PROGRESS IN MEDICINAL CHEMISTRY 25

G. P. ELLIS
G. B. WEST
EDITORS

Progress in Medicinal Chemistry 25

This Page Intentionally Left Blank

Progress in Medicinal Chemistry

Edited by

G.P. ELLIS, D.SC., PH.D., F.R.S.C.

Department of Applied Chemistry,
University of Wales Institute of Science and Technology,
P.O. Box 13,
Cardiff, CF1 3XF,
United Kingdom

and

G.B. WEST, B. PHARM., D.SC., PH.D., F.I.BIOL.

Department of Paramedical Sciences,
North East London Polytechnic,
Romford Road,
London, E15 4LZ,
United Kingdom

1988

ELSEVIER
AMSTERDAM · NEW YORK · OXFORD

ISBN 0-444-80965-1
ISBN Series 0-7204-7400-0

Published by:

Elsevier Science Publishers B.V. (Biomedical Division)
P.O. Box 211
1000 AE Amsterdam
The Netherlands

Sole distributors for the USA and Canada:
Elsevier Science Publishing Company, Inc.
52 Vanderbilt Avenue
New York, NY 10017
USA

Library of Congress Cataloging in Publication Data

Please refer to card number 62-27/2 for this series

Printed in The Netherlands

Preface

To commemorate the publication of the twenty-fifth volume of this Series, we have chosen six chapters covering a wide range of topics, from the latest in medicinal products to the usefulness of different types of evaluation of biological results.

In Chapter 1, the discovery of cyclosporins, a new generation of immunosuppressants, is described, thereby stimulating further the continuous search for chemicals of value in organ transplantation. For the design of new antineoplastic agents, factors such as electron distribution, partition coefficients and certain physical characteristics are important and these are discussed in Chapter 2, particularly relating to new 2-phenylnaphthalene-type ring systems. The topic of cancer chemotherapy is continued in Chapter 3 where the focus is on the synthesis of folic acid, aminopterin and methotrexate analogues in which an intact L-glutamic acid side-chain is present. We hope to include a complementary review on medicinal aspects of this topic in a future volume.

Chapter 4 surveys a new class of 5-substituted arylmethylbenzimidazole carbamates and increases the prospect that an orally active and powerful drug against the adult filarial worm may soon be discovered. The protective action offered by vitamin E against the damaging effects of hypoxia is discussed in Chapter 5, although this action is only one possible use for this vitamin in medicine.

Finally, from Chapter 6, the utility of multivariate statistics in different stages of the scientific process (like design, description and analysis) appears to be more informative and more valuable than the univariate approach.

As in all previous twenty-four volumes, we thank our authors for surveying the literature of their chosen fields so that we all benefit. We also offer our thanks to owners of copyright material who have allowed it to be reproduced in this volume. Finally, our publisher has, as always, given us every assistance in our aim to present timely reviews in medicinal chemistry.

October 1987

G.P. Ellis
G.B. West

This Page Intentionally Left Blank

Contents

Progress in Medicinal Chemistry – Vol. 25, edited by G.P. Ellis and G.B. West

1 Cyclosporins, Fungal Metabolites with Immunosuppressive Activities

ALBERT VON WARTBURG, Ph.D. and RENÉ TRABER, Ph.D.*

Pharmaceutical Division, Preclinical Research, Sandoz Ltd., CH-4002 Basel, Switzerland

* To whom correspondence should be addressed.

THE DISCOVERY OF CYCLOSPORINS

The fungal metabolite, cyclosporin A [1, 2], exerts a powerful immunosuppressive activity directed mainly against T-lymphocytes without affecting haemopoietic tissues. This natural compound, recently introduced under the trade name Sandimmun®, marks an important advance in the immunotherapy of bone marrow and organ transplantations. The history of cyclosporin A begins in 1970 with the isolation of two new strains of fungi imperfecti in the course of our microbiological screening programme. Both strains, taxonomically determined as *Cylindrocarpon lucidum* Booth and *Tolypocladium inflatum* Gams (originally classified as *Trichoderma polysporum* [Link ex Pers]Rifai), were isolated from soil samples collected in Wisconsin (U.S.A.) and in the Hardanger Vidda (Norway) [3]. In primary tests, extracts of cultures displayed a rather narrow spectrum of antifungal activities *in vitro*; however, *in vivo*, only marginal antimycotic effects were noticed. Based on our experience that microbial metabolites may often show unexpected, additional biological effects, a partially purified extract preparation from *T. inflatum* was submitted to a general pharmacological screening programme. This programme comprised a series of assays designed to recognize drug effects on the cardiovascular system and the central nervous system, and included also tests indicating inhibition of cell proliferation, tumour growth and the immune response. The very first experiments in mice carried out with this preparation (composed mainly of cyclosporins A and B) revealed high immunosuppressive and anti-inflammatory activities, surprisingly not linked with general cytostatic effects [4, 5]. These exciting results marked the starting point for extensive chemical, biological and pharmacological investigations.

First of all, the complex mixture of fungal metabolites was separated into single components, yielding pure cyclosporin A and several minor metabolites (1973). Subsequently, we established the structure, conformation and absolute configuration of cyclosporin A [2, 6]. Further purification processes with crude fungal extracts led to the isolation of a plethora of structurally closely-related congeners [7–10]. All 25 cyclosporins (designated as cyclosporins A to Z), isolated so far, represent neutral cyclic oligopeptides composed of 11 amino acids. Other characteristic features consist in the presence of a unique C_9-amino acid, the *N*-methylation of several amino acids and the occurrence of one or two amino acids of the D-series (*Figure 1.1*, *Table 1.2*). Based on the acquired structural information, the total synthesis of cyclosporin A was achieved in 1980 starting from natural chiral building blocks [11, 12]. Essential structure–activity relationships were recognized by comparing the biological effects of natural and synthetic cyclosporins, revealing the spatial and conformational requirements for immunosuppressive activity.

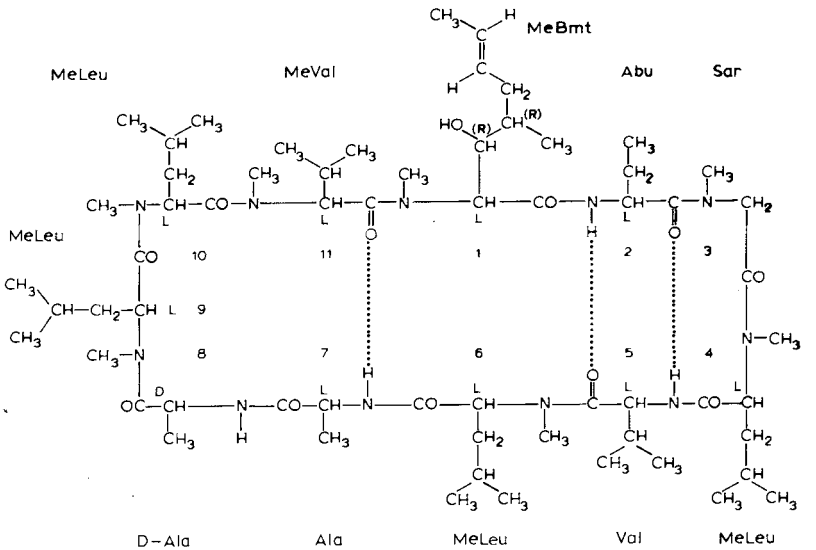


Figure 1.1. Cyclosporin A (1).

Parallel with chemical investigations, microbiological studies were intensified, especially the search for high producing mutants, as well as the improvement of media and culture conditions to enhance yields. A substantial feedback resulted from examination of the biosynthesis of cyclosporins. Yields and the composition of the metabolite mixture were significantly influenced by exogenously supplied amino-acid precursors.

Simultaneously, rapid progress in the pharmacological field was attained. Using pure, crystallized cyclosporin A, the immunosuppressive activity was confirmed by *in vitro* and *in vivo* assays. Toxicity studies again showed the selectivity of cyclosporin A for lymphocytes and the lack of effect on the haemopoiesis. Extensive animal studies in various transplantation models displayed the specific activity in preventing allograph rejection. First kidney transplantations in humans were reported in 1978 [13] and bone marrow transplantations were performed in the same year [14], demonstrating the strong immunosuppressive effect of cyclosporin A also in man. New important areas for the clinical use of cyclosporin A, still in the investigation phase, concern autoimmune disorders and, rather unexpectedly, parasitology.

Complementary research activities deal with the development of suitable galenical formulations, the elaboration of analytical methods for monitoring cyclosporin levels, the study of pharmacokinetics and metabolism and, last but not least, the elucidation of the intriguing mechanism of action of cyclosporin A.

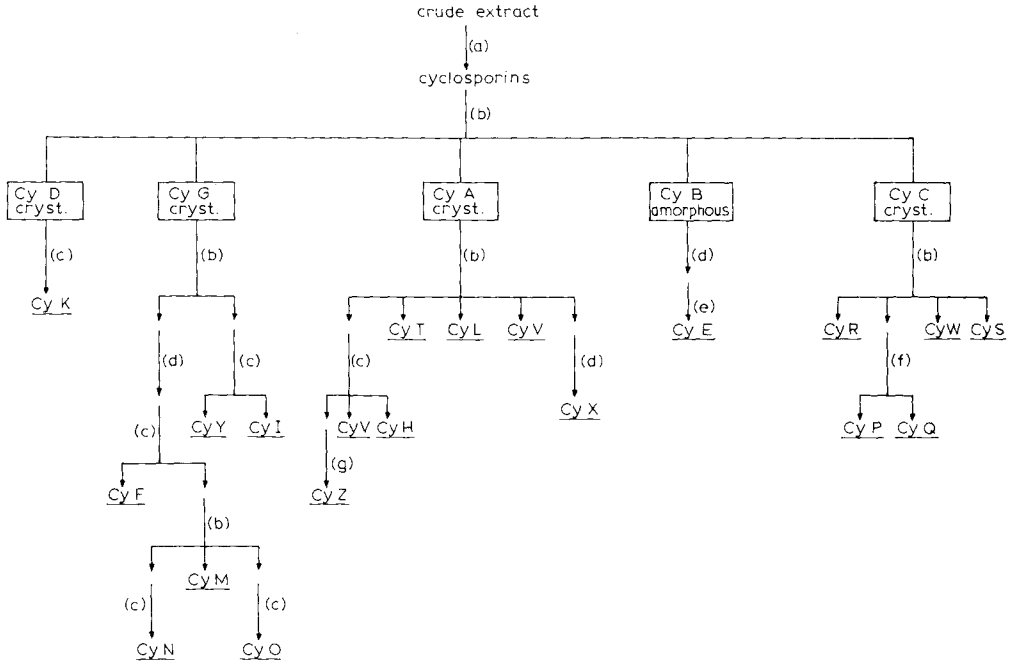


Figure 1.2. Isolation of the cyclosporins A–Z. (a) Sephadex LH-20, methanol; (b) silica-gel, water-saturated ethyl acetate; (c) silica-gel, hexane/acetone (2 :1); (d) silica-gel, chloroform/methanol (98 :2); (e) silica-gel, diethyl ether/methanol (95 :5); (f) silica-gel, methylene chloride/methanol (96 :4); (g) LiChroprep RP-18, methanol/water (4 :1).

PRODUCTION AND ISOLATION OF CYCLOSPORINS

In the beginning cyclosporins were encountered exclusively in *Cylindrocarpon lucidum* and *Tolypocladium inflatum*. Systematic screening procedures showed later that the related species *Tolypocladium geodes* as well as several other genera of lower fungi are also capable of synthesizing these secondary metabolites [15, 16]. For large-scale production, aerated submerged cultures of mutants selected from the original strain of *T. inflatum* (NRRL 8044) were used. *C. lucidum*, which grows only in surface culture, was less suitable. For inoculum propagation a yeast-malt agar, and for cultivation a medium containing glucose and casein peptone supplemented with trace elements and vitamins is recommended. After a fermentation time of 10–12 days the biomass was separated and the mycelium cake was extracted with methanol/water (9:1). In an alternative procedure, the culture broth was mixed with *n*-butyl acetate and then centrifuged. Concentration of the extracts *in vacuo* and removal of lipids furnished a crude complex mixture of metabolites. The complete separation of this material was achieved by systematic chromatography on silica gel. First, the quantitatively dominating cyclosporins A and C were isolated in a pure state, followed by the congeners B, D and G. Further processing of minor fractions and mother liquors unravelled additional cyclosporins, some of them occurring in only tiny amounts (*Figure 1.2*). In several cases, the yields of a desired trace component could be enhanced by adding specific amino acids to the culture medium (see section 'Directed Biosynthesis of Cyclosporins'). The course of the fractionation and purification procedure was followed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Many cyclosporins crystallized from acetone or diethyl ether. Definite proof of purity of the single components was established by HPLC-profiles and spectro-analytic evidence. Up to now, a total of 25 individual cyclosporins have been isolated from *T. inflatum*. Several additional congeners not yet obtained in a pure state are under investigation. The molecular formulae and some of the physicochemical data of all known cyclosporins are compiled in *Table 1.1*.

STRUCTURE ELUCIDATIONS

STRUCTURE OF CYCLOSPORIN A

The main metabolite, cyclosporin A, crystallizes from acetone as white prismatic needles. The lipophilic compound is soluble in most organic solvents

Table 1.1. PHYSICOCHEMICAL DATA OF CYCLOSPORINS A–Z

Metabolite (Cyclosporin:)	*Molecular formula*	*Melting point (°C)*	$[\alpha]_D^{20}$ *in* $CHCl_3$	$[\alpha]_D^{20}$ *in* CH_3OH
A	$C_{62}H_{111}N_{11}O_{12}$	148–151 cryst., acetone	– 244°	– 189°
B	$C_{61}H_{109}N_{11}O_{12}$	149–152 amorphous	– 238°	– 168°
C	$C_{62}H_{111}N_{11}O_{13}$	152–155 cryst., acetone	– 255°	– 182°
D	$C_{63}H_{113}N_{11}O_{12}$	148–151 cryst., acetone	– 245°	– 211°
E	$C_{61}H_{109}N_{11}O_{12}$	149–152 cryst., ether	– 179°	– 186°
F	$C_{62}H_{111}N_{11}O_{11}$	195–196 cryst., ether/ hexane 1:4	– 285°	– 205°
G	$C_{63}H_{113}N_{11}O_{12}$	196–197 cryst., ether/ hexane 1:1	– 245°	– 191°
H	$C_{62}H_{111}N_{11}O_{12}$	162–165 cryst., ether	– 177°	– 112°
I	$C_{62}H_{111}N_{11}O_{12}$	137–140 amorphous	– 220°	– 183°
K	$C_{63}H_{113}N_{11}O_{11}$	127–132 amorphous	– 293°	– 241°
L	$C_{61}H_{109}N_{11}O_{12}$	154–157 amorphous	– 292°	– 187°
M	$C_{63}H_{113}N_{11}O_{12}$	142–144 cryst., ether	– 211°	– 183°
N	$C_{62}H_{111}N_{11}O_{12}$	169–172 cryst., ether	– 234°	– 188°
O	$C_{60}H_{109}N_{11}O_{11}$	186–188 cryst., ether/ hexane 1:7	– 304°	– 228°
P	$C_{61}H_{109}N_{11}O_{13}$	157–160 amorphous	– 258°	– 181°
Q	$C_{60}H_{107}N_{11}O_{12}$	160–164 amorphous	– 247°	– 156°

Table 1.1. continued

Metabolite (Cyclosporin:)	*Molecular formula*	*Melting point (° C)*	$[\alpha]_D^{20}$ *in* $CHCl_3$	$[\alpha]_D^{20}$ *in* CH_3OH
R	$C_{60}H_{107}N_{11}O_{12}$	173–175 amorphous	– 175°	– 179°
S	$C_{60}H_{107}N_{11}O_{13}$	174–176 amorphous	– 199°	– 153°
T	$C_{61}H_{109}N_{11}O_{12}$	168–171 amorphous	– 245°	– 196°
U	$C_{61}H_{109}N_{11}O_{12}$	177–179 amorphous	– 207°	– 190°
V	$C_{63}H_{113}N_{11}O_{12}$	149–152 cryst., ether	– 225°	– 191°
W	$C_{61}H_{109}N_{11}O_{13}$	150–156 amorphous	– 219°	– 207°
X	$C_{62}H_{111}N_{11}O_{12}$	160–163 cryst., ether	– 233°	– 165°
Y	$C_{62}H_{111}N_{11}O_{12}$	172–175 amorphous	– 192°	– 179°
Z	$C_{61}H_{111}N_{11}O_{11}$	132–136 amorphous	– 297°	– 226°

but poorly in water. Titration and paper-electrophoresis indicated that cyclosporin A (1) contains neither free amino nor carboxylic groups. Elemental analysis and mass spectra (*m/z* 1201) are consistent with the formula $C_{62}H_{111}N_{11}O_{12}$. The UV spectrum displays only end absorption. IR and NMR spectra revealed the presence of a secondary hydroxyl group, confirmed by acylation reactions, and a C=C double bond, corroborated by hydrogenation of cyclosporin A to dihydrocyclosporin A (2). The high content of nitrogen, characteristic bands for amide groups (1630–1670 cm^{-1}) and a series of informative ^1H- and ^{13}C-NMR signals associated with amide protons, α-protons of amino acids and carbonyl groups pointed clearly to a peptide structure for cyclosporin A. Accordingly, hydrolysis with 6 M HCl furnished a mixture of amino acids which was separated by chromatography on cellulose into the following components: sarcosine, DL-alanine (2 mol), L-α-aminobutyric acid, L-valine, *N*-methyl-L-valine, *N*-methyl-L-leucine (4 mol) and an artifact (5) of an eleventh, hitherto unknown, *N*-methylated C_9-amino acid. The

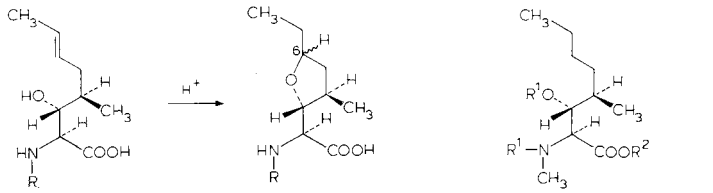
identification of the common amino acids was carried out by direct comparison and by gas chromatography of the corresponding trifluoroacetyl methyl ester derivatives. The two alanine units were isolated as a racemate. Cyclosporin A contains obviously one D-alanine; all the other chiral amino acids belong to the L-series.

Structure of the C_9-amino acid

The genuine unknown amino acid, originally designated as C_9-amino acid according to its carbon skeleton, contains both the OH-group and the double bond established in cyclosporin A. In the isolated artifact, these functional groups were absent. Circumstantial evidence for the structure of the intact C_9-amino acid and its transformation product (5) was received from the dihydro-C_9-amino acid (7) isolated in genuine form from the hydrolysis products of dihydrocyclosporin A (2). Informative fragmentation patterns in the mass spectrum and indicative NMR signals displayed by the corresponding *N,O*-bis(trifluoroacetyl) methyl ester derivative (8) revealed formulae (3) and (7) for the genuine C_9-amino acid and the dihydro derivative, respectively. The proposed positions of the functional groups and the assumed *trans*-geometry of the double bond were confirmed by high-resolution ^{1}H-NMR spectroscopy at 360 MHz. The instability of the C_9-amino acid towards strong acids and the ready formation of cyclic artifacts (actually a mixture of C-6 isomers) are explained by an internal acid-catalysed cyclization leading to tetrahydrofuran derivatives (5). Final proof of the stereochemical assignments and the absolute configuration ((2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid) emerged from X-ray analysis of cyclosporin A. According to IUPAC rules for amino-acid nomenclature, the C_9-amino acid is now designated (4*R*)-4-((*E*)-2-butenyl)-4,*N*-dimethyl-L-threonine (abbrev. MeBmt). Due to the sensitivity towards strong acids, the intact MeBmt was not accessible by hydrolysis of cyclosporin A. However the *N*-demethylated C_9-amino acid (Bmt) (4) was recently discovered as a metabolite produced by a blocked mutant of *T. inflatum* [17]. As expected, Bmt furnished the cyclic derivatives (6) by acidic treatment.

Sequence analysis

In summary, cyclosporin A is a cyclic undecapeptide composed of ten trivial amino acids and the unique MeBmt as the eleventh building block. All chiral amino acids belong to the L-series, with the exception of one D-alanine unit. Another striking feature is the *N*-methylation of seven amino acids responsible

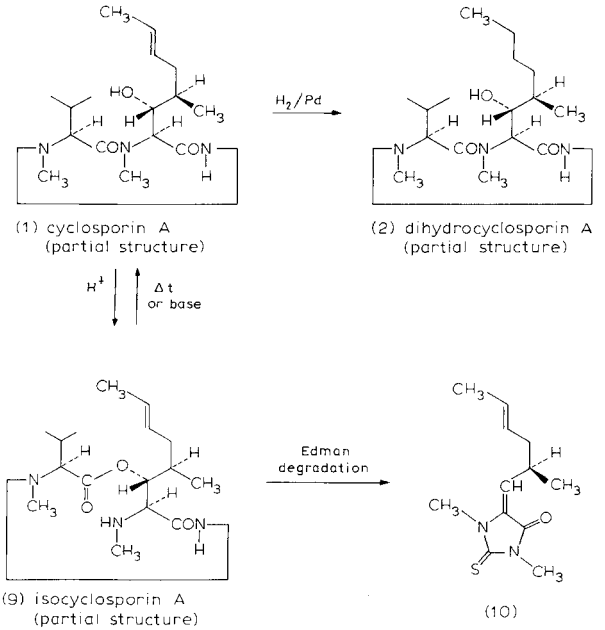


(3) R=CH_3 C_9—amino acid
(4) R=H *N*-demethyl-C_9—amino acid

cyclic artifacts
(5) R=CH_3
(6) R=H

(7) $R^1=R^2=H$ dihydro-C_9—amino acid
(8) $R^1=CF_3CO$ $R^2=CH_3$

for the lipophilic character. In addition, it can be inferred by the lack of free amino and carboxylic groups that cyclosporin A is a cyclic peptide. Sequence analysis using conventional methods seemed therefore not feasible. Attempts to open the assumed 33-membered ring selectively by hydrolysis failed, yielding only small, insignificant fragments. Acid-catalysed methanolysis of cyclosporin A took an unexpected course, furnishing not the parent linear peptide but a cyclic isomeric rearrangement product (9). Isocyclosporin A (9) lacks the OH-group of the MeBmt unit but contains the intact double bond and, surpris-



(1) cyclosporin A (partial structure)
(2) dihydrocyclosporin A (partial structure)
(9) isocyclosporin A (partial structure)
(10)

ingly, an ester and an *N*-methylamino group. Accordingly isocyclosporin A yielded a dihydro compound and *N*-acylderivatives. The isomerization reaction is reversible, because, in boiling dioxane or with base, isocyclosporin A reverts into cyclosporin A. These findings are consistent with an *N,O*-acylmigration. The foregoing amino acid (MeVal) migrates from the *N*-methylamino group of MeBmt to the neighbouring OH-group, giving rise to ester formation, ring enlargement and liberation of a free *N*-methylamino function. The presence of this basic group made isocyclosporin A a key intermediate which allowed a sequence analysis by a modified Edman degradation. Due to the lipophilic character, several adaptations had to be made, taking into account the presence of seven *N*-methylated amino acids, the occurrence of unselective, additional cleavage of peptide bonds and enhanced difficulties in the extraction of the primary formed thiazolinone intermediates. Using methyl isothiocyanate as suitable reagent, the ester linkage was easily cleaved under the reaction conditions and a stepwise release of amino acids could be attained. As anticipated, the methylthiohydantoin of anhydro-MeBmt (10) appeared as first building block, followed successively by the corresponding derivatives of α-aminobutyric acid, sarcosine, *N*-methylleucine, valine, *N*-methylleucine, alanine, alanine, *N*-methylleucine, *N*-methylleucine and finally *N*-methylvaline. The sequence of amino acids thus revealed in isocyclosporin A and hence in cyclosporin A is illustrated in *Figure 1.3* [2].

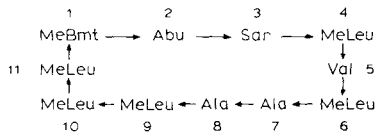
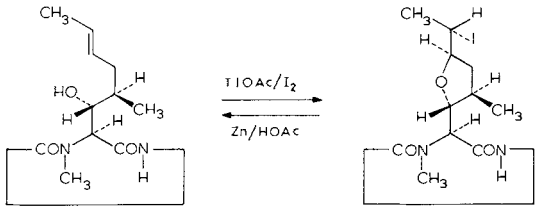


Figure 1.3. Amino-acid sequence in cyclosporin A.

Final proof for the inferred structure (1) for cyclosporin A and a first insight in the shape of the molecule resulted from X-ray analysis and high-resolution NMR spectra. The preparation of a crystallized derivative containing a heavy atom was achieved by an addition reaction using iodine and thallium(I) acetate. Instead of the expected iodoacetoxy derivative, the cyclic product (11) was obtained. Obviously, the reaction proceeded by a selective addition of iodine to the double bond of the MeBmt unit followed by an internal cyclization with the participation of the OH group. Iodocyclosporin A (11) reverted easily with Zn powder in acetic acid into genuine cyclosporin A by *trans*-elimination. X-ray analysis [6] revealed that iodocyclosporin A assumes a rather rigid backbone conformation. The amino acids 1–6 adopt an antiparallel, markedly twisted β-pleated sheet conformation, whereas the residues 7–11 form a loop.



(I) cyclosporin A
(partial structure)

(II) iodocyclosporin A
(partial structure)

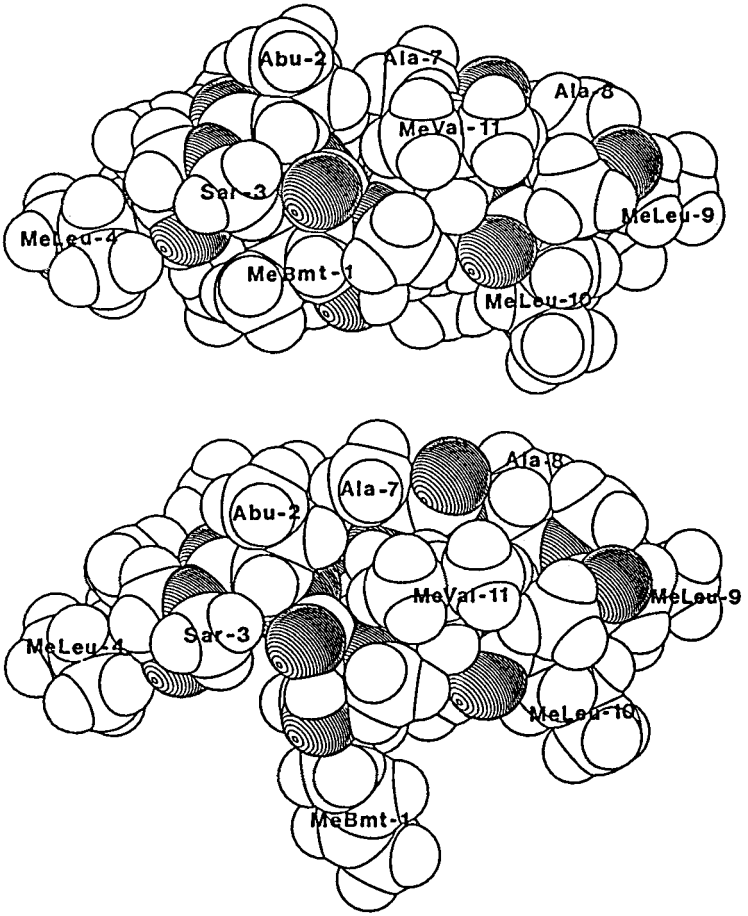
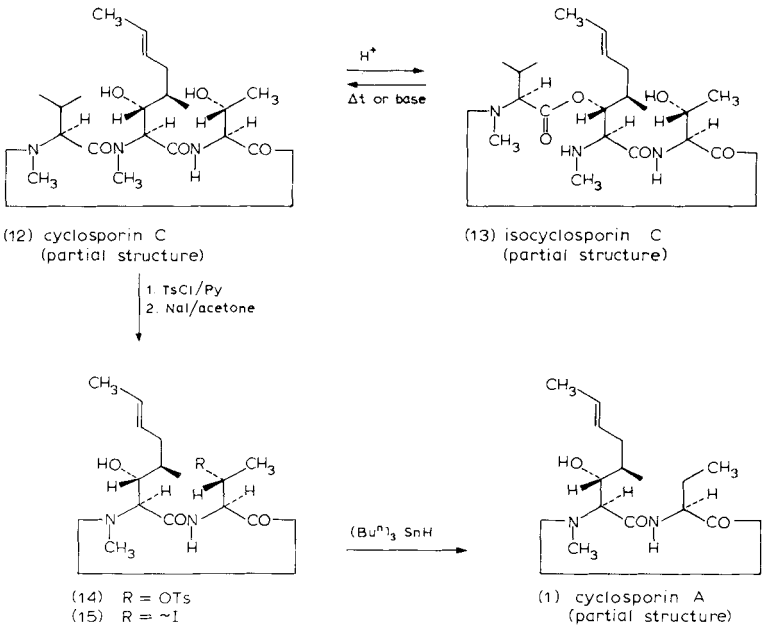


Figure 1.4. Space-filling models of cyclosporin A. (a) Solid-state conformation (X-ray analysis); (b) computer-generated conformation in apolar solvents (NMR measurements).

Stabilization is maintained by several hydrogen bonds. Interesting details include a single *cis*-amide linkage between MeLeu-9 and MeLeu-10. Furthermore, the L-configuration of MeBmt-1 was confirmed and the still unknown partial sequence of residues 7 and 8 was determined as L-alanine–D-alanine. The absolute configuration of iodocyclosporin A and hence that of cyclosporin A were deduced from the reliable identification of the L-amino-acid residues.

In order to acquire further understanding and for the development of a working hypothesis for drug–receptor interactions, additional conformational studies were later conducted using crystallized cyclosporin A [18]. In spite of different packing in the crystal and the structural divergencies in the side-chain of MeBmt-1, the backbone and most of the side-chain conformations of cyclosporin A and its iodo derivative (11) proved to be similar, both displaying a 'butterfly shape' of high internal stability. It is further noteworthy that the alkylene side-chain of MeBmt-1 in cyclosporin A is folded back into the ply of the β-pleated sheet, thus enabling the molecule to adopt a globular shape (*Figure 1.4*).

In apolar solvents ($CDCl_3$, C_6D_6), cyclosporin A assumes almost the same inflexible backbone conformation as in the solid state. This could be deduced from the interpretation of numerous spectral parameters [19]. The main differ-



(12) cyclosporin C (partial structure)

(13) isocyclosporin C (partial structure)

(14) R = OTs
(15) R = ~I

(1) cyclosporin A (partial structure)

ence lies in the orientation of the side-chain of MeBmt-1. In solution, the alkylene side-chain sticks out in a proboscis-like fashion (*Figure 1.4*).

Additional experiments in polar solvents will probably yield more information on the question which conformer interacts with cyclosporin receptors.

STRUCTURE OF CYCLOSPORIN C

The second major metabolite from *T. inflatum* is structurally closely related to cyclosporin A, as can be deduced by elemental analysis, mass spectrum (*m/z* 1217), IR and NMR spectra. Furthermore, the presence of the double bond and OH group of the unusual MeBmt was established. Sulphonic acids in methanol or dioxane effected the typical rearrangement reaction by *N,O*-acyl migration to the iso-compound (13). Hydrolysis furnished the same amino acids as cyclosporin A with the exception of L-α-aminobutyric acid, which is replaced in cyclosporin C (12) by L-threonine. The amino-acid sequence could be deduced by conversion of cyclosporin C into cyclosporin A via the corresponding tosylate (14) and iodo derivatives (15) [7]. Position 2 for L-threonine as well as the assumed twisted β-pleated sheet conformation of the molecule were confirmed by ^{13}C-NMR spectra.

STRUCTURES OF THE OTHER NATURAL CYCLOSPORINS

Structure elucidation of natural cyclosporins is generally based on elemental analysis and mass spectra, identification of the amino-acid building blocks and, if possible, on chemical correlation reactions. Furthermore, the paramount role of detailed interpretation of NMR spectra has to be emphasized. Extensive analysis of ^{1}H-, ^{13}C- and ^{15}N-NMR spectra of cyclosporin A in $CDCl_3$ and C_6D_6 at 360 MHz, including combined two-dimensional NMR-techniques, allowed the complete assignment of all H, all C and four N signals. Thus, each individual amino acid can be recognized and identified by its relevant chemical shifts [19]. In continuation of this fundamental work, assignments of the ^{13}C signals of the other natural cyclosporins could be achieved [10]. Comparison of the available NMR spectra enabled the detection of structural variations and the determination of the position of aberrant structural elements (for example, exchanged or demethylated amino acids) as well as the recognition of significant conformational deviations. In several cases, the proposed structures have been confirmed by X-ray analysis. One single exception concerns the structure of cyclosporin R regarding the correct position of the two leucine units. The alloted positions 6 and 10 are still speculative, due to complex NMR spectra.

The cyclosporins A to Z are all cyclic undecapeptides differing from each

Table 1.2. CHEMICAL STRUCTURES OF CYCLOSPORINS A–Z

Bmt = (2*S*,3*R*,4*R*,6*E*)-2-amino-3-hydroxy-4-methyl-6-octenoic acid (= (4*R*)-4-((*E*)-2-butenyl)-4-methyl-L-threonine); Abu = L-α-aminobutyric acid; Nva = L-norvaline; MeVal = *N*-methyl-L-valine; MeLeu = *N*-methyl-L-leucine.

Metabolite	*Amino-acid composition*										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
A	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
B	MeBmt	Ala	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
C	MeBmt	Thr	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
D	MeBmt	Val	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
E	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	Val
F	Desoxy-MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
G	MeBmt	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
H	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	D-MeVal
I	MeBmt	Val	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	Leu	MeVal
K	Desoxy-MeBmt	Val	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
L	Bmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
M	MeBmt	Nva	Sar	MeLeu	Nva	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
N	MeBmt	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	Leu	MeVal

Table 1.2 continued

Metabolite	Amino-acid composition										
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*
O	MeLeu	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
P	Bmt	Thr	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
Q	MeBmt	Abu	Sar	Val	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
R	MeBmt	Abu	Sar	MeLeu	Val	Leu(?)	Ala	D-Ala	MeLeu	Leu(?)	MeVal
S	MeBmt	Thr	Sar	Val	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal
T	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	Leu	MeVal
U	MeBmt	Abu	Sar	MeLeu	Val	Leu	Ala	D-Ala	MeLeu	MeLeu	MeVal
V	MeBmt	Abu	Sar	MeLeu	Val	MeLeu	Abu	D-Ala	MeLeu	MeLeu	MeVal
W	MeBmt	Thr	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	Val
X	MeBmt	Nva	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	Leu	MeLeu	MeVal
Y	MeBmt	Nva	Sar	MeLeu	Val	Leu	Ala	D-Ala	MeLeu	MeLeu	MeVal
Z	MeAmino-octanoic acid	Abu	Sar	MeLeu	Val	MeLeu	Ala	D-Ala	MeLeu	MeLeu	MeVal

other by variation of one or two amino acids. The most frequent diversity occurs in position 2; the majority of the known natural compounds contain residues such as L-alanine, L-threonine, L-valine or L-norvaline instead of the L-α-aminobutyric acid encountered in cyclosporin A. In other cases, the unusual MeBmt-1 is replaced by desoxyMeBmt, *N*-demethylMeBmt (= Bmt), *N*-methyl-L-2-aminooctanoic acid or *N*-methyl-L-leucine. Several cyclosporins differ from closely related compounds only by *N*-demethylation of an amino-acid residue. In these cases, for structural proof, a direct chemical correlation via the corresponding common *N*-permethylation product has been performed. Variations in the amino-acid sequence have been observed in all positions, with the exception of positions 3 and 8, so far. A striking modification giving rise to considerable conformational distortion was encountered in cyclosporin H, which contains *N*-methyl-D-valine in position 11, instead of the usual L-epimer. The structures of cyclosporins A–Z are indicated in *Table 1.2*.

BIOSYNTHESIS OF CYCLOSPORIN A

A non-ribosomal biosynthetic pathway is clearly indicated for cyclosporin A, considering the uncommon structural elements MeBmt, L-α-aminobutyric acid and D-alanine as well as the plethora of isolated congeners [20, 21]. Non-ribosomal biosynthesis directed by multienzyme thiotemplates have been reported for other small peptides of microbial origin, for example, gramicidin S [22] and enniatin [23]. Experimental data for cyclosporin A were obtained by feeding appropriate labelled precursors to cultures of *T. inflatum* strains. The distribution profile of the labelled atoms in cyclosporin A was determined by ^{3}H- or ^{13}C-NMR spectroscopy. In preliminary trials with several tritium and carbon-14 labelled precursors, [*methyl*-^{3}H]methionine proved to be the most suitable marker for the biosynthetic preparation of radiolabelled cyclosporin A for pharmacokinetic and metabolic studies [24].

In a more detailed investigation, the incorporation of carbon-13 from acetate, labelled either at the methyl or the carboxyl group, was determined [24]. Using [1-^{13}C]acetate, four enhanced NMR signals, assigned to the carbon atoms 1, 3, 5 and 7 of the C_9-amino acid (MeBmt), were displayed; with [2-^{13}C]acetate, equal C-13 enrichment corresponded to atoms 2, 4, 6 and 8. Obviously, the biosynthesis of the carbon skeleton of MeBmt proceeds by head-to-tail coupling of four acetate units. Further experiments with methionine labelled with tritium or carbon-13 demonstrated that the methyl in position 4 of MeBmt and the seven *N*-methyl groups in cyclosporin A originate from the *S*-methyl of methionine. The *C*-methyl transfer to the ketide chain proceeds in the form

of a CD_3-unit, as could be shown by feeding doubly marked [*methyl*-$^{13}CD_3$]methionine. The occurrence of *N*-methyl-2-aminooctanoic acid in cyclosporin Z is in accordance with the above results. The distribution pattern of carbon-13 obtained from feeding experiments is summarized in *Figure 1.5*.

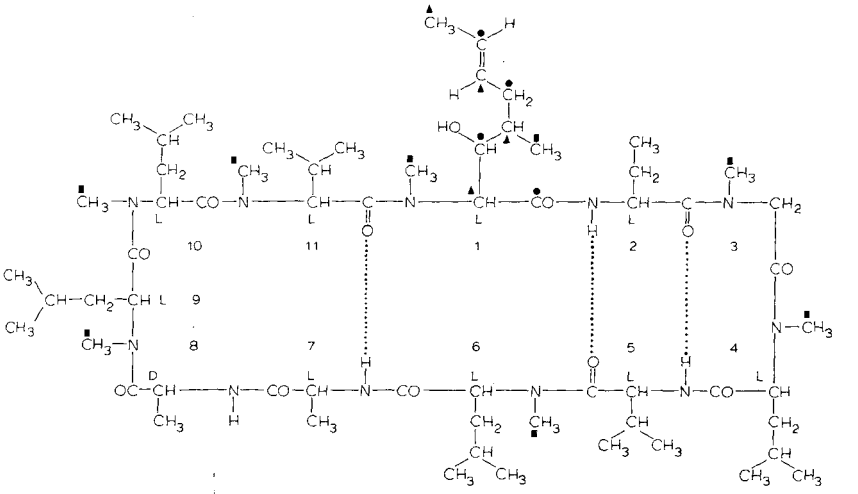


Figure 1.5. Distribution of ^{13}C in cyclosporin A after addition of [1-^{13}C]acetate (●), [2-^{13}C]acetate (▲) and [methyl-^{13}C]methionine (■).

Short-term experiments (10–30 min feeding periods), adding ^{14}C-labelled amino acids to cultures of *T. inflatum* effected selective incorporation of L-leucine, L-valine, DL-alanine and glycine into cyclosporins A and C [20]. Radiolabels were found exclusively in the respective amino acids. Cyclosporin C could be specifically marked by feeding [^{14}C]threonine. With [^{14}C]methionine, a selective labelling of the *N*-methyl moieties of cyclosporin A was observed. These results are in agreement with earlier findings that all *N*-methyl groups originate from methionine [24]. In contrast to prior results, [^{14}C]acetate was only weakly incorporated in short-term incubation periods and no enriched radioactivity in MeBmt could be determined. Summarizing the results so far obtained, it can be assumed that a multifunctional enzyme carries out *N*-methylation of the constituent amino acids in cyclosporin A after their activation as enzyme-bound thioesters, as is the case in enniatin biosynthesis [23]. Tentatively, a three-step reaction sequence in the biosynthesis of cyclosporin A has been proposed: synthesis of all building units; activation of the 11 amino acids; and finally *N*-methylation and peptide formation [20].

In more recent investigations, the assumed multienzyme involved in cyclosporin A biosynthesis could be isolated from *T. inflatum*. A partially purified enzyme fraction was indeed capable of forming enzyme-substrate complexes by thioester linkage. Although *de novo* synthesis (*in vitro*) of cyclosporin A has not yet been achieved, the formation of a partial sequence, namely, the diketopiperazine cyclo(DAla-MeLeu), from D-alanine and L-leucine was observed under consumption of ATP and *S*-adenosyl-L-methionine [25].

DIRECTED BIOSYNTHESIS OF CYCLOSPORINS

A strong influence on the biosynthesis of peptide metabolites exerted by exogenously supplied amino-acid precursors has been observed earlier both in prokaryotes and eukaryotes [26]. In several cases, enhanced yields of a desired metabolite can be obtained and a replacement of certain constitutional building elements by other amino acids may occur [27, 28].

In the cyclosporin series, addition of specific amino acids in excess to the fermentation medium effects a shift in the ratio of the metabolite composition and the incorporation of the surplus amino acid preferably in position 2 [21]. Systematic trials revealed that an amount of 8 g per litre is a favourable concentration.

Table 1.3. CYCLOSPORIN FORMATION AFTER EXOGENOUS ADDITION OF VARIOUS AMINO ACIDS AS PRECURSORS TO SHAKE CULTURES OF *T. INFLATUM*

–, amounts below 5 mg/l were not considered.

Precursor (8 g/l)	*Total cyclosporins* mg/l	*Ratio of cyclosporin components (%)*				
		A	*B*	*C*	*D*	*G*
0 = control	131	77	–	23	–	–
DL-α-Abu	249	100	–	–	–	–
L-Ala	113	51	13	36	–	–
L-Thr	672	59	–	41	–	–
L-Val	743	43	–	20	37	–
L-Nva	260	9	–	–	–	91

As can be deduced from *Table 1.3*, enrichment of the amino-acid pool by adding L-threonine or L-valine raises considerably the total cyclosporin production, accompanied by increased yields of cyclosporin C and cyclosporin D,

respectively. DL-α-Aminobutyric acid suppresses the biosynthesis of the minor congeners, cyclosporin A becoming the almost exclusive metabolite. Cyclosporin G, usually found only in tiny amounts, can be produced in high percentage by external supply of L-norvaline. Incorporation of 'foreign' amino acids in various positions can also be attained, as illustrated by the replacement of D-alanine by other amino acids with D-configuration. Addition of D-serine, for instance, results in the production of [DSer^8]cyclosporin A, a metabolite not yet encountered in nature. However, incorporation reactions occur only with a relatively limited number of specific amino acids. Many substrates used in systematic experiments were a failure; some compounds exerted negative effects by partial or total suppression of cyclosporin biosynthesis.

The incorporation of constitutional and foreign amino acids under replacement of 'inborn' building units demonstrates convincingly the low specificity in the biosynthesis of cyclosporins characteristic for a non-ribosomal biosynthetic pathway.

TOTAL SYNTHESIS OF CYCLOSPORIN A AND ANALOGUES

The acquired knowledge on the composition and sequence of amino acids in cyclosporin A combined with an insight into stereochemically relevant details formed a firm basis for synthetic approaches. The first total synthesis of cyclosporin A by R. Wenger [11, 12] passes through three major phases: (1) synthesis of the C_9-amino acid (MeBmt); (2) construction of the linear undecapeptide; and finally (3) the cyclization reaction.

SYNTHESIS OF (2*S*,3*R*,4*R*,6*E*)-3-HYDROXY-4-METHYL-2-METHYLAMINO-6-OCTENOIC ACID (MeBmt)

As outlined above, MeBmt contains three contiguous asymmetric carbon atoms and an (*E*)-*trans* double bond. The MeNH and OH groups are arranged in a *threo* configuration, whereas the OH and CH_3 groups are oriented in the *erythro* form.

For the synthesis of MeBmt, tartaric acid was chosen as starting material from the pool of natural chiral building blocks (*Figure 1.6*). In a first reaction sequence of seven steps (for details see Ref. 11), one hydroxy group of (*R*,*R*)-(+)-tartaric acid was replaced by a methyl group with inversion of configuration via the epoxide (16), thus providing the asymmetric centres C-3 and C-4 of MeBmt. The necessary elongation of the carbon chain of the

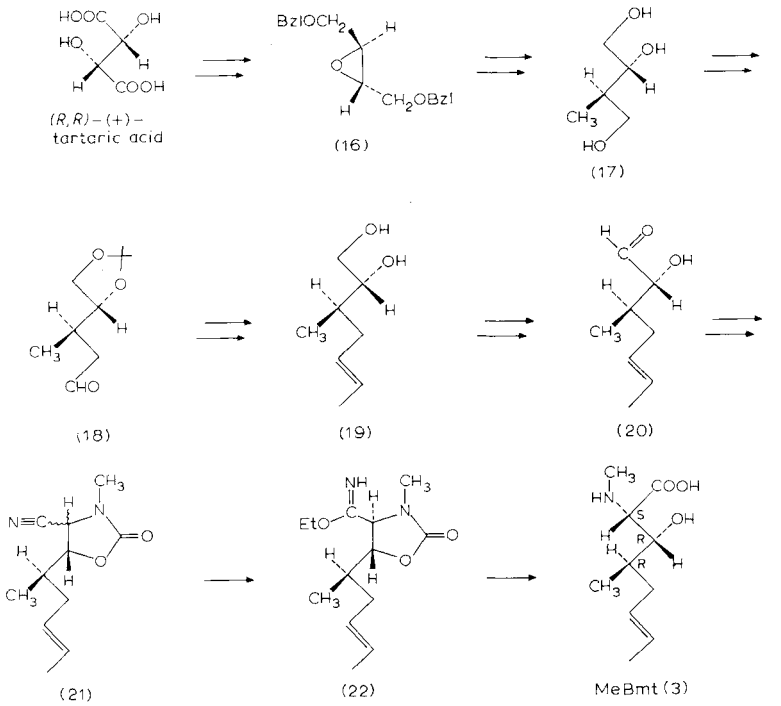
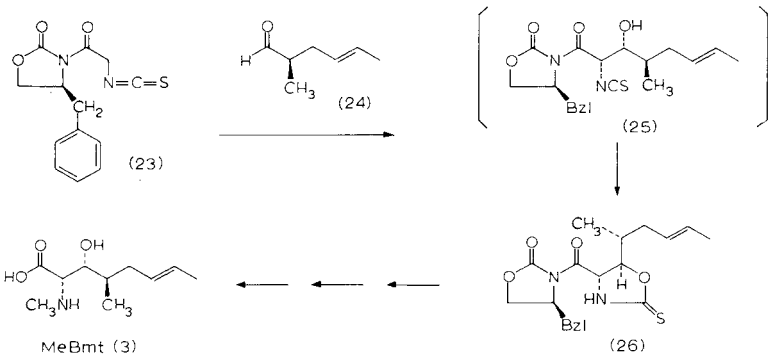


Figure 1.6. Synthesis of (2S,3R,4R,6E)-3-hydroxy-4-methyl-2-methylamino-6-octenoic acid (MeBmt).

resulting triol (17) was performed by selective tosylation, treatment with KCN and reduction of the obtained nitrile to the aldehyde (18). Introduction of the (*E*)-*trans*-butenyl moiety was effected by a modified Wittig reaction using ethyl(triphenyl)phosphonium bromide under controlled conditions to ensure the formation of the *trans*-olefin (19). This unsaturated diol was oxidized to the aldehyde (20), which in turn was submitted to a Strecker reaction with KCN and methylamine hydrochloride. The primary formed mixture of isomeric cyanamines was converted to the oxazolidine-2-one diastereoisomers (21). Treatment with potassium carbonate in ethanol yielded the thermodynamically more stable *trans* carboximidate (22). Hydrolysis and removal of the protecting groups furnished enantiomerically pure MeBmt with the desired *threo* configuration of the MeNH and OH groups. The overall yield from diethyl tartrate after 24 steps amounted to 7.8%.

Recently, a conceptually different synthesis of MeBmt using an asymmetric glycine aldol reaction was reported by Evans and Weber [29]. The key step consists in the stereochemically controlled condensation of the chiral glycine enolate synthon (23) with the (*R*)-aldehyde (24) mediated by stannous triflate (tin salt of trifluoromethanesulphonic acid). The desired *syn*-aldol adduct (25) was isolated in form of the heterocyclic compound (26). The sense of asymmetric induction in the aldol reaction was established by conversion of (26) over three steps into uniform MeBmt (3).



SYNTHESIS OF THE LINEAR UNDECAPEPTIDE

The strategy used for the synthesis of the linear peptide depended on the planned cyclization reaction. For this important final step, the peptide bond between L-alanine and D-alanine (positions 7 and 8) was chosen, since the formation of a linkage between non-methylated amino acids was regarded as less difficult than between *N*-methylated building blocks. In addition, it was assumed that intrachain hydrogen bonds between amide groups may stabilize the linear peptide in folded conformations similar to the ring structure of cyclosporin A, thus facilitating cyclization. To construct the linear undecapeptide, a fragment-condensation technique was used introducing the precious MeBmt unit towards the end of the synthesis. In general, the amino groups of the building blocks were protected with *t*-butoxycarbonyl residues (Boc) and the carboxy groups as benzyl esters (OBzl). For the activation of carboxy groups, a variation of the mixed pivalic anhydride method described by Zaoral [30] was used with adaptation for *N*-methyl amino acids. To remove the Boc

residues, the peptide intermediates were treated with trifluoroacetic acid, whereas the benzyloxy groups were cleaved by catalytic hydrogenation.

In a first reaction sequence, the tetrapeptide Boc-D-Ala-MeLeu-MeLeu-MeVal-OBzl (27) was constructed stepwise starting with Boc-D-Ala-OH. In a second series of reactions, the hexapeptide Boc-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (28) was synthesized by linking the dipeptide Boc-Abu-Sar-OBzl with the corresponding tetrapeptide. Each subunit was built up by stepwise elongation to minimize the risk of racemization. The next steps consist in the deblocking of the amino end of the hexapeptide (28) and the attachment of the amino acid MeBmt (C_9-amino acid). To avoid epimerization of the functional groups during carboxy activation and amide-bond formation, the isopropylidene derivative of MeBmt was utilized and the attachment effected with dicyclohexylcarbodiimide in the presence of *N*-hydroxybenztriazol. After removal of the isopropylidene protecting group, the resulting heptapeptide (29) was coupled with the above tetrapeptide (27) containing the amino acids 8–11 to yield the linear undecapeptide Boc-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-Abu-Sar-MeLeu-Val-MeLeu-Ala-OBzl (30).

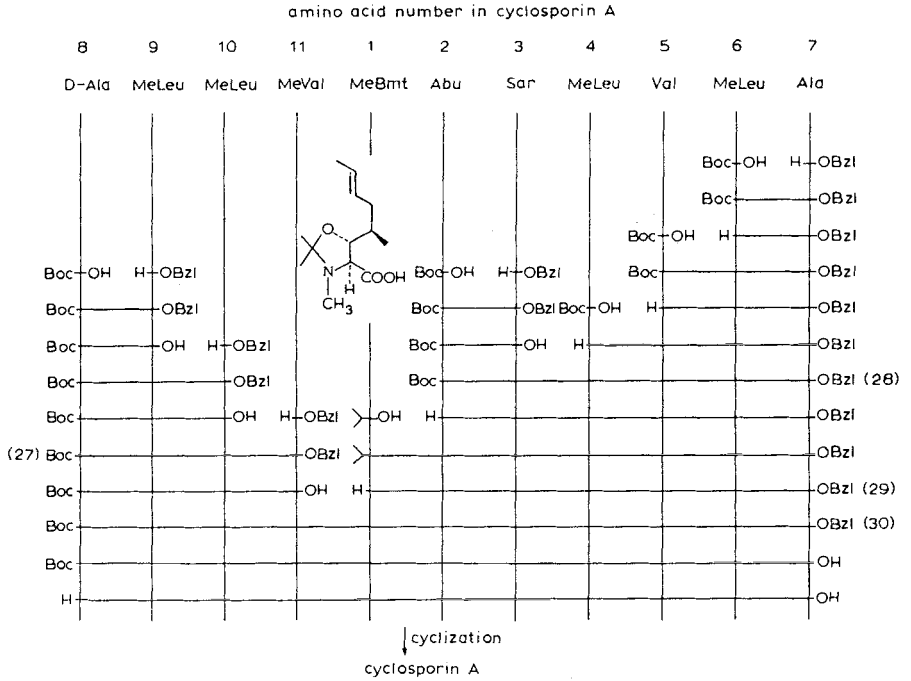


Figure 1.7. Synthesis of cyclosporin A.

CYCLIZATION OF THE LINEAR UNDECAPEPTIDE TO CYCLOSPORIN A

To effect the desired ring closure, both protecting groups of the linear undecapeptide (30) had to be removed. The benzyl ester was therefore cleaved by alkaline hydrolysis, and the Boc group by treatment with trifluoroacetic acid. Cyclization of the unprotected undecapeptide was then effected in dilute solution of methylene chloride with the reagent $BtOP(NMe_2)_3{}^+PF_6^-$ (= (1*H*-1,2,3-benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate) in the presence of *N*-methylmorpholine to furnish cyclosporin A in high yields. This reagent developed by Castro, Dormoy, Evin and Selve [31] proved to be superior to other condensing agents. The cyclization product was identical in all respects to natural cyclosporin A on comparison of physicochemical and spectroscopic data as well as in biological assays.

SYNTHESIS OF CYCLOSPORIN ANALOGUES

The reaction sequence developed by R. Wenger [12] for the synthesis of cyclosporin A allows in principle the exchange of any amino acid of the natural peptide chain. Using this versatile synthetic approach, numerous analogous cyclosporins have been prepared [32] in order to contribute new aspects concerning structure–activity relationships. The importance of the intact side-chain of MeBmt was further demonstrated with several synthetic compounds containing different amino acids in position 1 such as [MeThr^1]CyA [32], [MeAbu^1]CyA, [MeAbu^1,Sar^{10}]CyA and [MeLeu(3-OH)^1]CyA [33]. Supplementary variations were also made in position 2, e.g., [Ser^2]CyA and in position 3, e.g., [DPro^3]CyA, [LPro^3]CyA. Another modification influencing the ring conformation represents the synthetic [DAla^7]CyA, in which both alanine units belong to the D-series. Extensive studies dealt with the role of MeVal-11. In addition to the natural cyclosporin H ([DMeVal^{11}]CyA) and cyclosporin E (= [Val^{11}]CyA), several synthetic modifications were prepared, such as [MeLeu^{11}]CyA, [MeIle^{11}]CyA, [aMeIle^{11}]CyA and [MeAla^{11}]CyA.

Contracted cyclosporins lacking one or several amino acids represent also interesting compounds available only by synthesis. Removal of DAla-8 or replacement of the segment DAla-MeLeu-MeLeu-MeVal of cyclosporin A by 4-aminobutanoyl or 4-methylaminobutanoyl residues provided more constrained analogues. Although maintaining the β-pleated sheet portion of the cyclosporin molecule, these shortened analogues are inactive as immunosuppressants.

The implications for biological activity generated by structural modifications are discussed in the following section. In summary, it can be said that until now

no natural or synthetic compound exceeds cyclosporin A in immunosuppressive efficacy.

STRUCTURE–ACTIVITY RELATIONSHIPS

The original pharmacological screening programme for microbial metabolites included a mouse model in which inhibition of haemagglutinin formation against sheep erythrocytes and survival time following inoculation with murine leukaemia cell L1210 could be assessed [34]. In additional tests, the suppression of the humoral immune response was estimated by quantifying the antibody-forming cells in the spleen of mice immunized against sheep erythrocytes (Jerne's plaque-forming test) [4, 5]. The cell mediated immune response was checked in a delayed-type hypersensitivity reaction (DTH) by measuring the suppression of the oxazolone-induced skin reaction in mice and, *in vitro*, by quantifying the suppression of proliferation of lymphoid cells activated by surface alloantigens (MLR).

First structure–activity relationships could be established empirically by comparison of the data listed in *Table 1.4*. It is apparent that exchange of one or two amino acids in cyclosporin A exerts a more or less pronounced influence on the immunosuppressive activity. A deeper insight into the molecular shape of the molecules and an understanding of conformational changes induced by alterations in the sequence of amino acids emerged by extensive NMR-studies and various X-ray analyses. In general, conformational disturbances caused by breaking of existent hydrogen bonds or formation of new H-bonds result in a marked decrease in immunosuppressive activity. This is also true if effective binding to the cyclosporin receptor is prevented due to bulky substituents, especially in the important region comprising the amino acids in positions 11, 1, 2, 3 and 4.

The dominant role of the C_9-amino acid (MeBmt-1) for full immunosuppressive activity emerged at a very early stage of our investigations. Modification or replacement of this essential structural element in general results in a more or less dramatic decrease of immunosuppressive activity. Dihydrocyclosporin A, obtained by saturation of the double bond, still retains a major part of activity, but *O*-acyl derivatives as well as the 3′-deoxy analogues (cyclosporins F and K) and cyclosporin Z, containing *N*-methyl-2-aminooctanoic acid in position 1, are devoid of significant immunosuppressive effects. Removal of the *N*-methyl group is also combined with a considerable loss of efficacy, as illustrated by the naturally occurring cyclosporins L and P. Replacement of MeBmt-1 by mimetics, for example, common amino acids such

Table 1.4. IMMUNOSUPPRESSIVE ACTIVITY OF CYCLOSPORINS

nat = natural; dbs = by directed biosynthesis; ps = by partial synthesis; syn = by total synthesis; + + +, strong immunosuppressive activity; + +, moderate activity; +, weak activity; (+) or −, no significant activity.

Trivial name (Cyclosporin:)	*Cyclosporin (Cy)*	*Source*	*Activity*	*Ref.*
A	Cyclosporin A = Ciclosporin (*Sandimmun*®)	nat	+ + +	2
	[Dihydro-MeBmt^1]CyA	ps	+	2
	[(OAc)MeBmt^1]CyA	ps	(+)	9
L	[Bmt^1]CyA	nat	+	10
P	[Bmt^1,Thr^2]CyA	nat	+	10
F	[(3′-Desoxy)MeBmt^1]CyA	nat	(+)	9
K	[(3′-Desoxy)MeBmt^1,Val^2]CyA	nat	(+)	10
Z	[*N*-Me-2-aminooctanoic acid^1]CyA	nat	(+)	10
O	[MeLeu^1]CyA	nat	(+)	10
	[MeThr^1]CyA	syn	(+)	32
	[MeLeu(*threo*-3-OH)^1]CyA	syn	+	33
	[MeAbu^1]CyA	syn	−	33
B	[Ala^2]CyA	nat	+	8
C	[Thr^2]CyA	nat	+ +	7
D	[Val^2]CyA	nat	+	8
G	[Nva^2]CyA	nat	+ + +	9
	[Ser^2]CyA	syn	(+)	32
	[DMePhe^3]CyA	ps	(+)	35
	[DMeAla^3]CyA	ps	+	35
	[MeAla^3]CyA	syn	(+)	32
	[DPro^3]CyA	syn	(+)	32
	[Pro^3]CyA	syn	(+)	32
Q	[Val^4]CyA	nat	(+)	10
S	[Thr^2,Val^4]CyA	nat	(+)	10
M	[Nva^2,Nva^5]CyA	nat	+ +	10
U	[Leu^6]CyA	nat	+	10
Y	[Nva^2,Leu^6]CyA	nat	+	10
R	[Leu$^{6(?)}$,Leu$^{10(?)}$]CyA	nat	(+)	10
V	[Abu^7]CyA	nat	+ +	10
	[DAla^7]CyA	syn	(+)	32
	[DSer^8]CyA	dbs	+ +	36

(continued)

Table 1.4. continued

Trivial name (Cyclosporin:)	*Cyclosporin (Cy)*	*Source*	*Activity*	*Ref.*
X	[Nva^2,Leu^9]CyA	nat	+	10
I	[Val^2,Leu^{10}]CyA	nat	(+)	9
N	[Nva^2,Leu^{10}]CyA	nat	+	10
R	[Leu$^{6(?)}$,Leu$^{10(?)}$]CyA	nat	(+)	10
T	[Leu^{10}]CyA	nat	+ +	10
E	[Val^{11}]CyA	nat	(+)	8
W	[Thr^2,Val^{11}]CyA	nat	(+)	10
	[MeLeu^{11}]CyA	syn	(+)	32
	[MeIle^{11}]CyA	syn	+	32
	[aMeIle^{11}]CyA	syn	(+)	32
	[MeAla^{11}]CyA	syn	(+)	32
H	[DMeVal^{11}]CyA	nat	–	9

as *N*-methyl-L-leucine (cyclosporin O), *N*-methyl-L-threonine or *N*-methyl-L-α-aminobutyric acid, led to only marginal potent or inactive compounds (*Table 1.4*).

By contrast, substitution of L-α-aminobutyric acid in position 2 by other amino acids is quite well tolerated, as proven by the high potency of cyclosporins C (LThr^2) and G (LNva^2). However, shortened or branched side-chains of the substituting amino acid, as in cyclosporin B (LAla^2) and D (LVal^2), may diminish the immunosuppressive efficacy. The marked decrease in activity of the synthetic [Ser^2]cyclosporin A can be explained by a partially disturbed interaction with the cyclosporin receptor.

Natural metabolites with variation in position 3 have not yet been encountered. The synthetic compound [DMeAla^3]cyclosporin A is less active than cyclosporin A, whereas the introduction of *N*-methyl-D-phenylalanine, *N*-methyl-L-alanine, D-proline or L-proline furnished only minus variants (see *Table 1.4*). Reduction or loss of activity may be due either to induced conformational changes in the rigid backbone structure or to the prevention of efficient receptor binding caused by bulky side-chains. Cyclosporins Q and S, both containing valine instead of MeLeu in position 4, do not significantly suppress the immune response. Substitution of valine in position 5 by norvaline, as in cyclosporin M, is followed by a moderate drop of activity. On the other hand, *N*-demethylation of MeLeu-6 results in a significant deformation of the loop conformation; as expected, the cyclosporins containing L-leucine in

position 6 display only marginal to average immunosuppressive effects (cyclosporins U, Y and possibly R). Replacement of LAla-7 by the homologous L-α-aminobutyric acid (cyclosporin V) is allowed without a marked decrease of activity. This is also valid for the substitution of the unusual D-alanine at position 8 by D-serine, as illustrated by [DSer^8]cyclosporin A, a metabolite of directed biosynthesis. Unfavourable effects on the ring conformation are generally induced by *N*-demethylation of amino acids numbers 9 and 10 (MeLeu): cyclosporin X and the cyclosporins I, N, R and T display only marginal to average immunosuppression. Like MeBmt-1 the adjoining *N*-methyl-L-valine in position 11 represents also an essential building block of the cyclosporin molecule. *N*-Demethylation (valine instead of MeVal-11) encountered in cyclosporins E and W, may be responsible for the formation of an additional hydrogen bond between Val-11 and DAla-8 inducing severe conformational changes and a drastic decrease of activity. Synthetic variations of MeVal-11 led to a series of analogues (*Table 1.4*) which display only marginal immunosuppressive effects, with the exception of the moderately active *N*-methylisoleucine derivative. An even more profound interference with the active molecular conformation is reached by inversion of the configuration of *N*-methylvaline-11. It is not surprising that the natural cyclosporin H, which contains the antipode *N*-methyl-D-valine in position 11, is devoid of any immunosuppressive activity.

In summary, it can be concluded that the main traits of the overall shape of cyclosporin A are preserved in all congeners displaying high immunosuppressive activity. Significant deviations in the backbone conformation or in the space filling and spatial arrangement of the side-chains induce a decrease in activity to a greater or lesser extent.

MODE OF ACTION

The most important activity of cyclosporin A consists clearly in the marked suppression of antibody formation and cell-mediated immune response [4, 5]. In addition, the metabolite exhibits antiphlogistic effects, restricted to chronic inflammation processes, as well as antifungal and antiparasitic activities. Whether these different biological activities are inter-related or represent separate mechanisms is still an open question. Considering immunosuppression, early observations revealed that cyclosporin A acts selectively on lymphocytes, mainly on T cells, affecting rather the inductive phase than the proliferative phase of lymphoid cell populations. Apparently, cyclosporin A inhibits primary T-helper cell activation and blocks the formation of

lymphokines such as interleukin 2 [IL-2]. Regulation of the T-cell-derived lymphokine IL-2 can be effected, for instance, by blocking the biosynthesis of the mediator or by inhibition of precursor T cells from acquiring functional IL-2 receptors. Further evidence for the selective action on T cells results from the observation that cyclosporin A markedly decreases the secretion of γ-interferon, another T-cell product. On the other hand, α-interferon (leukocytes) and β-interferon (fibroblasts) are not affected. In the mechanism of action of cyclosporin A, the inhibition of the production of IL-2 and other lymphokines appears to account for the majority of its immunosuppressive effects. Studies on binding and the nature of receptors are still controversial, but point to transport of cyclosporin A through membrane and cytosol to nuclear elements of transcription. More detailed information on the mechanism of action of cyclosporin A is reported in several surveys published recently [37, 38].

In another approach to gather additional information on the mode of action of cyclosporin A, V. Quesiniaux and her colleagues [39] have generated monoclonal antibodies to probe the surface of cyclosporin molecules. These studies have not only enhanced our understanding of antibody-antigen recognition but have also furnished another exact method (besides HPLC) for monitoring the concentration of cyclosporin A in biological fluids by serological assays [40]. To raise specific antibodies against cyclosporins, several conjugates for immunization experiments were initially prepared, for example, by coupling natural cyclosporin C ([Thr^2]cyclosporin A), containing a reactive OH group, via a spacer (hemisuccinate) to a carrier protein [41]. More specific antisera with high titres were obtained with an immunogen produced by direct coupling of an activated ester of a synthetic lysyl-cyclosporin to carrier proteins [42]. Lymphocyte hybridomas were generated in the usual way by fusing spleen cells from immunized mice with myeloma cells. The screening of hybridomas, secreting anti-cyclosporin antibodies, was performed with an indirect solid-phase immunoassay (ELISA), which allowed the selection and characterization of approximately 200 monoclonal antibodies which recognize cyclosporins. By studying a series of 60 natural and synthetic cyclosporins, differently substituted in each single position of the peptide ring, it was possible to determine the contact residues directly involved in the epitope recognized by the McAbs on the cyclosporin molecule. Most of the McAbs recognized only limited clusters of amino-acid units on the cyclosporin molecule surface. One McAb, for instance, showing strong affinities (in the range of 10^{-10} to 10^{-11} mol/l), recognized mainly residues 2, 3, 4, 5 and 11, to a lesser extent position 1 and not at all amino acids 6, 7 and 8; the antibody binding site thus interacts mainly with the 'upper' side of the cyclosporin molecule. Other McAbs react with the 'top' or the 'front' side or 'transversally' from the top of

the molecule (at residue 2) to the bottom (at residue 6). Extensive investigations demonstrated that epitope recognition by antibody was strongly influenced by even small changes in the amino-acid sequence and conformation. On the other hand, the antigenic recognition pattern of a new cyclosporin derivative with a panel of McAbs may indicate the position of exchanged amino acids and the resulting conformational variations.

PHARMACOKINETICS AND METABOLISM

The pharmacokinetics and metabolism of cyclosporin A have been extensively investigated in humans and in animals [43, 44] mainly in order to design optimal therapeutic regimens. The determination of cyclosporin concentrations in biological fluids can be performed either by a sensitive and specific high-performance liquid chromatography method (HPLC) [45] or by radioimmunoassay (RIA) [41]. Tritium-labelled cyclosporin A with high biological stability is prepared biosynthetically by feeding submerged cultures of selected strains of *T. inflatum* with [*methyl*-^{3}H]methionine as precursor [24]. In [^{3}H]cyclosporin A, the protons of the seven *N*-methyl groups and the *C*-methyl of MeBmt-1 are labelled. For the determination of the intact drug, only the HPLC method is suitable to give specific values. Absorption of cyclosporin A from an olive-oil-based drinking solution is slow and peak blood levels are reached about 3–4 h after ingestion. The bioavailability of the drug is in the range of 20–50%. Due to its high lipid solubility, cyclosporin A is widely distributed throughout the body. After oral administration, the uptake by the liver is highest and is related to its drug metabolizing and excreting role. High concentrations are also found in fat tissue. Brain levels of radioactivity are extremely low, indicating that only little cyclosporin A crosses the blood-brain barrier. Within the blood, approx. 50% of cyclosporin A is associated with erythrocytes, 10–20% with leucocytes, and 30–40% is found in the plasma, predominantly bound to lipoproteins. Administration of [^{3}H]cyclosporin A to rats revealed a high tissue affinity of drug-related radioactivity (tissue-to-blood ratios 2:10) with relative slow elimination from tissue.

Cyclosporin A is slowly but extensively metabolized. The biotransformation pathway and the pattern of the generated metabolites are similar in humans and animals. Approximately 17 single metabolites have been detected so far, all of which are present in considerably lower plasma concentration than cyclosporin A itself [46]. Eleven ether-extractable compounds have been isolated from urine of dog and man and from rat bile and faeces using preparative HPLC and thin-layer chromatography [43]. Structural assignments for these

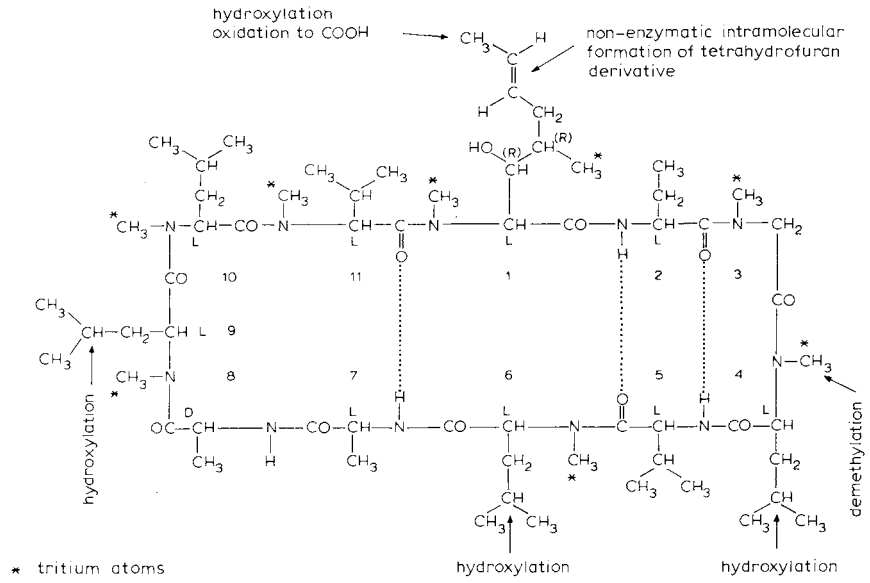


Figure 1.8. Site and type of biotransformation in cyclosporin A.

metabolites are based on spectroscopic evidence (^{1}H- and ^{13}C-NMR, MS) and the amino-acid analysis after hydrolytic cleavage. All characterized metabolites retained the intact cyclic oligopeptide structure of cyclosporin A. Structural modifications originated mainly from enzymatic oxidation processes (phase I reactions) at specific sites of the peptide subunits. Typical phase II reactions such as conjugation with sulphuric acid or glucuronic acid were not observed. Only 4 of the 11 amino acids of cyclosporin A are attacked by enzymes, namely MeBmt-1, MeLeu-4, MeLeu-6 and MeLeu-9. Transformation processes involved hydroxylation reactions, restricted to the terminal carbon atom of MeBmt and the C-4′ of the *N*-methylleucines, and oxidative *N*-demethylation occurring exclusively on MeLeu-4. Site and type of biotransformation in cyclosporin A are illustrated in *Figure 1.8*.

According to the assumed pathway for the biotransformation of cyclosporin A [43], the monohydroxylated compounds [8′-hydroxy-MeBmt^1]-cyclosporin A and [4′-hydroxy-MeLeu^4]cyclosporin A as well as the *N*-demethylated [Leu^4]cyclosporin A are considered as the primary metabolites. Further oxidation of these substrates produces several dihydroxy and additional *N*-demethyl derivatives. Recently, an acidic compound was isolated from rabbit and human bile. In this metabolite, the terminal methyl group of

MeBmt-1 is converted to a carboxylic acid [44]. Intermediate substrates for the reaction sequence may be represented by [8′-hydroxy-MeBmt^1]cyclosporin A containing an allylic OH-group and the corresponding α,β-unsaturated aldehyde. The ethylene side-chain of MeBmt-1 can also undergo a probably non-enzymatic reaction. Two metabolites have been found which display a tetrahydrofuran ring structure generated by an intramolecular addition of the OH-group to the double bond. An artifact of MeBmt originated by an analogous cyclization reaction has been encountered earlier among the hydrolysis products of cyclosporin A.

All identified metabolites of cyclosporin A display only low immunosuppressive activities and do not contribute to the clinical effect of cyclosporin A.

Clearance of cyclosporin A and its metabolites proceeds mainly through excretion of bile into faeces. After an oral dose of [^{3}H]cyclosporin A, only 4–6% of the radioactivity is excreted in urine within 96 h. Intact [^{3}H]cyclosporin A contributes only a small proportion of the excreted radioactivity (0.1–0.2% of the dose). The elimination half-life of cyclosporin A from blood, determined by HPLC, amounts to 15.8 ± 8.4 h.

FUTURE RESEARCH TRENDS

Cyclosporin A represents the prototype of a new generation of immunosuppressants and its impact on the practice of organ transplantation has been pointed out by numerous clinicians. Cyclosporin A may also play an important role in the treatment of diseases originated by autoimmune disorders. Additional investigations, currently performed, deal with the antiparasitic action of cyclosporin A, particularly against schistosomes and blood parasites. Like many other potent drugs, cyclosporin A is not entirely devoid of side-effects. The elucidation of the mechanism by which cyclosporin A induces clinical nephrotoxicity is therefore an important topic for further research work. Another still intriguing problem concerns its mode of action. A deeper insight into the *modus operandi* on the molecular level and supplementary information on structure–activity relationships emerging from on-going studies on receptor binding might help in the design of novel highly active cyclosporins with less secondary effects. The continuous search for new immunosuppressants, stimulated by the discovery of cyclosporins, presents a major challenge to medicinal chemists. The objective may be reached by systematic modification of the available natural and synthetic cyclosporins, by a persevering screening of microorganisms and plants as well as by a pure synthetic approach, based on rational drug design.

REFERENCES

1 A. von Wartburg and R. Traber, in Ciclosporin, Progress in Allergy, Vol. 38, ed. J.F. Borel (S. Karger, Basel, 1986) pp. 28–45.
2 A. Rüegger, M. Kuhn, H. Lichti, H.R. Loosli, R. Huguenin, Ch. Quiquerez and A. von Wartburg, Helv. Chim. Acta, 59 (1976) 1075.
3 M. Dreyfuss, E. Härri, H. Hofmann, H. Kobel, W. Pache and H. Tscherter, Eur. J. Appl. Microbiol., 3 (1976) 125.
4 J.F. Borel, C. Feurer, H.U. Gubler and H. Stähelin, Agents Actions, 6 (1976) 468.
5 J.F. Borel, C. Feurer, C. Magnée and H. Stähelin, Immunology, 32 (1977) 1017.
6 T.J. Petcher, H.P. Weber and A. Rüegger, Helv. Chim. Acta, 59 (1976) 1480.
7 R. Traber, M. Kuhn, A. Rüegger, H. Lichti, H.R. Loosli and A. von Wartburg, Helv. Chim. Acta, 60 (1977) 1247.
8 R. Traber, M. Kuhn, H.R. Loosli, W. Pache and A. von Wartburg, Helv. Chim. Acta, 60 (1977) 1568.
9 R. Traber, H.R. Loosli, H. Hofmann, M. Kuhn and A. von Wartburg, Helv. Chim. Acta, 65 (1982) 1655.
10 R. Traber, H. Hofmann, H.R. Loosli, M. Ponelle and A. von Wartburg, Helv. Chim. Acta, 70 (1987) 13.
11 R.M. Wenger, Helv. Chim. Acta, 66 (1983) 2308.
12 R.M. Wenger, Helv. Chim. Acta, 66 (1983) 2676; 67 (1984) 502.
13 R.Y. Calne, D.J.G. White, K. Rolles, D.P. Smith and B.M. Herbertson, Lancet, i (1978) 1183; R.Y. Calne, D.J.G. White, S. Thiru, D.B. Evans, P. McMaster, D.C. Dunn, G.N. Graddock, D.B. Pentlow and K. Rolles, Lancet, ii (1978) 1323.
14 R.L. Powles, A.J. Barrett, H. Clink, H.E.M. Kay, J. Sloane and T.J. McElwain, Lancet, ii (1978) 1327.
15 M. Dreyfuss, Sydowia, Annales Mycologici, 39 (1986) 22.
16 K. Sawai, T. Okuno, Y. Terada, Y. Harada, K. Sawamura, H. Sasaki and S. Takao, Agric. Biol. Chem., 45 (1981) 1223.
17 J.J. Sanglier and R. Traber, 15th IUPAC International Symposium on the Chemistry of Natural Products, Den Haag/NL, 17–22 August 1986, Poster presentation, Abstr. No. PC 29.
18 H.R. Loosli, H. Kessler, H. Oschkinat, H.P. Weber, T.J. Petcher and A. Widmer, Helv. Chim. Acta, 68 (1985) 682.
19 H. Kessler, H.R. Loosli and H. Oschkinat, Helv. Chim. Acta, 68 (1985) 661.
20 R. Zocher, N. Madry, H. Peeters and H. Kleinkauf, Phytochemistry, 23 (1984) 549.
21 H. Kobel and R. Traber, Eur. J. Appl. Microbiol. Biotechnol., 14 (1982) 237.
22 H. Kleinkauf and H. Koischwitz, in Multifunctional Proteins, ed. H. Bisswanger and E. Schmincke-Ott (Wiley, New York, 1980).
23 R. Zocher, U. Keller and H. Kleinkauf, Biochemistry, 21 (1982) 43.
24 H. Kobel, H.R. Loosli and R. Voges, Experientia, 39 (1983) 873.
25 R. Zocher, T. Nihira, E. Paul, N. Madry, H. Peeters, H. Kleinkauf and U. Keller, Biochemistry, 25 (1986) 550.
26 E. Katz, Cancer Chemother. Rep., 58 (1974) 83; E. Katz and A. Demain, Bacteriol. Rev., 41 (1977) 449.
27 H. Kobel and J.J. Sanglier, in Antibiotics and Other Secondary Metabolites, eds. R. Hütter, T. Leisinger, J. Nüesch and W. Wehrli, FEMS Symposium No. 5 (Academic Press, New York, 1978) pp. 233–242.

28 E. Beacco, M.L. Bianchi, A. Minghetti and C. Spalla, Experientia, 34 (1978) 1291.
29 D.A. Evans and A.E. Weber, J. Am. Chem. Soc., 108 (1986) 6757.
30 M. Zaoral, Collect. Czech. Chem. Commun., 27 (1962) 1273.
31 B. Castro, J.R. Dormoy, G. Evin and C. Selve, Tetrahedron Lett., 14 (1975) 1219.
32 R.M. Wenger, Angew. Chem. Inter. Edn. Engl., 24 (1985) 77.
33 D.H. Rich, M.K. Dhaon, B. Dunlap and S.P.F. Miller, J. Med. Chem., 29 (1986) 978.
34 S. Lazary and H. Stähelin, Experientia, 24 (1968) 1171.
35 D. Seebach, W. Murtiashaw, R. Naef, S. Shoda (ETH Zürich) and M. Krieger, P. Bollinger, A. Leutwiler and R. Wenger (Sandoz Ltd.), Work presented at the Autumn Meeting of the Swiss Chemical Society in Berne (October 18, 1985).
36 P. Bollinger, H.J. Bölsterli and H. Kobel, Sandoz Ltd., U.S. Patent 4.384.996 (1983); R. Wenger, R. Traber, H. Kobel and H. Hofmann, Sandoz Ltd., Fr. Demande FR 2,561,651 (1985) (C.A. 106 (1987): 50646t).
37 'Cyclosporin', Progress in Allergy, Vol. 38, ed. J.F. Borel (S. Karger, Basel, 1986) pp. 198–221.
38 R.M. Wenger, T.G. Payne and M.H. Schreier, in Progress in Clinical Biochemistry and Medicine, Vol. 3 (Springer-Verlag, Berlin, 1986) pp. 157–191.
39 V. Quesniaux, Transplant. Proc., Vol. 18, No. 6, Suppl. 5 (1986) 111; V. Quesniaux, R. Tees, M.H. Schreier, R.M. Wenger, P. Donatsch and M.H.V. van Regenmortel, in 'Ciclosporin', Progress in Allergy, Vol. 38, ed. J.F. Borel (S. Karger, Basel, 1986) pp. 108–122.
40 V. Quesniaux, R. Tees, M.H. Schreier, G. Maurer and M.H.V. van Regenmortel, Clin. Chem., 33 (1987) 32.
41 P. Donatsch, E. Abisch, M. Homberger, R. Traber, M. Trapp and R. Voges, J. Immunoassay, 2 (1981) 19.
42 V. Quesniaux, K. Himmelspach and M.H.V. van Regenmortel, Immunol. Lett., 9 (1985) 99.
43 G. Maurer, H.R. Loosli, E. Schreier and B. Keller, Drug Metabolism and Disposition, 12 (1984) 120; G. Maurer and M. Lemaire, Transplant. Proc., Vol. 18, No. 6, Suppl. 5 (1986) 25.
44 N.R. Hartman, L.A. Trimble, J.C. Vederas and I. Jardine, Biochem. Biophys. Res. Commun., 133 (1985) 964.
45 W. Niederberger, P. Schaub and T. Beveridge, J. Chromatogr., 182 (1980) 454.
46 A.J. Wood, G. Maurer, W. Niederberger and T. Beveridge, Transplant. Proc., Vol. 15, Suppl. 1 (1983) 2409.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry – Vol. 25, edited by G.P. Ellis and G.B. West

2 Structural Aspects of Antineoplastic Agents – A New Approach

C.C. CHENG, B.S., M.A, Ph.D.

Department of Pharmacology, Toxicology, and Therapeutics and Drug Development Laboratory, University of Kansas Cancer Center, University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.

INTRODUCTION

Although cancer was recognized as a disease as early as 1500 B.C. and herbal managements of cancer were recorded in many ancient medical writings, the use of drugs in cancer treatment had not been seriously considered prior to the 1930s. Application of androgens and oestrogens in the treatment of breast and prostatic cancers as well as other types of neoplastic growth, based on alteration of the hormonal status of the cancer patients, was initiated about 50 years ago [1–4]. Shortly thereafter, the discovery of antileukaemic activity by the use of nitrogen mustards [5–7] and analogues of folic acid [8–10] triggered the earnest search for cancer chemotherapeutic agents. Up to the present, several thousand synthetic compounds and numerous natural products have been evaluated biologically, and a total of some 40 drugs have been selected for anticancer therapeutic use.

The modes of action of these drugs may be classified as follows. (a) Inhibition of the biosynthesis of nucleic acids. For example, 6-mercaptopurine and 6-thioguanine block the purine biosynthesis and inhibit interconversion of purines; 5-fluorouracil blocks the function of thymidylate synthetase, and methotrexate blocks the function of folic reductase to deprive the bioavailability of one-carbon fragments. These two agents together inhibit the methylation of deoxyuridylic acid to thymidylic acid. Both hydroxyurea and arabinocytosine block the reduction of cytidylic acid to deoxycytidylic acid. Arabinocytosine also inhibits the function of DNA polymerase. (b) Interference with the functions of DNA. For example, procarbazine depolymerizes DNA; biological alkylating agents such as nitrogen mustard, cyclophosphamide, thio-TEPA, busulfan and nitrosoureas, together with cisplatin, cross-link with DNA; antibiotics such as adriamycin, dactinomycin, mitomycin and mithramycin bind with DNA to block the RNA synthesis. (c) Binding and modifying cell membrane receptors, for example, steroids, hormones and adrenal glucocorticoids. (d) Binding with tubulin and destroying spindle to produce mitotic arrest, for example, vinca alkaloids such as vinblastine and vincristine [11–16]. In reality, however, it should be stated that some of the aforementioned anticancer agents were not originally designed on the basis of biochemical aspects

but rather their activities were uncovered through random biological screening or from keen serendipitous observations, and their mechanisms of action were elucidated afterwards. On the other hand, some compounds designed on the basis of attractive biomechanistic rationale, such as PALA and related compounds [17–19] (designed as transition-state analogue in the *de novo* pyrimidine biosynthesis to interfere with the function of aspartate transcarbamylase), did not show the expected activity in clinical trials. The design of active-site-directed irreversible enzyme inhibitors [20] and organ-specific or target-oriented anticancer agents [21, 22] still awaits the realization of desired compounds.

Another method of study in drug design is the so-called QSAR (quantitative structure-activity relationship) approach, which involves mathematical correlation of a set of biological testing data with a set of approximately quantified structural features, with the ultimate expectation of applying mathematical models to the design of molecules with expected biological activities [23–27]. During the past decade, the computer-aided drug design methodologies have also gained popularity [28]. It should be remembered that, when dealing with life sciences, the "biological systems are not ready to lie quietly while we dissect and probe with neat, mathematical models and laws of physics" [25].

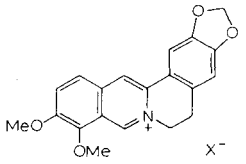
Design of antineoplastic agents as well as other agents of medicinal interest on the basis of characteristic common structural features observed among a large number of pharmacologically active compounds has been exercised in the author's laboratory [29–33]. Some of these postulations were found to be in accord with the energy conformation study and other theoretical and experimental investigations [34–44]. A potent anticancer drug, DHAQ (mitoxantrone), was designed and synthesized in the author's laboratory on the grounds of one of our working hypotheses as well as other information [30, 45–50].

Recently, a common but specific chemical structural pattern has been observed among a large number of biologically, pharmacologically, and clinically active compounds from both the natural and synthetic origin. This structural pattern may lead to the design of novel molecules with pertinent biomedical implications. A preliminary account of this observation has been published [51].

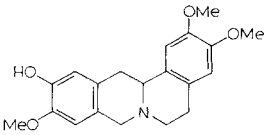
STRUCTURAL SIMILARITY BETWEEN SOME ANTINEOPLASTIC AGENTS AND POLYCYCLIC CARCINOGENIC HYDROCARBONS

The protoberberine alkaloids occupy a prominent seat among the isoquinoline alkaloids. They are widely distributed in higher plants, occurring in at least nine

botanical families (Annonaceae, Berberidaceae, Convolvulaceae, Fumariaceae, Lauraceae, Menispermaceae, Papaveraceae, Ranunculaceae, and Rutaceae). Most of these alkaloids are tetrahydroprotoberberines or as dihydropolyalkoxydibenzo[*a*,*g*]quinolizinium salts with alkoxy groups usually substituted at positions 2,3,9,10 and/or 11 [52, 53]. These alkaloids exhibited a variety of biological activity. For example, the antibacterial, antiprotozoal activity and many pharmacological actions of berberine (1) are well known [54–60]. The C-9 butyloxy or pentyloxy analogue of berberine has hypotensive activity [61]. 13-Ethylberberine is a potent alcohol dehydrogenase inhibitor [62]. 13-Allylberberine and a related alkaloid, dehydrocorydaline, are anti-gastric-ulcer agents [63, 64]. A tetrahydroprotoberberine, xylopinine (2), exhibits a long-lasting and relatively strong adrenergic α-blocking effect on the blood pressure of a variety of animals [65, 66].



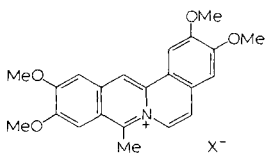
(1)



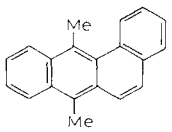
(2)

Coralyne (3), the hexadehydrocarolydine [67–70], was found to possess good antineoplastic activity against leukaemias P388 and L1210 [71–74]. The nucleus of coralyne strikingly resembles that of the potent carcinogenic and mutagenic polycyclic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (DMBA, (4)). Even the methyl group on coralyne is located at the position that corresponds to the methyl group on position 7 of DMBA. Moreover, DMBA was also found to exhibit good antineoplastic activity against adenocarcinoma CA755, sarcoma S180, and leukaemias P388 and L1210 in the U.S. National Cancer Institute experimental animal screens [75].

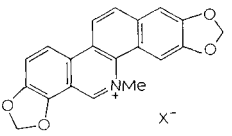
Another large group of isoquinoline alkaloids are the alkoxybenzo[*a*]-phenanthridines. They occur most frequently in the Fumariaceae, the Papaveraceae, and the Rutaceae families. Their close botanical relationship with the



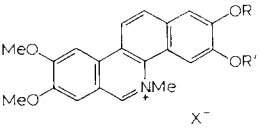
(3)



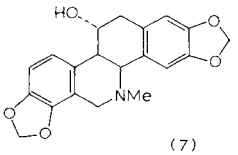
(4)



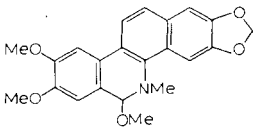
(5)



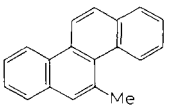
(6a) R + R' = CH_2
(6b) R = H; R' = Me



(7)



(8)



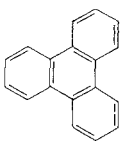
(9)

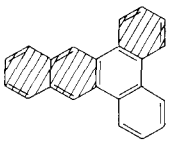
veraceae, and the Rutaceae families. Their close botanical relationship with the protoberberine alkaloids is therefore obvious. The alkoxybenzophenanthridines can be divided into two major classes: (a) those containing a completely aromatized system with oxygenated functions substituted mostly at positions 2,3,7, (or 9) and 8, such as sanguinarine (5) nitidine (6a), and fagaronine (6b), usually in the salt form; and (b) the hexahydrobenzophenanthridines containing a hydroxy function at position 11, as examplified by chelidonine (7). Both classes of compounds contain a methyl group substituted on the isoquinoline N-atom. These alkaloids also display a variety of antibacterial, antifungal, CNS-depressing, and other pharmacological actions [76–78]. Nitidine (6a), fagaronine (6b), and the closely related 6-methoxy-5,6-dihydronitidine (8) are active against leukaemias P388 and L1210. They also demonstrate excellent inhibitory action against Lewis lung carcinoma [79–85].

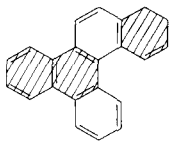
The benzo[*a*]phenanthridine ring system resembles the structure of chrysene, another carcinogen. It is intriguing to note that the carcinogenicity of chrysene is drastically increased by the addition of a methyl group at position 5 of that molecule [86–90]. The position of this methyl group (see structure 9) corresponds to that in the aforementioned benzophenanthridine alkaloids.

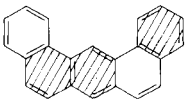
The structural resemblance between the two sets of antineoplastic agents and carcinogens led to a concern as to whether coralyne, nitidine and related antineoplastic alkaloids could also be carcinogenic. It has been reported repeatedly that a large number of clinically useful antineoplastic agents exhibit clastogenic, mutagenic, teratogenic, and carcinogenic effects in the animal studies [91–98], and there was a consensus that the antineoplastic action and carcinogenicity of a given compound may be inseparable. Consequently, a mutagenic evaluation of coralyne, nitidine and related compounds in these two groups was conducted with the Ames test [99–101] using DMBA as the reference standard. It was found that the antineoplastic alkaloids are not mutagenic, but the mutagenic response for DMBA was very high [75]. The results indicate that the presence of a quaternary nitrogen atom in these alkaloids could modify the overall electronic distribution of the ring system and the lodging of alkoxy groups at strategic positions interfere with the in vivo epoxidation-hydroxylation processes necessary for achieving the mutagenic and oncogenic action. The close relationship between mutagenicity and carcinogenicity of biologically active compounds has been discussed [99–106]. The information clearly implied that the antineoplastic action and mutagenicity (or carcinogenicity) of a compound can be separated by proper structural modification.

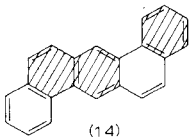
An examination of these structures reveals the following observation. A common tricyclic structural pattern can be perceived between the nucleus of DMBA (4) and that of 5-methylchrysene (9), which consists of a phenyl ring attached to position-2 of a naphthalene ring. The same structural pattern can also be seen in coralyne (3), nitidine (6a) and related alkaloids, except that the rings involved are not entirely composed of carbon atoms. In order to explore the generality of this structural pattern, an examination of the relationship between the chemical structure and the reported carcinogenicity of unsubstituted polycyclic aromatic hydrocarbons with 4–6 condensed ring systems was initiated [107–112]. Existing data indicated that triphenylene (10), perylene (16), pentacene (17), dibenzo[*c,g*]phenanthrene (18) and dibenzo[*e,l*]pyrene (20) possess very little or no carcinogenicity, whereas benzo[*b*]triphenylene (11), benzo[*a*]triphenylene (12), dibenz[*a,j*]anthracene (13), dibenz[*a,h*]-anthracene (14), benzo[*a*]pyrene (15), dibenzo[*def,p*]chrysene (19), and dibenzo[*b,def*]chrysene (21) are potent carcinogens. Compounds in the latter group contain at least one 2-phenylnaphthalene structural pattern (see the shaded areas). A list of many polycyclic aromatic hydrocarbons with different degrees of carcinogenicity and mutagenicity (Ames test score) was published recently [113]. The data strongly support the proposed structural pattern. For example, pyrene (22) and benzo[*e*]pyrene (23) were listed as inactive carcino-

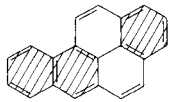

(10)

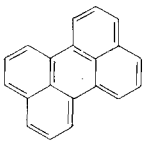

(11)

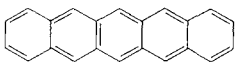

(12)

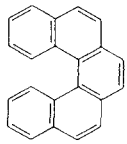

(13)

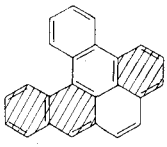

(14)

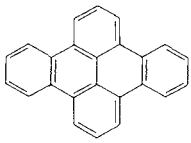

(15)

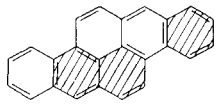

(16)

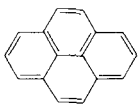

(17)


(18)

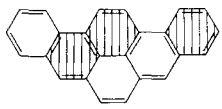

(19)


(20)


(21)


(22)


(23)


(24)

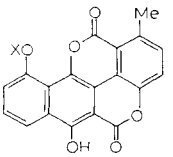
gens with Ames scores [100] at <0.002 and <0.006, respectively, whereas dibenzo[*a*,*i*]pyrene (24) was listed as a strong carcinogen, with an Ames score at 20.

An extensive search of biologically, pharmacologically, and clinically active compounds of both natural and synthetic origin was therefore conducted, which furnished the following additional examples.

ANTIBIOTICS WITH ANTINEOPLASTIC AND OTHER TYPES OF BIOLOGICAL ACTIVITY

CHARTREUSIN, GILVOCARCIN AND RELATED ANTITUMOUR ANTIBIOTICS

The antibiotic, chartreusin [113a], was isolated as greenish yellow crystals from *Streptomyces chartreusis* from an African soil and another *Streptomyces* sp. from a Michigan (U.S.A.) soil [114] and subsequently was found to be identical with antibiotics X-465A (soil from Virginia, U.S.A.) and X-3988 (soil from Brazil) [115]. Chartreusin (25a) possesses a condensed dicoumarin complex containing a D-digitalose and a D-fucose unit [116–118]. The presence of a 2-phenyl-substituted naphthalene feature in its aglycone portion, chartarin [119,120], is readily observed. Chartreusin inhibits the growth of a number of microorganisms, including bacterial strains resistant to streptomycin [114]. This antibiotic exhibits significant activity against ascitic P388, L1210 leukaemia, and B16 melanoma [121]. It binds to many AT and GC polymers, calf thymus DNA, transfer RNA, and ribosomal RNA [122].

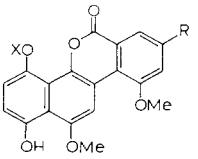


(25a) X = D-digitalosyl-D-fucosyl
(25b) X = D-(2-amino-2,6-dideoxy-3-*O*-methyl)-galactosyl-D-(6-deoxy-3-methyl)galactosyl
(25c) X = D-(6-deoxy-3-methyl)galactosyl

Two related antibiotics, elsamicin A (25b, BBM-2478) and elsamicin B (25c, BBM-28090), were isolated from an unidentified actinomycete strain J907-21 (soil from El Salvador). They have the same aglycone as that of chartreusin but contain different sugar moieties [123, 124]. All three antibiotics have similar antibacterial activity against both aerobic and anaerobic organisms. Elsamicin A was claimed to be 10–30-times more potent than chartreusin, but elsamicin B is a much less active antitumour agent.

The gilvocarcins are a group of antitumour antibiotics with very low mammalian toxicity (LD_{50} approx. 1000 mg/kg) [125]. They contain in common a benzonaphthopyranone ring system, which is similar to chartarin, the aglycone of chartreusin. Gilvocarcins V (26a) and M (26b) were isolated from *Streptomyces gilvotanareus* as yellow crystals [126] and are active against Gram-

positive bacteria and experimental tumours sarcoma S180 and leukaemia P388 [127, 128]. Later it was found that antibiotics toromycin (from *S. collinus* subsp. *albescens* and *S. arenae* 2064) [129, 130] and chrysomycin A (from Streptomyces A-419 [131] were identical with gilvocarcin V [130, 132–134] and chrysomycin B was identical with gilvocarcin M [134]. They all contain the same D-6-deoxygalactose side-chain and differ only in the small alkyl substitution. The viny group in (26a) is believed to be an important structural element for activity.



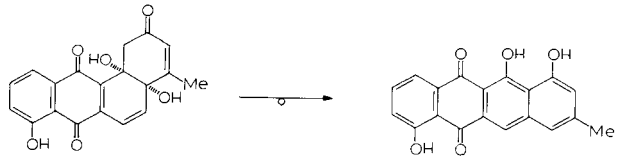
(26a) R = $CH{=}CH_2$, X = D-6-deoxygalactosyl
(26b) R = Me, X = D-6-deoxygalactosyl
(26c) R = Et, X = D-6-deoxygalactosyl
(26d) R = $CH{=}CH_2$, X = H
(26e) R = $CH{=}CH_2$, X = 3,6-dideoxy-3-(*N,N*-dimethylamino) *pseudo*altropyranosyl

The aglycones of gilvocarcins have been shown to be formed via the acetate pathway through a polyketide chain [135, 136]. The aglycone of gilvocarcin E (26c) was postulated as a biosynthetic intermediate [135]. Production of gilvocarcin V is repressed by inorganic nitrogen biosynthetically [137]. It is of interest to note that defucogilvocarcin V (26d), the aglycone of (26a), is itself an antimicrobial antibiotic isolated from *S. arenae* 2064 [138]. This information is in accord with the earlier suggestion that the carbohydrate side-chain is appended late in the biosynthesis [131, 134, 135]. Gilvocarcins inhibit DNA synthesis [139].

Ravidomycin (26e), isolated from *S. ravidus*, possesses potent antitumour activity against leukaemia P388, colon C38 and CD8F1 mammary tumour with low toxicity [140]. The structure of (26e) differs from gilvocarcin V only in the side-chain substitution [141, 142]. This antibiotic inhibits DNA and RNA syntheses, while protein synthesis is only slightly inhibited [143]. The biological activity of both ravidomycin and gilvocarcin V is reported to be activated in the presence of light [125, 144]. Side-chain modifications of ravidomycin were conducted [145]. In general, it can be said that the *C*-glycoside moiety of these antibiotics can be varied considerably without destruction of biological activity, but proper side-chain modification may aid in the transport of the molecule to the desired site.

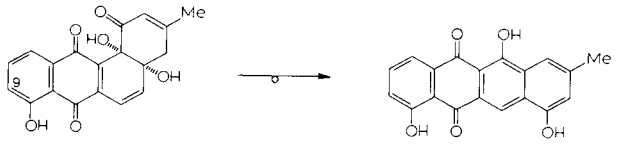
ISOTETRACENE ANTIBIOTICS (ANGUCYCLINES)

A yellow antibiotic SS-228Y (27), isolated from an actinomycete of the genus *Chinia* in a culture medium containing seaweed extract, has inhibitory activity against Gram-positive bacteria, Ehrlich carcinoma and the enzyme dopamine β-hydroxylase [146]. In the presence of either heat or light, this yellow antibiotic is readily converted to a red tetracenone, SS-228R (28), which shows diminished activity against bacteria and tumour cells but retains the enzyme inhibitory activity [146]. The originally purposed structures (27) and (28) were found to be slightly incorrect, and were later revised [147, 148] as (29) (for SS-228Y) and (30) (SS-228R), but the overall skeletons remain the same.



(27)

(28)



(29) (30)

The fact that the angular isotetracenone structure (29) is more biologically active than the straight tetracenone structure (30) is worth noting. It was later found that antibiotics containing the benz[*a*]anthraquinone or modified benz[*a*]anthraquinone-type ring systems are widespread. The aglycone of the antitumour antibiotic capoamycin (induces differentiation of mouse myeloid leukaemia cells and inhibits Ehrlich ascites carcinoma), isolated from *S. copoamus* in Japan, is identical (29) with a β-4-(2,4-decadienoyl)olivoside *C*-glycoside side-chain substituted at the C-9 position [149]. A related 'hydrated' structure (31), is the aglycone of the following antibiotics.

1. Aquayamycin. A strong inhibitor of tyrosine hydroxylase and dopamine β-hydroxylase; isolated from *S. misawanesis*; contains a hydroxylated dihydropyran *C*-glycoside substituted at C-9 [150–152].

2. Vineomycin A. (Os-4742A, P-1894B). Active against Gram-positive bacteria, collagen proline hydroxylase, and sarcoma S180; isolated from

S. matensis subsp. *vineus* and from *S. albogriseolus* subsp. No. 1894; contains two identical sets of *C*-glycosides (L-aculose and L-rhodinose) in addition to aquayamycin substituted at 3′-OH and through the C-9 glycoside of aquayamycin [152–156].

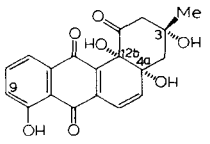
3. Sakyomicin A (and other sakyomicins). Active against Gram-positive bacteria; isolated from Nocardia sp. No. 53; contains a hydroxyl group at C-2 and a rhodinosyl group at 12b-OH [157].

4. Kerriamycin A (and other kerriamycins). Active against Gram-positive bacteria and Ehrlich ascites carcinoma; isolated from *S. violaceolatus* A32; contains a novel sugar, kerriose (2,6-dideoxyerythrohexopyran-3-ulose) in addition to aquayamycin, substituted through the C-9 glycoside of aquayamycin [158, 159].

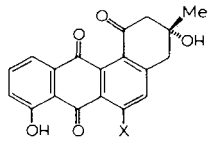
5. The saquayamycins A-D. Active against Gram-positive bacteria and P388 leukaemia cells sensitive and resistant to adriamycin; isolated from *S. nodosus* MH190-16F3; contains additional multiple glycosides than aquayamycin, substituted at 3′-OH and through C-9 of aquayamycin [160].

6. The urdamycins. Active against Gram-positive bacteria and stem cells of murine L1210 leukaemia; isolated from *S. fradiae* strain Tü 2717 in Tanzania; contain additional multiple glycosides than aquayamycin, substituted at 12b-OH, C-5 and through the C-9 glycoside of aquayamycin [161].

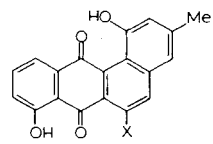
Capoamycins undergo the same skeletal rearrangement as SS-228Y in dilute base to the tetracenone [149b]. Similar structural rearrangements were reported for aquayamycin and related antibiotics in dilute base or by heat treatment [152].



(31)



(32a) X = H
(32b) X = OH

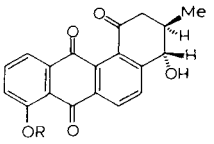


(33a) X = H
(33b) X = OH

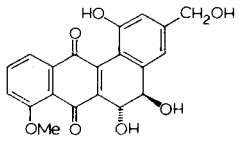
Among all the isotetracene antibiotics, tetrangomycin (32a), obtained from *S. rimosus*, possesses the simplest structure, and is the first antibiotic to be isolated [162, 163]. Compound (32a) differs from (31) only by the presence of a double bond (instead of a diol) between C-4a and C-12b. Base treatment of (32a) does not cause a skeletal rearrangement, but readily aromatizes it to the benz[*a*]anthraquinone derivative, tetrangulol (33a). The latter is an antibiotic per se, since it can also be isolated from the culture filtrates of *S. rimosus* without any alkaline treatment [162]. A related antibiotic rabelomycin (32b)

was isolated from *S. olivaceus* ATCC 21549 [164]. It is active against Gram-positive bacteria. Rabelomycin loses water readily to form the aromatized (33b) when treated with acid [164]. Compound (33b) is also the aglycone for benzanthrins A and B [165, 166], which were isolated from *Nocardia lurida* and possess inhibitory activity against Gram-positive bacteria as well as 9KB, 9PS, and 9ASK tumour cells in tissue culture. Both benzanthrins A and B contain a *C*-glycosidic sugar angolosamine at position 2. Benzanthrin B contains an additional angolosamine as an *O*-glycoside substituted at 1-OH and benzanthrin A contains an isomeric *O*-rhodosamine substituted at the same position [166].

Fujianmycins A and B were isolated from a *Streptomyces* species (IA-CAS-114) in Fujian, China [167]. The structure of fujianmycin A (34a) may be regarded as the 2,3-hydrated form of (33a), and fujianmycin B (34b) as its methyl ether derivative. The structure of antibiotic PD-116740 (35), isolated from an unidentified actinomycete species (WP-4669) and possessing activity against L1210 leukaemia *in vitro* and HCT-8 human colon adenocarcinoma cell line [168], may be visualized as the 3-hydroxylated 8-methyl-5,6-diol derivative of (33a). All these antibiotics contain the characteristic 2-phenylnaphthalene structural feature.



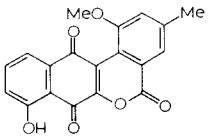
(34a) R = H
(34b) R = Me



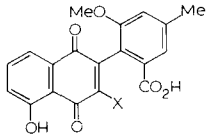
(35)

ANTICOCCIDIAL ANTIBIOTICS

Several synthetic compounds containing the naphthoquinone skeleton, such as 2-(4-cyclohexylcyclohexyl)-3-hydroxy-1,4-naphthoquinone [169], are effective in the treatment of coccidial infection. Two structurally related antibiotics WS-5995A (36) and WS-5995B (37a), isolated from *S. auranticolor* sp. nov. near Tokyo, efficiently protect *Eimeria tenella* (a species of coccidia) infection. An inactive component WS-5995C (37b) can be readily converted to the active (36) by simple dehydration [170–172]. Compound (36), the structure of which resembles the aforementioned benz[*a*]anthraquinone antibiotics, is the first example of a 5*H*-benzo[*d*]naphtho[2,3-*b*]pyran ring system found in nature.



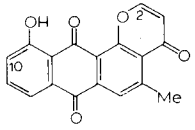
(36)



(37a) X = H
(37b) X = OH

THE PLURAMYCIN (IYOMYCIN) GROUP ANTIBIOTICS

A number of antibiotics, such as pluramycin [173], neopluramycin [174], hedamycin [175–179], kidamycin [176, 179–181], griseorubicin [182], largomycin [183], PD-121,222 [184], and SF-2330 [185], possess a common chromophore (38). The structure of (38) resembles that of benz[*a*]anthraquinone, with the exception that the angular ring unit is a γ-pyrone. Antibiotics in this group exhibit similar biological activities [181] and many have been reported to possess cytotoxic and antineoplastic action. Hedamycin (isolated from *S. griseoruber* C-1150) and kidamycin (isolated from *S. phaeoverticillatus*) are two of the most frequently studied antibiotics among this class. The former contains a bioxiranyl side-chain at position-2 and the latter has a 2-butenyl side-chain at that position. Both antibiotics contain the same *C*-glycosides at C-8 (angolosamine) and at C-10 (*N,N*-dimethylvancosamine). Other antibiotics contain similar but not identical side-chains and their solubility, distribution and biological potency, in general, depend on the nature and conformation of these substituents.



