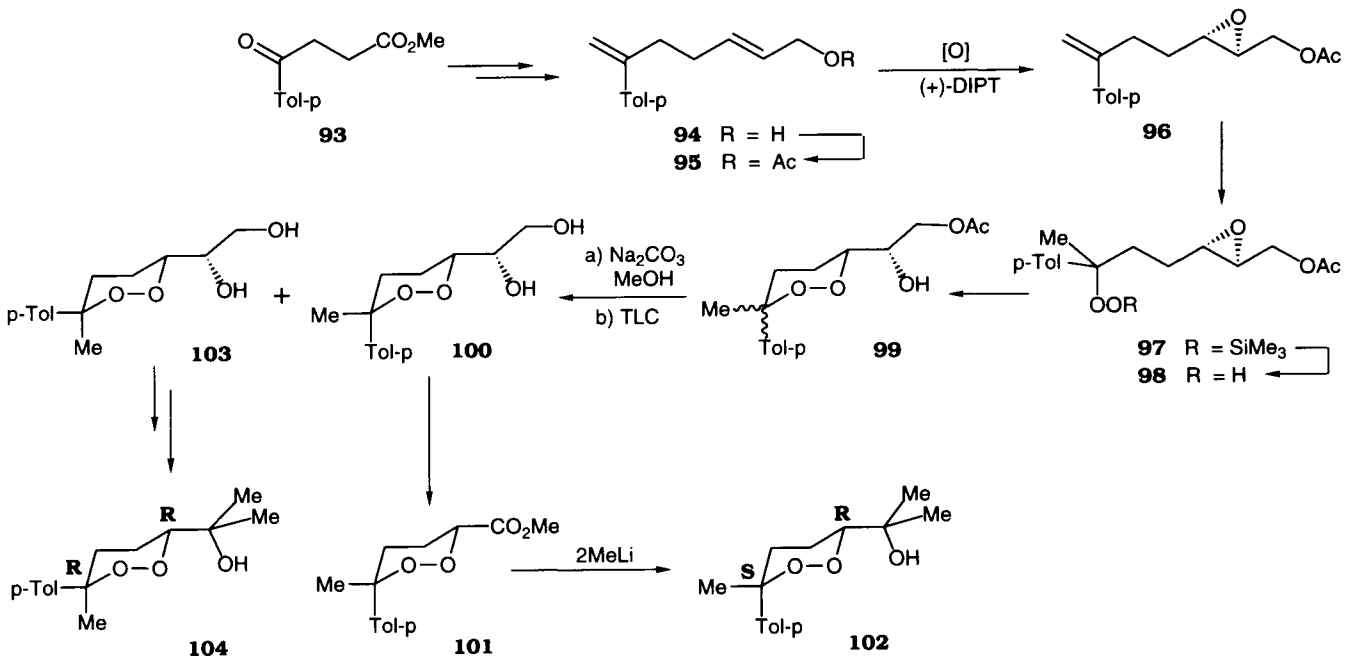
Finally, (−)-isopulegol (**37**) has been converted to **18** in 12 steps through the intermediacy of **41** (Constantino *et al.*, 1996). Hydroboration and



oxidation of the isopropenyl side-chain to the primary alcohol, protective benzylation and oxidation furnish the known cyclohexanone **41**. Attachment of the 2-oxobytyl side-chain to give **90**, Robinson annelation to **42**, and catalytic hydrogenation gives the *cis*-decalone **91**. Addition of methyllithium produces the epimeric tertiary alcohols **92**. Dehydration with *p*-toluenesulfonic acid delivers dihydroartemisinic acid (**44**) which is converted by the standard acid-catalysed double oxygenation protocol to **18**.

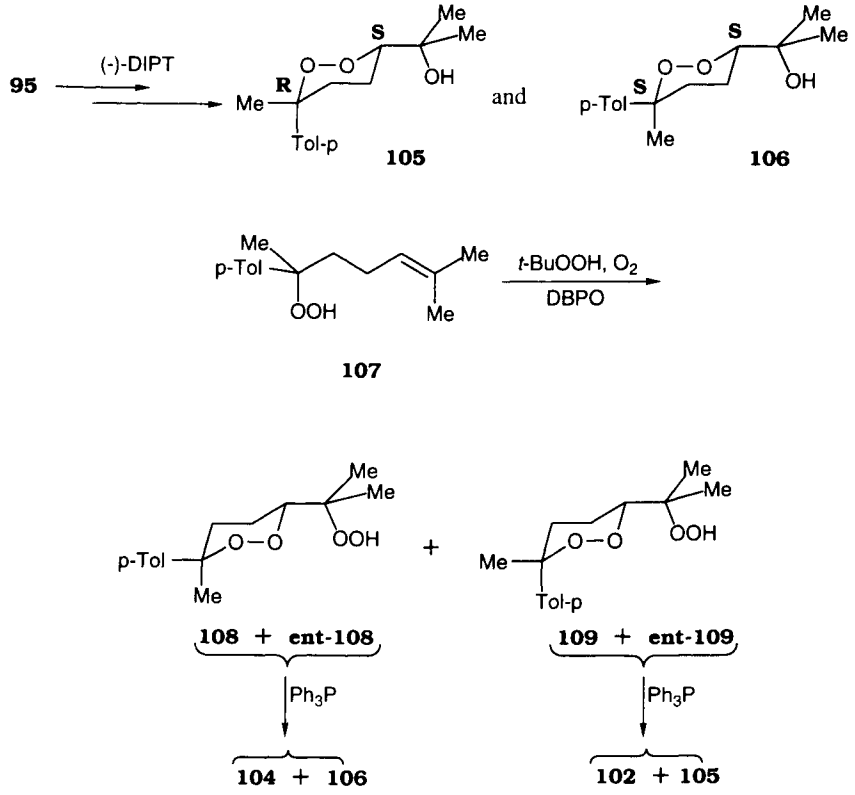
4.2.3 Syntheses of Yingzhaosu C

All four stereoisomers of yingzhaosu C have been synthesized from the dienol **94** (Xu and Dong, 1995). The known toluoyl ester, **93**, is prepared by


CO2Me
Tol-p
93
OR
94 R = H
95 R = Ac
[O]
(+)-DIPT
OAc
96
Me
p-Tol
OOR
97 R = SiMe3
98 R = H
a) Na2CO3
MeOH
b) TLC
99
100
103
p-Tol
Me
2MeLi
101
102
104
R
S

Friedel–Crafts acylation of toluene with succinic anhydride followed by methylation. Chain extension of **93** by two Wittig reactions and reduction yields **94**, which after protection as the acetate **95**, is asymmetrically epoxidized in the presence of L-(+)-diisopropyl tartrate ((+)-DIPT). The ene-epoxide **96**, obtained in high enantiomeric excess (>95%), is converted into the diastereomeric hydroperoxides **98** by fluoride-induced desilylation of the peroxides **97** resulting from the catalysed addition of oxygen and trimethylsilane to the double bond. Amberlyst-15 brings about efficient cyclization of **98** to the 1,2-dioxanes **99**. Saponification of **99** and chromatographic separation give the pure diastereomeric diols **100** and **103**. Finally, the diol portion of **100** is oxidatively cleaved and the intermediate acid converted to the methyl ester **101**. Addition of two equivalents of methyllithium followed by hydrolysis produces the dioxane-alcohol **102** having the 8*S*,12*R* configuration. Similar modification of the chain of **103** yields the 8*R*,12*R* diastereomer **104**.

The same sequence of operations carried out on **95**, but starting with epoxidation controlled by (−)-DIPT, furnishes the enantiomeric counterparts, the diastereomers **105** and **106**, possessing the 8*R*,12*S* and 8*S*,12*S*



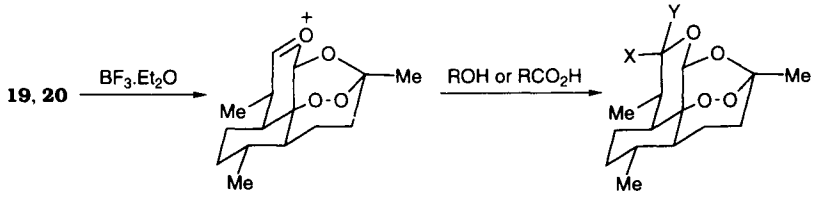
configurations, respectively. The ^{1}H NMR spectrum of **102** and **105** coincides with that of natural yingzhaosu C (= **31**). Since the latter displays an optical rotation of +2.89 in methanol, it can be deduced that it is a mixture of the two antipodes with **102** in slight excess.

A short synthesis of fully racemic yingzhaosu C has been reported (Boukouvalas *et al.*, 1995). Treatment of the olefinic hydroperoxide **107** with oxygen, excess *t*-butyl hydroperoxide and di-*t*-butyl peroxalate (DBPO), brings about efficient ring-closure to a diastereomeric mixture of the hydroperoxy-peroxides **108** and **109** and their enantiomers in a ratio of 1.7:1. Deoxygenation of the major diastereomers **108** and **ent-108** with triphenylphosphine gives racemic **104** and **106** of the non-natural configuration, whereas the minor isomers **109** and **ent-109** provide racemic yingzhaosu C, **102** and **105**.

5 Semi-synthetic Artemisinin Derivatives

An antimalarial drug must necessarily be as cheap and as easy to make as chloroquine (**5**) and be suitable for manufacture in quantities of several hundred tons per year. It is obvious from the preceding section that the synthesis of artemisinin (**18**) is neither a practical nor a commercial proposition. Consequently, much synthetic effort has been devoted to effecting relatively minor changes to plant-derived **18** with the aim of improving efficacy. An inherent drawback to **18** is its extremely low solubility in oils or water and its meagre bio-availability. β-Artemether (**21**) and sodium α-artesunate (**23**) represent the first improved and clinically used drugs, which are respectively administered as oily solutions by injection and as aqueous solutions *per os*.

Since the pioneering Chinese work of 1981 or thereabouts, the range of first-generation derivatives of **18** has been extended. For the most part, they are obtained, like the others, from dihydroartemisinin (**19** and **20**), obtained, as already mentioned, by reduction of **18** with sodium borohydride.



110

111 X = H, Y = OR, α series
112 X = OR, Y =H, β-series
113 X = H, Y = O_2CR, α series
114 X = O_2CRR, Y =H, β-series

TABLE 1

In vitro antimalarial activities against *Plasmodium falciparum* (IC_{50} values in ng ml^{-1})

Compound (Reference)	Sierra Leone D-6	Indochina W-2
18 (Brossi *et al.*, 1988)	10.53	6.54
21	4.49	3.34
115	4.07	2.94
5	10.84	99.97
6	33.78	5.75
18 (Lin *et al.*, 1987)	2.93	0.66
19, 20	0.41	0.69
116	1.74	0.92
117	4.07	1.38
18 (Lin and Miller, 1995)	2.30	1.02
21	0.87	0.30
117	4.87	2.08
120	0.29	0.09
121	0.51	0.23
122	0.50	0.21
123	0.46	0.14
124	0.56	0.23
125	0.25	0.05
126	0.92	0.34
127	0.69	0.32

Treatment of the lactols with a catalytic amount of boron trifluoride etherate generates the oxonium ion **110**. Typically, addition of alcohols (ROH) and acids (RCO_2H) annihilates the positive charge producing an epimeric mixture of the corresponding α- and β-ethers (**111** and **112**) and esters (**113** and **114**). Separation is effected by chromatography. In this way, a host of lactol esters, carbonates, ethers and the like are obtained.

As a complement to **21** and **23**, β-arteether (**115**=**22**) and potassium β-artelinate (**116**) were developed as drug candidates. It was assumed that **115**, on account of its ethyl substituent, would be more lipophilic than **21** and would accumulate better in the brain thereby benefiting patients afflicted with cerebral malaria (Brossi *et al.*, 1988). Secondly, it was supposed that **115** would break down in the body splitting off ethanol, and not methanol as **21** would do. In practice, both ethers undergo oxidative de-alkylation catalysed by cytochrome P-450 monooxygenases (Leskovac and Theoharides, 1991). Thus, the difference between an ethyl and methyl substituent is

TABLE 2
In vivo antimalarial activity against *Plasmodium berghei* in mice

Compound (Reference)	Dosage ($mg\,kg^{-1}$)[a]	Vehicle	Number of cures[b]
18 (Lin *et al.*, 1987)	640	Peanut oil	5/5
	160	Peanut oil	5/5
	40	Peanut oil	3/5, 2/5
23 (as acid)	640	5% $NaHCO_3$	5/5
	160	5% $NaHCO_3$	3/5
	40	5% $NaHCO_3$	2/5
117	640	5% $NaHCO_3$	5/5
	160	5% $NaHCO_3$	5/5
	40	5% $NaHCO_3$	5/5

[a]injected s.c. for 3 days from day 3.
[b]Survivors at day 60.