(38)

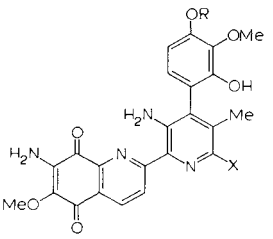
A related antibiotic, chromoxymycin [186], isolated from *S. rubropurpureus* No. 6362, is active against P388 leukaemia and B16 melanoma and exhibits antimicrobial activity against Gram-positive bacteria. The structure of chromoxymycin is very close to that of hedamycin, except that the C-12 quinone-carbonyl is reduced and attached to a 5-[(2-carboxyethyl)aminocarbonyl]-*N*-hydroxypyrrol-2-yl moiety, and that the *N,N*-dimethylvancosamine attached to C-10 is no longer in the boat conformation (as in hedamycin), but rather in the chair conformation. All the other substituents remain the same.

STREPTONIGRIN, LAVENDAMYCIN AND RELATED NATURAL AND SYNTHETIC COMPOUNDS

A dark-brown to black coloured antibiotic, streptonigrin, isolated from *S. flocculus* [187, 188], exhibits striking activity against a wide spectrum of experimental tumour systems, including sarcoma S180, Ehrlich carcinoma, adenocarcinomas Ca755 and EO771, Wagner and Ridgway osteogenic sarcomas, Walker 256 carcinosarcoma, Iglesias ovarian tumour and human HS-1 sarcoma and HEp3 epidermoid carcinoma. Streptonigrin also shows activity over a broad spectrum of bacteria, including representatives of the Gram-positive and Gram-negative bacteria and *Mycobacterium tuberculosis* 607 [189–191]. Chemotherapy with streptonigrin or its methyl ester has been conducted. Significant objective responses occurred (the compound being given either orally or parenterally) in malignant lymphoma, mycosis fungoides, squamous cell carcinoma of the cervix, malignant melanoma, carcinoma of the breast and miscellaneous neoplasms of the head and neck [192–197]. Clinical trials were discontinued recently, due mainly to the marked side-effect on the gastrointestinal tract and on the bone marrow [198].

The structure of streptonigrin was found to be a highly functionalized 2-(4-phenylpyridyl)quinolinedione (39) [199]. X-ray diffraction study of this antibiotic revealed that the pyridyl ring is nearly coplanar with the quinolinedione portion [200], probably due to the formation of an intramolecular hydrogen bond between the amino hydrogen substituted on the pyridine ring and the nitrogen atom of the quinoline ring. Two other antibiotics, rufochromomycin (isolated from *S. rufochromogenes* and *S. echinatus* [201]) and bruneomycin (isolated from *Actinomyces albus* var. *bruneomycini* [202, 203]), are identical with streptonigrin.

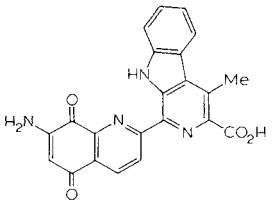
It is of interest to note that the *Streptomyces* species IA-CAS-114, which produces the fujianmycins (34a,b) [167], also produces streptonigrin [204]. In addition, it likewise provides a structurally related metabolite streptonigrone (39b), probably formed by decarboxylation of (39a), followed by oxidation. The



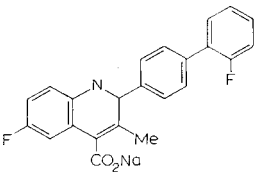
(39a) R = Me , X = CO_2H
(39b) R = Me , X = OH
(39c) R = H , X = CO_2H

biological activity of (39b) is much lower than (39a) [204]. Both streptonigrin and another antibiotic, demethylstreptonigrin (39c), were also isolated from *S. albus* [205]. Both the antibacterial and antitumour activities of (39c) are slightly weaker than those of (39a). Neither the separated quinolinedione moiety [206] nor the 4-phenylpyridine moiety [207, 208] of streptonigrin retained any of the originally reported biological activity.

Lavendamycin (40), a dark-red antibiotic isolated from *S. lavendulae* [209, 210], possesses a simpler but closely related structure to streptonigrin. The biosynthesis relationship between these two antibiotics, as postulated to be originated from β-methyltryptophan [211–213], is therefore further substantiated. The general antimicrobial inhibition pattern of lavendamycin is similar to that of streptonigrin, with lavendamycin being less potent. The only exception is with the fungi *Trichophyton rubrum, T. mentagrophytes* and *Microsporum canis*, where lavendamycin is more potent. Lavendamycin has no inhibitory effect against leukaemias P388 and L1210.



(40)



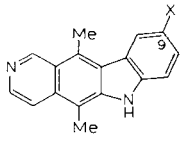
(41)

From over 200 synthetic compounds tested recently, several substituted 2-phenyl-4-quinolinecarboxylic acid derivatives were found to possess antineoplastic activity against leukaemias P388 and L1210, and against B16 melanoma. Among these, DuP-785 (41) was selected for phase I clinical trial for its additional inhibitory activity against a spectrum of xenografted human solid tumours in nude mice (including carcinomas of the breast, lung, stomach and colon) and for its solubility [214]. The structural similarity between these synthetic compounds and the aforementioned antibiotics can be readily visualized. Compound (41) is a potent inhibitor of dihydroorotate dehydrogenase in the *de novo* pyrimidine biosynthetic pathway [215].

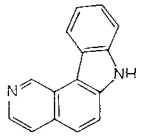
ALKALOIDS WITH ANTINEOPLASTIC AND OTHER TYPES OF BIOLOGICAL ACTIVITY

ELLIPTICINE AND RELATED NATURAL AND SYNTHETIC COMPOUNDS

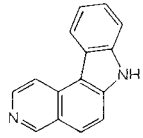
Ellipticine is a bright lemon yellow alkaloid isolated from the woody shrubs *Ochrosia elliptica* Labill, *O. maculata* Jacq., *O. moorei* F. Muell, *O. silvatica*, *O. sandwicensis* A. DC., *O. viellardii* and other *Ochrosia* species as well as from *Aspidosperma subincanum* Mart and *Excavatia coccinea* Mgf [216–223]. Most of the plants are found in Australia, the Andaman Islands, Madagascar, Hawaii, tropical Asia, Florida and Peru. Ellipticine is active against sarcoma S180, adenocarcinoma Ca755 and leukaemia L1210 and cytotoxic against human carcinoma of the nasopharynx [220, 221, 224]. It possesses powerful inhibitory action to the microsomal cytochrome *P*-450 monooxygenase, to the hydroxylation of benzo[*a*]pyrene, and to the mutagenicity of 3-methylcholanthrene [225, 226].



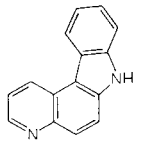
(42a) X = H
(42b) X = OMe
(42c) X = OH



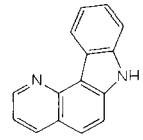
(43)



(44)



(45)

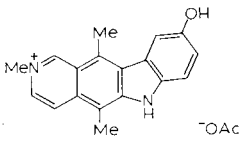


(46)

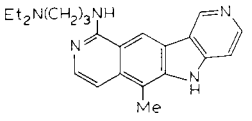
Ellipticine (42a) possesses a unique pyrido[4,3-*b*]carbazole ring [218]. The two methyl groups of the ellipticine molecule occupy positions equivalent to those of DMBA (4). In fact, structure–activity relationship study reveals that the presence of these methyl groups is essential for the expression of the inhibitory action against cytochrome *P*-450. The location of the pyridine ring appears to be the most important factor for the cytochrome *P*-450 binding, since isoellipticines (43), (44), (45) and (46) (none of which contains the characteristic '2-phenylnaphthalene' pattern) are devoid of such inhibitory activity [226].

The alkaloid, 9-methoxyellipticine (42b) [201, 221], possesses activity against several of the solid mouse neoplasms. It is less active than acronycine

(*q.v.*), but has a broader spectrum of activity than many clinically active agents [221]. Compound (42b) is active in human myeloblastic leukaemia [227]. The related 9-hydroxyellipticine (42c), which is a major metabolite of (42a) [225, 228], is 4-times more active than ellipticine against leukaemia L1210 (a minor metabolite, 7-hydroxyellipticine, is 6-times less active than ellipticine against the same leukaemia) [229] and less toxic [230]. Quaternization of the pyridine nitrogen of (42c) by methylation furnishes a highly active compound, 9-hydroxy-*N*-2-methylellipticinium acetate (47) [231], which displays markedly improved antitumour activity in advanced (osseous metastases) breast cancer, hepatomas, and non-Hodgkin's lymphomas without myelosuppression. Digestive disturbances, however, constitute a dose-limiting factor [232–234].



(47)



(48)

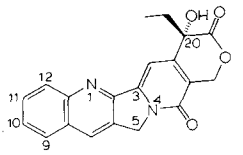
Numerous structural modification studies of ellipticine have been conducted. It is found that side-chain substitution has considerable influence on the biological activity to compounds of this type. A pyridopyrrolo[2,3-*g*]isoquinoline derivative, BD-40 (48) [235, 236], has demonstrated potent activity against many different experimental tumours including leukaemia L1210 and the Friend virus leukaemia. It is also active against the Moloney strain of murine sarcoma virus [237]. Phase I clinical study of (48) has been conducted [238, 239].

Ellipticine inhibits DNA, RNA and protein synthesis. The inhibition is not reversible by removal of the alkaloid. It has no appreciable effects on thymidine and uridine kinases or on RNA polymerase, but it markedly inhibits DNA polymerase activity [240, 241]. The actual mechanism of action of ellipticine and related compounds has not yet been elucidated. Ellipticine and derivatives have been found to interact with bacterial membranes [242]. Many investigators have categorized these compounds as DNA-interacting or intercalating agents [230, 235, 237, 243–246]. It has recently been postulated that mammalian DNA topoisomerase II may be a common target of these antitumour compounds [247].

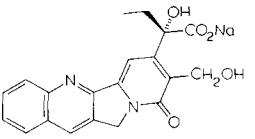
CAMPTOTHECIN AND RELATED COMPOUNDS

Isolated in low yield (0.005%) from the bark and stem wood of *Camptotheca acuminata* Decaisne (family Nyssaceae) native to China [248–250], and sub-

sequently from the Indian tree *Mappia foetida* Miers (family Olacaceae) *Nothapodytes foedita* Sleumer (family Icacinaceae) [251], from *Ophiorrhiza mungos* Linn. (Rubiaceae) [252], and from *Ervatmia heyneana* (Wall.) T. Cooke (Apocynaceae) [253], the pentacyclic alkaloid camptothecin, m.p. 287–288 °C, (49) [248, 254] displays potent antineoplastic activity [249] against B16 melanoma, carcinoma Ca-755, leukaemias K1964, L1210 and P388, and Walker carcinosarcoma 256 at low doses, also exhibits cytotoxicity against KB cell culture, leukaemias L1210 and L5178Y and the Novikoff hepatoma cell lines [253, 255–257]. Camptothecin inhibits DNA synthesis and certain species of RNA synthesis, but does not inhibit protein synthesis in mammalian cells [256–261].



(49)



(50)

Numerous partial and total syntheses of camptothecin have been reported, but none of these compounds containing less than the original pentacyclic rings possesses any antineoplastic activity (the sodium salt of camptothecin (50), which has a coplanar tetracyclic ring structure, should be regarded only as a prodrug-type compound of camptothecin). It has long been established that the α-hydroxylactone of the natural (20*S*)-camptothecin (49) is a critical substituent for antineoplastic activity, since 20-deoxycamptothecin, 20-chlorocamptothecin and the reduced camptothecin hemilactol are completely inactive [257, 262, 263] and the (±)-camptothecin is only about 50% as active as (49) [264].

Aside from the biological role lent by the α-hydroxylactone ring, which may have an alkylating function, the remaining portion of the molecule, which contains the characteristic '2-phenylnaphthalene' structural pattern, also exerts important influence on biological activity, since α-hydroxylactone-containing compounds possessing less condensed rings are completely inactive biologically.

Some ring-substituted (hydroxy or methoxy) camptothecin derivatives were either isolated in nature or obtained synthetically [253, 264–267]. The 9-methoxy- and 10-methoxycamptothecin are less active, but 10-hydroxycamptothecin is more active than the parent alkaloid against leukaemia P388 [253, 264]. Both camptothecin and its 10-hydroxy derivative are being used in mainland China for the treatment of liver carcinoma and tumours of the head

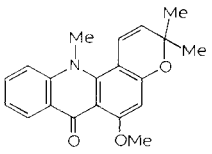
and neck [256]. The 11-hydroxycamptothecin has better antileukaemia activity and less toxicity than the parent compound [268], and the 12-hydroxy- and 12-methoxycamptothecin are reported to have superior antitumour activity in inhibiting Ehrlich ascites carcinoma [266]. The 12-carboxycamptothecin has excellent inhibitory activity against leukaemia L615 [266].

In addition to the antineoplastic activity, camptothecin was found to be an effective inhibitor of adenovirus replication [268, 269] and herpes virus replication [252, 270]. 10-Methoxycamptothecin is about 8-times more potent than camptothecin as an inhibitor of herpes virus [252]. A combination of camptothecin and dimethyl sulphoxide is very effective for the topical treatment of psoriasis [271]. Since, in the goldfish brain, camptothecin blocks RNA synthesis in eucaryotic cells by blocking the incorporation of uridine into RNA, this alkaloid can block the memory of conditioned avoidance and produces no measurable effect on retention of the learned response [272].

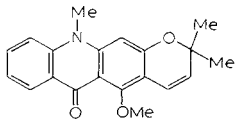
The mechanism of biological action for camptothecin is not yet entirely known. It has been suggested that this alkaloid may inhibit DNA formation or may catalyze the formation of DNA-damaging free radicals *in vivo* [256].

ACRONYCINE

The genus *Acronychia*, family Rutaceae, consists of more than 20 species of trees and shrubs in Australia and tropical Asia. The yellow acridone derivative acronycine (51) m.p. 175–176 °C, was originally isolated from the bark of the Australian yellowwood *A. baurei* Schott (*Bauerella australina* Borzi) and from *B. simplicifolia* (Endl.) Hartley [273–277]. This alkaloid, which is not very soluble in water but is soluble in most organic solvents, is a wide-spectrum antitumour agent and has been shown to be active against C-1498 myelogenous leukaemia (non-responsive to many clinically active anticancer drugs), B82, L-5178Y and AKR leukaemias (but inactive against leukaemia L1210), LPC-1 and X-5563 plasma cell tumours, Shionogi carcinoma 115 (an androgen-dependent tumour), adenocarcinoma 755 and Ridgway osteogenic sarcoma [276–280]. Acronycine is effective subcutaneously, intraperitoneally or orally. It blocks DMBA-induced adrenal apoplexy [276] and inhibits the growth of herpes virus [281].



(51)



(52)

Phase I and II clinical trials indicated that acronycine reduced pain of the spine in some patients with multiple myeloma [280, 282, 283]. Acronycine has been reported to cause leukopoenia and to have CNS-depressant activity [284]. Biochemically, acronycine inhibits incorporation of extracellular nucleosides into the RNA and DNA of leukaemia L-5178Y cell culture. There is, however, no evidence of interaction between acronycine and DNA or inhibition of template activity of DNA. This alkaloid does not inhibit nucleic acid synthesis in the cell, but rather inhibits the accumulation of extracellular uridine or thymidine, as nucleotides, in the intracellular precursor pool [285, 286]. Acronycine, acting primarily on membranous organelles [287], seems to interfere with the structure, function and/or turnover of cell membrane components, thereby changing the fluidity of the plasma membrane [288].

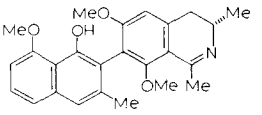
Several hydroxylated and methoxylated acronycine analogues were isolated from *Citrus depressa* (Rutaceae), a Chinese drug 'Chen-pi' used for treating heartache, abdominal pain, diarrhoea, tussis, vomiting, inappetence, and as an expectorant, and from *Glycosmis citrifolia* (Willd.) Lindl. (*G. cochinchinensis* Pierre), a wild shrub used in the treatment of scabies, boils and ulcers [289–291]. None of these compounds was reported to possess antineoplastic activity.

The angular acronycine (51) can be rearranged chemically to the linear isoacronycine (52) [292, 293]. It was found that, among some hundred derivatives of acronycine and isoacronycine synthesized, although several compounds have shown more potent cytotoxicity, none of the iso-series (e.g., linear isomers, which do not contain the characteristic structural pattern) has yielded any biologically active compounds [294]. The strict requirement for, and the dependence on, a specific structure was suggested as being more compatible with a drug receptor-type mechanism rather with a less precise type of action based on physical characteristics, such as lipophilicity or inadequate solubility [280].

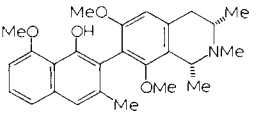
ANCISTROCLADIDINE, ANCISTROTECTORINE AND STRUCTURALLY RELATED NON-ALKALOIDS

Ancistrocladidine and ancistrotectorine

Two naphthalene-isoquinoline groups of alkaloids, ancistrocladidine (53) and ancistrotectorine (54), were isolated from *Ancistrocladus heyneanus* Wall [295], and *A. tectorius* (Lour.) Merr [296], respectively. The members of the plant family, ancistrocladaceae, are distributed in tropical Asia and Western Africa. Some are regarded as traditionally medicinal plants. The root of *A. tectorius* has been used to treat dysentery and malaria [296].



(53)

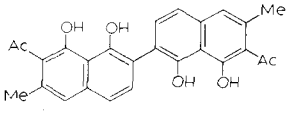


(54)

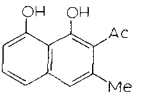
The tetra*ortho*-substitutions create a steric hindrance about the diaryl linkage, which results in a dihedral angle of 56.4° between the two aromatic rings [296].

Stypandrol

Stypandra imbricata R. Br. (Liliaceae), is known in Western Australia as 'blind grass', since ingestion of the plant by goats and sheep often causes initial weakness, incoordination, and paralysis of hind limb, which may progress to prostration and death. Blindness, due to irreversible retinal degeneration and optic nerve atrophy, could persist following recovery from acute intoxication [297–300].



(55)



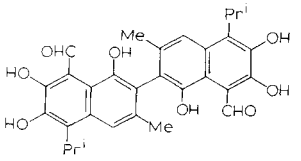
(56)

The compound responsible for the toxicity of this plant has been isolated and identified as a non-nitrogen-containing 2,2′-binaphthalenetetrol derivative, stypandrol (55) [301], hence it is not an alkaloid. The two naphthalene rings are not coplanar and the dihedral angle formed by the two aromatic rings is 44.3° [298]. The compound is a racemate, m.p. 241–242 °C.

The 'monomeric' dianellidin (56), which does not process the 2-aryl-naphthalene pattern, is non-toxic and without biological activity.

Gossypol

A yellow pigment found in the seed of cotton plant *Gossypium arboreum* L. (Malvaceae), from the woody stems and stem barks of Puerto Rico's *Montezuma speciosissima* Sesse and Moc. (Malvaceae) and from the bark and the flowers of *Thespesia populnea* (Malvaceae) found in Africa, Asia and Pacific Islands [302–304, 323], gossypol (57), m.p. 166–167 °C, is a symmetrically substituted hexahydroxy-2,2′-binaphthalenedialdehyde derivative [303–308].



(57)

Gossypol has long been known for its antioxidation property [309–312] and has been used as an effective stabilizer for vinyl compounds against polymerization in rubber industry [313]. This compound was reported to be toxic to nonruminant animals by reducing the oxygen-carrying capacity of blood, but apparently causes no ill effects in humans [308]. Gossypol was uncovered as a male contraceptive in China in 1972 [314–318]. By inhibiting the enzyme lactate dehydrogenase-X, the enzyme has a crucial role in both aerobic and anaerobic metabolism of sperm and sperm-generating cells [319].

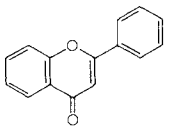
Due to its restricted rotation caused by the four *ortho* substituents in the structure of gossypol, the two naphthalene rings are not coplanar, resulting in the formation of a dihedral angle of 70° between the two aryl units [303, 304, 307]. The racemic gossypol has been resolved [320, 321]. As expected, the (−)-gossypol is the active component [322]; the (±)-isomer is only one-half as active and the (+)-isomer is inactive. However, all three isomers demonstrate the same degree of inhibitory action against the enzyme lactate dehydrogenase-X [321].

Some inhibitory activity of gossypol against mouse mammary adenocarcinoma 755 and borderline activity against leukaemia P388 were reported [323, 324]. The effective dose range of gossypol is rather narrow because of toxicity [324]. Gossypol has recently been found to be a specific inhibitor of DNA polymerase α [325], a major enzyme involved in DNA replication. This compound may also interfere with the DNA repair process [325].

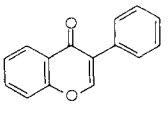
FLAVONES, ISOFLAVONES AND CHALCONES

Flavonoids are among the most abundant groups of low-molecular-weight compounds in the plant kingdom [326, 327]. They are ubiquitously present in almost every part of higher plants (many as flavonoid glycosides) including the leaves, flowers, fruits, seeds, nuts, stems, roots and the bark, and are responsible for much of the natural colouring in nature. In general, the toxicity of these flavonoids is low or even non-existent, as the average human's daily food intake contains about 1 g of various flavonoids [328–330].

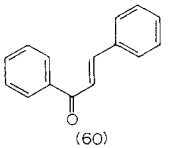
The parent nucleus of the flavonoids is flavone ((58), 2-phenylchromone or 2-phenylbenzopyran-4-one). Flavone and isoflavone ((59), 3-phenylchromone, the parent nucleus of the isoflavonoids) are the simplest oxygen-containing naturally occurring compounds that possess the '2-phenylnaphthalene'-type structure. The chalcones, represented by the nucleus (60), may be regarded as open-chain flavonoids and are usually hydroxylated. The interconversion of chalcone and flavonone catalyzed by chalcone isomerase is well known [326, 327, 331]. Chalcones can be precursors of both the flavonoids and the isoflavonoids [326–332].



(58)



(59)



(60)

A variety of biological properties are attributed to the flavones. The statement, "One can't help but wonder if some of the wisdom of herbal medicine can be attributed partly to the flavonoid content of the various plant decoctions and salves, etc. recommended for the treatment of various conditions for centuries" [328] is certainly adequate. For example, glepidotin A (61) and other prenylated flavonoids isolated from *Glycyrrhiza lepidota* (Leguminosae), or licorice, are antimicrobial agents [333]. The *Glycyrrhiza* species has been used by man for anti-inflammatory purposes for at least 4000 years [334]. Many flavonoids such as quercetin (62a), fisetin (62b), and baicalein (63) extracted from *Scutellaria baicalensis* G. (Gantsao), as well as the newly isolated podoverine A (64) and other podoverines from *Podophyllum versipelle*, possess anti-inflammatory activity [335, 336]. The anti-inflammatory activity of these flavonoids is connected with the inhibition of glyoxalase-I, and thus suppresses histamine release [335, 337]. Quercetin (62a) and its 3-L-rhamnosidyl derivative, quercetrin, have an inhibitory effect on virus multiplication (against HSV-I, pseudorabis virus and encephalitis in mice) [338–340]. It appears that there is a relationship between the antiviral effect and the cAMP-enhancing activity of flavonoids [340]. Hydroxylated flavonoids, particularly those possessing catechol-type substituted hydroxy groups in the phenyl ring (ring C), are larval (for example, *Heliothis zea*) growth inhibitors [341].

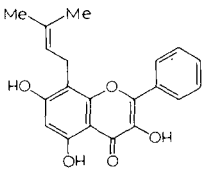
Flavonoids are highly potent inhibitors of lens aldose reductase, the enzyme which converts D-glucose and D-galactose to sorbitol and thus initiates cataract formation in diabetes [342–348a]. Most of these flavonoids contain at least one, preferably two (catechol-type), hydroxy groups in the phenyl ring (ring C) and at least three hydroxy or methoxy groups in ring A. The double

bond between carbon atoms 2 and 3 is necessary for activity. Plants, including *Brickellia arguta*, *Cichorium endivia* (endive), produce many such flavonoids [346–348]. Examples are quercetin (62a), orientin, rutin, myricitrin and 3-*O*-glycosides of patuletin (65). Glycosylation is not necessary for the anti-cataract activity, but it may favourably increase the water solubility for the parent flavones or flavonols [348].

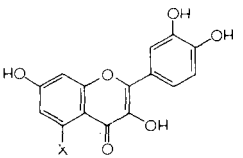
Quercetin and many 3-hydroxylated flavones, particularly those containing *o*- or *p*-quinol structure at ring C, such as (66), are strong antioxidants for unsaturated fatty acids and lipids of vegetable tissues [349–352]. The effect of antioxidant action is decreased when some of the hydroxy groups are methylated [353].

Two benzoflavones possess different actions toward the polycyclic aromatic hydrocarbons. 7,8-Benzoflavone (α-naphthoflavone, (67)) inhibits the metabolism (hence activation) of benzo[*a*]pyrene and DMBA [354–358]. Since the polycyclic aromatic hydrocarbons require metabolic activation from microsomal mono-oxygenase enzymes to become carcinogenic, 7,8-benzoflavone may be classified as an anticarcinogenic compound. On the other hand, 5,6-benzoflavone (β-naphthoflavone, (68)) and the methoxylated flavones, including quercetin pentamethyl ether, tangeretin (69a) and nobiletin (69b), are strong inducers of benzo[*a*]pyrene hydroxylase activity [359, 360]. Since these compounds activate the synthesis of cytochrome *P*-450 isozymes and extensively metabolize the carcinogenic polycyclic hydrocarbons such as DMBA, they also inhibit carcinogenesis, as shown by the absence in pulmonary adenoma formation [360].

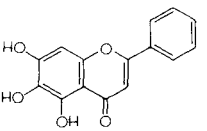
5,6-Benzoflavone, therefore, is likewise an anticarcinogenic agent. One may say that 7,8-benzoflavone acts directly, whereas the 5,6-isomer acts indirectly,



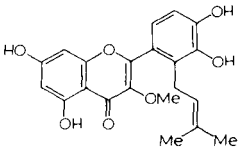
(61)



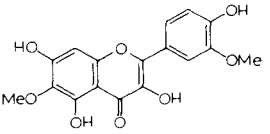
(62a) X = OH
(62b) X = H



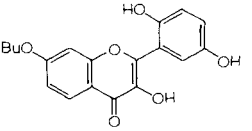
(63)



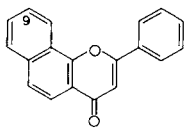
(64)



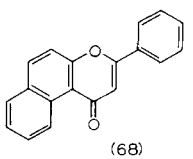
(65)



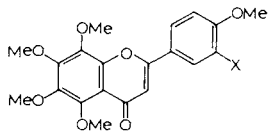
(66)



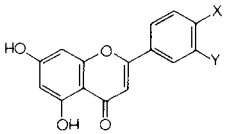
(67)



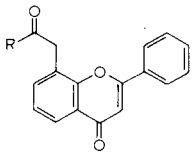
(68)



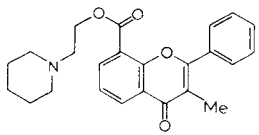
(69a) X = H
(69b) X = OMe



(70a) X , Y = H
(70b) X = OH, Y = H
(70c) X = H, Y = OH



(71a) R = $O(CH_2)_2NMe_2$
(71b) R = OH



(72)

against carcinogenesis. Interestingly, 7,8-benzoflavone also activates benzo-[*a*]pyrene hydroxylation by liver microsomes [361, 362] and the mechanism of activation of microsomal benzo[*a*]pyrene hydroxylation by flavonoids has been aptly studied [363]. 7,8-Benzoflavone is a potent inhibitor in preventing chromosomal breaks caused by DMBA and benzo[*a*]pyrene [364].

Both 7,8-benzoflavone and the oestrogen steroids protect the cytotoxic effect of the carcinogens DMBA and aflatoxin B_1 as well as protecting against the effect of cytotoxin sterigmatocystin [358]. 7,8-Benzoflavone, chrysin (70a) and apigenin (70b) inhibit the aromatization of androstenedione and testosterone to oestrogens, but 5,6-benzoflavone is not inhibitory [365]. 7,8-Benzoflavone is a potent competitive inhibitor of human aromatase cytochrome *P*-450 (oestrogen synthetase). Subsequently, it was found that both 7,8-benzoflavone and its 9-hydroxy derivative exhibit very high affinity for this aromatase, which led to the postulation that these non-steroids can occupy the steroid-ring binding site of the enzyme [366].

Apigenin (70b), luteolin (70c) and related polyhydroxyflavones can activate the nodulation genes of the nitrogen-fixation bacteria (genus *Rhizobium*) in the leguminous plants such as peas. This is the first time that simple flavonoids have been shown to be involved in gene regulation [367–369].

In accord with the structure–activity relationship studies on antioxidants, many flavones containing the phenolic hydroxy groups inhibit the mutagenic activity from the metabolic activation of carcinogenic polynuclear hydrocarbons. Flavones which lack the free phenolic groups or containing only the methoxy groups are inactive [370]. In contrast, flavones with methoxy groups possess good inhibitory activity against human nasopharyngeal carcinoma [371, 372]. The popular pentahydroxylated flavone quercetin (62a) was

reported to be a strong mutagen [373, 374], but this flavone has no carcinogenic activity [375, 376]. Quercetin also markedly suppresses the promoting effect of teleocidin on skin tumour formation initiated by DMBA [375].

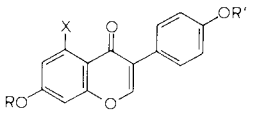
Among more than fifty 2-substituted benzopyran-4-one derivatives synthesized and evaluated for their antineoplastic activity, the 2-diethylaminoethyl ester of flavone-8-acetic acid (71a) was found to exhibit significant activity against the subcutaneously implanted colon adenocarcinoma 38, which is highly refractory to many established antitumour agents. Compound (71a) is only moderately active against leukaemias P388 and L1210 [377]. It does not cause myelosuppression, nausea, vomiting or major organ toxicity, but it has local anaesthetic and tranquillizing properties and cardiovascular side-effects such as hypotension and bradycardia [378–380]. Pharmacodynamic and drug degradation studies revealed that the ester is rapidly hydrolyzed *in vivo* to the free acid (71b) and 2-diethylaminoethanol [379, 380]. This aminoalcohol is believed to be responsible for the undesired side-effects, since the free acid (71b) possesses the same antitumour activity without the unwanted action [380–383]. Clinical evaluation of (71b) is underway.

Compounds with similar structural arrangement may have other types of biological activity. Flavoxate (72) has been used as a drug with a strong smooth muscle relaxant activity in the urinary tract [384–388]. This compound is devoid of potential mutagenicity [389].

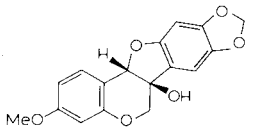
In contrast to the almost ubiquitous occurrence of the flavonoids in higher plants, the isoflavonoids have a very limited taxonomic distribution. They are in general confined to one group of plants – the sub-family Lotoideae of the Leguminosae [390, 391]. Occasionally they occur in a few other families [326]. The overall biological properties also differ from those of the flavonoids: flavonoids on the whole are innocuous substances [392], but isoflavonoids usually have oestrogenic, insecticidal, piscicidal and/or antifungal activities.

Isoflavones possessing the general structure (73) (genistein, 73a; prunetin, 73b; biochanin A, 73c; and formononetin, 73d) are weak oestrogens [393] and occasionally cause infertility problems in grazing animals [394, 395]. Phytoalexins such as pisatin (74), flemichapparin B (75a) and flemichapparin C (75b) constitute another type of isoflavonoids possessing antifungal activity [396–399]. Cajanone (76), an isoflavonone obtained from milled pigeon pea roots, possesses antifungal activity against *Fusarium oxysporum* [400]. Even simpler isoflavones, like genistein and luteone (77), demonstrate strong antifungal activity against *Cladosporium cucumerinum* and *C. herbarum* [401–403]. Accumulation of phytoalexins and other isoflavones is usually the result of plants responding to stress or infection by pathogens.

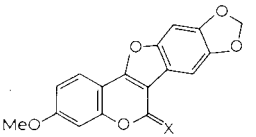
In addition to the flavone derivatives, some hydroxylated isoflavones, in-



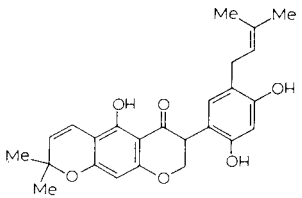
(73a) R, R' = H ; X = OH
(73b) R = Me ; R' = H ; X = OH
(73c) R = H ; R' = Me ; X = OH
(73d) R, R', X = H



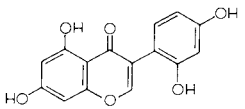
(74)



(75a) X = H_2
(75b) X = O



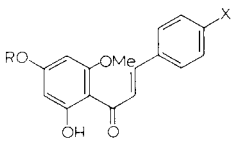
(76)



(77)

cluding genistein (73a) and prunetin (73b), also posess good antioxidant activity [404]. The antioxidant activity of these compounds may be related to the ease of formation of stabilized free radicals with *para*-quinonoid structures [404].

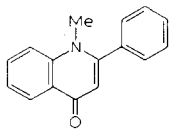
Chalcones, resembling the flavonoids, occur widely in green plants [326]. The presence of conjugated enone function confers its antibiotic (bacteriostatic, bactericidal) activity. Perhaps because of its bioprecursory relationship with both the flavonoids and the isoflavonoids, biological activities expressed by the chalcones encompass those of the two groups. Many substituted chalcones, therefore, are known for their inhibitory activity against microbes, parasites, and fungi, as well as having profound influence on the cardiovascular, cerebrovascular and neurovascular systems [326, 398, 405]. In addition, potent antimutagenic effect (100–700-times stronger than that of L-ascorbic acid) was uncovered in several alkoxylated chalcones (78) [406]. Chalcone (60) itself, which is not found in nature, possesses anti-inflammatory action [330, 407]. Inhibition of aldose reductase by chalcone derivatives has also been reported [342].



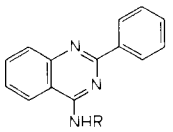
(78) R = Me, Et
X = OH, SMe

MISCELLANEOUS NITROGEN HETEROCYCLIC COMPOUNDS

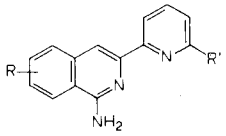
The aza analogue of flavone, 1-methyl-2-phenyl-4-quinolone (79), has repeatedly been reported to be isolated from *Balfourodendron riedelianum* [408], from the seeds of *Casimiroa edulis* [409], from the aerial parts of *Haplophyllum foliosum* [410], and from the stem barks of *Flindersia fournieri* [411]. Biological activity of a variety of nitrogen heterocyclic compounds has been indicated. 4-Amino-2-phenylquinazolines (80) possess bactericidal, anti-inflammatory, fungicidal, insecticidal and acaricidal action [412]. Antimycoplasmal activity was observed in 1-amino-3-(2-pyridyl)isoquinolones (81) [413]. Many 3-phenyl-substituted quinazolones, including methaqualone (82), are hyptonic, sedative, and anticonvulsive [414]. Rosoxacin (83), an isomeric quinolone derivative, has antibacterial activity [415]. 2-Phenylphthalazinones of type (84) are potent inhibitors of platelet aggregation and resulting in prevention of prostaglandin synthesis [416–418]. Ethidium bromide (85), a compound often used in DNA-intercalation studies [419–426], has antitrypanosomiasis activity [419, 420]. Even condensed nitrogen heterocyclic compounds such as monosubstituted benz[*a*]acridines exhibit biological activity: tetraphan (86) is analeptic [427, 428] and PAA-2056 (87) has antiamoebic activity [429, 430].



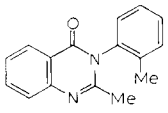
(79)



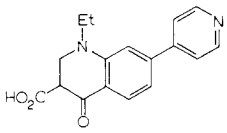
(80)



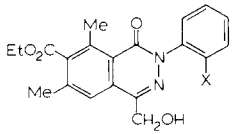
(81)



(82)

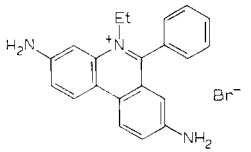


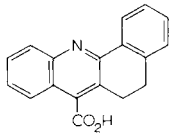
(83)

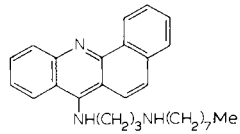


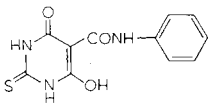
(84)

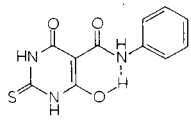
After some 1000 barbituric and thiobarbituric acids had been evaluated, the U.S. National Cancer Institute found a relatively simple thiobarbituric acid, merbarone (88), which showed exceptional antineoplastic activity [431]. It exhibits good inhibitory activity against leukaemias P388 and L1210, B16 melanoma, and M5076 sarcoma by either the i.p. or oral route [431, 432]. Merbarone induces DNA damage but does not inhibit RNA or protein synthesis [432]. The structure of merbarone could be postulated as assuming a

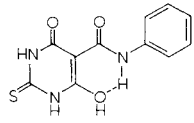

(85)


(86)


(87)

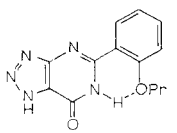

(88)

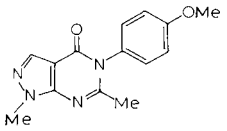

(88a)

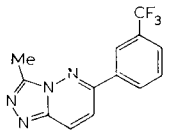

(88b)

'2-phenylnaphthalene'-type arrangement by hydrogen bond formation, shown as (88a) or (88b). The coplanar configuration has recently been verified [433]. It has been suggested [405, 406, 434] that certain uncyclized compounds with proper chain substituents, which 'imitate' biologically active ring structures, could behave as the cyclized compounds or even possess additional types of activity.

The '2-phenylnaphthalene'-type structural construction can also be surmised in the structurally related '2-phenylpurine'-type compounds for a reason which will be discussed later. Methylxanthines, such as caffeine and theophylline, inhibit a slow-reacting substance of anaphylaxis from passively sensitized human lung and human basophilic leucocytes. However, the methylxanthines are much less potent than the commonly used antiallergic drugs such as disodium cromoglycate (DSCG). Progressive structural modification resulted in the synthesis of several 2-phenyl-8-azapurine-6-ones which possess high and selective antiallergic activity [435–437]. The best compounds are those containing *ortho*-substituted phenyl groups which are capable of forming hydrogen bond to the N(1)-H of the purine ring [438]. 2-(*o*-Propoxyphenyl)-8-azapurin-6-one (89) is not only orally active but is 20–50-times more active than DSCG [431–434]. Other examples of compounds of this type include a pyrazolo[3,4-*d*]pyrimidine (90), which is active against lymphocytic leukaemia in mice [439]. The 4-methoxy group in the phenyl ring is a condition of the anticancer activity [439, 440]. A phenyl-substituted triazolopyridazine (91) has sedative and hypnotic activity [441, 442].


(89)

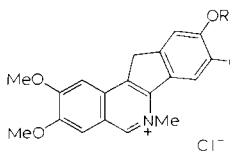

(90)


(91)

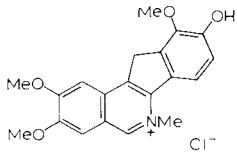
THE IMPORTANCE OF RING COPLANARITY TO ANTINEOPLASTIC ACTION

A generalization can now be made from the preceding examples of biologically active compounds. Although compounds possessing the '2-phenylnaphthalene'-type structural pattern exhibit a variety of biological activities, most antineoplastic compounds described contain a coplanar conformation. The coplanarity between the two ring units can be assured either through a condensed ring structure, as in coralyne, nitidine, fagaronine, chartreusin and related antibiotics, the gilvocarcins, the isotetracene antibiotics, the pluramycin-group antibiotics, ellipticine, camptothecin and acronycine; or through hydrogen-bond formation between the two ring units, as in streptonigrin and lavendamycin. Compounds containing a free rotating 'phenyl' unit may or may not assume the coplanar conformation, and thus may or may not possess the antineoplastic activity. Examples in the former case include DuP-785 and flavone-8-acetic acid, whereas ethidium bromide and many other compounds belong to the latter case, in spite of the fact that some are potent DNA intercalators.

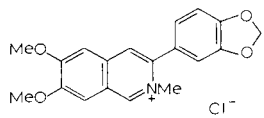
The importance of coplanarity to the antineoplastic activity of compounds of this type can be further illustrated by the following investigation. In a structure–activity relationship study on nitidine (6a), fagaronine (6b) and related benzophenanthridines, the indenoisoquinoline analogues ((92a) and (92b), respectively) of both alkaloids, a position isomer (93) resembling coralyne (3), and a free rotating compound (94) were synthesized and evaluated against leukaemia P388 [443, 444]. It was found that compounds (92a), (92b) and (93) retained the original activity, but compound (93) was not active [443–445]. These investigators also discussed the biological activity relationship between coralyne (3) and related protoberberine alkaloids in terms of structural planarity [446].



(92a) R + R' = CH_2
(92b) R = H, R' = Me



(93)

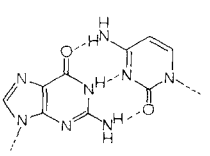


(94)

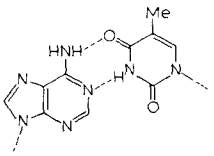
PATTERN RESEMBLANCE TO PERTINENT BIOMOLECULES

The repeated occurrences of the '2-phenylnaphthalene'-type molecular pattern among such a huge number of otherwise structurally unrelated natural and synthetic compounds with diverse biological activities suggest that this characteristic molecular arrangement could be intimately related to the structures of some biologically pertinent molecules which are of importance to the processes of life. Molecules containing such a pattern, together with the contribution of proper substituents and functional groups, may either mimic, modify or interfere with the ongoing biological events, and thus exhibit the observed desired or undesired biological and pharmacological properties.

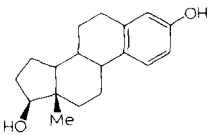
Two groups of biopertinent molecules fulfil the proposed requirements. The purine-pyrimidine (e.g., cytosine-guanine and adenine-thymine) base-pairs of the nucleic acids (represented as (95) and (96), respectively) and the steroid hormones (represented by oestradiol (97), an oestrogen; progesterone (98), a 'pregnancy hormone'; and testosterone (99), an androgen). The steroid hormones are depicted in an upside-down manner compared with the conventional drawings.



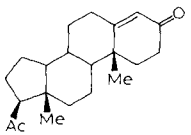
(95)



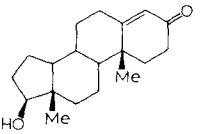
(96)



(97)



(98)



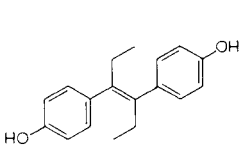
(99)

The similarity in molecular shapes among cytosine-guanine, progesterone, DMBA, and benzo[*a*]pyrene was illustrated by means of molecular models in 1962 [447]. Earlier, while dealing with alkylating agents and carcinogenic hydrocarbons, under a title of 'New Facts and Concepts: A General Survey', a prophetic statement was presented in a cancer conference as follows [448]: "... whether there is any significant association between the molecular planarity, which is a feature of the carcinogenic hydrocarbons (and the absence of which apparently entails biological inactivity), and the flatness of the

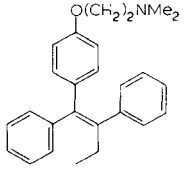
nucleotide plates. The purine: pyrimidine bonded pairs of the Crick–Watson model present planar structures of the same general order of size as the hydrocarbons, and it is a question whether these thoughts should not be further pursued, from the aspects of theoretical physics, reactivity, and biological incorporation, ...".

Many of the compounds mentioned in this search, particularly those related to cancer – carcinogens, antineoplastic agents and prophylactic anticarcinogens – are either planar or can assume the coplanar conformation at the '2-phenylnaphthalene' portion of the molecule. The structural resemblance between these compounds and the nucleic acid base-pairs may indicate that enzymes controlling the topological state of DNA (DNA topoisomerases [449–452]), which are important to the process of DNA replication and genetic recombination, could well be reasonable targets for these molecules. A number of recent publications [453–466] have already reported that many antineoplastic agents, including ellipticine and camptothecin, indeed interfere with the function of DNA topoisomerases.

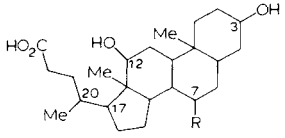
As early as 1932 it was noted that steroids and some carcinogenic polycyclic hydrocarbons had similar shapes and dimensions [467–469]. Although the steroid molecules are not altogether planar, the overall conformation of the steroids is closer to the planar structure [470]. Besides, many biomedically useful hormonal therapeutic agents, such as diethylstilbestrol (DES, 100), the antioestrogenic antineoplastic drugs, such as tamoxifen (101), or the previously mentioned flavones (inhibit the aromatization of androstenedione and testosterone to the oestrogens) and isoflavones (oestrogenic), could assume planar conformation and also possess the uncyclized '2-phenylnaphthalene' structural



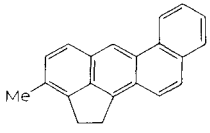
(100)



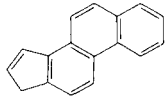
(101)



(102a) R = OH
(102b) R = H



(103)



(104)

pattern. Formation of the carcinogenic 3-methylcholanthrene (103) from cholic acid (102a) or 7-deoxycholic acid (102b) was reported more than 50 years ago [469, 471–474]. Correlation and interaction between carcinogenicity and the chemical structure in derivatives of cyclopenta[*a*]phenanthrene (104, an aromatized steroid) [475, 476], as well as potentiation of steroid binding to proteins by DMBA [477], were also reported.

In a study of the relationship of polynuclear aromatic hydrocarbons, steroids and carcinogenesis, it was noticed that there is usually a direct increase in carcinogenicity when the hydrocarbons become structurally more similar to steroids [478]. These investigators thus postulated that the polynuclear aromatic hydrocarbons may act at the same biological sites as steroid hormones [478]. The target tissue for oestrogens (oestrogen receptors) in rats was found to be located in the uterus, the ovaries and the mammary glands, but not in the small intestine or blood serum [479–482]. It is therefore conceivable to find that administration of carcinogenic polynuclear hydrocarbons such as benzo[*a*]pyrene, 2-hydroxybenzo[*a*]pyrene, methylcholanthrene and DMBA resulted in the tumour formation not at the site of local application but in the breast, the ovaries or the uterus in the rat [447, 483]. Tumour induction by oestrogens in female genital organs has also been reported [484–486].

A number of publications reporting the competitive binding of polynuclear aromatic hydrocarbons appeared in the literature [487–490]. It is of interest to note that 3,9-dihydroxybenz[*a*]anthracene, a potential metabolite of benz[*a*]anthracene, has both oestrogenic and antioestrogenic properties [487, 488, 490]. The effect of DMBA and hormones on neoplasia was adequately discussed [491].

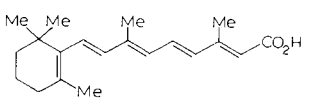
In contrast to the high degree of specificity of oestrogen receptors, the receptors for androgens are not as well-defined. Nevertheless, a paper published in London more than 200 years ago [492] reported that a high incidence of cancer of the scrotum and testicles was detected among chimney-sweeps. This disease could be associated with the chronic contact with soot. It is well known that the androgen, testosterone, is formed and accumulated mainly in the testes.

AN APPROACH TO THE TOTAL DRUG DESIGN

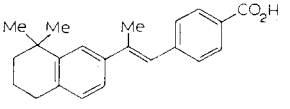
The preceding information clearly suggests that compounds containing the '2-phenylnaphthalene'-type ring systems as the nucleus *with proper substituents attached to both ring units* should be explored with synthesis and biological evaluation. These ring systems could either be carbocyclic or heterocyclic, with

nitrogen, oxygen and/or sulphur atoms placed at selected positions. For the design of antineoplastic agents, the coplanarity of two ring units may be achieved either by the creation of connecting (condensed) ring systems (for example, coralyne) or by introducing the hydrogen bond formation between the *ortho*-substituted hydroxy or amino groups on one ring unit and the electronegative atoms in the other ring system (for example, streptonigrin). In the selection of proper ring systems and side-chain substituents, factors including electron distribution, partition coefficient, physical characteristics and chemical reactivities should definitely be considered. Reasonable ring skeletal modifications, such as using the '5-phenylindene'-type (or '2-phenylpurine'-type, for example) in place of the '2-phenylnaphthalene'-type nucleus, should also be of interest. After all, the fact that both the DNA base-pair and the steroids contain a 5-membered ring unit at equivalent positions may not be purely coincidental.

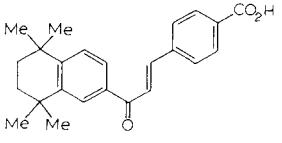
It should be emphasized that the postulated structural pattern is only provided as a necessary, but not sufficient, fundamental condition for designing compounds of this type. There are many other types of antineoplastic agent with different chemical structures which do not belong to this series at all. On the other hand, the characteristic '2-phenylnaphthalene' pattern can be found even in many biologically active compounds containing mainly chain structures. For example, the structure of retinoic acid is conventionally drawn as (105). Its characteristic pattern can either be visualized through an atomic model or be recognized from compounds (106) [493], (107) [494], and (108) [495]. All these compounds demonstrated potent and often stronger biological activity (growth inhibition and induction of differentiation) than retinoic acid.



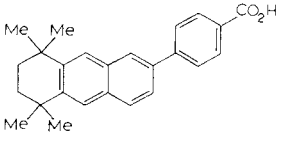
(105)



(106)



(107)



(108)

ACKNOWLEDGEMENTS

The author wishes to thank Miss Audrey Y. Cheng for examination of this manuscript and to Mrs. Van N. Dang for typing this manuscript.

REFERENCES

1 A.A. Loeser, Acta Unio Int. Cancrum, 4 (1939) 375.
2 P. Ulrich, Acta Unio Int. Cancrum, 4 (1939) 377.
3 C. Huggins and C.V. Hodges, Cancer Res., 1 (1941) 293.
4 C. Huggins, J. Am. Med. Assoc., 131 (1946) 576.
5 C.P. Rhoads, J. Mt. Sinai Hosp. (NY), 13 (1947) 299.
6 L.S. Goodman, M.M. Wintrobe, W. Damashek, M.J. Goodman, A. Gilman and M.T. McLennan, J. Am. Med. Assoc., 132 (1946) 126.
7 A. Haddow, G.A.R. Kon and W.C.J. Ross, Nature (London), 162 (1948) 824.
8 S. Farber, L.K. Diamond, R.P. Mercer, R.F. Sylvester and J.A. Wolff, N. Engl. J. Med., 238 (1948) 787.
9 D.R. Seeger, D.B. Cosulich, J.M. Smith, Jr. and M.E. Hultaquist, J. Am. Chem. Soc., 71 (1949) 1753.
10 M.C. Li, R. Hertz and D.B. Spencer, Proc. Soc. Exp. Biol. Med., 93 (1956) 361.
11 R.B. Livingston and S.K. Carter, Single Agents in Cancer Chemotherapy, (IFI/Plenum, New York, 1970).
12 A.G. Sartorelli and D.G. Johns, Antineoplastic and Immunosuppressive Agents (Springer-Verlag, Berlin, 1975).
13 S.K. Carter, M.T. Bakowski and K. Hellmann, Chemotherapy of Cancer, (John Wiley, New York, 2nd Edn., 1981).
14 S.K. Carter, E. Glatstein and R.B. Livingston, Principles of Cancer Treatment (McGraw-Hill, New York, 1982).
15 V.T. DeVita, Jr., S. Hellmann and S.A. Rosenberg, Cancer: Principles and Practice of Oncology (Lippincott, Philadelphia, 1985).
16 I.H. Krakoff, Ca-A Cancer J. Clin., 37 (1987) 93.
17 K.D. Collins and G.R. Stark, J. Biol. Chem., 246 (1971) 6599.
18 B. Ardalan, R.J. Glaser, T.W. Kensler, H.N. Jayaram, V.P. Tu, J.S. MacDonard and D.A. Cooney, Biochem. Pharmacol., 30 (1981) 2045.
19 P. Kafarski, B. Lejczak, P. Mastalerz, D. Duś and C. Radzikowski, J. Med. Chem., 28 (1985) 1555.
20 B.R. Baker, Design of Active-Site-Directed Irreversible Enzyme Inhibitors (John Wiley, New York, 1967).
21 Y.-C. Cheng, B. Goz, and Minkoff, eds., Development of Target-Oriented Anticancer Drugs (Raven Press, New York, 1988).
22 E.P. Goldberg, ed., Targeted Drugs (John Wiley, New York, 1983).
23 C. Hansch and T. Fujita, J. Am. Chem. Soc., 86 (1964) 1616.
24 S.M. Free and J.W. Wilson, J. Med. Chem., 7 (1964) 395.
25 W.P. Purcell, G.E. Bass and J.M. Clayton, eds., Strategy of Drug Design (John Wiley, New York, 1973).
26 R.F. Rekker, in Structure-Activity Relationships of Anti-tumor Agents, eds. D.N. Reinhoudt, T.A. Connors, H.M. Pinedo and K.W. van de Poll (Martinus Nijhoff Publishers, The Hague, 1983), p. 23.
27 N. Trinajstić, A. Sabljić and S. Nikolić, eds. Acta Pharmac. Jugoslavica, 36 (1986) 79–280 (Special Subject Issue on QSAR in Drug Research).
28 A.J. Hopfinger, J. Med. Chem., 28 (1985) 1133.
29 K.Y. Zee-Cheng and C.C. Cheng, J. Pharm. Sci., 59 (1970) 1630.
30 C.C. Cheng and R.K.Y. Zee-Cheng, Heterocycles, 15 (1981) 1275.

31 C.C. Cheng, J. Pharm. Sci., 60 (1971) 1596.
32 C.C. Cheng, J. Pharm. Sci., 63 (1974) 307.
33 C.C. Cheng, J. Theor. Biol., 59 (1976) 497.
34 G.H. Loew and R. Sahakina, J. Med. Chem., 20 (1977) 103.
35 A. Rosowsky, M. Chaykovsky, S.A. Yeager, R.A. St. Amand, M. Lin and E.J. Modest, J. Heterocycl. Chem., 8 (1971) 809.
36 L.B. Kier, J. Pharm. Pharmacol., 21 (1969) 93.
37 B. Pullman, J.-L. Coubeils, P. Courrière and J.-P. Gervois, J. Med. Chem., 15 (1972) 17.
38 D.C. Warhurst and S.C. Thomas, Trans. R. Soc. Trop. Med. Hyg. 67 (1973) 15.
39 P.-L. Chien, D.J. McCaustland, W.H. Burton and C.C. Cheng, J. Med. Chem., 15 (1972) 28.
40 P.-L. Chien and C.C. Cheng, J. Med. Chem., 16 (1973) 1093; 19 (1976) 170.
41 B.P. Das, R.T. Cunningham and D.W. Boykin, Jr., J. Med. Chem., 16 (1973) 1361.
42 J.W. Panter, D.W. Boykin, Jr. and W.D. Wilson, J. Med. Chem., 16 (1973) 1366.
43 D.J. McCaustland, P.-L. Chien, C.C. Cheng, J. Novotny, W.L. Schreiner and F.W. Starks, J. Med. Chem., 16 (1973) 1311.
44 L.-Q. Li, Z.-X. Qu, Z.-M. Wang, Y.-L. Zeng, G.-S. Ding, G.-J. Hu and X.-Y. Yang, Scienta Sinica, 22 (1979) 1220.
45 R.K.-Y. Zee-Cheng and C.C. Cheng, J. Med. Chem., 21 (1978) 291.
46 R.H. Adamson, Cancer Chemother. Rep., 58 (1974) 293.
47 C.C. Cheng, G. Zbinden and R.K.-Y. Zee-Cheng, J. Pharm. Sci., 68 (1979) 393.
48 C.C. Cheng, R.K.-Y. Zee-Cheng, V.L. Narayanan, R.B. Ing. and K.D. Paull, Trends Pharmacol. Sci., 2 (1981) 223.
49 R.K.-Y. Zee-Cheng and C.C. Cheng, Drugs Future, 8 (1983) 229.
50 C.C. Cheng and R.K.-Y. Zee-Cheng, Prog. Med. Chem., 20 (1983) 83.
51 C.C. Cheng, Med. Hypotheses, 20 (1986) 157.
52 P.W. Joffs, in The Alkaloids, Chemistry and Physiology, ed. R.H.F. Manske, Vol. IX (Academic Press, New York, 1967), p. 41.
53 M. Shamma, The Isoquinoline Alkaloids, Chemistry and Pharmacology, (Academic Press, New York, 1972), p. 269.
54 R.L. Varma, Indian Med. Gazz., 62 (1927) 84.
55 O. Stickl, Z. Hyg. Infektionstr., 108 (1928) 567.
56 T.V. Subbaiah and A.H. Amin, Nature (London), 215 (1967) 527.
57 A.H. Amin, T.V. Subbaiah and K.M. Abbasi, Can. J. Microbiol., 15 (1969) 1067.
58 S.M. Shanbhag, H.J. Kulkarni and B.B. Gaitonde, Jpn J. Pharmacol., 20 (1970) 482.
59 M. Sabiv and N.K. Bhide, Indian J. Physiol. Pharmacol., 15 (1971) 111.
60 F.E. Hahn and J. Ciak, in Antibiotics, eds. D. Gottlieb, P.D. Shaw and J.W. Corcoran. (Springer-Verlag, New York), Vol. 3 (1975), p. 577.
61 H. Fukuda, K. Watanabe and Y. Kudo, Chem. Pharm. Bull., 18 (1970) 1299.
62 S. Pavelka and J. Kovar, Collect. Czech. Chem. Commun., 40 (1975) 753.
63 S. Naruto and H. Kaneko, Yakugaku Zasshi, 92 (1972) 1017.
64 J. Iwasa, S. Narito, K. Nakamura and Y. Shoji, Japan 70-04992 (1970). Chem. Abstr., 72 (1970) 111 667d.
65 N. Nakanishi, Jpn J. Pharmacol., 12 (1962) 208.
66 S. Sasagawa, K. Kametani and S. Kiyofugi, Chem. Pharm. Bull., 17 (1969) 1.
67 W. Schneider and K. Schroeder, Ber., 53B (1920) 1459.
68 W. Awe, J. Thum and H. Wichmann, Arch. Pharm. (Weinheim, Ger.), 293 (1960) 907.
69 W. Awe, H. Halpaap and O. Hertel, Arzneim.-Forsch., 10 (1960) 936.

70 W. Wiegrebe, Angew. Chem., 78 (1966) 647; Dtsch Apoth.-Ztg., 106 (1966) 1546; Arch. Pharm. (Weinheim, Ger.), 301 (1968) 25.
71 K.-Y. Zee-Cheng and C.C. Cheng, J. Pharm. Sci., 61 (1972) 969; 62 (1973) 1572.
72 K.Y. Zee-Cheng, K.D. Paull and C.C. Cheng, J. Med. Chem., 17 (1974) 347.
73 M.J. Cho, A.J. Repta, C.C. Cheng, K.Y. Zee-Cheng, T. Higuchi and I.H. Pitman, J. Pharm. Sci., 64 (1975) 1825.
74 M. Shamma and J.L. Moniot, Isoquinoline Alkaloids Research, 1972–1977 (Plenum Press, New York, 1978), p. 250.
75 C.C. Cheng, R.R. Engle, J.R. Hudson, R.B. Ing, H.B. Wood, S.J. Yan and R.K.Y. Zee-Cheng, J. Pharm. Sci., 66 (1977) 1781.
76 S.A.E. Hakim, V. Mijović and J. Walker, Nature (London), 189 (1961) 201.
77 M. Onda, K. Abe and K. Yonezawa, Chem. Pharm. Bull., 16 (1968) 2005.
78 S.A. Vichkanova and V.V. Adgina, Antibiotiki, 16 (1971) 609.
79 M.E. Wall, M.C. Wani and H.L. Taylor, 162nd American Chemical Society Meeting, Washington, DC (1971) MEDI-34; J. Nat. Prod., 50 (1987) 1095.
80 W.M. Messmer, M. Tin-Wa, H.H.S. Fong, C. Bevelle, N.R. Farnsworth, D.J. Abraham and J. Trojánek, J. Pharm. Sci., 61 (1972) 1858.
81 K.Y. Zee-Cheng and C.C. Cheng, J. Heterocycl. Chem., 10 (1973) 85, 867; J. Med. Chem., 18 (1975) 66.
82 F.R. Stermitz, K.A. Larson and D.K. Kim, J. Med. Chem., 16 (1973) 939.
83 M. Tin-Wa, C.L. Bell, C. Bevelle, H.H.S. Fong and N.R. Farnsworth, J. Pharm. Sci., 63 (1974) 1476.
84 F.R. Stermitz, J.P. Gillespie, L.G. Amoros, R. Romero, T.A. Stermitz, K.A. Larson, S. Earl and J.E. Ogg, J. Med. Chem., 18 (1975) 708.
85 Z.-X. Huang and Z.-H. Li, Acta Chim. Sinica, 38 (1980) 535.
86 C.E. Dunlap and S. Warren, Cancer Res., 3 (1943) 606.
87 D. Hoffmann, W.E. Bondinell and E.L. Wynder, Science (Washington, DC), 183 (1974) 215.
88 S.S. Hecht, W.E. Bondinell and D. Hoffmann, J. Natl. Cancer Inst., 53 (1974) 1121.
89 S.S. Hecht, M. Loy, R.R. Maronpot and D. Hoffmann, Cancer Lett., 1 (1976) 147.
90 S. Amin, K. Huie, A.A. Melikian, J.M. Leszczynska and S.S. Hecht, Cancer Res., 45 (1985) 6406.
91 G. Obe, K. Sperling and H.J. Belitz, Angew Chem., Int. Ed. Engl., 10 (1971) 302.
92 J.F. Fraumeni and R.W. Miller, J. Natl. Cancer Inst., 48 (1972) 1267.
93 G.D. Stoner, M.B. Shimkin, A.J. Kniazeff, J.H. Weisburger, E.K. Weisburger and G.B. Gori, Cancer Res., 33 (1973) 3069.
94 S.M. Sieber and R.H. Adamson, in Pharmacological Basis of Cancer Chemotherapy, T.L. Loo, Symp. Chairman (Williams and Wilkins, Baltimore, 1975), p. 401.
95 D.D. Beal, J.L. Skibba, W.A. Croft, S.M. Cohen and G.T. Bryan, J. Natl. Cancer Inst., 54 (1975) 951.
96 J.P. Seiler, Chimia, 29 (1975) 8.
97 C.C. Harris, Cancer, 37 (1976) 1014.
98 R.W. Miller, J. Natl. Cancer Inst., 58 (1977) 471.
99 B.N. Ames, J. McCann and E. Yamasaki, Mutation Res., 31 (1975) 347.
100 J. McCann, E. Choi, E. Yamasaki and B.N. Ames, Proc. Natl. Acad. Sci. U.S.A., 72 (1975) 5135.
101 J. McCann and B.N. Ames, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 950.
102 G. Obe, K. Sperling and H.J. Belitz, Angew. Chem., 83 (1971) 301.

103 J.P. Seiler, Chimia, 29 (1975) 8.
104 E. Huberman and L. Sachs, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 188.
105 B.A. Bridges, Nature (London), 261 (1976) 195.
106 J. McCann, Chem. Technol., (1976) 682.
107 Survey of compounds which have been tested for carcinogenic activity. U.S. Department of Health and Human Services (formerly U.S. Department of Health, Education, and Welfare, indicated in earlier volumes). Eighteen volumes between 1951 and 1984.
108 J.W. Cook, I. Hieger, E.L. Kennaway and W.V. Mayneord, R. Soc. Proc. 111B (1932) 455.
109 A. Pullman and B. Pullman, Adv. Cancer Res., 3 (1955) 117.
110 Q.H. Dai, Sci. Sin. (Engl. Ed.), 23 (1980) 453.
111 L.V. Szentpály, J. Am. Chem. Soc., 106 (1984) 6021.
112 J.P. Lowe and B.D. Silverman, Acc. Chem. Res., 17 (1984) 332.
113 G.M. Blackburn and B. Kellard, Chem. Ind., (1986) 687.
113a J.A. Beisler, Prog. Med. Chem., 19 (1982) 247.
114 B.E. Leach, K.M. Calhoun, L.E. Johnson, C.M. Teeters, and W.G. Jackson, J. Am. Chem. Soc. 75 (1953) 4011.
115 J. Berger, L.H. Sternbach, R.G. Pollock, E.R. LaSalla, S. Kaiser and M.W. Goldberg, J. Am. Chem. Soc., 80 (1958) 1636.
116 L.H. Sternbach, S. Kaiser and M.W. Goldberg, J. Am. Chem. Soc., 80 (1958)1639.
117 E. Simonitsch, W. Eisenhuth, O.A. Stamm and H. Schmid, Helv. Chim. Acta, 47 (1964) 1459.
118 W. Eisenhuth, O.A. Stamm and H. Schmidt, Helv. Chim. Acta, 47 (1964) 1475.
119 T.R. Kelly, J.A. Magee and F.R. Weibel, J. Am. Chem. Soc., 102 (1980) 798.
120 F.M. Hauser and D.W. Combs, J. Org. Chem., 45 (1980) 4071.
121 J.P. McGovren, G.L. Neil, S.L. Crampton, M.I. Robinson and J.D. Douros, Cancer Res., 37 (1977) 1666.
122 W.C. Krueger, L.M. Pschigoda and A. Moscowitz, J. Antibiot., 39 (1986) 1298.
123 M. Konishi, K. Sugawara, F. Kofu, Y. Nishiyama, K. Tomita, T. Miyaki and H. Kawaguchi, J. Antibiot., 39 (1986) 784.
124 K. Sagawara, M. Tsunakawa, M. Konishi, H. Kawaguchi, B. Krishnan, C.H. He and J. Clardy, J. Org. Chem., 52 (1987) 996.
125 R.K. Elespuru and S.K. Gonda, Science (Washington, DC), 223 (1983) 69.
126 K. Takahashi, M. Yoshida, F. Tomita and K. Shirahata, J. Antibiot., 34 (1981) 271.
127 H. Nakano, Y. Matsuda, K. Ito, S. Ohkubo, M. Morimoto and F. Tomita, J. Antibiot., 34 (1981) 266.
128 M. Morimoto, S. Okubo, F. Tomita and H. Marumo, J. Antibiot., 34 (1981) 701.
129 K. Hatano, E. Higashide, M. Shibata, Y. Kameda, S. Horii and K. Mizuno, Agr. Biol. Chem., 44 (1980) 1157.
130 S. Horii, H. Fukase, E. Mizuta, K. Hatano and K. Mizuno, Chem. Pharm. Bull., 28 (1980) 3601.
131 F. Strelitz, F. Flon and I.N. Ashehov, J. Bacteriol., 69 (1955) 280.
132 T.T. Wei, J.A. Chan, P.P. Roller, U. Weiss, R.M. Stroshane, R.J. White and K.M. Bryne, J. Antibiot., 35 (1982) 529.
133 T.C. Jain, G.C. Simolike and L.M. Jackman, Tetrahedron, 39 (1983) 599.
134 U. Weiss, K. Yoshihira, R.J. Highet, R.J. White and T.T. Wei, J. Antibiot., 35 (1982) 1194.
135 K. Takahashi and F. Tomita, J. Antibiot., 36 (1983) 1531.
136 G.T. Carter, A.A. Fantini, J.C. James, D.B. Borders and R.J. White, Tetrahedron Lett., 25 (1984) 255; J. Antibiot., 38 (1985) 242.

137 K.M. Byrne and M. Greenstein, J. Antibiot., 39 (1986) 594.
138 R. Misra, H.R. Tritch, III and R.C. Pandey, J. Antibiot., 38 (1985) 1280.
139 F. Tomita, K. Takahashi and T. Tamaoki, J. Antibiot., 35 (1982) 1038.
140 S.N. Sehgal, H. Czerkawski, A. Kudelski, K. Pandev, R. Saucier and C. Vézina, J. Antibiot., 36 (1983) 355.
141 J.A. Findlay, J.S. Liu, L. Radics and S. Rakhit, Can. J. Chem., 59 (1981) 3018.
142 J.A. Findlay, J.S. Liu and L. Radics, Can. J. Chem., 61 (1983) 323.
143 K. Singh, J. Antibiot., 37 (1984) 71.
144 M. Greenstein, T. Monji, R. Yeung, W.M. Maiese and R.J. White, Antimicrob. Agents Chemother. 29 (1986) 861.
145 S. Rakhit, C. Eng, H. Baker and K. Singh, J. Antibiot., 36 (1983) 1490.
146 Y. Okami, J. Nat. Prod., 42 (1979) 583.
147 N. Imamura, K. Kakinuma, N. Ikekawa, H. Tanaka and S. Ōmura, J. Antibiot., 35 (1982) 602.
148 D.W. Cameron, G.I. Feutrill, G.L. Gibson and R.W. Read, Tetrahedron Lett. 26 (1985) 3887.
149 (a) Y. Hayakawa, T. Iwakiri, K. Imamura, H. Seto and N. Ōtake, J. Antibiot., 38 (1985) 957. (b) Y. Hayakawa, K. Furahata, H. Seto, and N. Ōtake, Tetrahedron Lett., 26 (1985) 3471.
150 M. Sezaki, T. Hara, S. Ayukawa, T. Takeuchi, Y. Okami, M. Hamada, T. Nagatsu and H. Umezawa, J. Antibiot., 21 (1968) 91.
151 S. Ayukawa, T. Takeuchi, M. Sezaki, T. Hara, H. Umezawa and T. Nagatsu, J. Antibiot., 21 (1968) 324.
152 M.Sezaki, S. Kondo, K. Maeda, H. Umezawa and M. Ohno, Tetrahedron, 26 (1970) 5171.
153 S. Ōmura, H. Tanaka, R. Ōiwa, J. Awaja, R. Masuma and K. Tanaka, J. Antibiot., 30 (1977) 908.
154 K. Okta and K. Kamiya, J. Chem. Soc., Chem. Commun., (1981) 154.
155 N. Imamura, K. Kakinuma, N. Ikekawa, H. Taneka and S. Ōmura, Chem. Pharm. Bull., 29 (1981) 1788.
156 K. Ohta, H. Okazaki and T. Kishi, Chem. Pharm. Bull., 30 (1982) 762.
157 H. Irie, Y. Mizuno, I. Kouno, T. Nagasawa, Y. Tani, H. Yamada, T. Taga and K. Osagi, J. Chem. Soc. Chem. Commun., (1983) 174.
158 Y. Hayakawa, T. Iwakiri, K. Imamura, H. Seto and N. Ōtake, J. Antibiot., 38 (1985) 960.
159 Y. Hayakawa, K. Furihata, H. Seto and N. Ōtake, Tetrahedron Lett., 26 (1985) 3475.
160 T. Uchida, M. Imoto, Y. Watanabe, K. Miura, T. Dobashi, N. Matsuda, T. Sawa, H. Naganawa, M. Hamada, T. Takeuchi and H. Umezawa, J. Antibiot., 38 (1985) 1171.
161 H. Drantz, H. Zähner, J. Rohr and A. Zeeck, J. Antibiot., 39 (1986) 1657.
162 M.P. Kuntsmann and L.A. Mitscher, J. Org. Chem., 31 (1966) 2920.
163 P.M. Brown and R.H. Thomson, J. Chem. Soc., Perkin Trans. 1, (1976) 997.
164 W.-C. Liu, W.L. Parker, D.S. Slusarchyk, G.L. Greenwood, S.F. Graham and E. Meyers, J. Antibiot., 23 (1970) 437.
165 R.J. Theriault, R.R. Rasmussen, W.L. Kohl, J.F. Prokop, T.B. Hutch and G.J. Barlow, J. Antibiot., 39 (1986) 1509.
166 R.R. Rasmussen, M.E. Nuss, M.H. Scherr, S.L. Mueller, J.B. McAlpine and L.A. Mitscher, J. Antibiot., 39 (1986) 1515.
167 R.W. Rickards and J.-P. Wu, J. Antibiot., 38 (1985) 513.
168 J.H. Wilton, D.C. Cheney, G.C. Hokanson, J.C. French, C.-H. He and J. Clardy, J. Org. Chem., 50 (1985) 3936.

169 E.F. Rogers (Merck), U.S. Patent 3,347,742 (1967); Chem. Abstr. 68 (1968) 6167.
170 H. Ikushima, E. Iguchi, M. Kohsaka, H. Aoki and H. Imanaka, J. Antibiot., 33 (1980) 1103.
171 H. Ikushima, M. Okamoto, H. Tanaka, O. Ohe, M. Kohsaka, H. Aoki and H. Imanaka, J. Antibiot., 33 (1980) 1107.
172 H. Tanaka, Y. Itoh, H. Ikushima, M. Okamoto, Y. Kawai and H. Imanaka, Tetrahedron Lett., 21 (1980) 4359.
173 K. Maeda, T. Takeuchi, K. Nitta, K. Yagishita, R. Utahara, T. Ōsato, M. Ueda, S. Kondō, Y. Okami and H. Umezawa, J. Antibiot., 9A (1956) 75.
174 S. Kondo, T. Wakashiro, M. Hamada, K. Maeda, T. Takeuchi and H. Umezawa, J. Antibiot., 23 (1970) 354.
175 H. Schmitz, K.E. Crook, Jr. and J.A. Bush, Antimicrob. Agents Chemother., 1966, (1967) 606.
176 U. Séquin, Tetrahedron, 34 (1978) 761.
177 A. Fredenhagen, W. Ritter, U. Séquin and M. Zehnder, Chimia, 35 (1981) 334.
178 A. Fredenhagen and U. Séquin, J. Antibiot., 38 (1985) 236.
179 A. Fredenhagen and U. Séquin, Helv. Chim. Acta, 68 (1985) 391.
180 N. Kanda, J. Antibiot., 24 (1971) 599.
181 M. Furukawa, I. Hayakawa, G. Ohta and Y. Iitaka, Tetrahedron, 31 (1975) 2989.
182 K. Dornberger, U. Berger and H. Knöll, J. Antibiot., 33 (1980) 1.
183 S.K. Gonda, K.M. Byrne, P.K. Herber, Y. Tondeur, D. Liberato and B.D. Hitton, J. Antibiot., 37 (1984) 1344.
184 H. Nadig, U. Séquin, R.H. Bunge, T.R. Hurley, D.B. Murphey and J.C. French, Helv. Chim. Acta, 68 (1985) 953.
185 J. Itoh, T. Tsuyuki, K. Fujita and M. Sezaki, J. Antibiot., 39 (1986) 780.
186 Y. Kawai, K. Furihata, H. Seto and N. Otake, Tetrahedron Lett., 26 (1985) 3273.
187 K.V. Rao and W.P. Cullen, Antibiot. Annu. 1959–1960, (1960) 950.
188 W.S. Marsh, A.L. Garretson and E.M. Wesel, Antibiot. Chemother., 11 (1961) 151.
189 J.J. Oleson, L.A. Calderella, K.J. Mjos, A.R. Reith, R.S. Thie and I. Toplin, Antibiot. Chemother., 11 (1961) 158.
190 M.N. Teller, S.F. Wagshul and G.W. Woolley, Antibiot. Chemother., 11 (1961) 165.
191 H.C. Reilly and K. Sugiura, Antibiot. Chemother., 11 (1961) 174.
192 W.L. Wilson, C. Labba and E. Barrist, Antibiot. Chemother., 11 (1961) 147.
193 C.A. Hackethal, R.B. Golbey, C.T.C. Tan, D.A. Karnofsky and J.H. Burchenal, Antibiot. Chemother., 11 (1961) 178.
194 E.W. Humphrey and F.S. Dietrich, Cancer Chemother. Rep., 33 (1963) 21.
195 R.D. Sullivan, E. Miller, W.Z. Zurek and F.R. Rodriguez, Cancer Chemother. Rep., 33 (1963) 27.
196 S.L. Rivers, R.M. Whittington and T.J. Medrek, Cancer Chemother. Rep., 46 (1965) 17; Cancer, 19 (1966) 1377.
197 M.N. Harris, T.J. Medrek, F.M. Golomb, S.L. Gumport, A.H. Postel and J.C. Wright, Cancer, 18 (1965) 49.
198 (a) D.T. Kaung, R.M. Whittington, H.H. Spencer and M.E. Patno, Cancer, 23 (1969) 597; 1280. (b) H.L. Davis, D.D. Von Hoff, J.T. Henney and M. Rozenzweig, Cancer Chemother. Pharmacol., 1 (1978) 83.
199 K.V. Rao, K. Biemann and R.B. Woodward, J. Am. Chem. Soc., 85 (1963) 2532.
200 Y.-Y.H. Chiu and W.N. Lipscomb, J. Am. Chem. Soc., 97 (1975) 2525.
201 Société des usines chimiques Rhône-Poulenc, Br. Patent, 872, 261 (1961); Chem. Abstr., 55 (1961) 25158a.

202 G.F. Gause, Vest. Acad. Med. Nauk SSSR, 4 (1965) 46.
203 E.S. Kudina, O.L. Olkhovatova, L.I. Muravieva and G.F. Gause, Antibiotiki, 11 (1966) 400.
204 A.J. Herlt, R.W. Rickards and J.-P. Wu, J. Antibiot., 38 (1985) 516.
205 K. Isshiki, T. Sawa, K. Miura, B.-S. Li, H. Naganawa, M. Hamada, T. Takeuchi and H. Umezawa, J. Antibiot., 39 (1986) 1013.
206 T.K. Liao, W.H. Nyberg and C.C. Cheng, Angew. Chem., 79 (1967) 100; J. Heterocycl. Chem., 13 (1976) 1063.
207 T.K. Liao, P.J. Wittek and C.C. Cheng, J. Heterocycl. Chem., 13 (1976) 1283.
208 P.J. Wittek, T.K. Liao and C.C. Cheng, J. Org. Chem., 44 (1979) 870.
209 T.W. Doyle, D.M. Balitz, R.E. Grulich, D.E. Nettleton, S.J. Gould, C.-H. Tann and A.E. Moens, Tetrahedron Lett., 22 (1981) 4595.
210 D.M. Balitz, J.A. Bush, W.T. Bradner, T.W. Doyle, F.A. O'Herron and D.E. Nettleton, J. Antibiot., 35 (1982) 259.
211 S.J. Gould and C.C. Chang, J. Am. Chem. Soc., 99 (1977) 5496; ibid, 102 (1980) 1702.
212 S.J. Gould and D.S. Darling, Tetrahedron Lett., (1978) 3207.
213 S.J. Gould, C.C. Chang, D.S. Darling, J.D. Roberts and M. Squillacote, J. Am. Chem. Soc., 102 (1980) 1707.
214 D.L. Dexter, D.P. Hesson, R.J. Ardecky, G.V. Rao, D.L. Tippett, B.A. Dusak, K.D. Paull, J. Plowman, B.M. DeLarco, V.L. Narayanan and M. Forbes, Cancer Res., 45 (1985) 5563.
215 S.-F. Chen, R.L. Ruben and D.L. Dexter, Cancer Res., 46 (1986) 5014.
216 A. Buzas, M. Osowiecki and O. Schindler, C. R. Hebd. Seances Acad. Sci., (1958) 1390.
217 S. Goodwin, A.F. Smith and E.C. Horning, J. Am. Chem. Soc., 81 (1959) 1903.
218 R.B. Woodward, G.A. Iacobucci and F.A. Hochstein, J. Am. Chem. Soc., 81 (1959) 4434.
219 G. Buechi, D.W. Mayo and F.A. Hochstein, Tetrahedron, 15 (1961) 167.
220 L.K. Dalton, S. Demerac, B.C. Elmes, J.W. Loder, J.M. Swan and T. Teitei, Aust. J. Chem., 20 (1967) 2715.
221 G.H. Svoboda, G.A. Poore and M.L. Montfort, J. Pharm. Sci., 57 (1968) 1720.
222 J.P. Cosson and M. Schmid, Phytochemistry, 9 (1970) 1353.
223 K.N. Kilminster, M. Sainsbury and B. Webb, Phytochemistry, 11 (1972) 389.
224 C.P. Bournique, G. Deysson and J. Le Men, Ann. Pharm. Fr., 30 (1972) 85.
225 P. Lesca, P. Lecointe, C. Paoletti and D. Mansuy, Biochimie, 60 (1978) 1011.
226 P. Lesca, P. Lecointe, D. Pelaprat, C. Paoletti and D. Mansuy, Biochem. Pharmacol., 29 (1980) 3231.
227 G.G. Mathe, N. Hayat, F. De Vassal, L. Schwarznberg, M. Schneider, J.R. Schlumberger, C. Jasmin and C. Rosenfeld, Rev. Eur. Etud. Clin. Biol. 15 (1970) 541.
228 M.M. Chien and J.P. Rosazza, Drug Metabol. Disposit., 7 (1979) 211.
229 V.K. Kansal and P. Potier, Tetrahedron, 42 (1986) 2389.
230 J.B. Le Pecq, N. Dat-Xuang, C. Gosse and C. Paoletti, Proc. Natl. Acad. Sci., U.S.A., 71 (1974) 5078.
231 C. Paoletti, J.B. Le Pecq, N. Dat-Xuong, P. Lesca and P. Lecointe, Current Chemother. Proc. 10th Int. Congr. Chemother., 2 (1978) 1195.
232 C. Paoletti, J.B. Le Pecq, N. Dat-Xuong, P. Juret, H. Garnier, J.-L. Amiel and J. Rouesse, Recent Results Cancer Res., 74 (1980) 107.
233 P. Juret, J.F. Heron, J.E. Couette, T. Delozier and J.Y. Le Talaer, Cancer Treat. Rep., 66 (1982) 1909.
234 A. Clasysse, A. Brugarolas, P. Siegenthaler, R. Abele, F. Cavalli, R. Dejager, G. Renard, M. Rosenoweig and H.H. Hansen, Eur. J. Cancer Clin. Oncol. 20 (1984) 243.

235 J.-C. Chermann, J. Gruest, L. Montagnier, F. Wendling, P. Tambourin, M. Perrin, F. Pochon, C. Ducrocq, C. Rivalle and É. Bisagni, C. R. Hebd. Seances Acad. Sci., 285D (1977) 945.
236 C. Ducrocq, É. Bisagni, C. Rivalle and J.M. Lhosta, J. Chem. Soc., Perkin Trans. 1, (1979) 142.
237 J.C. Chermann, R. Lidereau and L. Montagnier, Current Chemother. Proc. 10th Int. Congr. Chemother. 1 (1978) 1200.
238 P. Juret, A. Tanguy, A. Girard, J.Y. Le Talaer, J.S. Abbatucci, N. Dat-Xuong, J.B. Le Pecq and C. Paoletti, Eur. J. Cancer, 14 (1978) 205.
239 B. Blais, B. Gourmel, M. Marty, C. Gisselbrecht, E. Bisagni, M. Boiron and C. Dreux, J. Liq. Chromatogr., 6 (1983) 939.
240 B.K. Bhuyan, T.J. Fraser and L.H. Li, Cancer Res., 32 (1972) 2538.
241 L.H. Li and C.H. Corvie, Biochim. Biophys. Acta, 353 (1974) 375.
242 L. Lempereur, A.M. Sautereau, J.F. Tocanne and G. Laneelle, Biochem. Pharmacol., 33 (1984) 2499.
243 K.W. Kohn, M.J. Waring, D. Glaubiger and C.A. Friedman, Cancer Res., 35 (1975) 71.
244 B. Festy, J. Poisson and C. Paoletti, FEBS Lett., 17 (1971) 321.
245 A. Gouyette, R. Reynaud, J. Sadet, M. Baillargé, C. Gansser, S. Cros, F. Le Goffic, J.-B. Le Pecq, C. Paoletti and C. Viel, Eur. J. Med. Chem.-Chim. Ther., 15 (1980) 503.
246 C. Malvy and S. Cros, Biochem. Pharmacol., 35 (1986) 2264.
247 K.M. Tewey, G.L. Chen, E.M. Nelson and L.F. Liu, J. Biol. Chem., 259 (1984) 9182.
248 M.E. Wall, M.C. Wani, C.E. Cook, K.H. Palmer, A.T. McPhail and G.A. Sim, J. Am. Chem. Soc., 88 (1966) 3888.
249 R.E. Perdue, Jr., M.E. Wall, J.L. Hartwell and B.J. Abbott, Lloydia, 31 (1968) 229.
250 R.E. Perdue, Jr., R.L. Smith, M.E. Wall, J.L. Hartwell and B.J. Abbott, U.S. Agr. Res. Service Tech. Bull. No. 1415 (1970) 1.
251 T.R. Govindachari and N. Viswanathan, Indian J. Chem., 10 (1972) 453.
252 S. Tafur, J.D. Nelson, D.C. DeLong and G.H. Svoboda, Lloydia, 39 (1976) 261.
253 S.P. Gunsekera, M.M. Badaw, G.A. Gordell, N.R. Farnsworth and M. Chitnis, J. Nat. Prod., 42 (1979) 475.
254 A.T. McPhail and G.A. Sim, J. Chem. Soc. (B), (1968) 923.
255 R.C. Gallo, J. Whang-Peng and R.H. Adamson, J. Natl. Cancer Inst., 46, (1971) 789.
256 C.R. Hutchinson, Tetrahedron, 37 (1981) 1047.
257 J.L. Hartwell and B.J. Abbott, Pharmacol. Chemother., 7 (1969) 137.
258 H.B. Bosmann, Biochem. Biophys. Res. Commun., 41 (1970) 1412.
259 R.S. Wu, A. Kumar and J.R. Warner, Proc. Natl. Acad. Sci. U.S.A., 68 (1971) 3009.
260 S.B. Horwitz, C.-K. Chang and A.P. Grollman, Mol. Pharmacol., 7 (1971) 632.
261 D. Kessel, H.B. Bosmann and K. Lohr, Biochim. Biophys. Acta, 269 (1972) 210.
262 M.E. Wall, Biochem. Physiol. Alkaloid 4th Int. Symp., 1969 (1972) 77.
263 M.E. Wall and M.C. Wani, Annu. Rev. Pharmacol. Toxicol., 17 (1977) 117.
264 M.C. Wani, P.E. Ronman, J.T. Lindley and M.E. Wall, J. Med. Chem., 23 (1980) 554.
265 M.C. Wani and M.E. Wall, J. Org. Chem., 34 (1969) 1364.
266 P.-C. Pan, S.-Y. Pan, Y.-H. Tu, S.-Y. Wang and T.-Y. Owen, Hua Hsueh Hsueh Pao, 33 (1975) 71.
267 M.E. Wall, M.C. Wani, S.M. Natschke and A.W. Nicholas, J. Med. Chem., 29 (1986) 1553.
268 M.S. Horwitz and S.B. Horwitz, Biochem. Biophys. Res. Commun., 45 (1971) 723.
269 M.S. Horwitz and C. Brayton, Virology, 48 (1972) 690.
270 Y. Becker and U. Olshevsky, Isr. J. Med. Sci., 9 (1973) 1578.

271 C.-Y. Chiao and H.-S. Li, Chinese Med. J., (1974) 208; ibid, (1975) 355.
272 J.H. Neale, P.D. Klinger and B.W. Agronoff, Science (Washington, DC), 179 (1973) 1243.
273 G.K. Hughes, F.N. Lahey, J.R. Price and L.J. Webb, Nature (London), 162 (1948) 223.
274 F.N. Lehey and W.C. Thomas, Austr. J. Sci. Res., 2A (1949) 423.
275 P.L. MacDonald and A.V. Robertson, Aust. J. Chem., 19 (1966) 275.
276 G.H. Svoboda, G.A. Poore, P.J. Simpson and G.B. Boder, J. Pharm. Sci., 55 (1966) 758.
277 G.H. Svoboda, Lloydia, 29 (1966) 206.
278 P.W. Gout, B.P. Dunn and C.T. Beer, J. Cell. Physiol., 78 (1971) 127.
279 S.K. Carter, Cancer Chemother. Rep., [3] 2 (1971) 81.
280 K. Gerzon and G.H. Svoboda, The Alkaloids, 21 (1983) 1.
281 D.R. Brannon, D.R. Horton and G.H. Svoboda, J. Med. Chem., 17 (1974) 653.
282 K.J. Liska, J. Med. Chem., 15 (1972) 1177.
283 (a) T. Higuchi, A.J. Repta and D.W.A. Bourne, U.S. Patent 3,943,137 (1976), (b) Eli Lilly & Co., U.S. Patent 4,309,431 (1982).
284 G.H. Svoboda, M.J. Sweeney and W.D. Walkling, J. Pharm. Sci., 60 (1971) 333.
285 P.W. Gout, B.P. Dunn and C.T. Beer, J. Cell Physiol., 78 (1971) 127.
286 B.P. Dunn, P.W. Gout and C.T. Beer, Cancer Res., 33 (1973) 2310.
287 P. Tan and N. Auersperg, Cancer Res., 33 (1973) 2320.
288 R.S. Low and N. Auersperg, Exp. Cell. 131 (1981) 15.
289 T.-S. Wu, H. Furukawa and C.-S. Kuoh, Heterocycles, 19 (1982) 273.
290 T.-S. Wu and H. Furukawa, Heterocycles, 19 (1982) 825.
291 T.-S. Wu, H. Furukawa, C.-S. Kuoh and K.-S. Hsu, J. Chem. Soc., Perkin Trans. 1, (1983) 1681.
292 S. Funayama and G.A. Cordell, J. Nat. Prod., 48 (1985) 114.
293 S. Funayama and G.A. Cordell, J. Nat. Prod., 48 (1985) 938.
294 G.A. Cordell, Personal Communication, December 3, 1985.
295 T.R. Govindachari, P.C. Parthasarathy and H.K. Desai, Indian J. Chem., 11 (1973) 1190.
296 N. Ruangrungsi, V. Wongpanich, P. Tantivatana, H.J. Cowe, P.J. Cox, S. Funayama and G.A. Cordell, J. Nat. Prod., 48 (1985) 529.
297 H.W. Bennetts, J. West Aust. Dep. Agr., 12 (1935) 433.
298 S.L. Everest, in Poisonous Plants of Australia (Angus and Robertson, Sydney, 1974) p. 357.
299 C.R. Huxtable, P.R. Dorling and D.H. Slatter, Neuropath. Appl. Neurobiol. 6 (1980) 221.
300 D.C. Main, D.H. Slatter, C.R. Huxtable, I.C. Constable and P.R. Dorling, Aust. Vet. J., 57 (1981) 132.
301 S.M. Colegate, P.R. Dorling, C.R. Huxtable, B.W. Skelton and A.H. White, Aust. J. Chem., 38 (1985) 1233.
302 K.N. Campbell, R.C. Morris and R. Adams, J. Am. Chem. Soc., 59 (1937) 1723.
303 T.J. King and L.B. de Silva, Tetrahedron Lett., (1968) 261.
304 S.C. Datta, V.V.S. Murti and T.R. Seshadri, Indian J. Chem., 10 (1972) 263.
305 R. Adams, R.C. Morris, T.A. Geissman, D.J. Butterbaugh and E.C. Kirkpatrick, J. Am. Chem. Soc., 60 (1938) 2193.
306 D.A. Shirley and W.L. Dean, J. Am. Chem. Soc., 79 (1957) 1205.
307 A.B. Wood, F.V. Robinson and R.C.A. Lago, Chem. Ind. (London), (1969) 1738.
308 R. Adams, T.A. Geissman and J.D. Edwards, Chem. Rev., 60 (1960), 555.
309 H.A. Mattil, J. Biol. Chem., 90 (1931) 141.
310 H.D. Royce, Oil Soap (Chicago), 10 (1933) 123.
311 E.L. Hove and Z. Hove, J. Biol. Chem., 156 (1944) 611.

312 G. Katsui and K. Kato, Vitamins, 7 (1954) 746.
313 F.C. Pack, R.N. Moore and W.G. Bickford, U.S. Patent 2,685,597 (1953); Chem. Abstr., 48 (1954) 14297.
314 Nanjing Institute of Materia Medica, unpublished results (1972).
315 National Coordination Group on Male Antifertility Agents, Chinese Med. J., 4 (1978) 417.
316 S.-Z. Qian, Y. Xu, Z.-C. Chen, L.-M. Gao, S.-G. Sun, X.-C. Tang, Y.-E. Wang, L.-Y. Shen and M.-K. Zhu, Acta Pharm. Sinica, 14 (1979) 513.
317 T.H. Maugh, II, Science (Washington, DC), 212 (1981) 314.
318 S.-M. Tong, X.-H. Zhou and Y.-X. Zhou, Chinese Med. J., 95 (1982) 355.
319 C.-Y. G. Lee, Y.S. Moon, J.H. Yuan and A.F. Chen, Mol. Cell. Biochem. 47 (1982) 65.
320 Y.-K. Si, J. Zhou and L. Huang, Kexue Tongbao, 28 (1983) 640.
321 D.-K. Zheng, Y.-K. Si, J.-K. Meng, J. Zhou and L. Huang, J. Chem. Soc., Chem. Commun., (1985) 168.
322 Y.E. Wang, Y.D. Luo and X.C. Tang, Acta Pharm. Sinica, 14 (1979) 662.
323 S.D. Jolad, R.M. Wiedhopf and J.R. Cole, J. Pharm. Sci., 64 (1975) 1889.
324 P.N. Rao, Y.-C. Wang, E. Lotzova, A.A. Khan, S.P. Rao and L.C. Stephens, Cancer Chemother. Pharmacol., 15 (1985) 20.
325 L.J. Rosenberg, R.C. Adlakha, D.M. Desai and P.N. Rao, Biochim. Biophys. Acta, 866 (1986) 258.
326 J.B. Harborne, T.J. Mabry and H. Mabry, eds., The Flavonoids (Academic Press, New York, 1975).
327 J.B. Harborne and T.J. Mabry, eds., The Flavonoids: Advances in Research (Chapman and Hall, London, 1982).
328 (a) B. Havsteen, Biochem. Pharmacol., 32 (1983) 1141; (b) E. Middleton, Trends Pharmacol. Sci., (1984) 335.
329 K. Herrmann, Food Technol., 11 (1976) 433.
330 V. Cody, E. Middleton, Jr. and J.B. Harborne, eds., Plant Flavonoids in Biology and Medicine, (Alan R. Liss, New York, 1986).
331 K. Hahlbrock, in The Biochemistry of Plants, Vol. 7, E.E. Conn., ed. (Academic Press, New York, 1981), Ch. 14.
332 H.A.M. Al-Ani and P.M. Dewick, J. Chem. Soc. Perkin Trans 1, (1984) 2831.
333 L.A. Mitscher, G.S.R. Rao, I. Khanna, T. Veysoglu and S. Drake, Phytochemistry, 22 (1983) 573.
334 M.R. Gibson, Lloydia, 41 (1978) 348.
335 M. Ito, S. Ishimoto, N. Nishida, T. Shiramizu and H. Yunoki, Agr. Biol. Chem., 50 (1986) 1073.
336 H. Arens, B. Ulbrich, F. Fischer, M.J. Parnham and A. Römer, Planta Medica, (1986) 468.
337 E. Gillespie, Biochem. Biophys. Res. Commun., 98 (1981) 463.
338 (a) W.C. Cutting, R.H. Dreisbach and B.J. Neff, Stanford Med. Bull., 7 (1949) 137; (b) W.C. Cutting, R.H. Dreisbach and F. Matsushima, ibid, 11 (1953) 227.
339 A. Veckenstedt, I. Béládi and I Mucsi, Arch. Virol., 57 (1978) 255.
340 I. Musci and B.M. Prágai, Experientia, 41 (1985) 930.
341 C.A. Elliger, B.C. Chan and A.C. Waiss, Jr., Naturwissenschaften, 67 (1980) 358.
342 S.D. Varma and J.H. Kinoshita, Biochem. Pharmacol., 25 (1976) 2505.
343 P.F. Kador and N.E. Sharpless, Biophys. Chem., 8 (1978) 81.
344 A. Beyer-Mears and P.N. Farnsworth, Exp. Eye Res., 28 (1979) 709.
345 J. Okuda, I. Miwa, K. Inagaki, T. Horie and M. Nakayama, Biochem. Pharmacol., 31 (1982) 3807.

346 Y.R. Sharma and E. Cutlier, Exp. Eye Res., 35 (1982) 21.
347 M. Woeldecke and K. Herrmann. Z. Lebensm.-Unters. Forsch., 156 (1974) 153.
348 K.-L. A. Rosler, R.S. Goodwin, T.J. Mabry, S.D. Varma and J. Norris, J. Nat. Prod., 47 (1984) 316.
348a L.G. Humber, Prog. Med. Chem., 24 (1987) 299.
349 T.H. Simpson and N. Uri, Chem. Ind. (London), (1956) 956.
350 A.C. Mehta and T.R. Seshardi, J. Sci. Indian Res., 18B (1958) 24.
351 A. Letan, J. Food Sci., 31 (1966) 395; 518.
352 B.J.F. Hudson and J.I. Lewis, Food Chem., 10 (1983) 47.
353 D.C. Crawford, R.O. Sinnhuber and H. Aft, J. Food Sci., 26 (1961) 139.
354 L. Diamond and H.V. Gelboin, Science (Washington, DC), 166 (1969) 1023.
355 H.V. Gelboin, F. Wiebel and L. Diamond, Science (Washington, DC), 170 (1970) 169.
356 L. Diamond, R. McFall, J. Miller and H.V. Gelboin, Cancer Res., 32 (1972) 731.
357 N. Kinoshita and H.V. Gelboin, Cancer Res., 32 (1972) 1329; Proc. Natl. Acad. Sci. U.S.A., 69 (1972) 824.
358 A.G. Schwartz, Cancer Res., 34 (1974) 10.
359 L.W. Wattenberg, M.A. Page and J.L. Leong, Cancer Res., 28 (1968) 934.
360 L.W. Wattenberg and J.L. Leong, Proc. Soc. Exp. Biol. Med., 128 (1968) 940; Cancer Res., 30 (1970) 1922.
361 F.J. Wiebel, H.V. Gelboin, N.P. Buu-Hoï, M.G. Stout and W.S. Burnham, in Chemical Carcinogenesis, P.O.P. Tso and J.A. DiPaolo, eds. (Marcel Dekker, New York, 1974), p. 249.
362 F.J. Wiebel and H.V. Gelboin, Biochem. Pharmacol., 24 (1975) 1511.
363 M.-T. Huang, R.L. Chang, J.G. Fortner and A.H. Conney, J. Biol. Chem., 256 (1981) 6829.
364 A.S. Raj and M. Katz, Mutation Res., 110 (1983) 337.
365 J.T. Kellis, Jr. and L.E. Vickery, Science (Washington, DC), 225, (1984) 1032.
366 J.T. Kellis, Jr., S. Nesnow and L.E. Vickery, Biochem. Pharmacol., 35 (1986) 2887.
367 S.R. Long, in Plant Microbe Interactions, Vol. 1, eds. T. Kosage and E.W. Nester (MacMillan, N.Y., 1984).
368 S.R. Long and J. Frost, unpublished results.
369 J.L. Firmin, K.E. Wilson, L. Rossen and A.W.B. Johnston, Nature (London), 324 (1986) 90.
370 M.-T. Huang, A.W. Wood, H.L. Newmark, J.M. Sayer, H. Yagi, D.M. Jerina and A.H. Conney, Carcinogenesis, 4 (1983) 1631.
371 P.W. Le Quesne, M.P. Pastore and R.F. Raffauf, Lloydia, 39 (1976) 391.
372 M. Bittner, M. Silva, J. Vargas and F. Bohlmann, Phytochemistry, 22 (1983) 1523.
373 C.Y. Wang, A.M. Panukcu and G.T. Bryan, Med. Biol. Environ., 4 (1976) 565.
374 M. Nagao, N. Morita, T. Yahagi, M. Shimizu, M. Kuroyanagi, M. Fukuoka, K. Yoshihira, S. Natori, T. Fujino and T. Sugimura, Environ. Mutagen. 3 (1981) 401.
375 H. Nishino and A. Iwashima, Kyoto Pref. Univ. Med., 92 (1983) 1367.
376 B. Werner, W. Sprecher and H. Mitscheke, Verdauungskrankheiten, 3 (1985) 113.
377 G. Atassi, P. Briet, J.-J. Berthelon and F. Collonges, Eur. J. Med. Chem.-Chim. Ther., 20 (1985) 393.
378 D.J. Kerr, S.B. Kaye, J. Graham, J. Cassidy, M. Harding, A. Setanoians, J.C. McGrath, W.R. Vezin, D. Cunningham, G. Forrest and M. Soukop, Cancer Res., 46 (1986) 3142.
379 D.J. Kerr, S.B. Kaye, J. Cassidy, S. Dutta, A. Setanoians, G. Forrest, D. Cunningham, M. Soukop and W.R. Vezin, Br. J. Cancer, 52 (1985) 467.
380 J.A. Double, M.C. Bibby and P.M. Loadman, Br. J. Cancer, 54 (1986) 595.

381 J. Plowman, V.L. Narayanan, D. Dykes, E. Szarvasi, P. Briet, O.C. Yoder and K.D. Paull, Cancer Treat. Rep., 70 (1986) 631.
382 P.J. O'Dwyer, D. Shoemaker, D.S. Zaharko, C. Grieshaber, J. Plowman, T. Corbett, F. Valeriote, S.A. King, J. Cradock, D.F. Hoth and B. Leyland-Jones, Cancer Chemother. Pharmacol., 19 (1987) 6.
383 M.C. Bibby, J.A. Double, R.M. Phillips and P.M. Loadman, Br. J. Cancer, 55 (1987) 159.
384 P. Da Re, M. Verlicchi and I.J. Setnikar, J. Med. Pharm. Chem., 2 (1960) 263.
385 I. Setnikar, M.T. Ravasi and P. Da Re, J. Pharmacol. Exp. Ther. 130 (1960) 356.
386 M. Conti and I. Setnikar, Arch. Int. Pharmacodyn. 213 (1975) 186.
387 E. Pedersen, Urol. Int., 32 (1977) 202.
388 P. Cazzulani, R. Panzarasa, C. De Stefani and G. Graziani, Arch. Int. Pharmacodyn., 274 (1985) 189.
389 M. Veronese and D. Barzaghi, Arzneim.-Forsch., 37 (1987) 528.
390 M. Jay, P. Lebreton and R. Letoublon, Boissiera, 19 (1971) 219.
391 J.B. Harborne, in Chemotaxonomy of the Leguminosae, eds. J.B. Harborne, D. Boulter and B.L. Turner (Academic Press, London, 1971), p. 31.
392 W.B. Whalley, in The Pharmacology of Plant Phenolics, J.W. Fairbairn, ed., (Academic Press, London, 1959), p. 27.
393 J.D. Biggers, in ref. 392, p. 51.
394 A.H. Braden and I.H. McDonald, in Australian Glasslands, R.M. Moore, ed., (Australian National University Press, Canberra, 1970), p. 38.
395 T.J. Batterham, D.A. Shutt, N.K. Hart, A.W.H. Braden and H.J. Tweeddale, Austr. J. Agr. Res., 22 (1971) 131.
396 R.K.S. Wood, Proc. R. Soc., B181 (1972) 213.
397 B.J. Deverall, Proc. R. Soc., B181 (1972) 233.
398 A. Chowdhury, N. Mukherjee and N. Adityachaudhury, Experientia, 30 (1974) 1022.
399 D.A. Smith and S.W. Bank, Phytochemistry, 25 (1986) 979.
400 C.K. Wilkins and B.A. Bohm, Phytochemistry, 16 (1977) 144.
401 H. Fukui, H. Egawa, K. Koshimizu and T. Mitsui, Agr. Biol. Chem., 37 (1973) 417.
402 M.J. O'Neill, S.A. Adesanya and M.F. Roberts, Z. Naturforsch., 38c (1983) 693.
403 S. Tahara, S. Nakahara, J. Mizutani and J.L. Ingham, Agr. Biol. Chem., 48 (1984) 1471.
404 S.Z. Dziedzic and B.J.F. Hudson, Food Chem., 11 (1983) 161.
405 D.N. Dhar, The Chemistry of Chalcones and Related Compounds (John Wiley, New York, 1981).
406 T. Torigoe, M. Arisawa, S. Itoh, M. Fujiu and H.B. Maruyama, Biochem. Biophys. Res. Commun., 112 (1983) 833.
407 W.W. Busse, D.E. Kopp and E. Middleton, Jr., J. Allergy Clin. Immunol., 73 (1984) 801.
408 H. Rapoport and K.G. Holden, J. Am. Chem. Soc., 82 (1960) 4395.
409 D.L. Dreyer, J. Org. Chem., 33 (1968) 3577.
410 D.M. Razzakova, I.A. Bessonova and S.Y. Yunusov, Khir. Prir. Soedin, 8 (1972) 113.
411 F. Tillequin, M. Koch and T. Sevenet, Planta Med., 39 (1980) 383.
412 K.E. Nielsen and E.B. Pedersen, Acta Chem. Scand., B34 (1980) 637; Chimica Scripta, 18 (1981) 242.
413 P.J. Pijper, H. Van der Goot, H. Timmerman and W.T. Nauta, Eur. J. Med. Chem.-Chim. Ther., 19 (1984) 389; 393.
414 A. Mannschreck, H. Koller, G. Stühler, M.A. Davies and J. Traber, Eur. J. Med. Chem.-Chim. Ther., 19 (1984) 381.
415 B.M. Limson, R.K. Macasaet, J.J. Salem, C.G. Beling and H.A. Burnham, Curr. Ther. Res., 26 (1979) 842.

416 A. Sugimoto, S. Ito, Y. Eguchi, H. Tanaka, Y. Takashima and M. Ishikawa, Eur. J. Med. Chem.-Chim. Ther., 19 (1984) 223.
417 A. Sugimoto, H. Tanaka, Y. Eguchi, S. Ito, Y. Takashima and M. Ishikawa, J. Med. Chem., 27 (1984) 1300.
418 A. Sugimoto, K. Sakamoto, Y. Fujino, Y. Takashima and M. Ishikawa, Chem. Pharm. Bull., 33 (1985) 2809.
419 J.-B. LePecq, in Methods of Biochemical Analysis, Vol. 20, ed. D. Glick (Wiley-Interscience, New York, 1971), p. 41.
420 M. Waring, in Antibiotics, Vol. 3, eds. J.W. Corcoran and F.E. Hahn, (Springer-Verlag, New York, 1975), p. 141.
421 T.R. Krugh and C.G. Reinhardt, J. Mol. Biol., 97 (1975) 133.
422 T.R. Krugh, F.N. Wittlin and S.P. Cramer, Biopolymers, 14 (1975) 197.
423 D.J. Patel and L.L. Canuel, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 3343.
424 G.R. Pack and G. Loew, Biochim. Biophys. Acta, 519 (1978) 163.
425 C. Malvy, Anal. Biochem., 143 (1984) 158.
426 J. Röding, A. Naujok and H.W. Zimmermann, Histochem., 85 (1986) 215.
427 P. Ostmann, Dtsch. Med. Wochenschr., 51 (1925) 2030.
428 W. Radermacher, Munch. Kinderheik, 79 (1939) 49.
429 F.W. Short, E.F. Elslager, A.M. Moore, M.J. Sullivan and F.H. Tendick, J. Am. Chem. Soc., 80 (1958) 223.
430 E.F. Elslager, F.W. Short, M.J. Sullivan and F.H. Tendick, J. Am. Chem. Soc., 80 (1958) 451.
431 A.D. Brewer, J.A. Minatelli, J. Plowman, K.D. Paull and V.L. Narayanan, Biochem. Pharmacol., 34 (1985) 2047.
432 D.A. Cooney, J.M. Covey, G.J. Kang, M. Dalal, J.B. McMahon and D.J. Johns, Biochem. Pharmacol., 34 (1985) 3395.
433 A.D. Brewer, G. Ferguson and M. Parvez, Acta Crystallogr. C43 (1987) 144.
434 F. Bergman, Cancer Res., 2 (1942) 660.
435 B.J. Broughton, P. Chaplen, P. Knowles, E. Lunt, D.L. Pain, K.R.H. Woodridge, R. Ford, S. Marshall, J.L. Walker and D.R. Maxwell, Nature (London), 251 (1974) 650.
436 B.J. Broughton, P. Chaplen, P. Knowles, E. Lunt, S.M. Marshall, D.L. Pain and K.R.H. Woodridge, J. Med. Chem., 18 (1975) 1117.
437 A. Holland, D.S. Jackson, P. Chaplen, E. Lunt, S.M. Marshall, D.L. Pain and K.R.H. Woodridge, Eur. J. Med. Chem.-Chim. Ther., 10 (1975) 447.
438 S.R. Wilson, R.B. Wilson, A.L. Shoemaker, K.R.H. Woodridge and D.J. Hodgson, J. Am. Chem. Soc., 104 (1982) 259.
439 P. Finlander, S.V. Nielsen and E.B. Pederson, Chem. Scr., 22 (1983) 171.
440 P. Finlander and E.B. Pederson, Arch. Pharm. (Weinheim, Ger.), 318 (1985) 452.
441 C.A. Klepner, A.S. Lippa, D.I. Benson, M.C. Sano and B.Beer, Pharmacol. Biochem. Behav., 1 (1979) 457.
442 J.-C. Gaignault and D. Bidet, Ann. Pharm. Fr., 43 (1985) 321; 427.
443 M. Cushman, P. Mohan and E.C.R. Smith, J. Med. Chem., 27 (1984) 544.
444 M. Cushman and P. Mohan, J. Med. Chem., 28 (1985) 1031.
445 N. Kubová, E. Smékal, V. Kleinwächter and M. Cushman, Stud. Biophys., 114 (1986) 251.
446 M. Cushman, F.W. Dekow and L.B. Jacobson, J. Med. Chem., 22 (1979) 331.
447 C. Huggens and N.C. Yang, Science (Washington, DC), 137 (1962) 257.
448 A. Haddow, Can. Cancer Conf., 2 (1957) 361.
449 M. Gellert, Annu. Rev. Biochem., 50 (1981) 879.