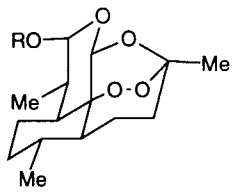
irrelevant. In fact, the antimalarial activity of each of them is similar and exceeds that of **18** some two-times (Table 1). It should be noted that the artemisinin congeners are far more effective than chloroquine (**5**) and mefloquine (**6**). Clearly, the co-development of **21** and **115** as drugs is hard to justify.

Sodium artesunate (**23**) was found wanting on account of its short half-life in plasma of about 30 minutes due to hydrolysis. In contrast, potassium β-artelinate (**116**), as an ether, is far more resilient to water than the ester **23** (Lin *et al.*, 1987). It also retains good artemisinin-like activity *in vitro* (Table 1). The acid **117** is superior to artesunic acid in the Rane Test (Table 2).

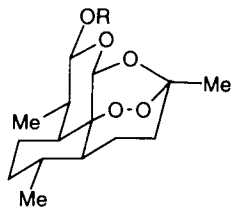
α-Artelinic acid (**118**) has been prepared by reacting **19** and **20** with methyl *p*-(iodomethyl)benzoate in the presence of fresh Ag_2O in methylene chloride. It is stable, water soluble and brings about rapid and effective clearance of *P. knowlesi* infections in the rhesus monkey (Vishwakarma *et al.*, 1992). α-Arteether (**119**) has been similarly prepared from dihydroartemisinin and ethyl bromide (Vishwakarma, 1990).

Several diastereomeric pairs (*R* and *S*) of β-(esterbenzyl) ethers (**120–127**) have been prepared by the Lewis acid-mediated procedure (Lin and Miller, 1995). Their *in vitro* activities greatly surpass that of β-artelinic acid (**117**), therefore suggesting that the corresponding free acids would be even more active (Table 1).

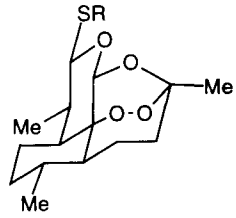
Further quests for improved dihydroartemisinins have been undertaken. Still more ethers and, for the first time, thioethers have been prepared and evaluated *in vivo* (Venugopalan *et al.*, 1995b). The β-configured propargyl



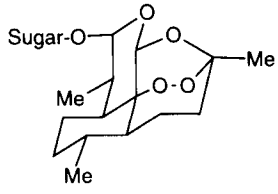
115 R = Et
116 R = $CH_2C_6H_4CO_2K$-p
117 R = $CH_2C_6H_4CO_2H$-p
120 R = $CHPhCO_2Et$ (**R**)
121 R = $CHPhCO_2Et$ (**S**)
122 R = $CHPhCH_2CO_2Et$ (**R**)
123 R = $CHPhCH_2CO_2Et$ (**S**)
124 R = $CH(C_6H_4NO_2$-p$)CH_2CO_2Et$ (**R**)
125 R = $CH(C_6H_4NO_2$-p$)CH_2CO_2Et$ (**S**)
126 R = $CHMeC_6H_4CO_2Me$-p (**R**)
127 R = $CHMeC_6H_4CO_2Me$-p (**S**)
128 R = $CHMeC{\equiv}CH$
129 R = $CMe_2C{\equiv}CH$



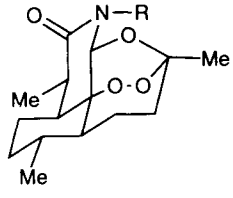
118 R = $CH_2C_6H_4CO_2H$-p
119 R = Et



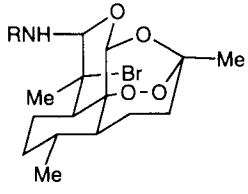
130 R = Cyclohexyl
131 R = Ph



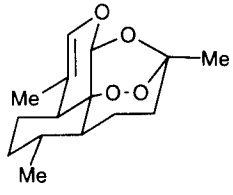
132 Sugar = D-glucose
133 Sugar = D-galactose



134 R = CH_2-pyridyl-*o*
135 R = CH_2CHO



136 R = Ph
137 R = C_6H_4F-*m*



138

ethers **128** and **129** together with the cyclohexylthio and phenylthio derivatives **130** and **131**, of the α-configuration, performed well, and slightly better than β-arteether (**115**) when administered both s.c. and p.o. for 5 days consecutively to mice infected with *P. berghei* K-173 (Table 3). In all cases, parasitaemia was completely cleared by day 7. High cure rates (90–100%)

TABLE 3
In vivo antimalarial activity against *Plasmodium berghei* K-173 in mice

Compound (Reference)	Dosage (mg kg^{-1})[a]	Route	Number of cures[b]
115 (Venugopalan *et al.*, 1995b)	5	s.c.	13/16
	2.5	s.c.	26/36
	20	p.o.	15/28
128	5	s.c.	6/6
	2.5	s.c.	10/11
	20	p.o.	5/5
129	5	s.c.	10/10
	2.5	s.c.	10/10
	20	p.o.	5/6
130	5	s.c.	11/12
	20	p.o.	6/18
131	5	s.c.	11/11
	2.5	s.c.	19/21
	25	p.o.	11/11

[a]Administered in 'Kardi' oil and Tween 80 for five consecutive days post infection.
[b]Survivors on day 28.

were achieved by day 28 at the lowest dosage. Curiously, the β-epimers corresponding to **130** and **131** were much less effective, giving cures of 17% and 50% at the 5 mg dose level.

In the same vein, various sugar derivatives (Lin *et al.*, 1992), lactams (Torok *et al.*, 1995), and amine analogues (Lin *et al.*, 1990) have been prepared by conventional methods. Activities against the W-2 and D-6 clones of *P. falciparum* are generally severalfold less than that displayed by **18**. Representative compounds are **132–137**. However, notable exceptions are **134**, **135**, and **137**. The IC_{50} values of the two lactams against a chloroquine-resistant strain of *P. falciparum* (FCR3) are 22 and 26 times smaller than that seen for **18**; the *m*-fluorophenylamine **137**, unlike the non-fluorinated analogue **136**, is about 4.5 times more active than **18** against the D-6 clone. The activities of **136** and **137** *in vivo* are insignificant. In contrast, the analogous *m*- and *p*-chloroaniline derivatives lacking the bromo substituent at C9 are about twice as effective as **18** in their action against *P. berghei* K 173 in the mouse (Yang *et al.*, 1995). Therefore, the presence of an additional substituent at C9 is deleterious to activity (Acton and Klayman, 1987; Li and Pan, 1993). Further examples are the (9*S*)- and (9*R*)-hydroxy-(10*R*)-(*m*-chlorobenzoyl) derivatives of dihydroartemisinin (Lin *et al.*, 1991). The former diastereomer has *in vivo* activity comparable to that of **18**, while the

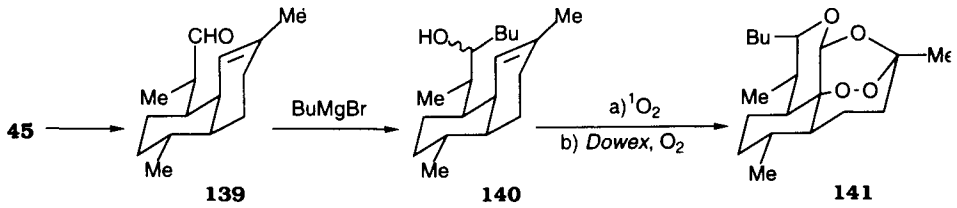
TABLE 4
In vitro antimalarial activity against *Plasmodium falciparum* (IC_{50} values in ng ml^{-1})

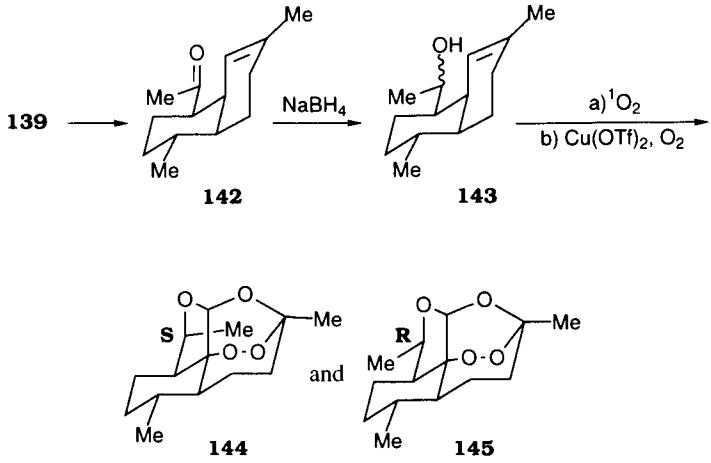
Compound (Reference)	Sierra Leone D-6	Indochina W-2
18 (Acton and Klayman, 1987)	1.0	1.1
25	5.5	3.9
150	11.1	7.1
151	2.7	2.0
149 (9*R*)	9.4	8.1
18 (Lin *et al.*, 1989)	2.35	2.60
19, 20	0.41	0.69
117	4.07	1.38
138	0.83	0.43
18 (Jung *et al.*, 1990b)	2.33	1.21
19, 20	0.11	0.04
81	0.58	0.15

latter showed no activity at dose levels of up to 320 mg kg^{-1}.

In vitro tests reveal that **138** has the same order of activity as dihydroartemisinin and three times the activity of artemisinin (**18**) (Lin *et al.*, 1989) (Table 4). In other tests, deoxoartemisinin (**81**), is some eight times more potent than **18** (Jung *et al.*, 1990b) (Table 4).

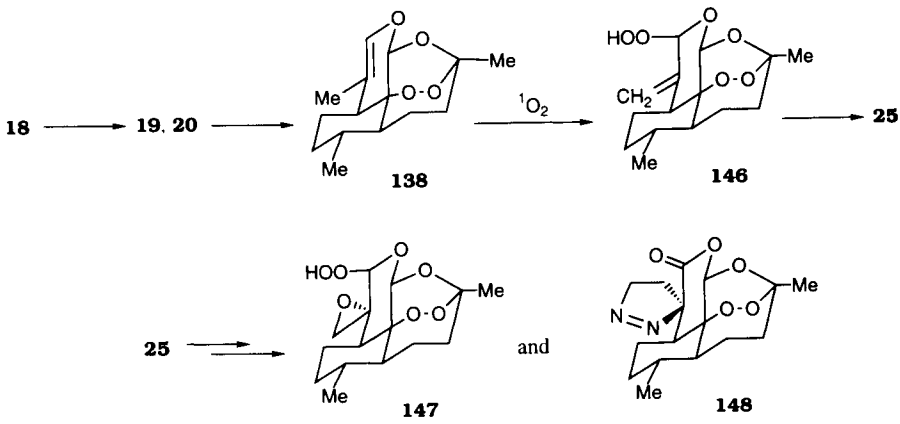
(10*R*)-10-Butyldeoxoartemisinin (**141**) has been concisely prepared from methyl dihydroartemisinate (**45**) by recourse to the acid-catalysed double oxygenation procedure (Jung *et al.*, 1990a). Reduction of **45** to the aldehyde **139** followed by addition of butylmagnesium bromide provides a mixture of the *erythro* and *threo* alcohols **140** in a ratio of 4:1. Successive submission of **140** to singlet oxygen and oxygen in hexane containing acidic Dowex resin for 4 h at room temperature gives **141** in 12% yield after chromatographic purification. Despite the absence of the lactone function, its *in vitro* activity is not enhanced and stays close to that of **18**. At a later date, an identical sequence starting from **139** was used to prepare the ethyl, phenyl and allyl analogues of **141** (Haynes and Vonwiller, 1992). The same methodology has been used to prepare (+)-carboxyalkyldeoxoartemisinins (Jung *et al.*, 1994).