450 L.F. Liu, CRC Critical Rev. Biochem. 15 (1983) 1.
451 T.C. Rowe, K.M. Tewey and L.F. Liu, J. Biol. Chem., 259 (1984) 9177.
452 G.L. Chen and L.F. Liu, Annu. Rep. Med. Chem., 21 (1986) 257.
453 K.M. Tewey, G.L. Chen, E.M. Nelson and L.F. Liu, J. Biol. Chem., 259 (1984) 9182.
454 G.L. Chen, L. Yang, T.C. Rowe, B.D. Halligan, K.M. Tewey and L.F. Liu, J. Biol. Chem., 259 (1984) 13560.
455 E.M. Nelson, K.M. Tewey and L.F. Liu, Proc. Natl. Acad. Sci. U.S.A., 81 (1984) 1361.
456 K.M. Tewey, T.C. Rowe, L. Yang, B.D. Halligan and L.F. Liu, Science (Washington, DC), 226 (1984) 466.
457 T. Rowe, G. Kupfer and W. Ross, Biochem. Pharmacol., 34 (1985) 2483.
458 W.E. Ross, Biochem. Pharmacol., 34 (1985) 4191.
459 Y. Pommier, R.E. Schwartz, L.A. Zwelling and K.W. Kohn, Biochemistry, 24 (1985) 6406.
460 Y. Pommier, J.K. Minford, R.E. Schwartz, L.A. Zwelling and K.W. Kohn, Biochemistry, 24 (1985) 6410.
461 L.A. Zwelling, Cancer Metastasis Rev., 4 (1985) 263.
462 M.D. Crespi, S.E. Evanier, J. Genovese and A. Baldi, Biochem. Biophys. Res. Commun., 136 (1986) 521.
463 T.C. Rowe, G.L. Chen, Y.-H. Hsiang and L.F. Liu, Cancer Res., 46 (1986) 2021.
464 L.E. Dillehay, S.C. Denstman and J.R. Williams, Cancer Res., 47 (1987) 206.
465 M.R. Mattern, S.-M. Mong, H.F. Bartus, C.K. Mirabelli, S.T. Crooke and R.K. Johnson, Cancer Res., 47 (1987) 1793.
466 R.B. Alexander, W.G. Nelson and D.S. Coffey, Cancer Res., 47 (1987) 2403.
467 O. Rosenheim and H. King, Chem. Ind. (London), 51 (1932) 464.
468 J.D. Bernal, Chem. Ind. (London), 51 (1932) 466.
469 H. Wieland and E. Dane, Z. Physiol. Chem., 216 (1933) 91.
470 J.P. Glusker, in Biochemical Actions of Hormones, ed. G. Litwack, Vol. VI (Academic Press, New York, 1979), Ch. 3, p. 122.
471 H. Wieland and E. Dane, Z. Physiol. Chem., 219 (1933) 240.
472 J.W. Cook and E.C. Dodds, Nature (London), 131 (1933) 205.
473 J.W. Cook and G.A.D. Haslewood, Chem. Ind. (London), (1933) 758; J. Chem. Soc. (1934) 428.
474 L.F. Fieser and M.S. Newman, J. Am. Chem. Soc., 57 (1935) 961.
475 M.M. Coombs, T.S. Bhatt and C.J. Croft, Cancer Res., 33 (1973) 832.
476 S. Kashino, D.E. Zacharias, R.M. Peck, J.P. Glusker, T.S. Bhatt and M.M. Coombs, Cancer Res., 46 (1986) 1817.
477 J.W. Barlow, L.C. Minasian and J.W. Funder, J. Steroid Biochem., 9 (1978) 1027.
478 N.C. Yang, A.J. Castro, M. Lewis and T.-W. Wong, Science (Washington, DC), 134 (1961) 386.
479 R.F. Glascock and W.G. Hoekstra, Biochem. J., 72 (1959) 673.
480 G.M. Stone, B. Baggett and R.B. Donnelly, J. Endocrinol., 27 (1963) 271.
481 D. Toft and J. Gorski, Proc. Natl. Acad. Sci. U.S.A., 55 (1966) 1574.
482 E.V. Jensen, T. Suzuki, T. Kawashima, W.E. Stumpf, P.W. Jungblut and E.R. DeSombre, Proc. Natl. Acad. Sci. U.S.A., 59 (1968) 632.
483 R.H. Ebright, J.R. Wong and L.B. Chen, Cancer Res., 46 (1986) 2349.
484 E.D. Nissen and D.R. Kent, Obstet. Gynecol., 46 (1975) 460.
485 P.K. Siiteri, Cancer Res., 38 (1978) 4360.
486 IARC Monographs on The Evaluation of the Carcinogenic Risk of Chemicals to Humans, Sex Hormones, Vol. 21 (International Agency for Research on Cancer, Lyon, 1979).

487 S.L. Schneider, V. Alks, C.E. Morreal, D.K. Sinha and T.L. Dao, J. Natl. Cancer Inst., 57 (1976) 1351.
488 C.E. Morreal, S.L. Schneider, D.K. Sinha and R.E. Bronstein, J. Natl. Cancer Inst., 62 (1979) 1585.
489 V. Ivanovic and I.B. Weinstein, Nature (London), 293 (1981) 404.
490 C.E. Morreal, D.K. Sinha, S.L. Schneider, R.B. Bronstein and J. Dawidzik, J. Med. Chem., 25 (1982) 323.
491 J.R. Curtis, Med. Hypotheses, 9 (1982) 489.
492 P. Pott, Chirurg. Obs., (1775) 63.
493 W.-R. Chao, C.T. Helmes and M.I. Dawson, Cancer Lett., 29 (1985) 43.
494 A.M. Jetten, K. Anderson, M.A. Deas, H. Kagechika, R. Lotan, J.I. Rearick and K. Shudo, Cancer Res., 47 (1987) 3523.
495 J. Eustache and B. Shroot, 194th American Chemical Society National Meeting, (September 4, 1987) MEDI Abstr. 8.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry – Vol. 25, edited by G.P. Ellis and G.B. West

3 Synthesis of Analogues of Folic Acid, Aminopterin and Methotrexate as Antitumour Agents

DAVID C. PALMER, Ph.D.[a,*] JERAULD S. SKOTNICKI, Ph.D.[b] and EDWARD C. TAYLOR, Ph.D.[c]

[a] *BOC Group Technical Center, 100 Mountain Avenue, Murray Hill, NJ 07974,* [b] *Wyeth Laboratories, P.O. Box 8299, Philadelphia, PA 19101 and* [c] *Department of Chemistry, Princeton University, Princeton, NJ 08544, U.S.A.*

* To whom correspondence should be addressed.

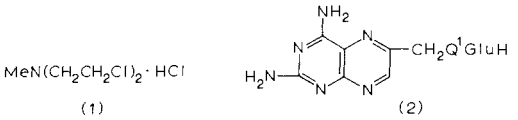
INTRODUCTION

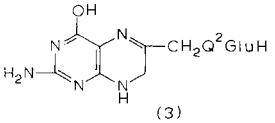
Of all the diseases that have afflicted mankind, none evokes more fear than the collective group known as cancer, acknowledged as the second most common cause of death among adults today. Enormous resources have been devoted to elucidating the mechanisms of carcinogenesis and to developing cures for those forms of cancer which are reasonably well understood. Among the modes of treatment, surgical removal of the tumour, radiation therapy and chemotherapy remain the primary programmes available to the clinician.

Modern chemotherapy was initiated 40 years ago when Goodman and Gilman reported their findings regarding the use of methylbis(β-chloroethyl)amine hydrochloride ((1), nitrogen mustard) for the treatment of Hodgkin's disease, lymphosarcoma and leukaemia [1,2]. Shortly thereafter, in 1949, a Lederle group described the first synthesis of the powerful antimetabolite, methotrexate (2) [3]. Since its introduction clinically in 1953, methotrexate has been the most widely used chemotherapeutic agent, useful either alone or in combination therapy for the treatment of acute lymphocytic leukaemia, choriocarcinoma, breast carcinoma, head and neck cancer, oat cell carcinoma, mycosis fungoides and osteogenic sarcoma [4].

Methotrexate acts by inhibition of dihydrofolate reductase, the enzyme requisite for the reduction of dihydrofolic acid (3) to 5,6,7,8-tetrahydrofolic acid (4). In turn, (4) is a precursor to a series of enzyme cofactors (5–7) essential for the transfer of one carbon unit necessary for the biosynthesis of purines and pyrimidines and hence, ultimately, DNA. As an inhibitor of dihydrofolate reductase, methotrexate kills cells during the S phase of the cell cycle, when the cells are in the log phase of growth. Unfortunately, this cytotoxicity is non-selective, and rapidly proliferating normal cells, e.g., gastrointestinal epithelium cells and bone marrow, are dramatically affected as well. In addition, recent use of high dose methotrexate therapy with leucovorin rescue has led to additional clinical problems arising from a dose-related nephrotoxic metabolite, 7-hydroxymethotrexate (8). Finally, the very polar nature of methotrexate renders it virtually impenetrable to the blood-brain barrier, which can necessitate direct intrathecal injection in order to achieve therapeutic doses for the treatment of CNS tumours.

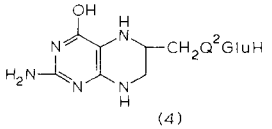
It is not surprising then that, despite its effectiveness, methotrexate therapy is underscored by serious side-effects and problems which have prompted ongoing research programmes to attempt the preparation of new analogues possessing better tumour-cell selectivity, lower toxicity, better transport properties, and improved lipid solubility and membrane permeability. These efforts have resulted in the preparation of thousands of analogues in which virtually every major area of the molecule has been varied and evaluated.

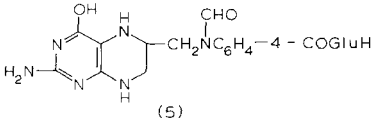




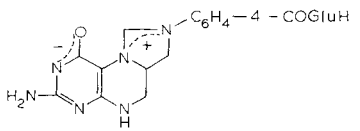
$Q^1 = MeNC_6H_4—4\text{-}CO$
$Q^2 = HNC_6H_4—4\text{-}CO$

DHFR

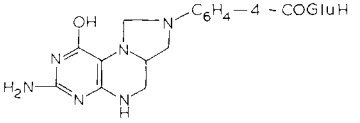




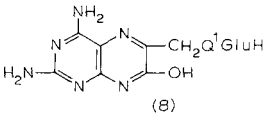
GAR transformylase → purines



AICAR transformylase → purines



thymidylate synthase → pyrimidines

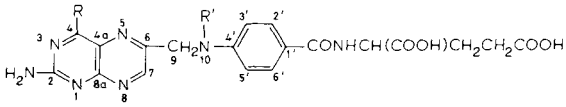


(8)

This review will focus on syntheses of folic acid, aminopterin and methotrexate analogues in which an intact L-glutamic acid side-chain is present, while simultaneously varying the pteridine ring moiety, the C-9,N-10 bridge and/or the benzoyl group. Analogues in which modifications have been made in the amino acid, including polyglutamates, will not be discussed. Several 'analogues' of folic acid are known in which the only remaining structural feature of the folic acid molecule is the *p*-aminobenzoylglutamic acid moiety. Since these compounds possess none of the additional structural units required for enzyme binding, they will also be omitted from this discussion. Further information regarding glutamic acid analogues can be obtained from an excellent and thorough series of papers from the Dana-Farber Cancer Institute [5], while the area of polyglutamate synthesis has been reviewed recently [6–8]. Further details regarding the mechanism of action, pharmacology, structure–activity relationships, X-ray structures, transport and clinical aspects of methotrexate therapy are outside the scope and intent of this review. For excellent reviews of these areas, the reader is directed to Refs. 9–30.

In order to avoid unnecessary repetition, and to stress new, unusual or critical applications of synthetic methodology in this area of heterocyclic chemistry, some synthetic schemes will be discussed in greater detail than others. The preparation of essential, commonly employed intermediates such as 6-formylpterin, 2,4-diamino-6-bromomethylpteridine, 2-amino-3-cyano-5-halomethylpyrazines, etc. (see next section) will be discussed separately, and only once. Several often-used strategies, such as the Boon–Leigh pteridine synthesis, and the Waller three-component synthesis, will also be detailed in this introductory section, and then subsequently referred to without further discussion.

The numbering system employed below for methotrexate (2), folic acid (9), and aminopterin (10) will be employed throughout this review. For convenience



(2) R = NH_2 R′ = Me
(9) R = OH R′ = H
(10) R = NH_2 R′ = H

in the presentation of formulae, and for the sake of consistency, the normal lactam or thiolactam (cyclic amide or cyclic thioamide) tautomer will be represented in the hydroxy or mercapto (lactim or thiolactim) form.

The following abbreviations will be routinely employed:

GluH	L-glutamic acid	Q	RNC_6H_4-4-CO
GluMe	dimethyl L-glutamate	Q^1	$MeNC_6H_4$-4-CO
GluEt	diethyl L-glutamate	Q^2	HNC_6H_4-4-CO
GluBu^t	di-*t*-butyl L-glutamate	Q^3	$AcNC_6H_4$-4-CO
MTX	methotrexate	guan	guanidine
FA	folic acid	DBP	dibenzoyl peroxide
AP	aminopterin	DBDMH	1,3-dibromo-5-5-dimethyl-hydantoin
DHFR	dihydrofolate reductase		
TS	thymidylate synthase	ECF	ethyl chloroformate
		Bu^{i}CF	isobutyl chloroformate
		NMM	*N*-methylmorpholine
		TEOF	triethyl orthoformate

The following nomenclature (which is not always in adherence with the many variants found in the literature) will be used to denote common variations in the C-9,N-10 bridge region:

normal	CH_2NR	bishomo	$CH_2CH_2CH_2NR$
homo	CH_2CH_2NR	11-methylene	CH_2NRCH_2
iso	$NRCH_2$	11-aza	CH_2NHNR
isohomo	$NRCH_2CH_2$	10-deaza-11-methylene	$CH_2CH_2CH_2$

Biological results will be discussed in those cases where the analogue has significant activity in tumour systems relative to MTX or AP.

PREPARATION OF COMMONLY EMPLOYED INTERMEDIATES

6-FORMYLPTERIN (11)

6-Formylpterin (11) is a key intermediate in the preparation (described in detail later in this review) of a variety of 6-substituted pterins (FA, AP, MTX, etc.). It has been prepared as follows:

(1) From naturally occurring pterins

A straightforward procedure for the preparation of (11) involves heating a solution of FA (9) in 40% hydrogen bromide containing excess bromine. The reaction is accompanied by the evolution of CO_2 and the formation of (11) (insoluble in organic solvents) together with 2,4,6-tribromoaniline (readily separated from (11) by extraction with organic solvents). This procedure gives isomer-free (11) in 50–60% yield, and is clearly the simplest route, given a ready source of FA [31,32].

A much more expensive route to (11) has been described which involves periodate cleavage of L-neopterin [33]. An analogous but synthetically uninteresting synthesis involves photolysis of either biopterin or neopterin in a phosphate buffer at pH 10 in the absence of oxygen. Subsequent introduction of oxygen results in oxidation of the intermediate 5,8-dihydropterin-6-carboxaldehyde to give (11) in unspecified yield [34].

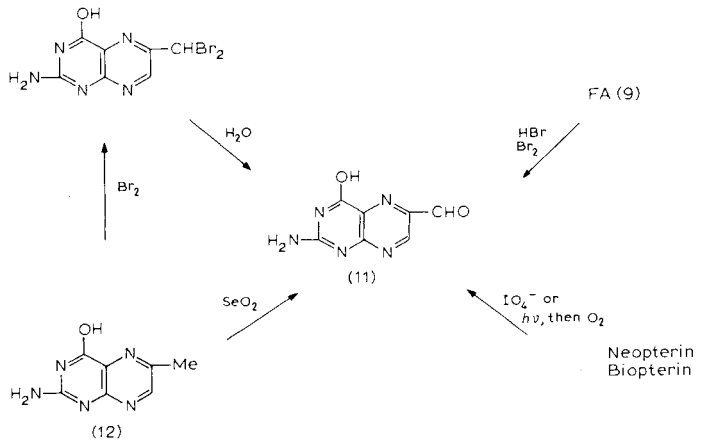
(2) From 6-methylpterin

Dibromination of the 6-methyl group of the 2-acetyl derivative of 6-methylpterin (12), followed by hydrolysis with aqueous sodium acetate, is reported to give (11) [35]. It should be noted, however, that isomer-free (11) is available by this procedure only when the precursor 6-methylpterin (12) is prepared by an unequivocal procedure; '6-methylpterin' derived from 2,4,5-triamino-6-hydroxypyrimidine and methylglyoxal inevitably consists of a mixture of the 6- and 7-regioisomers.

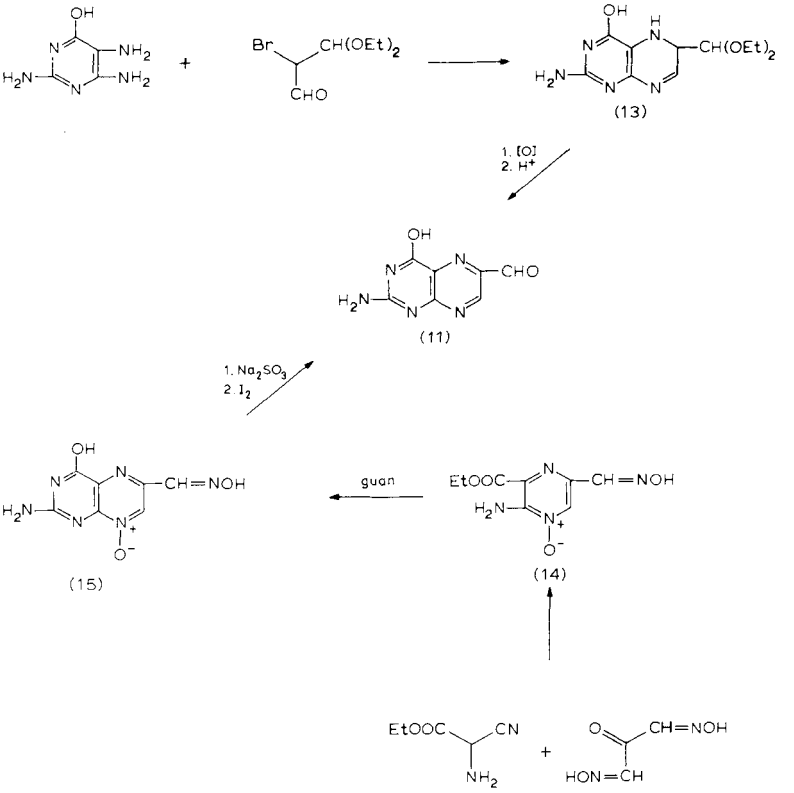
An early unequivocal synthesis of (11) which, however, is too involved to be practical, involved condensation of 2-amino-4-chloro-5-phenylazo-6-hydroxypyrimidine with the ethylene ketal of aminoacetone, followed by reduction of the phenylazo group, hydrolysis and cyclization to 6-methyl-7,8-dihydropterin. This latter intermediate was then oxidized with potassium permanganate to 6-methylpterin, which was converted to (11) with selenium dioxide in acetic acid (63% in the last step) [36] (*Scheme 3.1*).

(3) By total synthesis

One of the earliest syntheses of (11) involved condensation of α-bromo-β,β-diethoxypropanal with 2,4,5-triamino-6-hydroxypyrimidine followed by oxidation of the resulting 5,6-dihydropterin (13) and hydrolysis of the acetal [37]. This early procedure is clearly equivocal, however, since the initial condensation reaction is known to lead to a mixture of regioisomers. The first



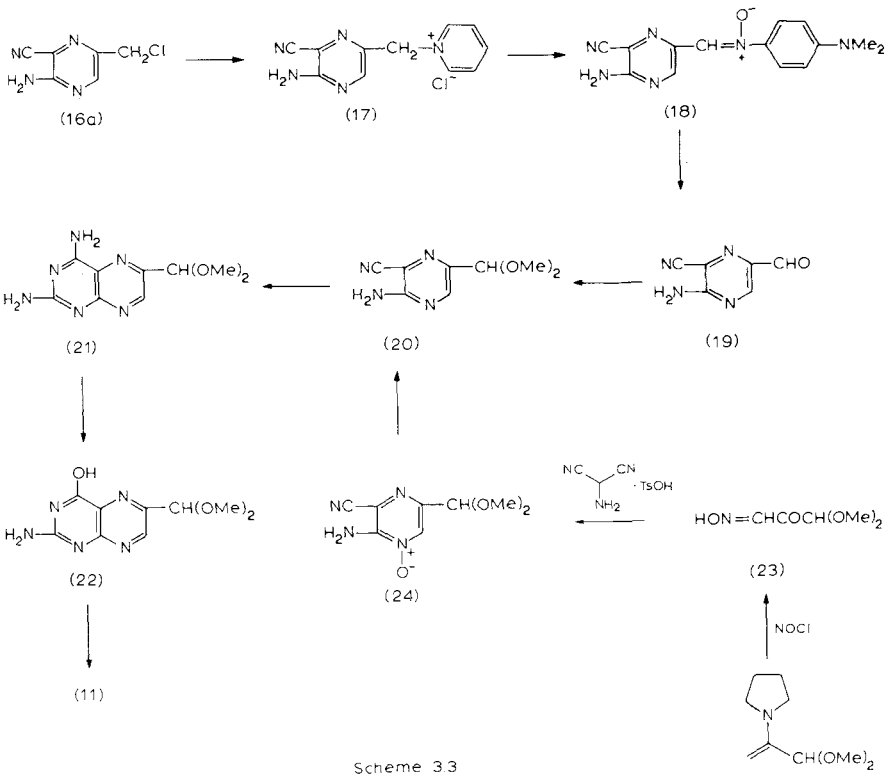
Scheme 3.1



Scheme 3.2

unambiguous synthesis of isomer-free (11) (see *Scheme 3.2*) involved condensation of ethyl α-aminocyanoacetate with diisonitrosoacetone to give ethyl 2-amino-5-oximinomethylpyrazine-3-carboxylate 1-oxide (14), which was cyclized with guanidine to pterin-6-carboxaldehyde oxime 8-oxide (15). Direct hydrolysis of the oxime was not possible, but conversion to (11) was achieved by reduction with sodium sulphite (which gave 6-aminomethylpterin) followed by oxidation with iodine [38].

The optimal synthetic route to isomer-free (11), however, is outlined in *Scheme 3.3* [39], and again exploits the above unequivocal approach to pteridine synthesis involving cyclization of unambiguously prepared pyrazine intermediates. Thus, 2-amino-3-cyano-5-chloromethylpyrazine (16a) (see below) was converted to the pyridinium salt (17), which was then condensed with *p*-nitrosodimethylaniline to give the nitrone (18) (the Kröhnke procedure for aldehyde synthesis). Acid hydrolysis gave 2-amino-3-cyanopyrazine-5-carbaldehyde (19). The corresponding dimethylacetal (20) was cyclized (with-



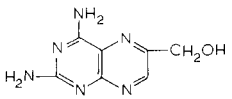
Scheme 3.3

out isolation) to 2,4-diamino-5-formylpterin dimethylacetal (21) with guanidine in anhydrous methanol; brief treatment with 5% sodium hydroxide gave the dimethylacetal of 6-formylpterin (22), which was converted to (11) with formic or trifluoroacetic acid. An alternative unequivocal route to the dimethylacetal (20) of 2-amino-3-cyanopyrazine-5-carboxaldehyde which, however, proceeded in significantly lower overall yield, involved the reaction of 3,3-dimethoxy-1-pyrrolidinopropene with nitrosyl chloride; addition of aminomalononitrile tosylate to the resulting oxime (23) gave 2-amino-3-cyano-5-(dimethoxymethyl)pyrazine 1-oxide (24), which was deoxygenated to (20) with trimethyl phosphite [40].

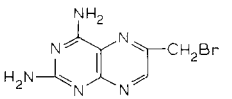
2,4-DIAMINO-6-HYDROXYMETHYLPTERIDINE (25) AND 2,4-DIAMINO-6-BROMOMETHYLPTERIDINE (26)

Almost all of the syntheses of (25) involve condensation of 2,4,5,6-tetraaminopyrimidine with dihydroxyacetone [41]. This condensation, however, also yields varying amounts of 2,4-diamino-6-methylpteridine, as well as the 7-substituted regioisomers of both the hydroxymethyl and methyl compounds; separation of pure, isomer-free (25) from the resulting mixture is a formidable undertaking. Despite various claims that subtle adjustments of pH, or the presence or absence of various buffers, favour formation of the desired 6-regioisomer, this synthetic approach cannot be considered reliable.

2,4-Diamino-6-bromomethylpteridine (26) has been prepared from the hydroxymethyl derivative either with HBr, or preferably by initial conversion to its HBr salt, followed by treatment with dibromotriphenylphosphorane in DMA. Under these conditions, both amino groups are converted to triphenylphosphilimines, which undergo hydrolysis upon acidic work-up [42].



(25)

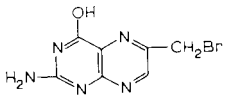


(26)

6-BROMOMETHYLPTERIN (27)

It has been claimed in a patent that this compound may be prepared by condensation of 2,4,5-triamino-6-hydroxypyrimidine with 2,3-dibromopro-

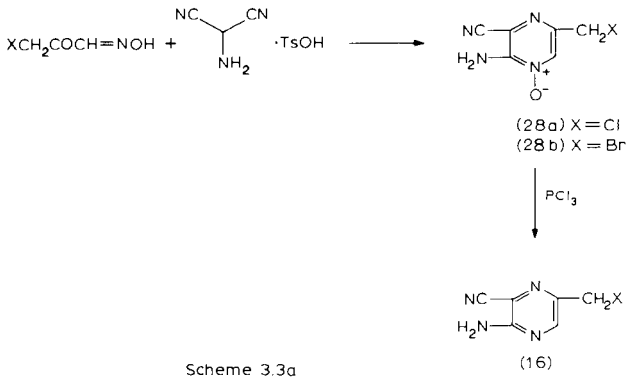
pionaldehyde [43]. However, not only is the product of this condensation impure (only 70% purity was claimed), but the crude product consists of a mixture of regioisomers. Alternatively, some monobromination of 6-methylpterin (12) has been achieved utilizing bromine in a sealed tube in the absence of solvent at 100–150 °C [44], or with bromine in 48% HBr [45]. The major product in each of these reactions, however, was the dibromo derivative. Monobromination of (12) also apparently takes place with NBS in the presence of benzoyl peroxide [46].



(27)

2-AMINO-3-CYANO-5-HALOMETHYLPYRAZINES (16a,b)

The above problems of concomitant formation of regioisomers (as well as products resulting from disproportionation of various dihydro intermediates) have been successfully surmounted by an unequivocal synthesis of 2-amino-3-cyano-5-halomethylpyrazines (16a,b) and their corresponding 1-oxides (28a,b), which can then be utilized for the construction of isomer-free 6-substituted pteridines and pterins (*Scheme 3.3a*). Thus, condensation of β-chloropyruvaldoxime or β-bromopyruvaldoxime, derived from diketene by chlorination or bromination, respectively, with aminomalononitrile tosylate in 2-propanol gives the 2-amino-3-cyano-5-halomethylpyrazine 1-oxides (28a) and (28b). Deoxygenation to (16a) and (16b) is readily accomplished with phosphorus trichloride [47].



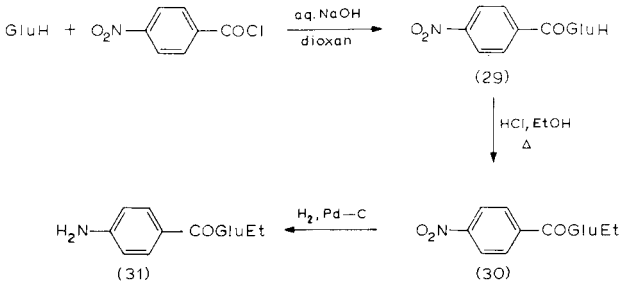
(16)

Scheme 3.3a

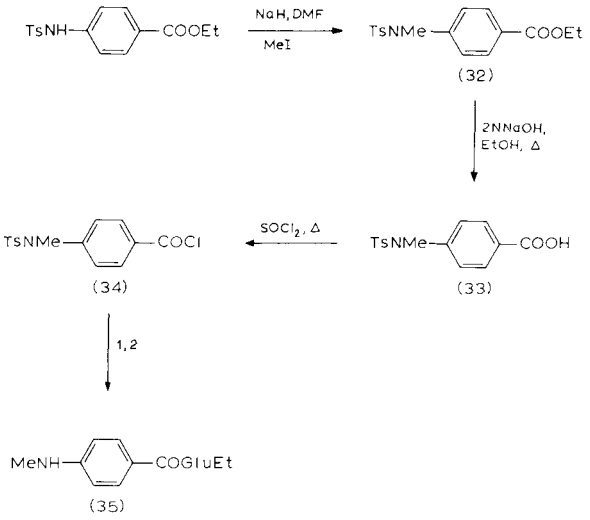
p-SUBSTITUTED BENZOYL-L-GLUTAMATES

Diethyl p-aminobenzoyl-L-glutamate (31)

Reaction of L-glutamic acid with *p*-nitrobenzoyl chloride under Schotten–Bauman conditions gave (29), from which the diester (30) was obtained using refluxing 5% ethanolic HCl. Catalytic reduction of the nitro group then yielded (31) [48] (*Scheme 3.4*).



Scheme 3.4



1. GluEt · HCl, $KHCO_3$, CH_2Cl_2 — H_2O; 2. 30% HBr — gl. AcOH

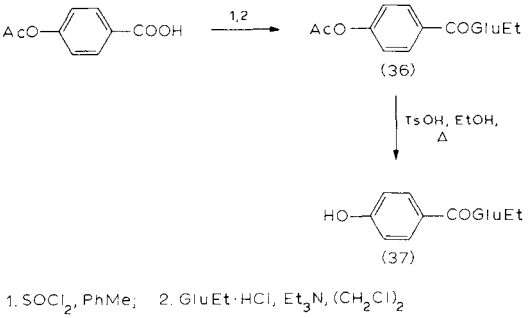
Scheme 3.5

Diethyl p-methylaminobenzoyl-L-glutamate (35)

Santi has described a high-yield synthesis (59.2% overall) of (35). The known ethyl *N*-tosyl-*p*-aminobenzoate [49] was first alkylated to generate (32). Saponification of (32) gave (33), which was converted to (34) with thionyl chloride. Acylation of diethyl L-glutamate with (34) in a two-phase system followed by cleavage of the tosyl protecting group with HBr in glacial HOAc gave (35) [50] (*Scheme 3.5*).

Diethyl p-hydroxybenzoyl-L-glutamate (37)

Treatment of *p*-acetoxybenzoic acid with thionyl chloride generated the acid chloride, which was used without purification to acylate diethyl L-glutamate. Deacetylation of the resulting triester (36) gave (37) [51] (*Scheme 3.6*).

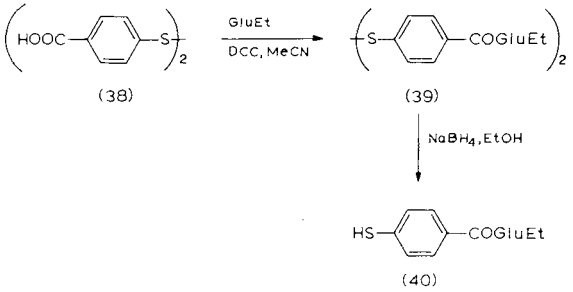


Scheme 3.6

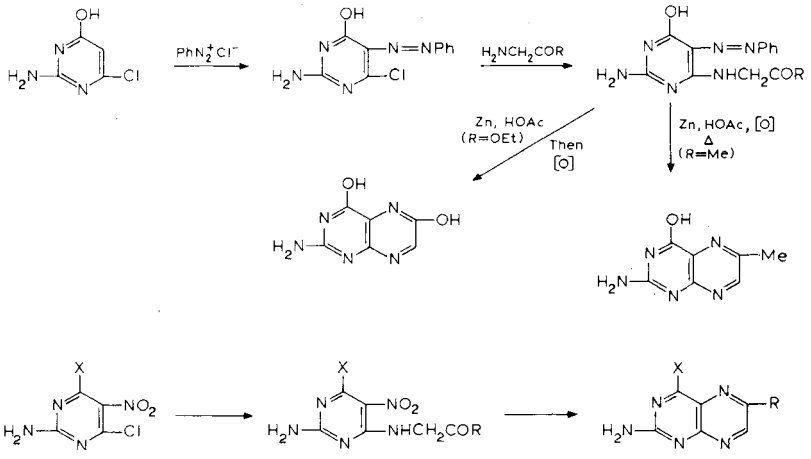
Diethyl p-mercaptobenzoyl-L-glutamate (40)

Condensation of 4,4-dithiobis(benzoic acid) (38) [52] with diethyl L-glutamate in the presence of DCC produced (39), from which (40) was readily obtained by reduction with sodium borohydride [53] (*Scheme 3.7*).

The need for an unambiguous synthesis of 6-substituted pteridines was early recognized by Boon and Leigh [54]. The key step of their synthetic approach involves condensation of 5-arylazo-4-chloropyrimidines with α-aminoketones or esters, followed by reduction (usually with Zn/HOAc) and thermal cyclization (see *Scheme 3.8*). The phenylazo moiety serves both as a precursor of the 5-amino group, and as an activating group for the critical nucleophilic displacement of the *ortho*-situated chloro group. A common extension of the



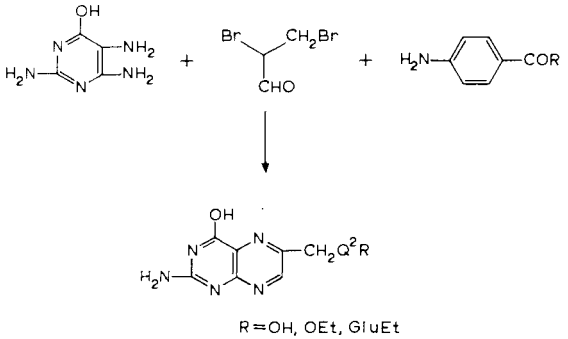
Scheme 3.7



Scheme 3.8

Boon and Leigh procedure involves use of more readily available (and more reactive) 5-nitro-4-chloropyrimidines.

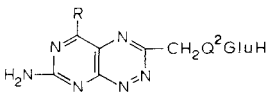
An early synthesis of folic acid developed by chemists at American Cyanamid involved condensation of 2,4,5-triamino-6-hydroxypyrimidine, 2,3-dibromopropionaldehyde and *p*-aminobenzoylglutamic acid (*Scheme 3.9*) [55a–d].This 'three-component' procedure has become known as the Waller condensation, and has been widely employed for the synthesis of analogues because of the ease with which the various components of the condensation can be varied. Products from this 'three-component' reaction, however, are mixtures of 6- and 7-substituted regioisomers.



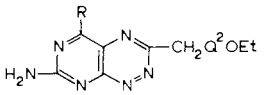
Scheme 3.9

7-AZA ANALOGUES

As part of a long-standing programme directed towards the synthesis of DHFR and TS inhibitors, workers at the Southern Research Institute have reported the preparation of 7-azaFA (41), 7-azaAP (42) and the corresponding thione (43). Patterned after their successful preparation of the 7-azapteroates (44a–c)

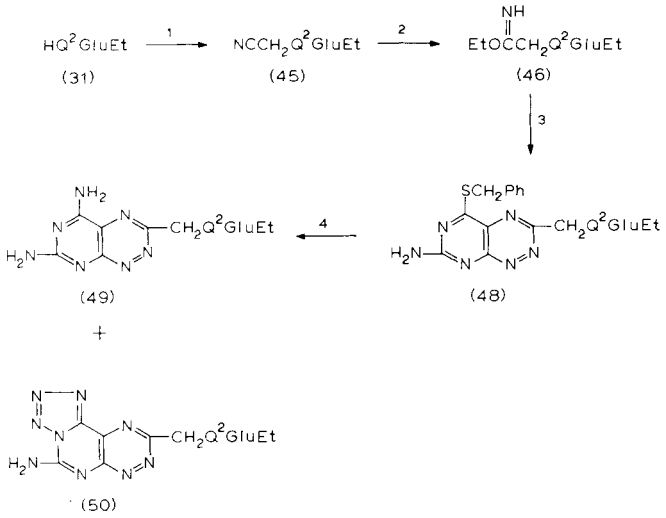


(41) R=OH
(42) R=NH_2
(43) R=SH



(44a) R=OH
(44b) R=NH_2
(44c) R=SH

[56a], the first approach to the key intermediate (48) is outlined in *Scheme 3.10* [56b]. Alkylation of diethyl *p*-aminobenzoylglutamate (31) with cyanomethyl *p*-toluenesulphonate [57a,b] in refluxing dioxan furnished the aminonitrile (45), which was converted to the known imino ether (46) [58] in the usual manner. This was not isolated, but reacted *in situ* with 2,5-diamino-4-benzylthio-6-hydrazinopyrimidine (47) [59] to produce (48). Hydrolysis of the benzylthio group with $KHCO_3$ in aqueous DMSO followed by saponification using oxygen-free NaOH at room temperature afforded (41) in 30.5% yield, isolated as the sesquihydrate [57]. Treatment of (48) with NaSH/EtOH followed by saponification as before yielded the corresponding thione (43) in *ca.* 37% yield



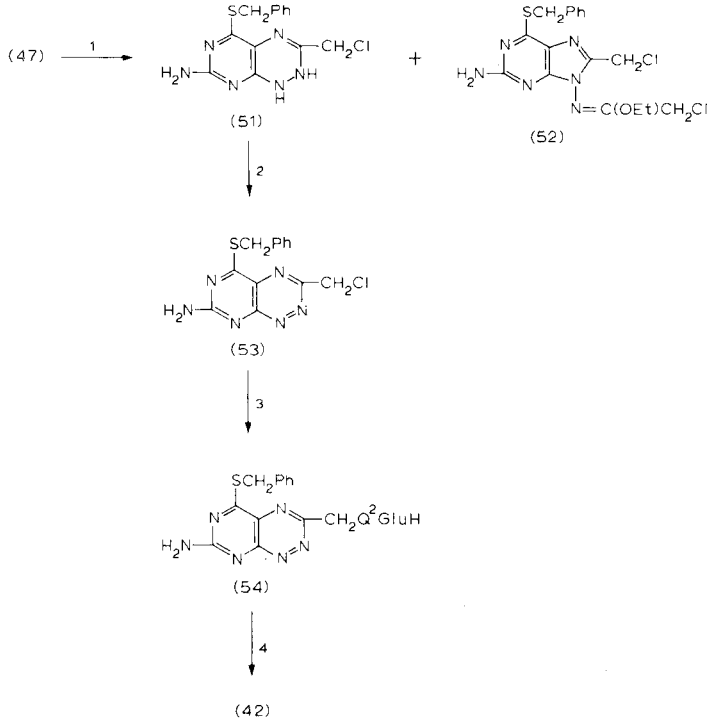
1. $NCCH_2OTs$, dioxan, Δ; 2. NaOEt, EtOH; 3. 2,5-diamino-4-hydrazino-6-benzylthiopyrimidine (47), EtOH, dioxan, r.t.; 4. NaN_3, aq. DMSO, 80°C.

Scheme 3.10

[60]. To avoid amidation of the esters during aminolysis of (48) (10% NH_3/EtOH), the benzylthio group was reacted with sodium azide in aqueous DMSO, which gave a separable mixture (*ca.* 2:1) of (49) and (50) [61]. Unfortunately, attempted saponification of (49) using oxygen-free NaOH gave little evidence for the presence of (42) by ^{1}H-NMR.

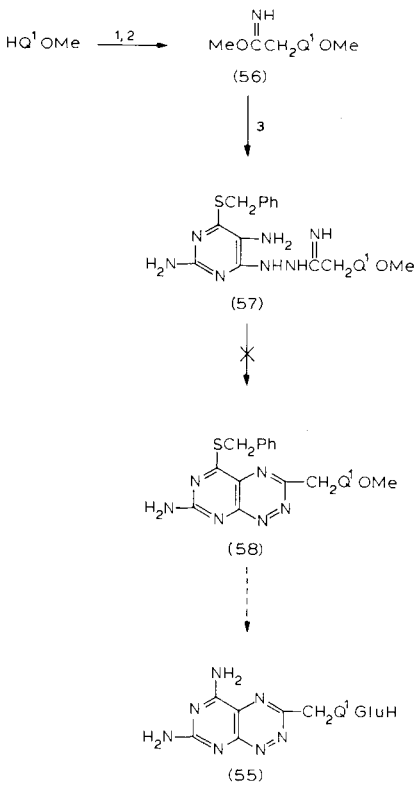
The successful synthesis of (42) was accomplished by the Southern Research Institute group about 1 year later by employing the complementary strategy shown in *Scheme 3.11* [62]. Thus condensation of (47) with ethyl ortho(chloro)acetate [63] gave a separable mixture of the dihydro 7-azapteridine (51) and the purine (52). Oxidation of (51) with silver oxide yielded the desired 7-azapteridine (53), which was treated with *p*-aminobenzoyl-L-glutamic acid under Finkelstein conditions to give (54). Incorporation of the 4-amino group was accomplished with sodium azide, thus completing the synthesis of (42).

Interestingly, attempts to adapt either *Scheme 3.10* or *Scheme 3.11* to the preparation of 7-azaMTX (55) were uniformly unsuccessful (*Scheme 3.12*). In the former case, the alkylation product of methyl *p*-(methylamino)benzoate [64] with cyanomethyl tosylate was converted to the imino ether (56) as previously described for (46). Reaction of (56) with (47) produced none of the



1. $ClCH_2C(OEt)_3$, HCl, r.t.; 2. Ag_2O, dioxan, r.t.; 3. KI, DMA, HQ^2GluH; 4. NaN_3, DMSO, 90 °C.

Scheme 3.11



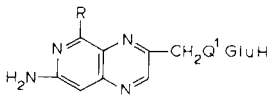
1. $NCCH_2OTs$; 2. NaOMe, MeOH; 3. (47), EtOH, dioxan

Scheme 3.12

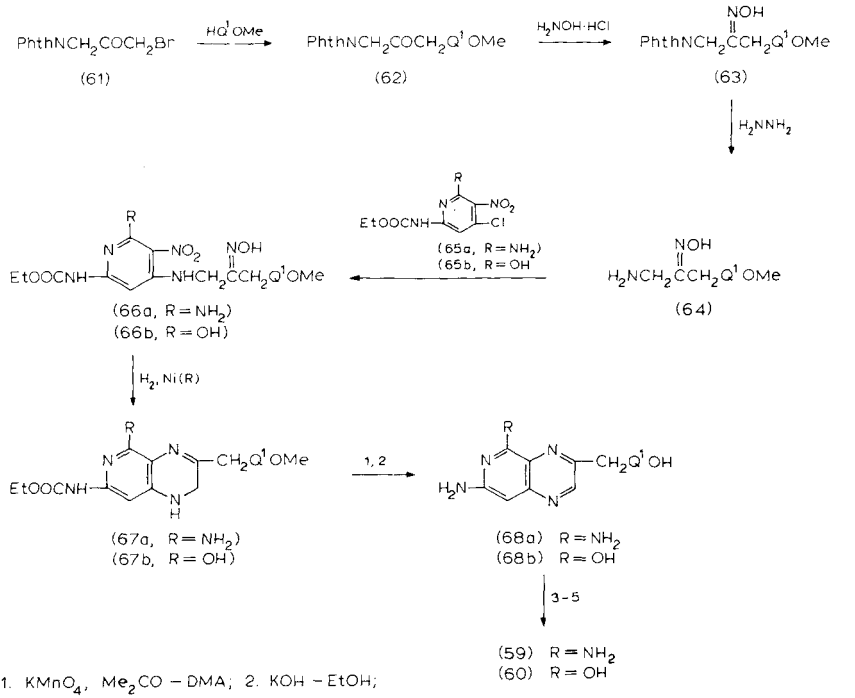
expected 7-azapteridine (58). The product isolated analyzed closely for the amidrazone (57), which could not be cyclized. In the latter case, all attempts to react methyl *p*-methylaminobenzoate with (53) were unsuccessful. There was no evidence of displacement of either the chloro or the benzylthio group [65]. Thus, 7-azaMTX (55) remains, to date, an elusive compound.

1-DEAZA ANALOGUES

Of the many thousands of derivatives of AP-FA-MTX prepared to date, several deaza analogues are among the most active and clinically most promising compounds known. Montgomery and co-workers have prepared the only representative 1-deaza analogues, namely 1-deazaMTX (59) and 1-deaza-N^{10}-methylFA (60) [66, 67]. Their approach, adapted from the Boon–Leigh synthesis of 6-substituted pteridines [54], is outlined in *Scheme 3.13*. Thus, methyl *p*-(methylamino)benzoate [64] was alkylated with *N*-3-(bromoacetonyl)phthalimide (61) [68] to give (62), which was converted to a mixture of *syn* and *anti* oximes (63) upon treatment with hydroxylamine hydrochloride. Hydrazinolysis of the phthaloyl group furnished the key amino oxime (64) [69], which, upon arylation with ethyl 6-amino-4-chloro-5-nitro-2-pyridinecarbamate (65a, R = NH_2) [70], yielded the triaminopyrimidine (66a, R = NH_2). Hydrogenation of (66a) with H_2/Ni(R) proved to be the most expedient approach to the dihydro-1-deazapteridine (67a, R = NH_2). Oxime hydrolysis followed by *in situ* hydrogenation of the nitro group with concomitant cyclization and subsequent dehydration also furnished (67a, R = NH_2) in good yields on a small scale. However, upon scale-up, hydrolysis of (66a, R = NH_2) yielded the corresponding ketone in only 27% yield. This low yield was shown



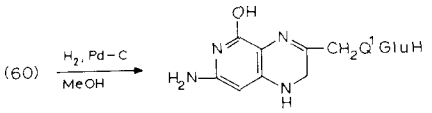
(59) R=NH_2
(60) R=OH



Scheme 3.13

to result from cleavage of the acetonyl side-chain via a Beckmann rearrangement. Careful oxidation of (67a, R = NH_2) with $KMnO_4$-acetone-DMA followed by alkaline hydrolysis afforded (68a, R = NH_2) in excellent yield. The synthesis of (59) was completed by acetylation with acetic anhydride, peptide coupling with GluEt/DCC and base hydrolysis. It should be noted that attempts to prepare 1-deazaAP by this route failed. Extensive decomposition of the AP analogue of (68a, R = NH_2) (NH vs. NMe, i.e., Q^2 vs. Q^1) was observed upon alkaline hydrolysis of the aromatic precursor.

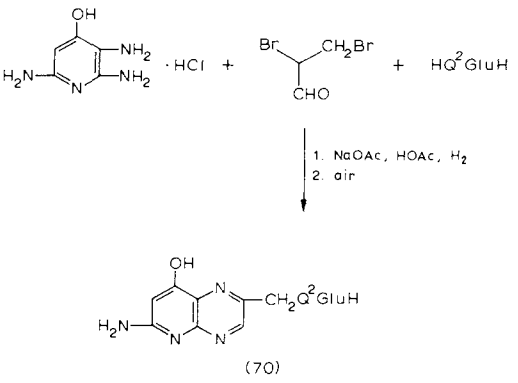
This same sequence, however, has been used successfully to prepare (60) [67]. Conversion of (65a, R = NH_2) to the corresponding pyridone (65b, R = OH) was accomplished with isoamyl nitrite-H_2SO_4. Arylation of (64) with (65b, R = OH) yielded (66b, R = OH) from which (60) was obtained as described (*vide supra*). Catalytic reduction of (60) then gave the 7,8-dihydro derivative (69).



(69)

3-DEAZA ANALOGUES

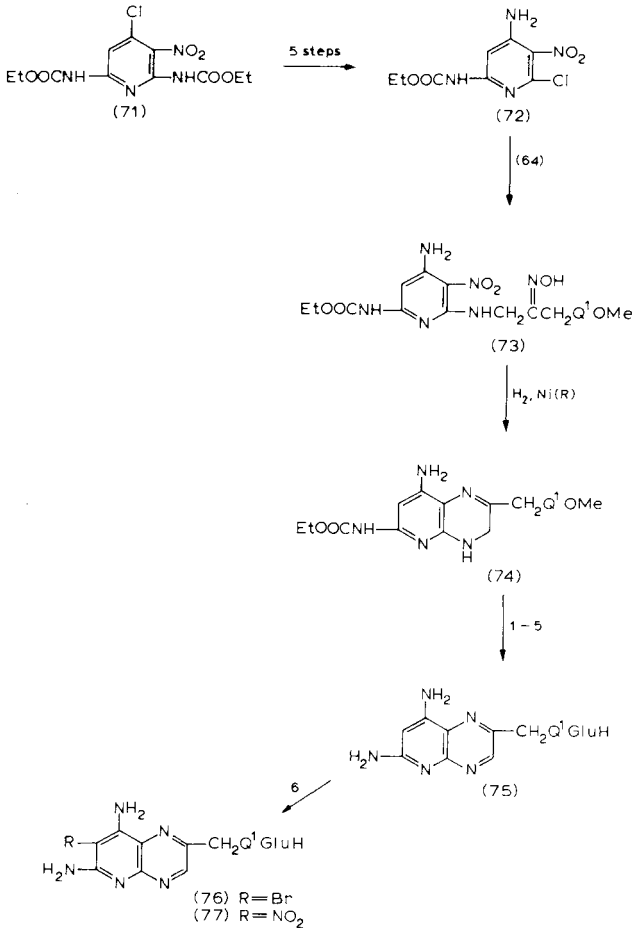
The first synthesis of a 3-deaza analogue, 3-deazaFA (70), was accomplished, albeit in poor yield, via the Waller condensation of 2,3,6-triamino-4-hydroxypyridine hydrochloride, 2,3-dibromopropionaldehyde and *p*-aminobenzoyl-L-glutamic acid, as shown in *Scheme 3.14*. This approach suffered from its equivocal nature as well as from a lengthy work-up involving repeated centrifugations and column chromatographic separations which were necessary to obtain (70) sufficiently pure to be characterized and evaluated [71].



(70)

Scheme 3.14

The only other member of this series, 3-deazaMTX (75), was prepared as shown in *Scheme 3.15*. The requisite chloronitropyridine (72), isomeric with (65a, R = NH_2), was readily available from the bisurethane (71) in five steps [70, 72]. Arylation of (64) with (72) gave (73), which was reduced to the 7,8-dihydro-3-deaza-N^{10}-methylpteroate (74). Conversion of (74) to (75) was accomplished in the same manner as described for the synthesis of (59) from (67a, R = NH_2) [66]. Bromination or nitration of (75) produced the corresponding 3-substituted 3-deaza analogues (76) and (77) in excellent yield [73].

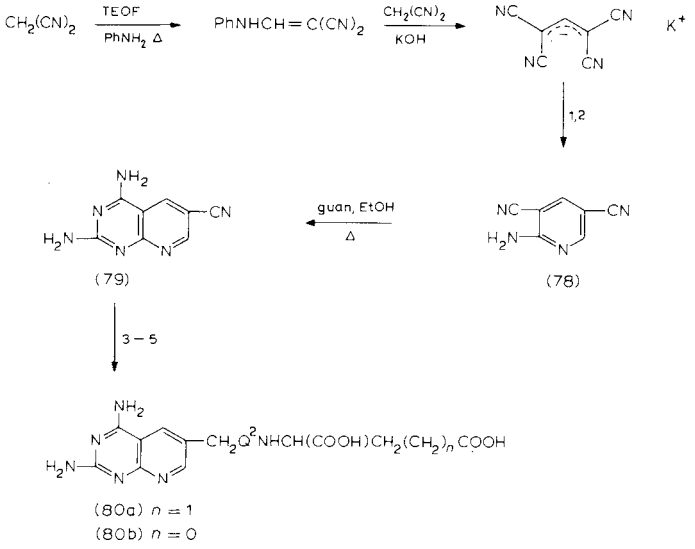


1. $KMnO_4$, Me_2CO; 2. KOH – EtOH; 3. Ac_2O;
4. GluEt, DCC; 5. NaOH; 6. Br_2 – HOAc – HCl or HNO_3, H_2SO_4.

Scheme 3.15

5-DEAZA ANALOGUES

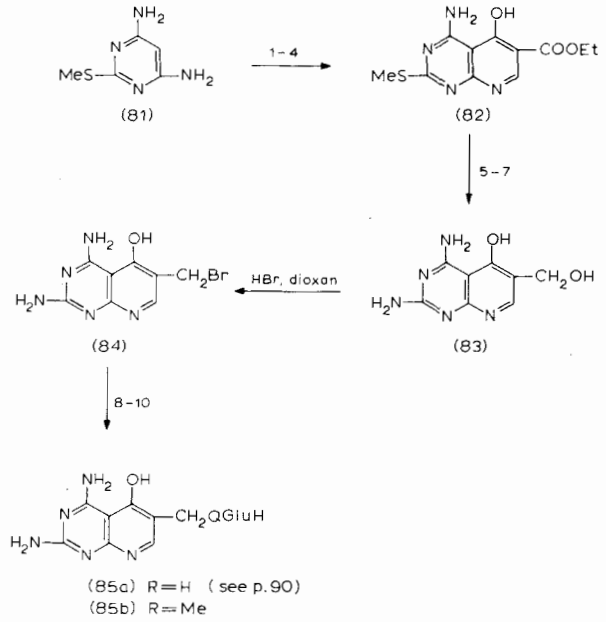
A group at Parke-Davis reported the preparation of 5-deazaAP (80a) in 1974 [74, 75]. This was the first synthesis of a member of this class of DHFR inhibitor. As shown in *Scheme 3.16*, the key intermediate, 2,4-diamino-5-deazapteridine-6-carbonitrile (79), was constructed in a straightforward manner in five steps from malononitrile. The use of chloroformamidine for the conversion of (78) to (79) proved to be less satisfactory than the (traditional) use of guanidine.



1. HCl, Me_2CO; 2. H_2, $PdCl_2$, Et_3N, DMF; 3. H_2, Ni(R)–HOAc; $HQ^2NHCH(COOEt)CH_2$–$(CH_2)_n$ COOEt, $n = 0,1$; 4. 2 M NaOH; 5. 1 M HCl.

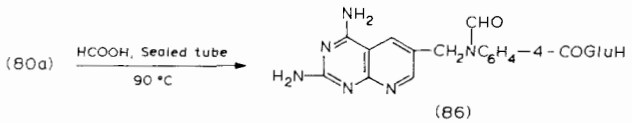
Scheme 3.16

About 6 years later, Broom and Srinivasan [76] described the synthesis of the 5-deaza-5-oxo analogues ((85a) and (85b), respectively) of AP and MTX (*Scheme 3.17*). Beginning with ethyl 4-amino-2-methylthio-5-oxopyrido-[2,3-*d*]pyrimidine-6-carboxylate (82), readily available in four steps from 2-methylthio-4,6-diaminopyrimidine (81) [77], they prepared the diaminoalcohol (83) in three steps. Conversion of (83) to the bromomethyl compound (84) was followed by reaction with (31) or (35) and saponification to generate the target compounds (85a) and (85b). At about the same time, it was reported that (80a)



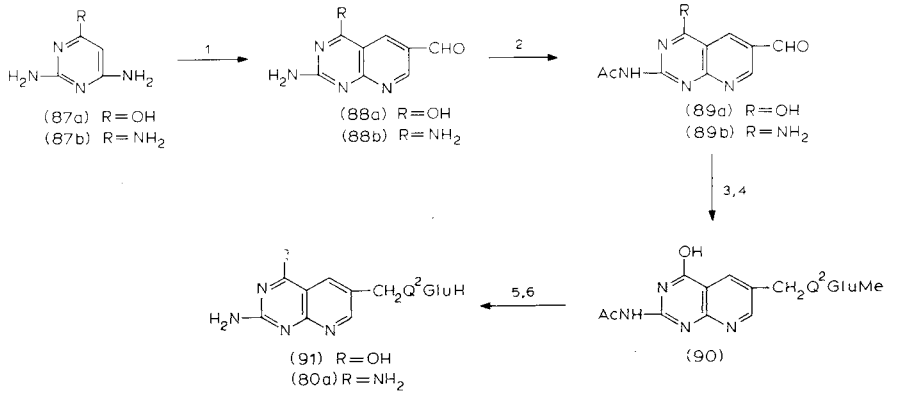
1. $EtOCH{=}C(COOEt)_2$; 2. Ac_2O; 3. Dowtherm A, Δ; 4. NaOEt; 5. LAH–THF; 6. mcpba; 7. liq NH_3, r.t; 8. HQ^2GluEt – or HQ^1GluEt – DMF; 9. 1 M NaOH; 10. 0.2 M HCl.

Scheme 3.17



could be converted to 10-formyl-5-deazaAP (86) using excess formic acid at 90 °C [78].

A Princeton group announced the first synthesis of L-5-deazaFA (91) via a path which to date remains the shortest and most direct route to this series [79, 80]. In one step, reaction of 2,4-diamino-6-hydroxypyrimidine (87a) with purified triformylmethane [81a,b] produced 2-amino-5-deazapterin-6-carboxaldehyde (88a), which was characterized as the acetamide (89a). Reaction of (89a) with dimethyl *p*-aminobenzoyl-L-glutamate in glacial HOAc afforded an intermediate imine which was reduced *in situ* with borane-triethylamine, yielding (90). Saponification of the amide and esters then completed the synthesis of (91) in 62% yield from (88a) (*Scheme 3.18*). Adaptation of this

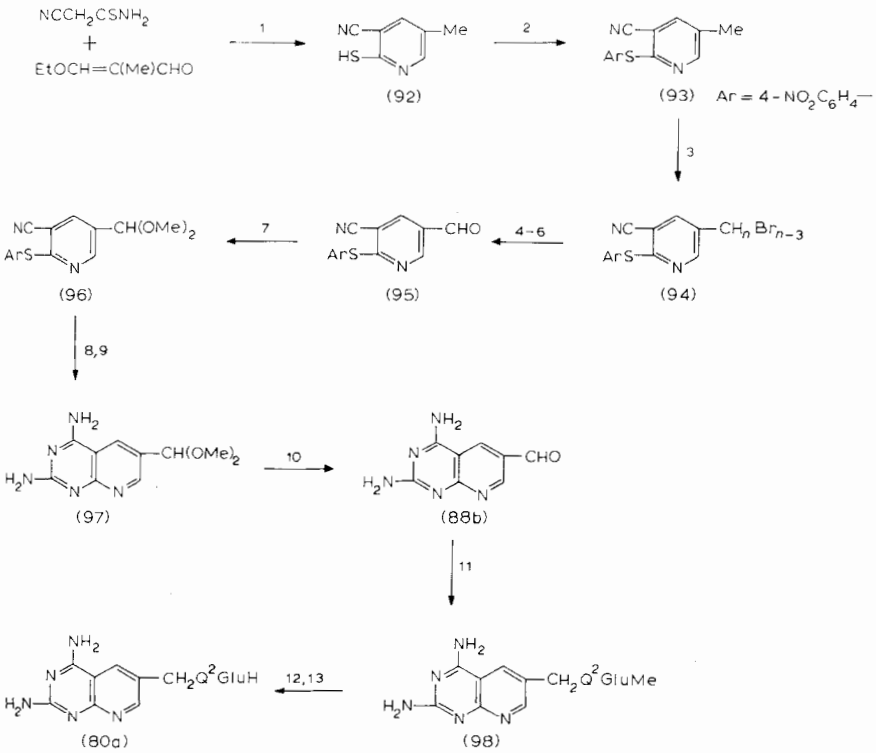


1. $CH(CHO)_3$, DMF, 55 °C; 2. Ac_2O; 3. HQ^2GluMe, HOAc; 4. BH_3, Et_3N; 5. 0·1 M NaOH; 6. 3 M HCl.

Scheme 3.18

strategy to the preparation of (80a) was unsuccessful. An attempt to generate the key intermediate (88b) from pure triformylmethane and 2,4,6-triaminopyrimidine (87b) failed, although a successful synthesis of (88b) from (87b) and impure triformylmethane has been described [82]. These failures necessitated an alternative strategy for the preparation of (80a) as shown in *Scheme 3.19* [80, 83]. Base-catalysed condensation of α-cyanothioacetamide with 3-ethoxymethacrolein gave the 2(1*H*)-pyridinethione (92), which was smoothly arylated on sulphur with *p*-nitrofluorobenzene. Free-radical bromination of (93) generated a complex mixture of mono-, di- and tribrominated products (94), from which the desired monobrominated material was isolated as the crystalline pyridinium salt. This, subjected to the Kröhnke procedure, yielded aldehyde (95) which was protected as its dimethylacetal. Subsequent ammonolysis of the arylthio group followed by guanidine cyclization gave (97), which, after brief exposure to 88% formic acid, afforded (88b). Conversion of (88b) to (80a) was achieved via reductive amination and saponification.

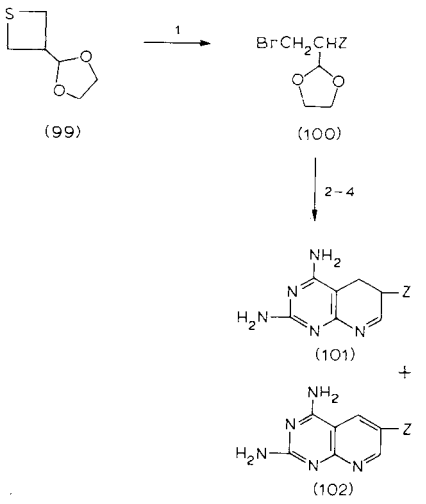
An attractive alternative strategy (*Scheme 3.20*) for the synthesis of (88b) focused on the conversion of a 3-formylthietane acetal (99) to an unsymmetrically substituted isobutyraldehyde acetal (100) via alkylative ring opening with *p*-methoxybenzyl bromide. Several subsequent steps produced a mixture of (101) and (102) which could not be fully aromatized to (102) owing to extraordinary insolubility, which, unfortunately, ultimately precluded synthesis of (88b) by this route.



1. $Me_2N(CH_2)_2OH$, EtOH; 2. 4-fluoronitrobenzene, Na_2CO_3, DMF; 3. NBS, PhH, DBP; 4. Pyridine; 5. $4\text{-}NOC_6H_4NMe_2$, K_2CO_3; 6. HCl; 7. Dowex 50W-X4, MeOH; 8. NH_3, $CuBr_2$, sealed tube; 9. guan, MeOH; 10. 88% HCOOH; 11. HQ^2GluMe, $NaBH_3CN$, HCl; 12. 0.47 M NaOH; 13. 0.47 M HCl.

Scheme 3.19

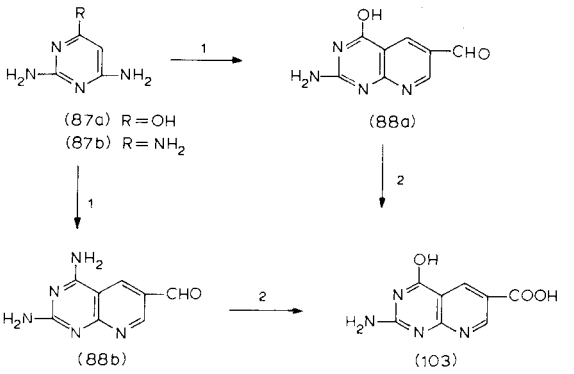
Compounds (80a), (91), 5-deazaMTX (104) and N^{10}-methyl-5-deazaFA (105) have been prepared by Montgomery's group via a route very similar to that described in *Scheme 3.18* [82]. In their approach, they reported that crude triformylmethane, i.e., $CH[CH=N^+Me_2]_3 \cdot 3Cl^-$ or its partially hydrolyzed derivatives could be employed in condensation reactions with (87a) or (87b), respectively, to yield either (88a) or (88b) directly. To establish the structures of these products unambiguously, both were converted ultimately to the known acid (103) as shown in *Scheme 3.21*. Reductive alkylation of (88b) with (31) using hydrogen and Raney nickel in 70% acetic acid, followed by saponification with NaOH/aq. DMSO, gave (80a). Methylation of (80a) then gave 5-deazaMTX (104). A similar reaction sequence using (88a) afforded (91)



1. 4-$MeOC_6H_4CH_2Br$, MeCN, r.t.; 2. NaI–$CH_2(CN)_2$, NaH–DMSO; 3. guan, EtOH; 4. 1 M HCl; Z = 4-$MeOC_6H_4CH_2SCH_2$

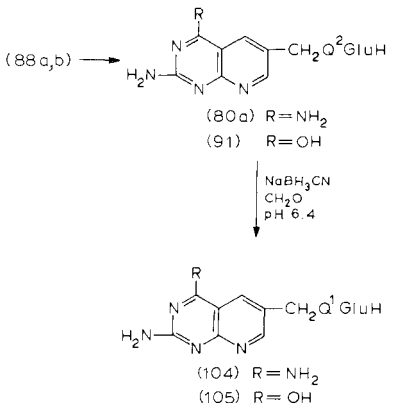
Scheme 3.20

which was methylated as before to give N^{10}-methyl-5-deazaFA (105). Although (91) could be produced directly, it was found to be more expedient to prepare it from the diethyl ester of 5-deazaAP using oxygen-free 1 M NaOH at reflux, which is noteworthy, since there was no evidence of any racemization in the product.



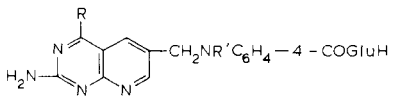
1. $CH(CH{=}\overset{+}{N}Me_2)_3 \cdot 3Cl^-$; 2. $KMnO_4$, OH^-

Scheme 3.21 (continued on p.111)

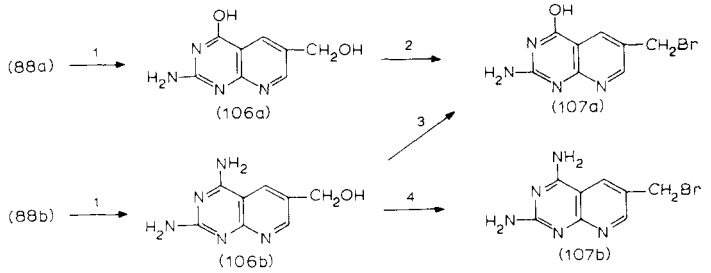


Scheme 3.21 (continued)

A recent patent issued to a Southern Research Institute group details the synthesis of (80a), (104) and N-10-substituted analogues as well as (91), (105) and N-10-substituted analogues [84] (*Scheme 3.21a*). The preparation of (80a), (104), (91) and (105) has already been described (*vide supra*). The



	R	R'		R	R'
(80a)	NH_2	H	(91)	OH	H
(104)	NH_2	Me	(105)	OH	Me
(104a)	NH_2	Et	(105a)	OH	Et
(104b)	NH_2	Pr	(105b)	OH	Pr
(104c)	NH_2	$CH_2CH=CH_2$	(105c)	OH	$CH_2CH=CH_2$
(104d)	NH_2	$CH_2C\equiv CH$	(105d)	OH	$CH_2C\equiv CH$

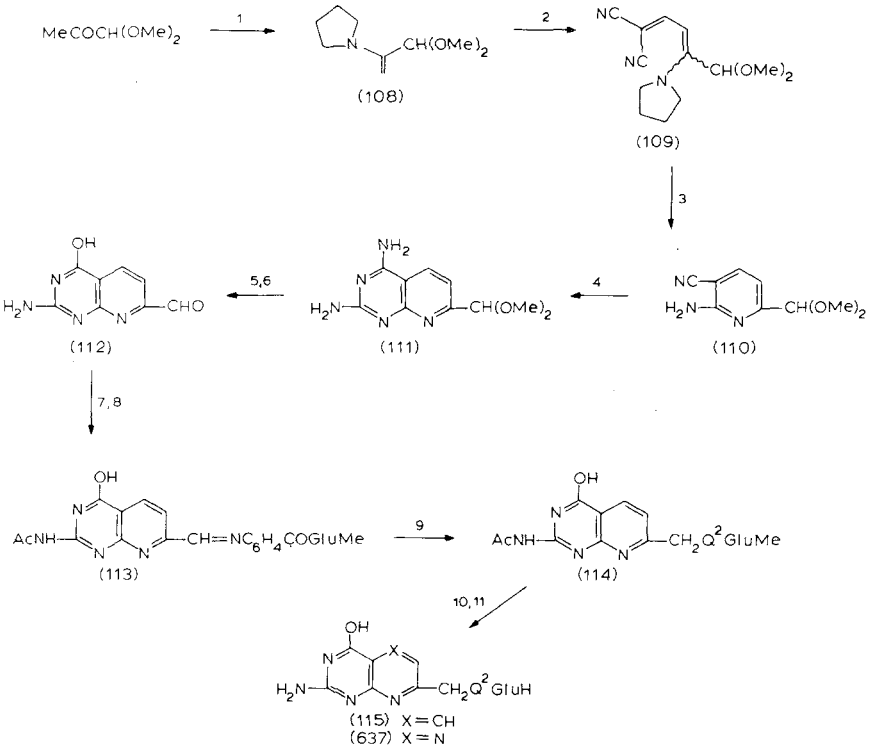


1. $NaBH_4$; 2. PBr_3; 3. 48% HBr; 4. Ph_3PBr_2.

Scheme 3.21a

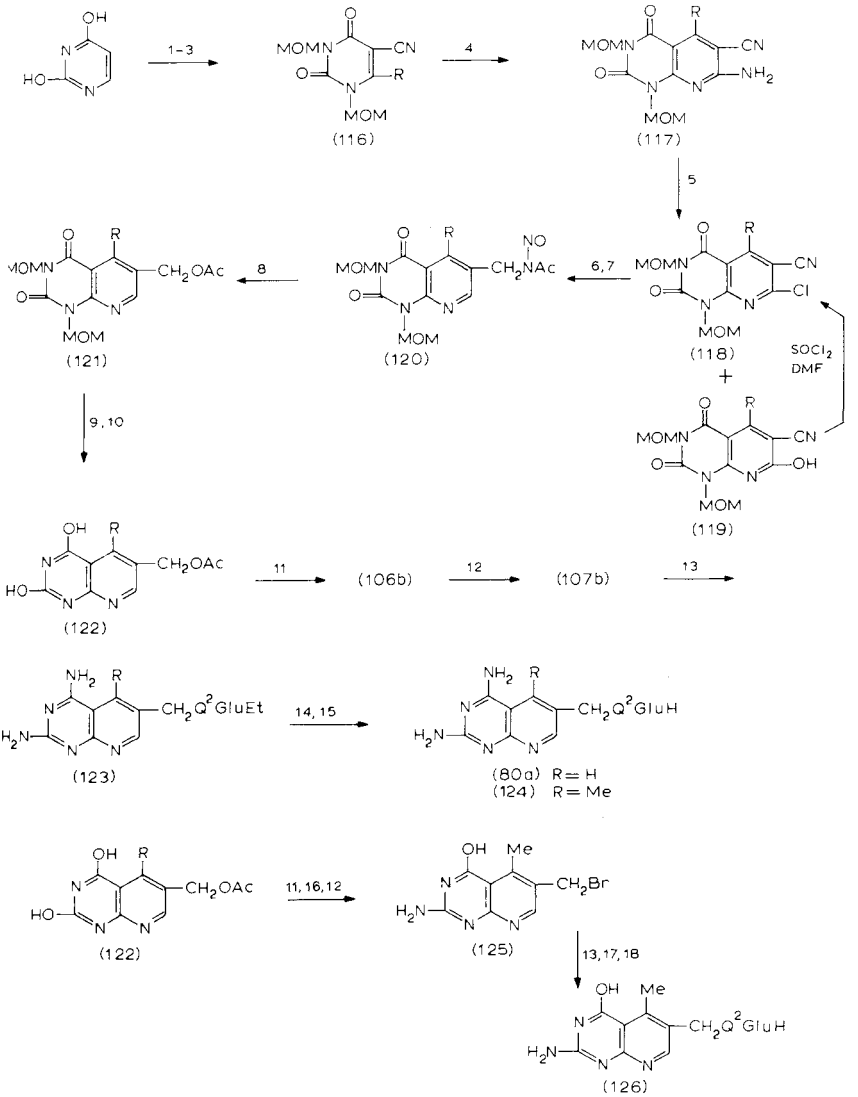
remaining derivatives (105a–d) and (104a–d) were prepared via alkylation of the corresponding *p*-(*N*-substituted amino)benzoyl glutamate esters [85a,b] with (107a) or (107b), followed by saponification. The bromomethyl compounds were, in turn, available from (88a) and (88b) via sodium borohydride reduction to the corresponding carbinols (106a) and (106b) and subsequent reaction with phosphorus tribromide [86], 48% hydrobromic acid [87], or dibromotriphenylphosphorane [42].

The synthetic routes to *N*-2′-acetyl-7-FA (643) described by Taylor and Dumas (*vide infra*) were readily adapted to the preparation of 5-deaza-7-FA (115) (*Scheme 3.22*) [40]. Reaction of pyruvaldehyde dimethylacetal with pyrrolidine using conditions described by Zoretic, Barcelos and Branchard



1. Pyrrolidine, $MgSO_4$, Et_2O; 2. $(NC)_2C{=}CHOMe$, THF; 3. NH_3, MeOH; 4. guan, BuOH, Δ; 5. NaOH, Δ; 6. HCl, Δ; 7. Ac_2O; 8. HQ^2GluMe; 9. $NaBH_4$; 10. 0.1 M NaOH; 11. 6 M HCl.

Scheme 3.22



1. $ClCH_2OMe$, K_2CO_3, DMF; 2. Br_2, HOAc; 3. NaCN, DMF, Δ; 4. $CH_2(CN)_2$, NaOEt, EtOH; 5. $NaNO_2$, HCl, Δ; 6. H_2, Ni(R), Ac_2O; 7. $NaNO_2$, Ac_2O, HOAc; 8. Ac_2O, Δ; 9. BCl_3, 10. Ac_2O, pyr; 11. $(Me_3Si)_2NH$, NH_3, TsOH; 12. HBr, dioxan; 13. (31), DMA; 14. NaOH, MeOH; 15. HCl; 16. 1 M NaOH, Δ; 17. NaOH; 18. HCl.

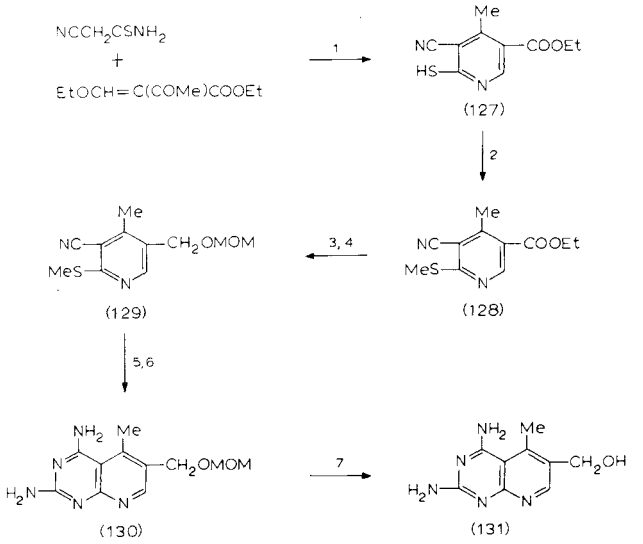
Scheme 3.23

[88] generated enamine (108), which was condensed with (methoxymethylene)malononitrile to provide (109) [89]. Methanolic ammonia smoothly transformed the butadiene to the 2,3,6-trisubstituted pyridine (110). Ring closure of the *o*-aminonitrile functionality in (110) required vigorous reaction conditions (guanidine, 1-butanol, reflux 24 h) to effect clean conversion to 2,4-diamino-5-deaza-7-dimethoxymethylpteridine (111). Sequential hydrolysis of the 4-amino group and the dimethylacetal then gave 5-deaza-7-formylpterin (112) in excellent yield. Acetylation of (112) and condensation with dimethyl *p*-aminobenzoyl-L-glutamate yielded the imine (113), which was reduced with sodium borohydride to afford (114) directly (this conversion involved the unexpected cleavage of the *N*-2′-acetyl group as well as imine reduction). Finally, saponification produced (115).

Very recently, a research group at the Sloan-Kettering Memorial Cancer Center reported a new approach to 5-deazaAP (80a), 5-deaza-5-methylAP (124) and 5-deaza-5-methylFA (126)[90, 91]. As an extension of their novel pyrimidine-to-pyridopyrimidine ring transformation [92] this strategy required four key steps – the pyrimidine-to-pyridopyrimidine ring transformation, removal of the 7-amino group, deprotection of the 1,3-*N*-methoxymethyl (MOM) substituents, and conversion of the resulting 2,4-dioxo derivative to the 2,4-diamino analogue (*Scheme 3.23*).

The starting protected 5-cyanouracil (116, R = H) [93] was obtained in a straightforward manner from uracil in three steps. Base-catalyzed reaction of (116) with malononitrile produced (117, R = H) in excellent yield. This transformation most probably proceeds via the ANRORC mechanism as described by van der Plas [94]. Diazotization of (117, R = H) produced a mixture of (118, R = H) and (119, R = H) from which the major component, (118, R = H) was isolated chromatographically. The minor component (119, R = H) was readily converted to (118, R = H) with thionyl chloride/DMF. Hydrogenolysis of the 7-chloro group with concomitant nitrile reduction gave the amine, isolated as the acetamide from which the *N*-nitroso derivative (120, R = H) was obtained in excellent yield using sodium nitrite in acetic anhydride. Refluxing (120, R = H) in acetic anhydride until nitrogen evolution ceased effected conversion to the acetate (121, R = H). Cleavage of the MOM protecting groups with BCl_3 followed by reacetylation furnished (122, R = H). Application of the recently reported silylation-amination procedure of Vorbrüggen and Krolikeiwicz [95] smoothly transformed (122, R = H) into 2,4-diamino-6-hydroxymethyl-5-deazapteridine (106b), from which the labile bromomethyl derivative (107b) was obtained with HBr in dioxane [77]. Alkylation of (31) with (107b) yielded (123, R = H) from which (80a) was obtained by saponification. The synthesis of 5-methyl-5-deazaAP (124) was accomplished in essentially the same manner starting with 5-cyano-6-methyluracil.

The preparation of 5-methyl-5-deazaFA (126) began with (122, R = Me), which was aminated as before, selectively hydrolyzed, and converted to 5-methyl-5-deaza-6-bromomethylpterin (125). Alkylation of (31) with (125) followed by saponification completed the synthesis of (126). An alternative route to 2,4-diamino-5-deaza-5-methyl-6-hydroxymethylpteridine (131) similar to previously described methods [74, 80] was also evaluated (*Scheme 3.24*).

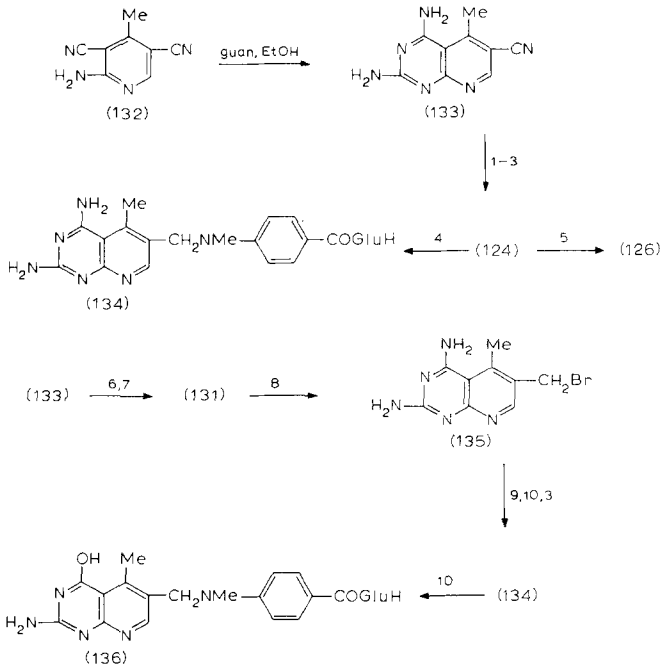


1. $Me_2NCH_2CH_2OH$, EtOH, Δ; 2. MeI, K_2CO_3, DMF; 3. LAH – Et_2O; 4. $ClCH_2OMe$, $PhNMe_2$; 5. mcpba; 6. guan, Ph_2O; 7. HCl – MeOH, Δ.

Scheme 3.24

Base-catalyzed condensation of cyanothioacetamide with ethyl β-(ethoxymethylene)acetoacetate furnished (127), which was quantitatively *S*-methylated to yield (128). Ester reduction and alcohol protection generated (129), which was oxidized to the corresponding sulphone with mcpba. Guanidine cyclization to (130) and deprotection then afforded (131), identical in all respects with material prepared by the previously described ring-transformation reaction.

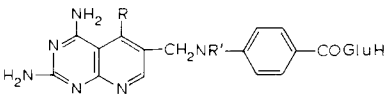
Chemists at the Southern Research Institute have also prepared (80a), (124) and (126), as well as 5-methyl-5-deazaMTX (134) and 5,N^{10}-dimethyl-5-deazaFA (136) [96]. They repeated Elslager's original synthesis of (80a) (see *Scheme 3.16*) with minor experimental changes, and subsequently found that



1. (31), H_2, Ni(R), HOAc; 2. NaOH–DMSO; 3. 1 M HCl; 4. CH_2O, $NaBH_3CN$; 5. 1 M NaOH, Δ; 6. HCOOH, Ni(R); 7. $NaBH_4$; 8. Ph_3PBr_2, DMA; 9. HQ¹GluMe; 10. 1 M NaOH.

Scheme 3.25

(132) could be converted into (124) analogously (see *Scheme 3.25*). Reductive methylation of (124) then generated 5-methyl-5-deazaMTX (134). Alternatively, a two-step reduction of (133) gave (131), which was converted to the hydrobromide salt of (135). The preparation of (134) from (135) was straightforward. Compounds (126) and (136) were obtained from (124) and (134), respectively, via selective hydrolysis of the 4-amino group in refluxing 1 M NaOH, conditions which apparently do not effect side-chain racemi-

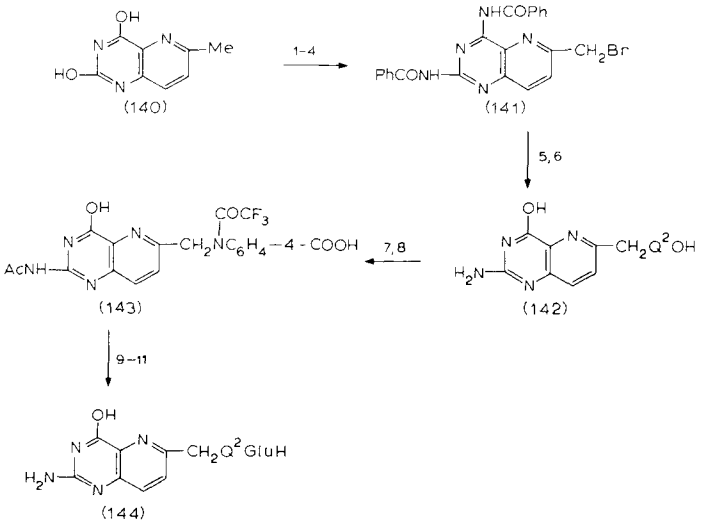


(137) R = Et R' = H
(138) R = Me R' = Et
(139) R = Et R' = Me

zation. Very recently, this methodology has been extended to include the preparation of 5-ethyl-5-deazaAP (137), 5-methyl-5-deaza-N^{10}-ethylAP (138) and 5-ethyl-5-deazaMTX (139) [97].

8-DEAZA ANALOGUES

8-DeazaFA (144) was initially synthesized by DeGraw, Kisliuk, Gaumont and Baugh in 1974 [98], although the pteroic acid precursor (142) had been described some 18 years earlier by an English group [99, 100]. Based on Rydon's earlier work, this synthesis is outlined in *Scheme 3.26*. Starting from 2,4-dihydroxy-6-methylpyrido[3,2-*d*]pyrimidine (140) [101], the bromomethyl derivative (141) could be obtained, but not without some difficulty (the bromination of (140) in large-scale reactions, > 5 g, was the problematic reaction). Fusion of (141) with ethyl *p*-aminobenzoate followed by ester and amide hydrolysis furnished (142). Protection of N-2 and N-10 as acetamido and trifluoroacetamido groups, respectively, gave (143), which was coupled with diethyl glutamate hydrochloride via the mixed anhydride using isobutyl chloro-

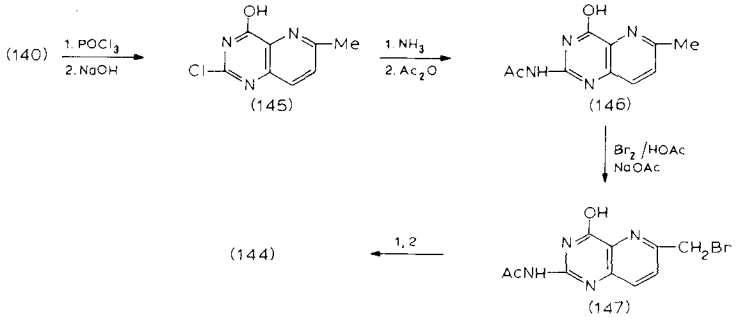


1. $POCl_3$, Δ; 2. NH_3; 3. PhCOCl, Et_3N; 4. DBDMH; 5. HQ^2OEt; 6. KOH – EtOH; 7. TFAA; 8. Ac_2O; 9. iBuCF, Et_3N; 10. GluEt·HCl; 11. NaOH, Δ.

Scheme 3.26

formate. Alkaline hydrolysis then produced (144). Alternatively, coupling the mixed anhydride with α-benzyl-γ-glutamyl Merrifield resin ester [102] and subsequent alkaline hydrolysis furnished (144) more efficiently.

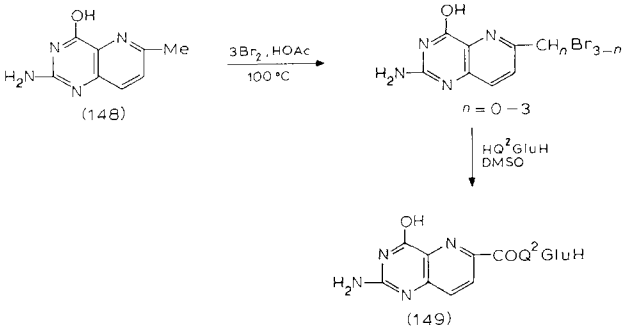
Montgomery's group has also prepared (144) using (140) as a starting material (*Scheme 3.27*) [103]. Selective hydrolysis of the derived dichloro



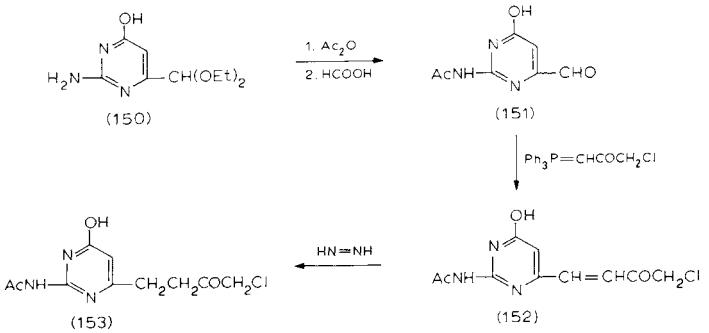
Scheme 3.27

derivative yielded (145), which was subjected to ammonolysis and acetylation. The resulting acetamide (146) was cleanly brominated to (147).

Attempts to brominate the free amine (148) were disappointing (*Scheme 3.28*). Either incomplete reaction was observed or else a mixture of mono-, di- and tribrominated compounds was obtained. Nonetheless, treatment of this crude mixture with *p*-aminobenzoylglutamic acid generated the amide (149), which presumably resulted from bromide displacement in the tribromomethyl precursor, followed by hydrolysis of the remaining geminal dibromide during work-up.



Scheme 3.28

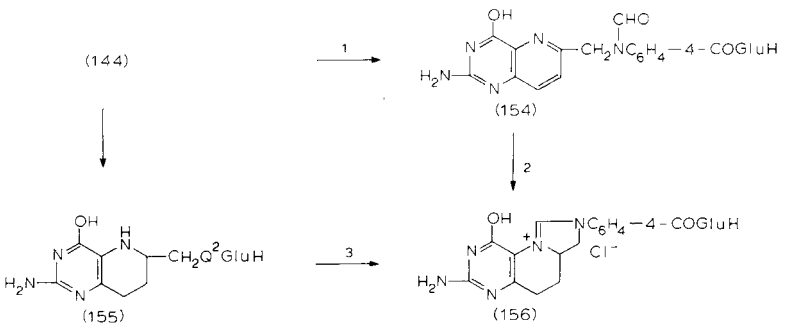


Scheme 3.29

An alternative approach was also briefly investigated (*Scheme 3.29*). Condensation of the aldehyde (151), readily available from (150) [104], with 1-chloro-3-(triphenylphosphoranylidene)-2-propanone afforded (152), which was reduced to (153) with diimide. This approach was abandoned in light of the poor yields obtained for both (152) and (153).

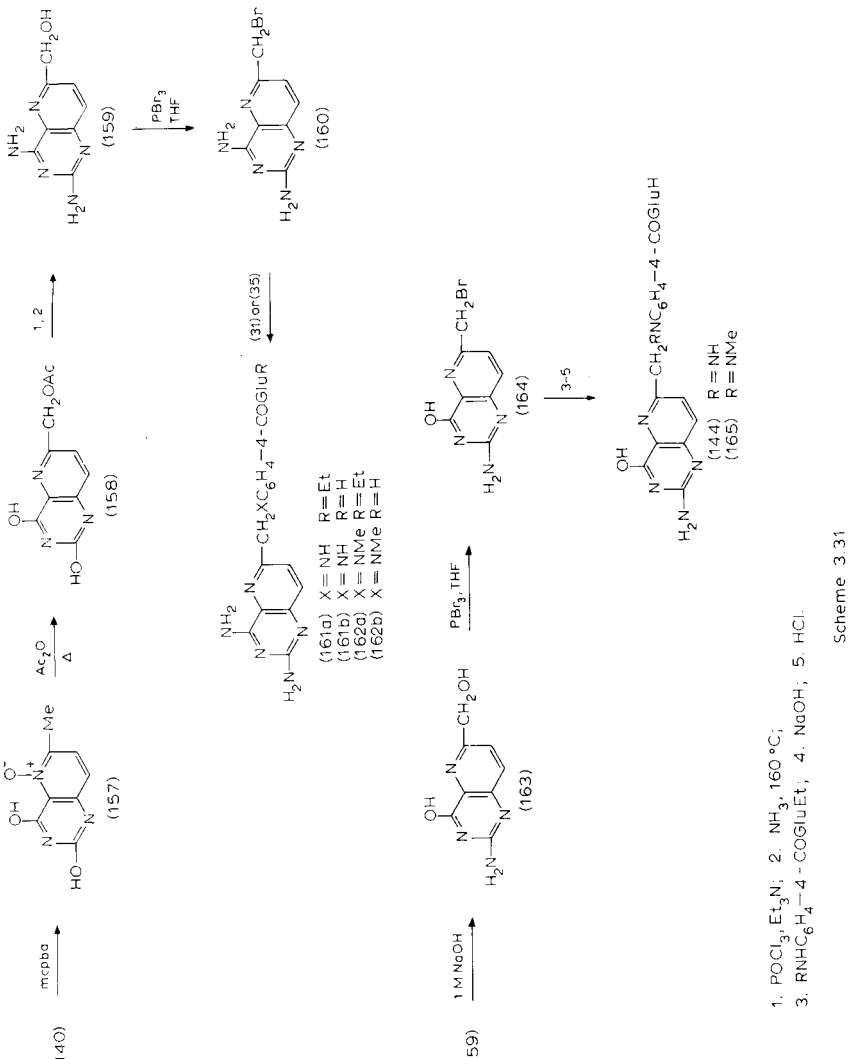
Catalytic reduction of (144) in the presence of Pt-HCOOH produced only (154), whereas in TFA, hydrogenation afforded the desired 5,6,7,8-tetrahydro derivative (155) as part of a complex mixture. The 5,10-methenyl analogue (156) was obtained conveniently from either (154) or (155) (*Scheme 3.30*) [103].

Recently, Broom's group has reported the synthesis of a series of 8-deaza compounds, including the AP (161b) and MTX (162b) analogues as well as (144) and 8-deaza-N^{10}-methylFA (165) [86]. It is noteworthy that their



1. H_2, Pt, HCOOH; 2. H_2, PtO_2, TFA; 3. 95% HCOOH

Scheme 3.30



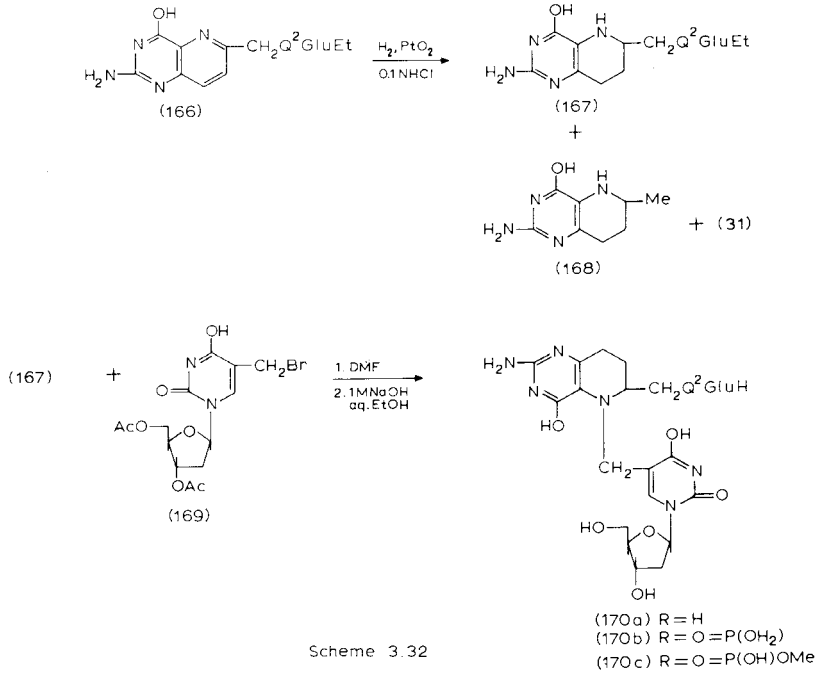
1. $POCl_3$, Et_3N; 2. NH_3, 160 °C; 3. $RNHC_6H_4$—4-COGluEt; 4. NaOH; 5. HCl.

Scheme 3.31

approach, outlined in *Scheme 3.31*, unlike either DeGraw's or Montgomery's synthesis, requires no protection/deprotection sequence at any point. Again, (140) was found to be the starting material of choice. Selective oxidation of N-5 with mcpba gave the *N*-oxide (157), which was rearranged to 6-(acetoxymethyl)-2,4-dioxopyrido[3,2-*d*]pyrimidine (158) in refluxing acetic anhydride. Chlorination with $POCl_3$-Et_3N followed by NH_3 at 160 °C in a sealed tube afforded (159) which was converted to the labile bromomethyl derivative (160) using PBr_3-THF. Condensation of (160) in DMA with (31) or (35) produced the diesters (161a) and (162a), which were saponified to (161b) and (162b), respectively.

When (159) was treated with NaOH, the 8-deazapterin carbinol (163) was obtained in good yield. Preparation of the bromomethyl derivative (164) was effected using PBr_3. Alkylation of (31) and (35) with (164) followed by saponification then yielded (144) and (165), respectively.

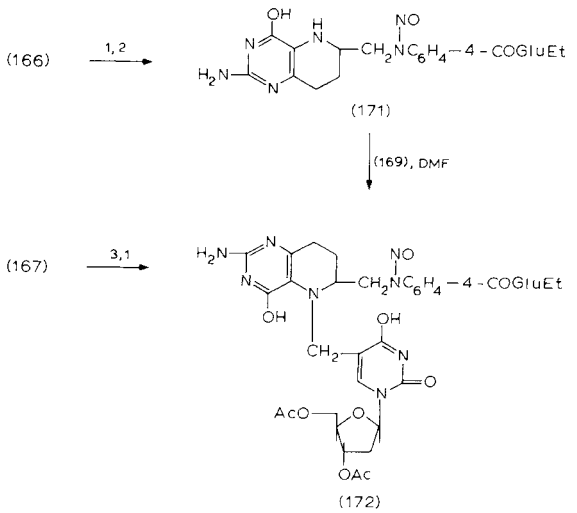
A short time later, these authors reported an elegant adaptation of this work for the synthesis of multisubstrate analogues (170a–c) of the proposed intermediate in the thymidylate synthase-mediated synthesis of thymidylic acid, as



Scheme 3.32

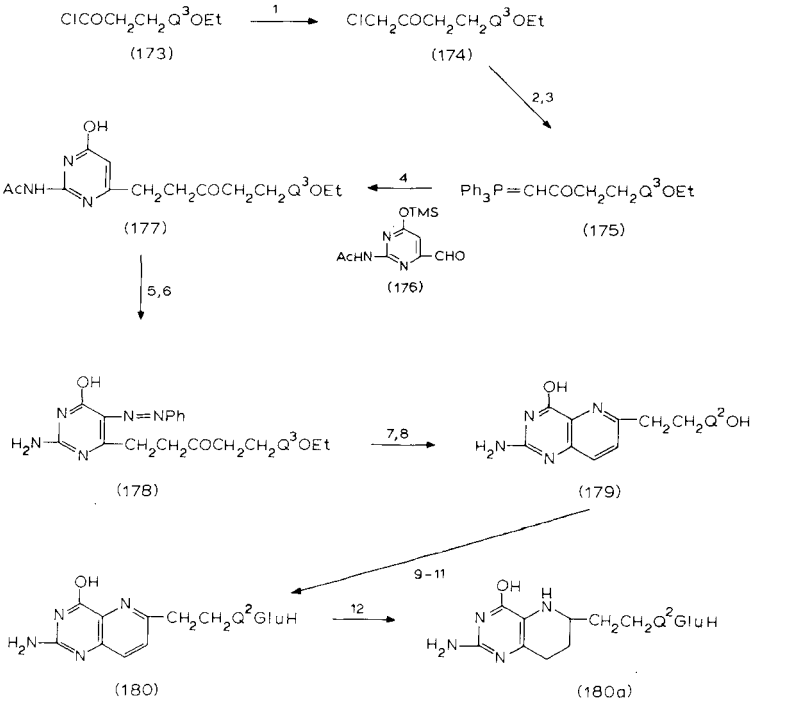
shown in *Scheme 3.32* [105a,b]. Reduction of (166) using Adams's catalyst in ethanol containing an equimolar amount of 0.1 M HCl led to the known 5,6,7,8-tetrahydro-8-deazaFA (167) together with (168) and (31), the products of hydrogenolysis [106]. After chromatographic purification, (167) was alkylated with (169) followed by saponification to yield (170a). Similarly, alkylation of (167) with (331) (see p. 151, 152) followed by deblocking with Zn-Cu couple and mild saponification gave (170b). Finally, (170c) was obtained from (167) and the corresponding 5′-methylated analogue of (331).

In addition to UV, ^{1}H-NMR and fast atom bombardment (FAB) mass spectrometry, the identity of (170) was confirmed by independent synthesis (*Scheme 3.33*). Thus, (166) was nitrosated [107] and reduced to give (171) [108], from which (172) was obtained via alkylation with (169). This was identical in all respects with the product obtained via direct nitrosation of the product from (167) and (169), and thereby confirmed the position of alkylation to be N-5 and not N-10.



Scheme 3.33

Several additional examples of 8-deaza compounds are known. DeGraw's approach to 8-deazahomoFA (180) is detailed in *Scheme 3.34* [109a,b]. Treatment of (173) with diazomethane-HCl produced the chloromethyl ketone (174), from which the ylide (175) was obtained by sequential reaction with triphenylphosphine and Na_2CO_3. Condensation of (175) with the silyl pyrimidine

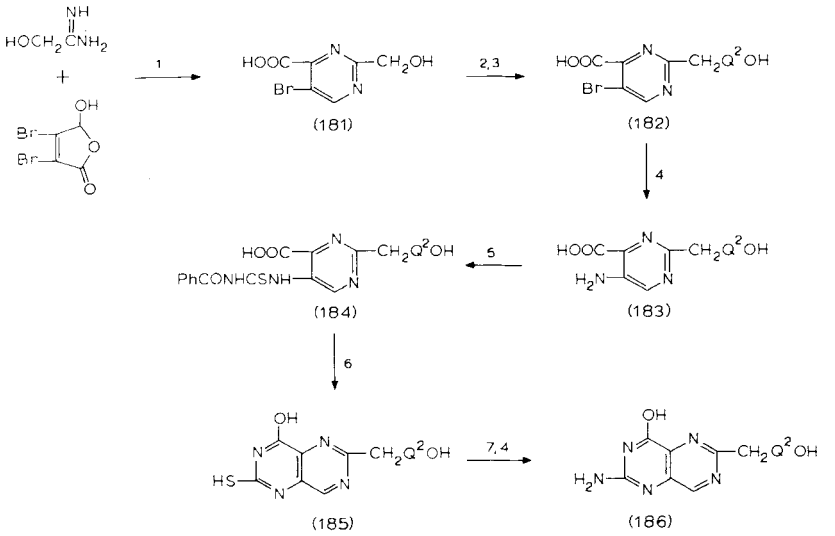


1. CH_2N_2, HCl; 2. Ph_3P, DME; 3. Na_2CO_3; 4. H_2, Rh–C; 5. HCl – EtOH; 6. $PhN_2^+Cl^-$; 7. H_2, Pd/C; 8. 10% NaOH; 9. Bu^tCF, Et_3N; 10. GluEt; 11. 0.1 M NaOH 12. H_2, PtO, 1 equiv. TFA.

Scheme 3.34

aldehyde (176) followed by catalytic hydrogenation afforded (177) uneventfully. Attempts to effect N-2 amide hydrolysis with either 0.1 M NaOH or 0.1 M NH_4OH were complicated by fragmentation of the β-acetamidoketone. However, HCl/EtOH followed by coupling with benzenediazonium chloride gave (178), which was hydrogenated and hydrolyzed to generate 8-deazahomopteroic acid (179). Incorporation of the glutamic acid moiety was accomplished uneventfully in the usual manner. Reduction of (180) over Adams's catalyst in the presence of 1 equivalent of TFA then afforded (180a).

There is one literature report of a pyrimido[5,4-*d*]pyrimidyl analogue (186) of pteroic acid, which can be viewed as an 8-deaza-7-aza derivative [110]. In this approach (*Scheme 3.35*), hydroxyacetamidine was condensed with mucobromic acid in a modified Budesinsky procedure to produce 2-hydroxy-

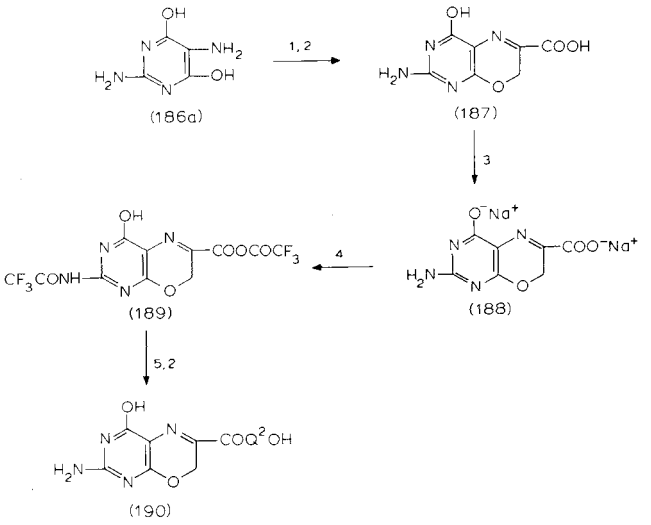


1. NaOMe, MeOH; 2. TsCl, NaOH; 3. HQ^2OH, DMF; 4. NH_3, sealed tube; 5. PhCONCS; 6. 3N KOH; Δ; 7. $KMnO_4$.

Scheme 3.35

methyl-5-bromopyrimidine-4-carboxylic acid (181)[111]. Introduction of *p*-aminobenzoic acid was then accomplished by sequential reaction of (181) with toluenesulphonyl chloride and *p*-aminobenzoic acid to yield (182). Reaction of (182) with ammonia gave (183), which was converted to the reactive intermediate (184) with benzoyl isothiocyanate. Brief exposure of (184) to hot KOH effected cyclization to (185). Introduction of the 2-amino group was accomplished indirectly via displacement of the labile sulphonate with ammonia to afford the target pteroic acid (186). There has not been a subsequent report detailing elaboration of (186) to the corresponding FA analogue.

Finally, Winchester, Zappone and Skinner have reported the synthesis of 7,8-dihydro-8-oxa-9-oxopteroic acid (190) as part of a programme to evaluate members of this class of heterocyclic compounds as DHFR inhibitors [112]. In their approach (*Scheme 3.36*), the key intermediate acid (187) was prepared from 2,5-diamino-4,6-dihydroxypyrimidine and ethyl bromopyruvate followed by saponification [113]. Owing to the extreme insolubility of (187) in most solvents, it was converted to the disodium salt (188) and treated with excess trifluoroacetic anhydride to yield the mixed anhydride (189) [114]. Coupling with *p*-aminobenzoic acid and careful base hydrolysis gave (190), albeit in low



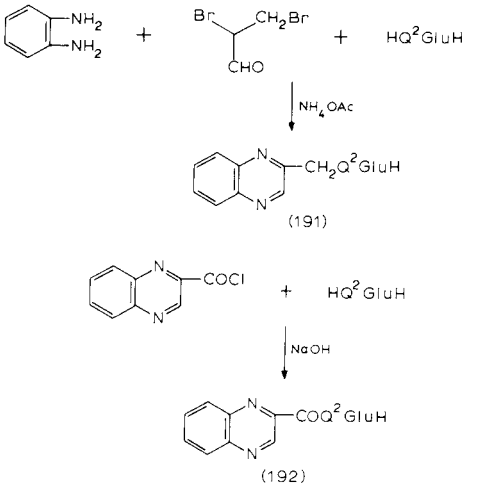
1. $BrCH_2COCOOEt$, EtOH, Δ; 2. NaOH; 3. 1 M NaOH; 4. TFAA; 5. HQ^2OH.

Scheme 3.36

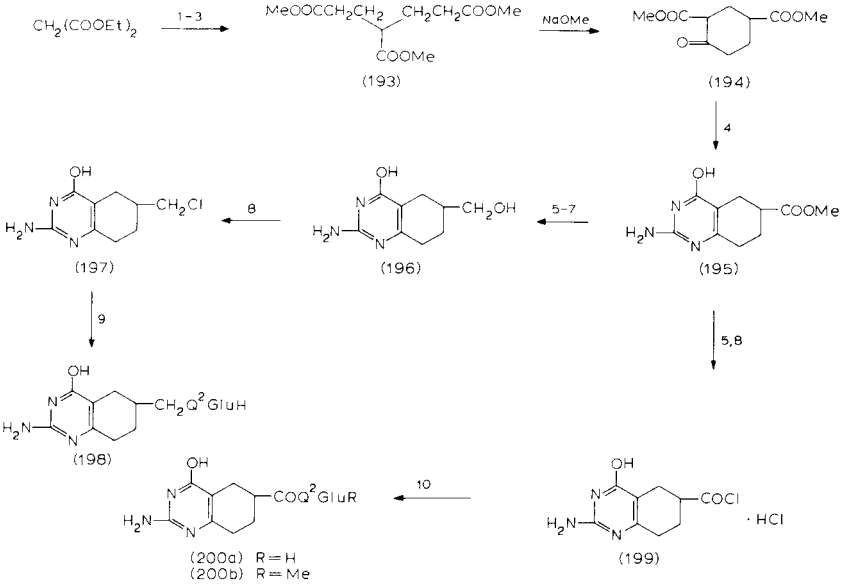
yield. Numerous factors portend against developing this approach for inclusion of fully elaborated side-chains, and alternative synthetic strategies are reportedly under investigation.

1,3-DIDEAZA ANALOGUES (QUINOXALINES)

There are no known examples of a 1,3-dideaza analogue, i.e., a quinoxaline derivative, although a group at Rockefeller Institute attempted a synthesis of this class of compounds almost 40 years ago [115] (*Scheme 3.36a*). Analogous to the Waller synthesis of FA (*vide supra*) [55], a mixture of *o*-phenylenediamine, 2,3-dibromoacrolein (generated *in situ*) and *p*-aminobenzoylglutamic acid was allowed to stand in an ammonium acetate buffer. After a lengthy work-up procedure a new compound was isolated which showed activity versus *L. casei* but which was clearly not the expected product (191), as evidenced by elemental analysis and glutamic acid determination. As part of this same study, the authors did prepare (192) from the reaction of quinoxaline-2-carboxylic acid chloride and *p*-aminobenzoyl-L-glutamic acid.



Scheme 3.36a



1. CH_2=CHCN, NaOMe; 2. 6MHCl; 3. MeOH, H_2SO_4, $(CH_2Cl)_2$; 4. guan, MeOH, NaOMe; 5. NaOH; 6. BuOH, TsOH; 7. $NaBH_4$, $AlCl_3$; 8. $SOCl_2$, pyr; 9. HQ^2GluH, Bu cellosolve, Δ; 10. HQ^2GluR, pyr.

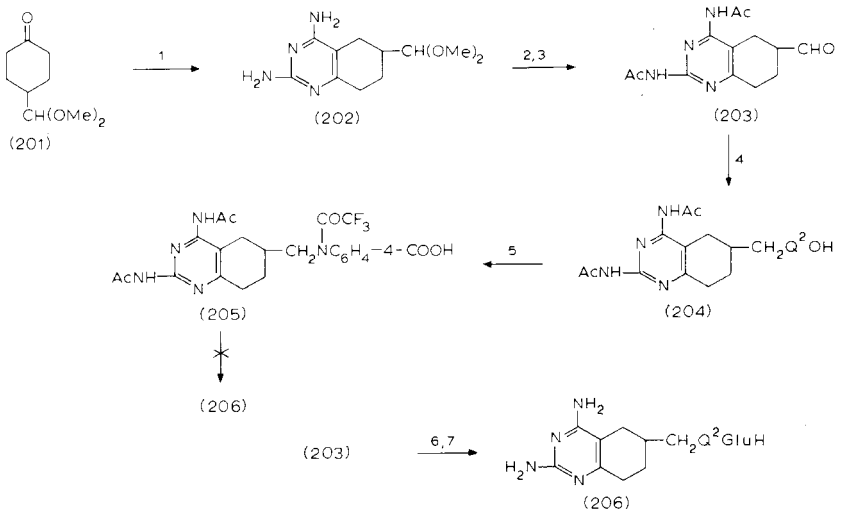
Scheme 3.37

See p. 127

5,8-DIDEAZA ANALOGUES (QUINAZOLINES)

Of the many classes of deazaFA derivatives investigated, none has received more attention or resulted in more promising candidate drugs than 5,8-dideaza (quinazoline) analogues. Historically, the first member of this group prepared was the tetrahydroquinazoline analogue (198) of FA, reported by a group from the Stanford Research Institute almost 30 years ago (*Scheme 3.37*, p. 126) [116]. Dimethyl 4-methoxycarbonylpimelate (193), obtained in three steps and in excellent yield from diethyl malonate, underwent smooth Dieckmann cyclization to yield (194), which was treated with guanidine to furnish the tetrahydroquinazoline (195). Saponification followed by reesterification with *n*-butanol produced the more readily soluble *n*-butyl ester, which was reduced to the carbinol (196) using sodium borohydride-aluminum chloride. Conversion of (196) into (197) was effected with thionyl chloride-pyridine, for which the amount of pyridine present was critical for success. Alkylation of *p*-aminobenzoyl-L-glutamic acid with (197) to furnish (198) was accomplished in refluxing butyl cellosolve.

Saponification of (195) and reaction with thionyl chloride gave the unstable acid chloride (199), which was used to acylate *p*-aminobenzoyl-L-glutamic acid

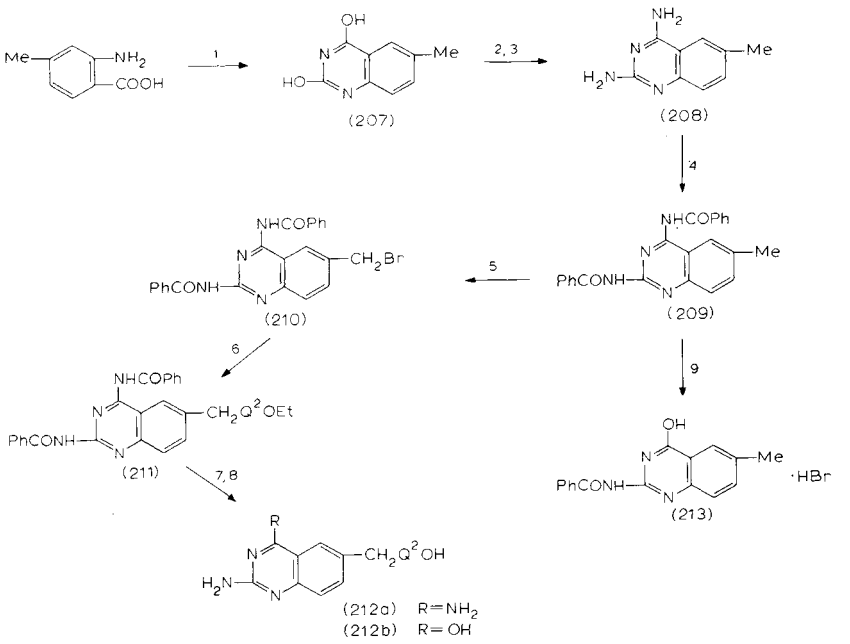


1. $NCN{=}C(NH_2)_2$, 180°C; 2. Ac_2O; 3. 90% HCOOH; 4. HQ^2OH, H_2, Pd–C, $MeOCH_2CH_2OH$; 5. TFAA; 6. HQ^2GluH, H_2, PtO_2, HOAc; 7. 0.4M NaOH, Δ.

Scheme 3.38

and its dimethyl ester, affording the 9-oxo analogues (200a) and (200b), respectively.

A similar approach was employed by this same group during their construction of 5,8-dideaza-5,6,7,8-tetrahydroAP (206) as shown in *Scheme 3.38* [117]. After much experimentation, it was found that the key 5,6,7,8-tetrahydroquinazoline acetal (202) was most conveniently prepared from (201) [118] in one step via thermal cyclization with *N*-cyanoguanidine. Following acetylation, the aldehyde (203) was liberated using 90% HCOOH. Reductive amination of (203) with *p*-aminobenzoic acid gave marginal yields of the 5,8-dideaza-5,6,7,8-tetrahydropteroic acid (204). This problem was shown to result from a facile deacetylation (up to 20%) during recrystallization from ethanol. Trifluoroacetylation of (204) yielded (205), which was converted to the acid chloride prior to reaction with diethyl L-glutamate. However, this reaction sequence did not yield material which could be hydrolyzed to (206).



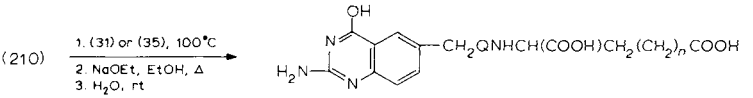
1. Urea, 195 °C; 2. $POCl_3$; 3. NH_3, PhOH, Δ; 4. PhCOCl, Et_3N, dioxan; 5. DMDBH, DBP, CCl_4, $h\nu$; 6. HQ^2OEt, Δ; 7. NaOEt, EtOH; 8. KOH, aq. EtOH; 9. Br_2, $CHCl_3$.

Scheme 3.39

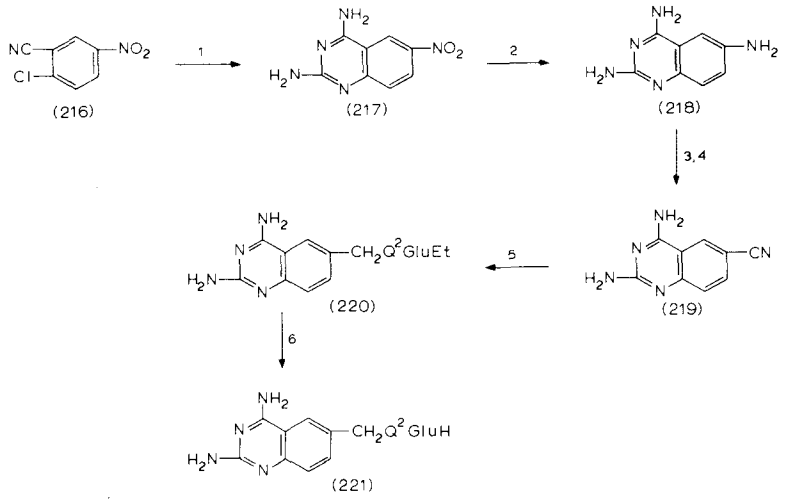
Instead, reductive amination of (203) with *p*-aminobenzoylglutamic acid and mild hydrolysis furnished (206). Interestingly, the reductive amination was found to be solvent-dependent in that considerable deacetylation occurred when the reaction was conducted in 2-methoxyethanol, whereas this problem was obviated by the use of glacial acetic acid.

As part of an extensive investigation of syntheses of deaza derivatives of pteroic acid, Rydon's group described the first synthesis of 4-amino-4-deoxy-5,8-dideazapteroic acid (212a) [99, 119]. In their approach (*Scheme 3.39*), 4-methylanthranilic acid was fused with urea to give (207) from which (208) was obtained in two steps. Attempts to brominate (208) directly were unsatisfactory, necessitating protection of the amino groups by benzoylation giving (209). Bromination of (209) with 1,3-dibromo-5,5-dimethylhydantoin furnished (210) uneventfully. Interestingly, bromination of (209) with bromine in chloroform gave only the hydrobromide salt (213) in quantitative yield, demonstrating the lability of the 4-substituent in 2,4-disubstituted quinazolines [99, 120]. Fusion of (210) with ethyl *p*-aminobenzoate produced (211), from which the desired diaminoquinazoline analogue (212a) was obtained in a straightforward manner. If the saponification was allowed to proceed to completion, the quinazoline pteroic acid analogue (212b) was produced instead.

A Parke-Davis group successfully extended this methodology to include preparation of the quinazoline analogues of FA (214a) and N^{10}-methylFA (214b), as well as the respective aspartic acid derivatives (215a) and (215b) [121]. Thus, alkylation of (31) with (210) followed by amide alcoholysis and hydrolysis generated (214a). Similarly, reaction of (35) with (210) furnished (214b). The requisite aspartate precursors led to (215a) and (215b), respectively. Repetition of this sequence using the unnatural D-isomers of glutamic and aspartic acids produced the corresponding D-isomers. It should be noted that the conditions used to remove the benzoyl protecting groups (sodium ethoxide in ethanol at reflux) may have effected side-chain racemization in view of some later results obtained during guanidine cyclization of *o*-aminonitriles containing a fully elaborated side-chain [122]. Efforts to extend this methodology to the preparation of the corresponding AP derivative (221) were ultimately frustrated by the lability of the 4-amino group towards hydrolysis. This problem was



(214a) R=H n=1
(214b) R=Me n=1
(215a) R=H n=0
(215b) R=Me n=0 Q: see p.90



1. guan, EtOH, Δ; 2. H_2, 10% Pd/C, DMF; 3. $NaNO_2$, HCl; 4. $CuSO_4 \cdot 5H_2O$, KCN; 5. (31), Ni(R), HOAc; 6. aq. Na_2CO_3.

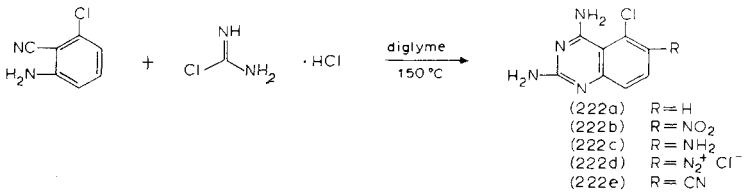
Scheme 3.40

obviated by the development of a new approach, shown in *Scheme 3.40* [123–125].

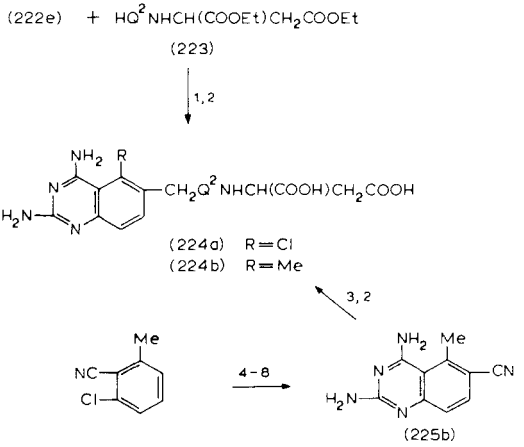
In this strategy, guanidine cyclization of (216) yielded (217), which was reduced to (218), diazotized and treated with cuprous cyanide to generate the key intermediate (219). Reductive amination of (219) with (31) gave (220), from which (221) was obtained by mild saponification.

Two important members of this series incorporating lipophilic substituents at C-5, namely the 5-chloro (chlorasquin, (224a)) and 5-methyl (methasquin, (224b)) derivatives, were prepared analogously (*Scheme 3.41*). Reaction of 6-chloroanthranilonitrile with chloroformamidine hydrochloride led to (222a, R = H), which was successively treated with fuming nitric acid/sulphuric acid (to give (222b, R = NO_2)), stannous chloride-HCl (to give (222c, R = NH_2)), sodium nitrite-HCl (to give (222d, R = $N_2^+ Cl^-$)), and cuprous cyanide (to give (222e, R = CN)). Reductive amination of (222e) with diethyl *p*-aminobenzoyl-L-asparate (223) in aqueous acetic acid ultimately furnished (224a) after saponification.

For (224b), 2,4-diamino-5-methyl-6-quinazolinecarbonitrile (225b) was readily available in five steps from 2-chloro-6-methylbenzonitrile via 2,4,6-triamino-5-methylquinazoline (225a; see *Scheme 3.46*). Reductive amination

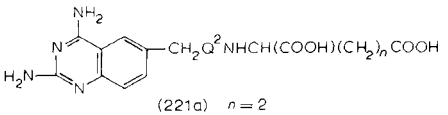


(222a) R = H
(222b) R = NO_2
(222c) R = NH_2
(222d) R = $N_2^+ Cl^-$
(222e) R = CN

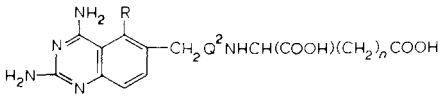


1. H_2, Ni(R), aq. HOAc; 2. Na_2CO_3; 3. (223), H_2, Ni(R); 4. $NO_3 \cdot NO_2$, −10°C;
5. guan, H_2CO_3, $EtOCH_2CH_2OH$, Δ; 6. $SnCl_2$, HCl; 7. $NaNO_2$, HCl; 8. $Cu_2(CN)_2$.

Scheme 3.41



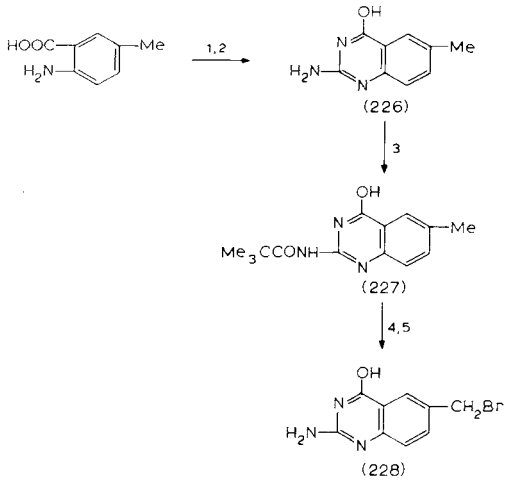
(221a) $n = 2$
(221b) $n = 1$
(221c) $n = 0$



(224a) R = Cl $n = 1$
(224b) R = Me $n = 1$
(224c) R = Cl $n = 0$
(224d) R = Me $n = 0$
(224e) R = Cl $n = 2$
(224f) R = Me $n = 2$

of (225b) with (223) and saponification to (224b) was straightforward. A rather extensive series of analogues related to (221) and (224) containing both natural and unnatural amino acids was prepared by this strategy (221a–c), (224a–f) [124]. Several improvements for the synthesis of (219), (222e) and (225) have been described [74, 125].

As part of their programme to synthesize and evaluate potential DHFR inhibitors, Acharya and Hynes described a more convenient and economical route to 2-amino-4-hydroxy-6-bromomethylquinazoline (228), a key intermediate in their approaches to 5,8-dideazaFA analogues [126] (*Scheme 3.41a*).

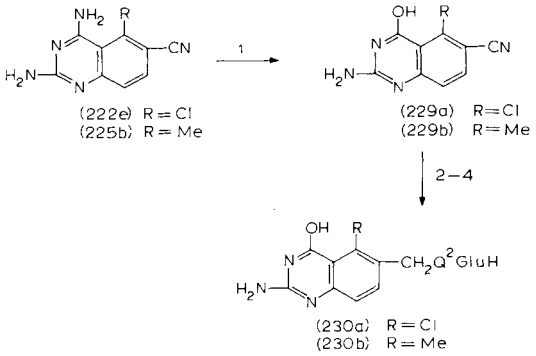


1. EtOH–HCl; 2– guan, Δ, $EtOCH_2CH_2OH$; 3. Me_3CCOCl, Et_3N, dioxan; 4. Br_2, DBP, CCl_4; 5. MeOH – HCl.

Scheme 3.41a

Esterification of 5-methylanthranilic acid followed by guanidine cyclization gave (226), which could not be brominated directly using either Br_2 or NBS owing to insolubility. Therefore, (226) was acylated with pivaloyl chloride, providing the soluble pivaloyl derivative (227), which underwent bromination uneventfully. Deprotection with methanolic HCl then afforded (228) in 34–39% overall yield from 5-methylanthranilic acid. Subsequent alkylation of (31) by (228), followed by saponification, yielded (214a). Similarly, (35) generated (214b).

A British group headed by T.R. Jones at the Institute of Cancer Research has concentrated on the synthesis and evaluation of quinazoline analogues of FA and AP as specific TS inhibitors. They prepared (214a), (214b), (221),

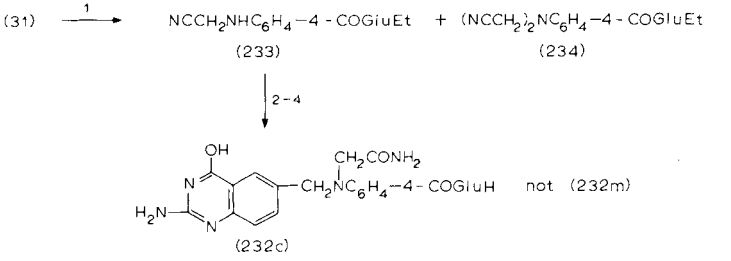


1. 2 M HCl, Δ; 2. (31), H_2, Ni(R), aq. HOAc; 3. NaOH; 4. HCl.

Scheme 3.42

(224e) and (224b) as previously described, and then further extended the methodology of Davoll and Johnson to include the previously unknown 5-substituted quinazoline FA analogues (230a) and (230b) [127] (*Scheme 3.42*). Concern regarding the purity of (229a) and (229b) prepared via acid hydrolysis of (222e) and (225b), respectively, was obviated by quantitative TLC studies using a system which permitted detection of the starting diamines at the 1% level. Reductive amination of (229a,b) with (31) and saponification then gave the target compounds (230a,b).

Recently, a series of new quinazoline derivatives (232) of FA, including aspartic acid and polyglutamic acid analogues, has been described [128]. These compounds were conveniently prepared by alkylation of (231), readily available from (31) and the appropriate alkylating agent, with (228) followed by saponifi-



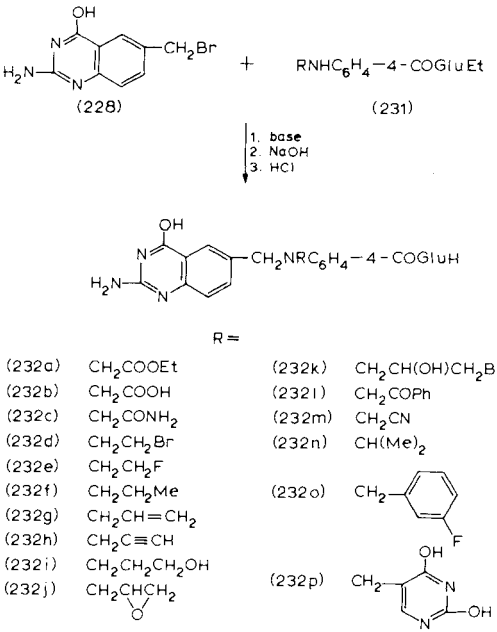
1. $BrCH_2CN$, K_2CO_3, EtOH; 2. (228), DMA, Δ; 3. NaOH, aq. MeCN; 4. HOAc.

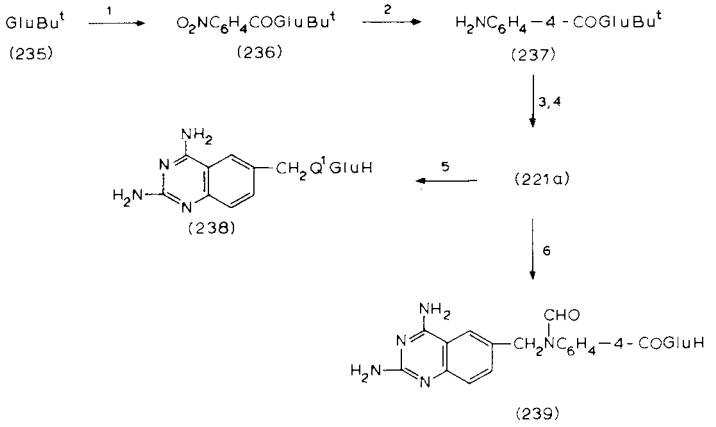
Scheme 3.42a

cation. A significant member of this series is the 10-propargyl analogue (232h), one of more promising candidates prepared to date, which is currently being evaluated in Phase I clinical trials [129, 130].

At about the same time, another group described its efforts to prepare the known 10-cyanomethyl analogue (232m) using essentially the same methodology described previously (*vide supra*) [131] (*Scheme 3.42a*). Alkylation of (31) with bromoacetonitrile gave a 2:1 mixture of (233) and (234) under optimum conditions. After chromatographic separation, (233) was alkylated with (228), from which the presumed target compound (232m) was obtained after saponification. The Institute of Cancer Research (ICR) workers showed later that the product of this reaction sequence was the amide (232c) and not the reported nitrile (232m) [132].

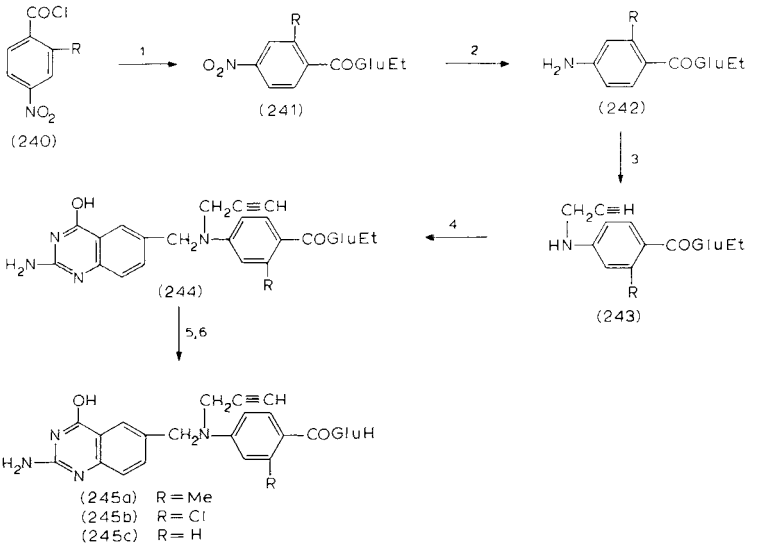
In 1985, Hynes, Harmon, Floyd, Farrington, Hart, Gale, Washtien, Susten and Freisheim described improvements in the synthesis of (221) (*Scheme 3.42b*) [133]. In their new strategy, di-*t*-butyl L-glutamate (235) [134] was acylated with *p*-nitrobenzoyl chloride and the resulting amide (236) reduced catalytically to (237). Reductive condensation of (237) with (219) followed by de-*t*-butylation with TFA then gave (221a). The primary advantage of this approach stems from the mild conditions used to deblock the *t*-butyl esters, in contrast to the normal saponification conditions used for ethyl esters,





1. $4-O_2NC_6H_4COCl$, $(CH_2Cl)_2$, Et_3N; 2. H_2, 10% Pd–C, EtOH; 3. (219), H_2, Ni(R), 70% HOAc; 4. TFA; 5. CH_2O, $NaBH_3CN$ (pH 6); 6. HCOOH, Ac_2O, r.t.

Scheme 3.42b



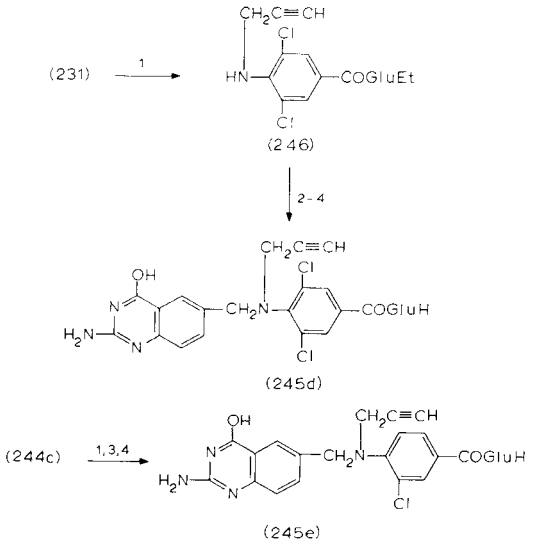
1. GluEt, pyr; PhMe, 10°C; 2. H_2, 10% Pd–C, EtOH or $Na_2S_2O_3$, $NaHCO_3$, aq. EtOH; 3. $BrCH_2C{\equiv}CH$, 2,6-lutidine, DMA; 4. (228), HBr, $CaCO_3$, DMF; 5. 1 M NaOH; 6. 0.1 M HCl.

Scheme 3.43

which can lead both to decomposition and to side-chain racemization. Reductive methylation of (221a) gave 5,8-dideazaMTX (238), while formylation with acetic-formic anhydride gave the known 10-formyl-5,8-dideazaAP (239) [135].

The ICR team has described additional examples of *N*-10 variants of (232) using its earlier methodology [132]. In particular, their efforts to prepare the 10-cyanomethyl derivative (232m) conclusively demonstrated that the previously reported synthesis [131] was in error, and that (232m) was only a minor impurity present in the carboxamide product (232c). Additional improvements in the synthesis of intermediates and known members of the series (232a–p) were also reported.

A further report from the ICR laboratory details a series of benzene ring-substituted derivatives of 5,8-dideaza-10-propargylFA (245a–d) (*Schemes 3.43, 3.44*) [136]. The 2′-methyl (245a) and 2′-chloro (245b) compounds were derived from the appropriate 4-nitrobenzoyl chlorides (242a,b) as follows. Acylation of diethyl L-glutamate with (240a,b) gave (241a,b), from which the respective amines (242a,b) were obtained reductively either catalytically (for (242a)) or using sodium dithionite (for (242b)). Alkylation of (242a,b) with propargyl bromide produced (243a,b), which were in turn alkylated with (228)·HBr to generate (244a,b). The desired diacids (245a,b) were then

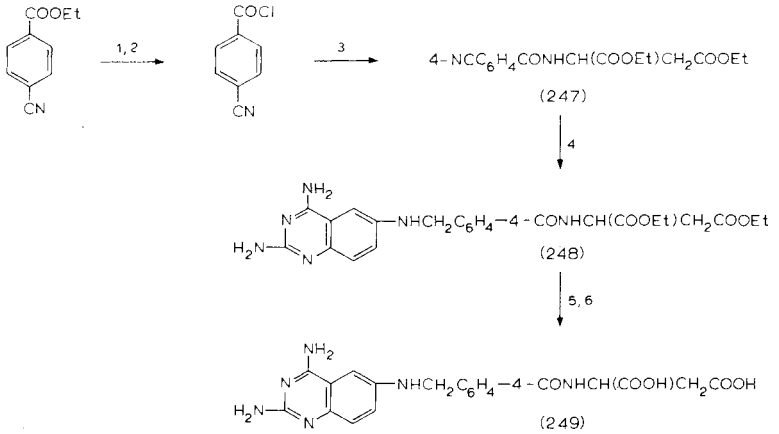


1. SO_2Cl_2, $CHCl_3$; 2. (228), HBr, $CaCO_3$, DMA; 3. 1 M NaOH; 4. 0.1 M HCl.

Scheme 3.44

available by saponification [128]. For the 3′,5′-dichloro analogue (245d), the methodology was essentially the same. Thus, (231, R = $CH_2C \equiv CH$) was chlorinated to (246), which upon alkylation with (228) · HBr and saponification afforded (245d). Finally, the 3′-monochloro derivative (245e) was most conveniently prepared via chlorination of (244c) followed by saponification. Very recently, the synthesis of amino-acid variants of (245c) have been described [137].

The noteworthy antitumour activity of quinespar (224, R = H) prompted Davoll and Johnson to prepare the N-9, C-10 analogue, isoquinespar (249) (*Scheme 3.45*) [138]. In their approach, ethyl *p*-cyanobenzoate was converted

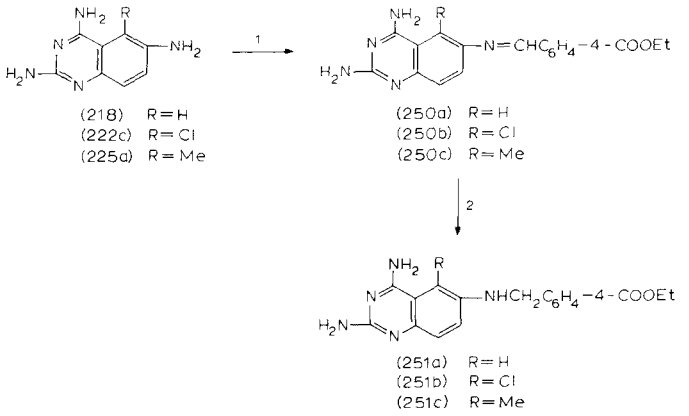


1. KOH, EtOH, PhH; 2. $SOCl_2$, Δ; 3. $H_2NCH(COOEt)CH_2COOEt$, $(CH_2Cl)_2$, Et_3N; 4. (218), H_2, Ni(R), aq. HOAc; 5. NaOH–EtOH; 6. HCl.

Scheme 3.45

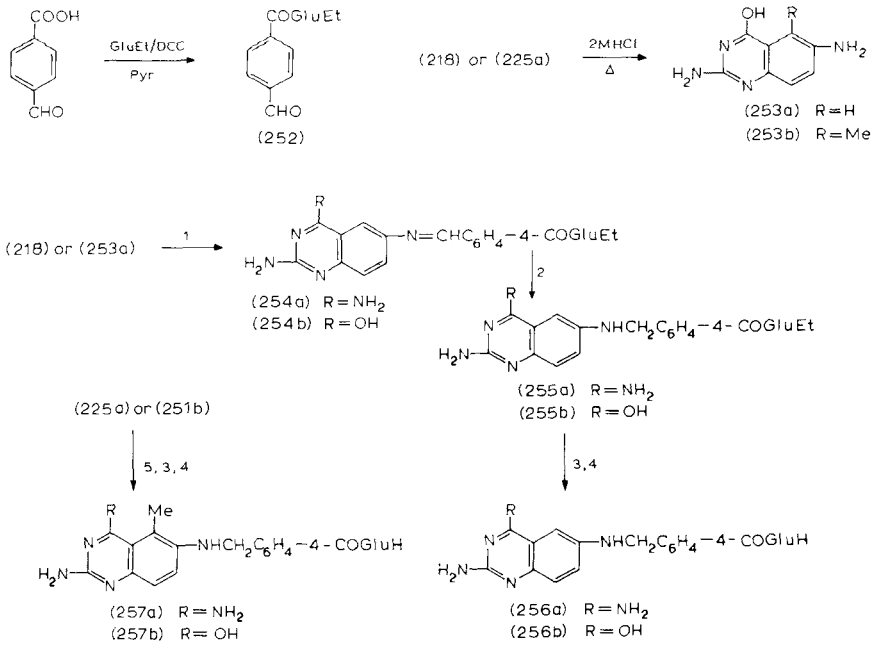
to the acid chloride, from which (247) was readily available by acylation of diethyl L-aspartate. Reductive coupling of (247) with 2,4,6-triaminoquinazoline (218) gave (248), from which (249) was obtained after saponification.

The first examples of quinazoline analogues of isopteroic acid and isoFA were described in 1975 by Hynes's team as part of their programme to synthesize and evaluate potential TS inhibitors (*Scheme 3.46*) [139, 140]. For the isopteroate esters (251a–c), the requisite triaminoquinazolines, i.e., (218, R = H), (222c, R = Cl) and (225a, R = Me) were condensed with ethyl *p*-formylbenzoate [141] and the resulting anils (250a–c) were then reduced with dimethylamine-borane [142]. Essentially the same methodology was



1. $4\text{-}OHCC_6H_4COOEt$, EtOH, Δ; 2. Me_2NH-BH_3, HOAc.

Scheme 3.46

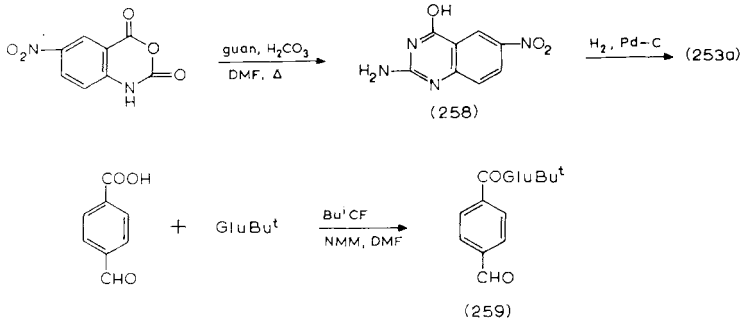


1. (252), DMSO, 4 Å sieves; 2. Me_2NH-BH_3, HOAc; 3. 0.1M NaOH; 4. 0.1M HCl; 5. (252), H_2–Ni(R), 70% HOAc.

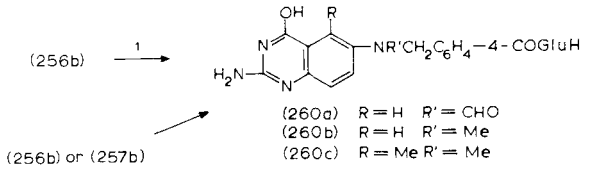
Scheme 3.47

adapted to the preparation of the isoAP analogues (256a) and (257a) and the corresponding isoFA derivatives (256b) and (257b) (*Scheme 3.47*). The necessary C-10-containing side-chain (252) was prepared by coupling *p*-formylbenzoic acid with diethyl L-glutamate. Acid hydrolysis of (218) and (225a) produced the other 6-amino precursors, (253a) and (253b), respectively. For the 5-methyl-substituted compounds, (257a) and (257b), direct reductive coupling of (252) with the respective quinazoline amine followed by saponification was a more expedient approach. Compound (256b) possessed sufficient activity to warrant further evaluation against colon cancer and other MTX-resistant tumours [143].

Improvements in the synthesis of (256b) which addressed the problems of contamination by (256a) as well as possible side-chain racemization during saponification have been reported by Hynes's group [144, 145]. Reaction of



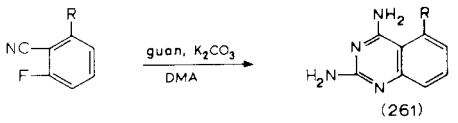
commercially available 5-nitroisatoic anhydride with guanidine carbonate yielded (258), from which (253a) was readily obtained by catalytic reduction. This approach obviated the presence of any (218) as a contaminant leading ultimately to the trace amount of (256a) previously observed. Coupling of *p*-formylbenzoic acid with (235) [134] gave the di-*t*-butyl ester (259) in excellent



1. HCOOH, Ac_2O; 2. CH_2O, $NaBH_3CN$.

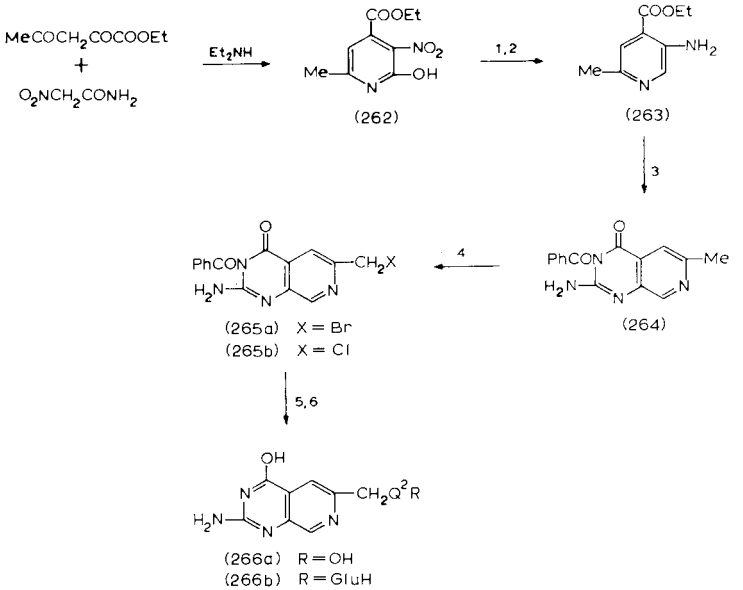
Scheme 3.47a

yield. Use of this compound in place of (252) in *Scheme 3.47* permitted final deblocking using TFA at room temperature, thereby precluding racemization. Application of these modifications afforded (256a,b) and (241a,b) in better overall yield and in better purity. Additionally, several new members of this series were now more conveniently available. For example, formylation of (256b) gave (260a) directly, while reductive methylation of (256b) and (257b) gave (260b) and (260c), respectively [145] (*Scheme 3.47a*). Finally, repetition of this reaction sequence (*Scheme 3.47*) substituting the unnatural D-isomer of (235) ultimately yielded the D-isomer of (256b).



(261)

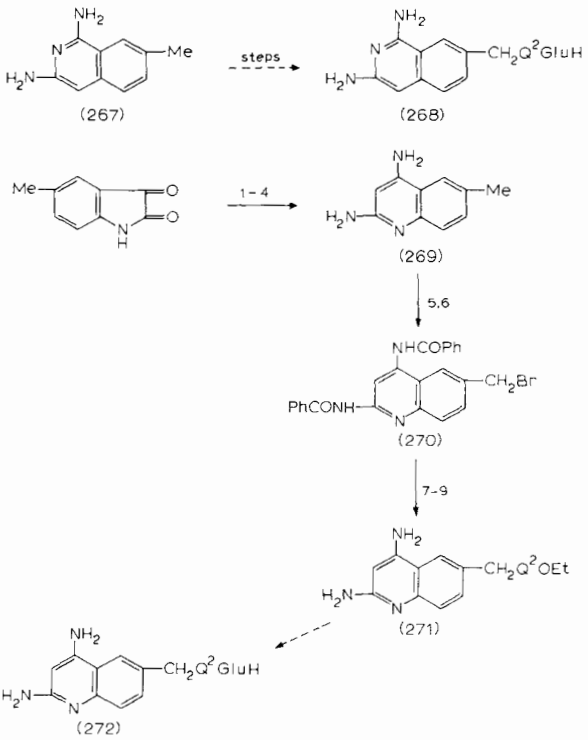
A very recent synthesis of 2,4-diamino-5-substituted quinazolines (261) has been described. Elaboration of these compounds into precursors for the preparation of isoFA and isoAP analogues is in progress [146].



1. $POCl_3$; 2. H_2, Pd–$BaCO_3$; 3. $PhCONHCH_2CN$, DMF; 4. DBDMH, $BrCCl_3$, 1 h; 5. HQ^2OEt; 6. NaOH.

Scheme 3.48

One other dideaza variation that has been attempted is the pyrido[3,4-*d*]-pyrimidine (266b), which can be considered as a 5,8-dideaza-7-aza analogue. The synthesis of the pteroic acid precursor has been completed as shown in *Scheme 3.48* [147]. Condensation of ethyl acetopyruvate [148] with nitroacetamide [149] generated the 2-pyridone (262), which was converted to the chloro compound and then subjected to reductive dechlorination and simultaneous reduction of the nitro group to produce (263). Treatment of (263) with benzoylcyanamide afforded (264), from which a 3 : 1 mixture of (265a) and (265b) was obtained after bromination with 1,3-dibromo-5,5-dimethylhydantoin in hot bromotrichloromethane. This crude reaction mixture was then fused with ethyl *p*-aminobenzoate and the resulting pteroate ester saponified to the pteroic acid analogue (266a). No further reports concerning this system or its elaboration to the FA analogue (266b) have yet appeared.



1. MeCOCOOH, KOH; 2. $SOCl_2$; 3. NH_3 – MeOH; 4. Br_2, KOH; 5. PhCOCl, Et_3N, dioxan; 6. Br_2 – $CHCl_3$; 7. HQ^2OEt; 8. NaOEt – EtOH; 9. HCl.

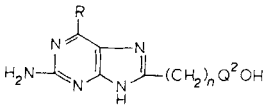
Scheme 3.49

1,5,8- and 3,5,8-TRIDEAZA ANALOGUES

To date, there is no example of a ring nitrogen trideaza analogue which has been carried beyond the pteroic acid stage. Rydon's group prepared 1,3-diamino-7-methylisoquinoline (267) as a precursor to 1,5,8-trideazaAP (268), but abandoned their efforts in view of the very slight and atypical biological activity of (267) [118, 150]. An approach to the isomeric 3,5,8-trideazaAP (272) was completed as far as the pteroic acid (271), as shown in *Scheme 3.49*, but was not carried further. The only other trideaza analogue, a 5,8,10-trideaza derivative, will be described in a later section.

PURINE ANALOGUES

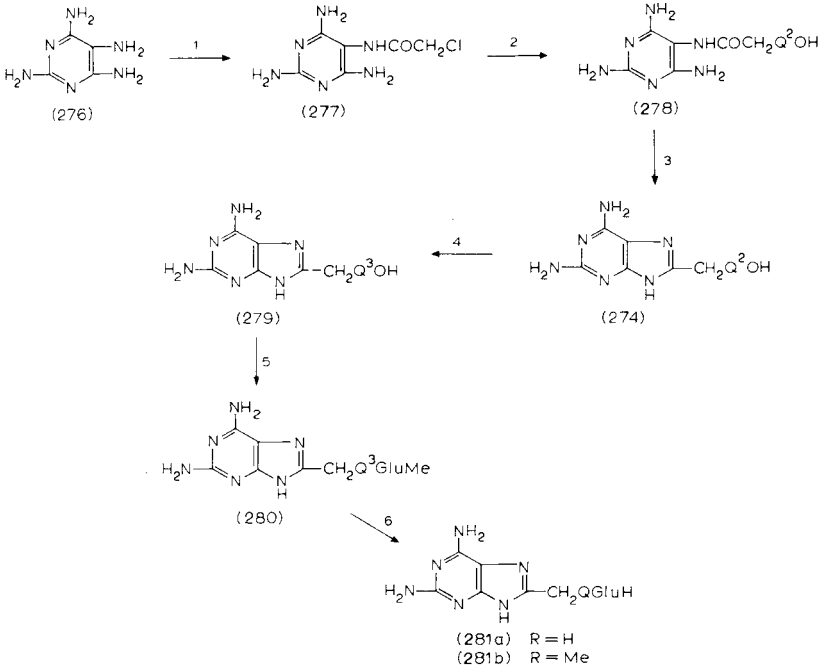
A group from the Midwest Research Institute has described two approaches to purine analogues of AP (281a) and MTX (281b) [151]. A shown in *Scheme 3.50*, the first, patterned after previous reports of the 'pteroic acid'



(273) R = OH $n = 1$
(274) R = NH_2 $n = 1$
(275) R = NH_2 $n = 2$

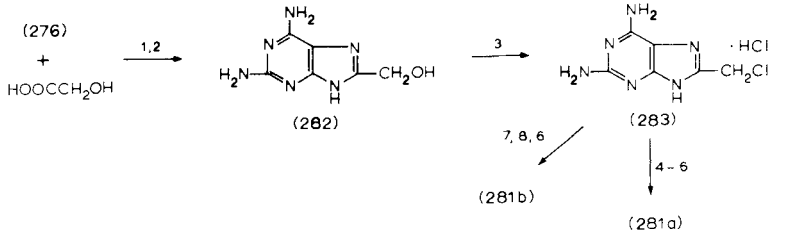
derivatives (273–275) [152, 153], began with acylation of 2,4,5,6-tetraaminopyrimidine (276) by chloroacetic acid. The resulting chloroacetamide (277) was then fused with *p*-aminobenzoic acid to yield (278), from which (274) was obtained via cyclization with hot potassium hydroxide solution. Acetylation of (274) gave (279) which was coupled with dimethyl L-glutamate via a mixed anhydride to furnish (280). Saponification then afforded (281a).

In their second approach (*Scheme 3.51*), the known 8-hydroxymethylpurine (282), available in two steps from (276) and glycolic acid [153], was treated with thionyl chloride. The resulting labile chloromethyl derivative (283) was used to alkylate *p*-aminobenzoyl L-glutamic acid after an *in situ* Finkelstein reaction furnishing (281a) directly. Use of (31) in this reaction sequence followed by saponification afforded no improvement in yield. Finally, reaction of (35) with (283) and ester hydrolysis yielded the purine MTX analogue (281b).



1. $ClCH_2COOH$, Δ; 2. HQ^2OH, H_2O, Δ; 3. 0.1M KOH, Δ; 4. Ac_2O;
5. ECF – Et_3N, GluMe · HCl, DMF; 6. 1M NaOH, r.t.

Scheme 3.50

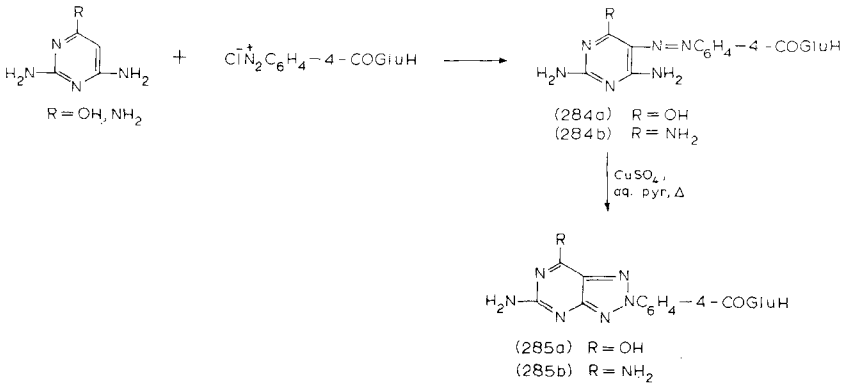


1. 100°C; 2. 2 M NaOH, Δ; 3. $SOCl_2$, DMF; 4. (31), KI – DMF; 5. NaOH;
6. HCl; 7. (35), KI – EtOH; 8. 1 M NaOH.

Scheme 3.51

PYRIMIDOTRIAZOLE ANALOGUES

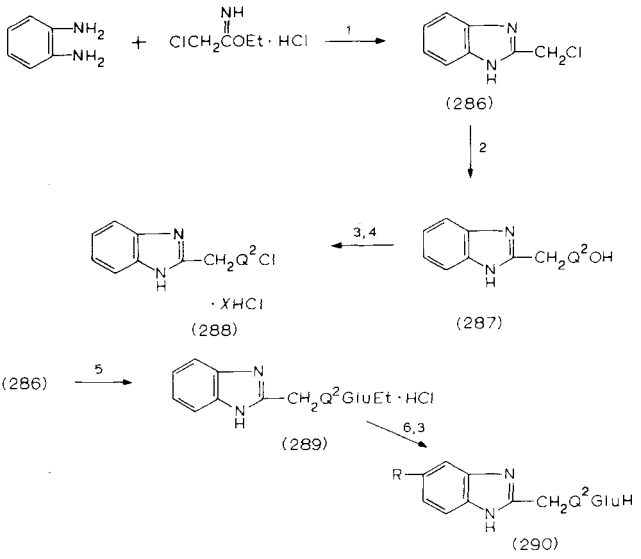
Over 35 years ago, a research group from Remington Rand described the synthesis of the v-triazole analogue of FA, (285a), followed shortly thereafter by a description of the v-triazole AP derivative (285b) (*Scheme 3.52*) [154, 155]. Coupling diazotized *p*-aminobenzoyl-L-glutamic acid with 2,4-diamino-6-hydroxypyrimidine gave the corresponding azo compound (284a), which was cyclized to (285a) using copper sulphate/pyridine. Repetition of this reaction sequence using 2,4,6-triaminopyrimidine ultimately afforded the AP analogue (285b).



Scheme 3.52

BENZIMIDAZOLE ANALOGUES

At about the same time, a group at Oxford reported their synthesis of a benzimidazole analogue of FA (290, R = H) (*Scheme 3.53*) [48, 156]. Initially, they envisioned that (290) could be obtained from acylation of glutamic acid with (288). The requisite acid chloride (288) was readily obtained in three steps from *o*-phenylenediamine via (286) and (287). Unfortunately, the extreme insolubility of (288) rendered it inactive towards basic solutions of glutamic acid or glycine. Therefore, an alternative approach was developed in which the chloromethylbenzimidazole (286) was used to alkylate (31) to yield (289), from which (290, R = H) was obtained following careful saponification. Repetition of this latter reaction sequence with 5-chloro-2-chloromethylbenzimidazole, available from 4-chloro-*o*-phenylenediamine dihydrochloride and chloroacetic acid, afforded (290, R = Cl) [156].



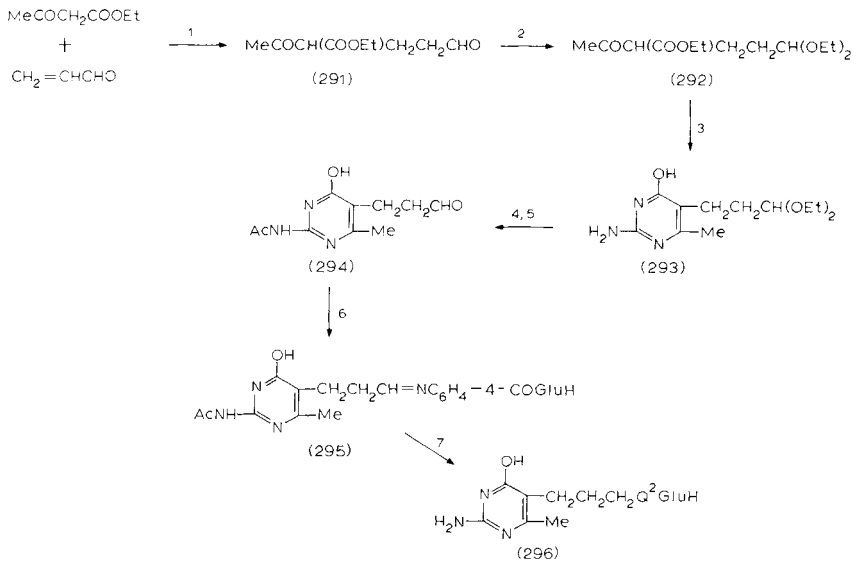
1. EtOH, Δ; 2. NaI, HQ^2OH, EtOH, Δ; 3. HCl; 4. PCl_5, $POCl_3$, Δ; 5. (31), NaI; EtOH, Δ; 6. NaOH–aq. EtOH.

Scheme 3.53

PYRIMIDINE ANALOGUES

B.R. Baker and co-workers reported the earliest synthesis of a pyrimidine analogue structurally related to FA (296) almost 25 years ago (*Scheme 3.54*) [157, 158]. Michael addition of ethyl acetoacetate to acrolein gave an acceptable yield of the glutaraldehyde (291), which was converted to the acetal (292) using conditions which did not effect conversion of the ketone to the enol ether. Guanidine cyclization of (292) produced (293), from which the protected aminoaldehyde (294) was obtained in two steps. Attempts to prepare the anil (295) in ethanol from (294) and *p*-aminobenzoyl L-glutamic acid were consistently frustrated by partial deacetylation of (294) and/or (295). This problem was circumvented using DMF as solvent, and the resulting anil (295) was reduced *in situ* to (296) without further problems.

About 2 years later, a Stanford Research Institute group described the synthesis of the first true pyrimidine analogue of FA (303) which incorporated all of the structural features of FA except the pyrazine ring (see *Scheme 3.55*) [159]. They envisioned that (303) could be assembled from appropriately protected derivatives of isocytosine, glyoxal and *p*-aminobenzoyl L-glutamic



1. NaOMe, EtOH; 2. NH_4Cl, EtOH; 3. guan, EtOH; 4. Ac_2O, Δ; 5. H_2O, Δ; 6. HQ^2GluH, DMF; 7. $NaBH_4$, MeOH.

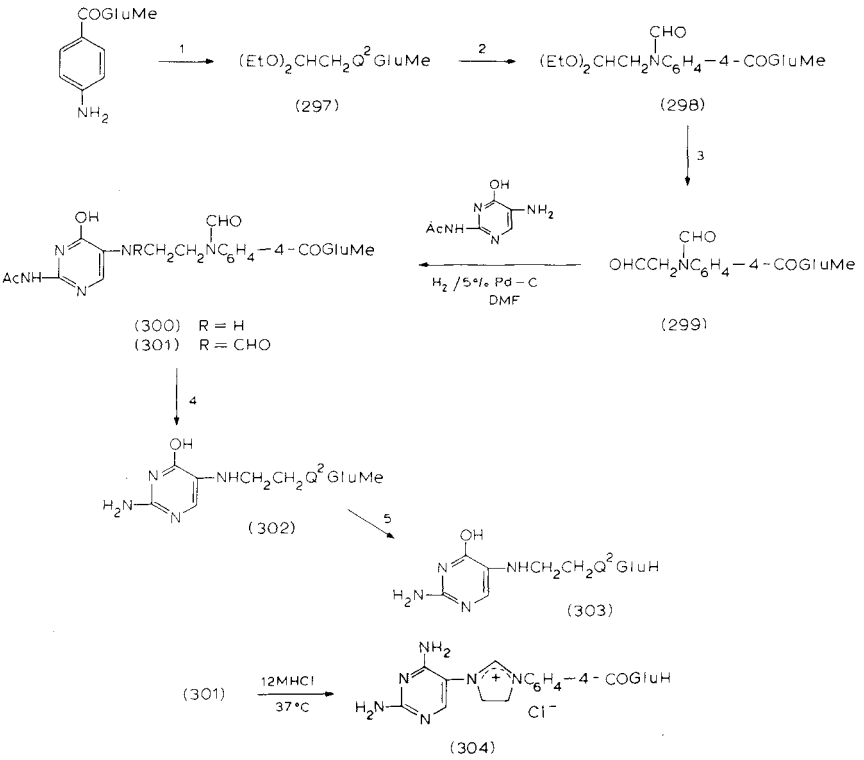
Scheme 3.54

acid. Thus, reductive amination of glyoxal hemiacetal with dimethyl *p*-aminobenzoyl L-glutamate yielded (297), from which the formamide (298) could be obtained only after considerable experimentation due to incomplete formylation with concomitant acetal hydrolysis. Once obtained, (298) proved to be a remarkably fragile compound, undergoing facile deformylation once the aldehyde was unmasked. Use of 98% formic acid accomplished this transformation to produce (299) with a minimum of deformylation.

Reductive amination of 2-acetamido-5-amino-4-hydroxypyrimidine, readily available from isocytosine via nitration, acetylation and reduction, with (299) generated (300), which was characterized as the crystalline bisformamide (301). Mild acid hydrolysis effected cleavage of all three amide bonds to afford (302), which was hydrolyzed to (303) under slightly more vigorous conditions.

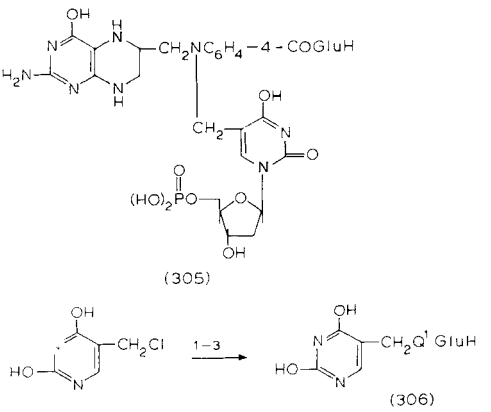
Attempts to hydrolyze (301) directly to (303) using 12 M HCl failed in that the remaining formyl group was found to cyclize with either N-7 or N-10 to afford the cyclic methylene analogue (304).

In 1967 Santi prepared (313) as an analogue of (305), a proposed intermediate in the TS-mediated conversion of 2′-deoxyuridine-5′-monophosphate

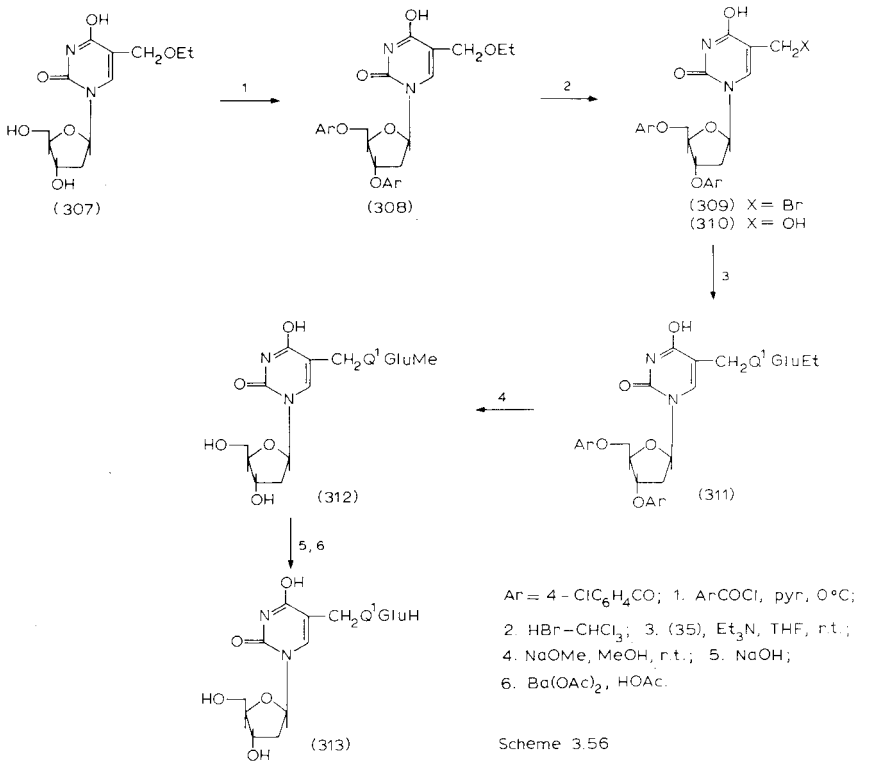


1. $(EtO)_2$**CH**CHO, H_2, 5% Pd/C; 2. HCOOH, Ac_2O, pyr; 3. 98% HCOOH; 4. 1 M HCl – MeOH, r.t.; 5. 12 M HCl, 37 °C.

Scheme 3.55



1. (35), Et_3N, aq. dioxan; 2. NaOH, aq. MeOH; 3. $Ba(OAc)_2$, HOAc.



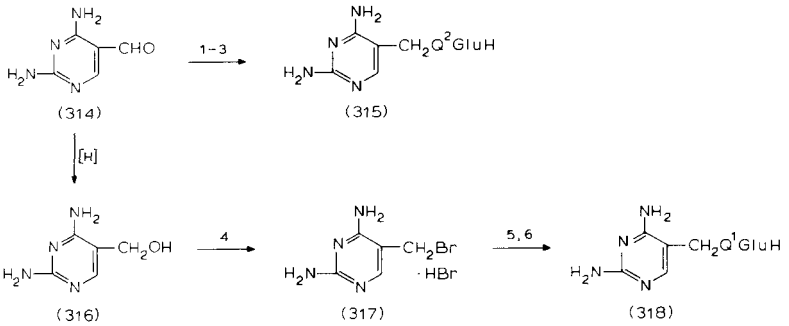
Scheme 3.56

(dUMP) to thymidine-5′-monophosphate (TMP) (*Scheme 3.56*) [50]. Initially, he synthesized (306) as a model system for the construction of (313). Thus, 5-chloromethyluracil was used to alkylate (35), from which (306) was obtained as a barium salt owing to the gelatinous nature of the free acid.

For (313), he began with the intact nucleoside 5-ethoxymethyl-2′-deoxyuridine (307) [160]. After protection of the 3′- and 5′-hydroxy groups with *p*-chlorobenzoyl chloride, the resulting diester (308) was converted into a separable mixture of (309) (major) and (310) (minor). Alkylation of (35) with crude (309) gave (311) together with small amounts of unreacted (35) and a product tentatively identified as (310). Cleavage of the *p*-chlorobenzoates with methanolic sodium methoxide with concomitant transesterification yielded (312), which was saponified and then treated with barium acetate to afford the barium salt of (313).

Shortly thereafter, two groups independently reported the synthesis of a monocyclic analogue structurally related to AP, namely, the 2,4-diamino-5-

pyrimidyl derivative (315) [161, 162]. Cheng's group at the Midwest Research Institute described two separate approaches to (315) (*Scheme 3.57*) [161].



1. HQ^2GluMe; 2. H_2, 10% Pd–C, DMF; 3. NaOH, H_2O, Δ; 4. 30% HBr, HOAc; 5. (35), DMF; 6. aq. NaOH, Δ.

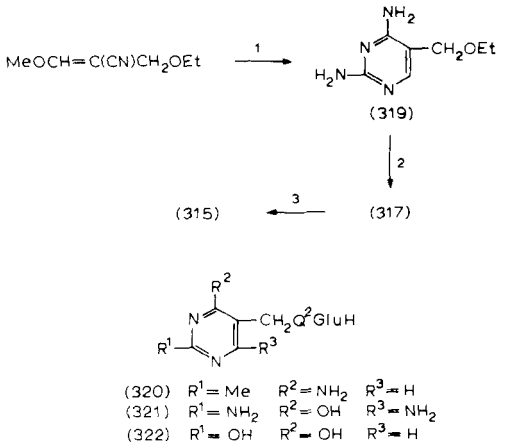
Scheme 3.57

In the first, the known aldehyde (314) [163] was condensed with dimethyl *p*-aminobenzoyl L-glutamate followed by *in situ* imine reduction and hydrolysis to furnish (315). Alternatively, reduction of (314) to the carbinol (316) was followed by conversion to the bromomethyl derivative (317). Alkylation of (31) with crude (317) and subsequent saponification then afforded (315) in better overall yields than their earlier efforts. Repetition of this latter reaction sequence using (35) gave the MTX analogue (318).

In their approach to (315), a Russian group adopted a different strategy to prepare (317) (*Scheme 3.58*) [162]. Thus, base-catalyzed cyclization of 2-ethoxymethyl-3-methoxyacrylonitrile with guanidine nitrate gave (319), from which (317) was obtained in excellent yield again using HBr in acetic acid. Alkylation of *p*-aminobenzoyl L-glutamic acid with (317) at pH 9.5–10.0 then yielded (315) directly. Several other pyrimidine analogues (320–322) were prepared from the respective bromomethylpyrimidines in a similar manner.

An interesting series of extended pyrimidine analogues was described by Davoll and Johnson (*Scheme 3.59*) [164]. Reductive amination of an appropriately substituted benzonitrile (323a–c) with *p*-aminobenzoyl aspartic acid followed by saponification gave (324a–c). The *m*-substituted compound (326) was prepared analogously starting with (325).

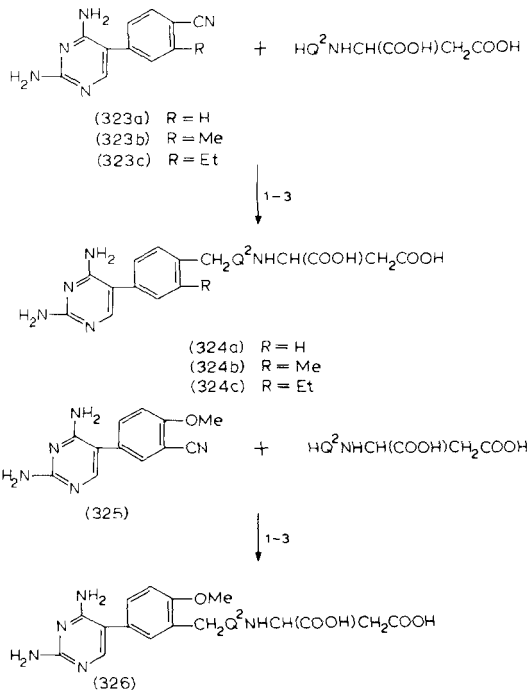
Very recently, Broom and Yang have prepared new, flexible multisubstrate analogues (330) of (170) which retain all of the appropriate groups necessary for enzymatic binding but do not suffer from the problems associated with



(320) R^1 = Me R^2 = NH_2 R^3 = H
(321) R^1 = NH_2 R^2 = OH R^3 = NH_2
(322) R^1 = OH R^2 = OH R^3 = H

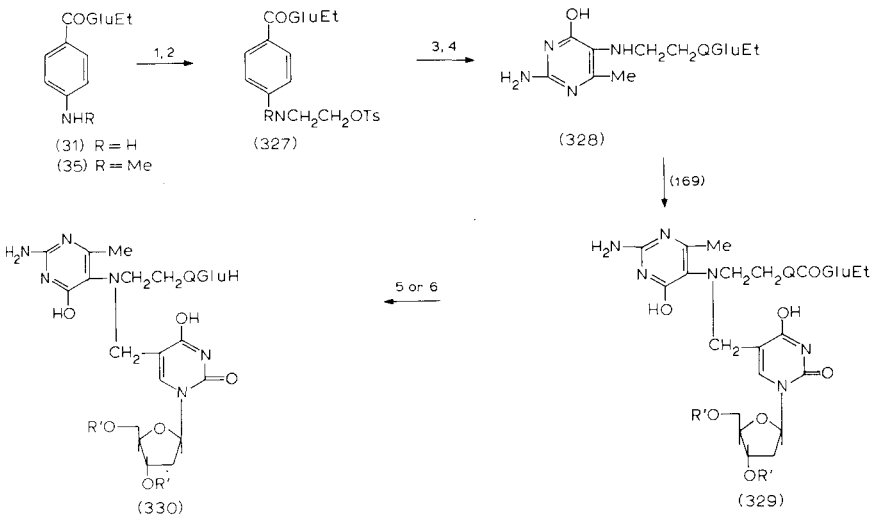
1. guan. HNO_3, NaOEt; 2. HBr, HOAc; 3. HQ^2GluH, NaOH.

Scheme 3.58



1. H_2, Ni(R), HOAc; 2. NaOH, EtOH; 3. HCl.

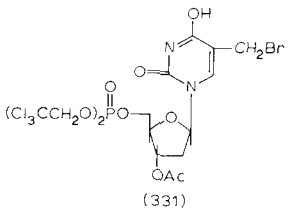
Scheme 3.59



1. Ethylene oxide, 50% HOAc; 2. TsCl; pyr; 3. 2,5-diamino-4-hydroxy-6-methylpyrimidine, DMF; 4. 30% HBr–HOAc; 5. 1 M NaOH – EtOH; 6. Zn–Cu couple, NaOH, EtOH.

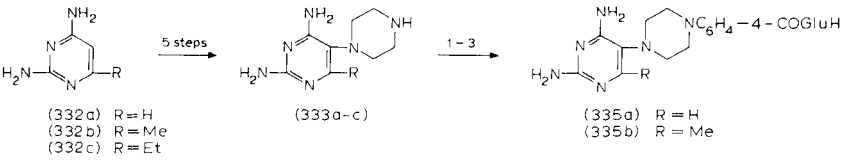
Scheme 3.60 (Q: see p. 90)

diastereomer formation at C-6 of (170) (*Scheme 3.60*) [165]. Using a convergent methodology, the side-chain-containing moiety (327, R = Ts) was readily assembled from (31) via sequential hydroxyethylation and tosylation. The corresponding analogue (327, R = Me) was also prepared in excellent yield in the same manner using (35). Alkylation of 2,5-diamino-4-hydroxy-6-methylpyrimidine (used in excess to minimize dialkylation) with (327, R = Ts) yielded (328, R = Ts) which was then detosylated using 30% HBr in acetic acid. Alkylation of the resulting free amine (328, R = H) with (169) gave (329, R′ = OAc), which was saponified to produce (330, R = R′ = H). Repetition of this reaction sequence with (327, R = Me) afforded (330, R = Me, R′ = H).



The nucleotide derivatives (330, R = H, R′ = PO_3^{2-} and R = Me, R′ = PO_3^{2-}) were obtained analogously from (328) (R = H, Me) and the selectively protected nucleotide (331) [166, 167]. The trichloroethyl groups were reductively cleaved with Zn-Cu couple, and saponification then gave the desired analogues.

As part of a long-standing programme for the synthesis of potential antifolates, chemists at Warner-Lambert have prepared the novel piperazinyl pyrimidines (335a,b) in which N-7 and N-10 have been linked via an ethylene bridge (*Scheme 3.61*) [168]. These compounds were available via arylation of



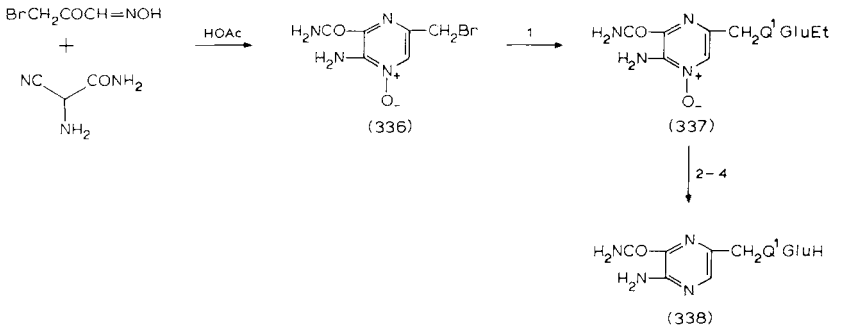
1. 4 – $FC_6H_4COGluEt$; 2. Saponification; 3. H^+.

Scheme 3.61

(333a–c), prepared from (332a–c) in five steps, with diethyl *p*-fluorobenzoyl L-glutamate (334). Preliminary biological evaluations suggest that this class may provide useful anticancer agents.

PYRAZINE ANALOGUES

A very recent report from a group at Burroughs-Wellcome details the only example of a monocyclic pyrazine analogue (338) of MTX (*Scheme 3.62*)



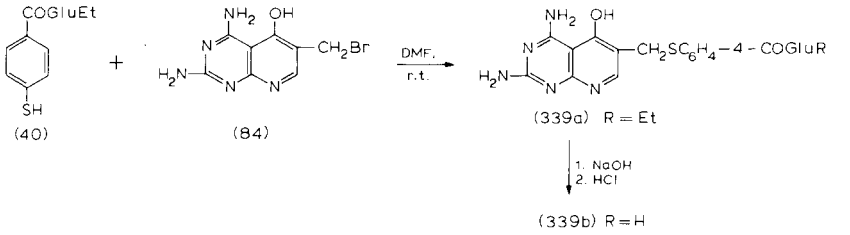
1. (35), K_2CO_3 – DMSO; 2. PCl_3; 3. 0.1 M NaOH; 4. 0.1 M HCl.

Scheme 3.62

[169]. Employing the classical Taylor pyrazine sequence, aminocyanoacetamide [170] was condensed with bromopyruvaldoxime [171] to yield the pyrazine carboxamide (336). Alkylation of (35) with (336) gave (337) which was deoxygenated with phosphorus trichloride and then saponified to furnish (338).

5-DEAZA-5-OXO-10-THIA ANALOGUES

During their work on 5-deaza-5-oxo analogues of AP, Broom's group also synthesized 5-deaza-5-oxo-10-thiaAP (339) via alkylation of the sodium salt of (40) with the bromomethyl enaminone (84) (see *Scheme 3.17*) [76]. As shown below, saponification of (339a) gave (339b) as expected (*Scheme 3.62a*).

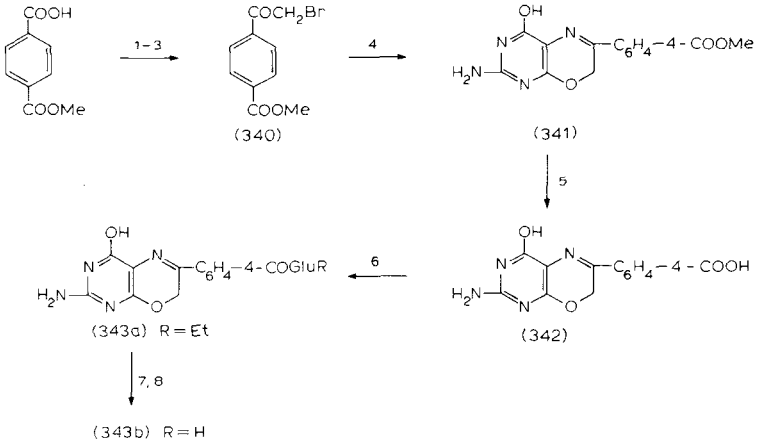


Scheme 3.62a

8-DEAZA-9,10-MODIFIED ANALOGUES

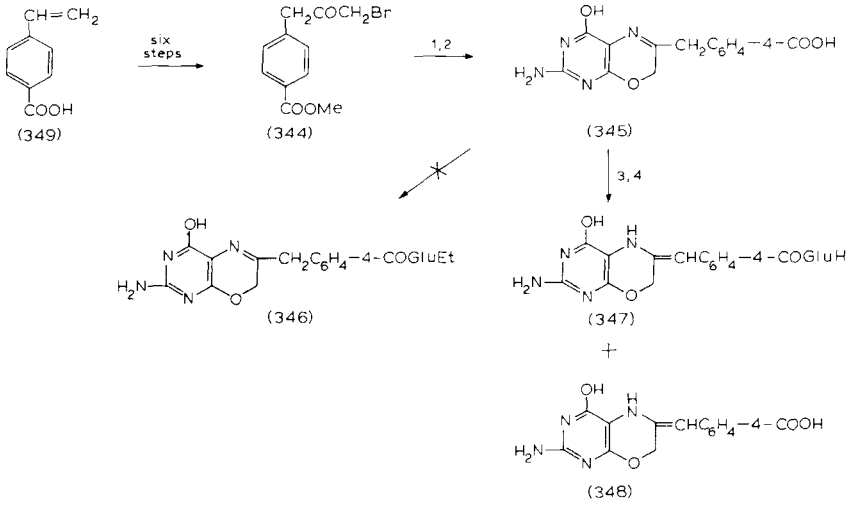
Winchester, Zappone and Skinner's synthesis of 7,8-dihydro-8-oxapteroic acid (190) represents the only example of this heterocyclic ring system incorporated into a precursor which could be elaborated to a FA analogue [112]. However, a recent report from Nair and co-workers describes this same ring system incorporated into potential DHFR inhibitors which have simultaneously been modified in the C-9,N-10 bridge region as well (*Scheme 3.63*) [172].

In one case, methyl terephthalic acid, obtained in two steps from *p*-formylbenzoic acid, was converted to the phenacyl bromide (340) in the usual manner. Alkylation of 2,5-diamino-4,6-dihydroxypyrimidine with (340) with subsequent cyclization produced the dihydrooxazine pteroate ester (341), which was hydrolyzed to (342) [173]. Coupling (342) with diethyl L-glutamate using isobutyl chloroformate [174] yielded the diester (343a) which was then saponified to the dihydrooxazine FA analogue without the C-9,N-10 bridge (343b).



1. $SOCl_2$, Δ, 2. CH_2N_2, 3. HBr; 4. (186a) (p.125) $NaHCO_3$, MeOH; 5. 0.1 M NaOH; 6. Bu^tCF, NMM, GluEt; 7. 0.1 M NaOH, MeCN; 8. HCl.

Scheme 3.63

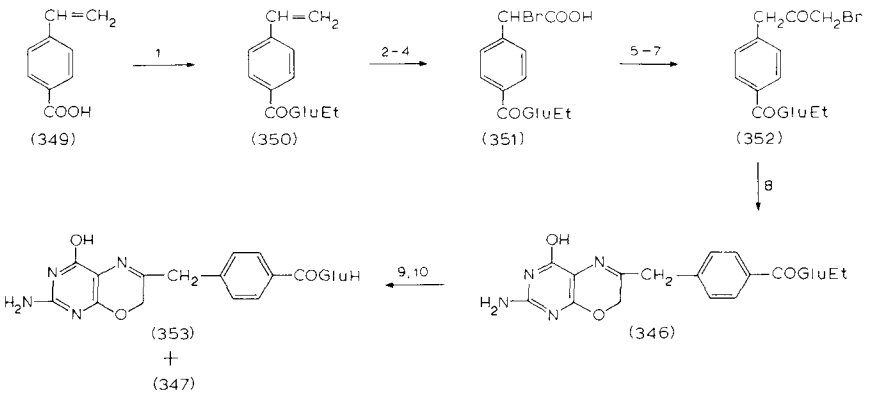


1. (186a), $NaHCO_3$, MeOH; 2. NaOH; 3. Bu^tCF, NMM; 4. NaOH – MeCN.

Scheme 3.64

Attempts to adapt this methodology to the preparation of the 9-methylene homologue (353) were ultimately frustrated by an untimely double bond isomerization (*Scheme 3.64*). Thus, *p*-vinylbenzoic acid was converted to the α-bromoketone (344) [175], which condensed smoothly with 2,5-diamino-4,6-dihydroxypyrimidine to yield (345) after saponification. Elaboration of the side-chain as before (*vide supra*) was presumed to generate (346). Unfortunately, after hydrolysis and work-up, the 6,9-dehydro isomer (347), together with the analogous 'pteroic acid' (348), were the only products isolated. Convincing NMR evidence to support these structures was presented. Ultimately, it was determined that the acidic reaction conditions of the mixed anhydride coupling effected this undesired double-bond isomerization, necessitating a modified synthetic strategy.

To that end, *p*-vinylbenzoic acid (349) was coupled with diethyl L-glutamate to produce (350), from which (351) was obtained in three straightforward steps (*Scheme 3.65*). Homologation of this α-bromo acid (351) to the α-bromoketone (352) was accomplished as before after debromination of (351) with zinc in acetic acid. Condensation of (352) with the requisite pyrimidine was achieved using MgO to scavenge the HBr and minimize the undesired double-bond isomerization. The resulting diester (346) was saponified to afford the target 5,6-dehydro analogue (353), together with a small amount of (347).

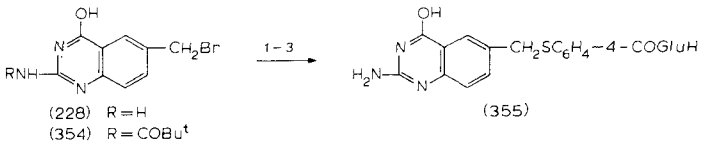


1. Bu^{t}CF, NMM, GluEt; 2. mcpba; 3. HBr; 4. Jones oxidation; 5. Zn–HOAc; 6. $SOCl_2$; 7. CH_2N_2; 8. (186a), MgO, MeOH; 9. NaOH–MeCN; 10. citric acid.

Scheme 3.65

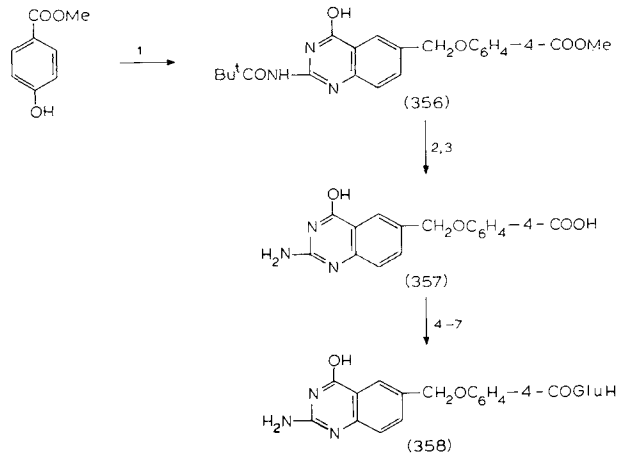
5,8-DIDEAZA-10-OXA; 10-THIA-, 10-PHENOXY-AP AND 9,10-MODIFIED ANALOGUES

As part of their synthetic programme for the preparation of quinazoline analogues of FA, Oatis and Hynes synthesized 5,8-dideaza-10-thiaFA (355) (*Scheme 3.66*) and 5,8-dideaza-10-oxaFA (358) [176]. For (355), 2-amino-4-hydroxy-6-bromomethylquinazoline (228) [126] was used to alkylate the sodium salt of 40 [53] followed by mild saponification to generate (355). Attempts to prepare (358) analogously using either (228) or the protected derivative (354) [126] via direct alkylation of diethyl 4-hydroxybenzoyl glutamate (37) resulted only in complex mixtures and necessitated an alternative synthetic strategy (*Scheme 3.67*).



1. (40), DMF, r.t.; 2. 0.2M NaOH; 3. HCl.

Scheme 3.66

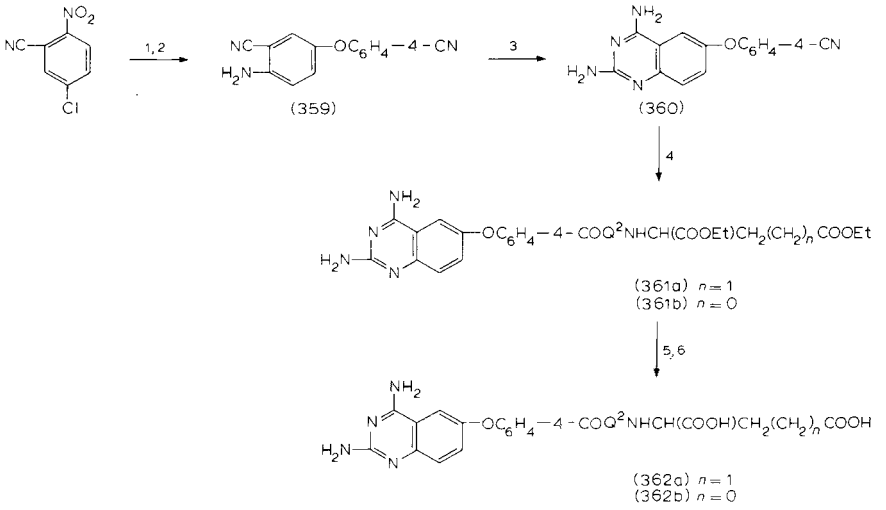


1. (354), $CsHCO_3$, DMF; 2. HCl–MeOH; 3. 0.2M NaOH; 4. Bu^iCF, NMM; 5. $H_2NCH(COOCH_2Ph)CH_2CH_2COO$ resin; 6. 2 M NaOH–dioxan; 7. 0.1 M HCl.

Scheme 3.67

This new approach involved alkylation of methyl 4-hydroxybenzoate with (354) using caesium bicarbonate as the acid scavenger [174]. The resulting amide ester (356) was sequentially deprotected and saponified to produce (357). Incorporation of the glutamic acid moiety was best accomplished using solid-phase peptide synthesis techniques and gave consistently better overall yields of (358) [102, 177].

During their extensive programme to develop new folate antagonists with reduced toxicological and cross-resistance liabilities, Elslager's group at Warner Lambert/Parke Davis described the synthesis of (362a), a quinazoline analogue of AP in which a phenoxy group has been interposed between the quinazoline ring and the C-9,N-10 bridge (*Scheme 3.68*) [74]. The requisite

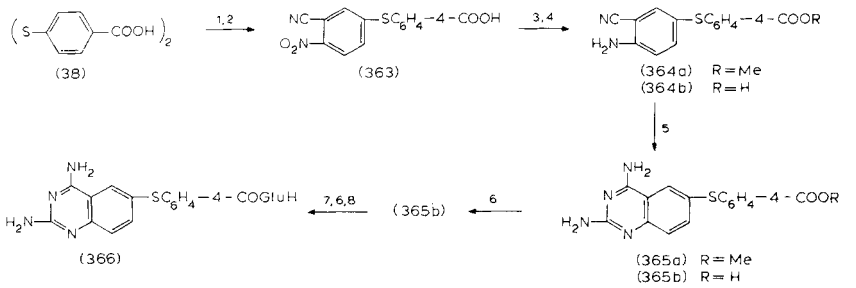


1. NaH, 4-HOC_6H_4CN, DMF; 2. $SnCl_2$,HCl–HOAc; 3. $ClC(=NH)NH_2$, diglyme; 4. (31), H_2–Ni(R), aq. HOAc; 5. 2 M NaOH, r.t.; 6. HCl.

Scheme 3.68

o-aminonitrile (359), readily available in two steps from 2-cyano-4-chloronitrobenzene, was cyclized with chloroformamidine hydrochloride to yield (360), from which (361a) was obtained via reductive amination with (31). Saponification of (361a) gave (362a). Similarly, the aspartate derivatives (361b) and (362b) were prepared from (223).

This same group also prepared (366), a non-classical quinazoline folate antagonist in which the C-9,N-10 bridge has been replaced by a sulphur atom



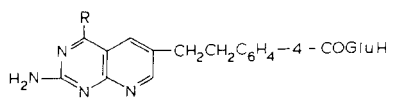
1. $NaBH_4$; 2. Et_3N, Me_2CO, 5-chloro-2-nitrobenzonitrile; 3. Me_2SO_4; 4 $SnCl_2$, HCl; 5 $ClC(=NH)NH_2 \cdot HCl$, DMSO, Δ; 6. NaOH; 7. GluEt, $HOBu^t$, DCC; 8. HCl.

Scheme 3.69

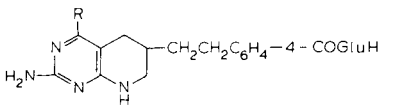
(*Scheme 3.69*) [74, 178]. Reduction of 4,4-dithiobis(benzoic acid) (38) [52] with sodium borohydride generated 4-mercaptobenzoic acid, which was arylated with 5-chloro-2-nitrobenzonitrile to generate (363). Methylation of (363) in the presence of 1,1′,1″-nitrilotris[2-propanol] followed by reduction to the *o*-aminonitrile (364a) was uneventful. Cyclization of (364a) with chloroformamidine hydrochloride gave (365a) quantitatively. After saponification, the resulting acid (365b) was coupled with diethyl glutamate in poor yield using DCC/*N*-hydroxybenzotriazole [179] to afford (366) after ester hydrolysis. An alternative approach to (365b) involving reduction of (363) to (364b) and then cyclization with chloroformamidine hydrochloride was abandoned in light of the poor yield (< 10%) encountered during the last step.

5,10-DIDEAZA ANALOGUES

Within the last year a report from Taylor's laboratories described several approaches to 5,10-dideaza derivatives, a class which has stimulated much interest in light of the exceptional and unique biological activity displayed by some representative members of this class of folate antimetabolites. In particu-

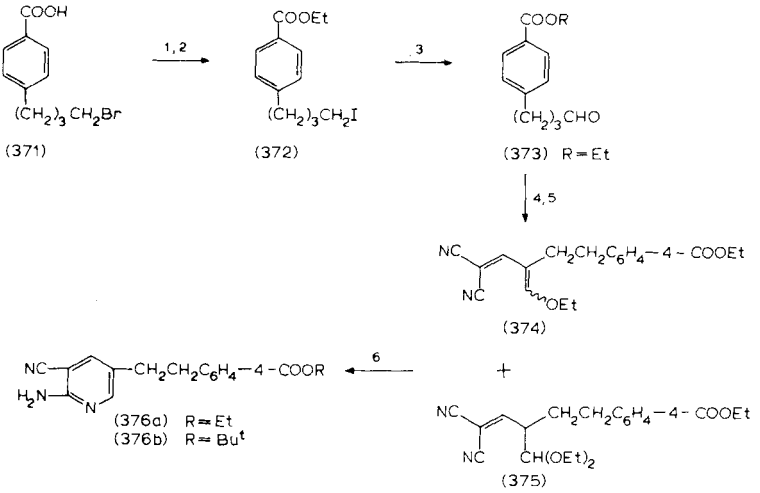


(367) R = OH
(368) R = NH_2



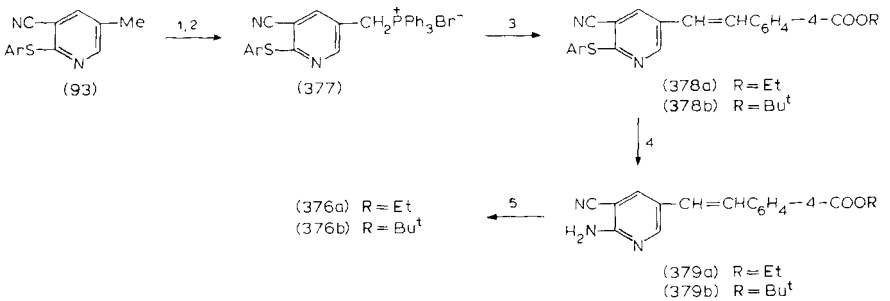
(369) R = OH
(370) R = NH_2

lar, they have completed the first syntheses of 5,10-dideazaFA (367), 5,10-dideazaAP (368), and the corresponding 5,6,7,8-tetrahydro derivatives (369) and (370) (*Schemes 3.70–3.73*) [180].



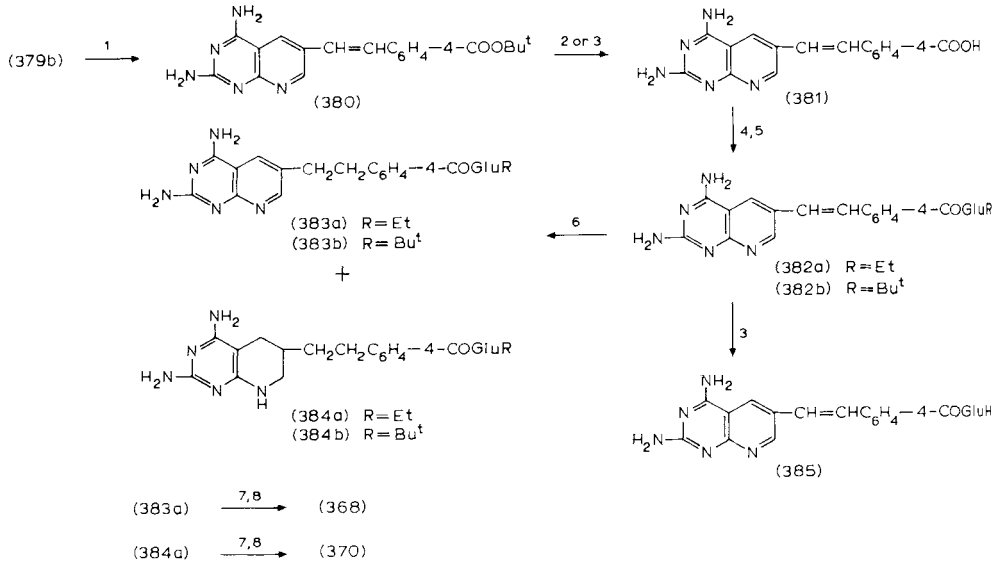
1. KI–Me_2CO; 2. EtOH, H_2SO_4; 3. $Me_3\overset{+}{N}\text{–}\bar{O}$; 4. $CH_2(CN)_2$, PhH, DL-alanine; 5. TEOF, Ac_2O, Δ; 6. NH_3–EtOH.

Scheme 3.70



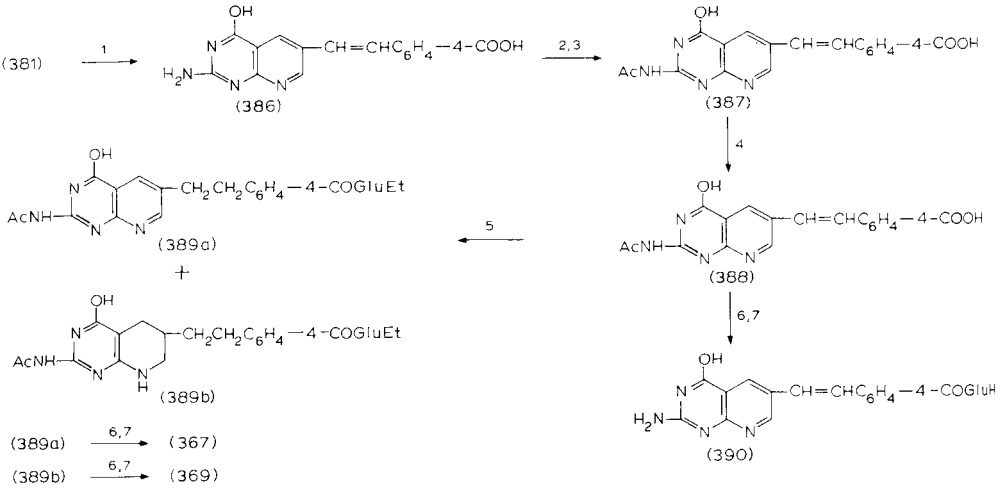
1. NBS, PhH, hν; 2. Ph_3P, PhH; 3. 4-$OHCC_6H_4COOR$, Et_3N; 4. liq. NH_3, $CuBr_2$; 5. H_2, 5% Pd–C, EtOAc.

Scheme 3.71



1. guan, EtOH, Δ; 2. 88% HCOOH; 3. HCl–$MeNO_2$; 4. $(PhO)_2POCl$, NMM, NMP; 5. GluR; 6. H_2, Pd–C, TFA; 7. NaOH–MeOH; 8. HOAc.

Scheme 3.72

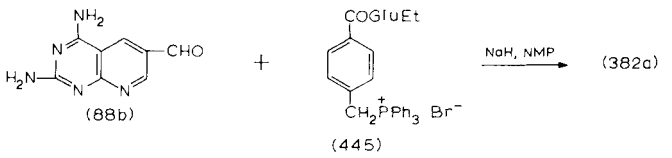


1. 1 M NaOH, Δ; 2. Ac_2O, DMAP; 3. NaOH; 4. PhO(PhNH)POCl, NMM, NMP, GluEt; 5. H_2, Pd/C, TFA; 6. NaOH–MeOH; 7. HOAc.

Scheme 3.73

Initially, it was expected that the aminonitrile (376) would be a viable precursor to (367)–(370) via cyclization to a fused 2,4-diaminopyrimidine [181]. Accordingly, two independent syntheses of (376) were developed. In the first (*Scheme 3.70*), 4-(4-bromobutyl)benzoic acid (371) was converted to the iodide and esterified to yield (372) from which the aldehyde (373) was obtained via oxidation with trimethylamine *N*-oxide. Sequential reaction of (373) with malononitrile, then triethyl orthoformate, gave a mixture of (374) and (375), which was converted to (376a) by treatment with ammonia. An alternative approach was patterned after previously developed methodology for 5-deazaAP (*Scheme 3.71*) [80]. The known pyridine (93) (*vide supra*) was treated sequentially with *N*-bromosuccinimide and triphenylphosphine to generate (377). Wittig reaction of (377) with ethyl *p*-formylbenzoate gave the *trans*-styryl derivative (378), which in turn was aminated with liquid ammonia/cuprous bromide to yield (379a). Finally, catalytic hydrogenation of (379a) afforded (376a).

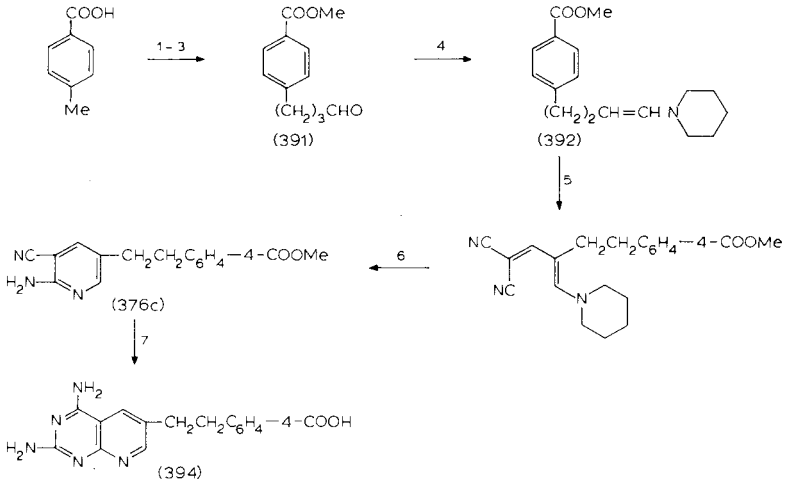
Surprisingly, all attempts to cyclize (376a) with guanidine, guanidine acetate or dimethylguanidine were frustrated by a combination of reduced electrophilicity of the nitrile grouping (relative to the analogous pyrazine) and increased electrophilicity of the benzoate carbonyl group (resulting in guanidine aroylation). Substitution of *t*-butyl-*p*-formylbenzoate in the Wittig reaction ultimately gave (376b), with which equally disappointing results were obtained. Using (379b) in the cyclization reaction, however, successfully led to (380) (*Scheme 3.72*). De-*t*-butylation was effected either with 88% formic acid or with HCl-nitromethane to produce the key pteroic acid analogue (381). Coupling of (381) with diethyl or di-*t*-butyl-L-glutamate using diphenylchlorophosphonate yielded (382a) and (382b), respectively. The structure of (382a) was unequivocally established by an independent synthesis from (88b) and the Wittig reagent prepared from diethyl *N*-[*p*-(bromomethyl)benzoyl]-L-glutamate (445) [182]. Considerable experimentation was necessary to determine suitable conditions to effect reduction of (382a). Having achieved this, the resulting separable mixture of (383a) and (384a) was saponified to afford (368) and (370), respectively. De-*t*-butylation of (382b) produced the 9,10-dehydro derivative (385).



The FA analogues (367) and (369) were produced via substantially the same methodology (*Scheme 3.73*). Thus, (381) was selectively hydrolyzed to (386), from which (387) was obtained uneventfully. Coupling (387) with diethyl L-glutamate to yield (388) was best accomplished using phenyl *N*-phenylphosphoramidochloridate. Catalytic hydrogenation, separation of (389a) and (389b), and saponification then gave (367) and (369), respectively. Finally, alkaline hydrolysis of (388) afforded the 9,10-dehydro derivative (390).

It is important to note that 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (369) is, as prepared above by catalytic hydrogenation of (388) (followed by saponification), a mixture of two diastereomers (DL and LL). Their separation was achieved by reaction of (369) with (+)-10-camphorsulphonic acid to give a mixture (DDL and DLL) of salts separated by fractional crystallization. Subsequent hydrolysis then gave (369) (isomer A, $[\alpha]_{589} = +31.09$) and (isomer B, $[\alpha]_{589} = -21.06$). The initially isolated mixture of diastereomers had $[\alpha]_{589} = +10.22$.

Although the emphasis in this review is on the chemistry of FA and MTX analogues, the extraordinary, unique antitumour activity of (369) deserves special mention. This compound (both isomers) is a potent inhibitor of cell growth in culture, without significant effect on DHFR or TS. The pattern of cytotoxicity reversal by various metabolic precursors is distinctly different from that seen with DHFR inhibitors; the primary site of its action has been identified as glycinamide ribonucleotide (GAR) transformylase. Compound (369) inhibits the formation of FGAR, the product of the GAR transformylase reaction, with precisely the same concentration dependence as it inhibits cell growth. Furthermore, intracellular pools of ATP and GTP, end-products of purine synthesis, are markedly suppressed by (369), strongly suggesting an apparent mechanism for its cytotoxicity. This compound (both isomers) shows dramatic and selective activity against a wide variety of solid tumours against which MTX has little or no activity; it is also active against MTX-resistant tumours. Results are typified by those found against subcutaneous B-16 melanoma (renowned for its resistance to other chemotherapeutic agents), where (369) produces complete inhibition of tumour growth at <6 mg/kg per day for 10 days without evidence of host toxicity up to >100 mg/kg per day. Apparently, all of the structural features of (369) combine to make it a uniquely effective FA antimetabolite. The presence of a 2-amino-4-hydroxypyrimidine 'A' ring and a fully reduced 'B' ring make (369) unlikely to be an inhibitor of DHFR; the absence of nitrogen atoms at positions 5 and 10 preclude (369) serving as a cofactor in any of the one-carbon transfers of folate metabolism; the presence of the normal *p*-benzoylglutamate moiety allows conversion to polyglutamate forms (believed to be the biologically effective form of (369)), and

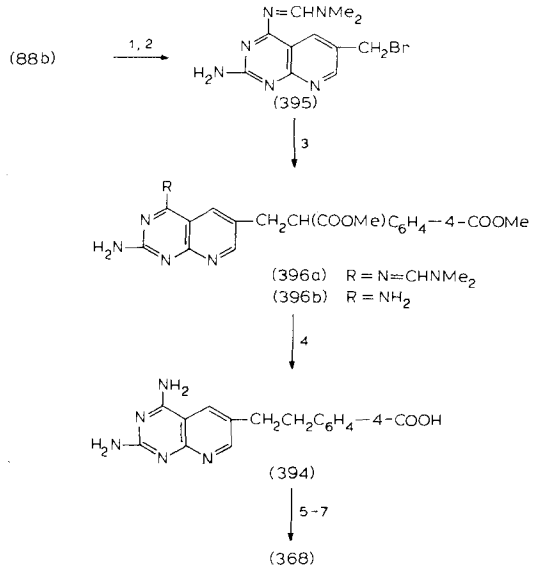


1. LDA(2 eq.), $MeOCH{=}CHCH_2Cl$; 2. $NaHCO_3$—MeI; 3. 1 M HCl, THF; 4. piperidine, K_2CO_3; 5. $EtOCH{=}C(CN)_2$, MeCN, r.t.; 6. NH_4OH, MeOH; 7. guan, $MeOCH_2CH_2OH$, 100 °C, 72h.

Scheme 3.74

the presence of carbon rather than nitrogen at position 5 avoids the chemical instability which otherwise plagues tetrahydrofolate derivatives.

About 1 year later a group of workers at SRI described their efforts to synthesize (368) [184]. Using methodology previously employed in their approach to 10-substituted 10-deazaAP analogues, *p*-toluic acid was smoothly converted to (391) in three steps (*Scheme 3.74*) [185]. After reaction with piperidine, the resulting enamine (392) was condensed with ethoxymethylenemalononitrile to give (393) in low yield. Enamine exchange of (393) with ammonia and concomitant cyclization generated (376c) [186]. Guanidine cyclization of (376c) afforded (394) directly, although the authors noted that this procedure was not easily reproduced. These results are in contrast to Taylor's previous observation regarding their inability to cyclize (376a) or (376b) under similar conditions (*vide supra*). In view of the many experimental difficulties encountered in this approach, an alternative preparation of (394) was sought to complete the synthesis of (368). Thus, the known 2,4-diamino-5-deazapterin-6-carboxaldehyde (88b) [80, 82] was converted to (395) [187], which was used to alkylate the dianion of dimethyl homoterephthalate to produce a mixture of (396a) and (396b) (*Scheme 3.75*). This mixture was not separated, but was immediately treated with a hot solution of sodium cyanide in DMSO [188] to effect ester dealkylation with concomitant decarboxylation



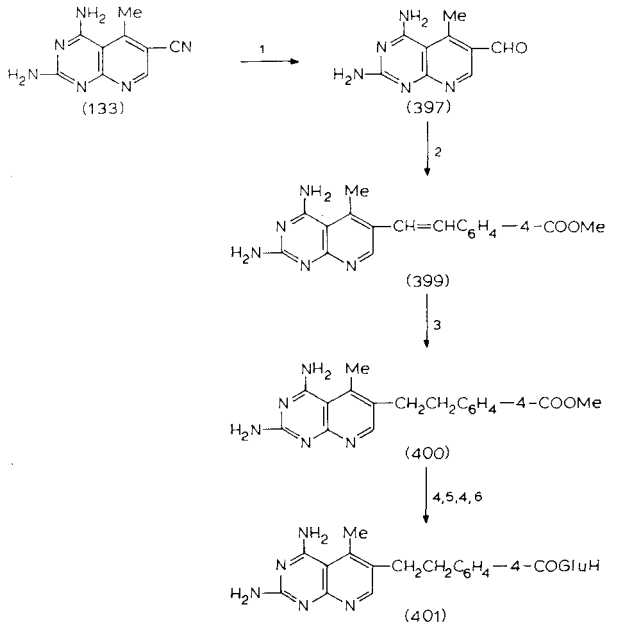
1. $NaBH_4$, MeOH; 2. Ph_3PBr_2, DMF; 3. KH, -40°C, 4-$MeOOCC_6H_4CH_2COOMe$; 4. NaCN-DMSO, 180°C Δ; 5. Bu^tCF, Et_3N, DMF, GluEt; 6. NaOH, $EtOCH_2CH_2OH$; 7. HOAc.

Scheme 3.75

of the benzylic carboxyl group to afford (394). Conversion of (394) to (368) was straightforward and gave material identical with that prepared by Taylor's group, as shown by ^{1}H-NMR (TFA-d).

The most recent work in this area, the result of intense synthetic efforts by both Montgomery's and Taylor's groups, has been directed at the preparation of alkyl analogues of (368)–(370) [97, 189]. Montgomery's efforts have focused on the synthesis of 5-alkyl compounds, namely, 5-methyl-5,10-dideazaAP (401) and 5-methyl-5,10-dideaza-5,6,7,8-tetrahydroFA (405) as shown in *Scheme 3.76* and *Scheme 3.77* [97]. For (401), the key aldehyde (397), readily prepared from (133), was condensed with (398) to yield (399), which was carefully reduced to (400). The choice of reaction conditions for the conversion of (399) to (400) was critical to avoid overreduction. Saponification of (400) and incorporation of the glutamic acid moiety then completed the synthesis.

Careful hydrolysis of the 4-amino group in (399) with concomitant saponification generated (402), which was coupled with dimethyl L-glutamate to (403).

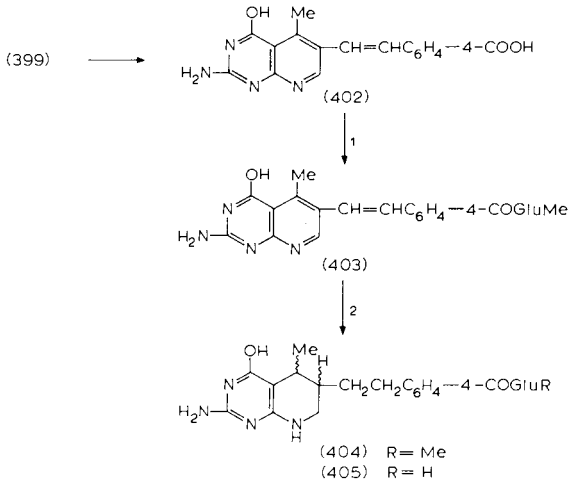


1. HCOOH, Ni(R); 2. base, 4-$MeOOCC_6H_4CH_2\overset{+}{P}Ph_3\bar{B}r$ (398); 3. H_2, 5% Pd-C, DMF, r.t.; 4. NaOH; 5. GluEt; 6. HCl.