Deoxoartemisinins with a contracted B-ring have been similarly prepared from the aldehyde **139** (Haynes *et al.*, 1995). The ketone **142** is formed by oxidative deformylation and then reduced with $NaBH_4$ to a mixture of the alcohols **143** dominated by the *S*-configured isomer. Double oxygenation as before, but accomplished in the presence of a catalytic amount of cupric triflate, gives the tetrahydrofuran derivatives **144** and **145**. The *S*-epimer **144** is about as active as β-artemether when tested *in vitro*, whereas the *R*-epimer **145** is 10 times less active. Many other ring-contracted artemisinin derivatives have been prepared by semi-pinacolic-type rearrangement of bromoacetal derived from anhydrodihydroartemisinin (**138**) (Venugopalan *et al.*, 1995a).

Artemisitene (**25**), hitherto only extractable in small amounts from the plant, can be conveniently prepared in high yield by oxygenation of anhydrodihydroartemisinin (**138**), which is readily obtainable from artemisinin

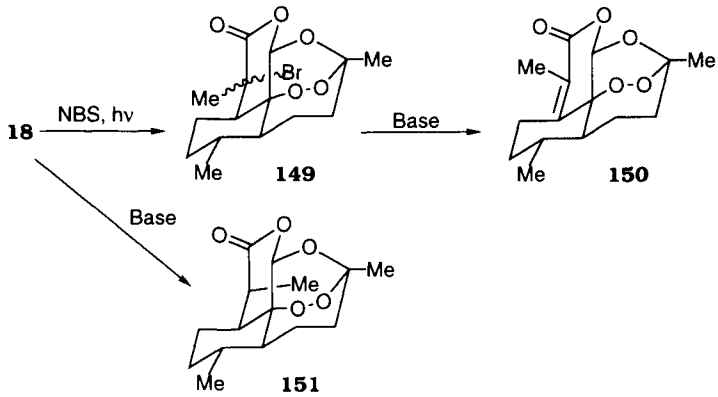


(**18**) by dehydration of dihydroartemisinin (**19** and **20**) (El-Feraly *et al.*, 1990). Photooxygenation of **138** yields the allylic hydroperoxide **146**, which on elimination of water gives **25**. Hence an alternative means of modifying the artemisinin skeleton is at hand. Functionalization of the double bond of **25** has provided a range of 9-substituted derivatives, some of which have *in vitro* activity like that of **18**, for example **147**, while others (e.g. **148**) are about 100 times weaker (Acton *et al.*, 1993).

Bromination of **18** with *N*-bromosuccinimide under irradiation with a street lamp produces the epimeric 9-bromoartemisinins (**149**) (Acton and Klayman, 1987). The mixture reacts selectively with base leaving the 9*R* epimer unaffected, while the 9*S* isomer eliminates hydrogen bromide affording iso-artemisitene (**150**). Simply treating **18** with lithium diethylamide at −78° C causes equilibration to 9-*epi*-artemisinin (**151**). The *in vitro* activities of **150** and **151** against the W2 and D6 clones of *P. falciparum* are less than that of **18** and bracket that of artemisitene (**25**) (Table 4). It is worth noting again that additional substitution or placing a double bond at C9 decreases activity 8–10 times.

A few functionalizations of other parts of the artemisinin skeleton have also been reported. Epoxidation and hydroxylation of **138** proceed as expected (Petrov and Ognyanov, 1991; Hufford *et al.*, 1993). The β-epoxide and the diol obtained by dihydroxylation of **138** on the α-face of the double bond are about one-tenth less active *in vitro* than artemisinin (Pu *et al.*, 1994).

Minuscule amounts of some fluorinated derivatives have been prepared from the products of the microbial oxidation of **115** (Pu *et al.*, 1995). *In vitro* tests show that such chemical changes do not significantly increase antimalarial activity. This latter commentary essentially sums up the situation for all the so-called first-generation artemisinin derivatives; hundredfold multiplications of activity are not seen. Nevertheless, desirable properties of clinical importance, such as better solubility and stability in water, as exhibited by artelinic acid and its sodium salt are attainable.



Many of the recent developments in the chemistry of artemisinin and related compounds have been reviewed (Jung, 1994).

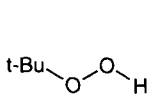
It is worth adding at this juncture, that most of the above-mentioned modifications of natural artemisinin bring small benefits which scarcely justify the inconvenience of the extra synthetic steps. Artemisinin should be considered merely as the prototype for a new class of peroxidic drugs.

6 Various Synthetic Hydroperoxides and Peroxides

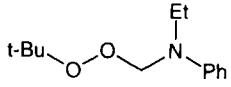
6.1 ACYCLIC PEROXIDES

In addition to the naturally occurring peroxides, several commercially available hydroperoxides and peroxides are endowed with mild antimalarial properties. Examples are hydrogen peroxide and *t*-butyl hydroperoxide (**152**) (Vennerstrom and Eaton, 1988). They destroy murine erythrocytes infected with *P. vinckei*. Their mode of action may entail a Fenton reaction with ferrous iron which generates lethal radicals that attack the parasite within the red blood cell. When the experiment is repeated with desferrioxamine, a powerful iron chelator, the parasiticidal effect is lost.

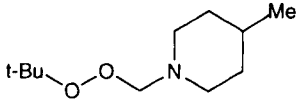
Although **152** only displays modest *in vitro* activity against the D-6 clone of *P. falciparum* ($IC_{50} = 203\ \mu M$), the attachment of amino groups through a methylene link, as illustrated by the aniline and piperidine derivatives **153** and **154**, reinforces the effect roughly tenfold (Vennerstrom, 1989). In the Rane test, however, no *in vivo* activity is observed.



152



153

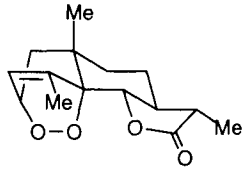


154

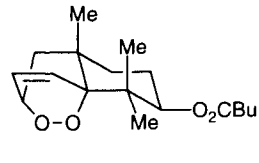
In general, diverse simple synthetic endoperoxides, peroxyesters, peroxides, peroxyacetals, and peroxycarbonates are only weakly active *in vitro* compared with **18** and devoid of activity *in vivo* (Vennerstrom *et al.*, 1989).

6.2 CYCLIC PEROXIDES

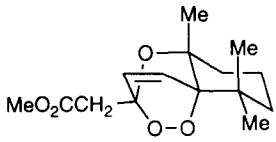
Peroxides derived from a sesquiterpene lactone, a related dihydroionol and tetrahydrobenzopyran have been prepared and tested to evaluate their



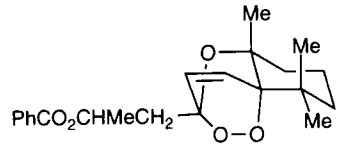
155



156



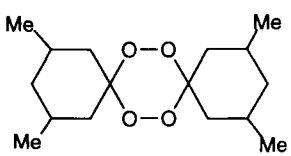
157



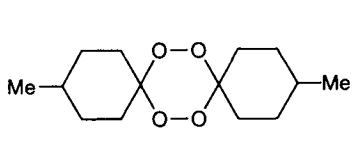
158

antimalarial properties. The idea was to add the peroxide bridge to the framework of the natural product and so endow it with antimalarial activity. Success was partial. The endoperoxide **155** obtained from α-santonin is inactive both in the Desjardins and Rane tests (Tani *et al.*, 1985). The structurally related peroxide **156** does better and shows an IC_{50} of 100 ng ml^{-1} and 57 ng ml^{-1} against the D-6 and W-2 clones, respectively, compared with <3.4 ng ml^{-1}, the mean for **18** (Keppler *et al.*, 1987). The bridged bicyclic 1,2,4-trioxanes **157** and **158** manifest much the same activity (Keppler *et al.*, 1988). IC_{50} values of 96 ng ml^{-1} and 39 ng ml^{-1} against the D-6 and W-2 clones are seen for **157**, whereas **158** displays values of 24 ng ml^{-1} and 99 ng ml, respectively. The reference value for **18** is 1 ng ml^{-1} for both clones.

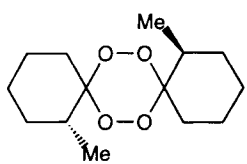
In contrast to the preceding results, the dispiro-1,2,4,5-tetraoxanes **159**, **160** and **161** are surprisingly potent (Vennerstrom *et al.*, 1992). They are easily prepared from the corresponding ketones by treatment with hydrogen peroxide in the presence of aqueous sulfuric acid. The IC_{50} values for **159–161**, namely, 50, 8.2 and 6.9 nM against the D-6 clone, are not far off that for **18**, which is 4.7 nM. The corresponding values are 13, 58, 3.4 and 2.2 nM against the W-2 clone. These *in vitro* activities are corroborated by results from the Rane test, with cures being obtained at dose levels of 320 mg ml^{-1} and 640 mg kg^{-1}



159



160



161

7 Synthetic Bridged Bicyclic Peroxides

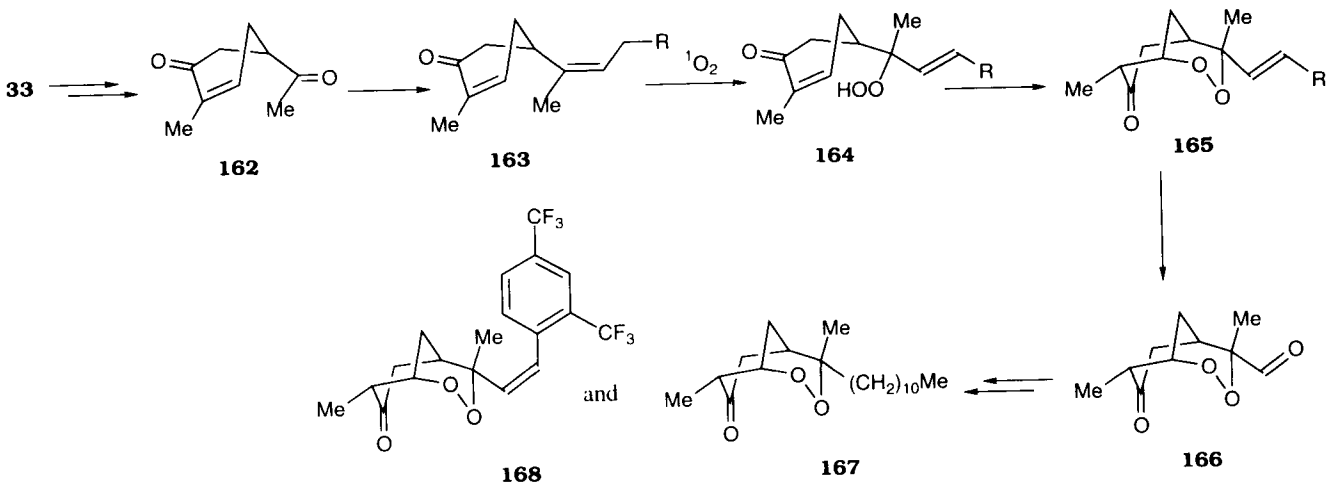
Taking yingzhaosu A (**17**) as a lead, chemists at Hoffmann LaRoche in Basel synthesized many analogues in which the side-chain was systematically varied (Hofheinz *et al.*, 1994). The choice of **17** over **18** was dictated by its simpler structure and its supposedly better stability. However, knowledge of its antimalarial potency was only anecdotal. The synthetic strategy was the same as that previously used for the synthesis of **17**. *R*-(−)-carvone (**33**) by acid-catalysed epoxidation, opening to the diol, furnishes the ketone **162** by oxidative cleavage with $NaIO_4$. Wittig olefination to **163** and reaction with singlet oxygen affords the allylic hydroperoxide **164**. Acid-catalysed ring-closure to **165** and ozonolysis provide the pivotal intermediate, the aldehyde **166**. A variety of alkyl and arylvinyl derivatives are accessible by conventional methods of chain-elongation; the most active are **167** and **168**. The latter, called arteflene, compares moderately well with artemisinin and its derivatives in both the Desjardins and Peters tests (Tables 5 and 6) (Jaquet *et al.*, 1994). At first sight, the IC_{50} value for **168** is somewhat high, especially against the non-critical sensitive strain (NF54). However, the levels against the resistant strains are much lower (Table 5). The ED_{90} values for the s.c.

TABLE 5
In vitro antimalarial activity against *Plasmodium falciparum* strains (IC_{50} values in ng ml^{-1})

Compound	K1	W-2	NF54
168	31.0	21.1	44.2
18	3.5	2.7	4.4
21	2.4	–	1.9
5	86.1	76.2	5.3
Resistant to	**5** and **9**	**5** and **9**	Sensitive

TABLE 6
In vivo antimalarial activity against *Plasmodium berghei* ANKA (mg kg^{-1} $day^{-1} \times 4$)

Compound	ED_{50} s.c.	ED_{90} s.c.	ED_{50} p.o.	ED_{90} p.o.
168	2.7	3.9	10.4	18.0
18	0.95	2.5	5.0	14.0
21	0.5	1.1	3.1	5.0
5	1.0	1.2	1.9	2.3


33
O
Me
Me
O
162
R
163
$^1O_2$
HOO
164
165
166
$(CH_2)_{10}Me$
167
CF_3
CF_3
and
168

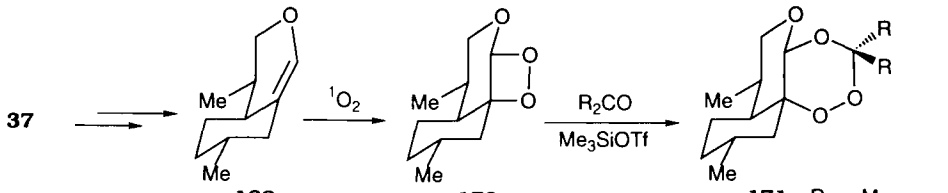
route and the critical p.o. route by **168** lie close to those of **18** and are therefore acceptable. Nevertheless, it must be admitted that artemether (**21**) still outperforms **168**.

8 Synthetic Tricyclic 1,2,4-Trioxanes

It gradually became obvious that not all parts of the artemisinin skeleton were required for activity. The lactone function in **18** seems to be detrimental because enhancements of 8–10 times result when it is absent in derivatives such as **19**, **20**, **81** and **138**. Some of the methyl substituents are superfluous since 6,9-desmethylartemisinin displays significant *in vitro* activity (Avery *et al.*, 1989). The methyl group at C3 is essential, as is the 1,2,4-trioxane ring itself, because the mammalian metabolite lacking the peroxide element, desoxyartemisinin (**24**), is inactive. On the other hand, the role of the third, non-peroxidic oxygen atom is a conundrum in the light of the activity of the bridged bicyclic peroxide, yingzhaosu A (**17**). Furthermore, some of the non-trioxane rings may well be redundant. The question then arises as to how much of the tetracyclic edifice of **18** can be demolished without impairing the artemisinin-like potency. In other words, what is the simplest molecule that embodies the pharmacophore? This question has been addressed in several studies and enlightening answers are beginning to emerge.

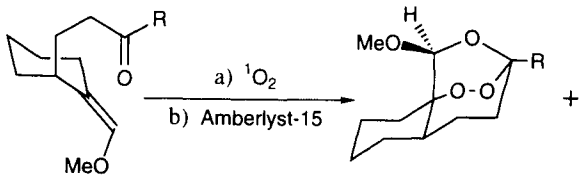
Two sets of tricyclic 1,2,4-trioxanes comprising the ABC and ACD rings were prepared. The AB ring portion of artemisinin, in the form of the dihydropyran **169**, is constructed in a few steps from ((−)-isopulegol (**37**). Dye-sensitized photo-oxygenation yields the dioxetane **170** which, on catalysis, combines with acetone or cyclopentanone to produce the seco-artemisinins **171** and **172** (Jefford *et al.*, 1988c).

An intramolecular variant of the preceding reaction furnishes the ACD analogues (Jefford *et al.*, 1988b, 1993b). Adjunction of singlet oxygen to the racemic enol ether **173** followed by treatment with Amberlyst-15 brings about cyclization to the *endo* and *exo* trioxanes **174** and **175**, together with the peroxide **176**. By the same method, the acetophenone precursor **177** gives the phenyl trioxanes, **178** and **179**.



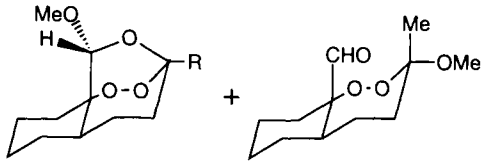
169 **170** **171** R = Me

172 R,R = $(CH_2)_4$



173 R = Me
177 R = Ph

174 R = Me
178 R = Ph



175 R = Me
179 R = Ph

176

All trioxanes, except peroxide **176**, are highly active *in vitro* and reveal some interesting trends when compared with chloroquine (**5**) and quinine (**1**) (Table 7). This result is not so surprising because all trioxanes retain some features of the artemisinin architecture. It is immediately clear, however, that rings B and D of artemisinin are not needed. The ABC analogues **171** and **172** are much more active against the resistant (W-2) than the sensitive clone (D-6). Between the two there is a significant difference. The substitution of a spiropentacyclic attachment is better than the gem-dimethyl grouping by improving potency. The ACD analogues **174** and **175** are also more effective against the resistant clone. Clearly, the absence of the lactone ring is no drawback to activity. Nevertheless, a fourth oxygen atom appears to be obligatory. The methoxy substituent takes on this role and exerts a subtle effect. In the *exo* isomer **175**, where it is disposed just like that of the B-ring oxygen atom in **18** or **19**, for example, activity is poor in the D-6, although a bit better in the W-2 clone. Paradoxically, the *endo* isomer **174**, in which the methoxy group has a non-artemisinin orientation, is markedly more active in both clones.

The inactivity of **176** demonstrates that the peroxide link alone is insufficient and that an intact trioxane is a minimum requirement for activity. The same differentiation between *exo* and *endo* isomers is seen for the phenyl derivatives **178** and **179**. They are also more active than their methyl homologues.

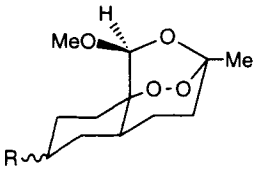
The preceding tricyclic ACD series has been successfully extended by using much the same intramolecular cyclization procedure as described above. It was found that *in vitro* activity can be modulated by varying substituents at

TABLE 7
In vitro antimalarial activity against clones of *Plasmodium falciparum* (ng ml^{-1})

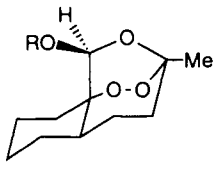
Compound (Reference)	Sierra Leone D-6		Indochina W-2	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
18 (Jefford *et al.*, 1993a)	2.2	–	1.1	–
171	28.7	59.8	6.2	25.8
172	2.3	30.4	2.0	3.3
174 (*endo*)	16.5	11.5	1.8	3.9
175 (*exo*)	754	875	9.7	16.9
176	2608	7547	1837	8132
5	0.9	–	23.4	
1	2.1	–	9.6	–

three key positions (Posner *et al.*, 1995c). Comparison of **180** and **181** with **174**, shows both to be about four times more active, thereby confirming the benefit of substitution on the cyclohexane ring. Similar improvement in activity occurs on replacing the methoxy in **174** by ethoxy, and particularly benzyloxy groups, as illustrated by **182** and **183**. The effects are most marked against the resistant W-2 clone. Methyl and phenyl groups at the bridgehead, as in **174** and **178**, ensure high activity, but **184** bearing the hydroxyethyl group is several times less active, while the benzyl derivative **185** is totally inactive.

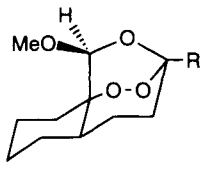
The effect of the hydroxyethyl group depends critically on where it is placed. It was found earlier that when attached to ring A, derivatives of substantially enhanced potency can be obtained (Posner *et al.*, 1992). Although the parent alcohol **186** is considerably weaker than artemisinin (**18**), certain of its esters and ethers are extremely active against the D-6 and W-2 clones. The benzoate **187** exhibits only 20% of the effect of **18** against the W-2 clone. In contrast, the carbamate **188** and the phosphate **189** are about seven times more powerful. The methyl ether **190** displays about 40% of the



180 R = Et
181 R = Ph



182 R = Et
183 R = CH_2Ph

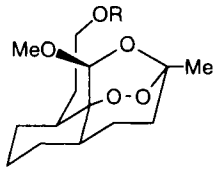


184 R = CH_2CH_2OH
185 R = CH_2Ph

activity of **18**, but the benzyl ether **191** outperforms **18** some three and a half times. Disappointingly, various sulfonates derived from **186** are about as active as **18** at best. The reason for the enhancement of activity is hard to formulate. Clearly, the pharmacophore has not been affected, but lipophilic appendages undoubtedly help it get to the receptor.

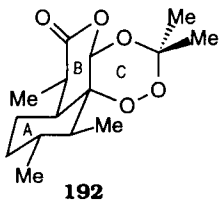
More benzylic ethers were prepared from **186** and also esters bearing potentially iron-chelating groups such as phenanthroline. No dramatic effects were observed on testing *in vitro*, with the sole exception of the *p*-fluoro derivative of **191**, which is about twice as active as **18** against the W-2 clone (Posner *et al.*, 1995b).

A set of ABC tricyclic analogues keeping the lactone functionality (**192–195**) has been obtained by exploiting the methodology previously used

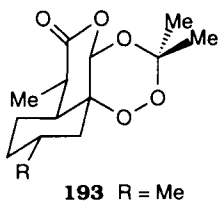


186 R = H
187 R = O_2CPh
188 R = O_2CNPh_2
189 R = $PO(OPh)_2$
190 R = Me
191 R = CH_2Ph

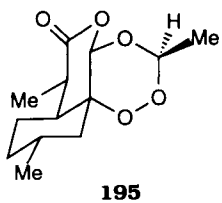
for synthesizing **18** via the silylvinyl derivative **64**, but lacking the oxobutyl side-chain (Avery *et al.*, 1994). A related method has been employed for making **189** (Imakura *et al.*, 1988). 4,5-Secoartemisinin (**192**), the closest in structure to **18**, retains only 14% of the activity of **18** against the W-2 clone. It therefore seems that either the rigidity of the whole molecule, or perhaps the boat conformation of the trioxane ring in **18**, is an important feature for activity. Removing the flanking methyl group on the A-ring, as in **193**, restores the activity to 75%, whereas removing both, as in **194**, loses it again, dropping it down to a mere 6%. Most significant is the inactivity of the monomethylated trioxane **195**.



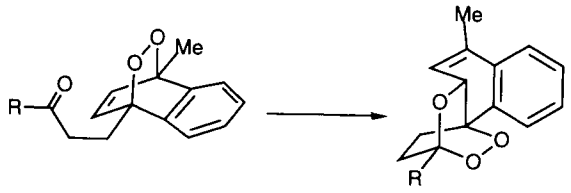
192



193 R = Me
194 R = H



195



196

197 R = H
198 R = Me
199 R = Bu
200 R = Ph

It was discovered that molecules bearing no resemblance to artemisinin could be quite potent. The intramolecular capture of a pendent ketone function by an endoperoxide provides tricyclic trioxanes of rigid geometry in which the trioxane ring is constrained in a boat-like conformation (Jefford *et al.*, 1986b). Catalysis of **196** with Amberlyst-15 gives the racemic bridged bicyclic trioxanes **197–200**. The nature of the substituent at the bridgehead is all-important as attested by *in vitro* tests (Grigorov *et al.*, 1996). Low activity is seen for **198** and **200** against the D-6 and W-2 clones – namely, IC_{50} values some 250–1000 times greater than for **18** (Table 8). The butyl substituent exerts the best effect: **199** has an IC_{50} of 14.0 ng ml^{-1} against both clones.