Scheme 3.76

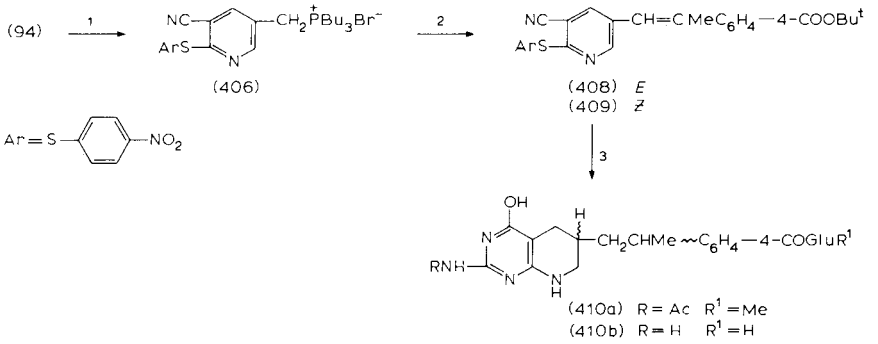
Reduction of the pyridine ring as well as the 9,10-double bond was achieved using Adams's catalyst in the presence of 1 equiv. of 0.1 M HCl in methanol. The resulting mixture of diastereomers (404) was then saponified to afford (405) as a mixture of four diastereomers.

Synthetic efforts from Taylor's laboratories have culminated in the preparation of the regioisomeric 10-methyl analogue, namely 10-methyl-5,10-dideaza-5,6,7,8-tetrahydroFA (410b) (*Scheme 3.78*) [189]. Their methodology was patterned after that used to obtain (369). Thus, (94) was converted to (406); the derived ylide condensed smoothly with (407) to generate a mixture of (408) and (409). The ylide derived from (377) was unreactive in this sequence. Conversion of (408)/(409) to (410b) was analogous to previous work (*Scheme 3.73*). Resolution of the four diastereomers of (410a) was achieved using an HPLC cyclobond column and led to isolation of the active diastereomer, the absolute configuration of which has not yet been determined.



1. GluMe; 2. H_2, PtO_2, 0.1 M HCl–MeOH.

Scheme 3.77

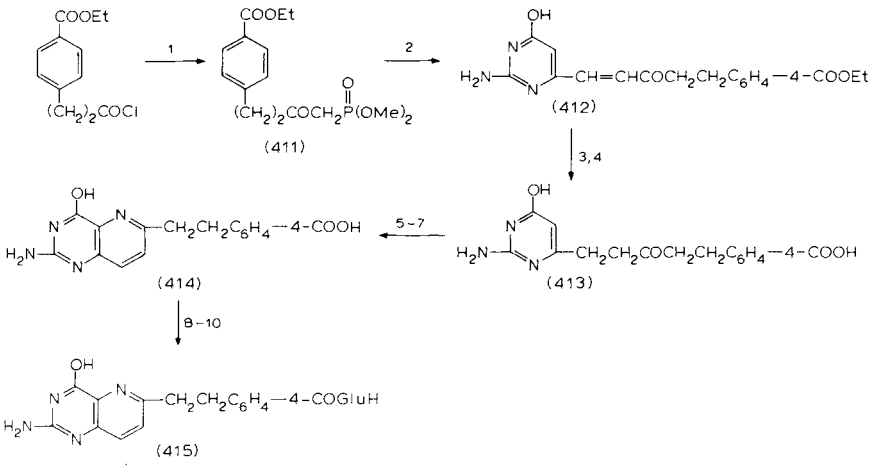


1. Bu_3P; 2. DBU, CH_2Cl_2, Δ, 4-$AcC_6H_4COOBu^t$ (407); 3. Steps in Scheme 3.72.

Scheme 3.78

8,10-DIDEAZA-, 8,10-DIDEAZA-5,10-METHYLENE-5,6,7,8-TETRAHYDRO, 8,10-DIDEAZA-10-OXA- AND 8,10-DIDEAZA-10-THIA ANALOGUES

A promising class of derivatives is the 8,10-dideaza series, the subject of intensive investigation. Adapting previously described methodology for 8-

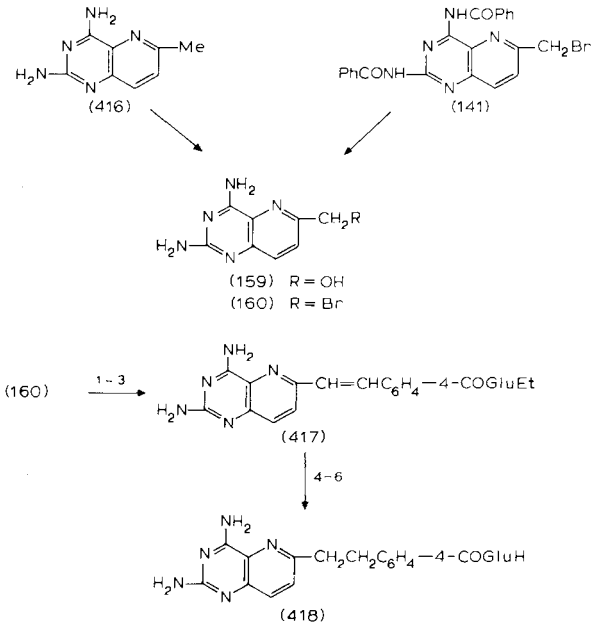


1. $(MeO)_2POCH_2Li$, -75°C; 2. NaH, (151); 3. H_2, Rh; 4. NaOH; 5. $PhN_2^+Cl^-$; 6. H_2, Pd-C; 7. [O], 8. Bu^tCF, Et_3N, GluEt; 9. 0.1 M NaOH; 10. HCl.

Scheme 3.79

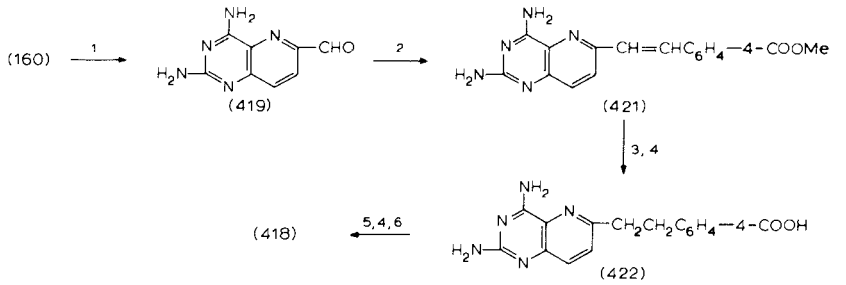
deaza and 8-deazahomo analogues to this series led to the preparation of 8,10-dideazaFA (415) (*Scheme 3.79*) [109a]. Inverse addition of lithiodimethylmethylphosphonate to *p*-ethoxycarbonylphenylpropionyl chloride produced (411) in good yield. This technique capitalized on acylation by the inherently more reactive acid chloride while minimizing exposure of the ester to the lithium reagent. Base-catalyzed condensation of (411) with (151) generated enone (412), which was reduced and saponified to (413). Diazo coupling of (413) and catalytic reduction afforded (414). Isolation of the aromatic 8-deazapteroic acid (414) was attributed to either disproportionation or air oxidation of the 7,8-dihydro intermediate. Peptide coupling of (414) and saponification then gave the desired target compound, (415).

Conceptually, a much different approach was used to prepare 8,10-dideazaAP (418) 3 years later (*Scheme 3.80*) [190a,b]. Here, (418) was assembled by a convergent pathway involving formation of the C-9,C-10 bond as the critical step. Until Broom's synthesis of (160) from (159) using PBr_3-THF [86], this labile material was accessible only with great difficulty from either (416) or (141) due to competitive dibromination in the former case [42] or the formation of complex, inseparable mixtures in the latter [98]. Once in hand, (160) was converted to the corresponding ylide, which was condensed with (252) [140] to yield the 9,10-dehydro derivative (417). Catalytic reduction of (417) followed by saponification produced (418).



1. Ph_3P; 2. NaOMe; 3. (252); 4. H_2, PtO_2–HOAc; 5. 1 M NaOH; 6. HCl.

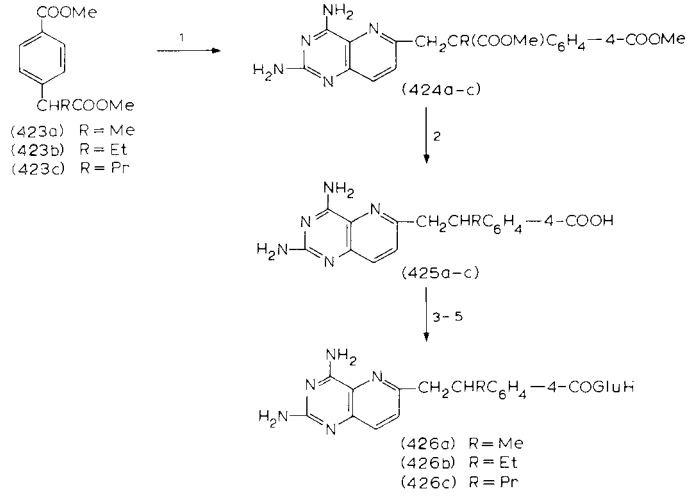
Scheme 3.80



1. Me_2CHNO_2, NaOMe; 2. 4-$MeOOCC_6H_4CH_2PO(OEt)_2$ (420), NaH–DMF; 3. H_2, PtO_2, HOAc–MeOH; 4. NaOH; 5. GluEt, couple; 6. H^+.

Scheme 3.81

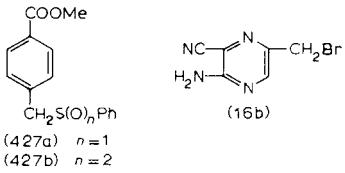
An alternative synthesis of (418), again involving construction of the C-9, C-10 bond as the critical step, was also evaluated (*Scheme 3.81*). In this approach, (160) was first oxidized to (419) using sodium methoxide/2-nitropropane, followed by condensation under Horner-Emmons conditions with phosphonate (420). The resulting 9,10-dehydro derivative (421) was converted to (422) via hydrogenation and saponification. Glutamate coupling of (422) and subsequent saponification then yielded (418). This route was not pursued for the synthesis of 10-alkyl analogues, since the preparation of secondary alkyl phosphonate homologues of (420) was not convenient.



1. KH, (160), DMF; 2. NaCN, DMSO, 170–180°C; 3. Bu^{i}CF, Et_3N, GluEt; 4. NaOH; 5. HCl.

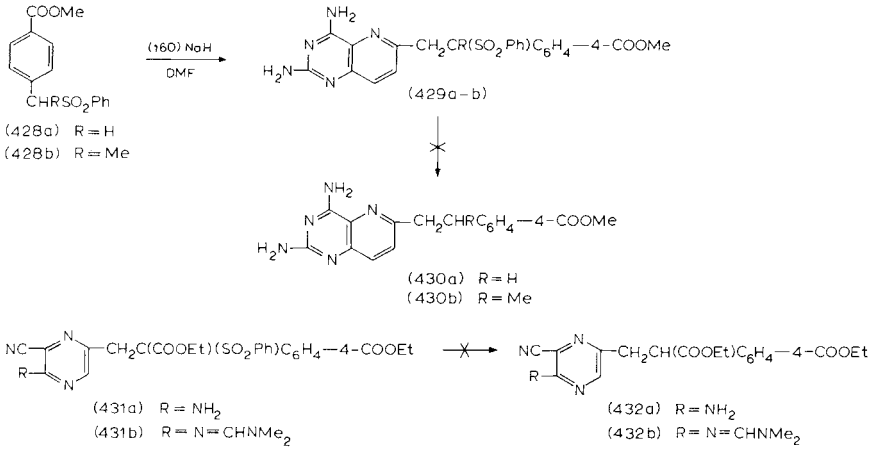
Scheme 3.82

A series of 10-alkyl homologues of (418) was described by the same group (*Scheme 3.82*) [191, 192). Interestingly, (160) was again the starting material of choice, although neither of the previous routes (*cf. Schemes 3.80, 3.81*) was



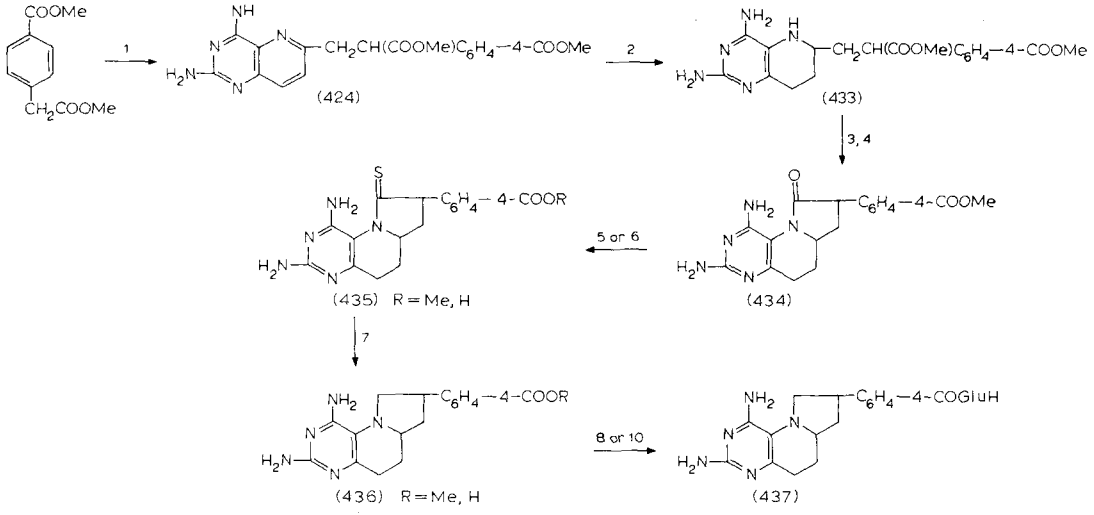
readily amenable to this homologation. Instead, (160) was used to alkylate the anion of the appropriate homoterephthalate (423a–c). The resulting vinylogous malonates (424a–c) were demethoxycarbonylated [188] to yield 10-alkyl-8,10-dideazapteroic acids (425a–c). Standard peptide coupling conditions and saponification afforded (426a–c).

Efforts to prepare (426, R = H or Me) via alkylation of a toluic acid dianion with (160) were unsuccessful. Similarly, monoanions derived from the analogous phenylthio (427a) and phenylsulphinyl (427b) esters were also found unacceptable. This behaviour was attributed to the lack of a suitable electon-withdrawing group to stabilize the adjacent anion. The Princeton group has also investigated alkylation of acidic methylene compounds with 2-amino-5-bromomethylpyrazine-3-carbonitrile (16b) and found that anions with $pK_a > 19$ do not react successfully [193]. DeGraw noted that alkylation of the sulphones (428a,b) with (160) afforded (429a,b), but unfortunately, all attempts to remove the phenylsulphonyl groups in (429) (Al-Hg, Na-Hg, Ni(R), $CrCl_2$) were futile. Ready desulphonylation of (428b) was possible (*Scheme 3.83*). Similar frustrations were encountered in attempts to desulphonylate the pyrazines (431a,b) [194].



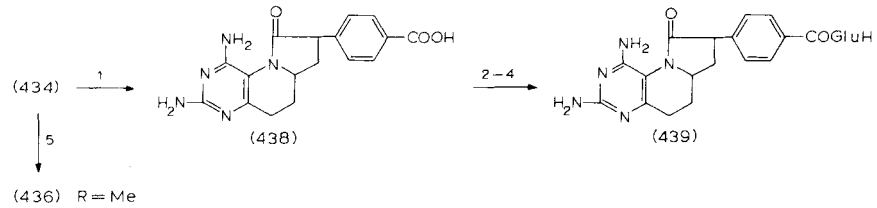
Scheme 3.83

An attempt to design an analogue incorporating structural features from the potent 8,10-dideaza series which could function as a dual inhibitor of DHFR and TS has resulted in the synthesis of 8,10-dideaza-5,10-methylene-5,6,7,8-tetrahydroAP (437) (*Scheme 3.84*) [195]. The anion of dimethyl homotereph-



1. KH, (160), DMF; 2. H_2, PtO_2, MeOH, TFA; 3. 10% K_2CO_3; 4. 170–180°C; 5. P_2S_5, pyr;
6. Lawesson's reagent; 7. Ni, $MeOCH_2CH_2OH$, 100°C; 8. Bu^tCF, Et_3N, GluEt·HCl; 9. 1 M NaOH, $MeOCH_2CH_2OH$; 10. HOAc.

Scheme 3.84 (continued on p.172)



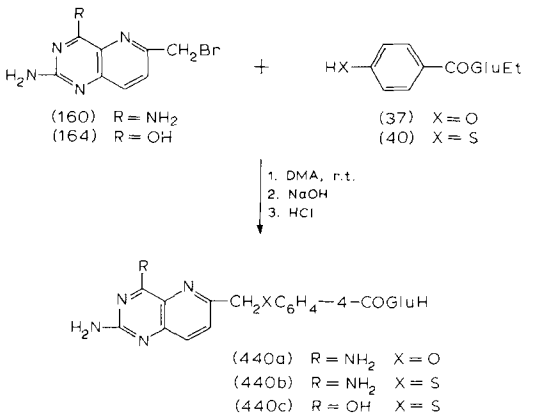
1. NaOH, $MeOCH_2CH_2OH$; 2. Bu^tCF, Et_3N, GluEt · HCl; 3. NaOH, $MeOCH_2CH_2OH$; 4. HOAc; 5. BH_3-THF, r.t.

Scheme 3.84 (continued from p.171)

thalate was alkylated with (160) to produce (424), which was reduced to (433), isolated as its trifluoroacetate salt. Neutralization of this salt and thermal cyclization generated the key lactam ester (434) in excellent yield. Conversion of (434) to the thiolactam (435) using P_2S_5 or Lawesson's reagent [196] required drastic conditions (refluxing pyridine) and was complicated by poor yields and undesired side-reactions (e.g., attack at the benzoate ester). Subsequent desulphurization of (435, R = Me) gave (436, R = Me). A more practical synthesis of (436, R = Me) was achieved in one step via direct lactam reduction of (434) using borane-THF under very mild reaction conditions. Saponification of (436, R = Me) to (436, R = H) and standard peptide coupling ultimately afforded (437). Saponification of (434) to (438), peptide coupling and resaponification gave the 5,10-oxomethylene analogue (439).

It is noteworthy that (434), (436) and (437), all theoretically capable of existing as mixtures of four diastereomers, appear to be single diastereomers based on 400 Mz NMR and HPLC data. The stereochemistry at C-6 and C-10 in these compounds is thought to reflect that of the thermodynamically more stable isomer created during the thermal ring closure, and should have hydrogens at C-6 and C-10 in a *trans* relationship to minimize unfavourable interactions and maximize the planarity of the ring system.

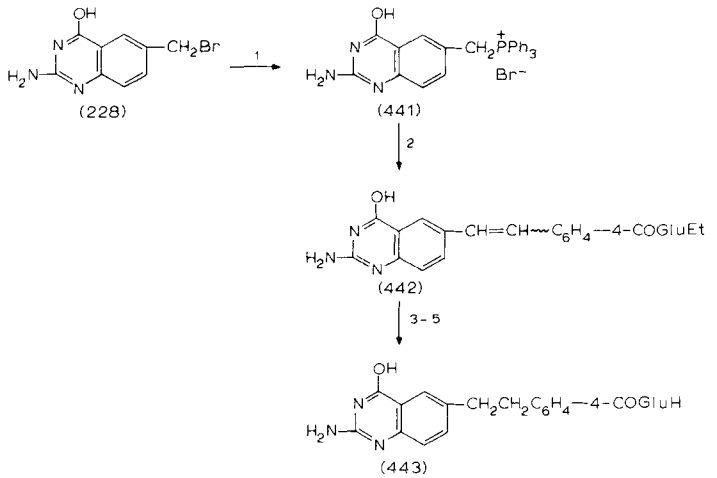
As part of their study of the effect of replacement of N-10 in 8-deazaFA (144) and 8-deazaAP (161b) by carbon, Broom and Srinivasan have prepared 8,10-dideaza-10-oxaAP (440a), 8,10-dideaza-10-thiaAP (440b), and 8,10-dideaza-10-thiaFA (440c) (*Scheme 3.85*) [86]. Condensation of either 2,4-diamino-6-bromomethyl-8-deazapteridine (160) or 6-bromomethyl-8-deazapterin (164) with the sodium salt of diethyl (*p*-hydroxybenzoyl)-L-glutamate (37) [51] or the sodium salt of diethyl (*p*-mercaptobenzoyl)-L-glutamate (40) [53] followed by saponification afforded (440a–c) uneventfully.



Scheme 3.85

5,8,10-TRIDEAZA ANALOGUES

The first member of this series, 5,8,10-trideazaFA (443), was prepared in 1977 (*Scheme 3.86*) [176]. The known bromomethylquinazoline (228) was quantita-

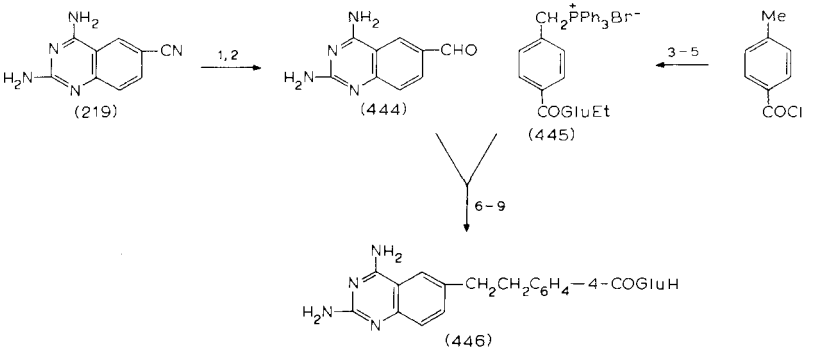


1. Ph_3P, DMF; 2. NaOEt, (252), DMF; 3. H_2, PtO_2, $MeSO_3H$;, 4. 0.1 M NaOH; 5. HCl.

Scheme 3.86

tively converted to (441) by treatment with triphenylphosphine. Subsequent ylide formation and condensation with (252) [140] gave (442) as a 50/50 mixture of *E* and *Z* isomers. Catalytic hydrogenation of this mixture and saponification then yielded (443).

Shortly after this report, the synthesis of the only other known member of this series, 5,8,10-trideazaAP (446) (*Scheme 3.87*) was described [182].



1. $PhNHNH_2$, Ni(R), H_2, 2. 4-$NO_2C_6H_4CHO$, aq. HOAc; 3. Br_2, $h\nu$, 160°C; 4. GluEt, Et_3N; 5. Ph_3P, PhMe; 6. NaH, DMF; 7. H_2, Pd–C, HOAc; 8. K_2CO_3, aq. EtOH; 9. 0.5 M HCl

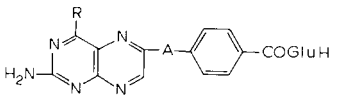
Scheme 3.87

Adopting an alternative convergent strategy, the two key halves of (446) were readily assembled as shown. Catalytic reduction of (219) with phenylhydrazine produced an intermediate phenylhydrazone which smoothly exchanged with *p*-nitrobenzaldehyde to give (444). Conversion of *p*-toluoyl chloride to the phosphonium salt (445) was straightforward. Wittig condensation of (445) with (444) followed by catalytic reduction and saponification afforded (446).

C-9,N-10 BRIDGE ANALOGUES

Although occupying only a small area of the entire MTX molecule, considerable attention has been given to changes in the C-9,N-10 bridge region in an attempt to modify lipophilicity, membrane transport properties and the ability of cofactor analogues to function as one-carbon transfer agents. Variations in this bridge region have included positional isomerism, homologation, isosteric substitution, alkylation and oxidation (see *Table 3.1*).

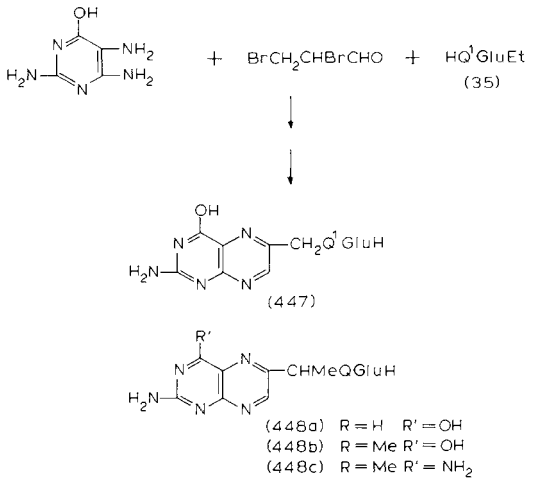
Table 3.1. CHANGES IN THE C-9,N-10 BRIDGE REGION



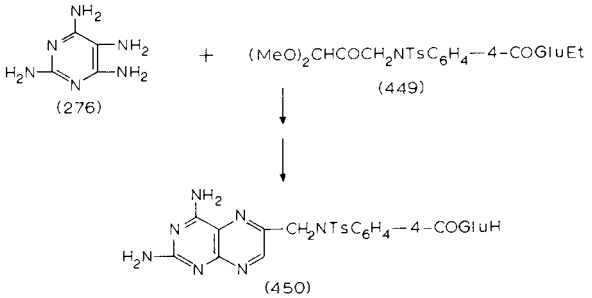
Cmpd	*R*	*A*	*Scheme 3.*
(447)	OH	CH_2NMe	88
(448a)	OH	CHMeNH	88
(448b)	OH	CHMeNMe	88
(448c)	NH_2	CHMeNMe	88
(450)	NH_2	CH_2NTs	89
(451a)	NH_2	$CH_2N(CH_2Ph)$	90
(451b)	NH_2	$CH_2N((CH_2)_7Me)$	90
(451c)	NH_2	$CH_2N(Pr)$	90
(451d)	NH_2	$CH_2N(CH_2C{\equiv}CH)$	90
(451e)	NH_2	CH_2NEt	90
(451f)	NH_2	CH_2NPh	90
(455)	OH	CONH	91
(456)	NH_2	CONH	91
(462)	OH	$NHCH_2$	92
(463)	NH_2	$NHCH_2$	92
(470)	OH	CH = CH	93
(471)	OH	CH_2CH_2	93
(478)	NH_2	CH_2CH_2	94, 95, 96, 98
(485b)	NH_2	CH_2CHMe	96, 97
(485c)	NH_2	CH_2CHEt	96, 97, 98
(485d)	NH_2	CH_2CMe_2	96
(498)	OH	CH_2O	99, 100
(499)	NH_2	CH_2O	99, 100
(511)	OH	CH_2S	100, 101
(512)	NH_2	CH_2S	100, 102
(521)	OH	CH_2CH_2NH	103, 104
(530)	OH	$(CH_2)_3NH$	105
(539)	OH	CH_2CH_2S	106
(544b)	NH_2	CH_2CH_2S	107
(553a)	OH	CH_2CH_2O	108, 109
(553b)	NH_2	CH_2CH_2O	109
(561a)	OH	CH_2NTsCH_2	110
(561b)	NH_2	CH_2NTsCH_2	110
(562)	NH_2	CH_2NMeCH_2	111
(566a)	OH	CH = NNH	113
(566b)	NH_2	CH = NNH	113
(567a)	OH	CH_2NHNH	113
(567b)	NH_2	CH_2NHNH	113
(572a)	OH	$CH_2CH_2CH_2$	114
(572b)	OH	$CH_2CHMeCH_2$	114
(573a)	NH_2	$CH_2CH_2CH_2$	114
(573b)	NH_2	$CH_2CHMeCH_2$	114

(a) Alkylation in the bridge region

An early structural modification of FA and AP involved alkylation at C-9 and/or N-10. 10-MethylFA (447) [197] was prepared by reaction of 2,4,5-triamino-6-hydroxypyrimidine, 2,3-dibromopropionaldehyde and diethyl *p*-methylaminobenzoylglutamate (35), followed by alkaline hydrolysis (the Waller condensation). Analogous utilization of 2,2,3-trichlorobutyraldehyde and the requisite *p*-(*N*-substituted amino)benzoylglutamate furnished (448a–c) (*Scheme 3.88*) [198]. The preparation of (450) by condensation of 2,4,5,6-tetraaminopyrimidine and the α-ketoacetal (449) was reported without details (*Scheme 3.89*) [199].



Scheme 3.88

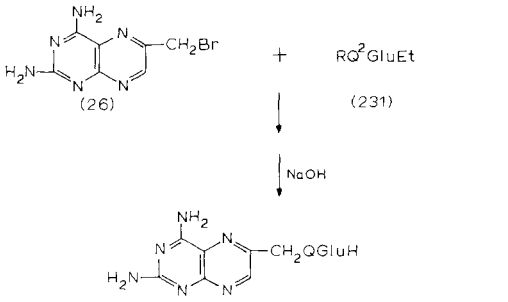


Scheme 3.89

More recently, the N-10 alkyl derivatives (451a–f) were prepared by alkylation of the appropriately functionalized anilines (231) with (26)·HBr, followed by saponification (*Scheme 3.90*) [73, 200, 201].

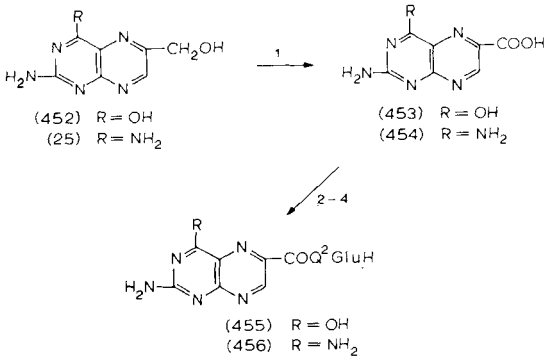
(b) 9-Oxo analogues

The syntheses of 9-oxoFA (455) and 9-oxoAP (456) are illustrated in *Scheme 3.91* [114]. Treatment of carboxylic acids (453) and (454), readily available by permanganate oxidation of the corresponding carbinols, with



(451a) R = CH_2Ph (451e) R = Et
(451b) R = $(CH_2)_7Me$ (451f) R = Ph
(451c) R = CH_2CH_2Me (451g) R = $CH_2CH_2CH_2Me$
(451d) R = $CH_2C{\equiv}CH$

Scheme 3.90



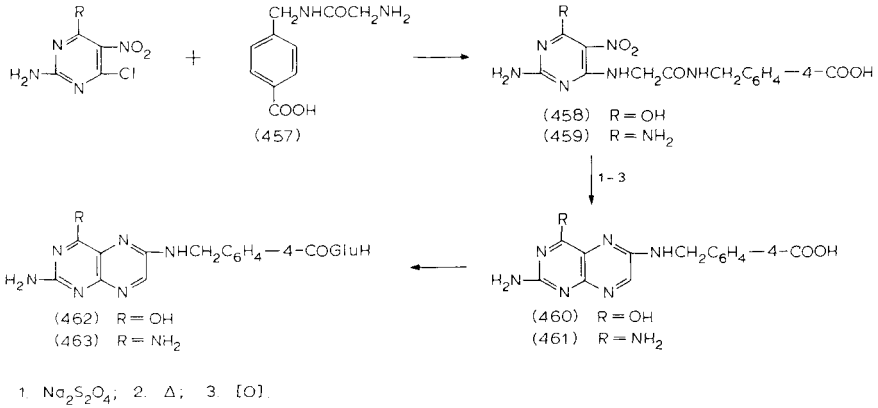
1. $KMnO_4$; 2. TFAA; 3. HQ^2GluH; 4. NaOH.

Scheme 3.91

trifluoroacetic anhydride gave mixed anhydrides which were condensed with *p*-aminobenzoyl L-glutamic acid to yield, after mild alkaline hydrolysis of the trifluoroacetyl protecting groups, (455) and (456), respectively.

(c) Interchange of the C-9, N-10 positions

Nair and Baugh have described the synthesis of isoFA (462) (*Scheme 3.92*) [202]. Arylation of (457) with 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine in refluxing aqueous ethanol gave (458). Reduction of (458) with sodium



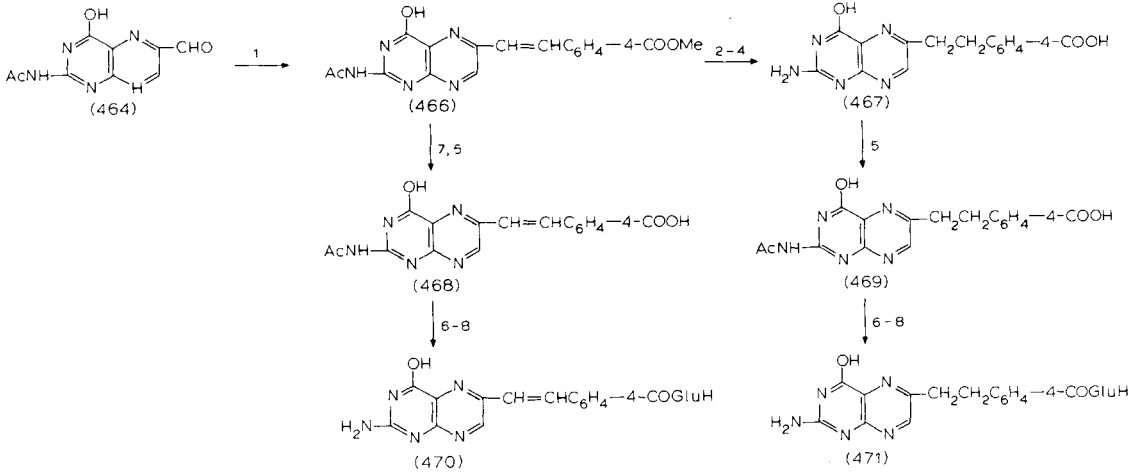
Scheme 3.92

dithionite, followed by thermal cyclization with concomitant dehydration, and then oxidation with manganese dioxide, afforded isopteroic acid (460). Peptide coupling under standard conditions yielded (462). In a similar fashion, isoAP (463) was prepared [203]. Commercially available *p*-formylbenzoic acid was converted to *p*-aminomethylbenzoic acid, which was coupled with N-CBZ glycine. Hydrogenolysis of the CBZ group furnished the key intermediate (457).

(d) 10-Deaza, 10-alkyl-10-deaza, 10-deaza-10-oxa, and 10-deaza-10-thia analogues

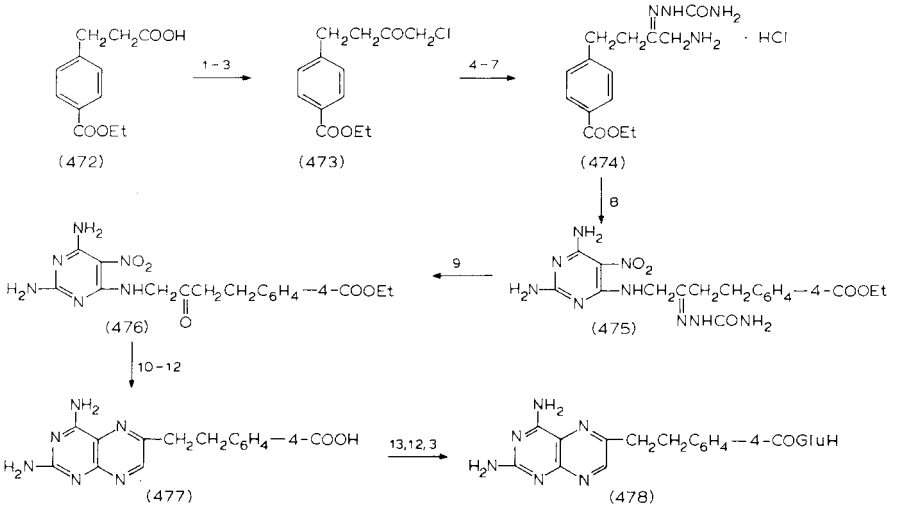
The first reported 10-deaza folate analogues were 10-deazaFA (471) and 10-deaza-9,10-dehydroFA (470) [179]. Treatment of 2-acetylpterin-6-carboxaldehyde [464) with ylide (465) furnished (466). Alkaline hydrolysis of (466) and reacetylation gave (468) which was converted to (470) under standard conditions. Catalytic hydrogenation of (466) with subsequent saponification and oxidation produced (467) which was converted analogously to (471) (*Scheme 3.93*).

The SRI group has examined a modification of the Boon–Leigh methodology in the synthesis of 10-deazaAP (478) (*Scheme 3.94*) [204-207]. The chloromethyl ketoester (473) was prepared in a straightforward manner by the Arndt–Eistert reaction, starting with (472). Treatment of (473) with sodium azide, followed by catalytic reduction and semicarbazone formation, provided the critical intermediate (474). Reaction of (474) with 2,4-diamino-6-chloro-5-nitropyrimidine, followed by cleavage of the semicarbazone with 90% trifluoroacetic acid, gave (476). Reduction of the nitro group using zinc in acetic



1. 4-$Ph_3P{=}CHC_6H_4COOMe$(465); 2. H_2, Pd; 3. KOH; 4. H_2O_2; 5. Ac_2O; 6. GluEt, DCC, pyr; 7. KOH; 8. HCl.

Scheme 3.93

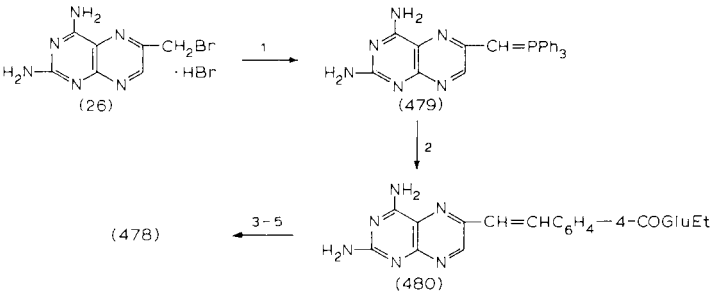


1. $SOCl_2$; 2. CH_2N_2; 3. HCl; 4. NaN_3; 5. H_2, PtO_2; 6. $H_2NNHCONH_2$; 7. Dowex 2 (Cl); 8. 6-chloro-2,4-diamino-5-nitropyrimidine; 9. 90% TFA; 10. Zn–HOAc; 11. H_2O_2; 12. 0.1 M NaOH; 13. Bu^tCF, GluEt.

Scheme 3.94

acid, and subsequent cyclodehydration, oxidation and saponification provided (477). Coupling of (477) with diethyl L-glutamate by the chloroformate procedure and mild saponification then afforded the target compound, (478).

An alternative approach [187, 208] for the synthesis of 10-deazaAP (478) is outlined in *Scheme 3.95*. Treatment of (26) · HBr with triphenylphosphine in

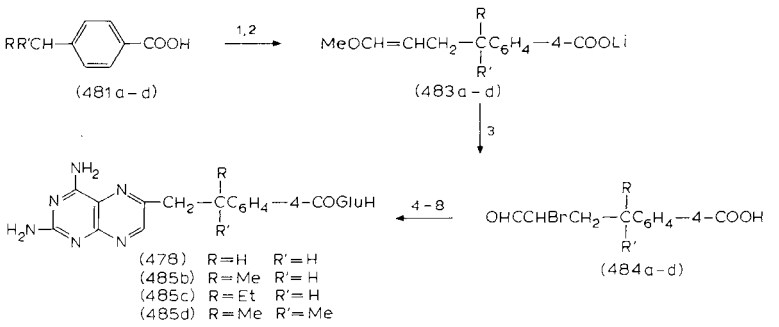


1. Ph_3P, NaOMe; 2. 4-$OHCC_6H_4COGluEt$; 3. H_2, PtO_2; 4. H_2O_2; 5. NaOH.

Scheme 3.95

DMA followed by sodium methoxide furnished ylide (479) which was immediately condensed with diethyl *N*-(*p*-formylbenzoyl)-L-glutamate to afford (480). Catalytic hydrogenation gave the 5,6,7,8-tetrahydro derivative; oxidation with hydrogen peroxide followed by hydrolysis of the ester groups then gave (478). This is the first report of the preparation and use of a pteridine ylide in the Wittig reaction.

An improved procedure for the synthesis of (478) has been reported recently [185, 207, 209]. An attractive feature of this approach is that it provides entry to related pharmacologically interesting 10-alkyl-10-deazaAP derivatives (*Scheme 3.96*). Treatment of *p*-toluic acid (481a) with two equivalents of LDA,

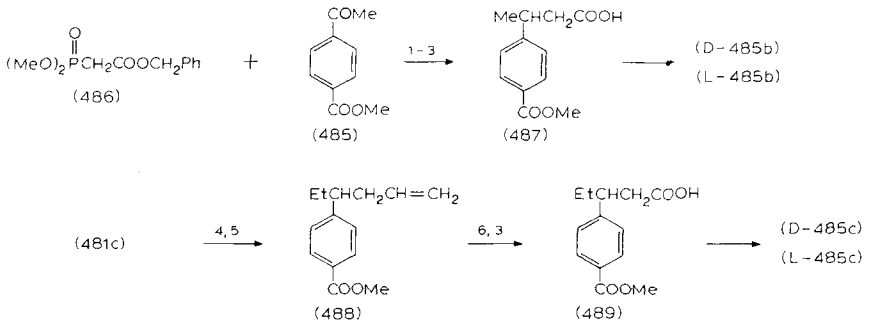


1. LDA(2eq.); 2. MeOCH=CHCH$_2$Cl(482); 3. Br_2,CH_2Cl_2; 4. (276); 5. KI_3; 6. Bu^{t}CF, GluEt, Et_3N; 7. NaOH; 8. HOAc.

Scheme 3.96

followed by alkylation of the resulting dianion with 3-methoxyallyl chloride (482), afforded the enol ether (483a). Bromination of (483a) with concomitant hydrolysis provided bromoaldehyde (484a), which was condensed with 2,4,5,6-tetraaminopyrimidine in dilute acetic acid. *In situ* oxidation (with KI_3) of the resulting dihydropteridine, glutamate coupling and saponification yielded (478). Analogous utilization of acids (481b–d) furnished 10-alkyl-10-deazaAP analogues (485b–d), respectively.

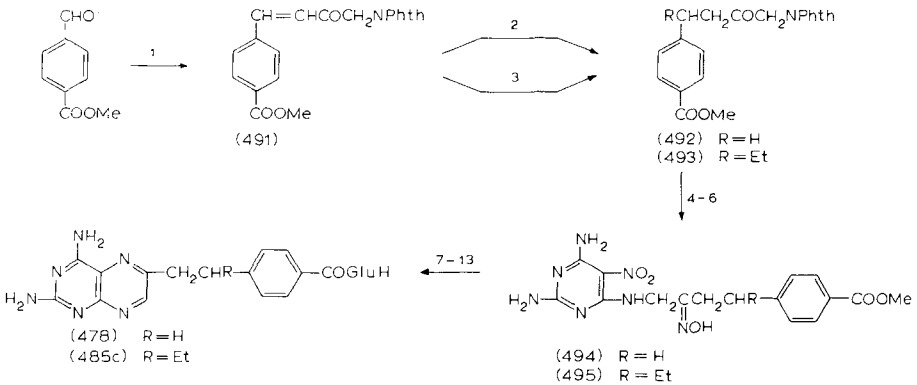
The SRI group has refined its methodology to provide the resolved C-10 diastereomers of 10-alkyl-10-deazaAP (*Scheme 3.97*) [210]. In one approach, the phosphonate (486) was condensed with ethyl *p*-acetylbenzoate, and the resulting acrylate ester was catalytically hydrogenated, with simultaneous cleavage of the benzyl ester, to generate racemic (487). Resolution of (487) was accomplished using quinine for the D-form and L-α-methylbenzylamine for the L-form. Attempts to extend this approach using ethyl *p*-propionylbenzoate were, however, frustrated by the production of complex mixtures and necessi-



1. NaH, DMF; 2. H_2, Pd-C; 3. resolve; 4. LDA, CH_2=$CHCH_2Ph$; 5. HCl-MeOH; 6. $NaIO_4$.

Scheme 3.97

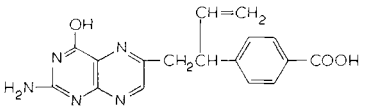
tated an alternative synthesis. Alkylation of the dianion of (481c) with allyl bromide, followed by esterification, gave (488), which was oxidized to the requisite acid (489) with sodium periodate. Resolution of (489) was accomplished using D- and L-α-methylbenzylamine. Conversion of D-(487), L-(487) and D-(489), L-(489) to D-(485b), L-(485b) and D-(485c), L-(485c), respectively, was effected using their previously described methodology (*cf. Scheme 3.94*) [204–207]. The authors noted a consistent upfield shift in the ^{1}H-NMR spectra for the C-7 proton signal with increasing steric bulk at C-10.



1. $PhthNCH_2COCH{=}PPh_3$ (490); 2. Zn–HOAc; 3. EtMgBr, CuBr; 4. H_2NOH; 5. H_2NNH_2; 6. 6-chloro-2,4-diamino-5-nitropyrimidine; 7. TFA–HCl; 8. $Na_2S_2O_4$; 9. NaOH; 10. aq. $KMnO_4$; 11. Bu^tCF, NMM, GluEt; 12. 0.1 M NaOH, MeCN; 13. HOAc.

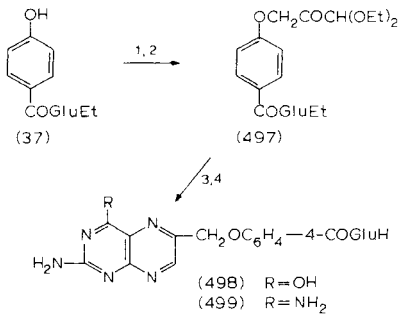
Scheme 3.98

The synthesis of several 10-deazaAP derivatives (*Scheme 3.98*) has been reported [211]. Condensation of methyl *p*-formylbenzoate with ylide (490) afforded the α,β-unsaturated ketone [491]. Conjugate reduction with zinc in acetic acid gave (492), which was contaminated with the corresponding alcohol if reaction times were not carefully controlled. Copper-catalyzed conjugate addition of ethyl magnesium bromide yielded mainly (493), together with minor products including the 1,2-adduct; the use of diethyl zinc was unsuccessful. Ketones (492) and (493) were protected as their respective oximes; hydrazinolysis, followed by reaction with 2,4-diamino-6-chloro-5-nitropyrimidine, furnished (494) and (495), respectively. Subsequent conversion of (494) and (495) to (478) and (485c), respectively, was accomplished as previously described (*cf. Scheme 3.94*). Recently, this methodology has been extended to the preparation of 10-vinyl-10-deazapteroic acid (496), which presumably will be fully elaborated [212].



(496)

The first report concerning the synthesis of 10-deaza-10-oxafolates described the preparation of 10-deaza-10-oxaFA (498) and 10-deaza-10oxaAP (499) by condensation of the ketoacetal (497) with the appropriate pyrimidine, followed by ester hydrolysis (*Scheme 3.99*) [51]. An improved, unambiguous synthesis [174] of (498) and (499) is outlined in *Scheme 3.100* and is a modification of the Boon–Leigh procedure. Reaction of (500) with methyl *p*-hydroxy-

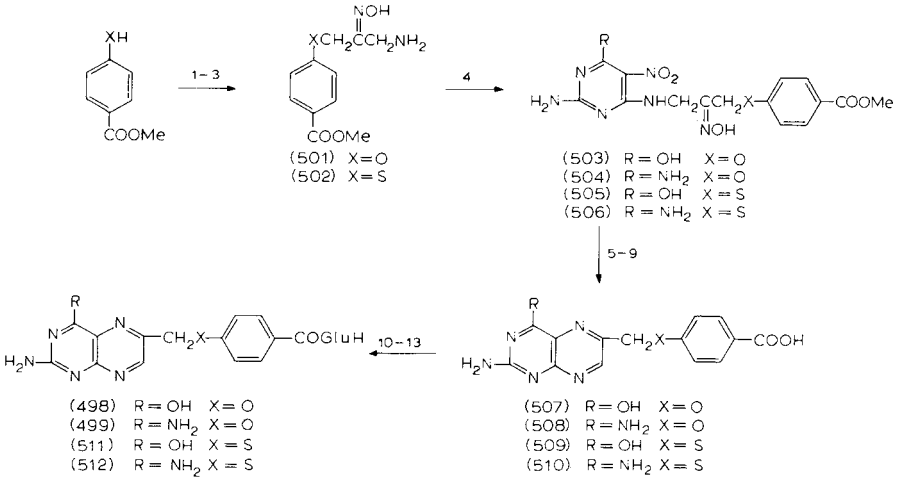


(37) (497)

(498) R=OH
(499) R=NH_2

1. 2,3-Epoxypropanal diethylacetal, pyr; 2. CrO_3;
3. 4-R-2,5,6-triaminopyrimidine; 4. NaOH.

Scheme 3.99

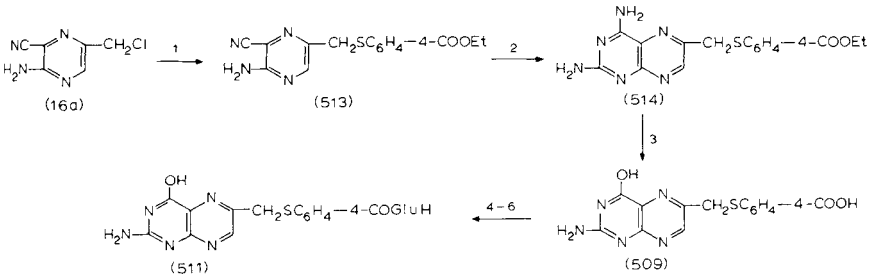


1. PhthNCH$_2$COCH$_2$Br (500), Cs_2CO_3; 2. H_2NOH; 3. H_2NNH_2; 4. 2-amino-4-chloro-5-nitro-6-R-pyrimidine; 5. H_3O^+; 6. $Na_2S_2O_4$; 7. pyridine·HCl, pyr; 8. [O]; 9. NaOH; 10. $H_2NCH(COOCH_2Ph)CH_2CH_2COO$-resin; 11. Bu^{t}OCF, NMM; 12. 2 M NaOH, dioxan; 13. 1 M HCl.

Scheme 3.100

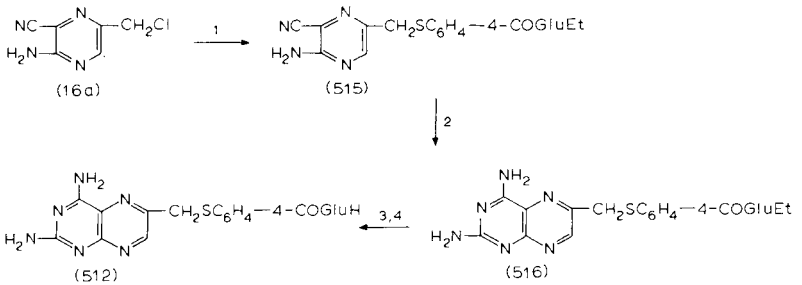
benzoate using caesium carbonate as the base, followed by oximation and hydrazinolysis, yielded (501). Interestingly, the authors noted that caesium carbonate was the only effective base for this conversion; all other bases examined (potassium *t*-butoxide, sodium bicarbonate, pyridine) gave rise to complex mixtures. The sulphur analogue (502) [213] was prepared in a similar manner from methyl *p*-mercaptobenzoate [214]; in this case, pyridine was an effective base. Condensation of (501) with the requisite chloronitropyrimidine then generated (503) and (504), which were converted to the target compounds (498) and (499). This strategy was also applied to the synthesis of 10-deaza-10-thiaFA (511) [213] and 10-deaza-10-thiaAP (512) [215]. Condensation of 2,4-diamino-6-bromomethylpteridine (26)·HBr with diethyl *p*-hydroxybenzoyl-L-glutamate (37) followed by saponification provided an alternative route to (499) [73].

A complementary approach to (511) and (512) (*Scheme 3.101* and *3.102*) [53, 216] was patterned after methodology developed in Taylor's laboratories [217, 218]. Alkylation of ethyl *p*-mercaptobenzoate with the chloromethylpyrazine (16a) furnished (513). Guanidine cyclization to give (514) followed by saponification resulted in concomitant hydrolysis of the 4-amino group to provide the pteroic acid analogue (509), which was converted to (511) in the usual manner. In order to avoid hydrolysis of the 4-amino group, the initial



1. $(4\text{-}EtOOCC_6H_4S^-)_2$, $NaBH_4$, EtOH; 2. guan; 3. NaOH; 4. Bu^{t}CF–Et_3N, GluEt; 5. 1 M NaOH; 6. H^+.

Scheme 3.101



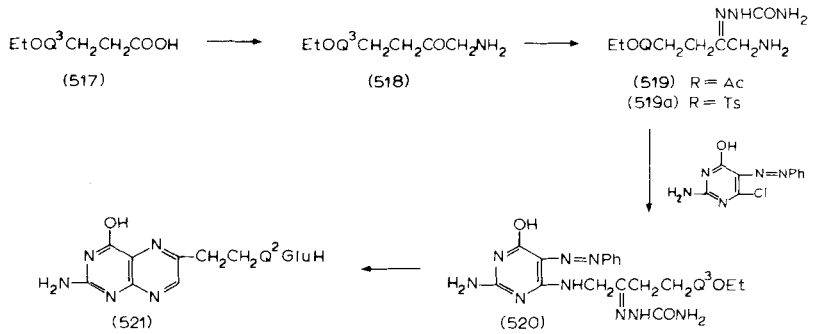
1. (40)/$NaBH_4$ – EtOH; 2. guan; 3. 0.02 M NaOH; 4. 0.1 M HCl.

Scheme 3.102

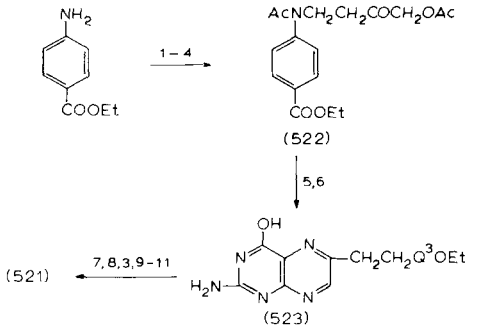
sulphide synthesis from (16a) was effected with (40). Guanidine annulation of the 2,4-diaminopyrimidine ring was now successful, but unfortunately also resulted in racemization of the glutamate moiety. Mild saponification thus gave racemic (512) [216].

Another strategy aimed at the preparation of folate derivatives with potentially improved therapeutic indices involved the synthesis of homologues of FA and AP through utilization of the Boon–Leigh and Waller reactions. For homoFA (521), the *N*-phenyl-*β*-alanine derivative (517) was elaborated to the aminoketone (518) via standard Arndt–Eistert methodology. The semicarbazone (519a) was arylated with 2-amino-4-chloro-6-hydroxy-5-phenylazopyrimidine to yield (520), from which (521) was obtained in a straightforward manner (*Scheme 3.103*) [219, 220].

About 8 years later, Friedman and co-workers [221] described an improved approach to (521) (*Scheme 3.104*). The key intermediate (522) was prepared



Scheme 3.103

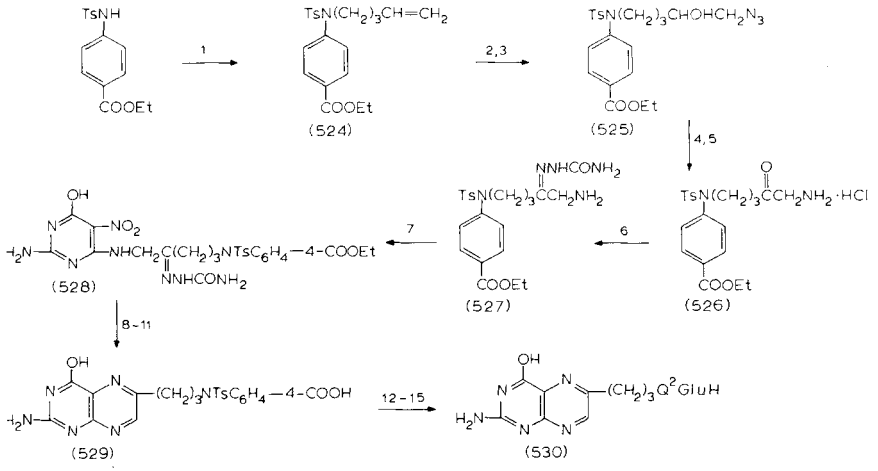


1. $CH_2{=}CHAc$; 2. Br_2–HOAc; 3. Ac_2O; 4. $Et_4\overset{+}{N}\,\bar{O}Ac$;
5. 6-hydroxy-2,4,5-triaminopyrimidine; 6. H_2O_2; 7. 10% NaOH;
8. TFAA; 9. Bu^tCF, Et_3N, GluEt; 10. OH^-; 11. H^+.

Scheme 3.104

in four steps from ethyl *p*-aminobenzoate. Condensation of 6-hydroxy-2,4,5-triaminopyrimidine with (522) under the standard Waller reaction conditions provided the fully aromatized homopteroate (523), which was then converted to (521).

In their synthesis of bishomoFA (530), Goodman's group demonstrated [222] a more direct and convenient approach to the aminoketones requisite for the Boon–Leigh methodology (*Scheme 3.105*). Thus, ethyl *p*-*N*-tosylamidobenzoate [49] was alkylated with 5-bromo-1-pentene. Epoxidation of the resulting vinyl tosylamine (524) followed by ring opening with sodium azide gave only (525), with no evidence of the isomeric azido alcohol. Oxidation of (525), followed by catalytic hydrogenation, then gave the α-aminoketone (526),

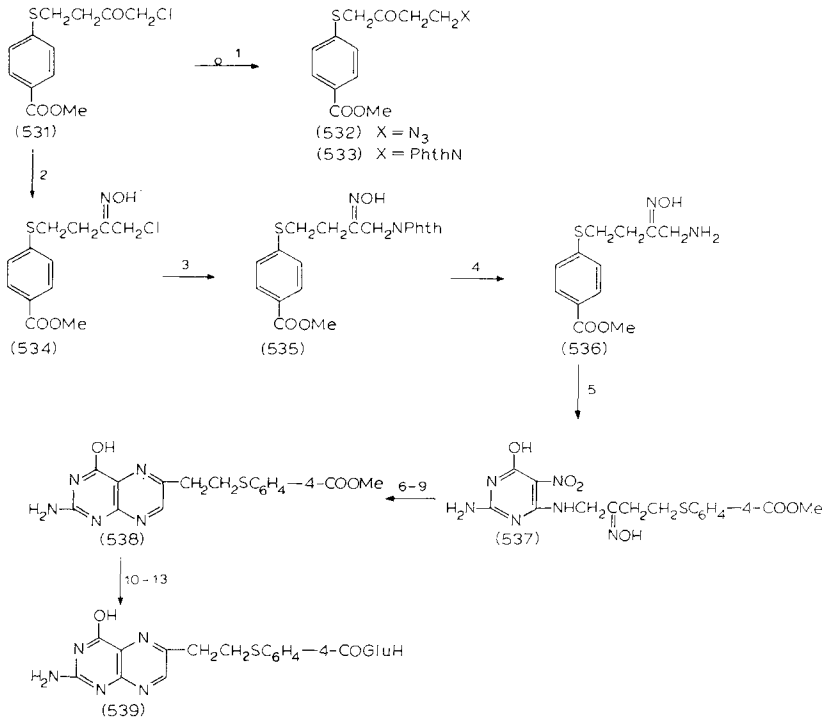


1. $CH_2{=}CH(CH_2)_3Br$; 2. mcpba; 3. NaN_3, H_2O; 4. CrO_3, HOAc; 5. H_2, 35%Pd-C, HCl; 6. $H_2NNHCONH_2$; 7. 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine; 8. HCl; 9. H_2, Pd-C; 10. H_2O_2; 11. NaOH; 12. Ac_2O; 13. Bu^tCF, Et_3N, GluEt; 14. KOH; 15. HBr-HOAc.

Scheme 3.105

isolated as its hydrochloride salt. Preparation of the semicarbazone (527) and conversion of this material to (530) followed the sequence of reactions outlined in *Scheme 3.105*. During the course of this work, homoFA (521) was prepared analogously from the appropriate semicarbazone (519a).

A synthesis of 11-thiohomoFA (539) [223] is outlined in *Scheme 3.106*. Chloromethylketone (531) was prepared via Michael addition of methyl *p*-mercaptobenzoate to chloromethylvinylketone. Reaction of (531) with sodium azide in aqueous acetone or with potassium phthalimide yielded only rearranged products resulting from initial base-catalyzed retro-Michael reactions. These products, (532) and (533) respectively, could not be elaborated further [223, 225], which necessitated the development of an alternative strategy involving protection of the ketone (to avoid enolization) prior to nucleophilic displacement. Thus, treatment of (531) with hydroxylamine afforded oxime (534). Chloride displacement was effected by treatment of (534) with potassium phthalimide in acetonitrile containing 18-*crown*-6 to give (535), which was deprotected to the primary amine (536). Treatment of (536) with 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine in ethanol using *N*-methylmorpholine as the proton scavenger afforded (537). Liberation of the ketone (trifluoroacetic acid-hydrochloric acid), followed by reduction (sodium dithionite), cyclodehydration, and aerobic oxidation furnished the methyl

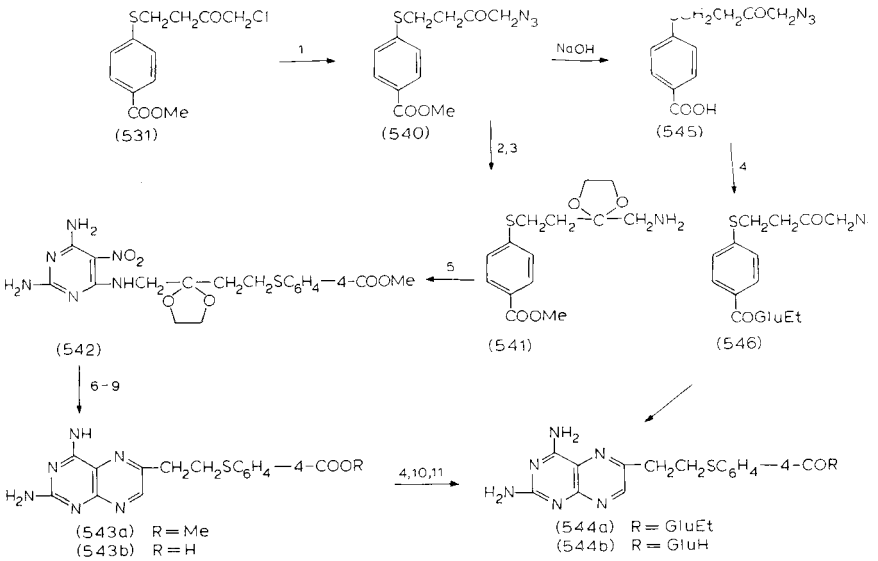


1. X^-, H_2O; 2. H_2NOH; 3. $PhthN^-K^+$, 18-*crown*-6, MeCN; 4. H_2NNH_2; 5. 2-amino-4-chloro-6--hydroxy-5-nitropyrimidine, *N*-ethylmorpholine; 6. TFA, HCl; 7 $Na_2S_2O_4$; 8. EtOH, pyr; 9. DMF, Δ; 10. NaOH; 11. Bu^{t}CF, NMM, GluEt; 12. 0.3 M NaOH; 13. HOAc.

Scheme 3.106

homopteroate analogue (538). Conversion of (538) to (539) was accomplished using standard methodology. The authors noted that solid-phase peptide coupling techniques were not suitable for the preparation of (539) because of the severe conditions required to cleave the product from the resin.

An analogous synthesis of (544b) is outlined in *Scheme 3.107* [224]. On reinvestigation, the undesired rearrangement reaction (*vide supra*) could be circumvented using strictly anhydrous conditions in aprotic solvents. Thus, treatment of (531) with sodium azide in acetone containing potassium iodide provided (540) in good yield. Ketalization followed by catalytic reduction gave primary amine (541), which was condensed with 6-chloro-2,4-diamino-5-nitropyrimidine to give (542). Deketalization with aqueous trifluoroacetic acid, followed by dithionite reduction, cyclization and aromatization gave (543a),

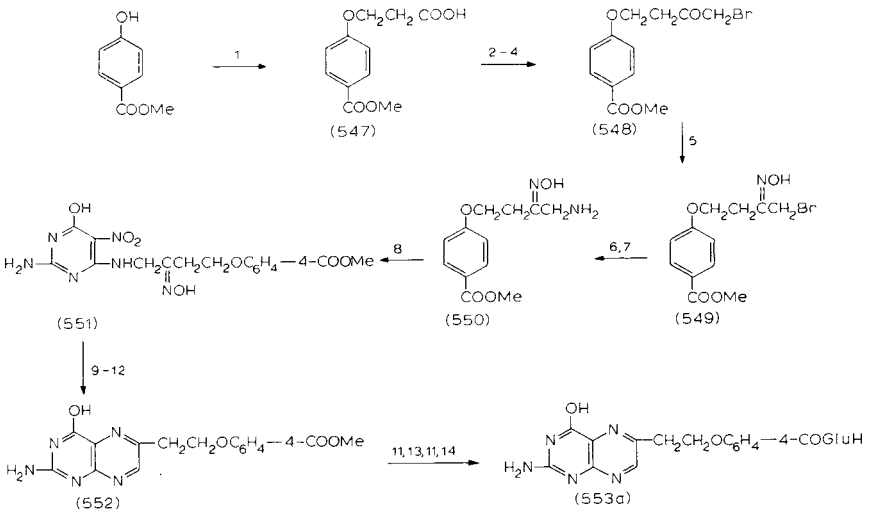


1. NaN_3, KI, Me_2CO; 2. $HO(CH_2)_2OH$, H^+; 3. H_2, Pd/C; 4. Bu^tCF, NMM, GluEt; 5. 6-chloro-2,4-diamino-5-nitropyrimidine; 6. aq. TFA; 7. $Na_2S_2O_4$; 8. pyridine·HCl, pyr; 9. DMF, Δ; 10. 1 M NaOH; 11. HOAc.

Scheme 3.107

from which (543b) was obtained by alkaline hydrolysis. Disappointingly, peptide coupling failed to give good yields of (544a). However, hydrolysis of (540) to (545) followed by coupling with diethyl L-glutamate gave (546), which was then converted to (544a). Saponification then furnished (544b). A synthesis of (539) by this alternative methodology was also reported.

11-OxahomoFA (553a) was prepared by Nair, Saunders, Chen, Kisliuk and Gaumont [226] using a related tactic, as outlined in *Scheme 3.108*. Treatment of methyl *p*-hydroxybenzoate with propiolactone afforded (547), which was subjected to an Arndt-Eistert reaction to give (548). Oximation to (549), conversion to (550), and arylation with 2-amino-6-chloro-4-hydroxy-5-nitropyrimidine then gave (551). Acidic hydrolysis of the ketal was followed by dithionite reduction, base-induced cyclization and air oxidation to give (552). Interestingly, attempted cyclization under acidic conditions (pyridine-hydrochloric acid) resulted in pteridine formation with concomitant loss of the side-chain. Peptide coupling was accomplished by the chloroformate method to give, after mild alkaline hydrolysis, the target folate surrogate (553a).

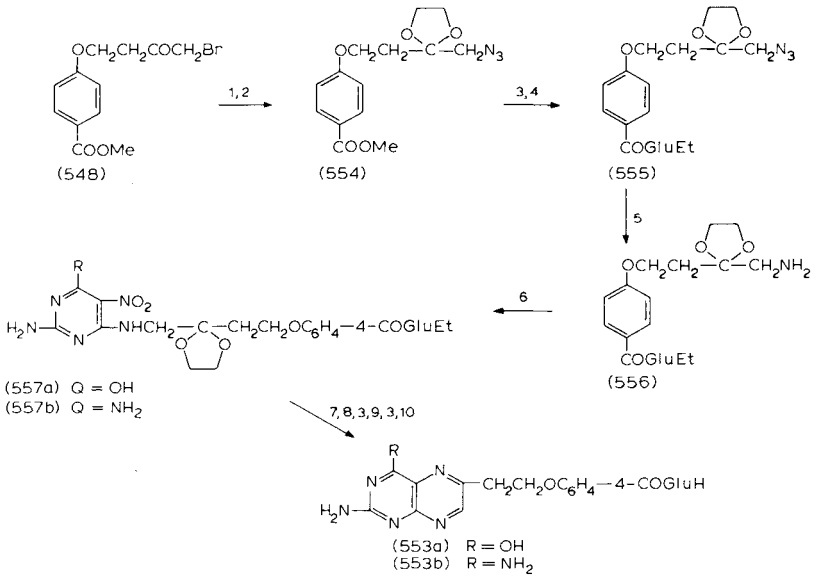


1. β-Propiolactone; 2. $SOCl_2$; 3. CH_2N_2; 4. HBr; 5. H_2NOH; 6. PhthN$\overset{+}{K}$, 18-*crown*-6, MeCN; 7. H_2NNH_2; 8. 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine; 9. aq. HCl; 10. $Na_2S_2O_4$; 11. NaOH; 12. DMF, Δ; 13. Bu^tCF, NMM, GluEt; 14. HOAc.

Scheme 3.108

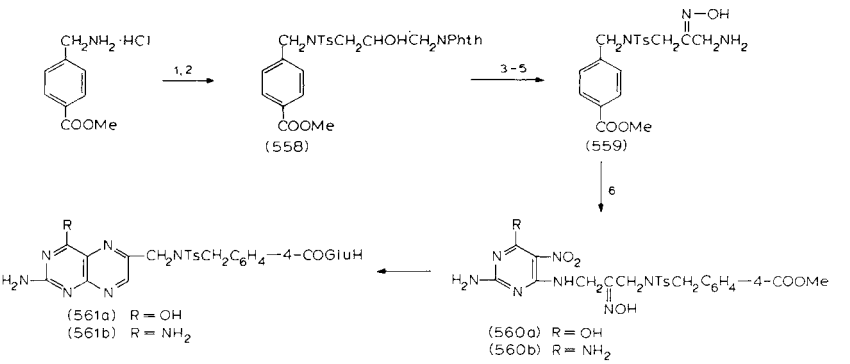
The following year, the same investigators reported an improved synthesis of (553a), as well as the preparation of 11-oxahomoAP (553b) (*Scheme 3.109*) [227]. Reaction of bromoketone (548) with sodium azide under anhydrous conditions afforded an α-azidoketone which was protected as its ethylene ketal (554). The glutamate moiety was introduced at this stage as previously described to furnish (555). Reduction yielded (556), which was converted to (557a) and (557b) by Boon–Leigh methodology. Sequential deketalization, nitro group reduction, cyclodehydration, air oxidation and saponification provided (553a) and (553b), respectively.

The Boon–Leigh approach has also been employed for the synthesis of N^{10}-tosyl-11-methyleneFA (561a) and N^{10}-tosyl-11-methyleneAP (561b) [228]. Tosylation of *p*-carbethoxybenzylamine hydrochloride, followed by fusion with 2,3-epoxypropylphthalimide with pyridine as the catalyst, afforded (558). Jones oxidation, oximation and hydrazinolysis yielded (559), which was arylated to give (560a) and (560b), respectively. Reductive cyclization, aromatization and peptide coupling were accomplished as previously described (see *Scheme 3.109*) to furnish (561a) and (561b) (*Scheme 3.110*). The only other known member of this series 10-methyl-11-methyleneAP (562), was prepared by alkylation of diethyl *p*-[(methylaminomethyl)benzoyl]-L-glutamate



1. NaN_3, KI, Me_2CO; 2. $(CH_2OH)_2$, H^+; 3. NaOH; 4. Bu^tCF, NMM, GluEt; 5. H_2, Pd/C; 6. 2-amino-4-chloro-5-nitro-6-R-pyrimidine; 7. TFA, HCl; 8. $Na_2S_2O_4$; 9. aq. $KMnO_4$; 10. HOAc.

Scheme 3.109



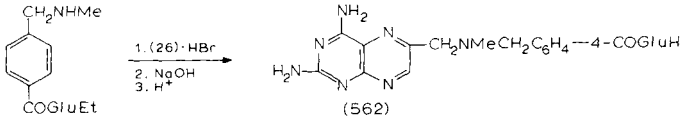
1. TsCl; 2. $PhthNCH_2CH(O)CH_2$, pyr; 3. Jones oxidation; 4. H_2NOH; 5. H_2NNH_2; 6. 2-amino-4-chloro-5-nitro-6-R-pyrimidine.

Scheme 3.110

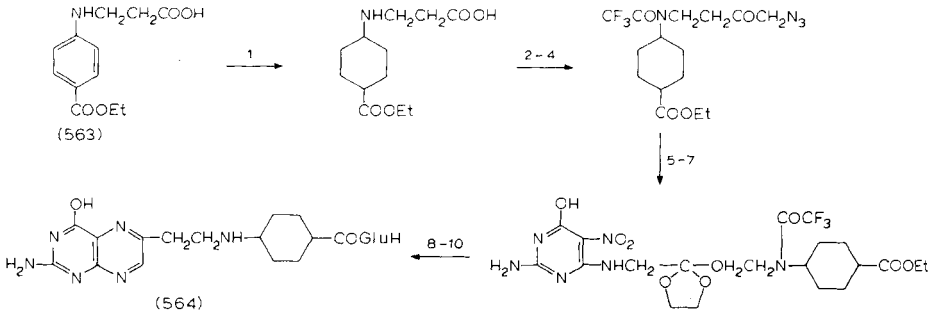
with (26)·HBr, followed by saponification (*Scheme 3.111*) [73].

The hexahydrohomoFA derivative (564) was synthesized starting with the β-alanine derivative (563), as summarized in *Scheme 3.112* [229].

11-AzaFA (567a) has been synthesized as outlined in *Scheme 3.113* [230]. Nitrosation of *p*-aminobenzoylglutamic acid followed by reduction afforded

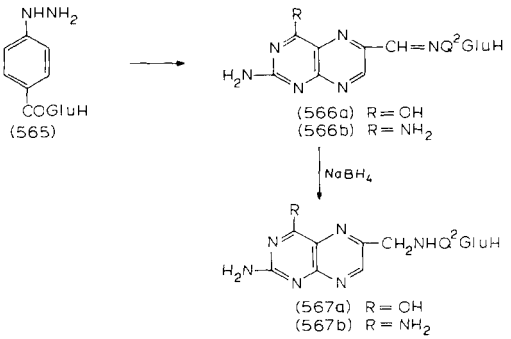


Scheme 3.111



1. H_2, PtO_2, HOAc; 2. TFAA; 3. Arndt-Eistert reaction; 4. NaN_3; 5. $(CH_2OH)_2$, H^+; 6. [H]; 7. 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine; 8. Bu^tCF, NMM, GluEt; 9. 0.1 M NaOH; 10. 1 M HCl.

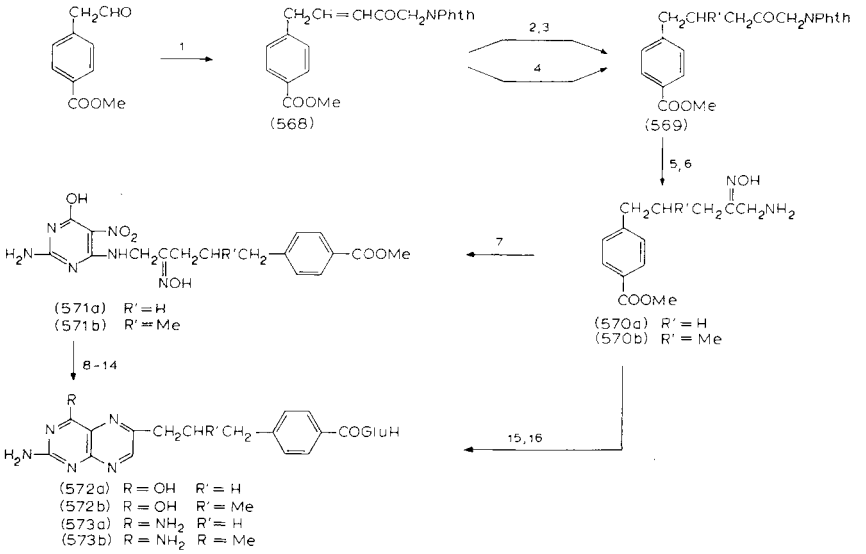
Scheme 3.112



Scheme 3.113

hydrazine (565), which was condensed with pterin-6-carboxaldehyde (11) to give the imine (566a). Reduction with sodium borohydride then gave (567a). 11-AzaAP (567b) was prepared analogously from 2,4-diaminopteridine-6-carboxaldehyde.

Very recently, the 10-deaza-11-methylene derivatives (572a), (572b), (573a) and (573b) (*Scheme 3.114*) have been reported [212]. Ylide (490) was condensed with *p*-carbomethoxyphenylacetaldehyde to yield (568), which was reduced and the resulting alcohol reoxidized to produce (569, R′ = H). Alterna-



1. $Ph_3P{=}CHCOCH_2NPhth$ (490); 2. Zn–HOAc; 3. Jones oxidation; 4. H_2, Pd; 5. H_2NOH; 6. H_2NNH_2; 7. 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine; 8. TFA, HCl; 9. $Na_2S_2O_4$; 10. NaOH; 11. $KMnO_4$; 12. Bu^tCF, GluEt; 13. $\bar{O}H$; 14. H^+; 15. 2,6-diamino-4-chloro-5-nitrosopyrimidine; 16. several steps.

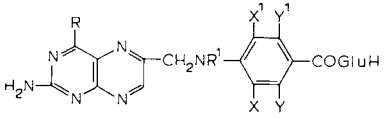
Scheme 3.114

tively, (569, R′ = H) could be obtained directly from (568) by carefully controlled catalytic hydrogenation using Pd. Oximation of (569, R′ = H) followed by hydrazinolysis gave (570a), which was reacted with 2-amino-4-hydroxy-5-nitro-6-chloropyrimidine to afford (571a). Conversion of this material to (572a) used previously described methodology (see *Scheme 3.98*). Conjugate addition of methyl Grignard to (568) provided the methyl derivative (569, R′ = Me) which was converted to (572b) as before. Reaction of (570a) and (570b) with 2,4-diamino-5-nitroso-6-chloropyrimidine gave, after appropriate elaboration, the AP analogues (573a) and (573b) respectively.

p-AMINOBENZOIC ACID UNIT ANALOGUES

Alteration of the *p*-aminobenzoic acid unit of FA and related compounds has provided many interesting analogues for chemotherapeutic evaluation. These

Table 3.2. *p*-AMINOBENZOIC ACID UNIT ANALOGUES: BENZENE MODIFICATIONS

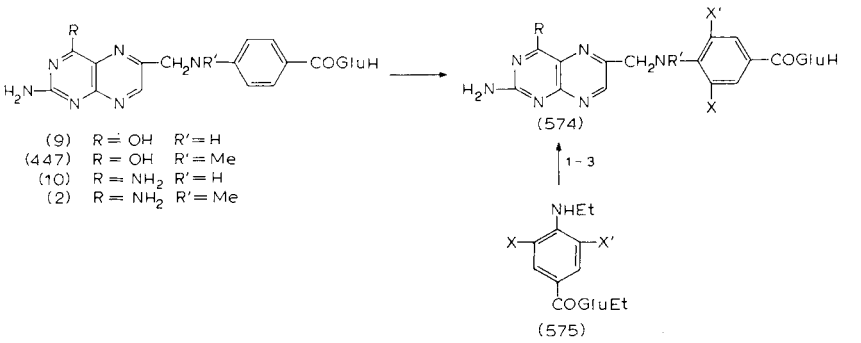


(574)

Cmpd	*R*	*R¹*	*X*	*X¹*	*Y*	*Y¹*	*Scheme 3.*	*Ref.*
a	OH	H	Cl	Cl	H	H	115	231, 237
b	OH	H	Br	Br	H	H	115	231
c	OH	Me	Cl	Cl	H	H	115	231, 233
d	NH_2	H	Cl	Cl	H	H	115	73, 231, 238
e	NH_2	Me	Cl	Cl	H	N	115	231, 233, 237
f	OH	Me	I	H	H	H	115	232
g	NH_2	Me	Cl	H	H	H	115	233
h	OH	Me	Cl	H	H	H	115	233
i	NH_2	Me	Br	H	H	H	115	233
j	OH	H	Br	H	H	H	115	233
k	NH_2	Me	Br	Cl	H	H	115	233
l	NH_2	H	I	H	H	H	115	234
m	NH_2	Me	Cl	Cl	H	H	115 [from (618)]	235
n	OH	H	Cl	Cl	H	H	115 [from (521)]	236
o	NH_2	Et	Cl	Cl	H	H	115	73
p	OH	H	NO_2	NO_2	H	H	115	232
q	NH_2	H	NO_2	NO_2	H	H	115	232
r	NH_2	Me	F	H	H	H	116	238
s	NH_2	Me	F	F	H	H	116	238
t	NH_2	H	F	H	H	H	117	239
u	NH_2	H	H	H	F	H	117	239
v	OH	H	Cl	H	H	H	118	232
x	OH	H	Me	H	H	H	118	240
y	OH	H	MeO	H	H	H	118	240
z	OH	H	H	H	Me	H	118	240
aa	OH	H	H	H	MeO	H	118	240
bb	OH	H	H	H	F	H	118	240
cc	OH	H	Et	H	H	H	119	241
dd	OH	H	Pr^i	H	H	H	119	241
ee	NH_2	H	H	H	N_3	H	120	242

include such structural changes as the introduction of substituents in the benzene ring, replacement of the benzene ring with heterocycles or (cyclo)-alkanes, insertion of a methylene group between the benzene ring and the glutamic acid residue, and replacement of the carbonyl of the amide function by SO_2 or CH_2. Early studies in this area involved the introduction of substituents into the benzene ring portion of the molecule. The compounds in this series which have been synthesized are compiled in *Table 3.2*.

Direct halogenation of folates (2), (9), (10) and (447) was accomplished to give 3′-halo or 3′,5′-dihalo derivatives, depending on reaction conditions (*Scheme 3.115*) [231–237]. In these procedures, halogenation is sometimes



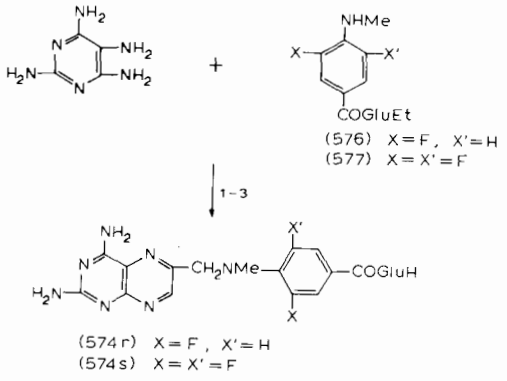
1. (26). HBr; 2. NaOH; 3. H^+.

Scheme 3.115

accompanied by side-reactions, including hydrolysis of the 4-amino group (of AP derivatives) or oxidative cleavage of the C-9,N-10 bond. Yields are generally only modest; purification of products is sometimes difficult. Interestingly, attempts to dibrominate MTX (2) [233] or diiodinate AP (10) [234] and folic acid (9) [232] were unsuccessful, due presumably to steric difficulties. Improved procedures for the preparation of 3′-chloroMTX (574g) and 3′,5′-dichloroMTX (574e) using *t*-butyl hypochlorite in acetic acid have been reported [237].

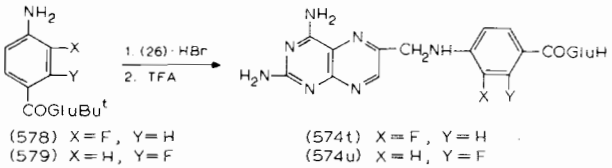
3′,5′-Dichloro-10-ethylAP (574*o*) was prepared by the reaction of (26) · HBr with diethyl 4-ethylamino-3,5-dichlorobenzoylglutamate (575), followed by saponification (*Scheme 3.115*) [73]. Nitration of FA and AP provided crude 3′,5′-dinitro derivatives, (574p,q) respectively [232].

Since direct fluorination of folates was not feasible, alternate syntheses were developed for the preparation of fluorinated derivatives of MTX (*Scheme 3.116*) [238] and AP (*Scheme 3.117*) [239]. In the former case,



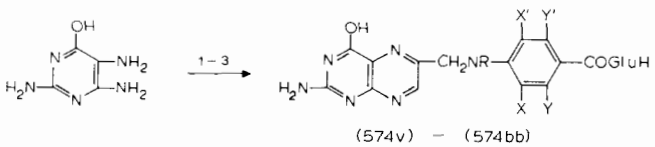
1. $BrCH_2CHBrCHO$; 2. NaOH; 3. H^+.

Scheme 3.116



Scheme 3.117

condensation of 2,4,5,6-tetraaminopyrimidine, 2,3-dibromopropionaldehyde and anilines (576) and (577) gave, after hydrolysis of the ethyl esters, analogues (574r) and (574s), respectively. Intermediates (576) and (577) were prepared in several steps under standard conditions starting with nitrobenzene precursors. For the AP analogues, alkylation of anilines (578) and (579) (prepared from the appropriate fluoronitrobenzoic acid and (235)) with (26)·HBr followed by deprotection furnished congeners (574t) and (574u), respectively.

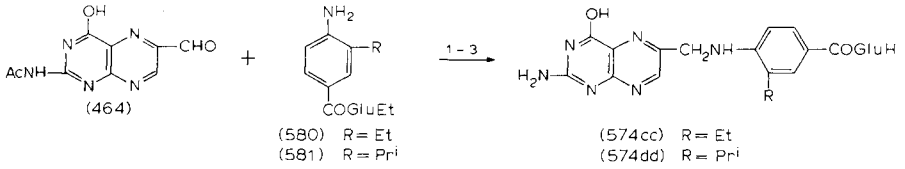


1. $BrCH_2CHBrCHO$ or $BrCH_2COCH_2Br$, $ArNH_2$; NaOH; 3. H^+

Scheme 3.118

Additional manipulations of the benzene ring, as illustrated in *Scheme 3.118*, include halogen substitution at the 2′-position [232, 240], as well as methyl and methoxy substitution [240].

An alternative route to alkyl-substituted FA derivatives is outlined in *Scheme 3.119* [241]. The requisite alkyl aniline intermediates (580) and (581)

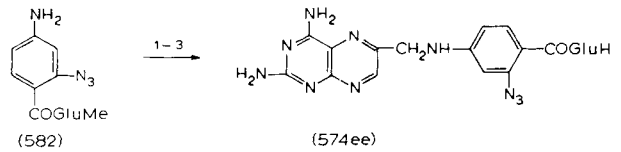


1. $NaBH_4$; 2. NaOH; 3. H^+.

Scheme 3.119

were prepared from nitrotoluene and nitrocumene, respectively, in several straightforward steps. Condensation of (580) and (581) with (464) afforded intermediate imines which were reduced with sodium borohydride; saponification then yielded (574cc) and (574dd) respectively.

A photoreactive analogue, 2′-azidoAP (574ee) was prepared as shown in *Scheme 3.120* [242]. Intermediate (582) was synthesized from 2-nitro-4-aminobenzoic acid by protection of the amino group, reduction, diazotization and peptide coupling.

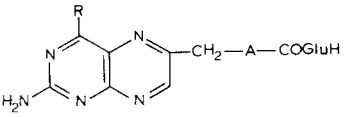


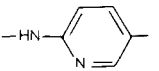
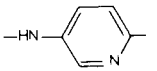
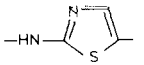
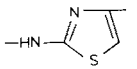
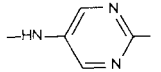
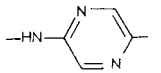
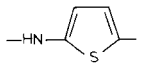
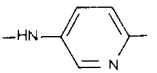
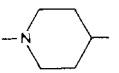
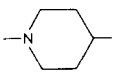
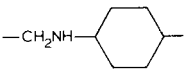
1. (26)·HBr; 2. NaOH. 3. H^+.

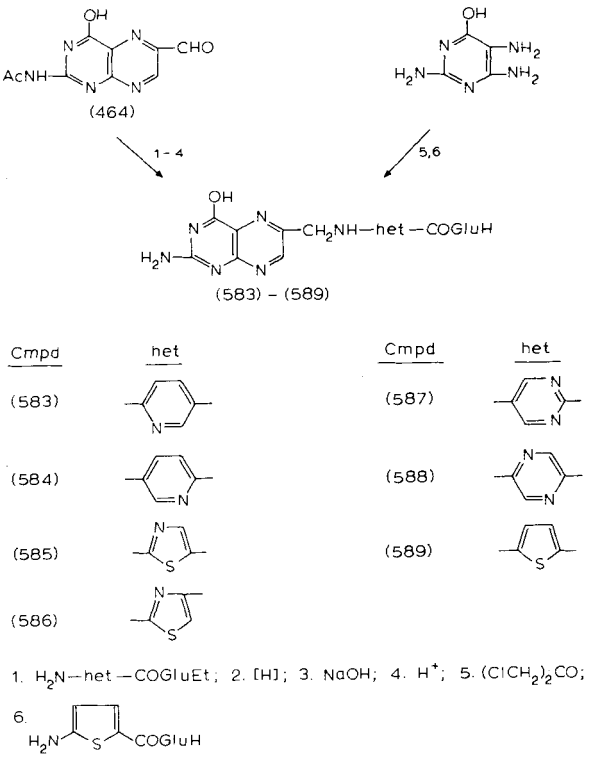
Scheme 3.120

Replacement of the benzene ring with heterocycles was also investigated (*Table 3.3*). Condensation of a suitably substituted heterocyclic amine with (464), followed by imine reduction and saponification, afforded (583)–(588) (*Scheme 3.121*) [243–246]. Thiophene derivative (589) was prepared by the condensation of 6-hydroxy-2,4,5-triaminopyrimidine, 1,1,2-trichloroacetone and *N*-(5-aminothienyl)-L-glutamic acid [247]. The pyridine analogue of AP,

Table 3.3. *p*-AMINOBENZOIC ACID UNIT ANALOGUES: HETEROCYCLIC MODIFICATIONS



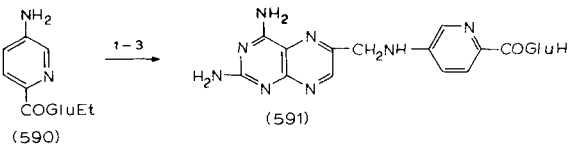
Cmpd	*R*	*A*	*Scheme 3.*
(583)	OH		121
(584)	OH		121
(585)	OH		121
(586)	OH		121
(587)	OH		121
(588)	OH		121
(589)	OH		121
(591)	NH_2		122
(592)	OH		123
(593)	NH_2		123
(564)	NH_2		112



Scheme 3.121

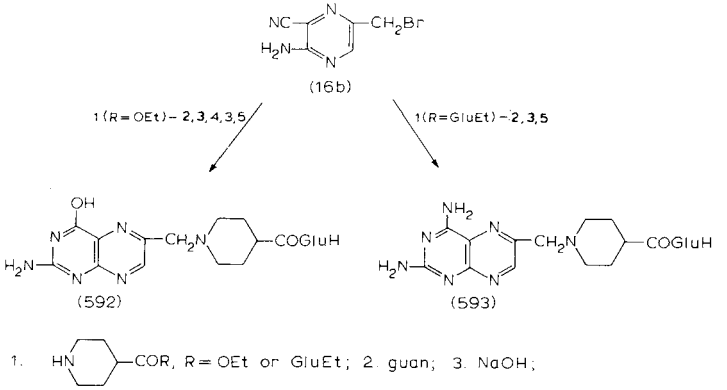
(591), was prepared by alkylation of (590) with (26) · HBr, followed by saponification (*Scheme 3.122*) [73, 248].

The isonipecotinoyl analogues of FA and AP, (592) and (593) respectively, were prepared from 2-amino-5-bromomethyl-3-cyanopyrazine (16b) by an extension of the strategy first developed by Taylor, as depicted in *Scheme 3.123* [249].

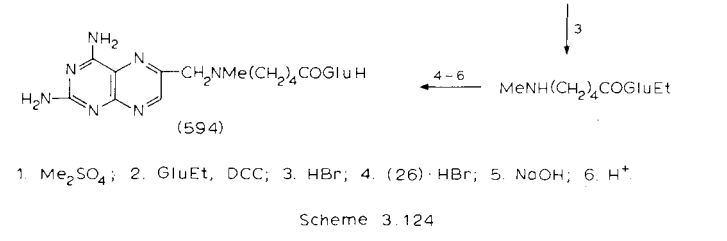


1. (26) · HBr; 2. NaOH; 3. H^+.

Scheme 3.122



Scheme 3.123

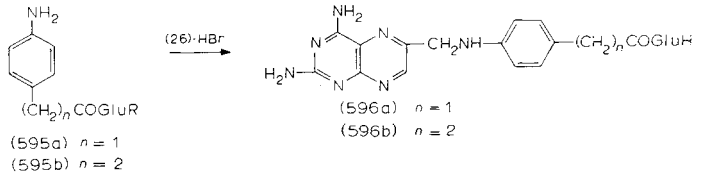


Scheme 3.124

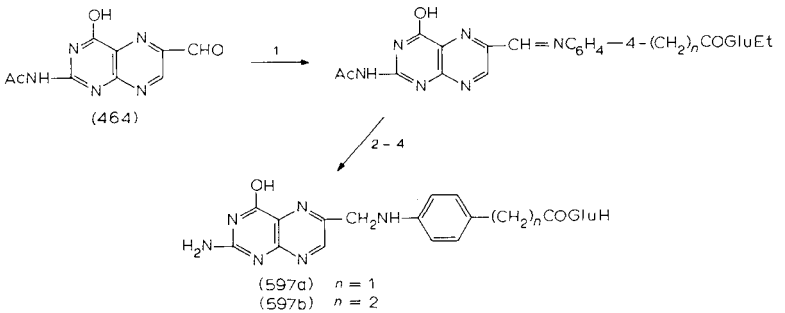
Replacement of the benzene ring with an *n*-butyl segment has been disclosed (*Scheme 3.124*) [73, 248]. Thus, methylation of 5-(*N*-tosylamino)valeric acid [250], followed by coupling with diethyl L-glutamate, detosylation, alkylation with (26) · HBr and hydrolysis afforded (594).

Workers at the Southern Research Institute have undertaken studies aimed at the investigation of compounds in which an aliphatic spacer was introduced in the *p*-aminobenzoic acid portion of the molecule to give phenylalkanoic acid derivatives. Homologues (596a) and (596b) (*Scheme 3.125*) were prepared by alkylation of the appropriate anilines (595a,b, R = H) with (26) · HBr [73, 248, 251]. The aspartic acid analogue of (596a) was also reported. Synthesis of the corresponding FA analogues (597a) and (597b) (*Scheme 3.126*) was carried out by a reductive alkylation sequence [252].

Rosowsky and Forsch [253] studied the effect of removing the AP amide carbonyl group (*Scheme 3.127*). Condensation of diethyl L-glutamate with

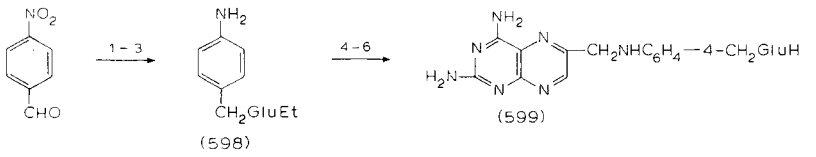


Scheme 3.125



1. (595a,b, R=Et); 2. $NaBH_4$; 3. NH_4OH, $HS(CH_2)_2OH$; 4. HCl.

Scheme 3.126

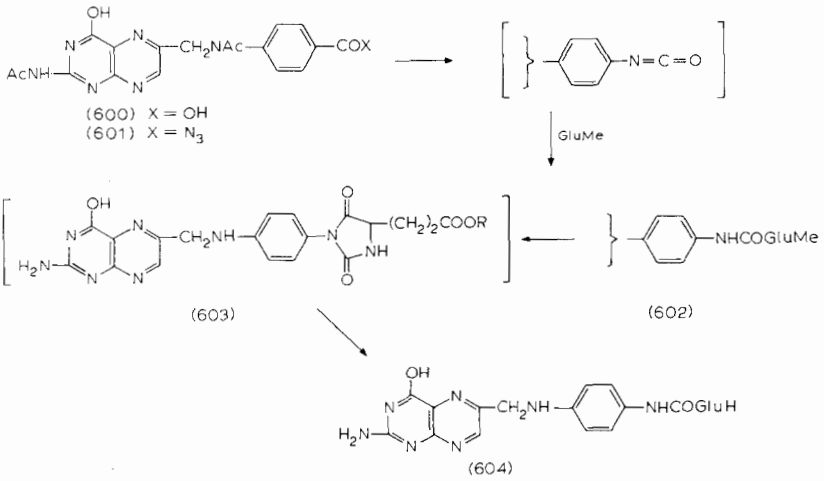


1. GluEt; 2. H_2, Pd/C; 3. $NaBH_4$; 4. (26)·HBr; 5. NaOH; 6. H^+.

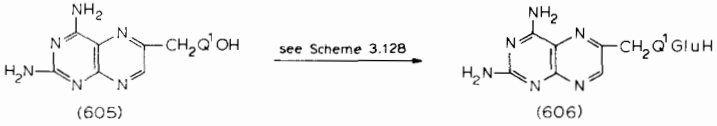
Scheme 3.127

p-nitrobenzaldehyde, followed by sequential catalytic hydrogenation and sodium borohydride reduction, afforded aniline (598), which was alkylated with (26)·HBr. Saponification then yielded (599). An alternative synthetic strategy employing the formyl derivative of the benzylic amino group of (598) offered no advantage.

The synthesis of analogues of FA and MTX in which a nitrogen atom has been interposed between the benzene ring and the glutamate side-chain has been reported (*Scheme 3.128*) [254, 255]. Treatment of 2,10-diacetylpteroic

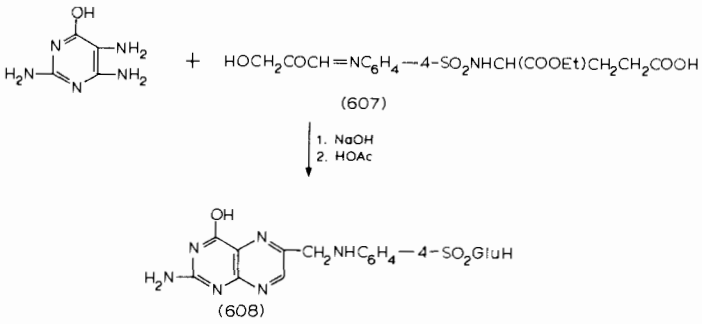


Scheme 3.128



Scheme 3.129

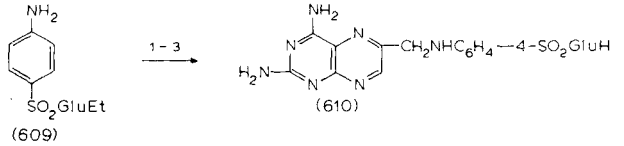
acid (600) with diphenylphosphoryl azide in DMF gave acyl azide (601), which underwent Curtius rearrangement in the presence of dimethyl L-glutamate to afford (602). Saponification of (602) furnished racemic (604), attributed to the intermediacy of hydantoin (603). Alternatively, reductive alkylation of (464)



Scheme 3.130

with the appropriate fully elaborated aniline gave N^{10}-deacetyl (602), from which racemic (604) was again isolated, despite the mild saponification conditions employed. Similarly, utilization of N^{10}-methylpteroic acid (605) in this sequence provided (606), an aza homologue of MTX (*Scheme 3.129*) [255]. The corresponding aspartic acids for both series were prepared as well.

The sulphonyl derivatives of FA and AP, (608) and (610), respectively, were also prepared. Thus, condensation of 6-hydroxy-2,4,5-triaminopyrimidine with sulphonyl glutamate (607) afforded (608) (*Scheme 3.130*) [256]. AP congener (610) was synthesized by alkylation of the requisite elaborated aniline (609) with (26)·HBr (*Scheme 3.131*) [73, 248].

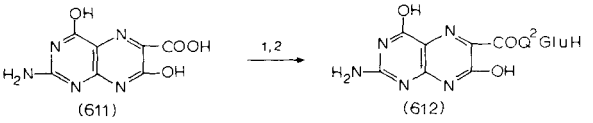


1. (26)·HBr; 2. NaOH; 3. HOAc.

Scheme 3.131

7-SUBSTITUTED ANALOGUES

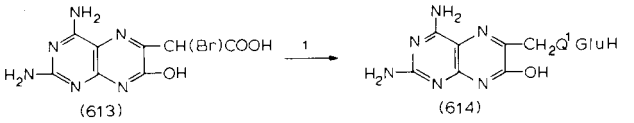
One of the earliest attempts to prepare analogues of FA as potential inhibitors involved the synthesis of 2-amino-4,7-dihydroxypteridine-6-carboxylyl-*p*-aminobenzoic acid (612) (in which the change from the structure of FA itself is exchange of the methylene bridge for a carbonyl group, and oxidation of position 7 to a lactam). This compound, which was a surprisingly effective inhibitor, was prepared from isoxanthopterin carboxylic acid (611) by *in situ* conversion to its acid chloride with a mixture of phosphorus oxychloride and phosphorus pentachloride, followed by addition of *p*-aminobenzoylglutamic acid (*Scheme 3.132*) [115].



1. $POCl_3$, PCl_5; 2. HQ^2GluH.

Scheme 3.132

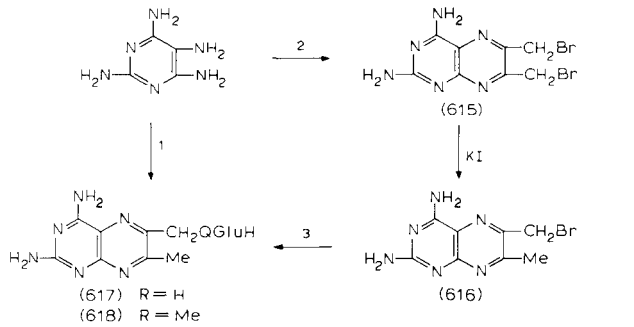
7-HydroxyMTX (614), which is an inactive metabolite of MTX, has been prepared by condensation of 2,4-diamino-7-hydroxypteridine-6-(α-bromo)-acetic acid (613) with *p*-methylaminobenzoylglutamic acid hydrobromide; decarboxylation takes place during the reaction to give (614) directly (*Scheme 3.133*) [257].



1. NaOAc, HQ^1GluH

Scheme 3.133

It was early recognized that *in vivo* oxidation of MTX at position 7 to (614) not only inactivates MTX, but thus produces a compound whose extreme insolubility leads directly (presumably by physical means) to kidney damage in patients treated with high doses of MTX. In an attempt to prevent this 7-hydroxylation reaction, 7-methylAP (617) and 7-methylMTX (618) were prepared (*Scheme 3.134*). The first synthesis of (617) involved condensation of 2,4,5,6-

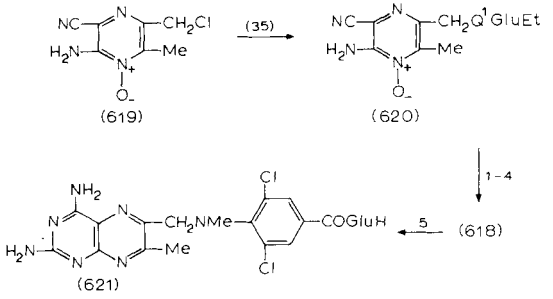


1. $BrCH_2CHBrAc$, HQ^2GluH; 2. $(BrCH_2CO)_2$; 3. RQ^1GluH.

Scheme 3.134

tetraaminopyrimidine with 3,4-dibromo-2-butanone and *p*-aminobenzoylglutamic acid in the presence of KI and I_2 (very low yield), and then alternatively by condensation of 2,4-diamino-6-bromomethyl-7-methylpteridine (616) (from reduction of 2,4-diamino-6,7-bis(bromomethyl)pteridine (615) with KI) with *p*-aminobenzoylglutamic acid. 7-MethylMTX (618) was prepared analogously utilizing *p*-methylaminobenzoylglutamic acid [257]. However, these prepara-

tions of (617) and (618) were equivocal in that the above reduction of the 6,7-bis(bromomethyl)pteridine (615) could not have been regiospecific. Furthermore, the reported UV spectrum of '7-methylMTX' resembled a pterin derivative much more than it did a 2,4-diaminopteridine derivative. 7-MethylMTX (618) was later prepared in an unequivocal fashion (*Scheme 3.135*) by application of the Taylor synthesis; i.e., by reaction of



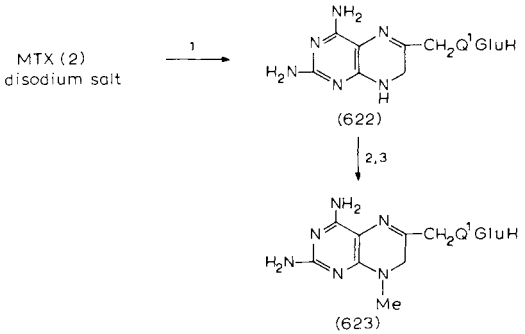
1. [H]; 2. guan; 3. NaOH; 4. HCl; 5. Cl_2, $HCONH_2$.

Scheme 3.135

2-amino-3-cyano-5-chloromethyl-6-methylpyrazine 1-oxide (619) with diethyl *p*-methylaminobenzoylglutamate (35) to give (620), followed by reduction of the *N*-oxide group, guanidine cyclization, and hydrolysis of the ester groups. Chlorination of (618) led to a further MTX analogue, the 7-methyl derivative of dichloroMTX (621) [235]. It should be pointed out that these latter analogues were optically inactive as a consequence of racemization of the L-glutamic acid side-chain during the guanidine cyclization step.

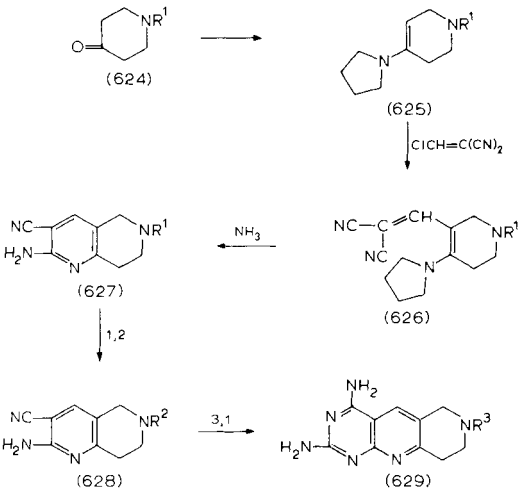
8-Methyl-7,8-dihydroMTX (623) was prepared by reduction of commercial disodium MTX with a large excess of sodium dithionite in aqueous sodium hydroxide to give 7,8-dihydroMTX (622), which was then treated first with 4.5 equiv. of *n*-butyllithium in hexane, followed by the addition of 3 equiv. of methyl iodide (*Scheme 3.136*) [258].

Related analogues (in the 5-deaza series) both of MTX and of FA are the 7,10-ethano derivatives (628) and (634), in which the 7-position is still blocked by alkyl substitution to prevent metabolic oxidation, but in which the steric requirements of a 7-methyl group are reduced by tying back the 7-alkyl group to the N-10 methyl group through an ethano bridge (*Scheme 3.137*) [259]. DL-7,10-Ethano-5-deazaAP (629) was prepared as follows. 1-[4-(*t*-Butoxycarbonyl)phenyl]-4-piperidone (624) was converted into the enamine (625) by reaction with pyrrolidine in THF in the presence of anhydrous magnesium



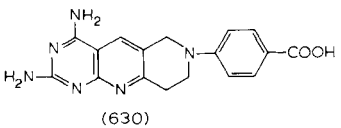
1. $Na_2S_2O_4$, aq. NaOH; 2. BuLi – DMSO; 3. MeI.

Scheme 3.136



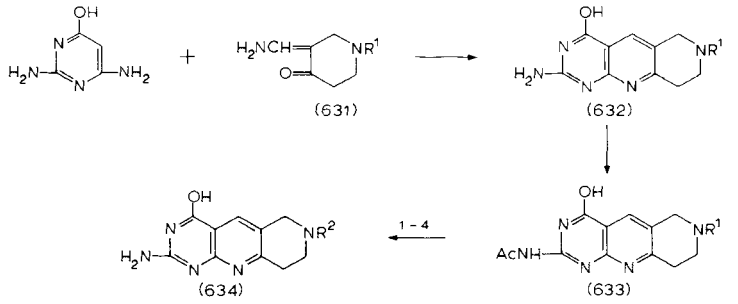
R^1 = 4-$Bu^tOOCC_6H_4$; R^2 = 4-$Bu^tGluCOC_6H_4$; R^3 = 4-$HGluCOC_6H_4$
1. HCl–$MeNO_2$; 2. (135); 3. $Me_2NC(=NH)NH_2$

Scheme 3.137



sulphate [88]. Conversion of (625) to the *o*-aminonitrile (627) was then best carried out by alkylation with (chloromethylene)malononitrile in the presence of triethylamine at $-10\,^{\circ}C$, followed by treatment of the resulting (626) with methanolic ammonia. The free carboxylic acid was obtained by de-*t*-butylation with dry HCl in nitromethane, and the requisite peptide (628) formed with di-*t*-butyl glutamate (235) in the presence of diphenylphosphorochloridate. Cyclization with *N,N*-dimethylguanidine in hot *t*-butanol followed by dide-*t*-butylation with dry HCl in nitromethane then gave 7,10-ethano-5-deazaAP (629). It was not possible to reverse the above sequence of reactions; i.e., to annulate the pyrimidine ring from (627) and then to form the peptide linkage (which would have given an optically active product) because of the extraordinary insolubility of 5-deaza-7,10-ethano-4-amino-4-deoxypteroic acid (630).

Since hydrolysis of the 4-amino group of (629) would have led to racemic 7,10-ethano-5-deazaFA, the pure L-isomer was obtained by use of a different strategy (see *Scheme 3.138*). Thus, 1-[4-(*t*-butoxycarbonyl)phenyl]-4-piperidone (624) was formylated with ethyl formate/potassium hydride-THF, and the resulting hydroxymethylene derivative was alkylated with dimethyl sulphate and treated with ammonia to give (631). This compound was then condensed with 2,4-diamino-6-pyrimidinol in aqueous acetic acid containing a catalytic amount of piperidine to give (632), which was acetylated to (633), treated with dry HCl in nitromethane to free the terminal carboxyl group, and coupled with diethyl L-glutamate in *N*-methylpyrrolidone, using phenyl *N*-phenylphosphoramidochloridate as the condensing agent. Hydrolysis of the coupled product with methanolic sodium hydroxide at room temperature then gave L-7,10-ethano-5-deazaFA (634).



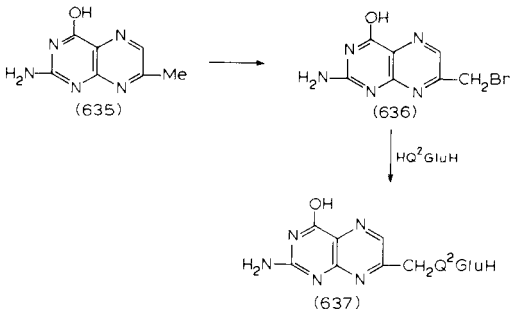
$R^1 = 4\text{-}Bu^tOOCC_6H_4$; $R^2 = 4\text{-}HGluCOC_6H_4$.

1. HCl–$MeNO_2$; 2. GluEt, PhO(PhNH)POCl, Et_3N, NMP; 3. OH^-; 4. H^+.

Scheme 3.138

7-ISOMERS OF MTX, FA AND RELATED COMPOUNDS

The first synthesis of 7-FA (637) involved condensation of *p*-aminobenzoylglutamic acid with 7-bromomethylpterin (636), which had been prepared by bromination of 7-methylpterin (635) (from 2,4,5-triamino-6-pyrimidinol and methylglyoxal) (*Scheme 3.139*) [260]. This 7-isomer of FA proved to be

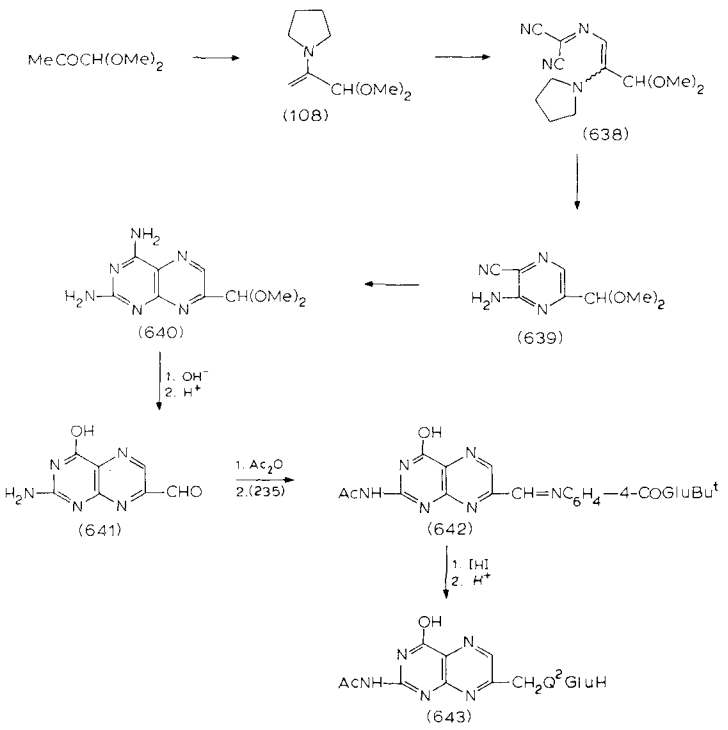


Scheme 3.139

extremely unstable, both in the solid state as the free acid and in alkaline solution; its instability may be responsible for the apparent absence of 7-FA in purified synthetic FA itself, even when the latter is prepared by what is now recognized as a non-regiospecific three-component condensation reaction.

An unequivocal synthesis of (637), protected as its *N*-2′-acetyl derivative (643), was successfully accomplished as shown in *Scheme 3.140* [40]. Pyruvaldehyde dimethylacetal was first converted into its enamine (108). Condensation of (108) with the *O*-tosyl derivative of oximinomalononitrile gave the azadiene (638), which was converted to 2-amino-3-cyano-6-dimethoxymethylpyrazine (639) with ammonia. This latter compound was condensed with guanidine and the product (640) hydrolyzed first with base (to remove the 4-amino group) and then with acid to give pterin-7-carboxaldehyde (641). Acetylation of (641) followed by condensation with di-*t*-butyl *p*-aminobenzoylglutamate (235), reduction and hydrolysis gave (643).

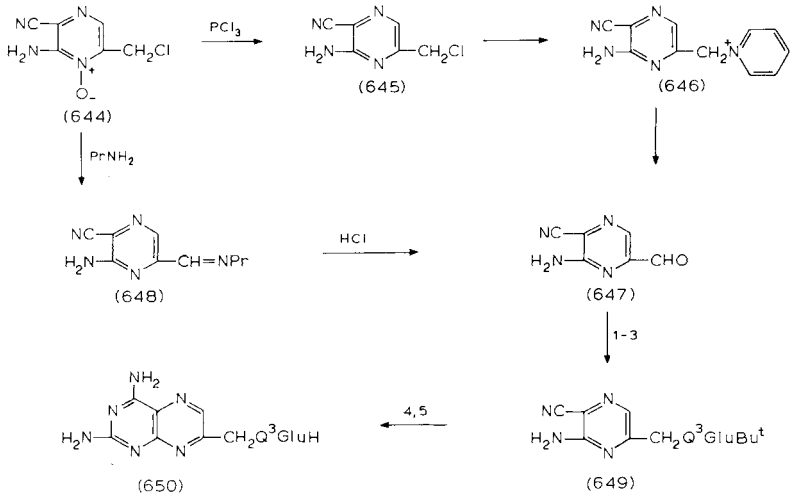
7-AP (protected by acetylation at N-10, (650)) was prepared in an unequivocal fashion by a different strategy starting with 2-amino-3-cyano-6-formylpyrazine (647), which was itself prepared by two independent procedures (*Scheme 3.141*) [261]. In the first, 2-amino-3-cyano-6-chloromethylpyrazine (645) was converted to (647) via (646) by the Kröhnke procedure in excellent overall yield. In the second, the 1-oxide (644) was treated with *n*-propylamine,



Scheme 3.140

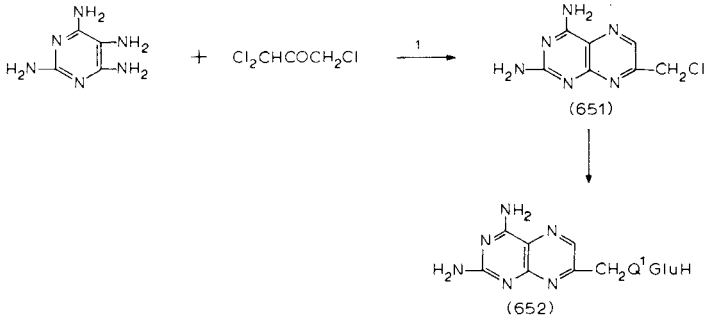
and the resulting imine (648) hydrolyzed with dilute HCl. The 10-acetyl derivative of 7-AP (650) was then prepared from (647) by condensation with di-*t*-butyl *p*-aminobenzoylglutamate (235), reduction of the resulting imine with sodium borohydride, protection of the fragile side-chain by acetylation, cyclization with guanidine, and hydrolysis. Attempts to hydrolyze the 4-amino group of (650) (which would have given N^{10}-acetyl-7-FA), or to remove the protecting acetyl group in (650), were unsuccessful.

The 7-isomer of MTX (652) accompanies MTX when the latter is prepared from 2,4,5,6-tetraaminopyrimidine, the sodium salt of *p*-methylaminobenzoylglutamic acid and 1,2-dibromopropionaldehyde. It was alternatively prepared in what was claimed to be a pure state by the procedure outlined in *Scheme 3.142*, involving condensation of 1,1,3-trichloroacetone with 2,4,5,6-tetraaminopyrimidine at pH 5.5–6.0 to yield isomer-free 2,4-diamino-7-chloromethylpteridine (651). This compound was condensed with *p*-methylaminobenzoylglutamic acid in a sodium acetate buffer at pH 3.0–3.5 to give



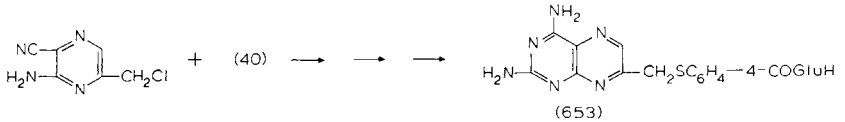
1. (235); 2. [H]; 3. Ac_2O; 4. guan; 5. H^+.

Scheme 3.141



1. pH 5·5–6·0.

Scheme 3.142

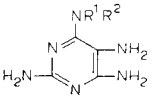


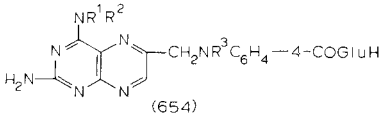
Scheme 3.143

(652) in an overall yield of 12–16% based on *p*-methylaminobenzoylglutamic acid [262].

The 7-isomer of 10-thiaAP (653) was prepared by taking advantage of the Taylor unequivocal synthesis of 2-amino-3-cyano-6-chloromethylpyrazine (645). Thus, condensation of the latter with the anion of diethyl *p*-mercaptobenzoylglutamate (40) [53], followed by guanidine cyclization and saponification, gave (653) (*Scheme 3.143*) [263].

A number of 4-alkyl derivatives of AP (654a–c) were prepared from 2,4,5-triamino-6-alkylaminopyrimidines, 2,3-dibromopropionaldehyde and *p*-aminobenzoylglutamic acid (*Scheme 3.144*) [264]. A corresponding derivative of





(654)

a) $R^1 = R^2 = Me$ $R^3 = H$ c) $R^1 = R^2 = -(CH_2)_5-$ $R^3 = H$
b) $R^1 = Me$ $R^2 = R^3 = H$ d) $R^1 = R^2 = R^3 = Me$

1. $BrCH_2CHBrCHO$, $4-NH_2C_6H_4COGluH$.

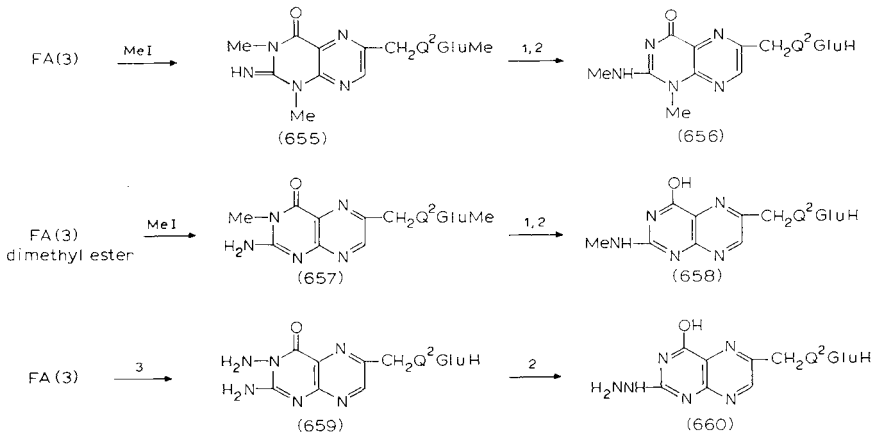
Scheme 3.144

MTX (654d) was prepared by an analogous three-component condensation of 2,4,5-triamino-6-dimethylaminopyrimidine, 2,3-dibromopropionaldehyde and *p*-methylaminobenzoylglutamic acid.

MISCELLANEOUS ANALOGUES

Methylation of FA with a 4-molar excess of methyl iodide gave a low yield of the tetramethyl derivative (655). A Dimroth rearrangement brought about by base at room temperature then gave (656), hydrolysis of the methyl esters having taken place under the conditions of the rearrangement. Alkylation of the dimethyl ester of FA with methyl iodide in DMA containing sodium hydride gave the mono-*N*-methylated derivative (657) (contaminated with some of (655)). Base-induced Dimroth rearrangement of (657) then gave the 2-methyl derivative of FA (658).

3-AminoFA (659) was prepared directly from FA by amination with hydroxylamine *O*-sulphonic acid in dilute sodium hydroxide. Rearrangement of (659) to the hydrazino analogue (660) was then smoothly effected under acidic conditions (a base-induced Dimroth rearrangement was not successful) (*Scheme 3.145*) [265].

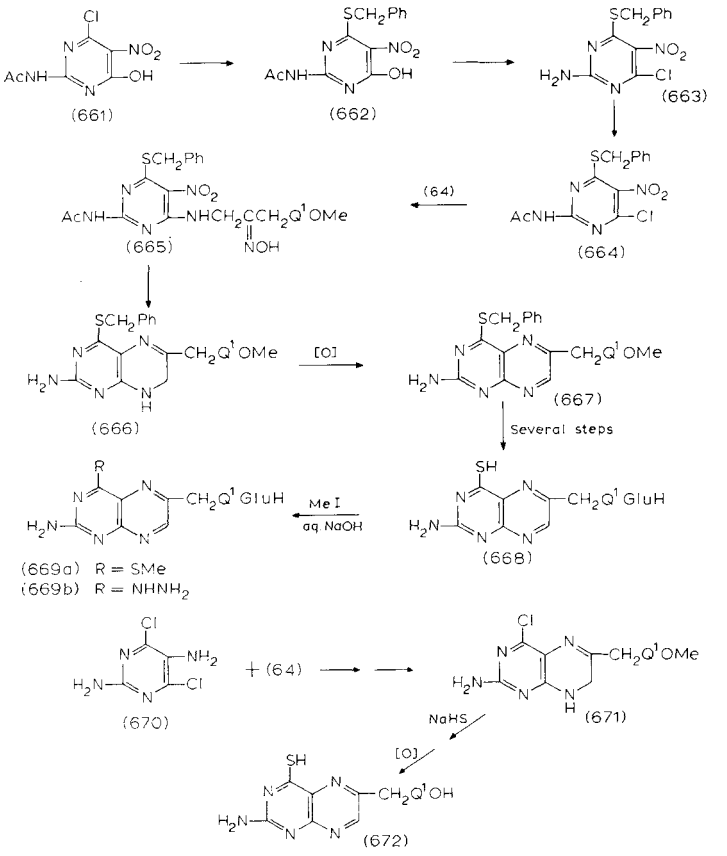


1. OH^-; 2. H^+; 3. H_2NOSO_3H.

Scheme 3.145

In view of the potent antitumour activity exhibited by 6-mercaptopurine, some 4-thio derivatives of FA and MTX have been prepared and examined for their biological activity. 2-Amino-4-chloro-5-nitro-6-pyrimidinol was first acetylated to (661) and then treated with α-toluenethiol to give (662), which was converted to (663) with phosphorus oxychloride. Acetylation of (663), followed by reaction of (664) with the α-aminooxime (64), gave (665) which was hydrolyzed to the corresponding α-amino ketone with 1 M HCl. Hydrogenation of the nitro group in the presence of Raney nickel was followed by spontaneous ring closure to give the dihydropteridine derivative (666), which was oxidized to (667) with potassium permanganate. The benzylthio grouping was then displaced with sodium hydrosulphide, the ester functionality in the resulting mercaptopteridine hydrolyzed with sodium hydroxide, the 4-mercapto group blocked by alkylation with methyl iodide, and the 2-amino group reacetylated with acetic anhydride. The requisite peptide linkage was then formed with diethyl L-glutamate and isobutyl chloroformate. Displacement of the 4-alkylthio grouping was then again carried out with sodium hydrosulphide in refluxing ethanol, and the acetyl blocking group removed with aqueous sodium hydroxide. This long series of reactions thus gave N^{10}-methyl-4-thioFA (668) (which proved to be uninteresting biologically). Methylation with methyl iodide in aqueous sodium hydroxide gave the corresponding methylthio derivative (669a), while displacement of the methylthio grouping with hydrazine gave the 4-hydrazino analogue (669b). An alternative synthesis of (672), one of the

intermediates in the above conversion of (667) to (668), involved reaction of 4,6-dichloro-2,5-diaminopyrimidine (670) with the α-aminooxime (64). Displacement of the 4-chloro substituent in the annulation product (671) with sodium hydrosulphide, followed by oxidation and hydrolysis, gave (672) (*Scheme 3.146*) [266, 267].



Scheme 3.146

5,6,7,8-TETRAHYDRO DERIVATIVES

It was found in 1960 [268] that the antibacterial activity of a variety of FA analogues was markedly increased by reduction to the 5,6,7,8-tetrahydro level.

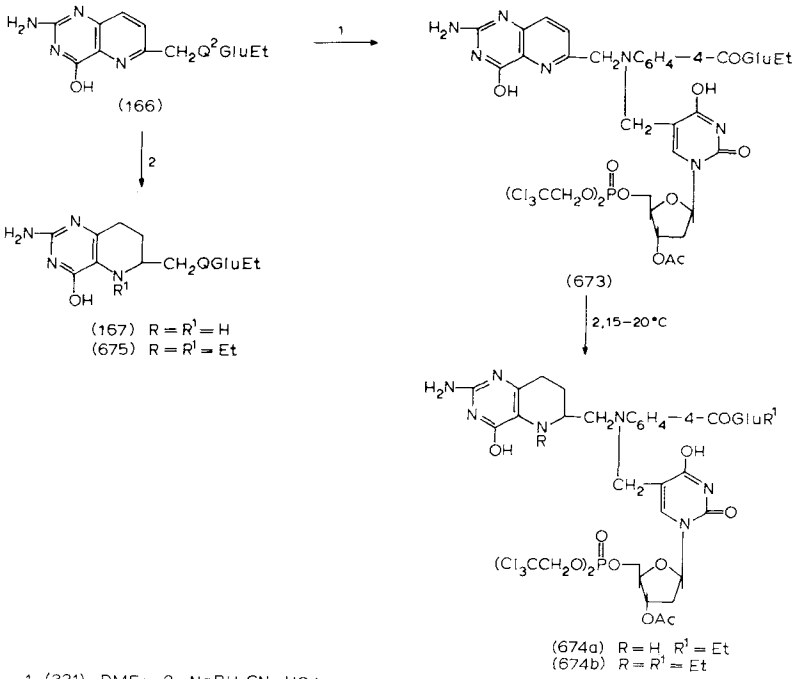
For example, the antibacterial (inhibitory) activity of both AP and N^{10}-formylAP against *Pediococcus cerevisiae* was increased more than 50-fold by hydrogenation; this inhibitory activity was reversed by leucovorin much more readily than by FA itself. The inhibitory activity of MTX was also increased in this system by hydrogenation. By contrast, in a cell-free enzyme system (tetrahydroFA-dependent methionine synthesis by *E. coli*, tetrahydroAP was inhibitory, whereas AP itself showed no significant activity. This work was followed up by a later paper [269] in which both dihydroAP and DL-tetrahydroAP were compared as inhibitors of *P. cerevisiae*. In this system, the dihydro derivative turned out to be twice as inhibitory as the tetrahydro analogue, and 100-times more inhibitory than AP itself, suggesting that these reduced analogues inhibited both DHFR and TS.

Interestingly, dihydroMTX and tetrahydroMTX were both less potent than MTX itself as inhibitors of DHFR, but proved to be more potent as inhibitors of TS; as in the case of the above AP derivatives, dihydroMTX was more inhibitory than the tetrahydro derivative in every system examined [270]. It is unsurprising, in view of our current knowledge of the folate coenzyme pathways, that the unnatural D-enantiomer of 5,10-methylene-5,6,7,8-tetrahydroFA had no activity either as a substrate for or as an inhibitor of DHFR [271].

A number of 5,10-bridged derivatives of 5,6,7,8-tetrahydroFA (designed as analogues of the 5,10-methylene cofactor) were prepared from tetrahydroFA by reaction with phosgene, thiophosgene and cyanogen bromide, and some 5-substituted derivatives were prepared by the addition of isocyanates and isothiocyanates to tetrahydroFA. The reaction of a number of aldehydes (other than formaldehyde) with tetrahydroFA in the presence of reducing agents led to 5-substituted and 5,10-bridged derivatives of 5,6,7,8-tetrahydroFA. In every case, these derivatives were inactive as inhibitors of any of the folate-dependent enzyme systems examined, and they were also inactive against L1210 leukaemia [272].

ADDENDUM

To further clarify questions concerning the structure of the ternary complex formed from TS, 5,10-methylene-5,6,7,8-tetrahydroFA (7) and deoxyuridine monophosphate, Broom's group recently described the synthesis of (674a), the fully protected precursor to the N-10 isomer of (170) (*Scheme 3.147*) [273]. Thus, (166) was alkylated with (331) to give (673), for which attempts to effect reduction to (674a) using Adams's catalyst in acidic ethanol [105a,b] were complicated by the formation of multicomponent mixtures. This problem was circumvented using sodium cyanoborohydride in acetic acid [274], although



Scheme 3.147

contamination with (674b) was observed once the reaction reached 50% completion. Interestingly, the authors found that problems previously encountered with benzylic cleavage during the conversion of (166) to (167) were completely obviated using 8 equiv. of sodium cyanoborohydride in acetic acid, whereas using a large excess of this reagent produced (675). Conversion of (674a) to the desired target will be accomplished using Zn-Cu couple to remove the trichloroethyl groups, followed by NaOH/EtOH [105a,b].

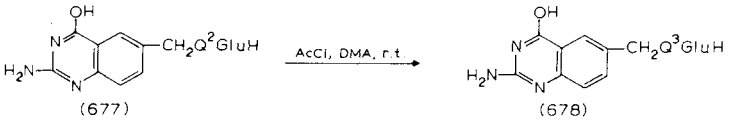
Nair, Nanarati, Nair, Kisliuk, Gaumont, Hsiao and Kalman have prepared the 5,8-dideazapteroic acid derivative (676), a useful precursor to polyglutamate analogues, from (228) and ethyl *p*-(*N*-propargyl)benzoate, followed by saponification. Similarly, (232q) and (232r) were obtained from (228) and (231) R = 2-butynyl or cyclopropylmethyl) (*Scheme 3.148*) [275].

Continuing interest in quinazoline analogues of FA has led to the synthesis of 10-acetyl-5,8-dideazaFA (678) via acetylation of (677) (*Scheme 3.149*) [276]. Improvements in the synthesis of the diethyl ester of (677) and in the formylation of (677) were also described.

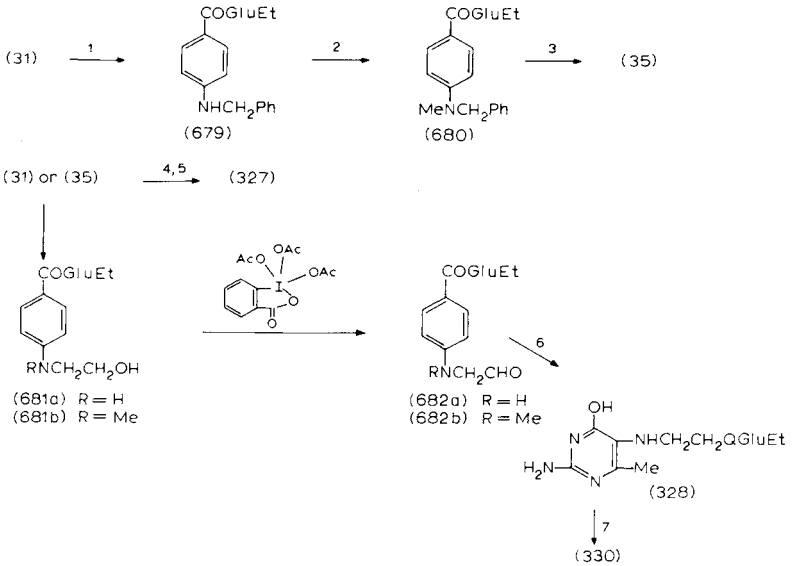
$R^1 = NC_6H_4{-}4{-}COOR^2$ ($CH_2C{\equiv}CH$); R^2=Et or H.

1. MgO, DMA, Δ; 2. NaOH, MeCN; 3. HCl.

Scheme 3.148



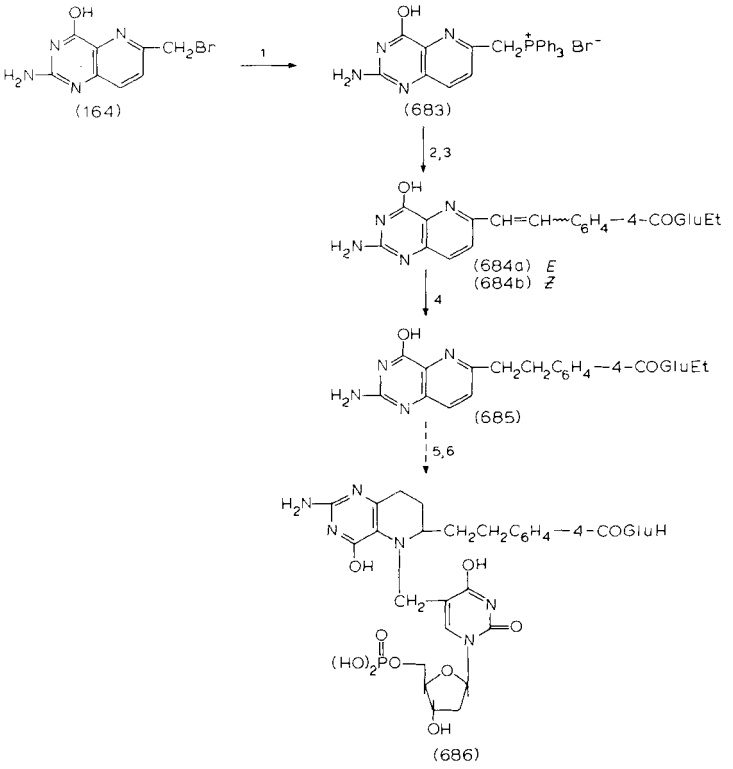
Scheme 3.149



1. PhCHO, $NaBH_3CN$, EtOH–HOAc, pH 4–5; 2. CH_2O, $NaBH_3CN$, MeCN–HOAc, pH 4–5; 3. H_2, Pd/C, EtOH,1 M HCl; 4. $I(CH_2)_2OH$, DMA; 5. TsCl, pyr; 6. 2,5-diamino-6-hydroxy-4-methylpyrimidine, $NaBH_3CN$, MeOH; 7. Several steps.

Scheme 3.150

Improvements in the synthesis of (330) (R = H or Me, R′ = PO_3^{2-}) have been described (*Scheme 3.150*) [277]. These include: a three-step, high-yield synthesis of (35) (see *Scheme 3.150*) from commercially available (31) via (679) and (680); an alternative approach to (327) (R = H, Me) using 2-iodoethanol in DMA; and the use of 2-acetamido-4-hydroxy-5-amino-6-methylpyrimidine to minimize *N,N′*-dialkylation during reaction with (327) (R = H, Me). This latter problem could be totally obviated by reductive alkylation of 2,5-diamino-4-hydroxy-6-methylpyrimidine with (681a) or (681b) in the presence of sodium cyanoborohydride to produce (328) (R = H, Me). The requisite side-chain aldehydes (682a) and (682b) were readily obtained from (681a) and (681b), respectively, via mild oxidation with periodinane [278]. One additional analogue, namely (330) (R = $CH_2C \equiv CH$), was prepared via this methodology.

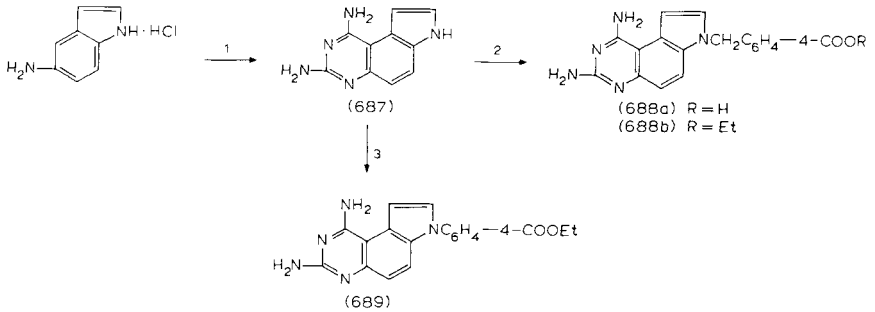


1. Ph_3P, DMF; 2. NaOMe, DMF; 3. (252), DMF; 4. H_2, PtO_2, HCl, EtOH; 5. (331), $NaHCO_3$–DMF; 6. Several steps.

Scheme 3.151

To delineate further the role of N-10 in the action and catalytic mechanism of TS, Samantham and Broom have also begun the synthesis of (686) in which N-10 has been replaced by a methylene group (*Scheme 3.151*) [279]. In this approach, 6-bromomethyl-8-deazapterin (164) [280] was converted into the phosphonium salt (683) and then treated successively with sodium methoxide and (252) to produce (684a) and (684b) as an 81 : 19 *E* : *Z* mixture, which was reduced uneventfully to (685). Alkylation of (685) with (331) and subsequent conversion to (686) has been reported to be in progress.

An unusual quinazoline isopteroic acid in which C-5 and N-9 were part of a fused pyrrole ring has been disclosed in the patent literature (*Scheme 3.152*)

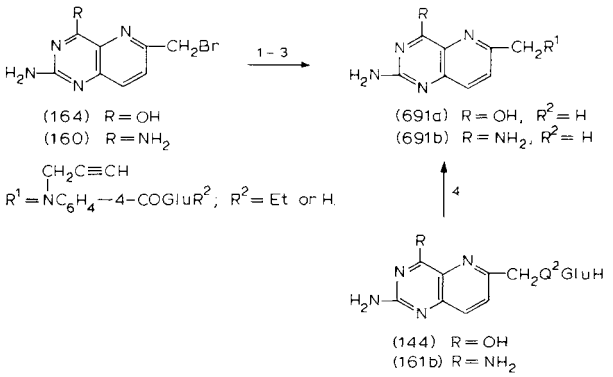


1. $NaN(CN)_2$, 1-octanol, Δ; 2. 4-$BrCH_2C_6H_4COOR$, NaH-DMF; 4. 4-FC_6H_4COOEt, NaH-DMF.

Scheme 3.152

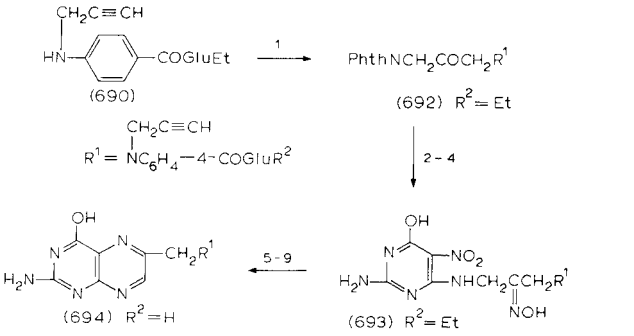
[281]. The requisite tricyclic 2,4-diaminoquinazoline precursor (687) was obtained in one step from 5-aminoindole hydrochloride and sodium dicyanamide [282]. Subsequent alkylation of (687) with (4-bromomethyl)benzoic acid yielded (688a). Alkylation of (687) with ethyl (4-bromomethyl)benzoate afforded (688b), while arylation of (687) with ethyl 4-fluorobenzoate generated the *N*-aryl analogue (689).

Very recently, 10-propargyl-8-deazaFA (691a) and 10-propargyl-8-deazaAP (691b) were prepared (see *Scheme 3.153*) and evaluated as TS inhibitors [282]. Alkylation of either (164) or (160) with (690), followed by mild saponification, afforded (691a) and (691b), respectively. It is interesting to note that (691a) and (691b) could be prepared via alkylation of (144) and (161b) with propargyl bromide in ethanol in good yield. This remarkable selectivity was attributed to selective solvation by the protic solvent.



1. R^1H(690, R^2=Et), DMA, 100°C; 2. NaOH, EtOH; 3. HCl; 4. $BrCH_2C{\equiv}CH$, EtOH, PhMe, Δ.

Scheme 3.153



1. $BrCH_2COCH_2NPhth$(500); 2. NH_2OH; 3. H_2NNH_2; 4. 2-amino-4-chloro-6-hydroxy-5-nitropyrimidine; 5. TFA, HCl; 6. $Na_2S_2O_4$; 7. OH^-; 8. $KMnO_4$; 9. H^+.

Scheme 3.154

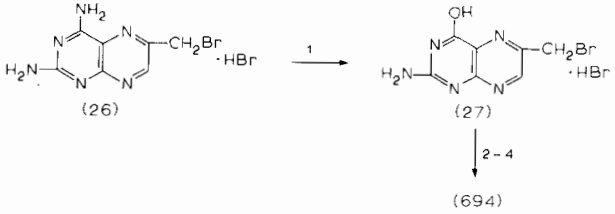
Two independent syntheses of 10-propargylFA (694) have recently been reported. In the first, the Boon–Leigh strategy was employed (*Scheme 3.154*) [284]. Thus, alkylation of diethyl [*p*-(*N*-propargylamino)benzoyl]-L-glutamate (690) with the phthalimide (500) provided the masked α-aminoketone (692), which was further elaborated to (694) via the oxime (693) under standard conditions. Various reduced derivatives of (694) were also prepared. An alter-

native synthesis of (694) (see *Scheme 3.155*) [285] involved hydrolysis of (26)·HBr with HBr to give (27)·HBr, which was then condensed with the elaborated aniline derivative (690).

Direct conversion of 10-propargylAP (451d) to (694) was unsuccessful due to the lability of the propargyl group under the requisite deamination conditions [284, 285].

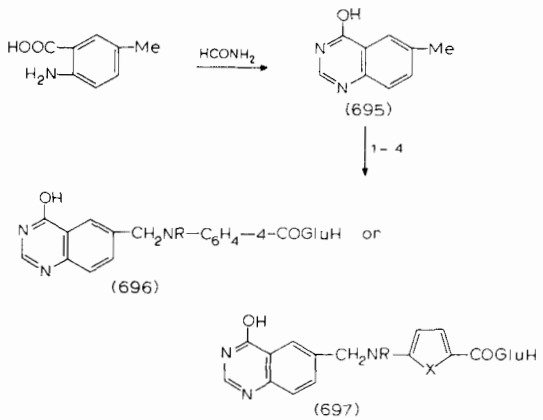
Recently, DeGraw and co-workers have described the preparation of 8-deazahomoFA (180) and the corresponding 5,6,7,8-tetrahydro derivative (180a) in a full paper [286].

An interesting series of 2-deaminoquinazolines containing a variety of modifications at N-10 (696) as well as the *p*-aminobenzoyl group (697) have been



1. HBr, 95°C; 2. (690); 3. 1 M NaOH; 4. HOAc.

Scheme 3.155



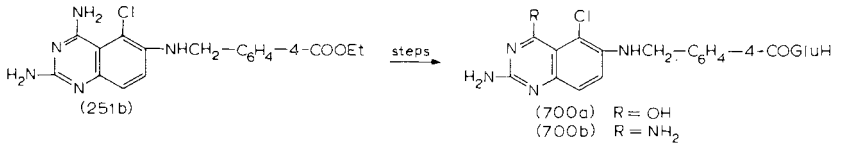
1. Bu^tCOOCH_2Cl; 2. Br^+; 3. GluEt; 4. NaOH–EtOH.

Scheme 3.156

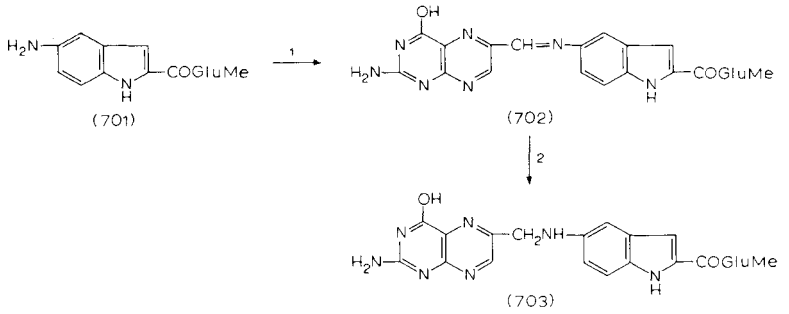
described in the patent literature (*Scheme 3.156*) [287]. For example, cyclocondensation of 5-methylanthranilic acid with formamide gave (695) which was sequentially protected on nitrogen and brominated. The product was then used to alkylate the requisite substituted *p*-aminobenzoyl- or substituted aminoheterocyclic glutamate moiety followed by saponification with concomitant deprotection to yield (696) and (697) analogues, respectively.

Further examples of quinazolines include the N-10 substituted 5,6,7,8-tetrahydro analogues (699) described by M.G. Nair and colleagues (*Scheme 3.157*) [288]. Although the chloromethyl quinazoline (197) had been used to prepare (198) [116], the present authors found that reaction of (197) with ethyl *p*-(*N*-propargylamino)benzoate resulted in extensive degradation of the propargyl amine, while the mesylate derived from (196) failed to react with ethyl *p*-aminobenzoate or the above propargyl amine. Accordingly, (196) was converted

Scheme 3.157



Scheme 3.158



1. (11), DMF - Δ; 2. $NaBH_4$/EtOH.

Scheme 3.159

to the corresponding bromomethyl salt (698), which was then converted to (699) via conventional methodology.

Improvements in the synthesis of (251b), the precursor to 5-chloro-5,8-dideazaFA (700a) and 5-chloro-5,8-dideazaAP (700b), have been reported and include the use of di-*t*-butyl glutamate to permit deblocking to the targets without racemization (*Scheme 3.158*) [289].

Substitution for PABA in a FA analogue by an indole ring has been accomplished by a Russian group (*Scheme 3.159*) [290]. Thus, acylation of dimethyl glutamate with 5-nitroindole-2-carbonyl chloride followed by catalytic hydrogenation furnished (701) which was condensed with (11) to yield imine (702). This was reduced with sodium borohydride to give (703) uneventfully.

Finally, while this manuscript was in press, a review by Rahman and Chhabra covering part of this material appeared [291].

REFERENCES

1 L.S. Goodman, M.M. Wintrobe, W. Dameshek, M.J. Goodman, A. Gilman and M. McLennan, J. Am. Med. Assoc., 132 (1946) 126.
2 A. Gilman and F.S. Philips, Science (Washington, DC), 103 (1946) 409.
3 D.R. Seeger, D.B. Cosulich, J.M. Smith, Jr. and M.E. Hultquist, J. Am. Chem. Soc., 71 (1949) 1753.
4 J.R. Bertino, in Cancer and Chemotherapy, eds. S.T. Crooke and A.W. Prestayko (Academic Press, New York, 1981) p. 359.
5a A. Rosowsky, J.H. Freisheim, R.G. Moran, V.C. Solan, H. Bader, J.E. Wright and M. Radike-Smith, J. Med. Chem., 29 (1986) 655 and references cited therein.
5b A. Rosowsky, H. Bader, M. Radike-Smith, C.A. Cucchi, M.M. Wick and J.H. Freisheim, J. Med. Chem., 29 (1986) 1703.
6 C.L. Krumdieck, T. Tamura and I. Eto, Vitam. Horm., 40 (1983) 45.
7 J.M. Covey, Life Sci., 26 (1980) 665.
8 J.R. Piper, G.S. McCaleb and J.A. Montgomery, J. Med. Chem., 26 (1983) 291.
9 R.L. Blakley and S.J. Benkovic, eds., Folates and Pterins, Vol. 1 and 2, Chemistry and Biochemistry of Folates (John Wiley & Sons, New York, 1984–1985).
10 B.-K. Chen, C. Horvath and J.R. Bertino, J. Med. Chem., 22 (1979) 483.
11 G.M. Crippen, J. Med. Chem., 23 (1980) 599.
12 J.M. Blaney, C. Hansch, C. Silipo and A. Vittona, Chem. Rev., 84 (1984) 333.
13a J.D. Prejean and J.A. Montgomery, Drug Metab. Rev., 15 (1984) 619.
13b J.A. Montgomery and J.R. Piper, in Folate Antagonists as Therapeutic Agents, Vol. 1, eds. F.M. Sirotnak, J.J. Birchall, W.B. Ensminger and J.A. Montgomery (Academic Press, New York, 1984) p. 219.
14 W.A. Bleyer, Cancer Treat. Rev., 4 (1977) 87.
15 W.A. Bleyer, Cancer (Philadelphia), 41 (1978) 36.
16 J.R. Bertino, in Ref. 4, p. 311.
17 J.R. Bertino, Cancer Res., 39 (1979) 293.
18 E. Frei, III, R.H. Blum, S.W. Pitman, J.M. Kirkwood, I.C. Henderson, A.T. Sharin, R.J.

Meyer, R.C. Bast, M.B. Garneck, L.M. Parker and G.P. Canellos, Am. J. Med., 68 (1980) 370.
19 W.D. Ensminger, G.B. Grinde and J.A. Hoglind, in Advances in Cancer Chemotherapy, ed. A. Rosowsky (Marcel Dekker, New York, 1981) p. 62.
20 N. Jaffe and S. Howell, in Ref. 19, p. 111.
21 J.C. White, Cancer Treat. Rep., 65, Suppl. 1 (1981) 3.
22 I.D. Goldman, Ann. N.Y. Acad. Sci., 186 (1971) 400.
23 F.M. Sirotnak, Pharmacol. Ther., 8 (1980) 71.
24 K.R. Harrap, B.T. Hill, M.E. Furness and L.I. Hart, Ann. N.Y. Acad. Sci., 186 (1971) 312.
25 D.V. Santi, J. Med. Chem., 23 (1980) 103.
26 A.L. Jackman, T.R. Jones and A.H. Calvert, in Experimental and Clinical Progress in Cancer Chemotherapy, ed. F.M. Muggia (Martinus Nijhoff, Boston, 1985) p. 155.
27 B. Roth and C.C. Cheng, Prog. Med. Chem., 19 (1982) 270.
28 R.C. Jackson, Pharmacol. Ther., 25 (1984) 61.
29 D.A. Mathews, R.A. Alden, J.T. Bolin, D.J. Filman, S.T. Freer, R. Hamlin, W.G.J. Hol, R.L. Kisliuk, E.J. Pastore, L.T. Plante, N.-H., Xuong and J. Kraut, J. Biol. Chem., 253 (1978) 6946.
30 G.C.K. Roberts, in Chemistry and Biology of Pteridines: Pteridines and Folic Acid Derivatives, ed. J.A. Blair (Walter de Gruyter, Berlin, 1983) p. 197.
31 H.H.W. Thijssen, Anal. Biochem., 54 (1973) 609.
32 P.H. Boyle and M.J. O'Mahony, J. Heterocycl. Chem., 21 (1984) 909.
33 M. Viscontini and J. Bieri, Helv. Chim. Acta, 54 (1971) 2291.
34 R. Mengel, W. Pfleiderer and W.-R. Knappe, Tetrahedron Lett., (1977) 2817.
35 S.I. Zav'yalov and A.G. Zavozin, Izv. Akad. Nauk. SSSR, Ser. Khim., (1981) 1669.
36 S.I. Zav'yalov and L.F. Ovechkina, Izv. Akad. Nauk. SSSR, Ser. Khim., (1978) 1935.
37 M. Sletzinger, D. Reinhold, J. Grier, M. Beachem and M. Tishler, J. Am. Chem. Soc., 77 (1955) 6365.
38 E.C. Taylor and K. Lenard, Liebigs Ann. Chem., 726 (1969) 100.
39 E.C. Taylor, R.N. Henrie III and R.C. Portnoy, J. Org. Chem., 43 (1978) 736.
40 E.C. Taylor and D.J. Dumas, J. Org. Chem., 46 (1981) 1394.
41 P.H. Boyle and W. Pfleiderer, Chem. Ber., 113 (1980) 1514 and references cited therein.
42 J.R. Piper and J.A. Montgomery, J. Org. Chem., 42 (1977) 208.
43 G. Carrara and V. D'Amato, U.S. Patent 2,710,866 (1955).
44 J.H. Boothe and C.W. Waller, J. Am. Chem. Soc., 70 (1948) 27.
45 J.H. Boothe, U.S. 2,584,538; Chem. Abst., 46 (1952) 96233.
46 Z.V. Pushkarera and L.V. Alekseeva, Zh. Obshch. Khim., 32 (1962) 1058.
47 E.C. Taylor, R.C. Portnoy, D.C. Hochstetler and T. Kobayashi, J. Org. Chem., 40 (1975) 2347 and references cited therein.
48 F.E. King, R.M. Acheson and P.C. Spensley, J. Chem. Soc., (1949) 1401.
49 B.R. Baker, D.V. Santi and H.S. Shapiro, J. Pharm. Sci., 53 (1964) 1317.
50 D.V. Santi, J. Heterocycl. Chem., 4 (1967) 475.
51 E.I. Fairburn, B.J. Magerlein, L. Stubberfield, E. Stapert and D.I. Weisblat, J. Am. Chem. Soc., 76 (1954) 676.
52 E. Campaigne and W.W. Meyer, J. Org. Chem., 27 (1962) 2835.
53 Y.H. Kim, Y. Gaumont, R.L. Kisliuk and H.G. Mautner, J. Med. Chem., 18 (1975) 776.
54 W.R. Boon and T. Leigh, J. Chem. Soc., (1951) 1497.
55a R.B. Angier, J.H. Boothe, B.L. Hutchings, J.H. Mowat, J. Semb, E.L.R. Stokstad, Y. SubbaRow, C.W. Waller, D.B. Cosulich, M.J. Fahrenbach, M.E. Hultquist, E. Kuh, E.H.

Northey, D.R. Seeger, J.P. Sickels and J.M. Smith, Jr., Science (Washington, DC), 103 (1946) 667.

55b C.W. Waller, B.L. Hutchings, J.H. Mowat, E.L.R. Stokstad, J.H. Boothe, R.B. Angier, J. Semb. Y. SubbaRow, D.B. Cosulich, M.J. Fahrenbach, M.E. Hultquist, E. Kuh, E.H. Northey, D.R. Seeger, J.P. Sickels and J.M. Smith, Jr., J. Am. Chem. Soc., 70 (1948) 19.

55c M.E. Hultquist, E. Kuh, D.B. Cosulich, M.J. Fahrenbach, E.H. Northey, D.R. Seeger, J.P. Sickels, J.M. Smith, Jr., R.B. Angier, J.H. Boothe, B.L. Hutchings, J.H. Mowat, J. Semb, E.L.R. Stokstad, Y. SubbaRow and C.W. Waller, J. Am. Chem. Soc., 70 (1948) 23.

55d R.B. Angier, E.L.R. Stokstad, J.H. Mowat, B.L. Hutchings, J.H. Boothe, C.W. Waller, J. Semb, Y. SubbaRow, D.B. Cosulich, M.J. Fahrenbach, M.E. Hultquist, E. Kuh, E.H. Northey, D.R. Seeger, J.P. Sickels and J.M. Smith, Jr., J. Am. Chem. Soc., 70 (1948) 25.

56a C. Temple, Jr., C.L. Kussner and J.A. Montgomery, J. Heterocycl. Chem., 8 (1971) 1099.

56b C. Temple, Jr., C.L. Kussner and J.A. Montgomery, J. Heterocycl. Chem., 10 (1973) 889.

57a S. Grudzinski, Acta Pol. Pharm., 24 (1967) 1.

57b S. Grudzinski, Acta Pol. Pharm., 24 (1967) 9.

58 F.C. Schaefer and G.A. Peters, J. Org. Chem., 26 (1961) 412.

59 C. Temple, Jr., C.L. Kussner and J.A. Montgomery, J. Org. Chem., 36 (1971) 3502.

60 C. Temple, Jr., C.L. Kussner and J.A. Montgomery, J. Org. Chem., 39 (1974) 2866.

61 For direct conversion of 4-azido-7-azapteridines to the corresponding 4-amino analogues see C. Temple, Jr., C.L. Kussner and J.A. Montgomery, J. Org. Chem., 36 (1971) 2974.

62 C. Temple, Jr., C.L. Kussner and J.A. Montgomery, J. Org. Chem., 40 (1975) 2205.

63 S.M. McElvain and J.W. Nelson, J. Am. Chem. Soc., 64 (1942) 1825.

64 F. Klaus and O. Baudisch, Chem. Ber., 51 (1918) 1044.

65 C. Temple, Jr. and J.A. Montgomery, J. Org. Chem., 28 (1963) 3038.

66a J.A. Montgomery, R.D. Elliott, S.L. Straight and C. Temple, Jr., Ann. N.Y. Acad. Sci., 186 (1971) 227.

66b R.D. Elliott, C. Temple, Jr., J.L. Frye and J.A. Montgomery, J. Org. Chem., 36 (1971) 2818.

67 R.D. Elliott, C. Temple, Jr. and J.A. Montgomery, J. Med. Chem., 17 (1974) 553.

68 D.P. Tschudy and A. Collins, J. Org. Chem., 24 (1959) 556.

69 R.D. Elliott, C. Temple, Jr. and J.A. Montgomery, J. Org. Chem., 35 (1970) 1676.

70 R.D. Elliott, C. Temple, Jr. and J.A. Montgomery, J. Org. Chem., 31 (1966) 1890.

71 B.S. Gorton, J.M. Ravel and W. Shive, J. Biol. Chem., 231 (1958) 331.

72 J.A. Montgomery and N.F. Wood, J. Org. Chem., 29 (1964) 734.

73 J.A. Montgomery, J.R. Piper, R.D. Elliott, C. Temple, Jr., E.C. Roberts and Y.F. Shealy, J. Med. Chem., 22 (1979) 862.

74 E.F. Elslager and J. Davoll, in Lectures in Heterocyclic Chemistry (Elsevier, Amsterdam), Vol. 2, (1974) S-97.

75 J. Davoll, E.F. Elslager, J. Blankley and E.P. Lewis, unpublished results (1973).

76 A. Srinivasan and A.D. Broom, J. Org. Chem., 45 (1980) 3746.

77 B.H. Riz Kalla and A.D. Broom, J. Org. Chem., 37 (1972) 3980.

78 G.K. Smith, W.T. Mueller, P.A. Benkovic and S.J. Benkovic, Biochemistry, 20 (1981) 1241.

79 This synthesis of L-5-deazafolic acid was first presented in detail in a plenary lecture delivered at the spring meeting of the Royal Society of Chemistry (London) at UWIST, Cardiff, Wales, in March, 1979.

80 E.C. Taylor, D.C. Palmer, T.J. George, S.R. Fletcher, C.P. Tseng, P.J. Harrington and G.P. Beardsley, J. Org. Chem., 48 (1983) 4852.

81a A. Arnold, Collect. Czech. Chem. Commun., 26 (1961) 3051.

81b A. Arnold and J. Zemlicka, Collect. Czech. Chem. Commun., 25 (1960) 1318.
82 C. Temple, Jr., R.D. Elliot and J.A. Montgomery, J. Org. Chem., 47 (1982) 761.
83 This synthesis of 5-deazaaminopterin was first presented in detail at the 8th International Congress of Heterocyclic Chemistry in Graz, Austria in August, 1981.
84 C.G. Temple, J.A. Montgomery and R.D. Elliott, US Patent 4,536,575 (1985).
85a A.H. Calvert, T.R. Jones, A.L. Jackman, S.J. Brown and K.R. Harrap, in Advances in Tumor Prevention, Detection and Characterization, Vol. 5. Human Cancer. Its Characterization and Treatment, ed. W. Davis, K.R. Harrap and G. Stathopoulos (Excerpta Medica, Amsterdam, 1980) p. 272.
85b T.R. Jones, A.H. Calvert, A.L. Jackman, S.J. Brown, M. Jones and K.R. Harrap, Eur. J. Cancer, 17 (1981) 11.
86 S. Srinivasan and A.D. Broom, J. Org. Chem., 46 (1981) 1777.
87 J.A. Montgomery, J.D. Rose, C. Temple, Jr. and J.R. Piper, in Chemistry and Biology of Pteridines. ed. W. Pfleiderer (Walter de Gruvter, Berlin, 1975) p. 485.
88 P.A. Zoretic, F. Barcelos and B. Branchard, Org. Prep. Proc. Int., 8 (1976) 211.
89 H. Kurihara and M. Mishima, J. Heterocycl. Chem., 14 (1977) 1077.
90 T.-L. Su, J.-T. Huang, J.H. Burchenal, K.A. Watanabe and J.J. Fox, J. Med. Chem., 29 (1986) 709.
91 The authors noted that (124, R = Me) as well as *N*-10 methyl derivatives have been recently described: J.R. Piper, G.S. McCaleb, J.A. Montgomery, Thirty-Sixth Southeastern Regional Meeting of the American Chemical Society, Raleigh, NC (Oct. 26, 1984).
92 T.-L. Su and K.A. Watanabe, J. Heterocycl. Chem., 21 (1984) 1543.
93a T.B. Johnson, J. Am. Chem. Soc., 42 (1920) 513.
93b G. Shaw, J. Chem. Soc., (1955) 1834.
94 For a review of this mechanism, see H.C. van der Plas, Acc. Chem. Res., 11 (1978) 462.
95 H. Vorbruggen and K. Krolikiewicz, Chem. Ber., 117 (1984) 1523.
96 J.R. Piper, G.S. McCaleb, J.A. Montgomery, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Med. Chem., 29 (1986) 1080.
97 J.R. Piper, G.S. McCaleb, J.A. Montgomery and F.M. Sirotnak, 8th International Symposium on Pteridines and Folic Acid Derivatives; Chemical, Biological and Clinical Aspects (June 15–20, 1986), Montreal, Canada. We thank Dr. J.R. Piper for providing us with a preprint of this work prior to publication.
98 J.I. DeGraw, R.L. Kisliuk, Y. Gaumont and C.M. Baugh, J. Med. Chem., 17 (1974) 470.
99 V. Oakes and H.N. Rydon, J. Chem. Soc., (1956) 4433.
100 V. Oakes, H.N. Rydon and K. Undheim, J. Chem. Soc., (1962) 4678.
101 W.J. Irwin and D.G. Wibberly, J. Chem. Soc., (1967) 1745.
102. C. Krumdieck and C.M. Baugh, Biochemistry, 8 (1969) 1568.
103 C. Temple, Jr., C.L. Kussner, J.D. Rose, D.L. Smithers, L.L. Bennett, Jr. and J.A. Montgomery, J. Med. Chem., 24 (1981) 1254.
104 W. Braker, E.J. Pribyl, J.T. Sheehan, E.R. Spitzmiller and W.A. Lott, J. Am. Chem. Soc., 69 (1981) 3072.
105a A. Srinivasan and A.D. Broom, Tetrahedron Lett., 23 (1981) 1431.
105b A. Srinivasan, U. Amarnath, A.D. Broom, F.C. Zou and Y.-C. Cheng, J. Med. Chem., 27 (1984) 1710.
106 These authors noted that (167) (as the acid, GluH) had been reported earlier by both DeGraw [98] and Montgomery [103]. In neither case was (167, GluH) isolated pure, nor was the structure confirmed by spectral data.

107 D.B. Cosulich and J.M. Smith, Jr., J. Am. Chem. Soc., 71 (1949) 3574.

108 E. Khalifa, A.N. Ganguly, J.H. Bieri and M. Viscontini, Helv. Chim. Acta, 63 (1980) 2254 have described the reduction and formylation of N^{10}-nitrosoFA.

109a J.I. DeGraw, V.H. Brown, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, in Chemistry and Biology of Pteridines, eds. R.L. Kisliuk and G.M. Brown (Elsevier, New York, 1979) p. 229.

109b J.I. DeGraw, personal communication.

110 D.H. Kim and R.L. McKee, J. Org. Chem., 35 (1970) 455.

111 Z. Budesinsky, Collect. Czech. Chem. Commun., 14 (1949) 223.

112 M.J. Winchester, L.J. Zappone and C.G. Skinner, J. Heterocycl. Chem., 18 (1981) 455.

113 M.J. Winchester, J. Heterocycl. Chem., 16 (1979) 1455.

114 This technique has been described by M.G. Nair and C.M. Baugh, J. Org. Chem., 38 (1973) 2185 to couple pterincarboxylic acid with amines.

115 D.W. Woolley and A. Pringle, J. Biol. Chem., 174 (1948) 327.

116 R. Koehler, L. Goodman, J. DeGraw and B.R. Baker, J. Am. Chem. Soc., 80 (1958) 5779.

117 J. DeGraw, L. Goodman, B. Weinstein and B.R. Baker, J. Org. Chem., 27 (1962) 576.

118 J. DeGraw, L. Goodman and B.R. Baker, J. Org. Chem., 26 (1961) 1156.

119 O.D. Bird, V. Oakes, K. Undheim and H.N. Rydon, in Pteridine Chemistry, eds. W. Pfleiderer and E.C. Taylor (Pergamon Press, Oxford, 1964) p. 417.

120 F.H.S. Curd, J.K. Landquist and F.L. Rose, J. Chem. Soc., (1947) 775.

121 J. Davoll, U.S. Patent 3,472,851 (1969); Chem. Abstr., 71 (1969) 124925.

122 H.G. Mautner and Y.-H. Kim, J. Org. Chem., 40 (1973) 3447.

123 J. Davoll, Br. Patent 1,104,576 (1968); Chem. Abstr., 69 (1968) 44206.

124a J. Davoll, U.S. Patent 3,471,498 (1969); Chem. Abstr., 71 (1969) 124925.

124b J. Davoll, Br. Patent 1,135,898 (1969); Chem. Abstr., 70 (1968) 47487.

125 J. Davoll and A.M. Johnson, J. Chem. Soc. (C), (1970) 997.

126 S.P. Acharya and J.B. Hynes, J. Heterocycl. Chem., 12 (1975) 1283.

127 T.R. Jones, Eur. J. Cancer, 16 (1980) 707.

128a T.R. Jones, K.R. Harrap and A.H. Calvert, Br. Patent 2,065,653 (1980).

128b T.R. Jones, K.R. Harrap and A.H. Calvert, U.S. Patent 4,564,616 (1986).

128c See also T.R. Jones, A.H. Calvert, A.L. Jackman, S.J. Brown, M. Jones and K.R. Harrap, Eur. J. Cancer, 17 (1981) 11.

128d T.R. Jones, A.H. Calvert, A.L. Jackman and K.R. Harrap, Abstracts, 8th International Congress of Heterocyclic Chemistry, Graz, Austria (Aug. 23–28, 1981) p. 67.

129a A.H. Calvert, D.L. Alison, S.J. Harland, A.L. Jackman, C.J. Mooney, J.E. Smith and K.R. Harrap, Br. J. Cancer, 48 (1983) 116.

129b D.L. Alison, D.R. Newell and A.H. Calvert, Br. J. Cancer, 48 (1983) 126.

130 Drugs Future, 9 (1984) 893 and references cited therein.

131 M.G. Nair, D.C. Salter, R.L. Kisliuk, Y. Gaumont, G. North and F.M. Sirotnak, J. Med. Chem., 26 (1983) 605.

132 T.R. Jones, A.H. Calvert, A.L. Jackman, M.A. Eakin, M.J. Smithers, R.F. Betteridge, D.R. Newell, A.J. Hayter, A. Stocker, S.J. Harland, L.C. Davies and K.R. Harrap, J. Med. Chem., 28 (1985) 1468.

133 J.B. Hynes, S.J. Harmon, G.G. Floyd, M. Farrington, L.D. Hart, G.R. Gale, W.L. Washtien, S.S. Susten and J.H. Freisheim, J. Med. Chem., 28 (1985) 209.

134 M. Kajtar and M. Hollosi, Acta Chim. Acad. Sci. Hung., 65 (1970) 403.

135 J.B. Hynes, D.E. Eason, C.M. Garrett, P.L. Colvin, Jr., K.E. Shores and J.H. Freisheim, J. Med. Chem., 20 (1977) 588 reported the synthesis of (239) by heating (221) with 97% formic acid.

136 T.R. Jones, M.J. Smithers, M.A. Taylor, A.L. Jackman, A.H. Calvert, S.J. Harland and K.H. Harrap, J. Med. Chem., 29 (1986) 468.
137 T.R. Jones, M.J. Smithers, R.F. Betteridge, M.A. Taylor, A.L. Jackman, A.H. Calvert, L.C. Davies and K.R. Harrap, J. Med. Chem., 29 (1986) 1114.
138 J. Davoll and A.M. Johnson, unpublished results (1973). See Ref. 41 in Ref. 74.
139 J.B. Hynes, J.M. Buck, L. D'Souza and J.H. Freisheim, J. Med. Chem., 18 (1975) 1191.
140 J.B. Hynes and C.M. Garrett, J. Med. Chem., 18 (1975) 632.
141 K.H. Slotta and R. Kethur, Chem. Ber., 71B (1935) 335.
142 L.T. Plante, J. Org. Chem., 36 (1971) 860.
143 D.J. Fernandes, J.B. Hynes, R.M. Cardenas and J.R. Bertino, Drugs Future, 6 (1981) 545.
144 J.B. Hynes, Y.C.S. Yang, J.E. McGill, S.J. Harmon and W.L. Washtien, J. Med. Chem., 27 (1984) 232.
145 The use of CH_2O-$NaBH_3CN$ for reductive methylation of folic acid was described by C. Temple, Jr., L.L. Bennett, Jr., J.D. Rose, R.D. Elliott, J.A. Montgomery and J.H. Magnum, J. Med. Chem., 25 (1982) 161.
146 J.B. Hynes, A. Pathak, C.H. Panos and C.C. Okeke, 8th International Symposium on Pteridines and Folic Acid Derivatives: Chemical, Biological and Clinical Aspects (June 15–20, 1986) Montreal, Canada. We thank Dr. J.B. Hynes for providing us with a preprint of this work prior to publication.
147 J.H. Maguire and R.L. McKee, J. Heterocycl. Chem., 16 (1979) 133.
148 C. Marvel and E. Dregar, in Organic Syntheses, Coll. Vol. I, ed. H. Gilman (John Wiley & Sons, New York, 1941) p. 238.
149 S. Brownstein, J. Org. Chem., 23 (1958) 113.
150 H.N. Rydon and K. Undheim, J. Chem. Soc., (1962) 4689.
151 L.T. Weinstock, B.F. Grabowski and C.C. Cheng, J. Med. Chem., 13 (1970) 995.
152 W.T. Caldwell and C.-S. Cheng, J. Am. Chem. Soc., 77 (1955) 6631.
153 B.R. Baker and D.V. Santi, J. Heterocycl. Chem., 4 (1967) 216.
154 F.R. Benson, L.W. Hartzel and W.L. Sawell, J. Am. Chem. Soc., 72 (1950) 1816.
155 L.W. Hartzel and F.R. Benson, J. Am. Chem. Soc., 76 (1954) 2263.
156 The authors noted an earlier communication describing (290) and the analogous sulphonamide derivative; i.e., -SO_2GluH, which did not, however, describe the method of preparation: P.C. Edwards, D. Starling, A.M. Mattocks and H.E. Skipper, Science (Washington, DC), 107 (1948) 119.
157 B.R. Baker and C.E. Morreal, J. Pharm. Sci., 51 (1962) 596.
158 B.R. Baker and C.E. Morreal, J. Pharm. Sci., 52 (1963) 840.
159 G.L. Tong, W.Lee and L. Goodman, J. Am. Chem. Soc., 86 (1964) 5664.
160 B.R. Baker, T.J. Schwan and D.V. Santi, J. Med. Chem., 9 (1966) 66.
161 L.T. Weinstock, D.E. O'Brien and C.C. Cheng, J. Med. Chem., 11 (1968) 1238.
162 V.M. Berezovskii, G.D. Glebova, E.M. Birinberg and N.A. Andreeva, Khim. Farm. Zh., 1 (1967) 5; Chem. Abstr., 68 (1968) 49546a.
163 H. Tieckelmann, R. Guthrie and J.G. Nairn, J. Org. Chem., 25 (1960) 1257.
164 J. Davoll and A.M. Johnson, Br. Patent 1,182,518 (1970); Chem. Abstr., 72 (1970) 90871.
165 A.D. Broom and I.-Y. Yand, 8th International Symposium on Pteridines and Folic Acid Derivatives; Chemical, Biological and Clinical Aspects (June 15–20, 1986) Montreal, Canada. We thank Dr. A.D. Broom for providing us with a preprint of this work prior to publication.
166 F. Eckstein and K.H. Scheit, Angew. Chem., Int. Ed. Engl., 6 (1967) 362.
167 A. Franke, K.H. Scheit and F. Eckstein, Chem. Ber., 101 (1968) 2998.

168 L.M. Werbel, J. Hung, J.A. Besserer, T.J. Boritzki, W.R. Leopold and D.W. Fry, 8th International Symposium on Pteridines and Folic Acid Derivatives; Chemical, Biological and Clinical Aspects (June 15–20, 1986) Montreal, Canada. We thank Dr. L.M. Werbel for providing us with a preprint of this work prior to publication.
169 O.W. Lever, Jr. and B.R. Vestal, J. Heterocycl. Chem., 22 (1985) 5.
170 A.H. Cook, I. Heilbron and E. Smith, J. Chem. Soc., (1949) 1440.
171 E.C. Taylor and R.C. Portnoy, J. Org. Chem., 38 (1973) 806.
172 M.G. Nair, O.C. Salter, R.L. Kisliuk, Y. Gaumont and G. North, J. Med. Chem., 26 (1983) 1164.
173 This approach to 7,8-dihydro-8-oxapterins has been described by S.C. Lin, G.P. Holmes, D.L. Dunn and C.G. Skinner, J. Med. Chem., 22 (1979) 741.
174 M.G. Nair and P.T. Campbell, J. Med. Chem., 19 (1976) 825.
175 M.G. Nair, M.K. Rozmyslovicz, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, in Ref. 30, p. 121.
176 J.E. Oatis, Jr. and J.B. Hynes, J. Med. Chem., 20 (1977) 1393.
177 B.F. Gisin, Helv. Chim. Acta, 56 (1973) 1476.
178 L.M. Werbel, L. Newton and E.F. Elslager, J. Heterocycl. Chem., 17 (1980) 497.
179 R. Struck, Y.F. Shealy and J.A. Montgomery, J. Med. Chem., 14 (1971) 693.
180 E.C. Taylor, P.J. Harrington, S.R. Fletcher, G.P. Beardsley and R.G. Moran, J. Med. Chem., 28 (1985) 914.
181 E.C. Taylor and A. McKillop, The Chemistry of Cyclic Enaminonitriles and *o*-Aminonitriles (Interscience, New York, 1970).
182 S.J. Yan, T. Weinstock and C.C. Cheng, J. Heterocycl. Chem., 16 (1979) 541.
183 G.P. Beardsley, E.C. Taylor, C.J. Shih, G.A. Poore, G.B. Grindey and R.G. Moran, Proc. Am. Assoc. Cancer Res., (1986) 1027.
184 J.I. DeGraw, H. Tagawa, P.H. Christie, J.A. Lawson, E.G. Brown, R.L. Kisliuk and Y. Gaumont, J. Heterocycl. Chem., 23 (1986) 1.
185 J.I. DeGraw, V.H. Brown, H. Tagawa, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Med. Chem., 25 (1982) 1227.
186 For a description of the utility of this exchange/cyclization to synthesize other heterocyclic *o*-aminonitriles, see [40] and M. Lang and J.-P. Fleury, Tetrahedron Lett., (1974) 3967.
187 J.R. Piper and J.A. Montgomery, J. Med. Chem., 23 (1980) 320.
188 A.P. Krapcho and A.J. Lovey, Tetrahedron Lett., (1973) 957.
189 E.C. Taylor, G.S.K. Wong, S.R. Fletcher, P.J. Harington, C.J. Shih and G.P. Beardsley, 8th International Symposium on Pteridines and Folic Acid Derivatives: Chemical, Biological and Clinical Aspects (June 15–20, 1986) Montreal, Canada.
190a J.I. DeGraw, L.F. Kelly, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Heterocyclic Chem., 19 (1982) 1587.
190b J.I. DeGraw, L.F. Kelly, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, in Ref. 30, p. 458.
191a J.I. DeGraw, L.F. Kelly and F.M. Sirotnak, U.S. Patent 4,532,241 (1985); Chem. Abstr., 104 (1985) 149407.
191b J.I. DeGraw, L.F. Kelly and F.M. Sirotnak, U.S. Patent 4,460,591 (1984); Chem. Abstr., 101 (1984) 211170; report the synthesis of (427a–c) as part of a series of 10-alkyl analogues.
191c J.I. DeGraw, L.F. Kelly and F.M. Sirotnak, GB Patent 2,160,525A (1985).
192 J.I. DeGraw, P.H. Christie, E.G. Brown, L.F. Kelly, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Med. Chem., 27 (1984) 376.
193 E.C. Taylor and J.L. LaMattina, J. Org. Chem., 42 (1977) 1523.

194 D.C. Palmer and E.C. Taylor, unpublished results.
195 J.I. DeGraw, W.T. Colwell, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Med. Chem., 29 (1986) 1786.
196 S. Scheibye, B.S. Pedersen and S.O. Lawesson, Bull. Soc. Chem. Belg., 87 (1978) 229.
197 D.B. Cosulich and J.M. Smith, Jr., J. Am. Chem. Soc., 70 (1948) 1922.
198 M.E. Hultquist, J.M. Smith, Jr., D.R. Seeger, D.B. Cosulich and E. Kuh, J. Am. Chem. Soc., 71 (1949) 619.
199 B.J. Magerlein and D.I. Weisblat, J. Am. Chem. Soc., 76 (1954) 3033.
200 J.C. Parham, M.A. Templeton, P.L. Chello and F.M. Sirotnak, J. Heterocyclic Chem., 16 (1979) 1645.
201 J.R. Piper, G.S. McCaleb, J.A. Montgomery, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Med. Chem., 25 (1982) 877.
202 M.G. Nair and C.M. Baugh, J. Med. Chem., 17 (1974) 223.
203 M.G. Nair, L.P. Mercer and C.M. Baugh J. Med. Chem., 17 (1974) 1268.
204 J.I. DeGraw, P. Tsakotellis, R.L. Kisliuk and Y. Gaumont, J. Heterocyclic Chem., 8 (1971) 105.
205 J.I. DeGraw, V.H. Brown, R.L. Kisliuk and Y. Gaumont, J. Med. Chem., 14 (1971) 866.
206 J.I. DeGraw, R.L. Kisliuk, Y. Gaumont, C.M. Baugh and M.G. Nair, J. Med. Chem., 17 (1974) 552.
207 J.I. DeGraw, V.H. Brown, R.L. Kisliuk and F.M. Sirotnak, in Ref. 109a, p. 225.
208 J.R. Piper and J.A. Montgomery, U.S. Patent 4,172,200 (1979); Chem. Abstr., 92 (1979) 76557.
209 J.I. DeGraw and F.M. Sirotnak, U.S. Patent 4,639,319 (1983).
210 J.I. DeGraw, P.H. Christie, H. Tagawa, R.L. Kisliuk, Y. Gaumont, F.A. Schmid and F.M. Sirotnak, J. Med. Chem., 29 (1986) 1056.
211 M.G. Nair, J. Org. Chem., 50 (1985) 1879.
212 M.G. Nair, T.R. Toghiyani, B. Ramamurthy, R.L. Kisliuk and Y. Gaumont, 8th International Symposium on Pteridines and Folic Acid Derivatives; Chemical, Biological and Clinical Aspects (June 15–20, 1986) Montreal, Canada. We thank Dr. M.G. Nair for providing us with a preprint of this work prior to publication.
213 M.G. Nair, P.T. Campbell and C.M. Baugh, J. Org. Chem., 40 (1975) 1745.
214 P.F. Wiley, J. Org. Chem., 16 (1951) 810.
215 M.G. Nair, P.T. Campbell, E. Braverman and C.M. Baugh, Tetrahedron Lett., (1975) 2745.
216 H.G. Mautner and Y.-H. Kim, J. Org. Chem., 40 (1975) 3447.
217 E.C. Taylor and T. Kobayashi, J. Org. Chem., 38 (1973) 2817.
218 E.C. Taylor, K.L. Perlman, Y.-H. Kim, I.P. Sword and P.A. Jacobi, J. Am. Chem. Soc., 95 (1973) 6413.
219 L. Goodman, J. DeGraw, R.L. Kisliuk, M. Friedkin, E.J. Pastore, E.J. Crawford, L.T. Plante, A. Al-Nahas, J.F. Morningstar, Jr., G. Kwok, L. Wilson, E.F. Donovan and J. Ratzan, J. Am. Chem. Soc., 86, (1964) 308.
220 J.I. DeGraw, J.P. Marsh, Jr., E.M. Acton, O.P. Crews, C.W. Mosher, A.N. Fujiwara and L. Goodman, J. Org. Chem., 30 (1965) 3404.
221 Y.-H. Kim, V. Grubliauskas and O.M. Friedman, J. Heterocycl. Chem., 9 (1972) 481.
222 C.W. Mosher, E.M. Acton, O.P. Crews and L. Goodman, J. Org. Chem., 32 (1967) 1452.
223 M.G. Nair, S.-Y. Chen, R.L. Kisliuk, Y. Gaumont and D. Strumpf, J. Med. Chem., 22 (1979) 850.
224 M.G. Nair, S.-Y. Chen, R.L. Kisliuk, Y. Gaumont and D. Strumpf, J. Med. Chem., 23 (1980) 899.

225 S.-Y. Chen and M.G. Nair, J. Org. Chem., 43 (1978) 4143.
226 M.G. Nair, C. Saunders, S.-Y. Chen, R.L. Kisliuk and Y. Gaumont, J. Med. Chem., 23 (1980) 59.
227 M.G. Nair, T.W. Bridges, T.J. Henkel, R.L. Kisliuk, Y. Gaumont and F.M. Sirotnak, J. Med. Chem., 24 (1981) 1068.
228 M.G. Nair, P.C. O'Neal, C.M. Baugh, R.L. Kisliuk, Y. Gaumont and M. Rodman, J. Med. Chem., 21 (1978) 673.
229 M.G. Nair, E.B. Otis, R.L. Kisliuk and Y. Gaumont, J. Med. Chem., 26 (1983) 135.
230 K. Slavik, V. Slavokova, K. Montyoka, E. Hermanova, J. Soucek, Z. Tomsova, M. Spundova and E. Novakova, Mol. Pharmacol., 5 (1960) 137.
231 D.B. Cosulich, D.R. Seeger, M.J. Fahrenbach, B. Roth, J.H. Mowat, J.M. Smith, Jr. and M.E. Hultquist, J. Am. Chem. Soc., 73 (1951) 2554.
232 D.B. Cosulich, D.R. Seeger, M.J. Fahrenbach, K.H. Collins, B. Roth, M.E. Hultquist and J.M. Smith, Jr., J. Am. Chem. Soc., 75 (1953) 4675.
233 R.B. Angier and W.V. Curran, J. Am. Chem. Soc., 81 (1959) 2814.
234 T.L. Loo, R.L. Dion, R.H. Adamson, M.A. Chirigos and R.L. Kisliuk, J. Med. Chem., 9 (1965) 713.
235 A. Rosowsky and K.K.N. Chen, J. Med. Chem., 17 (1974) 1308.
236 W.W. Lee, A.P. Martinez and L. Goodman, J. Med. Chem., 17 (1974) 326.
237 J.E. Martinelli and M. Chaykovsky, J. Org. Chem., 45 (1980) 527.
238 A.S. Tomcufcik and D.R. Seeger, J. Org. Chem., 29 (1961) 3351.
239 J. Henkin and W.L. Washtien, J. Med. Chem., 26 (1983) 1193.
240 H.J. Backer and A.C. Hautman, Rec. Trav. Chim., 70 (1951) 738.
241 E.C. Roberts and Y.F. Shealy, J. Med. Chem., 17 (1974) 219.
242 P.F. Holmes, J.G. Liehr and J. Henkin, Bioorg. Chem., 11 (1982) 281.
243 E.C. Roberts and Y.F. Shealy, J. Med. Chem., 14 (1971) 125.
244 E.C. Roberts and Y.F. Shealy, J. Med. Chem., 15 (1972) 1310.
245 E.C. Roberts and Y.F. Shealy, J. Heterocycl. Chem., 11 (1974) 547.
246 Y. Nakahara, I. Sekikawa and S. Kakimoto, J. Heterocycl. Chem., 12 (1975) 1073.
247 S.L. Gurina, Z.V. Pushkareva, N.V. Volkova, N.A. Andreeva and T.E. Zubova, Bioorg. Khim., 3 (1977) 1133; Chem. Abstr., 87 (1977) 184936k.
248 J.R. Piper and J.A. Montgomery, U.S. Patent 4,079,056 (1978).
249 I. Sekikawa and Y. Takakoshi, J. Heterocycl. Chem., 20 (1983) 807.
250 T. Sato and H. Wakatsuka, Bull. Chem. Soc. Japan, 42 (1969) 1955.
251 J.R. Piper and J.A. Montgomery, J. Heterocycl. Chem., 11 (1974) 279.
252 E.C. Roberts and Y.F. Shealy, J. Med. Chem., 16 (1973) 697.
253 A. Rosowsky and R. Forsch, J. Med. Chem., 25 (1982) 1454.
254 J.E. Martinelli, M. Chaykovsky, R.L. Kisliuk and Y. Gaumont, J. Med. Chem., 22 (1979) 874.
255 J.E. Martinelli, M. Chaykovsky, R.L. Kisliuk, Y. Gaumont and M.C. Gittelman, J. Med. Chem., 22 (1979) 869.
256 H.S. Forrest and J. Walker, J. Chem. Soc., (1949) 2002.
257 D. Farquhar, T.L. Loo and S. Vadlamudi, J. Med. Chem., 15 (1972) 567.
258 M. Chaykovsky, J. Org. Chem., 40 (1975) 145.
259 E.C. Taylor, J.S. Skotnicki and S.R. Fletcher, J. Org. Chem., 50 (1985) 1005.
260 C.W. Waller, M.J. Fahrenbach, J.H. Boothe, R.B. Angier, B.L. Hutchings, J.H. Mowat, J.F. Poletto and J. Semb, J. Am. Chem. Soc., 74 (1952) 5405.
261 E.C. Taylor, R.N. Henrie and D.J. Dumas, in Ref. 109a, p. 71.

262 D.C. Suster, L.V. Feyns, G. Ciustea, G. Botez, V. Dobre, R. Bick and I. Niculescu-Suvaz, J. Med. Chem., 17 (1974) 758.
263 K.-H. Kim and H.G. Mautner, Abstr. 172nd ACS National Meeting, San Francisco, CA (1976) MEDI 65.
264 B. Roth, J.M. Smith and M.E. Hultquist, J. Am. Chem. Soc., 72 (1950) 1914.
265 C. Temple, C.L. Kussner and J.A. Montgomery, J. Heterocycl. Chem., 14 (1977) 885.
266 R.D. Elliott, C. Temple, J.L. Frye and J.A. Montgomery, J. Heterocycl. Chem., 10 (1973) 1071.
267 R.D. Elliott, C. Temple, J.L. Frye and J.A. Montgomery, J. Med. Chem., 18 (1975) 492.
268 R.L. Kisliuk, Nature (London), 138 (1960) 584.
269 R.L. Kisliuk and M.D. Levine, J. Biol. Chem., 239 (1964) 1900.
270 S.B. Horwitz and R.L. Kisliuk, J. Med. Chem., 11 (1968) 907.
271 R.L. Kisliuk, D. Strumpf, Y. Gaumont, R.P. Leary and L. Plante, J. Med. Chem., 20 (1977) 1531.
272 C. Temple, Jr., R.D. Elliott, J.D. Rose and J.A. Montgomery, in Ref. 109a, p. 279.
273 D.I. Brixner and A.D. Broom, Abstr. 193rd ACS National Meeting, Denver, CO (1987) ORGN 60. We are grateful to Prof. Broom for a copy of the full manuscript prior to publication.
274 G.W. Gribble and P.W. Heald, Synthesis (1975) 650.
275 M.G. Nair, N.T. Nanarati, I.G. Nair, R.L. Kisliuk, Y. Gaumont, M.C. Hsiao and T.I. Kalman, J. Med. Chem., 29 (1986) 1754.
276 C.A. Caperelli and J. Conigliaro, J. Med. Chem., 29 (1986) 2117.
277 R.M. Slusher, I.-Y. Yang and A.D. Broom, Abstr. 193rd ACS National Meeting, Denver, CO (1987) ORGN 293. We are grateful to Prof. Broom for a copy of the full manuscript prior to publication.
278 D.B. Ross and J.C. Martin, J. Org. Chem., 48 (1983) 4155.
279 C.A. Samantham and A.D. Broom, Abstr. 193rd ACS National Meeting, Denver, CO (1987) ORGN 292. We are grateful to Prof. Broom for a copy of the full manuscript prior to publication.
280 The authors noted that an improved synthesis of (164) has been developed in their laboratories.
281 K.W. Ledig, U.S. Patent 4,118,561 (1978); Chem. Abstr., 90 (1979) 137852.
282 This approach to 2,4-diaminoquinazolines was described by A. Rosowsky and N. Papathanasopoulos, J. Org. Chem., 39 (1974) 3293 and by A. Rosowsky and E.J. Modest, J. Heterocycl. Chem., 3 (1966) 387.
283 D.I. Brixner, T. Ueda, Y.-C. Cheng, J.B. Hynes and A.D. Broom, J. Med. Chem., 30 (1987) 675.
284 M. Ghazala, M.G. Nair, T.R. Toghiyani, R.L. Kisliuk, Y. Gaumont and T.I. Kalman, J. Med. Chem., 29 (1986) 1263.
285 J.R. Piper, G.S. McCaleb and J.A. Montgomery, J. Heterocycl. Chem., 24 (1987) 279.
286 J.I. DeGraw, W.T. Colwell, V.H. Brown, M. Sato, R.L. Kisliuk, Y. Gaumont, J. Thorndike and F.M. Sirotnak, J. Med. Chem., 31 (1988) 150.
287 T.R. Jones, A.L. Jackman and D.R. Newell, Eur. Pat. Appl. EP 204, 529 (1986).
288 M.G. Nair, R. Dhawan, M. Ghazala, T.I. Kalman, R. Ferone, Y. Gaumont and R.L. Kisliuk, J. Med. Chem., 30 (1987) 1256.
289 J.B. Hynes, A. Kumar, A. Tomažič and W.L. Washtien, J. Med. Chem., 30 (1987) 1515.
290 M.S. Shengeliya, V.G. Arramenko, L.N. Kuleshova, Yu. A. Ershova, V.A. Chernov and N.N. Surorov, Khim. Farm. Zh., 20 (1986) 932.
291 L.K.A. Rahman and S.R. Chhabra, Med. Res. Rev., 8 (1988) 95.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry – Vol. 25, edited by G.P. Ellis and G.B. West

4 New Benzimidazole Carbamates as Antifilarial Agents*

SIYA RAM, Ph.D.

P.E.T. Facility, Nuclear Medicine Division, Department of Radiology, Duke University Medical Center, Durham, NC 27710, U.S.A.

INTRODUCTION

Despite much research and development in parasitology in the tropical countries, parasitic infections are still a major health hazard for humans and animals. For example, it has been estimated that approximately three hundred million people in the world are affected by filariasis, a disease caused by nematodes filarioidea. While this is only one type of parasitic disease, it is clearly one of the most important concerns for public health in a large geographic region. In this review, I discuss an approach to control filariasis by the use of a new class of pharmacological agents, benzimidazoles. There are

* Dedicated to the memory of my father, Sri Ram Sahai.

six species of human filariasis for which it is desirable to develop antifilarial agents: *Wuchereria bancrofti*, *Brugia malayi*, and *Onchocerca volvulus* are major human pathogens, responsible for practically all filaria-related deaths. *Mansonella ozzardi*, *Dipetalonema perstans* and Loa Loa are less prevalent species. The first three of these parasites are characterized below.

W. bancrofti and *B. malayi.* Both of these species affect mainly the lymphatic systems or connective tissues, causing elephantiasis or hydrocele. Geographically, *W. bancrofti* is found in Central Africa, Southeast Asia, Central and South America, while *B. malayi* is more prevalent in Southeast Asia. The microfilariae of both of these species are detectable at night and circulate in the peripheral blood.

O. volvulus. This is the causal organism of human onchocerciasis, which is a disease of the skin and eyes. This disease affects between 20 and 40 million people in the world, frequently causing blindness and skin pathology. The tropical zones most affected are Africa, Guatemala and Mexico. A mild form of the disease threatens to spread in tropical South America. The microfilariae circulate in the peripheral blood day and night. Many mosquitoes, such as *Culex*, *Anopheles*, *Mansonia*, *Aedes*, and *Simulium* Spp., are vectors for this and other types of filariasis.

MECHANISM OF ACTION OF BENZIMIDAZOLES

Benzimidazoles have been identified as antifilarial agents [1–5] based on an extensive series of studies in jirds (a kind of gerbil) and cats. The mechanism of action of benzimidazoles has been extensively studied in many laboratories and, in general, three possible modes of action have been suggested for these drugs.

(a) Inhibition of microtubular functions

It has been demonstrated by numerous workers that the majority of benzimidazoles inhibit the microtubulin [6–11] of tegumental or intestinal cells of nematodes or cestodes through degeneration of these cells. This mechanism is now well accepted as the mode of action for benzimidazole derivatives.

(b) Glucose transport inhibition

Vanden Bossche and de Nollin and others [12–14] have shown that benzimidazole carbamates, such as mebendazole, inhibit glucose uptake by helminths *in vivo* and *in vitro*; this enhances endogenous glycogen utilization and is accompanied by reduction in glycogenesis.

(c) Inhibition of fumarate reductase

Prichard [15] has shown that thiabendazole inhibits the NADH oxidation in the presence of fumarate in *Haemonchus contortus* homogenates, which are sensitive to benzimidazole derivatives. Later studies, however, in benzimidazole-resistant *H. contortus* [16, 17] provide contrary evidence.

PHARMACOLOGICAL EVALUATION OF ANTIFILARIAL ACTIVITY

In general, the following *in vivo* models are used for screening of antifilarial activity of new compounds.

Litomosoides carinii *of cotton rats*, Sigmodon hispidus

The *L. carinii* model is routinely used for primary screening and provides a reliable indication of the *in vivo* action of a compound. It was first used by Culbertson and Rose [18] for antifilarial screening. This parasitic infection is generally transmitted [19–21] in 3 weeks by the rat mites *Liponsyssus bacoti* and requires 8 more weeks to mature in the next animal until microfilariae appear in the blood. The adult filarial worms are found in the pleural cavity.

B. pahangi

This model was developed by Suswillo and Denham [22–24] for evaluation of filaricidal activity, in which jirds are infected by intraperitoneal implantation of ten female and five male *B. pahangi*. After 3 days, the compounds are administered either orally or by subcutaneous injection. The intraperitoneal injection of drugs may provide false positive results because some compounds remain concentrated at this site. This model is suitable for secondary screening of new drugs for micro- and macrofilaricidal activity.

D. viteae *in jirds*

The transmission of this infection has been described by Worms, Terry and Terry [25]. However, this model is unsuitable for routine screening of new drugs because microfilariae in blood are few and inconsistent. The adult worms are found under the skin and are difficult to observe if they have been killed by a drug. Diethylcarbamazine is ineffective on this parasite, even though it is the most effective compound for human filariasis.

Dirofilaria immitis

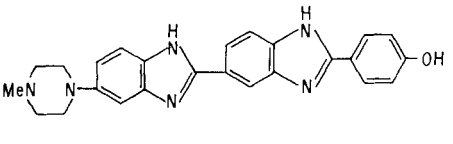
This model may be useful for secondary screening of new compounds. Otto and Maren [26] studied arsenicals in the treatment of this infection in dogs.

Experimental procedure

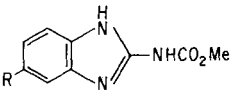
For evaluation of the antifilarial activity of a drug, a solution or suspension of the drug (in aqueous 1% (hydroxyethyl)cellulose and 0.1% Tween-80) is administered either intraperitoneally or subcutaneously to a group of three or four preinfected jirds [22, 27, 28]. The microfilariae are counted in blood drawn from the retro-ocular sinus [29] on the first day of dosing (day 0), and on day 4, 5 or 6 and at necropsy. After 55–70 days of the first dose of a compound, the surviving animals are killed and adult worms are counted in the pleural and peritoneal cavities. The number of surviving worms at autopsy is scored as a percentage relative to controls. The compounds are considered to be active if reduction of adult worm populations is greater than 60% or when the reduction of circulation *L. carinii* microfilariae exceeds 90%.

BENZIMIDAZOLE DERIVATIVES AS ANTIFILARIAL AGENTS

In 1971, the first benzimidazole derivative (1, HOE 33258) was reported to be an active microfilaricidal agent in *L. carinii* and *D. immitis* models [30]. After this report, very little effort was dedicated to this important problem for a decade. Recently, however, there has been renewed interest and activity which has shown some benzimidazole carbamates such as mebendazole (2), flubendazole (3), albendazole (4) and oxibendazole (5) to have significant macrofilari-



(1) HOE 33258



(2) R = PhCO	Mebendazole
(3) R = 4-FC_6H_4CO	Flubendazole
(4) R = SPr	Albendazole
(5) R = OPr	Oxibendazole

cidal activity against *B. pahangi* [1–3, 5] and *D. viteae* [4] in jirds and cats when the compounds were administered subcutaneously. Mebendazole was also found to be active against microfilariae of *D. perstans* and macrofilariae of *B. malayi* in humans [31, 58]. In the treatment of onchocerciasis in humans, flubendazole (3) was shown to be a safer and more effective drug than diethylcarbamazine (DEC) [3]. Nevertheless, poor water solubility and poor plasma and tissue absorption have limited the oral use of these agents in the treatment of filarial infections. With this background in mind, a programme to develop new efficacious antifilarial agents by systematic molecular modification of mebendazole (2) and flubendazole (3) was initiated by Townsend and coworkers at the University of Michigan.

MODIFICATION OF MEBENDAZOLE AND FLUBENDAZOLE

To explore further the structure–activity relationships of mebendazole (2) and flubendazole (3), four possible sites were chosen: (a) modification of the 2-methyl carbamate group (A); (b) modification of the 5-benzoyl group (B); (c) modification of keto group (C); and (d) modification of benzene ring (D) of the benzimidazole (*Figure 4.1*). In the process, a new class of micro- and macrofilaricidals was generated and the results of this innovative research along with reports from other laboratories are described here.

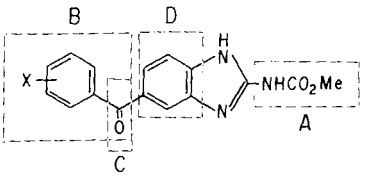


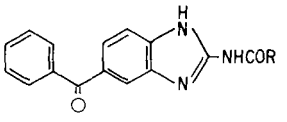
Figure 4.1. Potential sites of modification of the mebendazole and flubendazole.

MODIFICATION OF THE 2-METHYLCARBAMATE GROUP

In order to examine the antifilarial activity profiles of benzimidazole-2-carbamates by substitution of their methyl carbamate group by other functional groups such as alkyl or aryl ureas, a series of benzimidolyl ureas [32] were synthesized. Structure–activity relationship studies in this series revealed decreases in antifilarial activity against *B. pahangi* and *L. carinii* in jirds. However, 1-(5-benzoylbenzimidazol-2-yl)-3,3-dimethylurea (6) demonstrated 100% macrofilaricidal activity against both *B. pahangi* and *L. carinii* at 100 mg/kg when administered subcutaneously and was 87% microfilaricidal at

25 mg/kg against *L. carinii* at the time of necropsy. The 1-(5-benzoylbenzimidazol-2-yl)-3-arylureas (7, 8) exhibited marginal macrofilaricidal activity at 100 mg/kg against both *B. pahangi* and *L. carinii*, when injected subcutaneously. This decrease in antifilarial activity may be due to poor water solubility of ureas.

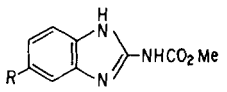
In another approach, where the 2-methyl carbamate moiety was replaced by a more lipophilic group such as SCO_2R, $SCONR^1R^2$ or $S(CH_2)_nCO_2R$ etc. [33]; more soluble compounds were generated. Surprisingly, the resulting compounds exhibited a complete loss of antifilarial activity. It is evident from these studies that the carbamate moiety is an essential pharmacophore for antifilarial activity.



(6) R = NMe_2
(7) R = PhNH
(8) R = 4-FC_6H_4NH

MODIFICATION OF THE 5-BENZOYL GROUP

Insertion of an NH group between the benzoyl group and benzimidazole moiety produced compounds (9, 10) [34]. These compounds were 100% effective against adult *B. pahangi* at 100 mg/kg subcutaneously; however, on reducing the dose to 25 mg/kg, compounds (9) and (10) were effective at only 86% and 60%, respectively. The decrease in antifilarial activity at lower dose demonstrates that these target compounds presumably possess poor water solubility and poor tissue distribution. In another approach, the NH group was incorporated between the phenyl group and keto function of mebendazole (2) and flubendazole (3). The resulting compounds (11, 12) [34] demonstrated significantly lower antifilarial activity than the parent compounds; this also may be due to poor solubility of these amide derivatives and further illustrates that



(9) R = 2-FC_6H_4CONH
(10) R = 4-FC_6H_4CONH
(11) R = 4-FC_6H_4NHCO
(12) R = 3-$CF_3C_6H_4NHCO$

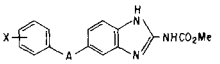
increasing the distance between either the 5-benzoyl and benzimidazole moiety or the phenyl and 5-carbonyl benzimidazole moiety is undesirable for macrofilaricidal activity.

MODIFICATION OF THE KETO GROUP

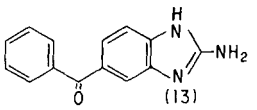
In this modification, the mebendazole (2) and flubendazole (3) metabolites, 2-amino-5-benzoylbenzimidazole (13) [32], methyl [5-α-hydroxyphenylmethylbenzimidazol-2-yl]carbamate (14) and methyl [5-α-hydroxy-4′-fluorophenylmethylbenzimidazol-2-yl]carbamate (16) were prepared [35] by reduction of the ketone functionality. Although, these compounds had been isolated and characterized in metabolism studies, they had not been evaluated for antifilarial activity. In addition to the partially reduced hydroxymethyl derivative (14), the fully reduced methyl (5-benzylbenzimidazol-2-yl)carbamate (15) was also prepared. It was of considerable interest that the compounds (14–16) significantly retained the macrofilaricidal activity profile (*Table 4.1*). On the other hand, metabolite (13) did not demonstrate any antifilarial activity, again indicating that the carbamate moiety is required for antifilarial activity. It was also interesting to note that the ketone-reduced derivatives (14–16) were considerably more soluble in water and ethanol than the parent drugs, mebendazole (2) or flubendazole (3).

It is understood from the activity profile analysis of metabolites (14–16) that the carbonyl moiety of mebendazole (2) and of flubendazole (3) is not absolutely necessary for potent macrofilaricidal activity. The further replacement of the α-hydroxy group by various other groups such as α-methoxy, α-ethoxy, resulted in a new class of benzimidazole-2-carbamates (17, 18) [35] which possess significant antifilarial activity and increased oral activity (*Table 4.1*). This presumably is a result of the compounds being more bioavailable *in vivo*. In other words, these compounds probably have better tissue absorption and water solubility than the parent drugs and this result also indicates that some level of steric hindrance is tolerable at the hydroxy position. It should be noted that these compounds (14, 16–18) have an asymmetric centre, and antifilarial activity data are obtained from a (±)-mixture of the compound, i.e., no attempts were made to separate the (+)- and (−)-isomers. The transposition of 2- and 5-positional groups of the above potent macrofilaricidal agents afforded methyl (2-α-substituted benzylbenzimidazol-2-yl)carbamates (19 and 20) [36]. Unfortunately, none of the compounds in this series has demonstrated any significant antifilarial activity, which further proves that the 2-carbamate moiety is an essential pharmacophore for antifilarial activity. Besides the above modification of the α-hydroxy group to α-alkoxy derivatives,

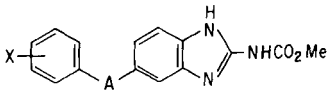
Table 4.1. ANTIFILARIAL ACTIVITY OF MEBENDAZOLE, FLUBENDAZOLE AND NEW CLASS OF BENZIMIDAZOLE CARBAMATES



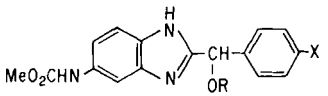
A	*X*	*Dosage (route) (mg/kg)*	*Antifilarial activity (% reduction of adult worms)* B. pahangi		L. carinii
CO (mebendazole)	H	6.25 × 5d (SC)	100		100
		1.56 × 5d (SC)	12.5		97
		100 × 5d (OR)	12		72
		25 × 5d (SC)	*D. viteae*	100	
		6.25 × 5d (SC)	*D. viteae*	78	
CO (flubendazole)	F	6.25 × 5d (SC)	100		100
		1.56 × 5d (SC)	100		100
		100 × 5d (OR)	0		84
		25 × 5d (SC)	*D. viteae*	100	
CHOH	H	25 × 5d (SC)	100		100
		6.25 × 5d (SC)	63		87
		25 × 5d (OR)			74
		100 × 1d (SC)	91		100
		25 × 1d (SC)	59		95
		100 × 5d (SC)	*D. viteae*	100	
CHOH	F	25 × 5d (SC)	*D. viteae*	95	
		25 × 5d (SC)	100		100
CH_2	H	100 × 5d (SC)	100		100
		100 × 5d (OR)	52		
CHOMe	H	25 × 5d (SC)	100		100
		6.25 × 5d (SC)	100		
		25 × 5d (SC)	*D. viteae*	92	
CHOEt	H	25 × 5d (SC)	100		100
		12.5 × 5d (SC)	50		
		50 × 5d (OR)	47		
		12.5 × 5d (SC)	*D. viteae*	100	



(13)



(14) X = H A = CHOH
(15) X = H A = CH_2
(16) X = F A = CHOH
(17) X = H A = CHOMe
(18) X = H A = CHOEt



(19) X = H or F R = H
(20) X = H or F R = Me or Et

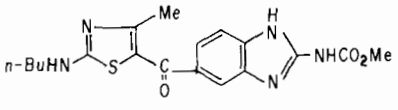
the replacement of the α-hydroxy group by other nucleophilic groups and other general keto group transformations were also studied. The resulting compounds exhibited potent macrofilaricidal activity with an increase in oral activity [35]. Hopefully, this novel class of compounds will provide a clinical candidate for the treatment of onchocerciasis.

MODIFICATION OF THE BENZENE RING OF THE BENZIMIDAZOLE RING

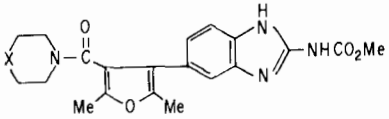
A replacement of the benzene ring of benzimidazole by heterocyclic rings such as pyrimidine, pyrazine, etc., was also investigated, since it was postulated that this molecular modification would perhaps provide more bioavailability and tighter binding of the molecule at the active site and thus lead to a more potent antifilarial compound. Unfortunately, in a series of purine-8-carbamates [37] designed as congeners of albendazole (4), all of these compounds were found to be only marginally active against both *B. pahangi* and *L. carinii*.

Recently a new benzimidazole-2-carbamate (21, CGI 13866) developed by Ciba-Geigy Pharmaceutical Company, has been reported which shows 49% macrofilaricidal activity when given orally and 100% microfilaricidal against *B. malayi* infection in monkeys [38]. A few benzimidazole-2-carbamates (22, 23) have been also reported in the literature [39–41] as micro- and macro-

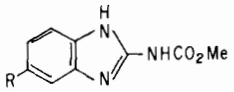
filaricidal against *L. carinii* in cotton rats at 30 mg/kg, when administered intraperitoneally for 5 days. Chauhan and Bhakuni [42] synthesized 2-methoxycarbonylaminothioxantheno[2,3-*d*]imidazol-10-one 5,5-dioxide (24) as a rigid analogue of fenbendazole sulphone (25), and mebendazole (2). However, this compound has been found to be inactive against both macro- and microfilarial worms of *L. carinii* at 30 mg/kg for 5 days.

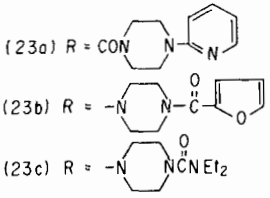


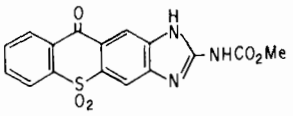
(21)



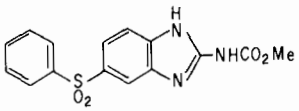
(22a) X = O
(22b) X = NMe







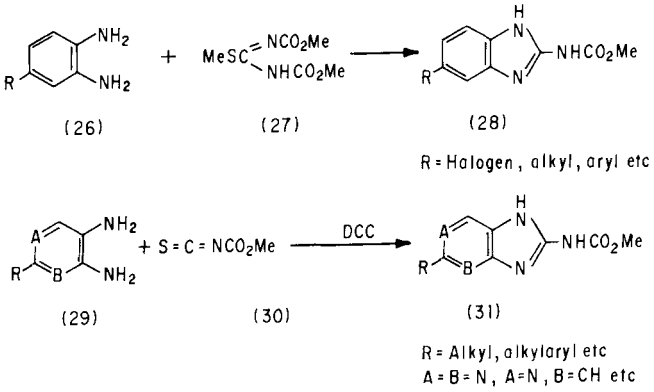
(24)



(25)

SYNTHESIS OF BENZIMIDAZOLE-2-CARBAMATES

In general, benzimidazole-2-methyl carbamate derivatives (28) can be synthesized by the reaction of an appropriate diamine (26) with 1,3-dicarbomethoxy-*S*-methylisothiourea (27) [43]. This is the most general and economic synthesis for benzimidazole carbamates. However, the same reaction was less than satisfactory when the aza analogues of *o*-phenylenediamines (29) were used. In order to synthesize the aza analogues of mebendazole (2) or flubendazole (3), the reactions of methoxycarbonyl isothiocyanate (30) with a wide variety of diamines such as 3,4-diaminopyridines, 4,5-diaminopyrimidines, *o*-phenylenediamines have been investigated in the presence of dicyclohexylcarbodiimide [44, 45] and were found to be quite successful in the synthesis of carbamates (31).



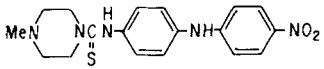
MISCELLANEOUS ANTIFILARIAL COMPOUNDS

During the last four decades, microfilaricidal diethylcarbamazine [46] and macrofilaricidal suramin [47, 48] have been used for the treatment of filariasis; however, both of these drugs possess unwanted side-effects such as mazzotti reaction, toxicity, etc. Suramin is highly active against the adult worm of *O. volvulus* [47] in humans, but it is very toxic to the kidney, liver and bone marrow and has other side-effects similar to DEC. DEC in the treatment of onchocerciasis produces severe mazzotti reaction (an allergic response due to rapid death of microfilariae) along with other side-effects such as pruritus and anaphylactic shock. However, the mass treatment of lymphatic filariasis with DEC was successful due to a lower and milder incidence of these adverse

effects. Recently, a review described the use of DEC and its congeners as filaricides [49]. Several analogues of suramin have been prepared [50]. The structure-activity relationship studies revealed that a symmetrical molecular structure is not an essential requirement for antifilarial activity. However, small structural modifications have a marked influence on the antifilarial activity.

Ivermectin possesses a broad spectrum of antiparasitic activities [51] and is a 22,23-dihydro analogue of the macrocyclic lactone, avermectin B_1. Ivermectin is highly effective against microfilariae of *O. volvulus* at very low doses (50–200 μg/kg) [52] and in comparison with DEC, induces only mild mazzotti reactions and other side-effects. Its effectiveness against *D. viteae* in *M. natalenis* [38] and *D. immitis* [53] is also documented.

A thiourea derivative CGP 6140 (32) [54], developed by Ciba-Geigy represents a new class of compound, which shows significant macrofilaricidal activity at 50–100 mg/kg in a variety of animal models. The further extension of this work generated two more new compounds, benzothiazole and benzoxazole derivatives (CGP 20376 and CGP 24914) which both possess potent micro- and macrofilaricidal activities [55]. However, CGP 20376 has also been found orally effective against *Brugia* infections at a dose of 6.25 mg/kg [38]. Further studies with these compounds in the treatment of onchocerciasis and other filarial infections are currently under evaluation.



(32) CGP 6140

The *N*-(5-nitro-2-furfurylidene)aminotetrahydro-2-(1*H*)-pyrimidone developed in China has exhibited both macro- and microfilaricidal activity in humans against lymphatic filariasis [56, 57]. Besides these compounds, various other drugs such as amodiaquine, primaquine, furazolidone and metrifonate, also have been tested as filaricidal in humans and were found to be ineffective [58]; however, levamisole [38, 59] has demonstrated some micro- and macrofilaricidal effects in humans at 100 mg/kg, but it is less effective than DEC and produces more severe side-effects. The mode of action of several antifilarial drugs has also been investigated and reviewed by Rew and Fetterer [60] and Subrahmanyam [38].

CONCLUSION

The present review demonstrates the extensive applicability of benzimidazole-2-carbamates in the treatment of filariasis. The generation of a new class of

5-α-substituted arylmethyl benzimidazole carbamates further increases the prospect that this direction will lead to a clinical candidate which would be orally active and more efficacious against the adult filarial worm than present agents.

ACKNOWLEDGEMENTS

I am grateful to Professors Leroy B. Townsend and Leonard D. Spicer for their criticism of the manuscript, and I also gratefully acknowledge the contributions by my associates at the University of Michigan, Ann Arbor, U.S.A., whose names appear in references. I thank Ms. Pricilla Torain for her efforts in preparing this manuscript. Support from the Department of Radiology, Duke University Medical Center is gratefully acknowledged.

REFERENCES

1 D.A. Denham, R.R. Suswillo and R. Rogers, Trans. R. Soc. Trop. Med. Hyg., 72 (1978) 546.
2 D.A. Denham, R. Samad, S.-Y. Cho, R.R. Suswillo and S.C. Skippins, Trans. R. Soc. Trop. Med. Hyg., 73 (1979) 673.
3 A. Dominguez-Vazquez, H.R. Taylor, A.M. Ruvalcaba-Macias, R.P. Murphy, B.M. Greene, A.R. Rivas-Alcala and F. Beltran-Hernandez, Lancet i (1983) 139.
4 D.A. Denham, Trans. R. Soc. Trop. Med. Hyg., 74 (1980) 829.
5 A.B. Reddy, U.R. Rao, R. Chandrashekar, R. Shrivastava and D. Subrahmanyam, Tropenmed. Parasitol., 34 (1983) 259.
6 M. Borgers, S. De Nollin, M. De Brabander and D. Thienpont, Am. J. Vet. Res., 36 (1975) 1153.
7 P. Kohler and R. Bachmann, Mol. Biochem. Parasitol., 4 (1981) 325.
8 C.M. Ireland, K. Gull, W.E. Gutteridge and C.I. Pogson, Biochem. Pharmacol., 28 (1979) 2680.
9 A. Verheyen, M. Borgers, D. Vanparijs and D. Thienpont, in Biochemistry of Parasites and Host-parasite Relationships, ed. H. Vanden Bossche (Elsevier, Amsterdam, 1976) pp. 605–618.
10 P.A. Friedman and E.G. Platzer, in The Host Invader Interplay, ed. H. Vanden Bossche (Elsevier/North-Holland Biomedical Press, Amsterdam, 1980) pp. 595–604.
11 K. Gull, P.J. Dawson, C. Davis and E.H. Byard, Biochem. Soc. Trans., 15 (1987) 59.
12 H. Vanden Bossche and S. De Nollin, Int. J. Parasitol., 3 (1973) 401.
13 H. Vanden Bossche, The Molecular Basis of Anthelmintic Action (Elsevier, Amsterdam, 1976) pp. 533–572.
14 M.S. Rahman and C. Bryant, Int. J. Parasitol., 7 (1977) 403.
15 R.K. Prichard, Nature (London) 228 (1970) 684.
16 R.K. Prichard, Int. J. Parasitol., 3 (1973) 409.