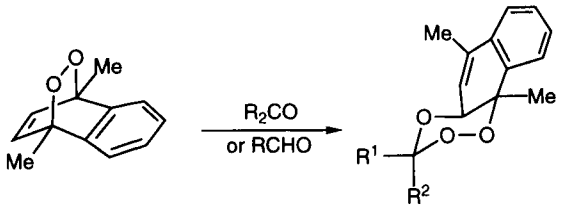
9 Synthetic Bicyclic 1,2,4-Trioxanes

The intermolecular variant of the preceding reaction conducted with the endoperoxide of 1,4-dimethylnaphthalene (**201**) and various carbonyl partners furnished *cis*-fused bicyclic trioxanes, of which **202–204** are representative. It was hoped that the attachment of groups which might have affinity for the parasitized red blood cell, e.g. **203** and **204**, would be particularly efficacious. Unfortunately, none shows useful antimalarial activity in experimental models (Jefford *et al.*, 1988a; Peters *et al.*, 1993a). These results clearly demonstrate that the trioxane entity is certainly necessary, but not sufficient in itself to confer activity.

The reaction of endoperoxides with aldehydes and ketones served as a simple means for preparing a great number and variety of *cis*-fused bicyclic trioxanes (Jefford *et al.*, 1986a; Jefford, 1991).

The endoperoxide **206**, obtained from 1,4-diphenyl-1,3-cyclopentadiene (**205**) by the cyclo-addition of singlet oxygen, provides an apt illustration. On catalysis with TMSOTf, it combines readily with acetone yielding the racemic *cis*-fused cyclopenteno-1,2,4-trioxanes **207** and **ent-207**.

Hundreds of racemic trioxanes were prepared in this way and screened in the Desjardins test. The racemic trioxanes **207–210** are noteworthy. They

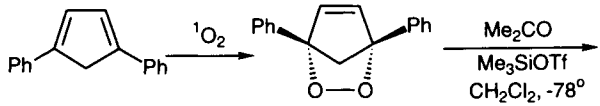


201

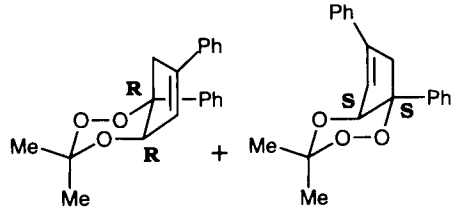
202 $R^1 = R^2 = Me$

203 $R^1 = CH_2C_6H_4OMe\text{-}4$, $R^2 = H$

204 $R^1 = (CH_2)_6C_6H_3Cl_2\text{-}3,4$, $R^2 = H$



205 **206**



207 **ent-207**

display extremely high activities, which, gratifyingly, are more acute against the chloroquine-resistant W-2 clone. In many cases the legendary activity of **18** is exceeded several times (Jefford *et al.*, 1993a). The results confirm that the pharmacophore must encompass the trioxane and *cis*-fused cyclopentene rings, and that it is sensitive to the nature of the C3 substituents (Table 8). The spirocyclic tetrahydropyran and cyclopentane moieties, and perhaps also the phenyl substituents, have the particularity of reinforcing activity. In another study, the higher homologues obtained by similar reaction with the endoperoxide of 1,4-diphenyl-1,3-cyclohexadiene proved to be poorly active, displaying IC_{50} values in the range of 100–1000 ng ml^{-1} against the W-2 clone. The conclusion is inescapable: three or four fused rings, a lactone function, or even a fourth oxygen atom are simply not required for artemisinin-like potency.

Confirmation of the above findings was secured by an *in vivo* study in which activities against parasites sensitive and resistant to chloroquine were assayed (Peters *et al.*, 1993b). The racemic 3,3-spirocyclic cyclopenteno-trioxanes (**209–212**) are all reasonably active; the spirohydropyranyl diphenyl deriva-

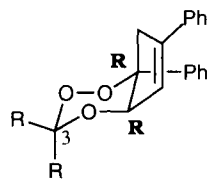
TABLE 8
In vitro antimalarial activity against *Plasmodium falciparum* clones (ng ml^{-1})

Compound (Reference)	Sierra Leone D-6		Indochina W-2	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
197 (Grigorov *et al.*, 1996)	Inactive	Inactive	Inactive	Inactive
198	114	662	256	1135
199	13.8	35.4	14.0	30.0
200	210	487	239	568
207 (Jefford *et al.*, 1993a)	5.94	19.29	5.31	8.35
208	5.72	12.23	3.91	5.00
209	2.21	12.52	0.10	0.39
210	1.42	2.32	0.49	1.08

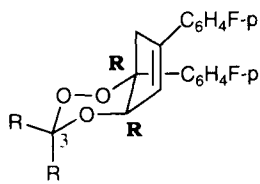
tive **209** performs better than the spirocyclopentyl analogue **210**, especially against *P. yoelii*, the resistant strain (Table 9). The ED_{90} value of **210** is unacceptably high. Substitution by fluorine at the *para* position on the phenyl groups corrects the situation dramatically. Activity improves for both, but strikingly more so for the spirocyclopentyl trioxanes. Comparison with the standards, chloroquine (**5**), artemisinin (**18**), and arteether (**115**), is favourable. The fully synthetic, structurally simple, racemic, fluorinated trioxane **212** approaches closely the kind of *in vivo* activity characteristic of the enantiomerically pure, tetracyclic natural substance. It will be seen later that the issue of configuration, which needs to be addressed, will prove to be unimportant. The reason why depends on the mechanism of action.

Recently, monocyclic trioxanes bearing a spirocyclopentyl or cyclohexyl grouping and a *p*-fluorophenyl substituent have been prepared and shown to have promising activity in a mouse model (Singh *et al.*, 1995).

A further comparative study confirmed the subcutaneous (s.c.), and more importantly, in the light of its future use as a drug, the oral (p.o.) efficacy



208 R,R = $(CH_2)_5$
209 R,R = $(CH_2)_2O(CH_2)_2$
210 R,R = $(CH_2)_4$



211 R,R = $(CH_2)_2O(CH_2)_2$
212 R,R = $(CH_2)_4$

TABLE 9
In vivo antimalarial activity against *Plasmodium berghei* and *P. yoelii* (mg kg^{-1} day^{-1} × 4 s.c.)

	P. berghei N		*P. yoelii* NS	
Compound (Reference)	ED_{50}	ED_{90}	ED_{50}	ED_{90}
209 (Peters *et al.*, 1993b)	6.8	20.2	7.2	15.5
210	12.8	24.5	30	170
211	11.5	19.5	6.6	11.0
212	2.5	6.8	4.5	7.6
5	1.8	3.1	2.4	56.0
18	0.9	2.3	5.8	10.0
115	0.3	0.5	1.7	2.2

of racemic **212** (Peters *et al.*, 1993c). The activities s.c. and p.o. for **212** are almost identical, whereas there are significant differences for the reference compounds, sodium artesunate (**23**), arteether (**115**), and **5** (Table 10). Little difference is seen for **212** even for the two strains. Sodium artesunate loses efficacy against both strains when administered by the oral route; as does **115**. In summary, **212** is about half as active *per os* as **115** against *P. berghei*, but almost three times more active than **23**. The activity of **212** is maintained against a wide spectrum of drug-resistant parasite lines. Its toxicity appears to be low, so **212** may be considered a serious candidate as a drug for treating polyresistant malaria in man.

10 **Mode of Action**

10.1 MECHANISTIC CONSIDERATIONS

The *cis*-fused cylopenteno-1,2,4-trioxanes, e.g. **210** and **212**, are remarkable in many ways. They are endowed with artemisinin-like activity, but their geometries are different from that of artemisinin (**18**). Not only is there no overlap, except in the crudest sense, between any parts of their structures, but the synthetic compounds are racemic whereas **18** is a single enantiomer. Furthermore, **18** is a rigid molecule in which the trioxane ring is locked in a boat conformation. NMR spectroscopy and PM3 calculations indicate that **210** is conformationally flexible and undergoes chair-to-chair or chair-to-boat inversions with little impediment (Jefford, *et al.*, 1995). Of course, it is reasonable to assume that **210** and **18**, as both are trioxanes, will have a common mode of parasiticidal action. An obvious first question, which needs

TABLE 10
In vivo activity against *Plasmodium berghei* N and *P. yoelii* NS (mg kg^{-1} $day^{-1} \times 4$)

Compound	*P. berghei*				*P. yoelii*			
	ED_{50} s.c.	ED_{90} s.c.	ED_{50} p.o.	ED_{90} p.o.	ED_{50} s.c.	ED_{90} s.c.	ED_{50} p.o.	ED_{90} p.o.
212 (Peters *et al.*, 1993c)	2.9	6.8	2.7	6.2	3.8	6.3	5.5	10.0
23	2.8	10.0	5.2	16.5	1.4	7.2	8.4	22.0
115	0.32	0.82	1.8	3.1	1.7	2.2	5.4	14.5
5 (sulfate)	1.8	3.2	1.7	3.2	3.0	60.0	3.9	32.0

to be answered, is does the configuration of the synthetic bicyclic trioxane matter? Does configuration correlate with activity?

The pure enantiomers, **210**, ***ent*-210**, **212**, and ***ent*-212**, were separated and identified by recourse to X-ray analysis of appropriate diasteromeric derivatives. They were examined *in vivo*, with reference to the usual control compounds, against the chloroquine-sensitive and -resistant strains in the Peters test (Table 11). For the less active trioxanes **210** and ***ent*-210**, the ED_{50} values are very similar for both administration routes against *P. berghei*. There is some divergence against *P. yoelii*, more so *per os* than s.c. The values for ***rac*-210** are intermediate between those of the enantiomers. For the more active fluorinated trioxane **212**, the ED values are more consistent, showing little variation. To all intents and purposes, it can be concluded that there is no significant difference in activity between the chiral forms. It is also worth noting that the synthetic fluoro-trioxane ***ent*-212** is four times more powerful than **18**, the standard for peroxidic activity, in the chloroquine-resistant strain. Moreover, its activity in the same strain is about the same by the oral and s.c. routes.

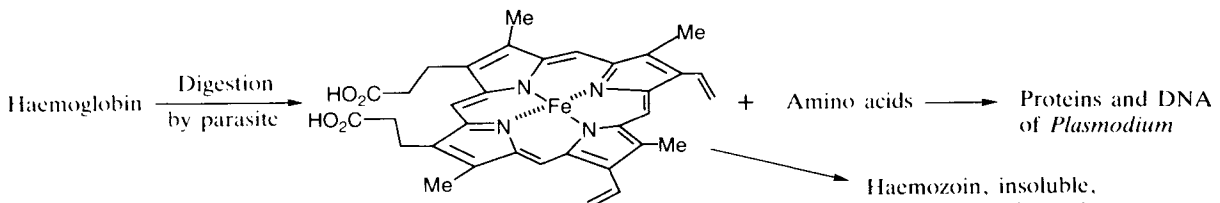
Apart from the superlative potency of these simple, *cis*-fused bicyclic trioxanes, the fundamental finding is the complete irrelevance of configuration to the mode of action in the animal model. By the same token, the receptor that interacts with the trioxane must be configurationally non-selective. It is now known that the receptor is haem (**213**), which, being achiral, fits the aforementioned criterion.