17 R.D. Romanowski, M.L. Rhoads, M.L. Colglazier and K.C. Kates, J. Parasitol., 61 (1975) 777.
18 J.T. Culbertson and H.M. Rose, J. Pharmacol. Exp. Ther., 81 (1944) 189.
19 J.A. Scott and J.B. Cross, Am. J. Trop. Med., 26 (1946) 849.
20 R.W. Williams and H.W. Brown, Science (Washington, DC), 102 (1945) 482 and 103 (1946) 224.
21 F. Hawking and P. Sewell, Br. J. Pharmacol., 3 (1948), 285.
22 R.R. Suswillo and D.A. Denham, J. Parasitol., 63 (1977) 591.
23 D.A. Denham, in Animal Models in Parasitology, ed. D. Owen (Macmillan, London, 1982) pp. 93–104.
24 D.A. Denham, E. Brandt and D.A. Liron, J. Parasitol., 67 (1981) 123.
25 M.J. Worms, R.J. Terry and A. Terry, J. Parasitol. 47 (1961) 963.
26 G.F. Otto and T.H. Maren, Science (Washington, DC), 106 (1947) 105; Am. J. Hyg., 50 (1949) 92.
27 J.W. McCall and H.H. Crounthamel, J. Parasitol., 62 (1976) 844.
28 J.W. McCall, J. Parasitol., 62 (1976) 585.
29 P.E. Thompson, L. Boche and L.S. Blair, J. Parasitol., 54 (1968) 834.
30 W. Raether and G. Lammler, Ann. Trop. Med. Parasitol., 65 (1971) 107.
31 K. Maertens and M. Wery, Trans. R. Soc. Trop. Med. Hyg., 69 (1975) 359.
32 S. Ram, M. Skinner, D. Kalvin, D.S. Wise, L.B. Townsend, J.W. McCall, D. Worth, D. Ortwine and L.M. Werbel, J. Med. Chem., 27 (1984) 914.
33 S. Ram, D.S. Wise and L.B. Townsend, J. Heterocycl. Chem., 22 (1985) 1269.
34 S. Ram, D.S. Wise, L.B. Townsend and L.L. Wotring, J. Med. Chem. (in preparation).
35 S. Ram, D.S. Wise and L.B. Townsend, U.S. Pat. filed November 6, 1983, Ser. No. 529765 (accepted).
36 S. Ram, D.S. Wise and L.B. Townsend, J. Heterocycl. Chem., 23 (1986) 1109.
37 S. Ram, W. Evans, D.S. Wise, L.B. Townsend and J.W. McCall, J. Med. Chem. (in preparation).
38 D. Subrahmanyam, in Filariasis Ciba Foundation Symposium 127 (Wiley, Chichester, 1987) p. 246.
39 S. Niwas, M.S. Akhtar, S. Kumar and A.P. Bharduri, Indian J. Chem., 24 (1985) 754.
40 S. Kumar, M. Seth, A.P. Bharduri, P.K.S. Visen, A. Misra, S. Gupta, N. Fatima, J.C. Katiyar, R.K. Chatterjee and A.B. Sen, J. Med. Chem., 27 (1984) 1083.
41 R. Dubey, S. Abuzar, S. Sharma, R.K. Chatterjee and J.C. Katiyar, J. Med. Chem., 28 (1985) 1748.
42 P.M.S. Chauhan and D.S. Bhakuni, Indian J. Chem., 25 (1986) 1146.
43 A.H.M. Raeymaekers, J.L.H. Van Gelder, L.F.C. Roevens and P.A.J. Janssen, Arzneim.-Forsch., 28 (1978) 586.
44 S. Ram, D.S. Wise and L.B. Townsend, Heterocycles, 22 (1984) 1789.
45 S. Ram, D.S. Wise and L.B. Townsend, Org. Prep. Proced. Int., 17 (1985) 215.
46 R.I. Hewitt, E. White, W.S. Wallace, H.W. Stewart, S. Kushner and Y. Subbarow, J. Lab. Clin. Med., 32 (1947) 1304.
47 L.V. Hoof, C. Henrard, E. Peel and N. Wanson, Ann. Soc. Belge Med. Trop., 27 (1947) 173.
48 G.C. Thooris, Bull. Soc. Pathol. Exot. Ses. Fil., 49 (1956) 1138.
49 S. Sharma, Pharm. Res., 3 (1986) 75.
50 Von P. Nickel, H.-J. Haack, H. Widjaja, U. Ardanuy, Ch. Gurgel, D. Duwel, H. Loewe and W. Raether, Arzneim.-Forsch., 36 (1986) 1153.

51 W.C. Campbell, M.H. Fisher, E.O. Stapley, G. Albers-Schonberg and T.A. Jacob, Science (Washington, DC), 221 (1983) 823.
52 K. Awadzi, K.Y. Dadzie, H. Shulz-Key, D.R.W. Haddock, H.M. Gilles and M.A. Aziz, Ann. Trop. Med. Parasitol., 79 (1985) 63.
53 W.C. Campbell, Parasitol. Today, 1 (1985) 1.
54 R.D. Walter and E.J. Albiez, Tropenmed. Parasitol., 35 (1984) 78.
55 J.J. Gallay and E. Schweizer (Ciba-Geigy A.-G.), U.K. Pat. GB 2,076,399 (1982); Eur. Pat. Appl. EP 41.046 (1981), Chem. Abstr., 96 (1982) 142841.
56 L.G. Goodwin, Trans. R. Soc. Trop. Med. Hyg., 78 (suppl.) (1984) 1.
57 Y. Xi, Y. Xu, J. Nie, M. Yan, and Z. Zhang, Zhongguo Yaoli Xuebao, 5 (1984) 207; Chem. Abstr. 101 (1984) 203931.
58 World Health Organization, Technical Report Series 702, Lymphatic Filariasis (1984).
59 M.V.V.L. Narasimham, S.P. Roychowdhury, M. Das and C.K. Rao, Southeast Asian J. Trop. Med. Public Health, 9 (1978) 571.
60 R.S. Rew and R.H. Fetterer, in Chemotherapy of Parasitic Diseases, eds. W.C. Campbell and R.S. Rew (Plenum Press, New York, 1986) pp. 321.

This Page Intentionally Left Blank

Progress in Medicinal Chemistry – Vol. 25, edited by G.P. Ellis and G.B. West

5 The Pharmacology of Vitamin E

M.J. KELLY, Ph.D.

Department of Applied Science, Leeds Polytechnic, Calverley Street, Leeds, LS1 3HE, U.K.

INTRODUCTION

Vitamin E (alpha-tocopherol) is still perhaps the least understood of all the body's vitamins in terms of its biological actions. This is despite abundant experimentation and numerous clinical studies spanning over 60 years. In some respects, the paucity of information has fuelled a continuing interest in vitamin E, but this gap in established scientific knowledge has also generated many speculative biological roles and doubtful potential therapeutic applications.

The lack of clear and well-defined deficiency symptoms in humans distinguishes this vitamin from the other fat-soluble vitamins. For example, a study, the 'Elgin project' [1–3], in which adult volunteers were fed a vitamin E-deficient diet for up to 3 years, showed that, although the levels of the vitamin in the blood were reduced by approx. 50%, the only symptoms seen were an increased susceptibility to peroxidation haemolysis and a slight decrease in the half-life of red blood cells. Such observations are in marked contrast to the prominent and relatively rapid deficiency signs seen in many animal studies [4]. However, treatment of human disorders bearing a resemblance to the animal deficiency diseases has proved disappointing, particularly in the case of muscular distrophy, a frequent sign of vitamin E deficiency in animals, where high doses of the vitamin given over considerable periods of time have been shown to be ineffective in alleviating symptoms [5]. This picture has led many pharmacologists and physicians to believe that the use of vitamin E in human adult subjects is unlikely to be beneficial in any measurable respect and is therefore not justified [6, 7].

It is against this background that some aspects of the pharmacology of vitamin E are examined in this review, because future therapies with this vitamin or its analogues will require a more rigorous scientific basis for their use. Many factors, such as its retention within the lipid components of the body, its interactions with other nutritional factors and its relationship to long-term age-related features, may obscure subtle signs of a deficiency. Therefore, further progress in unravelling the complex role of the vitamin in physiological processes will be difficult until its essential biochemical mechanisms in cellular metabolism have been established. In this review, efforts are made in this direction, and the reader is directed for a more comprehensive survey of the literature on vitamin E to the excellent reviews by Machlin [4, 8].

HISTORICAL CONTEXT

To try to give a perspective to current ideas on the mode of action of vitamin E, it might be prudent to examine the development of our knowledge of this vitamin. Its history is littered with fact and myth but for most people it is the vitamin of reproduction.

The existence of vitamins A, B, C and D was established when, in 1922, Evans and Bishop [9] showed that a factor found in lettuce and wheatgerm allowed reproduction in rats fed an otherwise nutritionally adequate semipurified diet containing the then-known vitamins. Absence of the factor, X, in the diet led to foetal death and resorption in the rat. The factor was designated a vitamin and given the letter E, the next in the series, by Sure [10] and Evans [11]. Early work concentrated on structural changes in the reproductive systems associated with vitamin E deficiency [12–14] and the vitamin therefore became known as the anti-sterility vitamin. However, subsequent work displayed more widespread deficiency signs with nutritional encephalomalacia [15], a condition with oedema and necrosis particularly in the cerebellum of the maturing brain of chicks, and muscular dystrophy [16], where there is extensive skeletal muscle necrosis in rabbits and guinea-pigs. Much of this work really required a purer and more potent form of the vitamin and in 1936, Evans, Emerson and Emerson [17] reported the isolation from wheatgerm oil of a chemical with marked biological activity. They provided its molecular chemical formula, $C_{29}H_{50}O_2$ and proposed the name α-tocopherol from the Greek 'tokos', childbirth, and the verb 'pherein', to bring forth. The ending of the word, 'ol', indicated that the chemical contained a hydroxy group (as a phenol) and shortly afterwards they showed the presence in vegetable oils of two other phenols with similar activity but lower potency, β- and γ-tocopherols [18]. Subsequently, the structural formula for the vitamin was discovered by Fernholz [19], and this aided a chemical synthesis in the Basel laboratories of Hoffmann-La Roche [20] in 1938.

Further developments began to lead to an improved understanding of the relevance of tocopherols to cellular processes. One significant early finding, concerning the mechanism of action of these agents, revealed their antioxidant properties but noted that this activity was not proportional to their biological activity [21]. The reversal of encephalomalacia in animals by vitamin E was reported [22], a capacity shared by synthetic antioxidants [23, 24], and the roles of other dietary factors in the vitamin's action were uncovered. The ability of lipids, high in unsaturated fatty acids, to inactivate vitamin E in the diet was deduced from a number of studies [25–27]. The ability of selenium to exert similar actions to vitamin E in deficiency states in animals and the contributory effect of sulphur-containing amino acids were noted [28].

Observations on deficiency symptoms in the cardiovascular system were also made [29, 30]. In chicks, exudative diathesis, a condition in which plasma moves from the capillaries into surrounding, particularly subcutaneous, tissue, was studied and found to be associated with lipid peroxidation [31]. In cardiac muscle, a necrotizing myopathy was found in vitamin-E-deficient mice [32], rats [33], rabbits [34] and ruminants [35]. Blood abnormalities, associated with increased susceptibility of erythrocytes to haemolysis, figure among reports of the effects of vitamin E deficiency [36, 37].

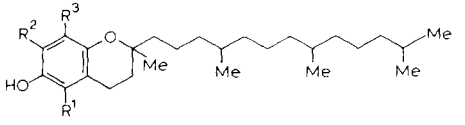
Other notable findings concerning vitamin-E deficient animals established hepatic lesions [38–40] and accumulation of lipopigment [41], while addition of the vitamin to the diet of rabbits evoked enhanced immune function [42]. These early studies in vitamin E deficiency states in animals produced sufficient diverse and interesting observations to generate much of the data currently available.

CHEMICAL PARAMETERS

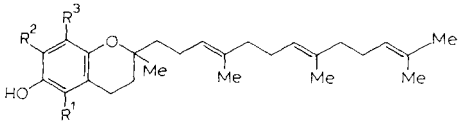
STRUCTURES

The pioneering work of Evans, Emerson and Emerson led to the isolation of α-tocopherol, a phenol exhibiting vitamin-E-like activity in biological systems. Now, eight compounds, usually oils, have been identified from plant sources, with similar vitamin-E-like actions. The tocopherols differ only in the number of methyl groups in the aromatic ring and therefore can be regarded as derivatives of 2-methyl-6-chromanol, onto which a 16-carbon isoprenoid chain is attached at C-2 and which is methylated at positions C-5, C-7 and C-8, respectively.

These compounds can be divided into two types, tocols and trienols. Although both types have the 6-chromanol ring structure and a phytol-like



(1)



(2)

side-chain, the trienols have a degree of unsaturation in the chain with double bonds at the 3′, 7′ and 11′ positions. This structure for the trienol can be inferred from the fact that its hydrogenation yields the tocol [43, 44]. The tocols (1) and trienols (2) are described by the presence or absence of methyl groups in the C-5, C-7 and C-8 positions of the chromanol ring (*Table 5.1*) and are called respectively α-, β-, γ- and δ-tocopherol or tocotrienol.

Table 5.1. STRUCTURES OF NATURAL TOCOPHEROLS AND TRIENOLS

Tocol	R^1	R^2	R^3	*Trienol*
α-Tocopherol	Me	Me	Me	α-tocotrienol
β-Tocopherol	Me	H	Me	β-tocotrienol
γ-Tocopherol	H	Me	Me	γ-tocotrienol
δ-Tocopherol	H	H	Me	δ-tocotrienol

The tocopherol structure indicates three positions of asymmetry at C-2 in the ring and C-4′ and C-8′ in the side-chain. Natural α-tocopherol, the most important vitamin of the group, has the (2*R*,4′*R*,8′*R*) configuration [45], as have the β and γ types [44].

The tocotrienols possess one centre of asymmetry, at C-2, in addition to the sites of geometrical isomerism at C-3′ and C-7′. The equivalent natural tocotrienols to the tocopherols mentioned above have a (2*R*)-3′-*trans*-7′-*trans* configuration [44–47]. Indeed, all natural tocopherols and tocotrienols have the *R* configuration at C-2 in the ring.

STRUCTURE–ACTIVITY RELATIONSHIPS

Methods for assessing vitamin-E-like activity are based on making animals deficient in the vitamin and then restoring some aspect of function. These procedures are time-consuming, requiring a minimum of 3 weeks, and involve quantifying protection against deficiency signs. For example, in the rat foetal-resorption test, female rats are fed a vitamin-E-deficient ration for 9–10 weeks before the animals are mated. Graded doses of vitamin E supplements are administered orally in five equal doses from the 4th–8th day after insemination. There is a narrow window in which vitamin E supplementation is effective.

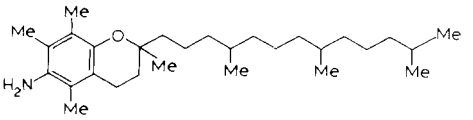
α-Tocopherol is the most active of the tocopherols in terms of traditional bioassay procedures using rat resorption-gestation, encephalomalacia, muscular dystrophy and erythrocyte haemolysis [4, 48]. In this form, the tocopherol is fully methylated in the benzene ring and any loss of methyl groups here, as

seen with β-, γ- and δ-tocopherols, reduces the biological activity significantly (*Table 5.2*). Tocol itself is claimed to have virtually no activity [49].

Table 5.2. BIOLOGICAL ACTIVITY OF THE TOCOPHEROLS [4]

	Activity (%)		
Structure	*Rat foetal resorption*	*Muscle dystrophy in chickens*	*Haemolysis in rats*
α-Tocopherol	100	100	100
β-Tocopherol	25–40	12	15–27
γ-Tocopherol	1–11	5	3–20
δ-Tocopherol	1	–	0.3–2

The presence of the hydroxy group in the ring of the tocopherols has been thought to be important in terms of its actions, but esterification did not alter or only slightly altered activity [50, 51], while replacement of the hydroxy with an amino group, as in α-tocopheramine (3), did not diminish its potency [52]. Shortening or elimination of the isoprenoid side-chain of vitamin E resulted in a sharp reduction in activity or no activity [53]. In the side-chain, a sequence of three isoprenoid units seems to be needed for maximum biological activity. Using chick exudative diathesis bioassay, 11-carbon and 13-carbon side-chain analogues of the vitamin had only partial activity and the 6-carbon side-chain structure was without effect [54].



(3)

In the rat foetal resorption and haemolysis tests, α-tocotrienol had respectively 29% and between 17–25% of the activity of vitamin E [4]. The tocotrienols also have a dependence on the presence of methyl groups in the benzene ring for their activity. Thus, 5,8-dimethyltocotrienol shows negligible activity compared with 5,7,8-trimethyltocotrienol and indeed (*RRR*)-α-tocopherol [55–57]. Unsaturation of the side-chain causes the activity to decrease [58].

These differences in biopotency are reflected in the standardization of the vitamin in terms of international units (iu). An international unit is equivalent

to the activity of 1 mg all-*rac*-α-tocopheryl acetate. This gives the equivalent tocopherol (all-*rac*-α-tocopherol, (±)-α-tocopherol) a potency of 1.1 iu and (*RRR*)-α-tocopheryl acetate and tocopherol potencies of 1.36 and 1.49, respectively. Recent studies using the rat resorption-gestation test have examined the eight stereoisomers of α-tocopherol and have shown that with respect to *RRR* which has 100% activity, *RRS* has 90%, *RSS* 73%, *SSS* 60%, *RSR* 57%, *SRS* 37%, *SRR* 31% and *SSR* has 21%. The activity of the *SSS* form suggests that the 4′-C is also important in terms of biological activity [59, 60].

THE ANTIOXIDANT THEORY

SIGNIFICANCE

The survival of living cells is determined by the conversion of nutrients into energy, a process dependent upon the intracellular reduction of oxygen. The process is normally accompanied by the generation of a small but significant

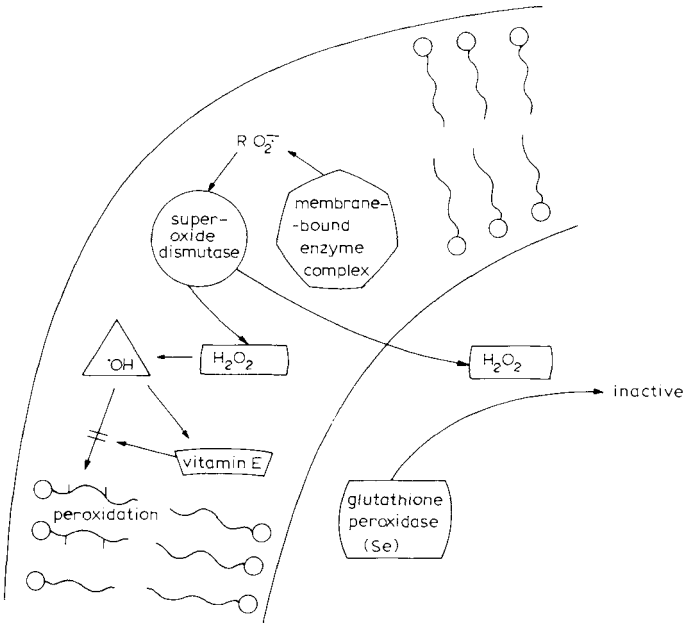


Figure 5.1. Schematic representation of the generation of oxygen intermediates in the membrane and the role of the protective mechanisms.

quantity of oxygen intermediates, which include superoxide (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^\bullet OH$). Although short-lived, these intermediates are highly reactive and therefore must be accommodated within the cell in order to preserve homeostasis. Enzymatic scavenging of these species constitutes the primary cellular defence against potential injury (*Figure 5.1*).

It has been known for some time that vitamin E can act as an antioxidant within the body [21, 61] and that the biological potency of the tocopherols is proportional to their antioxidant activity [62]. Synthetic antioxidants, which often have structures unrelated to that of the vitamin, are also capable of preventing the symptoms of vitamin E deficiency [23, 24, 63, 64]. The general proposal [63, 65], therefore, is that the function of vitamin E is one of an *in vivo* antioxidant, protecting membrane phospholipids from attack by free radicals generated within the cell.

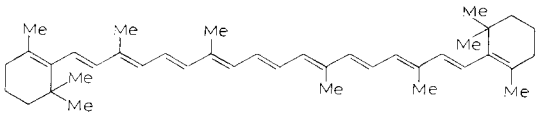
When the phytyl side-chain of vitamin E is replaced by a methyl group, vitamin E activity *in vivo* is lost [66, 67], although the antioxidant activity of the methyl-substituted compound *in vitro* is basically the same as that of the vitamin [66]. The chromanol ring of the vitamin may be located in the polar surface regions of the membrane, while the phytyl side-chain may interact with the polyunsaturated fatty acids of the phospholipids in the less polar internal parts of the membrane [65, 68]. The tocopherol is also likely to be located near free-radical-generating membrane-bound enzymes, but may also be able to move quickly through nonpolar portions of the membrane. Free radicals produced by these systems can interact with oxygen to yield superoxide, which can then react with hydrogen ions to produce the poisonous hydrogen peroxide. Within the aqueous phase of the cell, this material can be removed by glutathione peroxidase, which is a selenium-containing enzyme, whereas in the non-aqueous areas of the cell the hydrogen peroxide may interact with superoxide anions to form hydroxy radicals which are highly reactive. The presence of vitamin E in the membrane allows the action of these radicals to be quenched locally (*Figure 5.1*). Their presence would otherwise initiate peroxidation of polyunsaturated fatty acids in the membrane [69, 70].

PROTECTION

Vitamin E has been shown, in a wide range of studies, to be capable of affording considerable protection to cells exposed to free radical attack, either from pathological or drug-induced sources. In injured nervous tissue, vitamin E probably exerts its protective effect by scavenging free radicals, stabilizing cellular membranes and quenching the cascade of biochemical events that

follow necrosis in brain tissue [71]. Vitamin E may terminate brain peroxidation reactions by donation of a phenolic hydrogen, forming a quinone [72]. Indeed, CV-2619 (idebenone) is a novel quinone which has a protective effect on cerebral ischaemia [73] and can inhibit lipid peroxidation in a concentration-dependent manner in rat brain mitochondria [74].

A neuropathy caused by clioquinol (iodochlorohydroxyquin, chinoform) and enhanced by the formation of a clioquinol ferric chelate which initiates lipid peroxidation, leads to complete degeneration of retinal neuroblasts within a day. Vitamin E has a potent protective action against the effects of the chelate [75]. Peroxidative damage to DNA in rat brain, induced by methyl ethyl ketone peroxide, a potent initiator of lipid peroxidation, was inhibited by addition of vitamin E to the diet of rats [76].



(4)

Methyl Demeton (Meta-systox), an organophosphorus insecticide compound, caused a dose-dependent increase in lipid peroxidation in several regions of the rat central nervous system. This response was prevented when vitamin E was administered with the compound [77]. *N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a potent neurotoxin in dopaminergic neurones and produces a loss of striatal dopamine in mice. Four different antioxidants including vitamin E and β-carotene (4), were found to protect against this effect of MPTP [78]. This neurotoxin reduced brain-stem glutathione in mice and it is likely that MPTP metabolites directly damage dopaminergic nigrostriatal neurones and consume glutathione during their metabolism. This effect on glutathione can be prevented by pretreatment with large doses of vitamin E or β-carotene [79]. However, other workers [80] have shown that MPTP-induced dopaminergic neurotoxicity was not affected by antioxidants, including vitamin E, and suggest that the intraneuronal generation of superoxides is not a major factor in MPTP-like neurotoxicity. This work indicates that, if Parkinsonism is shown to be caused by a MPTP-like neurotoxin [81], treatment by antioxidants is unlikely to be effective.

It has previously been shown in mice that ageing is associated with a progressive and highly significant proliferation of the total number of lipofuscin granules in the cervical spinal cord. Continuous treatment for 8 months with either vitamin E or the synthetic antioxidant, butylated hydroxytoluene (BHT), diminished significantly the proliferation of lipofuscin granules in the spinal cord neurones. These results provide support for the theory of lipofuscin biogenesis [82].

Plasma malondialdehyde-like material, an indicator of lipid peroxidation, is increased in conditions of ischaemia, such as stroke [83, 84] and myocardial infarction [85]. Mitochondria extracted from hearts of vitamin-E-deficient rabbits showed a decreased mitochondrial function and an increased formation of oxygen radicals associated with a reduced superoxide dismutase activity. This was partially reversed by addition of vitamin E *in vitro* [86]. Measurement of *in vitro* susceptibility to lipid peroxidation in cardiac muscle from vitamin-E-deficient mice showed a highly significant negative correlation between the concentration of vitamin E and *in vitro* lipid peroxidation. The results indicate that short-term vitamin E deficiency may expose cardiac muscle to peroxidation injuries [87]. In rats, treatment for 2 days with isoprenaline increased lipid peroxide activity, as measured by malondialdehyde levels, in the myocardium. Vitamin-E-deficient animals were even more sensitive to this effect, and pretreatment with α-tocopheryl acetate for 2 weeks prevented the effect induced by isoprenaline. The authors [88] propose that free-radical-mediated increases in lipid peroxide activity may have a role in catecholamine-induced heart disease.

Ethanol-induced damage to mouse heart can be partially prevented by pretreatment with vitamin E [89] and this suggests that some of the toxic effects of ethanol on heart tissue are mediated through free radical mechanisms, leading to lipid peroxidation. Another study of cardiotoxicity, this time with adriamycin (doxorubicin) [89a], a widely used antitumour drug, showed that myocardial lipid peroxidation induced by the drug could be reduced by a combination of vitamin A, a singlet-oxygen quencher, and vitamin E, a free-radical scavenger. The lipid peroxidation appeared to lead to an increase in Ca^{2+} permeability, which could be counteracted by prenylamine, a Ca^{2+} antagonist [90].

Mice on a vitamin-E-deficient diet showed a negative correlation between the concentration of this vitamin and *in vitro* lipid peroxidation in skeletal muscle [87], and these results suggest that such a deficiency could expose skeletal muscle to peroxidation injuries. Another study has indicated that moderate exercise of several weeks duration could enhance the degradation and excretion of lipid peroxides [91].

In lung tissue, vitamin E can protect against paraquat-induced damage [92] and can scavenge radicals produced from the reaction of ozone with poly-unsaturated fatty acids [93]. In the liver, vitamin E can reduce lipid peroxide levels [94] in rats. Treatment of rats with the anti-cancer drug, 5-fluorouracil, increased liver and plasma lipoperoxide levels. Animals receiving vitamin E supplementation concomitantly with the drug treatment had liver and plasma lipoperoxide levels which were significantly lower than those which had received

only the anti-cancer drug [95]. An *in vitro* rat liver microsome model of lipid peroxidation illustrates the importance of two defence mechanisms against microsomal lipid peroxidation. The model shows a lag phase before lipid peroxidation commences; the existence of the lag is determined by the presence of a glutathione-dependent protein, while the length of the lag is affected by vitamin E. Such a model could prove useful in testing vitamin E analogues for their antioxidant properties. The authors suggest [96] that the glutathione may protrude from the membrane and scavenge radicals at some distance from the lipid/water interface but that vitamin E must act at the interface. A liposome model has also been used to test the vitamin's ability to suppress peroxidation of lipids. During this suppression, the vitamin molecule was found to be located in the 1-palmitoyl-2-arachidonylphosphatidylcholine-rich regions of membranes consisting of mixed phospholipids [97].

The formation of malondialdehyde-like material in human plasma *in vitro* can be reduced by incubation with vitamin E or BHT, and from these experiments it is concluded [98] that the level of antioxidant present in human plasma is not sufficient to counteract lipid peroxidation. Also in humans, increasing the polyunsaturated/saturated fat ratio in the diet was found not to influence the *in vivo* lipid peroxidation rate [99].

STRUCTURAL CONSIDERATIONS

A number of investigations have concentrated on the structural requirements for the antioxidant effect in various situations, both biological and chemical. The use of pyrene as a fluorescent probe [100] to test antioxidant activity with vitamin E showed that the interaction of membrane lipids with the vitamin-induced perturbation of the lipid organization, with some indication of restriction of movement of dye molecules due to a decrease in membrane fluidity. However, β-, γ- and δ-tocopherols were without effect on this model system. Electron spin resonance spectroscopy (ESR) was used to measure the interaction of tocopherol-like compounds with superoxide ion [101]. The results indicate that the side-chain does not affect the structure of the radical and that the ESR signal intensity of the chromanoxyl radicals from α-, β-, γ- and δ-tocopherols has an order of intensity similar to the biological potency of these agents. It is also suggested that the methyl group located in the C-5 position is important for the biological function and for the production of the free radical of vitamin E. Another study of the antioxidant potency of the tocopherols, using reactions with peroxyl radicals obtained either by autoxidation of styrene or by the flash photolysis of di-*t*-butyl ketone in an oxygen-saturated environment, gave a similar order of potency [102] and correspondence with biological

rank order [4]. One further chemical investigation, with peroxidizing lipids used to induce the formation of antioxidant radicals [103], revealed that (±)-α-tocopheroxyl radicals were formed in relatively high concentrations but were rapidly destroyed compared with (±)-δ-tocopheroxyl radicals, which were formed in rather low concentration and were destroyed rather slowly; (±)-β- and (±)-δ-tocopheroxyl radicals reacted in an intermediate way.

Further work on the structural requirements for the antioxidant effect has been performed with micelles and liposomes [104]. This group concluded that the antioxidant properties of vitamin E and its model compound without the phytyl side-chain were similar within micelles, liposomes and homogeneous solution but that the phytyl side-chain enhances the retention of vitamin E in liposomes and suppresses the transfer of the vitamin between liposomal membranes.

A study of the antioxidant capacities of tocopherols and their quinones in non-biological and biological systems concluded that in both systems, vitamin E and γ-tocopherol had similar antioxidant properties [105]. However it is suggested that the antioxidant capacity of vitamin E is enhanced by its metabolism to a quinone which, unlike the quinone from γ-tocopherol, functions as a biological antioxidant. An interesting observation in two biological model systems, rat liver microsomes and mitochondria, where the efficacy of lipid peroxidation inhibition by vitamin E, 2,2,5,7,8-pentamethylchroman-6-ol (PMC) and BHT was measured, showed that PMC and BHT could exert a destructive effect on biomembranes leading to an increase in their permeability to ions. It was concluded from the study [106] that the presence of the hydrocarbon tail in the vitamin molecule helps the structural stability of the membrane. Finally, a report [107] on a biological system, pulmonary artery endothelial cells, where the susceptibility to attack by hydrogen peroxide has been measured, showed that endogenous catalase, vitamin E and particularly Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), but not phytol, provided protection for the cells from hydrogen-peroxide-induced damage.

INVOLVEMENT WITH ARACHIDONIC ACID METABOLISM

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), a polyunsaturated fatty acid derived from dietary sources or by desaturation and chain elongation of the essential fatty acid linoleic acid, is found widely in the body. It is transported in a protein-bound state and stored in the phospholipids of cell membranes in all tissues of the body [108] from where it can be changed into biologically

active tissue hormones, eicosanoids, in response to appropriate stimuli [109]. The release step of the fatty acid from its storage position within phospholipid membranes, a Ca^{2+}-dependent process, appears to be rate-limiting in determining the amount of substrate available for eicosanoid synthesis.

Two main enzyme complexes, cyclo-oxygenase and lipoxygenase, compete for the released fatty acid. The cyclo-oxygenase system converts the arachidonic acid into a variety of active species. The endoperoxide prostaglandin (PGH_2) released from the enzyme system can be changed into metabolites, prostacyclin (PGI_2) and thromboxane (TXA_2), which are important cardiovascular factors. Prostacyclin synthetase, a membrane-bound enzyme in vascular tissue, synthesizes PGI_2, which is a potent vasodilator with platelet-aggregation inhibitory activity [110]. With a half-life of 5 min, PGI_2 is inactivated by a hydrolysis reaction to 6-keto-$PGF_{1\alpha}$ [111]. The membrane-bound thromboxane synthetase in platelets is responsible for the production of TXA_2 and also has a secondary catalytic activity [112] resulting in the decomposition of PGH_2 into malondialdehyde (MDA) and 17-carbon hydroxy fatty acids (HHT). TXA_2, a vasoconstrictor and stimulant of platelet aggregation, is hydrolysed to the relatively inactive metabolite, TXB_2 [113], with a half-life of 30 s. The other products of the cyclo-oxygenase complex, the prostaglandins (PG), with widespread pharmacological activity, are inactivated by chemical hydrolysis or by catalysis from 15-hydroxyprostaglandin dehydrogenase (PGDH) which generates 15-ketoprostaglandins [114]. Limitation of prostaglandin action to sites of production is determined by the activity of PGDH.

The lipoxygenase system also competes for released arachidonic acid in a way that seems to be tissue-selective, giving rise to hydroperoxy fatty acids (HPETE) which can be converted into leukotrienes or reduced to hydroxy fatty acid (HETE) products [115]. The basic scheme for these metabolic conversions involving arachidonic acid is presented in *Figure 5.2*. Both of the main enzymatic pathways of arachidonic acid metabolism are thought to involve free-radical-mediated reactions [108] and the antioxidant capacity of vitamin E could therefore allow the vitamin to modify the products of these pathways.

Vitamin E is known to protect against a lethal arachidonic acid infusion in rabbits [116] and to act synergistically with aspirin in depressing prostaglandin levels and mortality in chickens given *E.coli* infections [117]. In rat brain, where PGE_2 is known to inhibit neurotransmission, deficiency of vitamin E in the diet increased, and supplementation with vitamin E in the diet reduced, the production of PGE_2 [118]. Supplementation of a diet with α-tocopheryl acetate produced an enhanced immune response in aged mice, an effect which appeared to be mediated by a reduction in prostaglandin synthesis [119].

In view of the therapeutic use of vitamin E in intermittent claudication and

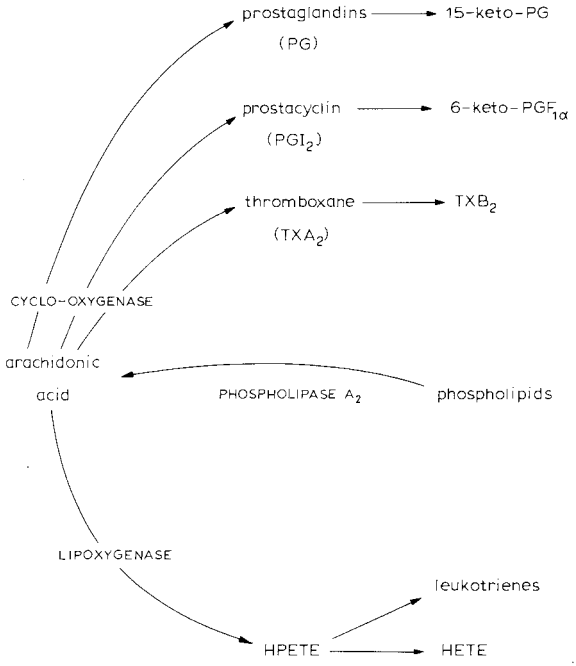


Figure 5.2. Outline of arachidonic acid metabolism.

its claimed beneficial actions in coronary heart disease, the vitamin's involvement in the regulatory processes associated with blood platelet aggregation seems worthy of consideration. Conditions in which platelet aggregation has an aetiological role, myocardial infarction, stroke, pre-eclampsia and the vascular complications of diabetes could be amenable to antioxidant nutrition [120]. Indeed, it has been proposed [121] that the increased incidence of myocardial infarction in the western world since the 1920's could be the result of elevated intakes of the unnatural dietary fatty acid, *trans-trans*-linoleic acid, in margarine and refined vegetable oils. This could result in a deficiency of the beneficial PGE_1 and an increase in the harmful TXA_2, as well as increasing vitamin E requirements.

Early work revealed evidence of enhanced prostaglandin synthesis in platelets of vitamin-E-deficient animals [122], and the vitamin is known to inhibit platelet aggregation both *in vivo* and *in vitro* [123]. Further studies have shown that vitamin E can suppress TXA_2 synthesis and enhance PGI_2 formation [124–126].

Experiments designed to investigate the effects of dietary vitamin E and synthetic antioxidants on PGI_2 and TXA_2 synthesis showed that the levels of PGI_2 in aortae were lowered in vitamin E deficiency, but the synthesis of TXA_2 in washed platelets was independent of the antioxidant status of the rabbits [127]. Different diets fed to growing male rats for 8 weeks showed a selective action on vascular eicosanoids [128]. Two diets were compared, one containing adequate amounts of vitamin E plus a fresh mixture of polyunsaturated oils and the other a diet where the oils had been altered (reduced vitamin E) by heating. Although all of the main nutritional parameters were similar in the two groups, the heat-treated-diet-fed animals had reduced vascular PGI_2 release and increased TXA_2 in their platelets. Addition of vitamin E (300 mg/kg) to the diet containing the heated fats neutralized their adverse effects on eicosanoid levels.

In human subjects, however, 8 weeks of vitamin E supplementation (800 IU/day) to the diet produced a lowered plasma PGI_2 level, as measured by 6-keto-$PGF_{1\alpha}$, compared with that in non-supplemented controls [129]. Addition of vitamin E to human platelets in concentrations which resemble normal plasma levels produced a moderately potent but consistent reduction in cyclo-oxygenase activity, with a dose-dependent response up to 1 mM. α-Tocopherol quinone was equally effective in this test [130].

The levels of cardiovascular factors could also be influenced by the ability of vitamin E to affect activation of arachidonic acid from membrane phospholipids by phospholipase A_2. Vitamin E, either given in the diet or by incubation with platelets themselves, was found to inhibit phospholipase A_2 in a dose-dependent manner. α-Tocopheryl acetate had little or no effect on the activity of this enzyme, but tocol, without methyl groups in the chroman ring, was more potent than either (+)- or (±)-α-tocopherol, suggesting that the methyl groups were not important for the inhibition but the hydroxy group in the ring was critical for activity [131].

Vitamin E is also capable of affecting the lipoxygenase enzyme complex. Supplementation of human subjects with the vitamin for 4 weeks doubled the plasma levels and elicited a transitory increase of lipoxygenase activity [132]. But other groups have shown an inhibitory action of vitamin E on lipoxygenase activity. Platelets from vitamin-E-deficient rabbits generated more 12-HETE than platelets from rabbits which had been supplemented with the vitamin [133]. Purified 5-lipoxygenase, an enzyme of importance in leukotriene synthesis, from potato tuber was found to be irreversibly, non-competitively inhibited by vitamin E [134]. The IC_{50} value was 5 mM, and evidence was found for binding to a single peptide. γ-Tocopherol was found to have a similar inhibitory action on the enzyme. Effective inhibitors but less potent were

(+)-α-tocopheryl acetate and (+)-α-tocopherol quinone, substances thought to be poor antioxidants. Similar findings were obtained by Grossman and Waksman [135], who showed that vitamin E could inhibit 15-lipoxygenase, but in this situation tocopheryl acetate was as potent as the vitamin itself. Both vitamin E and C have been shown to inhibit arachidonate-induced aggregation of human polymorphonuclear and mononuclear blood leukocytes, a mechanism which is thought to involve the lipoxygenase pathway [136].

Recently, Strivastava [137] has suggested a mechanism for the vitamin's anti-aggregatory action in platelets that does not involve the arachidonic acid cascade system. The author proposed that the inhibition of aggregation could be due to inhibition of intracellular mobilization of Ca^{2+} from the dense tubular system of the cytoplasm.

A number of studies have examined the involvement of the vitamin with arachidonic acid metabolism in disease states or after various drug therapies. Diabetic patients tend to develop microvascular complications which are thought to be related to platelet hyperaggregability. Increased platelet sensitivity is likely to be the result of an imbalance of PGI_2 and TXA_2 production. Karpen, Cataland, O'Dorisio and Panganamala [138] have shown that platelets from diabetic patients synthesize less vitamin E than those from control subjects. It was suggested that the low platelet vitamin E levels could be a contributing factor to the increased thromboxane synthesis in response to chemical challenge in such type I diabetic subjects. Although the level of the vitamin in platelets is reduced, the plasma level is not affected in diabetes [139]. Lowered platelet vitamin E was also found by other investigators [140], and the rate of platelet aggregation was significantly increased in diabetics with proliferative retinopathy. There was also enhanced production of TXB_2, a metabolite of TXA_2, in these patients. Animal experiments, using streptozotocin to induce diabetes, too, have shown changes in the eicosanoid levels. Endogenous release of PGI_2 by isolated aorta was significantly greater in rats receiving 100-times the normal vitamin E requirement than in untreated diabetic rats. Vitamin E supplementation at both 10- and 100-times nutritional requirements significantly depressed thrombin-stimulated synthesis of TXA_2 in washed platelets. The results suggest an ameliorating influence of the vitamin on the PGI_2/TXA_2 balance in diabetes [141]. Other workers have found that aortic rings from non-diabetic rats produced more prostacyclin than those from diabetic rats, and they showed that a vitamin-E-deficient diet decreased PGI_2 production [142].

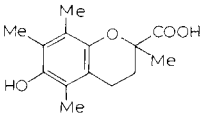
Oral vitamin E, 300 mg and 600 mg daily for 2 weeks, administered to type II and IV hyperlipoproteinaemia patients increased the serum vitamin E concentration 2- fold and suppressed the normally elevated plasma lipid peroxide

levels found in this condition [143]. The same group, working with rabbits, showed that an atherogenic diet given for 1 week could also elevate plasma lipid peroxide and, in addition, a 90% reduction in arterial PGI_2 generation could be measured. However, supplementation of this diet with 100 mg vitamin E per day prevented the increase in plasma lipid peroxides and protected the PGI_2-generating system in arteries. Another antioxidant, ascorbic acid (vitamin C), protected prostacyclin production from the damaging effects of a cholesterol-rich diet in a study of experimental atherosclerosis in rabbits [144].

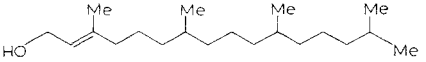
It is also possible that vitamin E can nullify some of the main adverse effects of oestrogen on blood platelets. Administration of ethynyloestradiol to female rats increased lipid synthesis, mainly lanosterol, and thrombin-induced platelet aggregation. Treatment of the rats with α-tocopheryl acetate depressed the enhanced lipid synthesis and aggregation induced by the oestrogen. *In vitro*, too, platelet aggregation was enhanced by the addition of lanosterol, and this response was almost completely antagonised by preincubation with α-tocopheryl acetate [145].

Further evidence for the involvement of vitamin E in arachidonic acid metabolism comes from work by Valentovic, Gairola and Lubawy [146], who showed that vitamin E deficiency in rats increased hepatic lipid peroxidation and decreased aortic PGI_2 synthesis. Inhalation of cigarette smoke by the rats increased platelet TXA_2 by over 90% and reduced aortic PGI_2 by between 26–33%. However, these effects were independent of the presence of vitamin E in the diet.

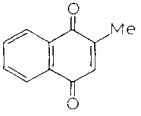
Another group [147, 148] has produced perhaps the most complete analysis of the interaction of vitamin E with both the cyclo-oxygenase and lipoxygenase pathways within a single model system. The group has also used this information in a clinical setting in the treatment of respiratory distress syndrome of the adult, a condition which has become an important cause of death in shock states when circulatory failure has been controlled. In the group's isolated perfused rabbit lung model, addition of arachidonic acid or its liberation by a Ca^{2+} ionophore (A23187) produced raised pulmonary vascular resistance, caused by products of the cyclo-oxygenase complex, and increased vascular permeability, the result of the action of products of the lipoxygenase system. An interesting analysis of the structure–activity relationship of the vitamin E molecule was performed, using a chroman-ring (in Trolox)(5), the side-chain (phytol)(6), and related structures containing a similar ring (2-methyl-1,4-naphthoquinone, vitamin K-3)(7) and ring with side-chain (2-methyl-3-phytyl-1,4-naphthoquinone, vitamin K-1)(8). Compounds with the side-chains, vitamin K-1 and phytol, increased the arachidonate-induced rise in pulmonary vascular resistance and vascular permeability, whereas the ring structures,



(5)

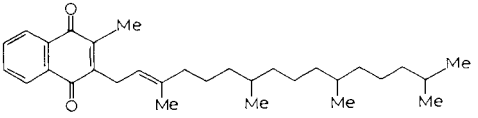


(6)



(7)

Trolox and vitamin K-3, reduced both parameters. The antioxidative portion of the vitamin molecule is thought to be the chroman-ring, and therefore similar structures might be expected to antagonize arachidonate-induced changes. The side-chain part of the structure, however, may have some form of membrane effect which induces an opposing response. Vitamin E itself had a selective effect, increasing pulmonary arterial pressure but having no action on vascular permeability. Clinically, nearly 40% of the vitamin-E-treated but only 17% of non-tocopherol-treated patients survived the respiratory shock reaction.



(8)

VITAMIN E AND OXYGEN

HYPEROXIA AND VITAMIN E

One situation in which oxygen intermediates are likely to be present in quantities greater than normal is hyperoxia. A study with hyperoxia-induced lipid peroxidation in the lungs of vitamin-E-deficient rats [149] revealed that the vitamin and glutathione peroxidase had mutually dependent roles in the protective system arranged against lipid peroxidation and that gluthathione peroxidase was of primary importance in neutralizing peroxidation induced by

hyperoxia. Treatment with (±)-α-tocopherol (100 mg/kg) at 1 and 24 h of life in rabbits completely abolished the effect of hyperoxia, that is, compromise of the pulmonary surfactant system [150]. In rabbit, isolated lungs infusion of the lipid peroxide, *t*-butyl hydroperoxide, produces vasoconstriction by stimulating the pulmonary synthesis of thromboxane and hyperoxia reduces prostacyclin synthesis. However, antioxidant treatment with vitamin E did not reverse this effect [151].

Hyperoxia was found to encourage the accumulation of primary and end-products of lipid peroxidation together with a significant lowering of the vitamin E content of rat brain tissue [152]. The consequence of hyperoxia was epileptiform seizures, which were prevented by vitamin E or synthetic antioxidant pre-injection. Other workers [153] have found that protection against hyperoxia is directly related to the level of vitamin E or selenium supplementation. However, some [154] have indicated no beneficial effects for vitamin E in reducing oxygen toxicity.

Several workers have examined the effect of oxygen on the developing retina and the protective actions of vitamin E on retinopathy of prematurity. The primary effect of oxygen is one of vascular closure, while the secondary response, which occurs after removal from increased oxygen, is retinal neovascularization [155]. The phenomenon was investigated *in vitro* using tissue culture techniques. Immature retinal vascular cells were adversely affected by hyperoxia, while mature cells were not, suggesting that the younger cells were deficient in free-radical-scavenging enzymes [156]. Incubation with vitamin E partially retarded the hyperoxic injury to vascular cells and this property was shared by γ-aminobutyric acid. The ability of vitamin E to bind to interstitial retinol-binding protein (IRBP) may provide an explanation for its capacity to suppress retinopathy of prematurity [157]. The distribution of IRBP within the subretinal space explains the gestational age-dependent efficacy of vitamin E in suppressing the development of severe retinopathy of prematurity [158]. In infants of less than 27 weeks gestational age, a large proportion of the spindle cell apron is not transretinal to IRBP, and therefore failure in vitamin E therapy in young infants can occur. However, 28 weeks gestation is the critical age at which complete suppression of severe retinopathy of prematurity with vitamin E supplementation occurs, and this corresponds to a surge of retinal maturation which increases the amount of overlap between IRBP in the subretinal space and spindle cells with the nerve fibre layer. A final area where hyperoxia has been implicated in damage in premature babies is intraventricular haemorrhage. Vitamin E is said to protect endothelial cell membranes from oxidative damage and reduces the risk of extension into the ventricles [159, 160].

ISCHAEMIA

Rather surprisingly, the protective effect of vitamin E has also been investigated in situations like ischaemia where the oxygen supply is likely to be reduced, although in many cases damage is associated with reperfusion after a period of deprivation. A number of studies have looked at ischaemia in heart tissue. Vitamin E prevents contractility disorders of the heart, provoked by stress and reoxygenation, in rats [161]. Treatment of rabbits with vitamin E and sodium nucleinate decreased lipid peroxidation in myocardial infarction [162]. In rat myocardium [163], vitamin E enhanced the power and efficiency of myocardial energy consumption. The vitamin also showed a protective effect in stress-induced myocardial lesions in rats [164]. Administration of the vitamin before stress prevented the corticosterone elevation and increased noradrenaline neuronal uptake [165]. 1.5 h of ischaemia in isolated hearts from rabbits induced a significant reduction of mitochondrial superoxide dismutase and of reduced glutathione/oxidized glutathione ratio. These alterations were associated with massive tissue and mitochondrial calcium accumulation. Vitamin E showed a protective effect on mitochondrial function, but failed to improve the recovery of mechanical function during reperfusion [166]. Vitamin E appears to have no effect on aerobically-perfused rabbit isolated heart, but it can attenuate hypoxia-induced rise in resting tension, depletion of adenosine triphosphate and creatine phosphate reserves, release of lactate, deterioration in mitochondrial function and release of enzyme. This protective effect of vitamin E also persisted during reoxygenation [167]. Drugs that have been shown to protect heart muscle during conditions of oxygen deprivation include verapamil and propranolol, both cardiodepressants. Methylprednisolone is also protective and, like vitamin E, is a membrane stabilizer [168].

Vitamin E was found to offer protection in a model of endothelaemia induced by intravenous administration of H_2O_2 and in leg ischaemia produced by ligature of the common femoral artery in rats [169].

In rats, hepatic ischaemia is associated with reduced ATP levels but normal lipid peroxide formation. Reperfusion gives a slow recovery of ATP levels, a reduction in endogenous vitamin E and glutathione, but an increase in lipid peroxidation. Vitamin-E-treated animals showed accelerated ATP synthesis with a suppression of the increased lipid peroxidation [170, 171]. Ischaemia of liver tissue reduced the metabolism of xenobiotics. Vitamin E was protectant against this effect [172]. The protective effect is related to an increase in catalytic activity of cytochrome *P*-450, to antioxidant and membrane-stabilizing properties [173]. In kidney tissue, prophylactic injection of vitamin E and synthetic antioxidants prevented the development of lesions during acute renal ischaemia and subsequent reperfusion. These effects were related therefore to the vitamin's antioxidant ability.

CEREBRAL ISCHAEMIA

There have been a large number of investigations of the potential protective effect of vitamin E in cerebral ischaemia. One group [175], using a perfusion method in a canine model of complete brain ischaemia, have established that vitamin E can protect brain tissue from the effects of cerebral ischaemia. Combination treatment with the vitamin, mannitol and dexamethasone proved more effective than the protection given by the individual agents [176]; the speed and degree of recovery of brain electrical activity also being greater when the drugs were given together [177]. After a period of severe ischaemia with cerebral blood flow reduced to 10% of normal, electrical activity does not recover in control animals but vitamin-E-treated dogs show a reasonable recovery rate [178]. This suggests that the vitamin scavenges the active oxygens produced after recirculation and thereby suppresses lipid peroxidation, preventing irreversible damage to the cells.

In rat forebrain subjected to decapitation ischaemia, there was a decrease in the levels of vitamin E, making the brain more susceptible to peroxidation attack when the tissue was subsequently reperfused [179]. Another study using rats indicated that reoxygenation was required for lipid peroxides to accumulate in the brain [180]. In spontaneously hypertensive rats where the effects of incomplete global cerebral ischaemia were measured [181], peroxide levels remained unchanged during ischaemia but rose in blood and cerebral tissue after reperfusion. The rise in peroxide after reperfusion corresponded to a decline in neurological function. Administration of vitamin E 30 min before ischaemia suppressed the peroxide changes and neurological decline during reperfusion. Cerebral reoxygenation after ischaemia propagates peroxidative reactions within esterified polyunsaturated fatty acids. The modification by vitamin E of reoxygenation-induced lipid peroxidation suggests free radical mediation [182].

Chemiluminescence has been used to investigate lipid peroxidation on the brains of rats. Cerebral hypoxia was induced by arterial hypoxaemia ($P_a^{O_2}$ 17–22 mmHg) with normocapnia ($P_a^{CO_2}$ 28–38 mmHg) and normotension (MABP 100–140 mmHg). Mixed gas (O_2/N_2, 4/96%) was used as a replacement for obtaining lowered $P_a^{O_2}$. The chemiluminescence findings indicate that vitamin E and betamethasone act on the breakdown of lipid hydroperoxide and that mannitol acts on hydroxy radicals in lipid peroxidation [183–185]. A recent study has found that phenytoin is superior to vitamin E as a protectant in a mouse cerebral infarction model [186] and that this effect is enhanced by combination with mannitol and vitamin E.

Cerebral vasospasm following aneurysmal subarachnoid haemorrhage is one

of the most important causes of cerebral ischaemia and is the leading cause of death and disability after aneurysm rupture. However, it is claimed that there is little evidence that vitamin E is of any significant clinical use [187], although the 'Sendai cocktail' (mannitol, dexamethasone and vitamin E) has been used successfully in a patient with subarachnoid haemorrhage to facilitate placement of balloon catheters [188].

HYPOXIA AND VITAMIN E

The vitamin has been shown to be able to protect animals from the lethal effects of anoxia and hypoxia. Rats [189] and rabbits [190] fed on vitamin-E-supplemented diets survived longer in hypoxia than non-supplemented animals. A similar protective effect has been demonstrated *in vitro* with cardiac muscle [167]. In hypoxic Langendorff-perfused rabbit heart, the presence of vitamin E protected the muscle from the deleterious effects of hypoxia, possibly by improving mitochondrial function [168]. However, in clinical studies, the use of vitamin E in ischaemic heart disease has met with little success [191, 192], although the results have been controversial [193].

Clinical investigations of the use of vitamin E in treating vascular disorders associated with hypoxia have been more successful [194]. In intermittent claudication, Boyd, Ratcliffe, Jepson and James [195] reported a 78% improvement over a 6 month trial period, while Haeger [196, 197] observed significant increases in walking distance in patients with intermittent claudication receiving 300 mg α-tocopherol acetate per day for 2–5 years. This condition currently represents the most potentially useful clinical area for administration of the vitamin in pharmacological doses.

POSSIBLE MODES OF ACTION

For some time, the effects of and responses to vitamin E have been interpreted in terms of an antioxidant mechanism of action. However, several observations have raised the question as to whether other mechanisms could be involved. For example, the effects of selenium and vitamin E on growth and polyunsaturated fatty acid synthesis in cultured mouse fibroblasts could not be reproduced by artificial antioxidants [198, 199]. The specific requirement of (+)-α-tocopherol for the phenotypic differentiation of the rotifer [200] may not be through an antioxidant mechanism. The effects of vitamin E on differentiation of neuroblastoma cells [201] and metamorphosis of various species [202] are likely to be due to a growth-factor-like action. A study on the interaction

between vitamin E and unsaturated phospholipids, using a tissue culture technique, indicated that the vitamin could exert a controlling influence on the linoleyl and arachidonyl residues within membrane phospholipids which could not be explained on the basis of an antioxidant function [203]. In addition, it has been noted that, although there are no significant differences in antioxidant activity between chirally different forms of vitamin E, there are large differences in biological activity [204].

SYMPATHETIC NERVOUS SYSTEM

Work on the sympathetic nervous system of rats [205] has shown that vitamin E increases the content and turnover rate of noradrenaline in heart and interscapular brown adipose tissue, suggesting that the vitamin could act in a specific role in the pathway for catecholamine biosynthesis. Obviously, one possibility is that vitamin E could be acting as an antioxidant, protecting lipids in the membrane of the sympathetic vesicle, or it could be acting as a co-factor for one of the enzymes in the biosynthesis of noradrenaline. Dopamine β-hydroxylase catalyses the conversion of dopamine to noradrenaline and is located within the membrane of catecholamine storage vesicles. The enzyme is a mixed function oxidase requiring molecular oxygen and a co-reductant. Although vitamin C can act in this capacity *in vitro*, it is possible that vitamin E takes this role *in vivo*, having easy access to the enzyme on the inside of the membrane.

In guinea-pig isolated portal vein, hypoxia reduces spontaneous muscle activity and contractile responses to electrical stimulation but does not affect contractile responses to a range of noradrenaline concentrations. However, substitution of glucose in the bathing solution by sucrose, a substrate unavailable to the cells for energy generation, produced a marked enhancement of the effect of hypoxia, with spontaneous activity reduced by 76%, electrically induced activity by 80% and noradrenaline responses by 85% [206]. Although the activity and responses of the portal vein were unaffected by the presence of vitamin E in normoxia, the vitamin significantly protected the functioning of the vein in hypoxic conditions (*Figure 5.3*). This effect was dose-dependent within the range 10–168 μM and was most marked when glucose was replaced by sucrose in the bathing solution [207]. A study of the mechanism of this protection, using four spasmogenic agents, methoxamine, acetylcholine, histamine and potassium, each with a different mode of action, produced responses which were significantly reduced by hypoxia and absence of glucose in the bathing solution. However, vitamin E failed to protect against the effect of hypoxia using any of these spasmogenic agents [208]. The findings suggested

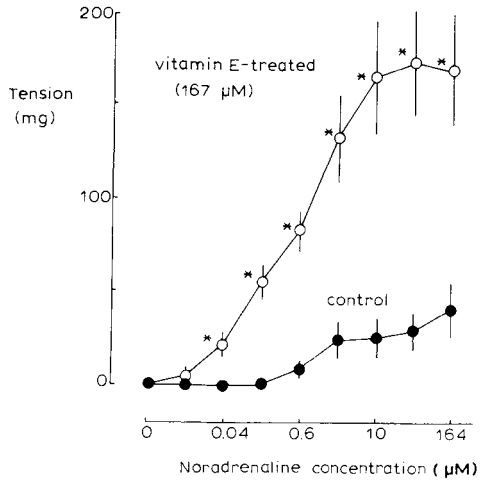


Figure 5.3. Noradrenaline cumulative concentration–effect curves from guinea-pig hypoxic portal vein [211]. All tissues were maintained in glucose-free physiological salt solution in hypoxic conditions. The vertical axis shows the mean force of contraction in response to each noradrenaline concentration on the horizontal axis. The statistical significance of differences between control (n = *20) and vitamin-E-treated* (n = *25) muscle responses is shown by* *, P < *0.001. Vertical lines indicate the standard errors for each mean.*

that the mechanism of protection might be specific for noradrenaline, possibly by changing the handling of this transmitter substance. This hypothesis was investigated by using drugs, cocaine, hydrocortisone and tyramine, known to interfere with the uptake and removal of noradrenaline from sympathetic synapses. However, they did not affect the response to vitamin E and these data therefore indicate that the protective effect of the vitamin is relatively specific for spontaneous activity, responses to electrical stimulation and exogenously administered noradrenaline but is not involved with the extracellular synaptic disposition of noradrenaline [209].

It is also possible that the protective action of vitamin E in hypoxia might be the result of activation of an anaerobic energy-producing pathway. Incubation of the tissues with iodoacetate, a glycolysis inhibitor, abolished the action of the vitamin [210]. The possibility of an activation of glycogen breakdown by the vitamin has been investigated [211]. Lactic acid, the end-product of anaerobic glycolysis, accumulated in the hypoxic portal vein, but its production was not increased by the presence of vitamin E, suggesting that this was not the primary site for the protective mechanism. Interestingly, vitamin E can

maintain 2,3-diphosphoglycerate levels in stored human red blood cells, giving a 6-fold time improvement over control cells [212], and this chemical can facilitate oxygen release by red blood cells.

CALCIUM INVOLVEMENT

The involvement of Ca^{2+} in the mechanism of action of the vitamin has been investigated by several groups. Both verapamil and vitamin E have been shown to protect cardiac and vascular tissue against the deleterious effects of oxygen deprivation [168, 213, 214]. Using spontaneous activity in guinea-pig portal vein as the model, a comparison of the pharmacological response to each agent was made in normoxia and hypoxia (*Table 5.3*). The results show that, whereas

Table 5.3. COMPARISON OF THE EFFECT OF VERAPAMIL AND VITAMIN E ON THE GUINEA-PIG ISOLATED PORTAL VEIN IN NORMOXIA AND HYPOXIA [211]
Figures are mean tension levels (mg) for each situation. Each hypoxic control was significantly different from its normoxic control and each drug treatment was significantly different from its hypoxic control.

Normoxic control	*Hypoxic control*	*Hypoxia + drug*	
253	140	240	vitamin E
231	96	33	verapamil
203	105	37	vitamin E + verapamil

verapamil causes a further decline in activity in hypoxia, vitamin E enhances this response, giving levels of tension comparable with those found in normoxia. Therefore, since vitamin E does not produce the same pharmacological profile as the extra-cellular calcium antagonist, verapamil, it is unlikely that this is the mode of action of the vitamin in this vascular model. Vitamin E was found, however, to reduce the rate of calcium uptake by mitochondria in cells and it is possible that this could form the basis of an explanation of the protective action and the maintenance of residual muscle activity by the vitamin in hypoxic conditions. The lack of effectiveness of the vitamin in protecting hypoxic vascular tissue when muscle stimulants other than noradrenaline are present tends to argue against a general antioxidant mechanism, and elevated intracellular calcium levels have been proposed as one mechanism for stimulating contractile activity in smooth muscle [215].

In rat isolated hepatocytes [216], vitamin E protected against the toxic effect

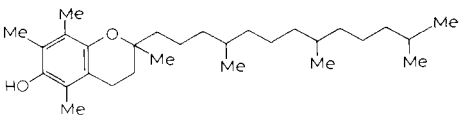
of the calcium ionophore, A23187, in a Ca^{2+}-free medium. However, when calcium was present the protectant effect was not apparent.

Hall and Wolf [217] have proposed a hypothesis, concerning the pathogenesis of post-traumatic central nervous system ischaemia, which integrates an injury-induced rise in intracellular Ca^{2+}, the increased synthesis of vasoactive prostanoids and progressive microvascular lipid peroxidation. The model used anaesthetised cats with a contusion injury to the lumbar spinal cord. Antioxidants, vitamin E and selenium, were compared with various Ca^{2+} antagonists, cyclo-oxygenase inhibitors, a thromboxane synthetase inhibitor and the stable prostacyclin analogue. The most impressive preservation of post-traumatic spinal cord blood flow was provided by the antioxidants.

Further instances where vitamin E and Ca^{2+} appear to interact involve skeletal and cardiac muscle. Feeding rats a diet enriched with vitamin E or addition of vitamin E to a suspension of sarcoplasmic reticular membranes of rat and rabbit skeletal muscles protects Ca^{2+}-dependent ATPase against thermal inactivation aggravated by the action of free fatty acids [218, 219]. The formation of malondialdehyde in heart tissue and in coronary perfusate of vitamin-E-deficient rat heart was measured during sudden changes in Ca^{2+} concentration. There was an absence of malondialdehyde after Ca^{2+} alterations in normal animals, whereas it could be detected in vitamin-E-deficient rats after Ca^{2+} changes. The extent of protection offered by the vitamin seemed to be related to its tissue concentration and the authors suggest a role for vitamin E against oxidative stress in heart tissue [220].

STRUCTURAL REQUIREMENTS FOR ACTIVITY

In intestinal tissue, vitamin E has little or no effect in normoxia. A study [221] using rabbit intestine showed that vitamin E had a weak inhibitory action which was direct and independent of adrenergic or cholinergic nervous influences. In guinea-pig colon, the vitamin was without action in normoxia. In hypoxia, where the spontaneous contractile activity was replaced by quiescence, vitamin E delayed the decline in activity [222]. However, once hypoxia had been achieved, vitamin E was able to induce an increase in contractile activity.



(9)

This effect was seen only in hypoxia, not normoxia, was dose-dependent within the range 0.2–200 μM, and provided a simple *in vitro* model for investigating the mode of action of the vitamin. By examining the structure-activity relationship of the response, compounds with a phytyl side-chain, phytol and vitamin K-1 (phytomenadione), of similar length to vitamin E (9) were found to be also active, but compounds that had structures which resembled the chroman ring of the vitamin, vitamin K-3 (menaphthone) and Trolox, were antagonists of the responses to the phytol side-chain effects.

In heart tissue, phytol also had an effect similar to that of vitamin E, while Trolox did not (*Table 5.4*) [223], suggesting that the phytyl side-chain was

Table 5.4. EFFECT OF VITAMIN E, PHYTOL AND TROLOX ON ATRIAL HEART RATE IN HYPOXIA [223]

	Control hypoxia	*Hypoxia + drug*
Vehicle	51	50
Vitamin E	47	77*
Phytol	47	73*
Trolox	50	43

* Statistically significant difference from control value, expressed in beats per minute.

necessary for activity. Several tocopherol-like compounds have been investigated for their cardiovascular therapeutic potential. Two isoprenoids, 5-nicotinoxymethyl-γ-tocopheryl nicotinate and decaprenoic ethyl ester, were studied in rats [224]. The agents did not affect blood pressure in normotensive animals, but significantly reduced blood pressure in spontaneously hypertensive rats and DOCA/salt hypertensive animals. This anti-hypertensive effect was mild and long-lasting but its mechanism was uncertain. Another potential antihypertensive drug (TF80, an α-tocopheryl ester of fusaric acid) was studied in normotensive and hypertensive rats [225] and found also to have a mild and long-lasting antihypertensive action. The agent was found not to be a diuretic and did not antagonize the rise in blood pressure produced by noradrenaline, $PGF_{2\alpha}$ or angiotensin II in spontaneously hypertensive rats.

To investigate the mode of action of vitamin E and related structures using the colon model, two hypotheses were tested: (1) that their antioxidant properties contribute to the response; and (2) that the vitamin was acting as an agonist on a 'receptor' with structural requirements for producing activity in hypoxia. Testing the various available tocopherols on the colon model

revealed that the order of potency was markedly different from previous biopotency methods [4] and also different from the order of measured antioxidant capacity [226]. The ratios of potency are shown in *Table 5.5*. One

Table 5.5. COMPARISON OF BIOPOTENCY OF THE TOCOPHEROLS, USING THE COLON MODEL AND RAT FOETAL RESORPTION, WITH THEIR ANTIOXIDANT CAPACITY

Adapted from Refs. 4, 226 and others.

	Antioxidant capacity	*Rat foetal resorption*	*Colon model*
(±)-α-Tocopherol	100	100	100
(±)-β-Tocopherol	71	32	–
(±)-γ-Tocopherol	66	6	553
(±)-δ-Tocopherol	27	1	1028

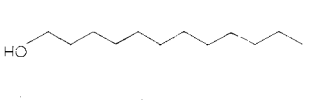
can see that, although the figures for the standard potency determination (rat foetal resorption) differ from the antioxidant measurement, there is the same order of potency, whereas with the colon model this order is dramatically different, offering little support to an antioxidant mode of action. In addition, the antioxidant capacity of the chromanol ring structure, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), is about 40% of that of vitamin E [226], but in the colon it has no activity and indeed antagonizes the action of the vitamin. The results of a study of the structural requirements for activity in the colon model are shown in *Table 5.6*. The results indicate that the chains themselves can confer activity and that a certain length is required for this action. The ring structures on their own have no activity and are antagonists of the actions of the chains. Analysis of these relationships gives little support to the antioxidant hypothesis in the colon model system. Tests on vitamin E and nine isoprenologues with respect to correction of the normalized activity index of the thyroid gland also showed an effect which was in reverse order to the antioxidant potency [227, 228].

Other studies have shown biological actions from these kinds of structure and indicate interrelationships between the chemicals in terms of their potencies. Tomita [229] found that the substances, vitamin A, vitamin K, vitamin E, β-carotene, ubiquinone (15), phytol and squalene (16), from green-yellow vegetables could suppress the growth of tumour cells and enhance T-cell cytotoxicity, but β-carotene, which does have both ends of the chain substituted with a bulky β-ionone ring on each end-group did not. Hydrophobic chain

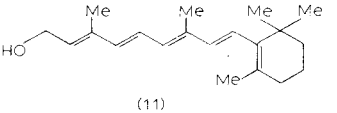
Table 5.6. STRUCTURE-ACTIVITY RELATIONSHIP FOR COMPOUNDS CONTAINING THE PHYTOL SIDE-CHAIN AND CHROMANOL RING STRUCTURES RELATED TO VITAMIN E

Compound		*Activity in hypoxia*	*No activity in hypoxia*	*Mean EC_{50} value in hypoxia (μM)*	*Antagonist*
Vitamin K-1	(8)	*	–	21	–
Vitamin E	(9)	*	–	10	–
Phytol	(6)	*	–	1	–
Lauryl alcohol	(10)	*	–	7	–
Vitamin A	(11)	*	–	20	–
Decanol	(12)	–	*	–	–
Allyl alcohol	(13)	–	*	–	–
Vitamin C	(14)	–	*	–	–
Vitamin K-3	(7)	–	*	–	*
Trolox[a]	(5)	–	*	–	*

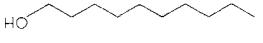
[a] 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.



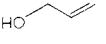
(10)



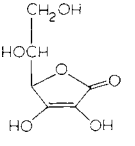
(11)



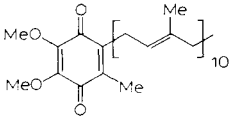
(12)



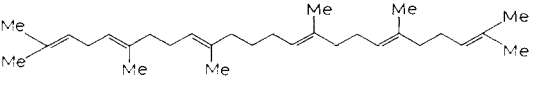
(13)



(14)



(15)



(16)

molecules of a certain length are capable of interfacing with the cell membrane (for example, lectin-induced stimulation of immune response). In this case, it did not seem to matter whether the chain was saturated or not to obtain the augmentation of the anti-tumour rejection. Vitamin E has been found to protect cultured human lymphoblastoid cells from the toxic effect of retinol. This effect was unrelated to lipid peroxidation and may be mediated by a membrane-stabilizing action [230]. In an investigation of the age-related accumulation of lipofuscin, vitamin E was found to interact with vitamin A ester metabolites *in vivo* in a manner more complex than simply acting as an antioxidant protectant [231]. Another situation where the antioxidant effects of vitamin E were of lesser importance than a different pharmacological effect was with its interactions with retinol and total vitamin A in the rat [232]. Retinyl palmitate hydrolase was inhibited by vitamin E and vitamin K-1. *N,N'*-Diphenyl-*p*-phenylenediamine, the most effective synthetic vitamin E substitute known, had little effect on the hydrolase. Vitamin E affects vitamin A metabolism in several tissues, and this suggests that it may be a physiological effector of tissue retinol homeostasis.

An attempt was made to analyse the mechanism of action of the vitamin using pharmacological methodology in the colon model [222]. The mechanism of the response was investigated by using known agonists and antagonists of contractile responses in intestinal smooth muscle. Responses to vitamin E, phytol and vitamin K-1, were not antagonized by the serotonin blocker, methysergide, or the antihistamine, mepyramine, but were all antagonized by the acetylcholine muscarinic receptor antagonist, atropine. However, vitamin K-3, the compound that antagonized the responses to vitamin E, phytol and vitamin K-1, could not block the effects of acetylcholine in hypoxia. This suggested that the stimulant agents were acting at a point before the muscarinic receptors in the colon and possibly through the release of acetylcholine. Physostigmine, an acetylcholinesterase inhibitor, potentiates responses to acetylcholine in the colon and it potentiated the responses to vitamin E, phytol and vitamin K-1. Hexamethonium, the ganglionic nicotinic receptor blocker, did not affect the responses to the agonists in hypoxia. These data indicate that the responses to vitamin E, phytol and vitamin K-1 in hypoxia are mediated through release of acetylcholine, probably from post-ganglionic nerve endings. These release sites can be blocked by both vitamin K-3 and Trolox in what appears to be a competitive manner (unpublished results). A possible scheme for these interactions is shown in *Figure 5.4*.

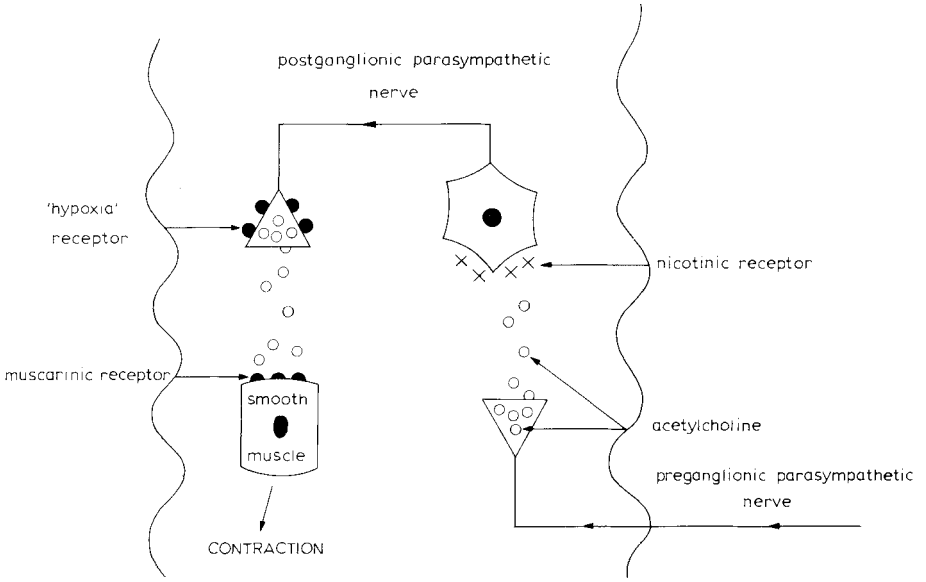


Figure 5.4. Schematic representation of possible sites of action of vitamin E and related chemicals in hypoxic colon.

MUSCLE ACTIVITY

In Refsum's disease, characterized by cerebral ataxia, retinitis pigmentosa, muscle weakness and peripheral neuropathy, there is accumulation of phytanate and it is treated by dietary management with a low phytanate and phytol intake [233]. However, the clinical improvement is incomplete and, since similar symptoms seen in abetalipoproteinaemia can be prevented and reversed by vitamin E therapy, this possibility was investigated using a model of rat skeletal muscle damage in vitamin-E-deficient rats. Using phytol, the phytanate was found to augment the muscle damage induced by vitamin E deficiency. It is suggested that the phytanate may displace vitamin E from the membranes, thereby enhancing the risk of free-radical damage, a theory supported by elevated muscle malondialdehyde content. Such a competitive process between vitamin E and phytanate may operate and be important in the pathogenesis of Refsum's disease and therefore this disease could lend itself to vitamin E treatment. The action of vitamin E has also been examined on rat neuromuscular junction [234, 235], where the vitamin was found to leave miniature end-plate potential frequency and resting membrane potential

unchanged but, when deficient, to produce a slight tendency towards increased miniature end-plate potential frequency. Electrophysiological measurement of facilitation and depression at the neuromuscular junction indicated that the vitamin was without effect. However, recently we have examined the potential protective action of vitamin E in skeletal muscle hypoxia using the rat phrenic nerve diaphragm preparation. The phrenic nerve was stimulated and the contractions of the diaphragm muscle were recorded. In normoxia, such responses will continue for many hours, but in hypoxia the contractions steadily decline and cease after about 20 min. While vitamin E (10–100 μM) had no effect on the activity of the nerve muscle preparation in normoxia, in hypoxia it significantly increased the time taken for muscle exhaustion, by between 50–100% (*Table 5.7*). It is not yet known whether the site of this protection is

Table 5.7. EFFECT OF VITAMIN E ON RESPONSES OF RAT PHRENIC NERVE-DIAPHRAGM PREPARATION IN HYPOXIA

Treatment	*Concentration (μM)*	*Time taken for abolition of contractions in hypoxia (min) (mean ± S.E.M. and* n*)*	
Vehicle		20.18 ± 1.37	$n = 16$
Vitamin E	1	32.70 ± 4.37	$n = 10$
Vitamin E	30	30.33 ± 3.11	$n = 6$
Vitamin E	100	40.42 ± 4.15	$n = 6$

the nerve ending or the muscle. In rats, pinched nerve endings could be damaged by phospholipases. Vitamin E could protect against this effect by restoring transmembrane potentials, probably through binding to fatty acid products [236]. The chroman compound shared some but not all of these properties [237] and the 6-hydroxy groups and the phytol side-chain are necessary for the action [238]. Certainly the hydroxy group of tocopherol is more important than that of cholesterol in influencing its interactions with phospholipids [239].

VITAMIN-E-BINDING SITES

Vitamin-E-binding sites have been demonstrated in a number of tissues. Catignani [240] has shown that the vitamin can bind to a cytoplasmic protein, with a molecular weight of about 31000, extracted from rat liver. Similar cytoplasmic proteins have been reported for 1,25-dihydroxycholecalciferol [241, 242], retinol [243, 244] and retinoic acid [245, 246]. The vitamin-E-

binding protein from rat liver required an intact chromanol ring and a free hydroxy group for specific binding.

The membranes of isolated adrenocortical cells from the rat have been found to contain binding sites for (+)-α-tocopherol which have specificity, saturability, time and temperature dependence, together with reversibility of binding [247]. Measurements of binding constants indicate that these sites may be physiologically significant and it is known that the adrenal gland has high concentrations of vitamin E as well as being a rich source of polyunsaturated fatty acids [248]. Competitive binding of tocopherol analogues suggested that the specificity of binding in adrenocortical cells is similar to that in rat liver cytoplasm.

In human erythrocyte samples, specific binding sites have also been described [249]. In this situation, there appears to be a high-affinity–low-capacity site and a low-affinity–high-capacity binding site, both of which have a protein component. The binding of vitamin E in perfused rabbit heart was distributed mainly to the nuclei of the muscle cells, although mitochondria, too, had significant binding capacity [250]. The vitamin was also found to be capable of stimulating RNA synthesis.

The vitamin can be carried by the high-affinity receptor for low-density lipoprotein in fibroblasts [251]. It has been suggested to act *in vivo* as a specific enzyme inhibitor (of lipoxygenase). A vitamin-E-lipoxygenase complex *in vivo* could terminate the initiation of free radicals and other oxidized products. The binding of the vitamin to the enzyme is probably through the hydrophobic chain and involves one peptide [135].

POSSIBLE VITAMIN E RECEPTORS AND FUTURE EXPERIMENTS

In conclusion, there seems to be enough evidence to suggest that vitamin E is capable of acting on cells and producing effects in cells which are not related exclusively to an antioxidant mechanism of action. In some situations, as in the colon model described earlier, it appears to act at sites which are accessible to simple straight-chain structures which have no antioxidant activity and whose potency is determined by their chain length. In one original scheme of the membrane interaction of the vitamin [252], the methyl groups of the phytyl tail were seen to have an attractive hydrophobic interaction with 'pockets' provided by the double bonds of arachidonic acid, but in this colon model, the methyl groups do not appear essential for initiation of a response. Some recent evidence from a related field may raise interesting questions about the mode of action of vitamin E and related structures. This work [253–255], using firefly luciferase, shows that a pure soluble protein can bind to and be inhibited by

a diverse range of simple anaesthetic molecules at concentrations similar to those that cause anaesthesia. The anaesthetics appear to bind to a common site, probably a hydrophobic pocket, which may be the site of anaesthetic inhibition. The mechanism of action of anaesthesia could be explained by interaction with a protein rather than by the more generally accepted lipid interaction. It is possible that similar protein sites of action or 'receptors' exist for the tocopherols and related chemicals. These sites may have hydrophobic regions of fixed size which could be linked to intracellular events such as release of transmitter or mobilization of ions leading to physiological or pharmacological effects. It is notable that, in all of the muscle types tested, cardiac, skeletal, vascular and intestinal smooth muscle, in hypoxia vitamin E is capable of offering some protection. These effects are seen within a physiological concentration range and could represent a mechanism by which the cells of the body are able to protect themselves from some of the consequences of hypoxia. This may be through stimulation of a 'vitamin E receptor', which might also respond to vitamins A and K, and, because the effect is proportional to concentration, it might be amenable to dietary or pharmacological enhancement.

Further work on the pharmacology of vitamin E will need to establish whether the agents tested so far, on the colon model, with structural similarities to the natural vitamin are capable of protecting the functioning of other tissues from the deleterious effects of hypoxia. If an order of potency for such a series of agonists could be established which was similar in different muscle types, this would provide further evidence for a more generalized 'hypoxia receptor'. Experiments using the chroman ring structure of vitamin E and similar ring configurations in hypoxic colon indicate that such agents are antagonists of protective responses. It would be interesting if agents like Trolox and vitamin K-3 could be found to inhibit responses to vitamin E and similar chain structures in all of these muscle types. In pharmacological terms, the potency of such antagonists could then be measured and quantified in order of pA_2 values, and the nature of such antagonism could be assessed as competitive or non-competitive, using vitamin E as the agonist.

The hypothesis that 'hypoxia receptors' exist, and can be stimulated by vitamin E and agents in the body which have a structural similarity to the vitamin but which are not necessarily antioxidants, might be useful as a way of investigating the body's physiological and biochemical response to hypoxia. The mechanism of this response could also offer a pharmacological perspective in which new anti-hypoxia drugs could be generated.

ACKNOWLEDGEMENTS

The author would like to thank I.C.I. Pharmaceuticals for library/information services, Hoffman-La Roche for materials, and Jan for typing the text.

REFERENCES

1 M.K. Horwitt, C.C. Harvey, G.D. Duncan and W.C. Wilson, Am. J. Clin. Nutr., 4 (1956) 408.
2 M.K. Horwitt, Am. J. Clin. Nutr., 8 (1960) 451.
3 M.K. Horwitt, C.C. Harvey, B. Century and L.A. Witting, J. Am. Diet. Assoc., 38 (1961) 231.
4 L.J. Machlin, in Handbook of Vitamins; Nutritional, Biochemical and Clinical Aspects, ed. L.J. Machlin (Marcel Dekker, New York, 1984) pp. 99–145.
5 G.M. Berneske, A.R. Butson, E.N. Gauld and D. Levy, Can. Med. Assoc. J., 82 (1960) 418.
6 H.G. Mandel and V.H. Cohn, in The Pharmacological Basis of Therapeutics, eds. A.G. Gilman, L.S. Goodman, T.N. Rall, F. Murad (MacMillan, New York, 1985) Ch. 67, pp. 1573–1591.
7 British National Formulary, ed. C.F. George (British Medical Association and The Pharmaceutical Society, London) No. 12, 1986, pp. 310–311.
8 L.J. Machlin (ed.) Vitamin E: A Comprehensive Treatise (Marcel Dekker, New York, 1980).
9 H. M. Evans and K.S. Bishop, Science (Washington, DC), 56 (1922) 650.
10 B. Sure, J. Biol. Chem., 58 (1924) 693.
11 H.M. Evans, Proc. Natl. Acad. Sci. U.S.A., 11 (1925) 373.
12 H.A. Mattill, J.S. Carman and M.M. Clayton, J. Biol. Chem., 61 (1924) 729.
13 K.E. Mason, J. Exp. Zool., 45 (1926) 159.
14 F.B. Adamstone and L.E. Card, J. Morphol., 56 (1934) 339.
15 A.M. Pappenheimer and M. Goettsch, J. Exp. Med., 53 (1931) 11.
16 M. Goettsch and A.M. Pappenheimer, J. Exp. Med., 54 (1931) 145.
17 H.M.Evans, O.H. Emerson and G.A. Emerson, J. Biol. Chem., 113 (1936) 319.
18 O.H. Emerson, G.A. Emerson, A. Mohammed and H.M. Evans, J. Biol. Chem., 122 (1937) 99.
19 E.J. Fernholz, J. Am. Chem. Soc., 60 (1938) 700.
20 P. Karrer, H. Fritzche, B.M. Ringier and H. Salomon, Nature (London), 141 (1938) 1057.
21 H.S. Olcott and O.H. Emerson, J. Am. Chem. Soc., 59 (1937) 1008.
22 H. Dam, J. Glavind, O. Bernth and E. Hagens, Nature (London), 142 (1938) 1157.
23 H. Dam and H. Granados, Acta Physiol. Scand.,10 (1945) 162.
24 E.P. Singsen, R.H. Bunnell, L.D. Matterson, A. Kozoff and E.L. Jungherr, Poult. Sci., 34 (1955) 262.
25 L.L. Modsen, C.M. McCay and L.A. Maynard, Proc. Soc. Exp. Biol. Med., 30 (1953) 1434.
26 C.G. Mackenzie, J.B. Mackenzie and E.V. McCollum, J. Nutr., 21 (1941) 1157.
27 N.G. Shimotori, G.A. Emerson and H.M. Evans, J. Nutr., 19 (1940) 547.
28 K. Schwarz and C.M. Foltz, J. Am. Chem. Soc., 79 (1957) 3292.
29 H. Dam and J. Glavind, Nature (London), 142 (1938) 1077.

30 H. Dam and J. Glavind, Nature (London), 143 (1939) 810.
31 H. Dam, Pharmacol. Rev., (1957) 1.
32 F.I. Dessau, L. Lipchuck and S. Klein, Proc. Soc. Exp. Biol. Med., 87 (1954) 522.
33 G.J. Martin and F.B. Faust, Exp. Med. Surg., 5 (1947) 405.
34 O.B. Houchin and P.W. Smith, Am. J. Physiol., 141 (1944) 242.
35 T.W. Gullickson and C.E. Calverley, Science (Washington, DC), 104 (1946) 312.
36 P. Gyorgy and C.S. Rose, Science (Washington, DC), 108 (1948) 716.
37 P. Gyorgy and C.S. Rose, Ann. N.Y. Acad. Sci., 52 (1949) 231.
38 F.B. Adamstone, Arch. Pathol., 31 (1941) 613.
39 H.Popper, P. Gyorgy and H. Goldblatt, Arch. Pathol., 37 (1944) 161.
40 Z. Menschik and T.J. Szczesniak, Anat. Rec., 103 (1949) 349.
41 R.D. Lillie, F.S. Daft and W.H. Sebrell, Public Health Rep., 56 (1941) 1255.
42 E. Segagni, Minerva Pediatr., 7 (1955) 985.
43 J.F. Pennock, F.W. Hemming and J.D. Kerr, Biochem. Biophys. Res. Commun., 17 (1964) 542.
44 O. Isler, J. Mayer, J. Metzger, R. Ruegg and P. Schudel, Angew. Chem., 75 (1963) 1030.
45 R.S. Cahn, C.K. Ingold and V. Prelog, Experientia, 12 (1956) 81.
46 P. Schudel, H. Mayer, J. Metzger, R. Ruegg and O. Isler, Helv. Chim. Acta, 46 (1963) 2517.
47 H. Mayer, J. Metzger and O. Isler, Helv. Chim. Acta, 50 (1967) 1376.
48 S. Kasparek, in Ref. 8, pp. 7–65.
49 J. Bunyan, J. Green, E.E. Edwin and A.T. Diplock, Biochem. J., 75 (1960) 460.
50 V. Demole, O. Isler, B.H. Ringier, H. Salomon and P. Karrer, Helv. Chim. Acta, 22 (1939) 65.
51 T. Tatsuta, Vitamins, 45 (1972) 247.
52 L.I. Smith, W.B. Renfrew and J.W. Opie, J. Am. Chem. Soc., 64 (1942) 1082.
53 P. Karrer and K.S. Yap, Helv. Chim. Acta, 24 (1941) 640.
54 P.B. Kingsley and G.F. Combs, Proc. Soc. Exp. Biol. Med., 166 (1981) 1.
55 M. Joffe and P.L. Harris, J. Am. Chem. Soc., 65 (1943) 925.
56 M.H. Stern, C.D. Robeson, L. Weisler and J.G. Baxter, J. Am. Chem. Soc., 69 (1947) 869.
57 R.J. Ward, Br. J. Nutr. 12 (1958) 226.
58 T. Tatsuta, Vitamins, 44 (1971) 185.
59 H. Weiser and M. Vecchi, Int. J. Vitam. Nutr. Res., 51 (1981) 100.
60 H. Weiser and M. Vecchi, Int. J. Vitam. Nutr. Res., 52 (1982) 351.
61 H.S. Olcott and H.A. Matill, Chem. Rev., 29 (1941) 257.
62 B. Century and M.K. Horwitt, Fed. Proc., 24 (1965) 906.
63 H. Dam, Pharmacol. Rev., 9 (1057) 1.
64 L.J. Machlin, J. Am. Oil Chem. Soc., 40 (1963) 368.
65 J.A. Lucy, in Tocopherol, Oxygen and Biomembrane, eds. E. de Duve and O. Hayaishi (Elsevier/North-Holland Biomedical Press, New York, 1978) pp. 109–119.
66 G.W. Burton, Y. Le Page, E.J. Gabe and K.U. Ingold, J. Am. Chem. Soc., 102 (1980) 7791.
67 J.A. Howard and K.U. Ingold, Can. J. Chem., 41 (1963) 1744.
68 I. Molenaar, C.E. Hulstaert and M.J. Hadonk, in Ref. 8, pp. 372–389.
69 P.B. McCay and M.M. King, in Ref. 8, pp. 289–317.
70 P.B. McCay, Annu. Rev. Nutr., 5 (1985) 323.
71 M. Halks-Miller, M. Henderson and L.F. Eng, J. Neuropathol. Exp. Neurol., 45 (1986) 471.
72 L.J. Willmore and J.J. Rubin, Brain Res., 296 (1984) 389.

73 A. Nagaoka, M. Suno, M. Shibota and M. Kakihana, Nippon Yakurigaku Zasshi, 84 (1984) 303.
74 M. Suno and A. Nagaoka, Biochem. Biophys. Res. Commun., 125 (1984) 1046.
75 K. Ohtsuka, N. Ohishi, G. Eguchi and K. Yagi, Experientia, 38 (1982) 120.
76 F.W. Summerfield and A.L. Tappel, Mutat. Res., 126 (1984) 113.
77 K. Tayyaba and M. Hasan, Acta Pharmacol. Toxicol., 57 (1985) 190.
78 T.L. Perry, V.W. Yong, R.M. Clavier, K. Jones, J.M. Wright, J.G. Foulks and R.A. Wall, Neurosci. Lett., 60 (1985) 109.
79 V.W. Yong, T.L. Perry and A.A. Krisman, Neurosci. Lett., 63 (1986) 56.
80 G. Martinovits, E. Melamed, O. Cohen, J. Rosenthal and A. Uzzan, Neurosci. Lett., 69 (1986) 192.
81 J.L. Cadet, Med. Hypotheses, 20 (1986) 87.
82 P. Constantinides, M. Harkey and D. McLaury, Virchows Arch. A: Pathol. Anat. Histol., 409 (1986) 583.
83 M.T. Santos, J. Walles, J. Agnor and J. Vilches, J. Clin. Pathol., 33 (1980) 973.
84 F. Violi, C. Alessandri, L. Iuliano, S. Frattaroli, A. Chiselli and F. Balsano, Stroke, 16 (1985) 14.
85 J.C. Dousset, M. Trouilh and M.J. Foglietti, Clin. Chim. Acta, 129 (1983) 319.
86 F. Flamigni, C. Guarnieri, R. Toni and C.M. Caldarero, Int. J. Vit. Nutr. Res., 52 (1982) 402.
87 A. Salminen, H. Kainulainen, A.U. Arstila and V. Vihko, Acta Physiol. Scand., 122 (1984) 565.
88 P.K. Singal, R.E. Beamish and N.S. Dhalla, Adv. Exp. Med. Biol., 161 (1983) 391.
89 J.E. Redetzki, K.E. Griswold, C. Nopajaroonsri and H.M. Redetzki, J. Toxicol. Clin. Toxicol., 20 (1983) 319.
89a J.R. Brown and S.H. Imam, Prog. Med. Chem., 21 (1984) 169.
90 J. Milei, A. Boveris, S. Llesuy, H.A. Molina, R. Storino, D. Ortega and S.E. Milei, Am. Heart J., 111 (1986) 95.
91 M. Suzuki, S. Katamine and S. Tatsumi, J. Nutr. Sci. Vitaminol., 29 (1983) 141.
92 D.M. Skillrud and W.J. Martin, Am. Rev. Respir. Dis., 129 (1984) 995.
93 D. Giamalva, D.F. Church and W.A. Pryor, Biochem. Biophys. Res. Commun., 133 (1985) 773.
94 N. Sakuma, Hokkaido Igaku Zasshi, 59 (1984) 549.
95 I.D. Capel, D. Leach and H.M. Dorrell, Anticancer Res., 3 (1983) 59.
96 K.E. Hill and R.F. Burk, Biochem. Pharmacol., 33 (1984) 1065.
97 H. Mowri, S. Nojima and K. Inoue, J. Biochem., 95 (1984) 551.
98 C. Cordova, F. Violi, A. Ghiselli, C. Alessandri and S. Frattaroli, Thromb. Haemostasis, 55 (1986) 296.
99 K.W. Buckingham, J. Nutr., 115 (1985) 1425.
100 T. Ohyashiki, H. Ushiro and T. Mohri, Biochem. Biophys. Acta, 858 (1986) 294.
101 T. Ozawa and A. Handki, Biochem. Int., 6 (1983) 685.
102 G.W. Burton, K.H. Cheeseman, T. Doba, K.U. Ingold and T.F. Slater, Ciba Found. Symp., 101 (1983) 4.
103 P. Lamberlet and J. Leoliger, J. Chem. Phys. Lipids, 35 (1984) 185.
104 E. Niki, A. Kawakami, M. Sato, Y. Yamamoto, J. Tsuchiya and Y. Kamiya, J. Biol. Chem., 260 (1985) 2191.
105 J.A. Lindsey, H.F. Zhang, H. Kaseki, N. Morisaki, T. Sato and D.G. Cornwell, Lipids, 20 (1985) 151.

106 E.A. Serbinova, R.A. Bakalova, TsS. Stoichev and V.E. Kagan, Biull. Eksp. Biol. Med., 102 (1986) 419.
107 N. Suttorp, W. Toepfer and L. Roka, Am. J. Physiol., 251 (1986) 671.
108 P.W. Ramwell, E.M. Leovey and A.L. Sintentos, Biol. Reprod., 16 (1977) 70.
109 A.J. Marcus, J. Lipid Res., 19 (1978) 793.
110 S. Moncada, R. Gryglewski, S. Bunting and J. Vane, Nature (London), 263 (1976) 663.
111 P. Isakson, A. Raz, S. Denny, E. Pure and P. Needleman, Proc. Natl. Acad. Sci. U.S.A., 71 (1977) 101.
112 F. Sun, Biochem. Biophys. Res. Commun., 74 (1977) 1432.
113 M. Hamberg, J. Sevensson and B. Samuelsson, Proc. Natl. Acad. Sci. U.S.A., 72 (1975) 2994.
114 E. Anggard, Ann. N. Y. Acad. Sci., 180 (1971) 200.
115 B. Samuelsson, S. Hammarstrom, R.C. Murphy and P. Borgeat, Allergy, 35 (1980) 375.
116 S. O'Regan and C. Turgeon, Pathol. Res. Pract., 176 (1983) 297.
117 R.O. Likoff, D.R. Guptill, L.M. Lawrence, C.C. McKay, M.M. Mathias, C.F. Nockels and R.P. Tengerdy, Am. J. Clin. Nutr., 34 (1981) 245.
118 M. Meydani, S.N. Meydani, J.B. Macauley and J.R. Blumberg, Prostaglandins Leukotrienes Med., 18 (1985) 337.
119 S.N. Meydani, M. Meydani, C.P. Verdon, A.A. Shapiro, J.B. Blumberg and K.C. Hayes, Mech. Ageing Dev., 34 (1986) 191.
120 M.F. McCarthy, Med. Hypotheses, 19 (1986) 345.
121 W. Martin, Med. Hypotheses, 15 (1984) 305.
122 W.C. Hope, L.J. Machlin, R.J. Filipski and F.M. Vane, Prostaglandins, 10 (1975) 557.
123 M. Stuart, Ann. N. Y. Acad. Sci., 393 (1982) 277.
124 J. Green and J. Bunyan, Nutr. Abstr. Rev., 39 (1969) 321.
125 A.C. Chan and M.K. Leith, Am. J. Clin. Nutr., 34 (1981) 2341.
126 G.A. Fitzgerald and A.R. Brash, Ann. N. Y. Acad. Sci. 393 (1982) 209.
127 A.C. Chan, E.T. Pritchard and P.C. Choy, J. Nutr., 113 (1983) 813.
128 E. Giani, I. Masi and C. Galli, Lipids, 20 (1985) 439.
129 S.L. Swartz, W.C. Willett and C.H. Hennekens, Prostaglandins Leukotrienes Med., 18 (1985) 105.
130 R. Mower and M. Steiner, Prostaglandins Leukotrienes Med., 10 (1983) 389.
131 C.E. Douglas, A.C. Chan and P.C. Choy, Biochim. Biophys. Acta, 876 (1986) 639.
132 A.C. Chan, C. Raynor, C. Douglas, J. Patrick and M. Boland, Am. J. Clin. Nutr., 44 (1986) 278.
133 K.A. Pritchard, N.J. Greco and R.V. Panganamala, Thromb. Haemostasis, 55 (1986) 6.
134 P. Reddanna, M. Rao and C.C. Reddy, FEBS Lett., 193 (1985) 39.
135 S. Grossman and E.G. Waksman, Eur. J. Biochem., 16 (1984) 281.
136 S. Villa, A. Lorica, G. Morazzoni, G. de Gaetano and N. Semeraro, Agents Actions, 19 (1986) 127.
137 K.C. Strivastava, Prostaglandins Leukotrienes Med., 21 (1986) 177.
138 C.W. Karpen, S. Cataland, T.M. O'Dorisio and R.V. Panganamala, Diabetes, 33 (1984) 239.
139 C.W. Karpen, S. Cataland, T.M. O'Dorisio and R.V. Panganamala, Diabetes, 34 (1985) 526.
140 J. Watanabe, F. Umeda, H. Wakasugi and H. Ibayashi, Thromb. Haemostasis, 51 (1984) 313.
141 V.A. Gilbert, E.J. Zebrowski and A.C. Chan, Horm. Metab. Res., 15 (1983) 320.

142 B. Spitz, H. Deckmyn, R. VanBree, R. Pijnenborg, J. Vermylen and F.A. Van Assche, Am. J. Obstet. Gynecol., 151 (1985) 116.
143 A. Szczeklik, R.J. Gryglewski, B. Domagala, R. Dworski and M. Basista, Thromb. Haemostasis, 54 (1985) 425.
144 J.R. Beetens, M.C. Coene, A. Veheyen, L. Zonnekeyn and A.G. Herman, Prostaglandins, 32 (1986) 335.
145 M. Ciavatti, E. Davenas, D. Blache and S. Renaud, Contraception, 30 (1984) 279.
146 M.A. Valentovic, C. Gairola and W.C. Lubawy, J. Toxicol. Environ. Health, 15 (1985) 493.
147 W. Seeger, H. Wolf, G. Stahler, H. Neuhof and L. Roka, Prostaglandins, 23 (1982) 175.
148 H.R.D. Wolf and H.W. Seeger, Ann. N. Y. Acad. Sci., 393 (1982) 392.
149 K. Sato, Bull. Chest. Dis. Res. Inst. Kyoto Univ., 18 (1985) 15.
150 J.A. Ward and R.J. Roberts, Pediatr. Res., 18 (1984) 329.
151 G.H. Gurtner, J.R. Michael, I.S. Farrukh, A.M. Sciuto and N.F. Adkinson, J. Appl. Physiol., 59 (1985) 953.
152 V.E. Kagan, R.A. Kopaladze, L.L. Prilipko and V.M. Savov, Biull. Eksp. Biol. Med., 96 (1983) 16.
153 L.N. North, M.M. Mathias and C.L. Schatte, Aviat. Space Environ. Med., 55 (1984) 617.
154 J.K. Timtim and J.J. McNamara, Hawaii Med. J., 42 (1983) 160.
155 A. Patz, Curr. Eye Res., 3 (1984) 159.
156 B.J. Tripathi and R.C. Tripathi, Curr. Eye Res., 3 (1984) 193.
157 A.T. Johnson and F.L. Kretzer, Prog. Clin. Biol. Res., 190 (1985) 251.
158 F.L. Kretzer, A.R. McPherson and H.M. Hittner, Albrecht von Graefes Arch. Klin. Exp. Ophthalmol., 224 (1986) 205.
159 M.L. Chiswick, M. Johnson, C. Woodhall, M. Gowland, J. Davies, N. Toner and D.G. Sims, Br. Med. J., 287 (1983) 81.
160 M.L. Chiswick, M. Johnson, C. Woodhall, M. Gowland, J. Davies, N. Toner and D.G. Sims, Ciba Found. Symp., 101 (1983) 186.
161 F.Z. Meerson, G.T. Sukhikh and K.D. Pletsttyi, Biull. Exsp. Biol. Med., 99 (1985) 646.
162 A.A. Engibarian, K.G. Karagezian and S.S. Ovakimian, Vopr. Med. Khim., 31 (1985) 43.
163 S.D. Artamonov, M.A. Danilov, A.A. Lubiako and N.A. Onishchenko, Farmakol. Toksikol. (Moscow), 48 (1985) 28.
164 M.H. Ayobe, A.R. Sabh, A.M. Malak Saleh and S. Abdel Tawab, J. Egypt. Public Health Assoc., 60 (1985) 109.
165 V.A. Petrova, V.V. Malyshev, B.N. Manukhin and F.Z. Meerson, Vopr. Med. Khim., 31 (1985) 115.
166 R. Ferrari, C. Ceconi, S. Curello, A. Cargnoni, E. Condorelli and R. Raddino, Acta Vitaminol. Enzymol., 7 (1985) 61.
167 C. Guarnieri, R. Ferrari, O. Visioli, C.M. Caldarera and W.G. Nayler, J. Mol. Cell. Cardiol., 10 (1978) 893.
168 W.G. Nayler, Br. J. Pharmacol., 62 (1978) 449.
169 J. Hladovec, Physiol. Bohemoslov., 35 (1986) 97.
170 S. Marubayash, K. Dohi, K. Ochi and T. Kawasaki, Surgery, 99 (1986) 184.
171 S. Marubayashi, K. Dohi, K. Yamada and T. Kawasaki, Biochim. Biophys. Acta, 797 (1984) 1.
172 G.G. Voronov and P.I. Lukienko, Vopr. Med. Khim., 29 (1983) 90.
173 G.G. Voronov and P.I. Lukienko, Biull. Eksp. Biol. Med., 95 (1983) 33.
174 M.V. Bilenko, L.N. Shelenkova, G.Ia. Dubar and A.K.L. Velena, Biull. Eksp. Biol. Med., 96 (1983) 8.

175 S. Fujimoto, K. Mizoi, T. Yoshimoto and J. Suzuki, No To Shinkei, 35 (1983) 305.
176 S. Fujimoto, K. Mizoi, M. Oba and J. Suzuki, No Shinkei Geka, 12 (1984) 171.
177 J. Suzuki, S. Fujimoto, K. Mizoi and M. Oba, Stroke, 15 (1984) 672.
178 S. Fujimoto, K. Mizoi, T. Yoshimoto and J. Suzuki, Surg. Neurol., 22 (1984) 449.
179 K. Abe, S. Yoshida, B.D. Watson, R. Busto, K. Kogure and M.D. Ginsberg, Brain Res., 273 (1983) 166.
180 S. Yoshida, R. Busto, M. Santiso and M.D. Ginsberg, J. Cereb. Blood Flow Metab., 4 (1984) 466.
181 M. Yamamoto, T. Shima, T. Uozumi, T. Sogabe, K. Yamada and T. Kawasaki, Stroke, 14 (1983) 977.
182 S. Yoshida, R. Busto, B.D. Watson, M. Santiso and M.D. Ginsberg, J. Neurochem., 44 (1985) 1593.
183 S. Imaizumi, T. Kayama, T. Yoshimoto and J. Suzuki, No To Shinkei, 37 (1985) 161.
184 J. Suzuki, S. Imaizumi, T. Kayama and T. Yoshimoto, Stroke, 16 (1985) 695.
185 K. Mizoi, J. Suzuki, S. Imaizumi and T. Yoshimoto, Neurol. Res., 8 (1986) 75.
186 S. Imaizumi, J. Suzuki, H. Kinouchi and T. Yoshimoto, No To Shinkei, 38 (1986) 135.
187 N.F. Kassell, T. Sasaki, A.R. Colohan and G. Nazar, Stroke, 16 (1985) 562.
188 A. Takahashi, J. Suzuki, T. Sugawara and T. Yoshimoto, No Shinkei Geka, 14 (1986) 179.
189 E.L. Hove, K. Hickman and P.L. Harris, Arch. Biochem., 8 (1945) 395.
190 I.R. Telford, O.B. Wiswell and E.L. Smith, Proc. Soc. Exp. Biol. Med., 87 (1954) 162.
191 T.W. Anderson, Can. Med. Assoc. J., 110 (1974) 401.
192 T.W. Anderson and D.B. Reid, Am. J. Clin. Nutr., 27 (1974) 1174.
193 A. Vogelsang and E.V. Shute, Nature (London), 157 (1946) 772.
194 J. Marks, Vitam. Horm., 20 (1962) 573.
195 A.M. Boyd, A.H. Ratcliffe, R.P. Jepson and G.W.H. James, J. Bone J. Surg., Br. Vol., 31B (1949) 325.
196 K. Haeger, Vasa, 2 (1973) 280.
197 K. Haeger, Am. J. Clin. Nutr., 27 (1974) 1179.
198 A.S.M. Giasuddin and A.T. Diplock, Arch. Biochem. Biophys., 196 (1979) 270.
199 A.S.M. Giasuddin and A.T. Diplock, Arch. Biochem. Biophys., 210 (1981) 348.
200 J.J. Gilbert, Am. J. Clin. Nutr. 27 (1974) 1005.
201 K.N. Prasad, S. Ramanujam and D. Gaudreau, Proc. Soc. Exp. Biol. Med., 161 (1979) 570.
202 E.S. Vanderzaut, Ann. Entomol. Soc. Am., 60 (1967) 1062.
203 A.T. Diplock, Ciba Found. Symp., 101 (1983) 45.
204 S.R. Ames, Ciba Found. Symp., 101 (1983) 66.
205 W.A. Behrens, G. Zaror-Behrens and R. Madere, Int. J. Vitam. Res., 56 (1986) 135.
206 M.J. Kelly and W. Richardson, Br. J. Pharmacol., 74 (1981) 941.
207 M.J. Kelly, W. Richardson and A.J. Sweetman, Methods Find. Exp. Clin. Pharmacol., (1988) in press.
208 M.J. Kelly, W. Richardson and A.J. Sweetman, Br. J. Pharmacol., 83 (1984) 417.
209 M.J. Kelly, W. Richardson and A.J. Sweetman, Methods Find. Exp. Clin. Pharmacol., (1988) in press.
210 M.J. Kelly, G.S. Lovett, W. Richardson and A.J. Sweetman, Br. J. Pharmacol., 77 (1982) 523.
211 W. Richardson, Ph.D. Thesis (CNAA) 1984.
212 R.B. Dawson, W.E. Ottinger, W.M. Chiu, A.A. Salib, D.S. Fagan and J.R. Keath, Prog. Clin. Biol. Res., 195 (1985) 349.
213 W.G. Nayler, A. Grau and A. Slade, Cardiovasc. Res., 10 (1976) 650.