The parasite within the red blood cell feeds on ingested haemoglobin utilizing the peptide part for the synthesis of proteins and DNA. The unwanted prosthetic group, haem, is normally discarded and, because it is toxic to the parasite, polymerized oxidatively to the insoluble malarial pigment, haemozoin (Slater *et al.*, 1991; Slater and Cerami, 1992). Artemisinin, like chloroquine and quinine, acts as a blood schizonticide by

TABLE 11

In vivo activity against *Plasmodium berghei* N and *P. yoelii* NS ($mg\,kg^{-1}\,day^{-1} \times 4$)

	P. berghei				*P. yoelii*			
	s.c.		p.o.		s.c.		p.o.	
Compound	ED_{50}	ED_{90}	ED_{50}	ED_{90}	ED_{50}	ED_{90}	ED_{50}	ED_{90}
***rac*-210** (Jefford *et al.*, 1995b)	4.0	8.0	25.0	75.0	5.8	13.0	10.0	28.0
210	2.3	5.5	20.0	25.0	5.0	9.0	6.0	19.0
***ent*-210**	4.2	8.0	40.0	400	6.0	11.0	23.0	68.0
***rac*-212**	2.5	6.0	2.5	6.0	4.5	7.6	5.6	10.0
212	2.1	3.6	2.6	4.8	1.8	3.4	1.4	5.0
***ent*-212**	1.8	3.2	2.1	3.6	1.5	2.8	1.1	3.1
18	0.9	2.3	–	–	5.8	10.0	–	–
5	1.8	3.1	–	–	2.4	56.0	–	–


Haemoglobin
Digestion
by parasite
HO2C
HO2C
Me
Me
Me
Me
N
N
Fe
N
N
+
Amino acids
Proteins and DNA
of Plasmodium
Haemozoin, insoluble,
non-toxic to Plasmodium

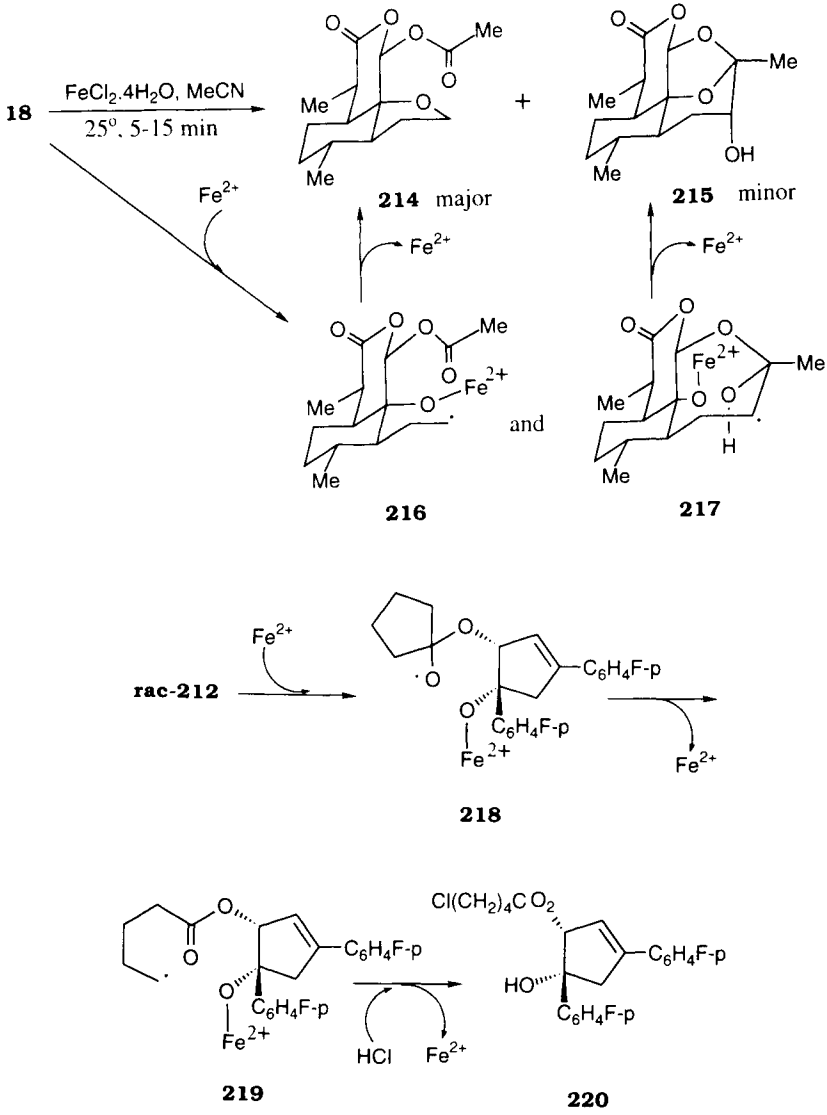
213

interfering with this detoxification process. It can be assumed that artemisinin and synthetic trioxanes such as **212** either sequester haem, thereby potentiating its toxicity, or react with it to produce a toxic species which, in turn, kills the parasite. Much evidence has been accumulated in favour of the latter alternative. Artemisinin reacts with haem to form first a radical which then evolves, in the absence of parasite, to an adduct of unspecified structure (Meshnick *et al.*, 1991; Hong *et al.*, 1994). In the intraerythocytic parasite, alkylation of proteins associated with the membrane occurs (Asawamahasakda *et al.*, 1994). Further evidence gleaned from model studies with tricyclic trioxanes such as the tosylate of **186** has suggested that a carbon-centred radical is the lethal agent (Posner and Oh, 1992; Posner *et al.*, 1994, 1995d).

Another view that has prevailed is that artemisinin (**18**) and related peroxidic antimalarials (e.g. ***rac*-212**) are oxidants which can transfer an atom of oxygen to haem to generate an $O{=}Fe^{2+}$ species. The latter then kills the parasite by monooxygenation. At one time it was thought that the metabolite **24** might have arisen from **18** by such a transfer in the parasiticidal event. The ultimate distinction between these contending eventualities, alkylation versus oxygenation, will be made on the basis of the chemical structure of the product that artemisinin actually forms with the malarial protein. In the meantime, model studies carried out with $FeCl_2.4H_2O$ in acetonitrile as a substitute for haem have provided some valuable clues (Jefford *et al.*, 1995a, 1996). In just a few minutes, $FeCl_2$ acts as a catalyst towards **18** by isomerizing it cleanly and exclusively into the tetrahydrofuran **214** and the hydroxydeoxyartemisinin **215**. The same result is obtained when the experiment is repeated in the presence of cyclohexene; not a trace of epoxide is found. The rearrangement most likely passes through the ferric C-centred radicals **216** and **217** which arise by coordination of ferrous ion to the peroxide bond with concomitant transfer of an electron to break the O—O bond. Closure to **214** and **215** occurs by loss of ferrous ion.

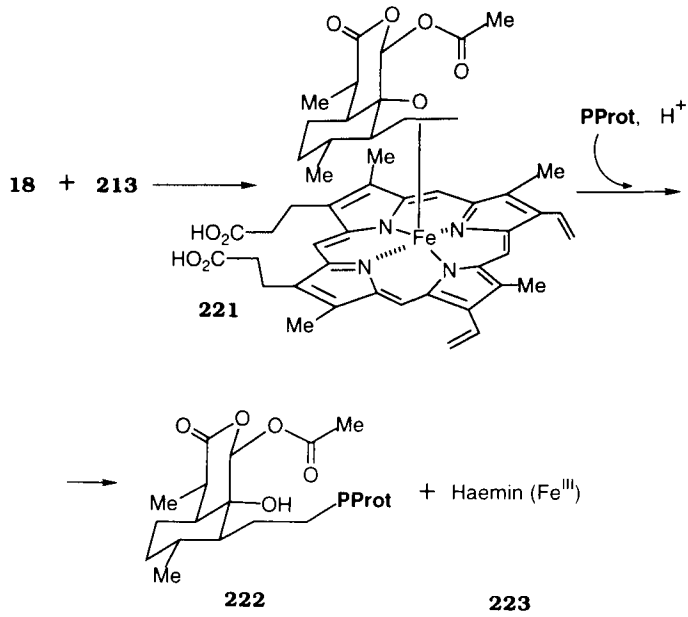
The bicyclic trioxane ***rac*-212** isomerizes in the same way, but because of its more flexible geometry opens up by adding a molecule of hydrogen chloride. The essential product is the alkyl chloride **220** arising through the oxy and alkyl radicals **218** and **219**. The chloride ion captures **219**, freeing ferrous ion to complete the reaction. As before, conducting the reaction in solvent containing cyclohexene leads to no epoxidation.

In both cases, the driving force for rearrangement to the primary, active and, therefore, lethal alkyl radical is the acquisition of thermodynamic stability through ester formation. This chemical incident constitutes the basis of parasiticidal action for many peroxidic antimalarials. It is important to emphasize that, under the foregoing experimental conditions, there is no sign of any deoxygenated products such as **24**, for example. Consequently, in similar model experiments with **18** employing $FeBr_2$ and tetrahydrofuran, where considerable amounts of **24** are formed, the assumption that $O{=}Fe^{2+}$



is the stoichiometric counterpart must be treated with circumspection (Posner *et al.*, 1995a, 1996).

It is by no means certain that haem will behave like ferrous ion, but there are good mechanistic parallels (Meshnick *et al.*, 1996). Inside the parasite or nearby, once it is liberated by proteolysis of haemoglobin, haem (**213**) will behave like a reagent rather than as a Fe^{2+}/Fe^{3+} redox couple. The parasite and artemisinin are the reactants. First, **18** and **213** combine to generate the aggressive, ferric alkyl radical **221**, which then attacks the protein of the parasite (**PProt**). Protonation liberates the alkylated protein



222 and haemin (**223**) in which iron is now raised to the ferric state. As a result, the parasite is disabled and **223** is ready to precipitate as haemozoin. A similar course will be followed by **212**.

Although the experiments have yet to be performed, the 1,2,4,5-tetraoxanes **159–161**, and related antimalarial endoperoxides, should rearrange to the more stable ester derivatives on treatment with ferrous chloride. Yingzhaosu A (**17**) and C (**31**), and their synthetic analogues **167** and **168** should be susceptible to cleavage by ferrous iron, and therefore by haem, as in their case the excision of a conjugated ketone fragment provides the stabilized element (Jefford *et al.*, 1996).

The ferrous chloride-promoted isomerization reaction could find application as a diagnostic test for antimalarial activity. The product composition and the ease of reaction should be enough to tell whether a particular peroxide passes through a non-stabilized primary C-centred radical, and would thus be a drug candidate. Clearly, the candidate must fulfil other criteria in order to bring its mechanistic potential to bear.

10.2 MOLECULAR CONSIDERATIONS

In addition to the mechanistic desiderata for radical formation, enunciated above, molecular properties will also be important. The peroxide must be able to penetrate the cell membrane and coordinate intimately with the surface of haem in order to effect the required transfer of an electron.

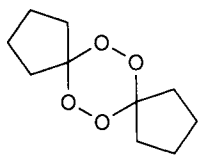
Conformation, electrostatic and other forces evidently come into play in the activation by haem. As an aid to understanding the nature of the interaction, several computer-assisted approaches have been undertaken. Molecular modelling studies of the docking of artemisinin onto haemin and haem have been performed by using the SYBIL program (Shukla *et al.*, 1995). In the most stable arrangement, the endoperoxide bridge lies close to the central iron of haem. Deoxyartemisin docks differently. In another study carried out with **212** and haem, but using the CATALYST program, similar closeness of fit between iron and the peroxide link is found. In addition, π–π interactions are required for close docking (Grigorov *et al.*, 1996).

The pharmacophore has also been elicited by comparative field analysis of a large set of artemisinin derivatives. It transpires that steric considerations outweigh electrostatic ones (Avery *et al.*, 1993). For sets of bicyclic trioxanes like **212**, the pharmacophore has been formulated by CATALYST in terms of features such as hydrogen bonding and hydrophobicity (Grigorov *et al.*, 1996).

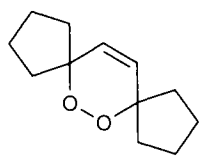
Finally, the molecular electrostatic potentials (MEP) of artemisinin (**18**) and some related molecules have been obtained by high-level computational studies (Bernardinelli *et al.*, 1994). The most negative zone lies on top of the peroxide bond of **18** and extends over the adjacent carbonyl function. In an inactive peroxide (**176**), the MEP is displaced towards the methoxy group.

11 Design of Future Peroxidic Antimalarial Drugs

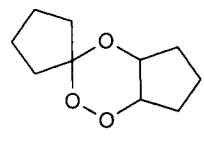
In the light of the previous discussion, the criteria for an effective peroxidic antimalarial agent can now be enumerated. As chirality is not an issue, the ideal molecule need not be chiral which would at the same time avoid certain regulatory problems pertaining to drug safety. Lipophilicity is desirable. Conformational mobility is important to ensure a close contact with haem. To facilitate charge transfer from iron to the drug, the lowest unoccupied orbital (LUMO) of the O—O sigma bond should lie close to the frontier. Most importantly, a masked ester or carbonyl group is a *sine qua non* to provide the driving force to create the alkyl radical. The radical itself must not be secondary or tertiary or stabilized by a substituent. The trioxane or



224



225



226

1,2-dioxane ring should be constrained, so that its rupture by coordination with haem is easy. As an illustration, the three minimum structures **224–226** fulfil most of the above criteria. They are all predicted to exhibit useful antimalarial activity.

12 Concluding Remarks

It is clear than more studies are necesary to further define the mechanism of alkylation. The adduct formed between haem and artemisinin and related peroxides needs to be characterized. The non-reactivity or the different reactivity of the ferric species, haemin or haematin, towards the peroxide bond in active trioxanes should also be verified. More amenable metalloporphyrins such as manganese tetraphenylporphin instead of the notoriously unstable haem will constitute useful models. Similar experiments, model and otherwise, conducted in the presence of simple peptide surrogates or with the parasite itself, will be necessary to identify the alkylated protein as a chemical entity and to confirm that mono-oxygenation does not occur. Recently, it has been shown in two animal models that artemether and arteether are neurotoxic at high dose (Brewer *et al.*, 1994). This latter effect appears to be related to drug action, but nonetheless needs to be established. In the same vein, the reported antitumour action of dihydroartemisinin in the presence of ferrous sulfate relates to its power of alkylation (Moore *et al.*, 1995) and prefigures similar activity for synthetic trioxanes, peroxides and tetroxanes. In view of their alkylating properties, antimalarial peroxides may have therapeutic potential against other parasites such as *Toxoplasma gondii* and *Pneumocystis carinii*.

Finally, there is no doubt that peroxidic antimalarials are well established as a new drug class. However, the possibility of the development of resistance by the malarial parasite should not be ignored. Synergistic effects with conventional nitrogen-containing antimalarials or drugs, such as atovaquone, which by themselves are therapeutically inadequate, are worth exploring.

It also seems likely that chloroquine, because of its diminishing efficacy for treating malaria, will be gradually replaced, not by another quinoline-based antimalarial or even a first-generation derivative of artemisinin, but by a simple peroxidic antimalarial, such as **212**, or another similar structure designed according to the above mechanistic principles.

References

Acton, N. and Klayman, D. L. (1985). *Planta Med.*, 441–442.
Acton, N. and Klayman, D. L. (1987). *Planta Med.*, 266–268.
Acton, N. and Roth, R. J. (1992). *J. Org. Chem.* **57**, 3610–3614.
Acton, N., Karle, J. M. and Miller, R. E. (1993). *J. Med. Chem.* **36**, 2552–2557.

Ager, A., Jr (1984). *In* 'Handbook of Experimental Parasitology: Antimalarial Drugs' (Peters, W. and Richards, W. H. G., eds), pp. 226–227. Springer Verlag, Berlin.

Anonymous (1994). *Trop. Dis. Res. News* **46**, 5.

Asawamahasakda, W., Ittarat, I., Pu, Y., Ziffer, H. and Meshnick, S. R. (1994). *Antimicrob. Agents Chemother.* **38**, 1854–1858.

Avery, M. A., Jennings-White, C. and Chong, W. K. M. (1989). *J. Org. Chem.* **54**, 1792–1795.

Avery, M. A., Chong, W. K. M., Jennings-White, C. (1992). *J. Am. Chem. Soc.* **114**, 974–979.

Avery, M. A., Gao, F., Chong, W. K. M., Mehrotra, S. and Milhous, W. K. (1993). *J. Med. Chem.* **36**, 4264–4275.

Avery, M. A., Gao, F., Chong, W. K. M., Hendrickson, T. F., Inman, W. D. and Crews, P. (1994). *Tetrahedron* **50**, 957–972.

Bernardinelli, G., Jefford, C. W., Maric, D., Thomson, C. and Weber, J. (1994). *Int. J. Quant. Chem.* **21**, 117–131.

Bhonsle, J. B., Pandey, B., Deshpande, V. H. and Ravindranathan, T. (1994). *Tetrahedron Lett.* **35**, 5489–5492.

Black, R. H., Canfield, C. J., Clyde, D. F., Peters, W. and Wernsdorfer, W. H. (1981). *In* 'Chemotherapy of Malaria', 2nd edn (Bruce-Chwatt, L. J., ed.), pp. 56–88. World Health Organization, Geneva.

Boukouvalas, J., Pouliot, R. and Fréchette, Y. (1995). *Tetrahedron Lett.* **36**, 4167–4170.

Brewer, T. G., Grate, S. J., Peggins, J. O., Weina, P. J., Petras, J. M., Levine, B. S., Heiffer, M. H. and Schuster, B. G. (1994). *Am. J. Trop. Med. Hyg.* **51**, 251–259.

Brossi, A., Venugopalan, B., Dominguez Gerpe, L., Yeh, H. J. C., Flippen-Anderson, J. L., Buchs, P., Luo, X. D., Milhous, W. and Peters, W. (1988). *J. Med. Chem.* **31**, 645–650.

Brown, P. (1992). *New Scientist* **136** (1845), 37–41.

Butler, A. R. and Wu, Y. L. (1992). *Chem. Soc. Rev.*, 85–90.

China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials (1982a). *J. Tradit. Chin. Med.* **2**, 9–16.

China Cooperative Research Group on Qinghaosu and its Derivatives as Antimalarials (1982b). *J. Tradit. Chin. Med.* **2**, 45–50.

Clara, F. (1990). *Med. Hyg.* **48**, 11–15.

Constantino, M. G., Beltrame, M., Jr and da Silva, G. V. J. (1996). *Synth. Commun.* **26**, 321–329.

Courtneidge, J. L. (1992). *J. Chem. Soc. Commun.* 381–383.

Desjardins, R. E., Canfield, C. J., Haynes, D. E. and Chulay, J. D. (1979). *Antimicrob. Agents Chemother.* **16**, 710–718.

Duran-Reynal, M. L. (1946). 'The Fever Bark Tree. The Pageant of Quinine'. Doubleday, New York.

Dussault, P. H., Anderson, T. A., Hayden, M. R., Koeller, K. J., Niu, Q. J. (1996). *Tetrahedron* **52**, 12,381–12,398.

El-Feraly, F. S., Ayalp, A., Al-Yahya, M. A., McPhail, D. R. and McPhail, A. T. (1990). *J. Nat. Prod.* **53**, 66–71.

Grigorov, M., Weber, J., Tronchet, J. M. J., Jefford, C. W., Milhous, W. K. and Maric, D. (1997). *J. Chem. Inf. Comput. Sci.*, **37**, 124–130.

Haynes, R. K. and Vonwiller, S. C. (1990). *J. Chem. Soc., Chem. Commun.* 451–453.

Haynes, R. K. and Vonwiller, S. C. (1992). *Synlett*, 481–483.

Haynes, R. K., Vonwiller, S. C. and Wang, H. J. (1995). *Tetrahedron Lett.* **36**, 4641–4642.

Hesp, B. and Willard, A. K. (1984). *In* 'Enzyme Chemistry', (Suckling, C. J., ed.), pp. 119–161. Chapman and Hall, London.

Hien, T. T. and White, N. J. (1993). *Lancet* **341**, 603–608.

Hofheinz, W., Bürgin, H., Gocke, E., Jaquet, C., Masciadri, R., Schmid, G., Stohler, H. and

Urwyler, H. (1994). *Trop. Med. Parasitol.* **45**, 261–265.

Hong, Y. L., Yang, Y. Z. and Meshnick, S. R. (1994). *Mol. Biochem. Parasitol.* **63**, 121–128.

Howells, R. E. (1982). *Brit. Med. Bull.* **38**, 193–199.

Hufford, C. D., Khalifa, S. I., McPhail, A. T., El-Feraly, F. S. and Ahmad, M. S. (1993). *J. Nat. Prod.* **56**, 62–66.

Imakura, Y., Yokoi, T., Yamagishi, T., Koyama, J., Hu, H., McPhail, D. R., McPhail, A. T. and Lee, K. H. (1988). *J. Chem. Soc., Chem. Commun.*, 372–374.

Jakupovic, J., Grenz, M. and Schmeda-Hirschmann, G. (1988). *Phytochemistry* **27**, 2997–2998.

Jaquet, C., Stohler, H. R., Chollet, J. and Peters, W. (1994). *Trop. Med. Parasitol.* **45**, 266–271.

Jefford, C. W. (1991). *In* 'Dioxygen Activation and Homogeneous Catalytic Oxidation' (Simándi, L. I., ed.), pp. 555–564. Elsevier Science Publishers B.V., Amsterdam.

Jefford, C. W., Ferro, S., Moulin, M. C., Velarde, J., Jaggi, D., Kohmoto, S., Richardson, G. D., Godoy, J., Rossier, J. C., Bernardinelli, G. and Boukouvalas, J. (1986a). *In* 'New Trends in Natural Products Chemistry 1986'. Studies in Organic Chemistry, Vol. 26. (Atta-ur-Rahman and Le Quesne, P. W., eds), pp. 163–183. Elsevier Science Publishers B.V., Amsterdam.

Jefford, C. W., Favarger, F., Ferro, S., Chambaz, D., Bringhen, A., Bernardinelli, G. and Boukouvalas, J. (1986b). *Helv. Chim. Acta* **69**, 1778–1786.

Jefford, C. W., McGoran, E. C., Boukouvalas, J., Richardson, G., Robinson, B. L. and Peters, W. (1988a). *Helv. Chim. Acta* **71**, 1805–1812.

Jefford, C. W., Velarde, J. A. and Bernardinelli, G. (1988b). *Tetrahedron Lett.* **30**, 4485–4488.

Jefford, C. W., Wang, Y. and Bernardinelli, G. (1988c). *Helv. Chim. Acta* **71**, 2042–2052.

Jefford, C. W., Misra, D., Rossier, J. C., Kamalaprija, P., Burger, U., Mareda, J., Bernardinelli, G., Peters, W., Robinson, B. L., Milhous, W. K., Zhang, F., Gosser, D. K. and Meshnick, S. R. (1993a). *In* 'Perspectives in Medicinal Chemistry' (Testa, B., Kyburz, E., Fuhrer, W. and Giger, R., eds), Chap. 29, pp. 459–473. Verlag Helvetica Chimica Acta, Basel.

Jefford, C. W., Velarde, J. A., Bernardinelli, G., Bray, D. H., Warhurst, D. C. and Milhous, W. K. (1993b). *Helv. Chim. Acta* **76**, 2775–2788.

Jefford, C. W., Favarger, F., Vicente, M. G. H. and Jacquier, Y. (1995a). *Helv. Chim. Acta* **78**, 452–458.

Jefford, C. W., Kohmoto, S., Jaggi, D., Timári, G., Rossier, J.-C., Rudaz, M., Barbuzzi, O., Gérard, D., Burger, U., Kamalaprija, P., Mareda, J., Bernardinelli, G., Manzanares, I., Canfield, C. J., Fleck, S. L., Robinson, B. L. and Peters, W. (1995b). *Helv. Chim. Acta* **78**, 647–662.

Jefford, C. W., Vicente, M. G. H., Jacquier, Y., Favarger, F., Mareda, J., Millasson-Schmidt, P., Brunner, G. and Burger, U. (1996). *Helv. Chim. Acta* **79**, 1475–1487.

Jung, M. (1994). *Curr. Med. Chem.* **1**, 35–49.

Jung, M., Li, X., Bustos, D. A., El-Sohly, H. N. and McChesney, J. D. (1989). *Tetrahedron Lett.* **30**, 5973–5976.

Jung, M., Bustos, D. A., El-Sohly, H. N. and McChesney, J. D. (1990a). *Synlett*, 743–744.

Jung, M., Li, X., Bustos, D. A., El-Sohly, H. N., McChesney, J. D. and Milhous, W. K. (1990b). *J. Med. Chem.* **33**, 1516–1518.

Jung, M., Freitas, A. C. C., McChesney, J. D. and El-Sohly, H. N. (1994). *Heterocycles* **39**, 23–29.

Kamchonwongpaisan, S., Nilanonta, C., Tarnchompoo, B., Thebtaranonth, C., Thebtaranonth, Y., Yuthavong, Y., Kongsaeree, P. and Clardy, J. (1995). *Tetrahedron Lett.* **36**, 1821–1824.

Keppler, J. A., Philip, A., Lee, Y. W., Musallam, H. A. and Carroll, F. I. (1987). *J. Med. Chem.* **30**, 1505–1509.

Keppler, J. A., Philip, A., Lee, Y. W., Morey, M. C. and Carroll, F. I. (1988). *J. Med. Chem.* **31**, 713–716.

Klayman, D. L. (1985). *Science* **228**, 1049–1055.

Kremsner, P. G. (1990). *J. Antimicrob. Chemother.* **25**, 9–14.

Lansbury, P. T. and Nowak, D. M. (1992). *Tetrahedron Lett.* **33**, 1029–1032.

Leskovac, V. and Theoharides, A. D. (1991). *Comp. Biochem. Physiol.* **99C**, 383–390.

Li, Y. and Pan, J. P. (1993). *Chin. Chem. Lett.* **4**, 99–102.

Li, Y., Yu, P., Chen, Y., Li, L., Gai, Y., Wang, D. and Zheng, Y. (1981). *Acta Pharm. Sinica* **16**, 429–439.

Liang, X. T. (1985). *In* 'Advances in Chinese Medicinal Materials Research' (Chang, H. M., Yeung, H. W., Tso, W. W. and Koo, A., eds), pp. 427–432, World Scientific Publishing Co., Singapore.

Liang, X. T., Yu, D. Q., Wu, W. L. and Deng, H. C. (1979). *Acta Chim. Sinica* **37**, 215–230.

Lin, A. J and Miller, R. E. (1995). *J. Med. Chem.* **38**, 764–770.

Lin, A. J., Klayman, D. L. and Milhous, W. K. (1987). *J. Med. Chem.* **30**, 2147–2150.

Lin, A. J., Lee, M. and Klayman, D. L. (1989). *J. Med. Chem.* **32**, 1249–1252.

Lin, A. J., Li, L. Q., Klayman, D. L., George, C. F. and Flippen-Anderson, J. L. (1990). *J. Med. Chem.* **33**, 2610–2614.

Lin, A. J., Li, L. Q., Milhous, W. K. and Klayman, D. L. (1991). *Med. Chem. Res.* **1**, 20–23.

Lin, A. J., Li, L. Q., Andersen, S. L. and Klayman, D. L. (1992). *J. Med. Chem.* **35**, 1639–1642.

Liu, J. M., Ni, M. Y., Fan, J. F., Tu, Y. Y., Wu, Z. H. and Zhou, W. S. (1979). *Acta Chim. Sinica* **37**, 129–143.

Liu, H. J., Yeh, W. L. and Chew, S. Y. (1993). *Tetrahedron Lett.* **34**, 4435–4438.

Luo, X. D. and Shen, C. C. (1987). *Med. Res. Rev.* **7**, 29–52.

Meshnick, S. R., Thomas, A., Ranz, A., Xu, C. and Pan, H. (1991). *Mol. Biochem. Parasitol.* **49**, 181–189.

Meshnick, S. R., Taylor, T. E. and Kamchonwongpaisan, S. (1996). *Microb. Rev.* **60**, 301–315.

Milhous, W. K., Weatherly, N. F., Bowdre, J. H. and Desjardins, R. E. (1985). *Antimicrob. Agents Chemother.* **27**, 525–530.

Miller, K. D., Lobel, H. O., Satriale, R. F., Kuritsky, J. N., Stern, R. and Campbell, C. C. (1986). *Am. J. Trop. Med. Hyg.* **5**, 451–458.

Moore, J. C., Lai, H., Li, J. R., Ren, R. L., McDougall, J. A., Singh, N. P. and Chou, C. K. (1995). *Cancer Lett.* **98**, 83–87.

Oaks, S. C., Jr, Mitchell, V. S., Pearson, G. W. and Carpenter, C. C. J. (eds) (1991). 'Malaria. Obstacles and Opportunities'. National Academy Press, Washington, D.C.

Osdene, T. S., Russell, P. B. and Rane, L. (1967). *J. Med. Chem.* **10**, 431–434.

Peters, W. (1980). *In* 'Malaria. Epidemiology, Chemotherapy, Morphology and Metabolism' (Kreier, J. P., ed.), Vol. 1, pp. 145–283. Academic Press, New York.

Peters, W. (1987). 'Chemotherapy and Drug Resistance in Malaria'. Academic Press, New York.

Peters W. (1994). *In* "Science and Medicine in the 21st Century", pp. 107–132. The King Faisal Foundation, Riyadh, Saudi Arabia.

Peters, W., Portus, J. H. and Robinson, B. L. (1975). *Ann. Trop. Med. Parasitol.* **69**, 155–171.

Peters, W., Robinson, B. L., Rossier, J. C. and Jefford, C. W. (1993a). *Ann. Trop. Med. Hyg.* **87**, 1–7.

Peters, W., Robinson, B. L., Rossier, J. C., Misra, D. and Jefford, C. W. (1993b). *Ann. Trop. Med. Parasitol.* **87**, 9–16.

Peters, W., Robinson, B. L., Tovey, G., Rossier, J. C. and Jefford, C. W. (1993c). *Ann. Trop. Med. Parasitol.* **87**, 111–123.

Petrov, O. and Ognyanov, I. (1991). *Collect. Czech. Chem. Commun.* **56**, 1037–1041.

Posner, G. H. and Oh, C. H. (1992). *J. Am. Chem. Soc.* **114**, 8328–8329.

Posner, G. H., Oh, C. H., Gerena, L. and Milhous, W. K. (1992). *J. Med. Chem.* **35**, 2459–2467.

Posner, G. H., Oh, C. H., Wang, D., Gerena, L., Milhous, W. K., Meshnick, S. R. and Asawamahasakda, W. (1994). *J. Med. Chem.* **37**, 1256–1258.

Posner, G. H., Cumming, J. N., Ploypradith, P. and Ho, C. H. (1995a). *J. Am. Chem. Soc.* **117**, 5885–5886.

Posner, G. H., McGarvey, D. J., Oh, C. H., Mumar, N., Meshnick, S. R. and Asawamahasakda, W. (1995b). *J. Med. Chem.* **38**, 607–612.

Posner, G. H., Oh, C. H., Gerena, L. and Milhous, W. K. (1995c). *Heteroatom Chem.* **6**, 105–116.

Posner, G. H., Wang, D., Cumming, J. N., Oh, C. H., French, A. N., Bodley, A. L. and Shapiro, T. A. (1995d). *J. Med. Chem.* **38**, 2273–2275.

Posner, G. H., Park, S. B., González, L., Wang, D., Cumming, J. N., Klinedinst, D., Shapiro, T. A. and Bachi, M. D. (1996). *J. Am. Chem. Soc.* **118**, 3537–3538.

Pu, Y. M., Yagen, B. and Ziffer, H. (1994). *Tetrahedron Lett.* **35**, 2129–2132.

Pu, Y. M., Torok, D. S., Ziffer, H., Pan, X. Q. and Meshnick, S. R. (1995). *J. Med. Chem.* **38**, 4120–4124.

Qinghaosu Research Group (1980). *Sci. Sinica* **23**, 380–388.

Ravindranathan, T., Kumar, M. A., Menon, R. B. and Hiremath, S. V. (1990). *Tetrahedron Lett.* **31**, 755–758.

Rieckmann, K. H. (1984). *In* 'Antimalarial Drugs' (Peters, W. and Richards, W. H. G., eds), Vol. II, pp. 443–470. Springer Verlag, New York.

Roth, R. J. and Acton, N. (1989). *J. Nat. Prod.* **52**, 1183–1185.

Rücker, G. (1995). *Pharmazie in unserer Zeit* **24**, 189–195.

Rücker, G., Walter, R. D., Manns, D. and Mayer, R. (1991). *Planta Med.* **57**, 295–296.

Russell, P. F. (1955). 'Man's Mastery of Malaria'. Oxford University Press, London.

Schmid, G. and Hofheinz, W. (1983). *J. Am. Chem. Soc.* **105**, 624–625.

Schuurkamp, G. J., Spicer, P. E., Kereu, R. K., Bulungol, P. K. and Rieckmann, K. H. (1992). *Trans. Roy. Soc. Trop. Med. Hyg.*, **86**, 121–122.

Shukla, K. L., Gund, T. M. and Meshnick, S. R. (1995). *J. Mol. Graph.* **13**, 215–222.

Singh, C., Misra, D., Saxena, G. and Chandra, S. (1995). *Bioorg. Med. Chem. Lett.* **5**, 1913–1916.

Slater, A. F. G. and Cerami, A. (1992). *Nature* **355**, 167–169.

Slater, A. F. G., Swiggard, W. J., Orton, B. R., Flitter, W. D., Goldberg, D. E., Cerami, A. and Henderson, G. B. (1991). *Proc. Natl Acad. Sci. USA* **88**, 325–329.

Stefanovič, M., Jokič, A., Behbud, A. and Jeremič, D. (1972). *Eighth Int. Symp. on Chemistry of Natural Products, New Delhi*, Abstr. C 57.

Sweeney, T. R. and Strube, R. E. (1979). *In* 'Burger's Medicinal Chemistry' (Wolff, M. E., ed.), Part II, pp. 333–413. John Wiley and Sons, New York.

Tani, S., Fukamiya, N., Kiyokawa, H., Musallam, H. A., Pick, R. O. and Lee, K. H. (1985). *J. Med. Chem.* **28**, 1743–1744.

Torok, D. S., Ziffer, H., Meshnick, S. R., Pan, X. Q. and Ager, A. (1995). *J. Med. Chem.* **38**, 5045–5050.

Vennerstrom, J. (1989). *J. Med. Chem.* **32**, 64–67.

Vennerstrom, J. and Eaton, J. W. (1988). *J. Med. Chem.* **31**, 1269–1277.

Vennerstrom, J., Acton, N., Lin, A. J. and Klayman, D. L. (1989). *Drug Design and*

Delivery **4**, 45–54.
Vennerstrom, J., Fu, H. N., Ellis, W. Y., Ager, A. L., Jr, Wood, J. K., Andersen, S. L., Gerena, L. and Milhous, W. K. (1992). *J. Med. Chem.* **35**, 3023–3027.
Venugopalan, B., Bapat, C. P., Karnik, P. J., Chatterjee, D. K., Iyer, N. and Lepcha, D. (1995a). *J. Med. Chem.* **38**, 1922–1927.
Venugopalan, B., Karnik, P. J., Bapat, C. P., Chatterjee, D. K., Iyer, N. and Lepcha, D. (1995b). *Eur. J. Med. Chem.* **30**, 697–706.
Vishwakarma, R. A. (1990). *J. Nat. Prod.* **53**, 216–217.
Vishwakarma, R. A., Mehrotra, R., Tripathi, R. and Dutta, G. P. (1992). *J. Nat. Prod.* **55**, 1142–1144.
Vonwiller, S. C., Warner, J. A., Mann, S. T. and Haynes, R. K. (1995). *J. Am. Chem. Soc.* **117**, 11,098–11,105.
Wernsdorfer, W. H. (1991). *Parasitol. Today* **7**, 297–298.
Wernsdorfer, W. H. (1994). *Acta Trop.* **56**, 143–156.
White, N. J. (1987). *Lancet* **1**, 680–681.
Woerdenbag, H. J., Lugt, C. B. and Pras, N. (1990). *Pharm Weekblad Sci.* **12**, 169–181.
Woerdenbag, H. J., Pras, N., van Uden, W., Wallart, T. E., Beekman, A. C. and Lugt, C. B. (1994). *Pharm. World and Science* **16**, 169–180.
Xu, X. X. and Dong, H. Q. (1995). *J. Org. Chem.* **60**, 3039–3044.
Xu, X. X., Zhu, J., Huang, D. Z. and Zhou, W. S. (1986). *Tetrahedron* **42**, 819–828.
Xu, X. X., Zhu, J., Huang, D. Z. and Zhou, W. S. (1991). *Tetrahedron Lett.* **32**, 5785–5788.
Yang, Y. H., Li, Y., Shi, Y. L., Yang, J. D. and Wu, B. A. (1995). *Biorg. Med. Chem. Lett.* **5**, 1791–1794.
Ye, B. and Wu, Y. L. (1990). *J. Chem. Soc. Chem. Commun.*, 726–727.
Zaman, S. S. and Sharma, R. P. (1991). *Heterocycles* **32**, 1593–1638.
Zhou, W. S. and Xu, X. X. (1994). *Acc. Chem. Res.* **27**, 211–216.

SUBJECT INDEX

CUMULATIVE INDEX OF AUTHORS

CUMULATIVE INDEX OF TITLES