214 P.L. DaLuz, L.F.M. Debarros, J.J. Leite, F. Pileggi and L.V. Decourt, Am. J. Cardiol., 45 (1980) 269.
215 M.A. Movsesian, Prog. Cardiovasc. Dis., 25 (1982) 211.
216 M.W. Fariss, G.A. Pascoe and D.J. Reed, Science (Washington, DC), 227 (1985) 751.
217 E.D. Hall and D.L. Wolf, J. Neurosurg., 64 (1986) 951.
218 L.V. Tabidze, V.B. Ritov, V.E. Kagan and IuP. Kozlov, Biull. Eksp. Biol. Med., 96 (1983) 48.
219 L.K. Ananieva, I.I. Ivanov, L.V. Tabidze and V.E. Kagan, Biokhimiia, 49 (1984) 60.
220 R.H.M. Julicher, L. Sterrenberg, J.M. Koomen, A. Bast and J. Noordhoek, Naunyn-Schmiedebergs Arch. Pharmakol., 326 (1984) 87.
221 B. Nagorna-Stasiak and M. Wawrzenska, Pol. Arch. Weter, 24 (1984) 215.
222 M.J. Kelly, J. Pharm. Pharmacol., 38 (1986) 66.
223 P.J. Garnett and M.H. Todd, Br. J. Pharmacol., 80 (1983) 706.
224 T. Igarashi, Y. Nakajima, N. Mori and T. Saeki, Clin. Exp. Hypertens., 4 (1982) 177.
225 M. Nishikibe, M. Shioya, A. Nakajima and J. Nagura, Nippon Yakurigaku Zasshi, 87 (1986) 609.
226 G.W. Burton, K.H. Cheeseman, T. Doba, K.U. Ingold and T.F. Slater, Ciba Found. Symp., 101 (1983) 4.
227 H. Weiser, U. Achterath and W. Boguth, Acta Agric. Scand. Suppl., 19 (1973) 208.
228 H. Weiser and R.M. Salkeld, Acta Vitaminol. Enzymol., 31 (1977) 143.
229 Y. Tomita, Nutr. Cancer, 5 (1983) 187.
230 H. Pasantes-Morales, C.E. Wright and G.E. Gaull, J. Nutr., 114 (1984) 2256.
231 M.L. Katz, C.M. Drea and W.G. Robinson, Jr., Mech. Ageing Dev., 35 (1986) 291.
232 J.L. Napoli, A.M. McCormick, B. O'Meara and E.A. Dratz, Arch. Biochem. Biophys., 230 (1984) 194.
233 S. Krywawych, M.J. Jackson, C. Forte, P.J. Garrod, D.K. Walker and D.P. Brenton, J. Inherited Metab. Dis., 8 (1985) 111.
234 C. Bruno, C. Cuppini, R. Cuppini and G. Cavalera, Boll. Soc. Ital. Biol. Sper., 62 (1986) 891.
235 C. Bruno, C. Cuppini, R. Cuppini and G. Cavalera, Boll. Soc. Ital. Biol. Sper., 62 (1986) 895.
236 A.N. Erin, N.V. Gorbunov, V.I. Brusovannik, V.A. Tiurin and L.L. Prilipks, Biokhimiia, 50 (1985) 998.
237 N.G. Davitashvili, A.N. Erin and L.L. Prilipko, Biokhimiia, 51 (1986) 472.
238 A.N. Erin, V.I. Skrypin, L.L. Prilipko and V.E. Kagan, Biull. Eksp. Biol. Med., 102 (1986) 25.
239 M.Z. Lai, N. Duezguenes and F.C. Szoka, Biochemistry, 24 (1985) 1646.
240 G.L. Catignani, Biochem. Biophys. Res. Commun., 67 (1975) 66.
241 H.C. Tsai and A.W. Norman, J. Biol. Chem., 248 (1973) 5967.
242 P.F. Brumbaugh and M.R. Haussler, J. Biol. Chem., 249 (1974) 1258.
243 M.M. Bashor, D.O. Toft and F. Chytil, Proc. Natl. Acad. Sci., U.S.A., 70 (1973) 3483.
244 D.E. Ong and F. Chytil, Biochem. Biophys. Res. Commun., 59 (1974) 221.
245 B.P. Sani and D.L. Hill, Biochem. Biophys. Res. Commun., 61 (1974) 1276.
246 D.E. Ong and F. Chytil, J. Biol. Chem., 250 (1975) 6113.
247 A.E. Kitabchi, J. Wimalasena and J. Barker, Biochem. Biophys. Res. Commun., 96 (1980) 1739.
248 A.E. Kitabchi and R.H. Williams, J. Biol. Chem., 243 (1968) 3248.

249 A.E. Kitabchi and J. Wimalasena, Biochim. Biophys. Acta, 684 (1982) 200.
250 C. Guarnieri, F. Flamigni and C.R. Caldarera, Ital. J. Biochem., 29 (1980) 176.
251 M.G. Traber and H.J. Kayden, Am. J. Clin. Nutr., 40 (1984) 747.
252 A.T. Diplock and J.A. Lucy, FEBS. Lett., 29 (1973) 205.
253 N.P. Franks and W.R. Lieb, Nature (London), 310 (1984) 599.
254 N.P. Franks and W.R. Lieb, Nature (London), 316 (1985) 349.
255 N.P. Franks and W.R. Lieb, Proc. Natl. Acad. Sci. USA, 83 (1986) 5116.

Progress in Medicinal Chemistry – Vol. 25, edited by G.P. Ellis and G.B. West

6 Multivariate Data Analysis and Experimental Design in Biomedical Research

LARS STÅHLE, M.D.[a] and SVANTE WOLD, Ph.D.[b]

[a] Department of Pharmacology, Karolinska Institute, Box 60 400, S-104 01 Stockholm, and [b] Research Group for Chemometrics, Institute of Chemistry, University of Umeå, S-901 87 Umeå, Sweden

INTRODUCTION

Biomedical research at the beginning of the century usually involved relatively few measurements on many subjects. While measurement was expensive, the cost for experimental subjects was often low. The methods for data analysis developed during the same period – *t*-tests, analysis of variance, multiple regression – were optimized for this situation with many subjects and few measurements.

Today the situation is the reverse. Multiple measurements are often made on each subject. By taking as many measurements as possible, it is hoped that the complete picture of each subject will be less error-prone than each individual measurement considered separately. As an illustration, the development of chromatographic separation methods and detection techniques like spectrophotometry has made it possible to identify many substances in one blood sample from a patient. At the same time, the cost of each experiment has increased. Consequently, new methods for data analysis are needed, methods that can utilize the information in the data also when the subjects are few and the measurements are many.

In medicinal chemistry, it is customary to take a large number of measurements to characterize drugs pharmacologically and chemically. For example, a physicochemical description of a drug molecule in quantitative terms is best made by a number of measurements in order to include information about properties relevant to the drug effect (see Ref. 1). Likewise, the pharmacological effects of the drug have to be assessed in an array of pharmacological model systems in order to make possible reasonable predictions about therapeutic value and side-effects. Hence, using statistical terminology, drug data are multivariate. The methods for multivariate data analysis discussed below have been shown to be suitable for these kinds of data.

However, it should be emphasized that the statistical methods presented here are no cures for poor data. Irrelevant or erroneous measurement and poorly planned experiments will still be irrelevant, erroneous and poorly planned in spite of any statistical analysis. There are, however, many examples of excellent data that have been seriously mutilated by poor statistical analysis. The aim of this chapter is to present multivariate statistical methods for design and

analysis of experiments that can substantially facilitate the research process.

First, three data sets which serve as illustrative examples will be presented. Most calculations presented in this review require simple computer programs. In Appendix A, algorithms (that is, calculation methods) for the various methods are given for those interested in the details.

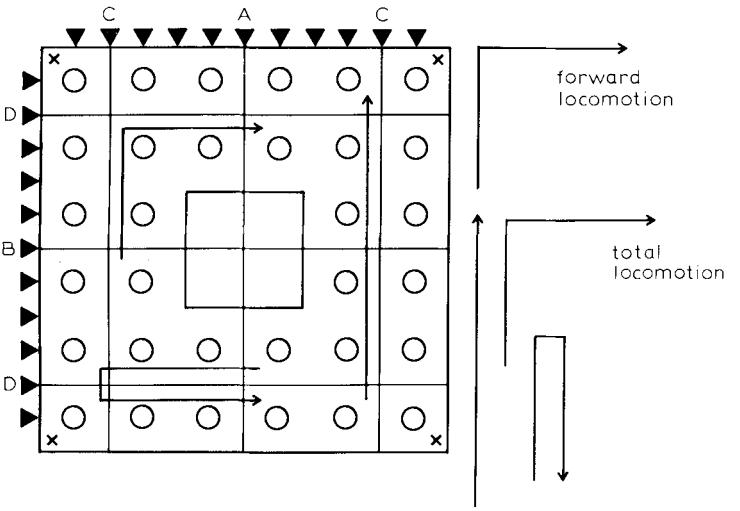


*Figure 6.1. Schematic drawing of the holeboard apparatus used to record behaviour of rats. The box is 70 * 70 cm and has 32 holes in the floor (circles). The position of photobeams recording nonspecific behavioural activity (variable x_1) are indicated by black triangles. These photobeams also record the two locomotion variables (x_2 and x_3) indicated by arrows. The frequency and accumulated duration of hole visits, corner visits and raising on the hind-legs (variables x_4–x_9) are recorded by separate photocells [2]. The 10th variable is the ratio between the activity during the second and the first half of the 10 min recording period.*

DOSE-RESPONSE DATA: EXAMPLE OF BHT

In order to study the behavioural effects in rats of the dopamine agonist 2-amino-6-allyl-5,6,7,8-tetrahydro-4*H*-thiazolo[4,5-*d*]azepine · 2HCl (BHT 920; Boehringer Ingelheim, BHT for short) a recording device, the holeboard (*Figure 6.1*) with infrared photobeams covering an open field was used [2]. The apparatus allows ten different measurements (which will be labelled $x_1 - x_{10}$) of behaviour to be recorded simultaneously. The rats ($n = 23$)

were treated with BHT 920 (0, 0.05 or 0.1 mg/kg s.c.) 30 min before the start of a 10 min recording period. Thus, for each rat we have information about its drug treatment and one value on each of the ten behavioural measures.

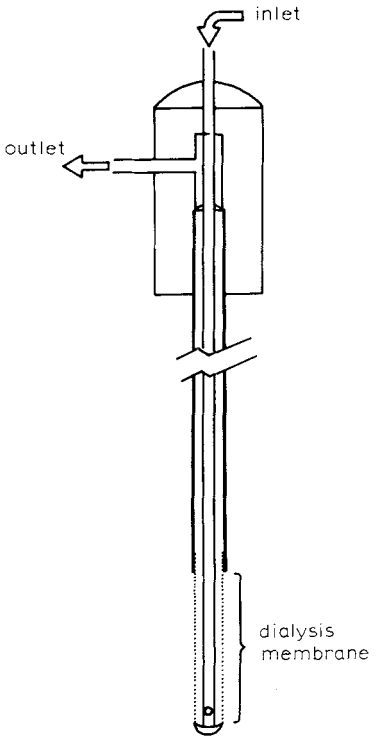


*Figure 6.2. Schematic drawing of the microdialysis probe used to sample the concentration of dopamine, DOPAC, 5HIAA and HVA in the extracellular space in rat brains. The fluid is delivered by a pump and passes via the vertical inlet and the inner cannula down to a 3.0 * 0.5 mm part of the probe, where a semipermeable membrane surrounds the inner cannula (stippled lines). The dialysis process occurs when the fluid passes between the inner cannula and the dialysis membrane. The perfusion medium flows inside the outer cannula to the horizontal outlet and is collected in 20 min (40 µl) fractions.*

TIME–RESPONSE DATA: EXAMPLE OF AMPHETAMINE

The recently developed microdialysis method [3], which is a technique to sample the extracellular space, was used to study the effects of the dopamine

releaser and reuptake-blocker, amphetamine, on the levels of dopamine and its metabolites DOPAC (3,4-dihydroxyphenylacetic acid) and HVA (homovanillic acid) and the serotonin metabolite 5HIAA (5-hydroxyindolacetic acid). The microdialysis probe (*Figure 6.2*, Carnegie Medicin AB, Stockholm, Sweden) was inserted into a densely dopamine-innervated area in the brain (the striatum) of rats. The dialysis probe was perfused with a Ringer solution at a speed of 2 μl/min and the content of dopamine, DOPAC, 5HIAA and HVA in 20-min fractions (40 μl) was analysed by liquid chromatography with electrochemical detection. The effects of amphetamine were followed for 2 h, that is, six fractions of 20-min dialysates were collected and analysed.

STRUCTURE–ACTIVITY RELATIONSHIPS: EXAMPLE OF PEPTIDES

Certain pentapeptides potentiate the contractile response of isolated guinea-pig ileum to bradykinin [4, 5]. The effects on bradykinin potentiation of varying the amino-acid sequence in the pentapeptide were investigated [6]. Each of the five amino acids was regarded as a substituent. By constructing a particular scale from the physicochemical properties of the amino acids, the variation in amino-acid sequence in the pentapeptides can be quantitatively described [6, 7].

UNIVARIATE VERSUS MULTIVARIATE METHODS

The most commonly employed univariate statistical methods are analysis of variance (ANOVA) and Student's *t*-test [8]. These methods are parametric, that is, they require that the populations studied be approximately normally distributed. Some non-parametric methods are also popular, as, for example, Kruskal-Wallis ANOVA and Mann-Whitney's *U*-test [9]. A key feature of univariate statistical methods is that data are analysed *o*ne *v*ariable *a*t a *t*ime (OVAT). This means that any information contained in the relation *between* the variables is not included in the OVAT analysis. Univariate methods are the most commonly used methods, irrespective of the nature of the data. Thus, in a recent issue of the European Journal of Pharmacology (Vol. 137), 20 out of 23 research reports used multivariate measurement. However, all of them were analysed by univariate methods.

The OVAT approach can be illustrated by the BHT 920 example. Analysing OVAT implies, for example, that the univariate one-way ANOVA method should be applied to each of the ten variables separately. When the BHT data

Table 6.1. MEAN AND STANDARD DEVIATION FOR EACH OF THE TEN HOLE-BOARD VARIABLES MEASURED ON RATS TREATED WITH THE DOPAMINE AGONIST BHT 920

	x_1	x_2	x_3	x_4	x_5	x_6	x_7	x_8	x_9	x_{10}	*dose*	n
mean	1126	33	68	110	105	67	63	19	36	0.88	none	11
sd	208	9	16	20	26	32	63	9	19	0.21		
mean	606	17	30	74	79	40	69	11	22	0.62	0.05	6
sd	293	10	17	29	31	21	45	10	20	0.48		
mean	262	2	7	35	26	42	108	3	5	0.33	0.1	6
sd	39	2	3	8	9	41	107	2	4	0.27		

were analysed in this way, all variables but two were found to be dose-dependently affected by treatment with BHT (*Table 6.1*).

Intuitively, this approach is associated with a risk of spurious significant differences between the treatment groups. If, say, 1000 variables instead of 10 had been measured, significant differences between the groups would be expected to appear by chance. This is so because there is always a risk (for example, 5% for each variable) that a variable by chance will differ significantly, and the more variables there are, the larger is this risk. Hence, the risk of making such a mistake is also larger when ten variables are measured than is the risk with only one variable. This argumentation has been intuitive. Some simple calculations of probabilities may help to clarify.

Assume that the Student's *t*-test is applied to test whether or not there is an effect in one variable (for example, x_4 = HOLE COUNT) of treatment with 0.05 mg/kg BHT compared with controls. The significance level 5% is then chosen, that is, 5% false positives (type I errors or α-errors) are accepted in the research. When one more measurement (x_5 = HOLE TIME) is taken on the same set of data and Student's *t*-test is applied again to the data, the risk for at least one false positive is no longer $1.0 - 0.95 = 5\%$ but $1.0 - 0.95^2 = 9.75\%$. When 10 variables are measured, as in the BHT 920 example, the risk is $1.0 - 0.95^{10} = 40.13\%$ (a more detailed table has been published elsewhere [10]). This phenomenon is usually referred to as the increased risk of type I errors.

It is easy to compensate for this increased risk of type I errors by dividing the probability level (usually 5%) by the number of tests (Bonferoni method [8]). For instance, if ten variables have been recorded and the maximum acceptable risk for type I errors is 5%, the significance level for each individual *t*-test should be adjusted to $5\%/10 = 0.5\%$. The corrected significance level will

then be $1.0 - 0.995^{10} \approx 5\%$ as required. For anyone with practical experience of statistics, this approach is accepted as less useful, even when the number of variables is moderate (for example, ten) because only significances that are obvious anyway are those that are detected.

The Bonferoni method with OVAT rests on the assumption that the variables analysed are independent. This is, however, seldom true. In fact, the chance that variables are independent and uncorrelated is very small unless it is possible to have full control over the variables in an experiment. If this is the case, it is possible to choose an experimental design that makes the intervariable correlations zero (*vide infra*).

When there are intervariable correlations, another source of error associated with the OVAT approach appears, the so-called type II error. This means that a true difference is spuriously undetected. The Bonferoni adjustment of p-values is one rich source of increased type II errors in univariate analysis of multivariate data. This is easily realized if a situation is considered where the effect of a drug has been recorded on one relevant variable and nine irrelevant variables. The Bonferoni adjustment would in this case obscure the truly significant change in the relevant variable by the compensation for the irrelevant variables.

A second source of type II errors appears when the difference between two groups is not to be found in any single variable as such but in the relation between the variables. Consider the small set of simulation data summarized in *Table 6.2*, in which the effect of a treatment of one group is compared with that of a control group on two measured responses.

With the information given in *Table 6.2* there is no evidence that the groups are significantly different. In fact, most researchers would consider such a result as good evidence that the treatment has no effect. Now consider the same data in a scatter plot (*Figure 6.3*).

Table 6.2. UNIVARIATE SUMMARY STATISTICS (MEAN ± S.E.M.) FROM THE SIMULATED DATA ILLUSTRATED IN *FIGURE 6.5* CONSISTING OF A CONTROL GROUP AND A TREATMENT GROUP

The p values refer to a two-sided t-test with 20 degrees of freedom.

	Variable 1	*Variable 2*
Control	4.91 ± 0.80	0.75 ± 0.09
Treatment	6.26 ± 0.83	0.58 ± 0.09
t_{20}	1.12	1.31
p	> 0.2	> 0.2

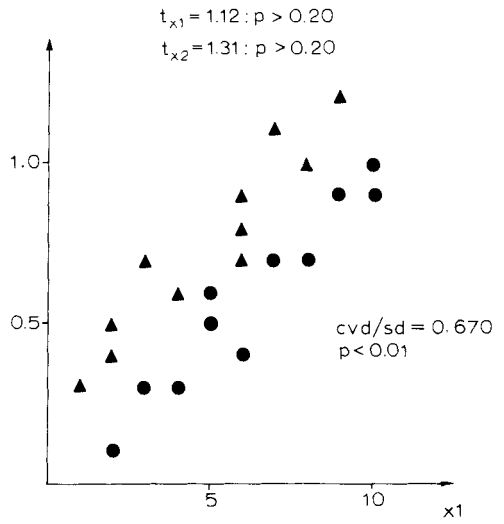


Figure 6.3. Scatter plot of simulation data illustrating an experiment comparing the results of a control group with those of a treatment group. Because of the strong correlation between the two variables, the difference between the groups is readily detected by eye and by multivariate statistics, but not by Student's t-test applied to each variable separately.

It is seen that the difference between the groups is detected only if both variables are considered simultaneously, as, for example, by the PLS-method described below. There is no way to handle such data by univariate analysis. The OVAT-approach has thus two considerable drawbacks:
(1) there is an increased risk of type I errors (risk of spurious differences);
(2) there is an increased risk of type II errors (risk that real differences are not seen).
In other words, the application of univariate statistical methods to multivariate data often results in a considerable loss of information and, hence, a loss of power. This is because the assumptions on which the univariate analysis rely are seldom fulfilled (for example, independence between variables).

How can multivariate methods be used to avoid the problems associated with the OVAT approach? In general, multivariate methods use the information contained in the relation between the variables (correlations or covariances) and therefore data like those in *Figure 6.3* present no problem. The risk of type I errors is kept under control in multivariate analysis by considering all variables simultaneously. To consider all variables simultaneously involves a

change in the way the researcher asks statistical questions. When comparing a treatment group with a control group, the multivariate question is, 'Has the treatment an effect?'. This is easily illustrated in a three-dimensional co-ordinate system (*Figure 6.4*). Assume that the control group is represented by a swarm of points in this three-dimensional space and let the treatment group be represented by another swarm of points. The multivariate question, 'Has the treatment an effect?' can now be translated into 'Is the location of the point swarm representing the treatment group different from the location of the control group?'. This is different from the univariate approach, where, instead, the projections of the two point swarms onto the co-ordinate axes are analysed one at a time.

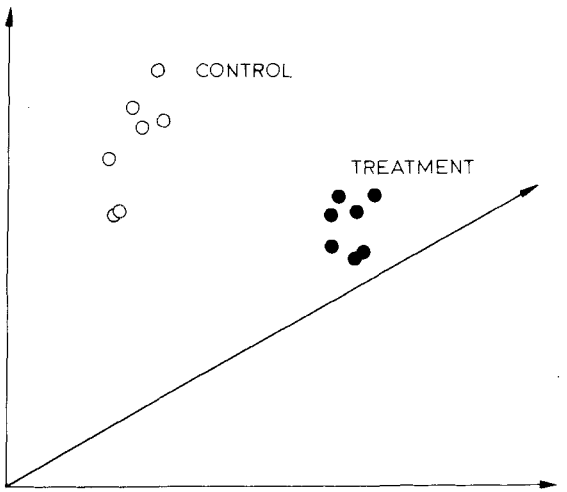


Figure 6.4. Scatter diagram of the results of two groups of subjects (open and filled spheres) in a three-dimensional measurement space. The groups differ in location. The difference between the groups can be fully described only by considering all three variables simultaneously.

The answers produced by a multivariate analysis are consequently different from those obtained from a univariate method. The answer to the question 'Has the treatment an effect' can be 'probably' or 'probably not' at a certain significance level. If the answer is that there is probably a difference between the groups, it is also possible to obtain some information concerning in which variables this change has occurred. It is, however, not possible to discuss the variables separately. An everyday example illustrates this point. The weight of

an individual is dependent upon, among other things, how tall and how fat he or she is. If the weight of young boys is compared with the weight of middle-aged men, it will (probably) be found that the latter group is more heavy on average. This increase in weight is, of course, the consequence of *both* the increased length and the increased amount of adipose tissue on the men. Neither factor can be independently singled out as *the* cause.

There are apparently many multivariate statistical methods partly overlapping in scope [11]. For most problems occurring in practice, we have found the use of two methods sufficient, as discussed below. The first method is called principal component analysis (PCA) and the second is the partial least-squares projection to latent structures (PLS). A detailed description of the methods is given in Appendix A. In the following, a brief description is presented.

PCA [12–19] can be used to obtain an overview of a set of data organized in a table $\boldsymbol{X}$ with n rows (subjects) and p columns (variables). By means of PCA, most of the variation in $\boldsymbol{X}$ is summarized in a few principal components (*Figure 6.5*). More specifically, the first PC is the main axis of the shape of the data scatter. Hence, the first PC explains the largest part of the variance in $\boldsymbol{X}$. if $\boldsymbol{X}$ contains similar objects, PCA can be used to formulate a model of the

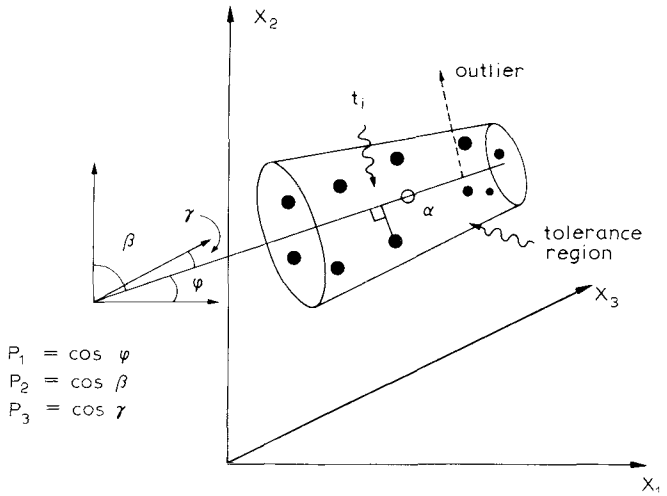


Figure 6.5. Scatter diagram of a set of data with measurements taken on three variables. The average point is **a**. *The first principal component is the line that best, of all possible lines, fits the data. This fitting is done by least squares, that is, the sum of the squared distances from the points perpendicular on to the line is minimized. The score,* t_i, *of the* i*th subject is the distance from* **a** *to the point on the principal component onto which a data point perpendicularly projects. The* k*th element of the loading vector* **p** *is the cosine between the PC and* k*th variable.*

dominant structure in $\boldsymbol{X}$. In addition, a tolerance region can be constructed around the model of $\boldsymbol{X}$ by means of the residual variance (that is, variance not used in the model). Subjects outside the tolerance region are dissimilar to the class of subjects which constitute $\boldsymbol{X}$. PLS [19–21] is used to relate $\boldsymbol{X}$ to other data about the same subjects (*Figure 6.6*) which have been organized in another table $\boldsymbol{Y}$ with n rows (same as the rows of $\boldsymbol{X}$) and q columns (variables). Thus, PLS is used in much the same way as simple linear regression is used to relate two variables (for example, dose and effect).

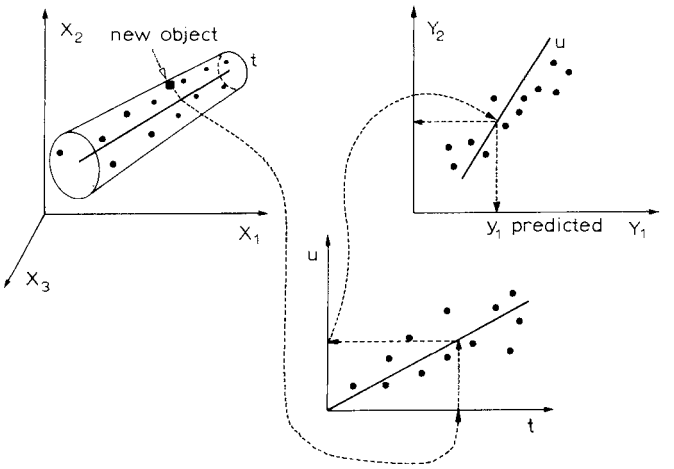


*Figure 6.6. Illustration of the PLS methodology to relate two co-ordinate systems (***X** *and* **Y***) via score vectors (*t *and* **u***, respectively). The upper left co-ordinate system contains the measurements* **X** *and the upper right co-ordinate system contains the external information,* **Y**. *The points in the two co-ordinate systems represent the same set of subjects. By fitting a line in each co-ordinate system to the points and then increasing the correlation between the* t*-scores and the* u*-score (lower middle plot) by tilting both lines, the PLS solution is obtained. The* **Y** *values of a new subject inside the tolerance region in* **X** *can be predicted by following the path indicated by the dotted line.*

As can be seen from *Figures 6.5* and *6.6*, there are several similarities between PLS and PCA. For example, both methods make a linear model of the data table $\boldsymbol{X}$ by means of a score vector, $\boldsymbol{t}$ (one score for each object), and a loading vector, $\boldsymbol{p}$, which measures the importance of the variables. However, in PCA, neither $\boldsymbol{t}$ nor $\boldsymbol{p}$ is influenced (computationally) by anything but the variation in the measurements. Hence, if it is attempted to relate the measurements $\boldsymbol{X}$ to some external event (for example, drug treatment) via the PC t-scores, it must be realised that, unless this external event *is* a sufficiently large

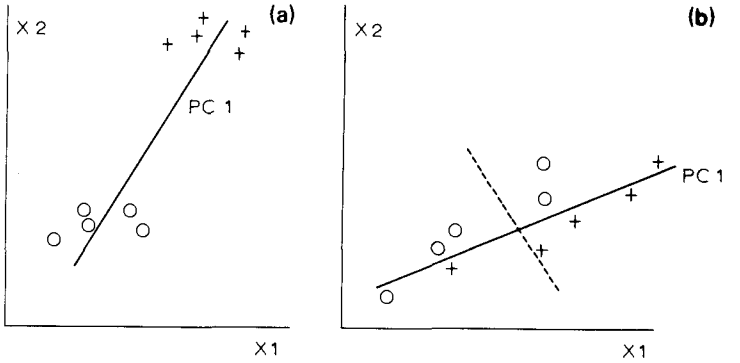


Figure 6.7. Scatter diagram of two groups of subjects (crosses (treatment) and circles (control)) in a two-dimensional measurement space. In (a), the difference between the groups is the largest source of variation in the data and the first PC will describe the difference. In (b), the difference between the groups does not constitute the largest source of variation in the data and PCA will therefore not detect the difference. By projecting the data onto the dotted line, it can be seen that the two groups are completely separated.

source of variation in $\boldsymbol{X}$ to cause a *significant* PC, there is a risk that no relation between the event and the data will be detected (*Figure 6.7*). This is in contrast to PLS were the score vector *by construction* is influenced by the external event. Therefore the relation between $\boldsymbol{X}$ and $\boldsymbol{Y}$ can be detected, even when $\boldsymbol{Y}$ is not the largest source of variation in $\boldsymbol{X}$. The method to relate PC t-scores to an external event (often called PC regression, PCR) relies upon the assumption that a large source of variation is the one of interest in the regression. There is no particular reason to make this assumption; on the contrary, experience shows that the assumption is often not fulfilled [22]. In PLS, the score vector $\boldsymbol{t}$ and the $\boldsymbol{X}$-variable weights ($\boldsymbol{w}$) and loadings ($\boldsymbol{p}$) are influenced by the external event tabulated in $\boldsymbol{Y}$ ($\boldsymbol{w}$ and $\boldsymbol{p}$ both measure the importance of the $\boldsymbol{X}$-variables; for details see Appendix A). One difference between PCR and PLS is that PLS does not require that the information in $\boldsymbol{X}$, which is of interest for the relation to $\boldsymbol{Y}$, is a large source of variation in $\boldsymbol{X}$.

A comparison between PLS and multiple regression (MR) by a simple example is also useful in understanding the features of PLS. Let $\boldsymbol{X}$ be two-dimensional as, for example, the size of the thigh and the upper arm in cm measured on a number of more or less athletic men. Let $\boldsymbol{Y}$ be one-dimensional as, for example, the maximum weight in kg that each of these men can lift. Thigh-size and arm-size correlate quite strongly and both correlate positively with the performance in weight-lifting. Thus, this set of data, when represented

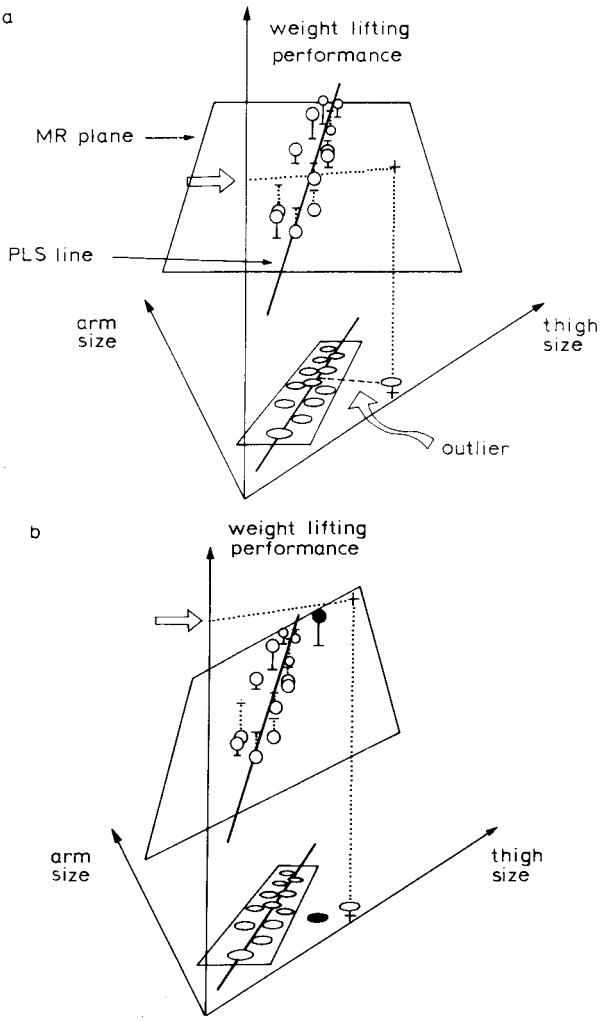


Figure 6.8. Illustration of the similarities and differences between MR and PLS. The arm size and thigh size of 12 men are plotted against their weight-lifting performance (spheres). The ellipses are the projections of the spheres onto the arm size–thigh size plane. In PLS, a model of **X** *(that is, in the arm size–thigh size plane) is made and a tolerance region is defined. The PLS line fitted to the spheres, which is the same as the regression line between* **t** *and* **u** *in* Figure 6.6, *is used to predict the weight-lifting performance of the men. In MR, a plane is fitted directly to the spheres using 100% of the information in* **X** *to predict* **Y**. *In (a), the MR plane gives equal weight to arm size and thigh size as do the PLS line. PLS, but not MR, will detect the female as an outlier. In (b), one outlier (black sphere) has tilted the MR plane and gives a large weight to thigh size. This will lead to an erroneous prediction of the weight-lifting capacity of the female by MR.*

in a three-dimensional co-ordinate system (*Figure 6.8a*), has a sausage-like appearance. In MR, a plane is fitted to the data (*Figure 6.8a*) while, in PLS, a line is fitted (*Figure 6.8a*). The MR solution is used under the assumption that all the information in $\boldsymbol{X}$ is 100% relevant. In contrast, the PLS model utilizes less than 100% of $\boldsymbol{X}$ to predict $\boldsymbol{Y}$ and includes the construction of a tolerance region in the $\boldsymbol{X}$-plane on the basis of the residuals, $\boldsymbol{E}$ (that is, the variance not used to predict $\boldsymbol{Y}$, see Appendix A). The tolerance region makes it possible to judge whether or not a new object is an outlier or whether the PLS model can be safely used to predict the weight-lifting capacity of this new individual. Now, let a woman join the men and measure her thigh size and arm size. Using the MR solution to predict her performance, it would happily suggest, on the basis of her considerable thigh size and her less considerable arm size, that her weight lifting performance is slightly above that of the average athletic man (*Figure 6.8a*). Although this may be a correct prediction, it is easily realized that it is not always so, for the reason that the relation between weight-lifting capacity and arm and thigh size is probably somewhat different in females. When PLS is used, it will be found that this new weight-lifter is different from the men who were used to make the PLS model. She falls outside the tolerance region and will therefore be labelled as an outlier (*Figure 6.8a*).

The ability to detect outliers is not the only feature that makes PLS differ from MR. Because of the sausage-like appearence of the data scatter in *Figure 6.8a*, the fitting of a plane to the data by MR also means that MR assigns more properties to the data than there really are. The position of the MR-fitted plane is well determined only along the main axis of the data scatter. In the other direction, the position of the plane may be strongly influenced by random features of single subjects. Assume that among the athletic men there was one man with particularly large thighs who was also very strong (*Figure 6.8b*). This would cause the MR-fitted plane to tilt so that the thigh-size is given a larger influence than in the previous MR model without this new subject (*Figure 6.8a*). With the new model, the female weight-lifter would, by MR, be predicted to be the next world champion (*Figure 6.8b*).

Thus, PLS (but not MR) assumes that data $\boldsymbol{X}$ may contain a structure irrelevant to the relation with $\boldsymbol{Y}$. This is the philosophical difference between MR and PLS. In general, PLS does not fit more dimensions to a set of data than those that improve the predictive ability of the model. This is ensured by the cross-validation procedure (see Appendix A).

PLS provides a great flexibility (and thus requires some responsibility) in the way external information can be included in $\boldsymbol{Y}$. One simple and very useful design is when the subjects are grouped in some way. In the BHT 920 example, the subjects can be divided into three different treatment groups. In this case,

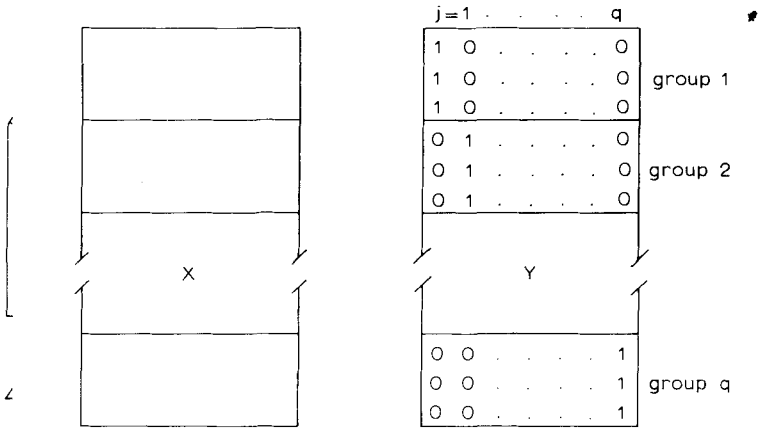


Figure 6.9. Illustration of ANOVA, or discriminant analysis, design of PLS by means of 0-1 indicator variables in **Y**.

Y is constructed so that there are as many columns as there are groups and let each column represent one group. The objects belonging to the *j*th group get a 1 in the *j*th column and 0 in the others (*Figure 6.9*). This design is usually referred to as PLS discriminant analysis [23] or analysis of variance like PLS design [24].

m-Way tables constitute a particular type of data. In the amphetamine example, a number of compounds (dopamine, DOPAC, 5HIAA and HVA) were measured in brain dialysates every 20 min for 2 h after injection of amphetamine (2 mg/kg). The observed data can be organised in a three-way $(10 * 4 * 6)$[a] table: objects * compound * time (*Figure 6.10*). This three-way table can then be unfolded to the usual two-way table ***X*** with $n = 10$ rows and $p = 4 * 6 = 24$ columns. The unfolded ***X***-table can be analysed by the usual PCA or PLS algorithm [25, 26]. After the PLS solution has been calculated, the weights (***w***) and the loadings (***p***) of ***X*** are then simply folded back and form a two-way table (compound * time). A smoothing procedure can be added by subjecting the two-way table of weights to PCA and then use the PC model of the weight table [25]. In general, any *m*-way table can be unfolded $m - 2$ times to give a two-way table [26].

In all PLS models in this chapter, the observed data are put in ***X***, and external information (like group membership and drug treatment) is put in ***Y***. With

[a] * Means 'multiply by' in computer science.

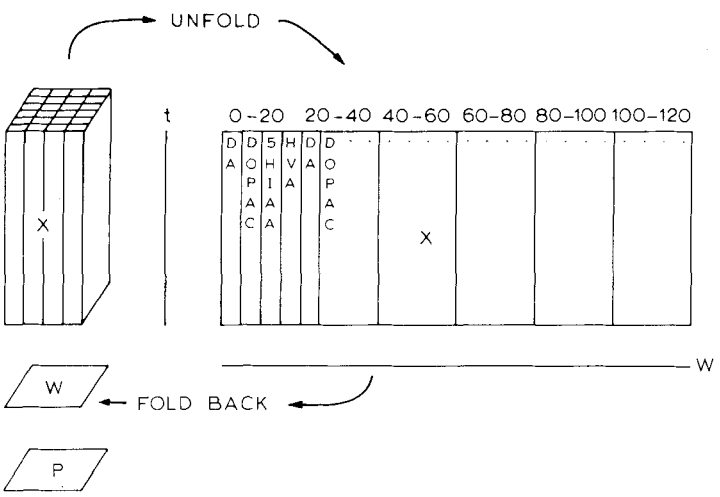


Figure 6.10. Illustration of an unfolding of a three-way table, **X**, *into a two-way table. In the amphetamine example, the three-way table has 10 rows, 4 columns, and 6 sheets of columns, which gives a 10 rows and 24 columns large two-way table after unfolding. The weight table,* **W**, *is also unfolded to form the usual row vector,* **w**. *After applying the PLS or PCA algorithm to the data, both* **X** *and* **w** *are folded back.*

classical linear regression in mind, it may seem awkward to predict, say, the drug treatment from the responses. This design is analogous to multivariate calibration in chemistry [27] and inverse regression commonly has better predictive properties than classical regression [28]. With the inverse approach used in the present paper, the possibilities to detect outliers in observations are improved, due to the possibility to define a tolerance region around the model of $\boldsymbol{X}$.

MULTIVARIATE DATA ANALYSIS

DATA INSPECTION

All data should be inspected by calculating variable means, standard deviations, skewness and similar parameters. Even better is to plot the data in all potentially useful ways, in particular various kinds of scatterplots of, for example, intervariable correlations and residuals. These methods will not be penetrated further in this paper because they can be found easily in any

statistical textbook [8, 29]. Instead, it will be shown how PCA and PLS can add a new type of information about a set of data. In particular, their ability to open relevant windows in the measurement space (M-space) is emphasized.

PCA of a data set, and of relevant subsets, provides insight into the shape and variation in the data. The number of significant PCs provides information about the number of dimensions that are required to cover the systematic variation in the data. For example, a spindle-shaped data scatter in a three-dimensional M-space has one significant dimension (*Figure 6.5*) while a T-shaped scatter (*Figure 6.11*) requires two dimensions to be accurately

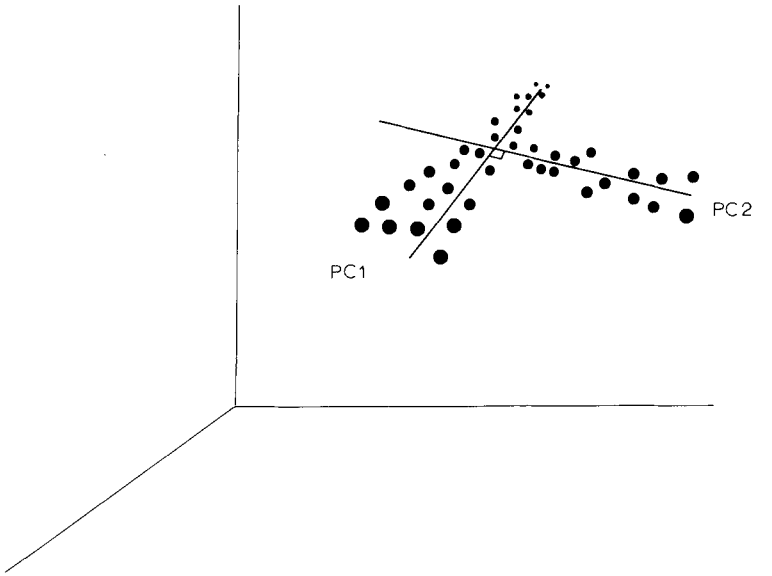


Figure 6.11. Scatter diagram of a set of data on which three measurements have been taken. The scatter consists of two parts. The first part goes in a direction almost perpendicular to the plane of the paper and away from the reader, while the second part goes from the left to the right. These two directions can be accurately described by two principal components (or PLS components) that are orthogonal to each other. Together, they span a two-dimensional plane.

described. By plotting the *t*-scores of the significant PCs, it can be seen in what way the shape of the data scatter differs from randomly scattered points. This procedure may be understood as opening a window in the measurement space through which the significant variation in the data can be observed.

Another useful property of PCA is its ability to detect outliers. By definition,

outliers are positioned far away from the other points in the measurement space and they may therefore be a sufficiently large source of variation to give rise to a PC. Such PCs are seldom significant in cross-validation and it is, for this reason, a good idea to extract one or two PCs beyond the last significant PC in order to see if the last PCs are defined by outliers. In *Figure 6.12*, the BHT

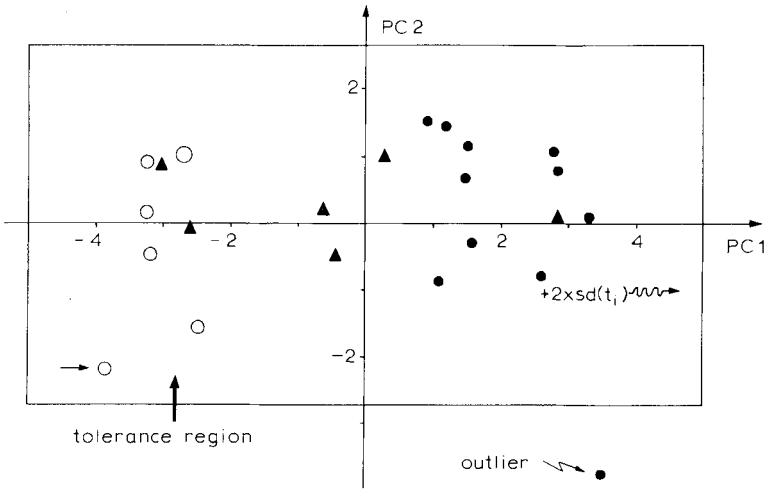


Figure 6.12. Scatter plot of the t-*scores of the first two PCs calculated from the BHT 920 data. Black circles are controls, black triangles are rats given BHT 920 0.05 mg/kg and open circles are rats given BHT 920 0.1 mg/kg. The outlier in the lower right quadrant has largely influenced the second PC. The subject in the lower left quadrant pointed at by an arrow is an outlier in a direction at an angle with* PC_1 *and* PC_2. *The tolerance region is roughly bounded in the direction of* PC_1 *by* $\pm 2 * sd(t_{i1})$.

example is analysed by plotting the scores on the first two PCs (only the first PC is significant according to cross-validation). Most data points lie within the tolerance region. One point is, however, a strong outlier (in the lower right quadrant) and it has strongly influenced the second PC. The corresponding loading plot is given in *Figure 6.13*. This plot shows that many variables contribute to the first PC, namely variables 1–5 and 8–10. Both cross-validation and jackknifing show that variables 6 and 7 are non-significant (*Table 6.3*). The large loadings of variables 6 and 7 on the second PC are also non-significant (*Table 6.3*). Inspection of the raw data reveal that the outlier previously discussed has unusually large values on these two variables. These findings explain why this subject is dissimilar to the other subjects and therefore responsible for the second PC.

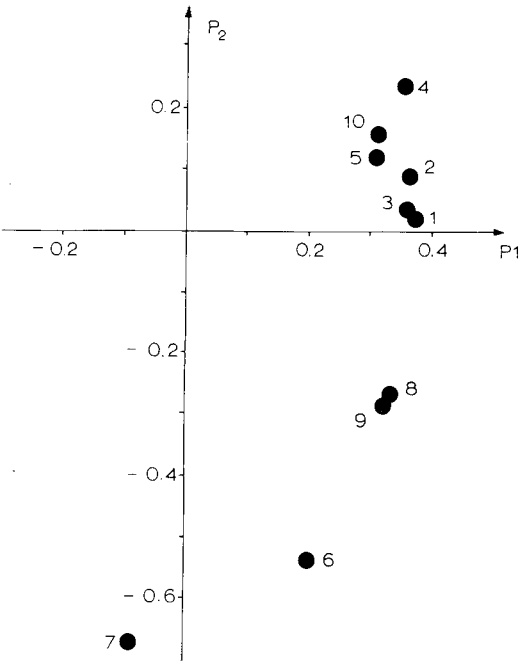


Figure 6.13. Scatter plot of the loadings of the variables on the first two PCs calculated from the BHT data. Each point represents the loadings of a variable. Note that the directions in this plot correspond to directions in Figure 6.12.

Table 6.3. LOADINGS WITH JACKKNIFE ESTIMATES OF STANDARD DEVIATION AND CROSS-VALIDATION cvd-sd FROM PCA OF THE BHT 920 EXAMPLE
The results from the first two components are given.

Variable	$p_{1k} \pm sd$	$cvd\text{-}sd_{1k}$	$p_{2k} \pm sd$	$cvd\text{-}sd_{2k}$
x_1	0.373 ± 0.018	0.348	0.037 ± 0.067	1.038
x_2	0.367 ± 0.031	0.336	0.091 ± 0.056	0.983
x_3	0.368 ± 0.022	0.361	0.040 ± 0.102	1.085
x_4	0.358 ± 0.043	0.506	0.238 ± 0.119	0.843
x_5	0.310 ± 0.055	0.721	0.120 ± 0.198	1.073
x_6	0.201 ± 0.113	0.987	−0.539 ± 0.212	1.023
x_7	−0.088 ± 0.144	1.104	−0.670 ± 0.128	1.045
x_8	0.335 ± 0.033	0.590	−0.274 ± 0.113	0.830
x_9	0.327 ± 0.025	0.624	−0.280 ± 0.152	0.863
x_{10}	0.314 ± 0.041	0.701	0.158 ± 0.201	1.031

It should be noted that other points may be outliers in some direction not spanned by the first two PCs. It is therefore important to calculate the residuals for each individual subject in order to get a picture of which the outliers are. In the BHT 920 example, the object in the lower left quadrant pointed at by an arrow (*Figure 6.12*) is also an outlier, but in a direction different from the previous outlier.

Data can also be inspected by PLS in situations where it is suspected that some external factor influences the data. Assume, for instance, that a new kind of experiment is done in the laboratory. For some reason, it is suspected that the skill in performing the experiment increases with time and that the skill of the experimenter influences the results. A PLS analysis with the experimental results in $\boldsymbol{X}$ and experience (the number of experiments previously performed) in $\boldsymbol{Y}$ can then be used to check this possibility.

DATA DESCRIPTION, MULTIVARIATE CHARACTERIZATION

In many situations it is useful to describe a set of data after reducing it to its significant PCs. The PCs will contain the *largest* sources of variation in the data. The peptide example illustrates this use of PCA. In quantitative structure–activity relationship (QSAR) studies, the physicochemical properties of substituents can be quantified by compiling a large amount of information on each substituent (for example, pK_a, Hansch π, MW, $\log P$ where P = the partition coefficient between octanol and water, and so on) and proceeding to find those properties that vary most between the substituents by PCA. This is particularly important in QSAR because the aim of synthesizing new drug molecules is to find substances with properties different from those of previously synthesized drugs. Hence, the substituents on the new molecules must differ substantially from those of previously synthesized drugs. By means of PCA, substituents are described in such a way that the t-scores quantify the properties which vary most between them.

In the peptide example, twenty measures of physicochemical properties of amino acids were compiled (*Table 6.4*). Plots of the first three PCs of these data are given in *Table 6.14*. The use of the PC t-scores in the design of QSAR experiments are illustrated by the peptide example in the Experimental design section.

Another use of PCA in multivariate characterization is the formulation of a class model. If there are several classes of subjects in a study, a PC model can be made of each class with surrounding tolerance volumes. New subjects are assigned to a class if it is inside the tolerance volume of this class. This simple but efficient classification scheme is called SIMCA (soft independent modelling of class analogy) and it is described in detail elsewhere [17, 18].

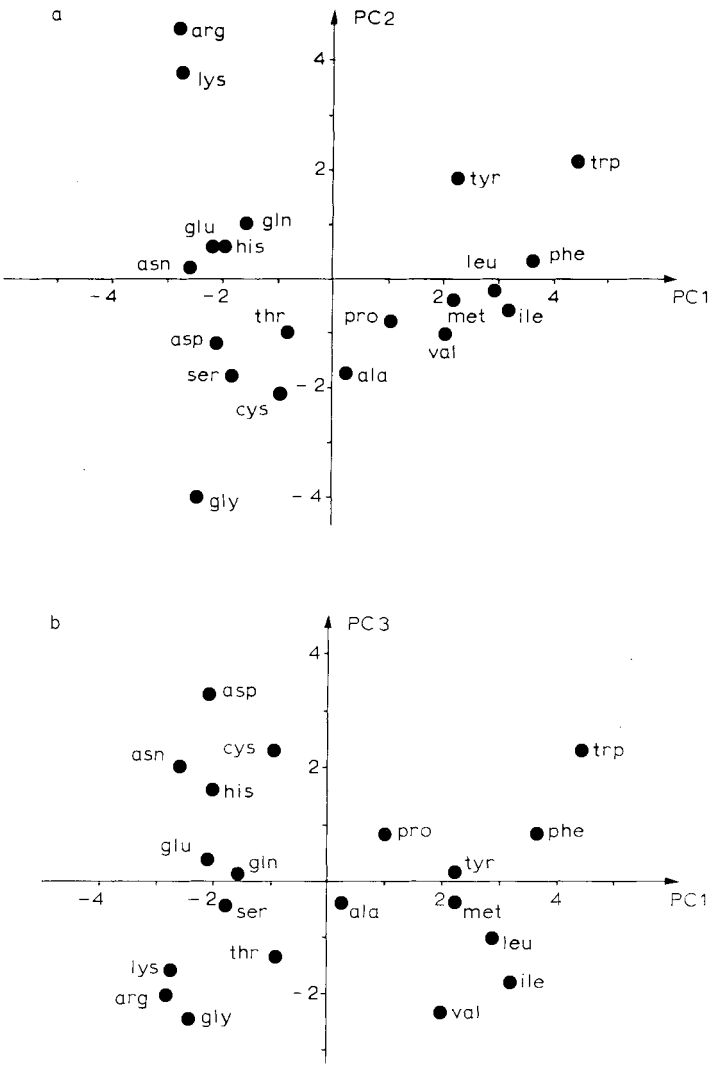


Figure 6.14. Scatter plot of t-*scores of (a) PC_1 versus PC_2 and (b) PC_1 versus PC_3 calculated from the physicochemical properties of amino acids in the peptide example.*

Table 6.4. PHYSICOCHEMICAL VARIABLES USED TO CHARACTERIZE THE AMINO ACIDS

NMR, nuclear magnetic resonance; R_f, rate of flow; dG, difference in free energy.

1. Molecular weight
2. pK_{COOH} (COOH on C_α)
3. pK_{NH_2} (NH_2 on C_α)
4. p*I*, pH at isoelectric point
5. Substituent van der Waals volume
6. ^{1}H-NMR for C_α–H (cation)
7. ^{1}H-NMR for C_α–H (dipolar)
8. ^{1}H-NMR for C_α–H (anion)
9. ^{13}C-NMR for C=O
10. ^{13}C-NMR for C_α–H
11. ^{13}C-NMR for C=O in tetrapeptide
12. ^{13}C-NMR for C_α–H in tetrapeptide
13. R_f for 1-*N*-(nitrobenzofurazono)amino acids in acetate/pyridine/water
14. Slope of plot of $1/R_f - 1$ *vs.* mol% in H_2O in paper chromatography
15. d*G* of transfer of amino acids from organic solvent to water
16. Free energy of transfer from vapor phase to water
17. R_f, salt chromatography
18. Log*P*, *P* = partition coefficient octanol-water
19. Log*D*, *D* = partition coefficient of acetyl-amide derivatives of amino acids in octanol-water
20. $dG = RT \ln f$; *f* = fraction (ratio) buried to accessible amino acids in 22 proteins

HYPOTHESIS TESTING

Two non-parametric methods for hypothesis testing with PCA and PLS are cross-validation and the jackknife estimate of variance. Both methods are described in some detail in the sections describing the PCA and PLS algorithms. Cross-validation is used to assess the predictive property of a PCA or a PLS model. The distribution function of the cross-validation test-statistic cvd-sd under the null-hypothesis is not well known. However, for PLS, the distribution of cvd-sd has been empirically determined by computer simulation technique [24] for some particular types of experimental designs. In particular, the discriminant analysis (or ANOVA-like) PLS analysis has been investigated in some detail as well as the situation with $\boldsymbol{Y}$ one-dimensional. This simulation study is referred to for detailed information. However, some tables of the critical values of cvd-sd at the 5% level are given in Appendix C.

PLS can be applied to data obtained from experiments designed to test some hypothesis. The BHT example is a part of a study investigating behavioural and neurochemical effects of dopamine agonists. The hypothesis tested was whether BHT has behavioural effects (that is, the null-hypothesis that the behaviour of BHT-treated animals does not differ from the behaviour of vehicle treated control animals is used). The analysis of the BHT data would in traditional statistics have been analysed by MR or by ten independent simple

linear (or non-linear) regressions. When analysed by PLS, the behavioural variables x_1–x_{10} (i.e., the effect variables) of the BHT 920 data are set up in the matrix $\boldsymbol{X}$ and the drug treatment is put in the one-dimensional matrix $\boldsymbol{Y}$. The first PLS dimension was significant according to cross-validation showing that there are changes in behaviour measured by the holeboard (and summarized in $\boldsymbol{X}$) which can be related to the drug treatment (summarized in $\boldsymbol{Y}$). The relation between $\boldsymbol{X}$ and $\boldsymbol{Y}$ can be visualized by plotting the *t*-scores against dose ($\boldsymbol{Y}$) as in *Table 6.15*. The second PLS component was not significant according to cross-validation. A plot of the *t*-scores of the first two PLS components (*Figure 6.16*) shows that the first component is similar to the first PC (*Figure 6.12*), whereas the second PLS component is rather different from the second PC, which was strongly influenced by an outlier (*vide supra*).

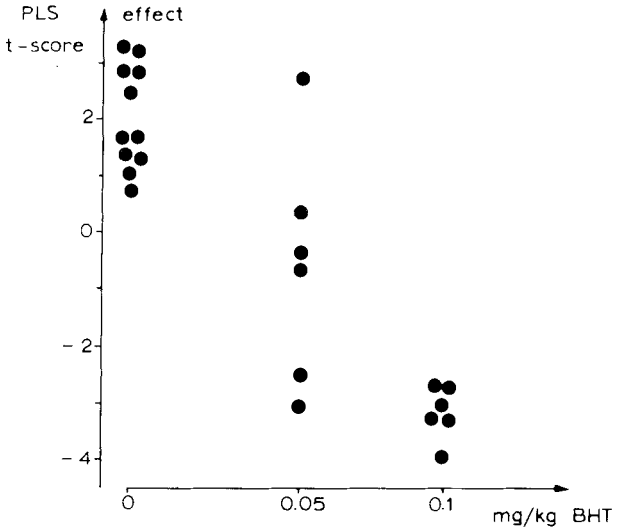


Figure 6.15. Plot of the t*-scores (drug effect) of the first PLS component calculated from the BHT data against dose BHT. By convention the dose has been plotted as the* x*-axes and effect (*t*-score) as the* y*-axis. This is opposite to the standard PLS plots (see* Figure 6.6*).*

The jackknife estimate of variance can be used to assess the significance of the weight and loading coefficients in a PLS model. This is a valuable source of information in case interpretation of the parameters is warranted. The weights with jackknifed standard deviations from the BHT example are given in *Table 6.5*. The weights of the first PLS dimension suggest that the behavioural effect of these doses of BHT is to suppress most aspects of a rat's

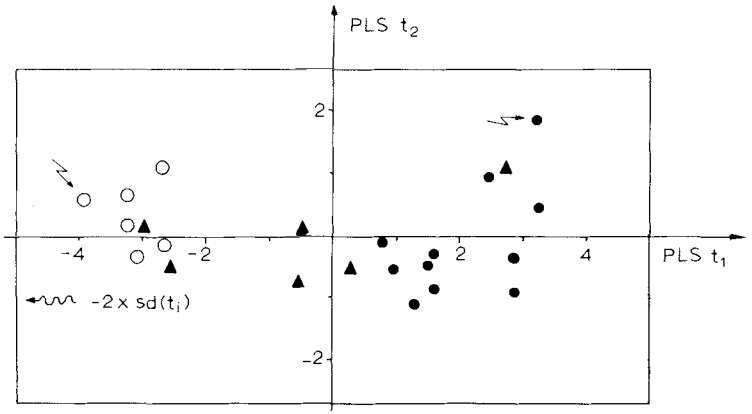


Figure 6.16. Scatter plot of the t-*scores of the first two PLS components calculated from the BHT data. Black circles are controls, black triangles are rats given 0.05 mg/kg of BHT 920 and open circles 0.1 mg/kg. The subjects pointed at by arrows are outliers in a direction at an angle with PLS_1 and PLS_2. See also* Figure 6.12.

Table 6.5. WEIGHTS AND JACKKNIFE ESTIMATES OF STANDARD DEVIATION FOR THE FIRST TWO PLS DIMENSIONS OF THE BHT 920 DATA

Variable	w_{1k}	w_{2k}
x_1	0.389 ± 0.025	−0.125 ± 0.148
x_2	0.372 ± 0.006	−0.072 ± 0.121
x_3	0.379 ± 0.028	−0.141 ± 0.162
x_4	0.383 ± 0.033	−0.167 ± 0.086
x_5	0.361 ± 0.054	−0.419 ± 0.183
x_6	0.153 ± 0.116	0.344 ± 0.353
x_7	−0.111 ± 0.136	0.099 ± 0.280
x_8	0.291 ± 0.040	0.341 ± 0.231
x_9	0.288 ± 0.043	0.291 ± 0.248
x_{10}	0.279 ± 0.049	0.338 ± 0.360

behaviour in the holeboard. Hence, BHT has effects similar to those of other dopamine agonists, such as apomorphine, when given in low doses [2]. Since none of the weight coefficients of the second PLS dimension was significant, no attempt has been made to interpret these weights.

As a second example of hypothesis testing with PLS, the amphetamine data are used. These data illustrate a control-treatment study. Such data are traditionally handled by univariate Student's *t*-tests applied to each variable separately. By means of the present methodology, the data are put in a three-way table, which is then unfolded (*Figure 6.10*) to the usual two-way table, $\boldsymbol{X}$, which has 10 rows and $4 * 6 = 24$ columns. A one-dimensional $\boldsymbol{Y}$ is

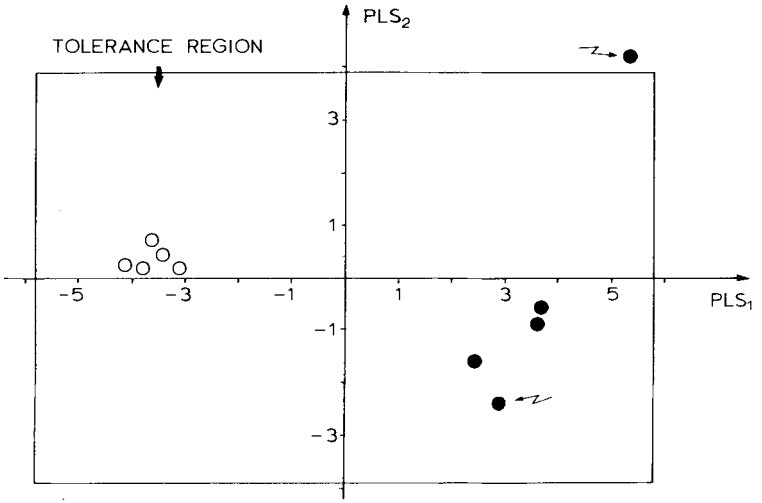


Figure 6.17. Scatter plot of the t-*scores of the first two PLS dimensions calculated from the amphetamine data. Open circles are controls and black circles are amphetamine-treated animals. Two outliers are pointed at by arrows. The tolerance region is roughly bounded in the direction of* PLS_1 *by* $\pm t_{0.025} * sd(t_{i1})$.

Table 6.6 TABLE OF WEIGHTS FOR AMPHETAMINE EXAMPLE

The upper six rows are the weights resulting directly from the PLS analysis and the last six rows are the weights as modelled by PCA with scores (fifth column) and loadings (middle row) as indicated.

Sample	*DA*	*DOPAC*	*5HIAA*	*HVA*	*PC* t-*score*
	original weights				
00– 20	0.210	−0.253	0.091	−0.247	0.410
20– 40	0.186	−0.230	0.061	−0.228	0.373
40– 60	0.188	−0.259	0.007	−0.254	0.408
60– 80	0.177	−0.259	−0.043	−0.252	0.403
80–100	0.191	−0.259	−0.048	−0.256	0.412
100–120	0.179	−0.260	−0.092	−0.248	0.402
Loadings	0.470	−0.632	−0.012	−0.617	
	PC model of weights				
00– 20	0.192	−0.259	−0.005	−0.253	
20– 40	0.175	−0.235	−0.004	−0.230	
40– 60	0.192	−0.258	−0.005	−0.252	
60– 80	0.189	−0.254	−0.005	−0.248	
80–100	0.193	−0.260	−0.005	−0.254	
100–120	0.189	−0.254	−0.005	−0.248	

used to quantify control, given 0, and treatment, given 1 as elements in $\boldsymbol{Y}$ (such 0-1 indicator variables are often referred to as dummy variables [8]). The data are then analysed by PLS and the first component is found to be highly significant. A plot of the first two t vectors is given in *Figure 6.17*. In addition to this analysis, the PLS weight vector (w) was folded back to form a two-way table $\boldsymbol{W}$ (*Figure 6.10*) which was then subjected to PCA. The first PC explains 97% of the variance in $\boldsymbol{W}$ and was highly significant. *Table 6.6* shows the close similarity between $\boldsymbol{W}$ and the one-component PC model of $\boldsymbol{W}$.

EXPERIMENTAL DESIGN

ASSESSING THE EFFECT OF FACTORS; FACTORIAL DESIGN

In order to investigate how a variable is influenced by a number of experimentally controllable factors, the most efficient way to obtain this knowledge is to use a statistically derived experimental design. There is, however, an intuitive method analogous to the OVAT approach of data analysis. This intuitive method consists of varying *o*ne *f*actor *a*t a *t*ime (OFAT) in the experimental design space (D-space), keeping all other controllable factors at a fixed level (*Figure 6.18*). It is often stated that if only one factor at a time is changed and all other factors are held constant, things are under control and the researcher knows what she or he is doing. Unfortunately, the best way to get almost as little information as possible out of an experiment (while still performing it) is the OFAT approach. This is easily realized if the results from the experiments illustrated in *Figure 6.18a* are considered. In these experiments,

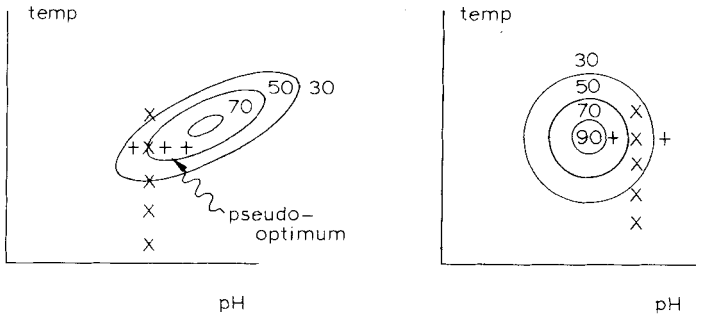


Figure 6.18. Illustration of the OFAT approach to experimental design. × represent experiments changing the temperature at a fixed pH and + represent experiments changing the pH at the previously found optimum temperature. The OFAT methodology will get stuck on a pseudo-optimum when the effect of changing pH is dependent on the temperature, as in (a). In the unusual case when the factor effects are independent of one another, as in (b), the OFAT approach works.

the influence of pH and temperature on the purity of the product of a chemical reaction is investigated. The OFAT method starts at a reasonable point in D-space (chosen by intuition, practical chemical experience, theoretical considerations, literature information, and so on) and then the temperature is varied, with the pH held constant, until the optimal temperature is found. Then the pH is varied at the optimum temperature, until the optimal pH has been located. It can be seen that, because the effect of changing pH varies with the temperature (and *vice versa*), the OFAT approach does not lead to the global optimum but to a point (a pseudo-optimum) somewhere half-way up the hill. From this point, the process cannot be improved irrespective of which direction is taken *out of the four possible directions allowed by the OFAT method.* The only way to improve the process is to move at an angle with the factor axes. There are, of course, situations when the OFAT methodology works (*Figure 6.18b*) because of a complete independence and lack of correlation between the effects of the factors. This is, however, rare.

Instead of changing one factor at the time, all factors should be changed simultaneously. This may sound chaotic but it is not, provided that the change in the factors is made *in a systematic way.* The most commonly used way to vary a number of factors is factorial design [29]. Using a chemical reaction as an example, this method proceeds as follows.

Starting with the same point as before, it is decided how much pH and temperature should be varied. One low (−) and one high (+) level of each factor is thus defined. Then $2^2 = 4$ experiments are performed as indicated in

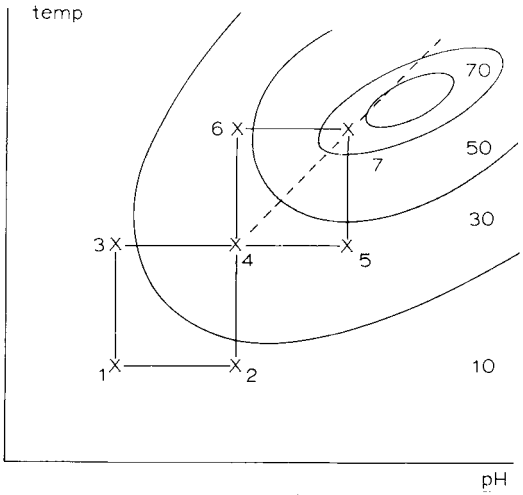


Figure 6.19. Sequential application of complete factorial designs in the same situation as in Figure 6.18a. *An alternative approach is the method of steepest ascent indicated by the dotted line.*

Figure 6.19 (experiments 1–4). The novelty compared with the OFAT approach is the experiment at the point 4, where both factors are high. This point gives information about the *interaction* between pH and temperature, that is, the particular influence of the combination of high temperature and high pH. With the results from these four experiments, the search for the optimum is continued, for example, by running three new experiments (5–7) in a factorial design using the best point from the previous set of experiments as the low level combination of pH and temperature. Alternatively, the method of steepest ascent [29] could have been used. Following the latter methodology, new experiments are done along the dotted line indicated in *Figure 6.19* until the response is not further improved and then a new factorial experiment is done about the best point along this line. The important thing here is not so much which method is used but the *principle* that all factors are allowed to vary over the experimental range in such a way that interaction effects can be detected. There is yet another method to find optimal conditions, called the simplex method [30, 31]. The simplex method is not different from the previous methods with respect to general principles and will therefore not be elaborated upon.

When three factors can be varied (for instance, the reaction time of the chemical reaction example can be added), a larger number of experiments must be made, namely $2^3 = 8$ in the factorial design (*Figure 6.20*). In the three-factor experimental design, a new type of interaction term appears, the three-factor interaction, which is the particular result of doing the synthesis at high pH and high temperature and using a long reaction time. In general, experiments

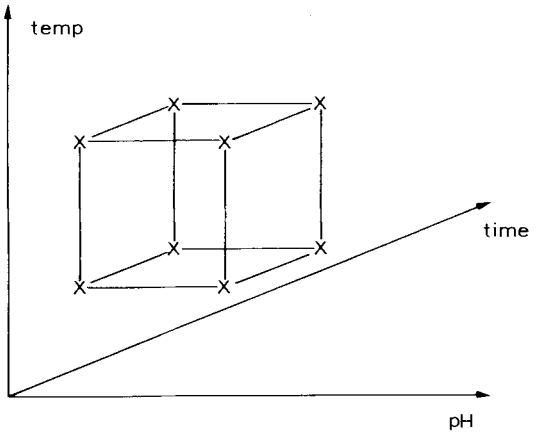


Figure 6.20. Illustration of a 2^3 complete factorial design using the same chemical example as in Figure 6.18 *and* Figure 6.19 *with the additional possibility to change the reaction time.*

involving k factors allow estimation of all kinds of interaction terms up to k-factor interactions in a 2^k factorial design. It is seen that even a moderate number of factors will increase the number of experiments beyond what is reasonable for most researchers, for example, 8 factors will require 256 experiments for the first complete factorial design. Luckily, experience shows that statistically significant high-order interactions rarely occur and the number of experiments can therefore be reduced without taking a serious risk. Consider the 2^5 factorial design resulting in 32 experiments which involves:

1* basepoint (all factors at the low level);
5* main factor effects;
10* two-factor interactions;
10 three-factor interactions;
5 four-factor interactions;
1 five-factor interaction.

If it is suspected that the probability of significant three- and higher-order interactions is negligible, it will suffice to make 16 (those marked with an asterisk) instead of 32 experiments which is a considerable gain in resources and time. This experimental design is called a half-fractional factorial design [29].

Thus, an efficient way to cut down the number of experiments is to use fractional factorial designs [29]. These make it possible to assess the main

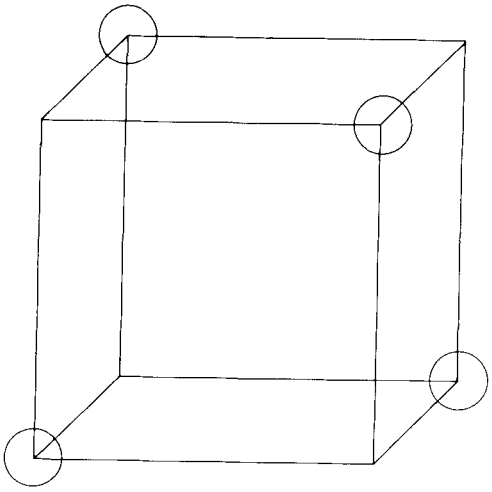


Figure 6.21. Visualization of an optimal 2^{3-1} fractional factorial design. The circles indicate factor combinations used in the experiments. It can be seen that this choice of experiments will span the three-dimensional D-space.

effect of each factor as well as low-order interactions. At the same time, D-space is properly spanned by the experiment. The easiest design to visualise is the three-factor design reduced to $2^{3-1} = 4$ experiments (*Figure 6.21*). The more complicated $2^{4-1} = 8$ design is shown in *Figure 6.22*. The price paid for making fractional instead of complete factorial designs is that high-order

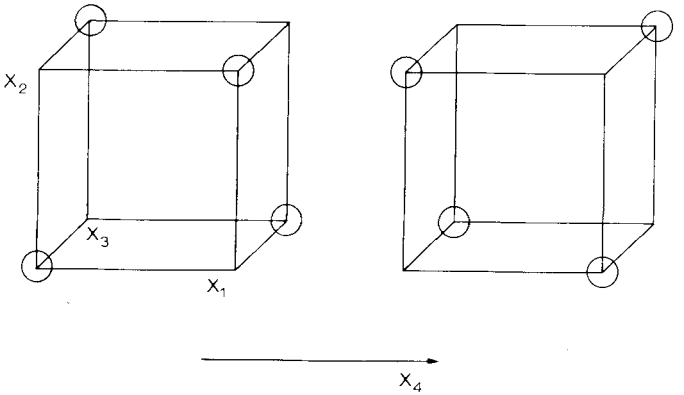


Figure 6.22. Visualization of an optimal 2^{4-1} fractional factorial design. The design is constructed by first taking the 2^{3-1} design of Figure 6.21 *as the low level of the fourth factor and then the 2^{3-1} design complementary to the design in* Figure 6.21 *(at the right-hand side of this figure) is taken as the high level of the fourth factor.*

interaction effects are confounded with the main factor effects and with low-order interactions. However, in case it is found that one factor has no effect, it is possible to use this information and construct more complete designs from the data (*Figure 6.23*) provided that the experiment is carefully planned. Methods to construct complicated fractional factorial designs are given in [29].

An application of experimental designs of particular interest to medicinal chemists is their use in quantitative structure activity relation studies (QSAR) as indicated above in the section on data description, multivariate characterization. In QSAR, the problem is to change the substituents of a drug molecule systematically so that the pharmacological properties of the drug are improved and to establish, whenever possible, the relation between the chemical properties of a class of similar molecules and their pharmacological effects. As before, the intuitive method is to change one substituent at a time (COST). The COST approach has the same drawbacks as OVAT and OFAT, as discussed by Hellberg [1]. The more efficient approach is to employ a factorial or a fractional factorial design. Considering the vast amount of measurements of physicochemical properties of substituents, it is realized that these cannot form a basis

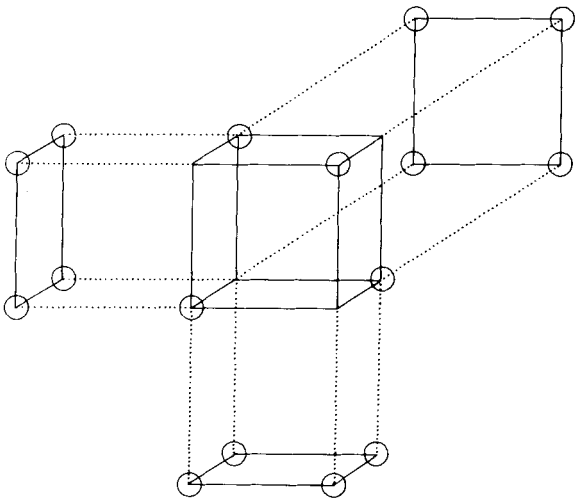


Figure 6.23. If one of the three factors investigated in a 2^{3-1} fractional factorial design turns out to have no effect, a complete factorial design in the remaining two factors can be reconstructed from the data. If the design used in this figure is employed, this result will hold, irrespective of which of the three factors turns out to be insignificant. The same result holds for the 2^{4-1} fractional factorial design in Figure 6.22, *which will collapse to a complete 2^3 design in the remaining factors if any one turns out to be insignificant.*

for even a fractional factorial design because they are too many. Furthermore, many measurements tend to be indicators of the same underlying property, for example, MW and log$P$ will both be indicators of lipophilicity in a series of hydrocarbons. This will result in multicollinearity and severe problems in choosing substituents for new syntheses.

A better approach to the problem of choosing substituents is to characterize the substituents by PCA, as described above, in order to capture the largest sources of distinguishable chemical properties. If the number of significant PCs are three and there are two substituent sites on the drug molecule, it will suffice to synthesise $2 * 2^3 = 16$ new compounds in order to cover the experimental domain by a factorial design in the PC t-scores. It should be noted that, even though it is preferable to have exactly two levels on each PC, this is often impossible in practice. Instead, the substituents should be chosen as to make the experimental design come as close as possible to a theoretically satisfactory design. Again, it is the *principle* of spanning the chemical property space as well as possible that matters and not to rigidly conform to a theoretical model. As

long as the effects of various substituents and the interactions between them can be reliably assessed, there is a solid ground for planning new experiments.

The peptide example illustrates these points. The PC *t*-scores of the first three principal components of the physicochemical properties of amino acids are plotted in *Figure 6.14*. This space is well spanned by arg, asp, gly, ile and trp (*Figure 6.24*), which may therefore form a basis for construction of a fractional factorial design of a QSAR as previously suggested [6].

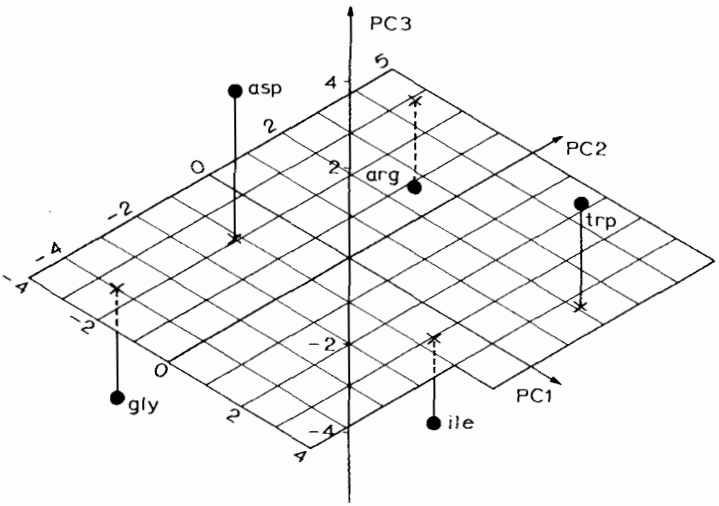


Figure 6.24. Choice of five amino acids that will efficiently span the three-dimensional D-space defined by the first three PCs from the analysis of the physicochemical properties of the amino acids illustrated in Figure 6.14.

MULTI-FACTOR AND/OR MULTI-RESPONSE EXPERIMENTS

In many cases, both a number of factors are varied in experiments and many response variables are measured. In such situations, there are often correlations between response variables. This suggests that it may be useful to try to find out to what extent the response variables can be independently affected by the factors and interactions between the factors. One way to analyse this problem is to use PCA or PLS and determine how many significant dimensions that can be extracted in designed studies. As an example, the influence of various mobile phase factors in a HPLC system for separating catecholamines and some catechol- and indolamine metabolites was investigated measuring the retention

time of the amines (Ståhle, unpublished data). In this system, the pH, the percentage methanol, the (phosphate) buffer strength and the concentration of an ion-pair reagent could be varied. It was found that the PLS model had only two significant dimensions. One dimension selectively affected the retention time of the catecholamines, while the other dimension changed the retention time of all compounds in the same direction and to the same extent. This result shows that catecholamines can be moved in the chromatogram independently of the metabolites. Within the catecholamines, no independent moves are possible and the situation is the same for the metabolites. From the result of the PLS analysis it may be concluded that a new principle in the chromatographic separation must be introduced if the attempt is to be made to selectively affect the retention time of, for instance, the indolamine metabolite, 5-hydroxyindolacetic acid, relative to the metabolites of the catecholamines.

Similarly, in QSAR, the aim is to improve the pharmacological properties of a drug and this usually involves an increase in selectivity for one type of receptor by reducing the drug affinity for one or more side-effect-related receptors. A good example of a class of drugs that affect an array of receptors is the antipsychotic phenothiazines, which are antagonists on dopamine-, muscarinic-, serotonin-, histamine- and α_1-receptors [32]. A QSAR study attempting to reduce the affinity of phenothiazines for all receptors but the dopamine receptors might be analysed by PLS in order to determine whether the substituents used really allow an independent increase in the affinity for dopamine receptors and/or a decrease in the affinity for the other receptors.

CONCLUSIONS

This chapter constitutes an attempt to demonstrate the utility of multivariate statistics in several stages of the scientific process. As a provocation, it is suggested that the multivariate approach (in experimental design, in data description and in data analysis) will *always* be more informative and make generalizations more valid than the univariate approach. Finally, the multivariate strategy can be really enjoyable, not the least for its capacity to reveal hidden treasures in data that in a univariate analysis look like a set of random numbers.

ACKNOWLEDGEMENTS

The present study was supported by grants from the Swedish Medical and Natural Science Research Councils and from the Karolinska Institute.

APPENDIX A

ALGORITHMS

In this appendix PCA and PLS will be described in some detail. Readers who are less interested in the mathematical and statistical details can restrict themselves to *Figures 6.5–6.8*, *6.25* and *6.27*, which illustrate the main concepts of PCA and PLS used in practice.

Principal components analysis

This method is applicable when data are to be inspected and characterized. PCA is easily understood by graphical illustrations, for example, by a two-dimensional co-ordinate system with a number of points in it (*Figure 6.25*). The first principal component (PC) is the line with the closest fit to these points [12]. Unless the point swarm has, for example, the shape of a circle, the position of the first PC is unambiguous. Because the first PC is the line of closest fit, it is also the line that explains most of the variation (maximum variance) in the data [13]. Therefore it is called the principal component.

The term 'closest fit' is used to denote a least-squares fit. In PCA, the sum of the squared distances from the points perpendicularly to the fitted line (*Figure 6.25*) is minimized.

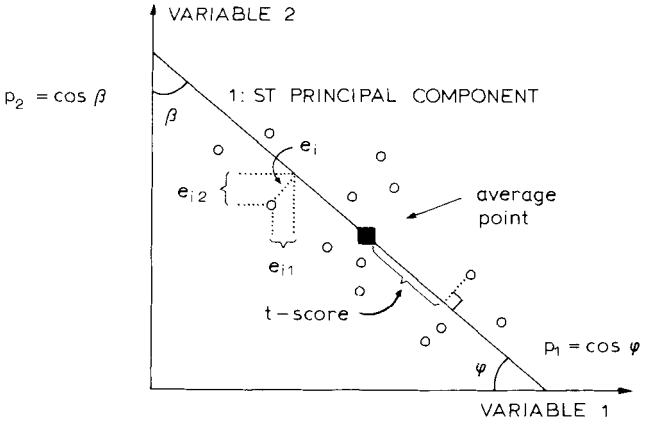


Figure 6.25. The first principal component (PC) is the line that best fits the data points by minimizing the sum of the squared distances from the points perpendicular to the PC. By the Pythagorean theorem ($e_{i\,1}^2 + e_{i\,2}^2 = e_i^2$)*, it can be seen that this is equivalent to Eqn. (2). The* t*-score of a subject is the distance from the average point to the projection of the subject onto the PC. The* k*th element of the loading vector* **p** *is the cosine of the angle between the* k*th variable and the PC.*

Let the observed data be organized in a table (matrix) $\boldsymbol{X}$ with n rows (subjects) and p columns (variables). The indices i (i:$i = 1..n$) and k (k:$k = 1..p$) are used for rows and columns respectively (*Figure 6.26*). In

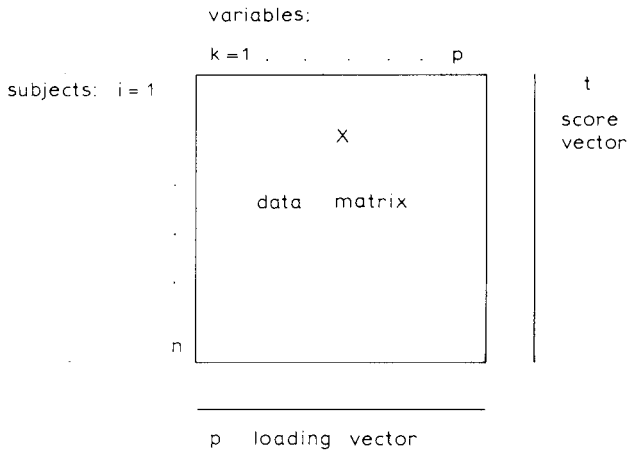


Figure 6.26. Illustration of the matrix and vectors used in PCA.

matrix notation (for readers unfamiliar with matrix algebra, the necessary concepts and formulae are given in Appendix B), a one-dimensional PC model is written:

$$\boldsymbol{X} = \boldsymbol{\alpha} + \boldsymbol{tp} + \boldsymbol{E} \tag{1}$$

where $\boldsymbol{\alpha}$ is the row vector of variable means and $\boldsymbol{t}$ is the score vector (column vector, see *Figure 6.26*), with elements t_i being the projection of the ith subject perpendicular on to the PC (*Figure 6.25*). The row vector, $\boldsymbol{p}$ (*Figure 6.26*) is called the loading vector, with elements P_k, being the cosine of the angle between the kth variable axis and the PC. The matrix, $\boldsymbol{E}$, is the matrix of residuals having the property that

$$\text{RSS} = \sum_{i=1}^{n} \sum_{k=1}^{p} e_{ik}^2 \tag{2}$$

is minimized, which is equivalent to minimizing the sum of the squared perpendicular distances to the PC, as can be seen in *Figure 6.5*. To calculate the PC-model, the NIPALS algorithm [14] has been used for several reasons. It is fast, it uses raw data-input, it tolerates missing values [16] and it can be used with cross-validation [33]. The algorithm is easily written in matrix

notation (the sign := denotes assignment, as in the computer language Pascal):

A. start guess of $\boldsymbol{t}$, for example, the first column in $\boldsymbol{X}$.

B. $\boldsymbol{p}' := \boldsymbol{X}'\boldsymbol{t} \div \boldsymbol{t}'\boldsymbol{t}$ (3)

C. $\varphi_p := \sqrt{\boldsymbol{p}\boldsymbol{p}'}$ (4)

D. $\boldsymbol{p} := \boldsymbol{p} \div \varphi_p$ (5)

E. $\boldsymbol{t} := \boldsymbol{X}\boldsymbol{p}' \div \boldsymbol{p}\boldsymbol{p}'$ (6)

F. repeat B–F until two successive $\boldsymbol{t}$ are sufficiently similar. Steps C and D are scaling of $\boldsymbol{p}$ to unit length (which means that the elements of p are scaled so that $\sqrt{\Sigma p_k^2} = 1$).

Because of the denominators in steps B and E, it is possible to do the computations only over non-missing values [16]. This means that by means of the NIPALS algorithm all information can be used, making deletion of incomplete observations unnecessary.

When the first PC has been calculated, there may still be information of interest left in $\boldsymbol{X}$. This can be illustrated in a three-dimensional space by a data scatter shaped like a flattened spindle (*Figure 6.27*). Such data have two

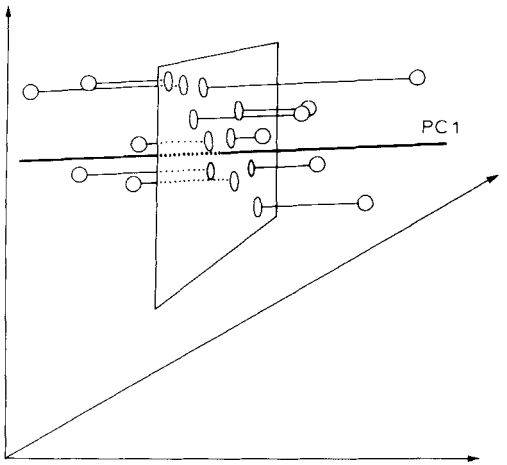


Figure 6.27. Illustration of calculation of the error matrix, **E**, *by subtracting* **tp** *from* **X**, *which corresponds to projecting the data points onto a plane perpendicular to the PC. This plane goes through the average point.*

significant dimensions: the length and the width of the spindle. Hence, there is non-random variation in the residuals when the first dimension has been fitted. To fit the second PC, the substitution $\boldsymbol{X} := \boldsymbol{E}$ is first made, that is, the dimension of the first PC is subtracted from $\boldsymbol{X}$, which corresponds to projecting the data points in M-space onto a plane perpendicular to the PC (*Figure 6.27*). To calculate the next dimension, the NIPALS-procedure above is repeated using the new $\boldsymbol{X}$ matrix. In principle, it is possible to calculate as many PCs as the number of variables or the number of objects minus one (whichever is smallest). However, usually only a few PCs are statistically significant. The significance testing is discussed below.

It should be noted that the handling of missing values presents a problem when dimensions higher than one are calculated [16]. If the fraction of missing values is reasonably small (25%), this problem can be solved by a particular methodology described elsewhere [19].

When the significant PC's have been extracted from $\boldsymbol{X}$, the information left in the error matrix, $\boldsymbol{E}$, can be used to estimate the residual variance of the model. This corresponds to constructing a tolerance volume around the PC model. This can be illustrated only with three-dimensional data and a one-dimensional PC model (*Figure 6.5*). There are no mathematical restrictions on estimating the residual variance in higher-dimensional models, but the human ability to visualize and understand spaces with more than three dimensions is limited. The residual variance can be calculated as

$$\text{var} := \sum_{i=1}^{n} \sum_{k=1}^{p} (e_{ik}^2) \div ((p - a)(n - a - 1)) \tag{7}$$

where n is the number of subjects, p is the number of variables and a is the number of dimensions in the PC model. The residual variance of each individual subject is

$$\text{var}_i := \sum_{k=1}^{p} (x_{ik} - (\alpha_k + \sum_{d=1}^{a} t_{id} p_{dk}))^2 \div (p - a) \tag{8}$$

where d ($d{:}d = 1..a$) is the index for PC dimensions. By forming a ratio between (8) and (7), it is possible to estimate whether this subject is an outlier, since this ratio is an approximate F-test with $(p - a)$ and $(p - a)(n - a - 1) \div 2$ degrees of freedom (*Figure 6.5*).

To assess whether a principal component is significant (that the shape of the point swarm differs sufficiently from that of a spheroid), cross-validation is employed [33–36]. In cross-validation, a part (say 1/4th or 1/5th) of the data is held out in a patterned way (deletions in diagonal bands in $\boldsymbol{X}$ as in

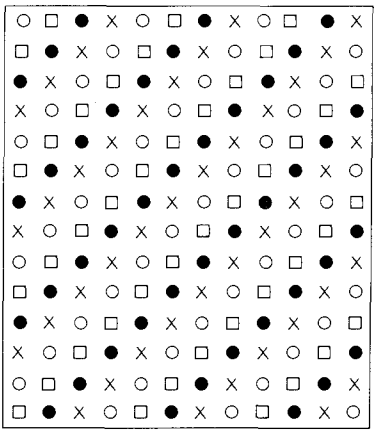


Figure 6.28. Deletion pattern in **X** *used in cross-validation of the number of components in a PC model of* **X**. *In this example,* g = 4 *cross-validation rounds were calculated. In the first round, elements marked by open circles are deleted. In the next round, open squares are deleted, then filled circles and finally crosses are deleted.*

Figure 6.28) and the PC-model is calculated for the reduced data-set. Because the PC model of $\boldsymbol{X}$ is the product of $\boldsymbol{t}$ and $\boldsymbol{p}$, the model predicts the held-out elements (the element x_{ik} is predicted as $t_i p_k$). Hence, by comparing the prediction of the held-out elements with their actual values, an estimate of the predictive power of the model is obtained. The usual estimator of the predictive power in PCA and PLS is prediction error sum of squares (PRESS), defined as:

$$\text{PRESS} = \sum_{i=1}^{n} \sum_{k=1}^{p} (x_{ik} - t_i p_k)^2 \tag{9}$$

where the summation is made over held-out elements. By reinserting the held-out elements, holding out another part of the data and repeating the procedure until each element in $\boldsymbol{X}$ has been held out once and once only, the total PRESS will get exactly one contribution from each element in $\boldsymbol{X}$. By comparing PRESS with the residual sum of squares (RSS)

$$\text{RSS} = \sum_{i=1}^{n} \sum_{k=1}^{p} x_{ik}^2 \tag{10}$$

before the model has been calculated, it can be tested if the model performs better than chance. Thus, the more

$$\text{cvd-sd} = q\sqrt{\text{PRESS} \div \text{RSS}} \tag{11}$$

is below 1.0 the better is the model. The correction factor, q, (initially 1.0) is used to avoid the bias appearing when higher dimensions are fitted. An advantage with cross-validation is that it is non-parametric as opposed to, for example, chi-square tests on the residuals which require a multinormal distribution [15].

In addition to assessing the significance of a PC dimension, there is also a need to assess the significance of the elements of the loading vector ($\boldsymbol{p}$) in case there should be a need for interpretation of $\boldsymbol{p}$. In principle, there are two ways to assess the significance of each loading. One is to apply the cross-validation not only to the whole matrix, $\boldsymbol{X}$, but also to each variable. Thus the more

$$\text{cvd-sd}_k := \sqrt{\text{PRESS}_k \div \text{RSS}_k} \tag{12}$$

is below 1.0 the more significant is the kth variable contributing to the variance of the PC. The relation between the variable cvd-sd_k and the total cvd-sd is

$$\text{cvd-sd} = \sqrt{\sum_{k=1}^{p} \text{cvd-sd}_k^2} \tag{13}$$

A second possibility is to use some estimate of the variance of the loadings. This can be done by the jackknife method due to Quenouille and Tukey (see [37]) or by Efron's bootstrap method [38] (the colourful terminology stems from the expressions 'jack of all trades and master of none' and 'lifting yourself up by your own bootstraps'). The use of the bootstrap to estimate the variance of the loadings in PCA has been described [39] and will not be elaborated upon further. The jackknife method is used partly because it is a natural side-product of the cross-validation and therefore computationally non-demanding and partly because the jackknife estimate of variance is used later on in conjunction with PLS.

The jackknife method is based on an idea similar to cross-validation. The calculation of the statistical model is repeated g times holding out $1/g$th of the data each time. In the end, each element has been held out once and once only (exactly as in cross-validation). Thus, a number of estimates of each parameter is obtained, one for each calculation round. It has been proposed that the quantity

$$\text{VAR}(\pi) := \frac{(g-1)}{g} \times \sum_{s=1}^{g} (\pi_s - \pi_{(\cdot)})^2 \tag{14}$$

is an estimate of the variance of π [37] where π is the parameter, and π_s is the sample estimate of π with elements held out during the sth calculation and $\pi_{(\cdot)} = \sum_{s=1}^{g} \pi_s \div g$. It has been shown theoretically that the jackknife estimate is conservative in expectation [40], which has been confirmed in computer

simulation studies [41]. Because of this conservativeness, the jackknife can be used with some confidence for interpretative purposes. The application of the jackknife to estimating the variance of the loadings is straightforward. The statistical formula (14) is used simply by substituing π: = p_k for the kth variable.

Plots

Because the first two or three PC's usually describe the major variation in a data matrix $\boldsymbol{X}$, plots of the first two or three score vectors $\boldsymbol{t}_d$ give a graphical display of the dominant patterns in $\boldsymbol{X}$. As an example, the first two PC's of the amphetamine data are plotted in *Figure 6.29*. The first three PCs of the peptide

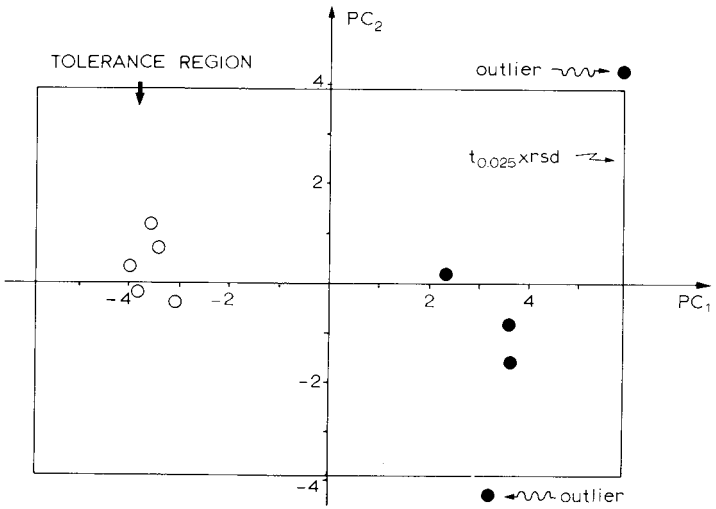


Figure 6.29. Scatter plot of the t*-scores of the first two PCs calculated from the amphetamine data. Open circles are controls and black circles are amphetamine-treated animals. Two outliers are pointed at by arrows. The tolerance region is roughly bounded in the direction of* PC_1 *by* $\pm t_{0.025} * sd(t_{i1})$.

data are plotted in *Figure 6.14* and first two PCs of the BHT 920 data are plotted in *Figure 6.12*.

In addition to the score plot, the loading vectors can also be plotted as exemplified by the BHT example (*Figure 6.13*). Note that directions in the score plots and the loading plots correspond to one another, for example, the directions in *Figure 6.12* correspond to those in *Figure 6.13*.

Scaling

Both PCA and PLS are sensitive to the scaling of the variables. It is therefore customary to scale the data variable-wise to zero mean and unit variance. However, the possibility to employ a different scaling whenever this seems to be appropriate should be kept in mind. For instance, a well-known important variable may be given a larger variance, for example, 3.0 instead of 1.0. Another situation in which a different scaling may be considered is when the variables are obviously blocked in such a way that each block contains closely related variables. Each block may then be given equal variance whereby the blocks have equal chances to influence the direction of the PC or PLS dimension.

Partial least squares (PLS) analysis

While PCA can reveal structure in a set of data viewed in isolation, PLS can be used to disclose structure in the data in view of external information [19–21]. The amphetamine example was analysed by PCA above without using the knowledge about the treatment that each subject received. In PLS, such information can be included. This corresponds to tilting the PC so that the score vector better describes the relation between the treatment and the changes in the measured responses. In *Figures 6.12* and *6.17*, the PLS scores of the BHT data and the amphetamine data are plotted, respectively. The strategy used in PLS is to add a new matrix, ***Y***, in addition to the matrix of measurements ***X***. The matrix ***Y*** contains external information such as treatment of each

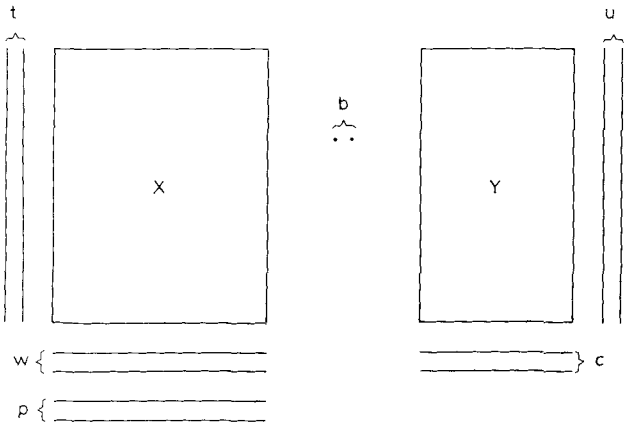


Figure 6.30. Illustration of the matrices and vectors used in a PLS model. In this example, two PLS dimensions are indicated by the presence of two vectors each of **t**, **u**, **w**, **p** *and* **c**, *as well as two inner relation regression coefficients,* b.

subject. Thus, PLS analysis closely corresponds to multiple regression, but with the difference that PLS can cope with collinearities in $\boldsymbol{X}$, a large number of $\boldsymbol{X}$ variables and several $\boldsymbol{Y}$ variables (see also *Figure 6.8*). In more detail, the constituents of the PLS model are (see also *Figure 6.30*):

(a) the $(n * p)$ matrix $\boldsymbol{X}$ of observations which may be regarded as a set of n points in the p-dimensional measurement space (M-space);

(b) the $(n * q)$ matrix, $\boldsymbol{Y}$, containing information external to $\boldsymbol{X}$, such as experimental design variables, for example, dose or group membership (0–1 variables). $\boldsymbol{Y}$ may be regarded as a set of n points in a q-dimensional space. The index j ($j : j = 1..q$) is used for the variables of $\boldsymbol{Y}$.

(c) an $(n * 1)$ score vector, $\boldsymbol{t}$, which is the estimated latent variable (LV) of $\boldsymbol{X}$. The ith element of $\boldsymbol{t}$ is the projection of the ith subject in M-space onto the LV.

(d) a $(1 * p)$ weight vector, $\boldsymbol{w}$, which determines the direction of the LV $\boldsymbol{t}$ in M-space.

(e) a $(1 * p)$ loading vector, $\boldsymbol{p}$, which is used to calculate the residuals $\boldsymbol{E}$ in the model of $\boldsymbol{X}$.

(f) an $(n * 1)$ score vector, $\boldsymbol{u}$, which is the estimated LV of $\boldsymbol{Y}$. The elements of $\boldsymbol{u}$ are the projections of the points in $\boldsymbol{Y}$ onto $\boldsymbol{u}$.

(g) a $(1 * q)$ weight vector, $\boldsymbol{c}$, which determines the direction of $\boldsymbol{u}$ in $\boldsymbol{Y}$-space.

(h) a scalar, b, which is the regression coefficient between $\boldsymbol{t}$ and $\boldsymbol{u}$, that is, b connects $\boldsymbol{X}$ and $\boldsymbol{Y}$ and is therefore called the inner relation.

In PCA, $\boldsymbol{t}$ and $\boldsymbol{p}$ are used to model $\boldsymbol{X}$. In PLS, $\boldsymbol{X}$ (assuming that $\boldsymbol{X}$ has been scaled to zero mean) is similarly modelled by:

$$\boldsymbol{X} = \boldsymbol{tp} + \boldsymbol{E} \tag{15}$$

In addition, there is a model of $\boldsymbol{Y}$:

$$\boldsymbol{Y} = b\boldsymbol{tc} + \boldsymbol{F} \tag{16}$$

where $\boldsymbol{F}$ is the matrix of residual errors in $\boldsymbol{Y}$. It is seen that the LV $\boldsymbol{t}$ of $\boldsymbol{X}$ is the common part of the two matrices, $\boldsymbol{X}$ and $\boldsymbol{Y}$. These and other features of the PLS model are illustrated in *Figure 6.6*. The algorithm [19, 21] proceeds as follows:

A. Make a starting guess of $\boldsymbol{u}$ (*e.g.*, y_1)

B. $\boldsymbol{w}' := \boldsymbol{X}'\boldsymbol{u} \div \boldsymbol{u}'\boldsymbol{u}$ (17)

C. $\varphi_w := \sqrt{\boldsymbol{w}\boldsymbol{w}'}$ (18)

D. $\boldsymbol{w} := \boldsymbol{w} \div \varphi_w$ (19)

E. $\boldsymbol{t} := \boldsymbol{X}\boldsymbol{w}' \div \boldsymbol{w}\boldsymbol{w}'$ (20)

F. $\boldsymbol{c}' := \boldsymbol{Y}'\boldsymbol{t} \div \boldsymbol{t}'\boldsymbol{t}$ (21)

G. $\varphi_c := \sqrt{\boldsymbol{c}\boldsymbol{c}'}$ (22)

H. $\boldsymbol{c} := \boldsymbol{c} \div \varphi_c$ (23)

I. $\boldsymbol{u} := \boldsymbol{Y}\boldsymbol{c}' \div \boldsymbol{c}\boldsymbol{c}'$ (24)

J. Repeat B–J until two successive $\boldsymbol{u}$ are sufficiently similar

K. $\boldsymbol{p}' := \boldsymbol{X}'\boldsymbol{t} \div \boldsymbol{t}'\boldsymbol{t}$ (25)

L. $\varphi_p := \sqrt{\boldsymbol{p}\boldsymbol{p}'}$ (26)

M. $\boldsymbol{p} := \boldsymbol{p} \div \varphi_p$ (27)

N. $\boldsymbol{t} := \boldsymbol{t}\varphi_p$ (28)

O. $\boldsymbol{w} := \boldsymbol{w}\varphi_p$ (29)

P. $b := \boldsymbol{t}'\boldsymbol{u} \div \boldsymbol{t}'\boldsymbol{t}$ (30)

The scalars, φ, are used to scale weights and loadings to unit length in the steps A–B, G–H and L–M. The steps L–O are optional because it is also possible to have $\boldsymbol{w}$ with unit length instead of $\boldsymbol{p}$.

In the same way as in PCA, more than one dimension can be calculated. This is done by computing

$$\boldsymbol{E} := \boldsymbol{X} - \boldsymbol{t}\boldsymbol{p} \tag{31}$$

$$\boldsymbol{F} := \boldsymbol{Y} - b\boldsymbol{t}\boldsymbol{c} \tag{32}$$

and then substitute $\boldsymbol{X} := \boldsymbol{E}$ and $\boldsymbol{Y} := \boldsymbol{F}$ and repeat the steps A–P with the new $\boldsymbol{X}$ and $\boldsymbol{Y}$.

The significance of each PLS dimension is assessed by cross-validation. The deletion pattern is, however, different from cross-validation of PCA. In PLS, whole subjects are deleted, that is, every gth row of $\boldsymbol{X}$ and $\boldsymbol{Y}$ is held out. The $\boldsymbol{Y}$ values of the held-out subjects are then calculated using the PLS model and the $\boldsymbol{X}$ values of the held-out subjects.

$$\text{PRESS}_j := \sum_{i=1}^{n} (y_{(i)j} - bt_{(i)}c_j)^2 \tag{33}$$

using step E (18) $t_{(i)} := \Sigma x_{(i)k} w_k$ to calculate the score of the held-out ith subject. In the same way as with PCA, this compares

$$\text{RSS}_j := \sum_{i=1}^{n} y_{ij}^2 \tag{34}$$

with PRESS_j to get

$$\text{cvd} \div \text{sd}_j := \sqrt{\text{PRESS}_j \div \text{RSS}_j} \tag{35}$$

If the value of cvd ÷ sd_j is below 1.0, this indicates that the PLS component is significant for the jth $\boldsymbol{Y}$ variable. Note that PRESS and RSS only involve $\boldsymbol{Y}$. This is so because the PLS model is formulated as prediction of $\boldsymbol{Y}$ from $\boldsymbol{X}$. In a recent simulation study, it has been shown how the 5%-level varies with the number of objects and the percentage variance in $\boldsymbol{X}$ explained by the first PLS dimension [24].

APPENDIX B

SOME ELEMENTARY MATRIX ALGEBRA

In the present study, matrix algebra (see [42] for a more detailed description) is used as a shorthand for otherwise tedious formulae. In matrix algebraic notation, small boldface letters denote vectors (for example, $\boldsymbol{x}$) and capital boldface letters denote matrices (for example, $\boldsymbol{X}$). Vectors can be either row vectors or column vectors (see *Figure 6.31*, in which some operations are

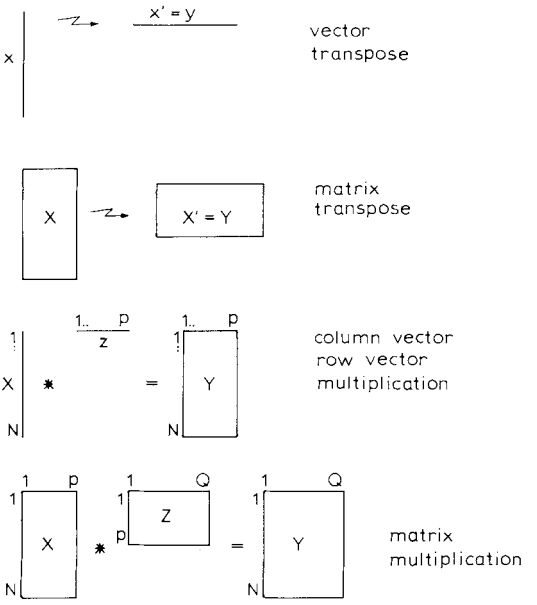


Figure 6.31. Illustration of matrix algebraic operations.

illustrated). A matrix is the same as a table with rows and columns. Each row in a matrix can be regarded as a row vector and each column as a column vector. The following operations on vectors and matrices are used in the present study:

Multiplication of a matrix by a scalar:

$$\boldsymbol{Y} := a\boldsymbol{X} \Leftrightarrow y_{ij} := ax_{ij} \tag{M1}$$

is equivalent to multiplication of each element in $\boldsymbol{X}$ by the scalar a.

Transposition of a vector

$$\boldsymbol{y} := \boldsymbol{x}' \tag{M2}$$

is equivalent to transforming the column vector, $\boldsymbol{x}$, to the row vector, $\boldsymbol{y}$ (*Figure 6.31*).

Transposition of a matrix

$$\boldsymbol{Y} := \boldsymbol{X}' \Leftrightarrow y_{ij} := x_{ji} \tag{M3}$$

is equivalent to transforming, in order, the columns of $\boldsymbol{X}$ to the rows of $\boldsymbol{Y}$ (*Figure 6.31*).

Addition of matrices

$$\boldsymbol{Y} := \boldsymbol{X} + \boldsymbol{Z} \Leftrightarrow y_{ij} := x_{ij} + z_{ij} \tag{M4}$$

is addition of corresponding elements in $\boldsymbol{X}$ and $\boldsymbol{Z}$.

Norm (length) of a column vector

$$a := \sqrt{\boldsymbol{x}'\boldsymbol{x}} \Leftrightarrow a := \sqrt{\sum_{i=1}^{n} x_i^2} \tag{M5}$$

is the square root of the sum of the squared elements of $\boldsymbol{x}$.

Scalar product of a row vector ($\boldsymbol{x}$) and a column vector ($\boldsymbol{z}$)

$$a := \boldsymbol{x}\boldsymbol{z} \Leftrightarrow a := \sum_{i=1}^{n} x_i z_i \tag{M6}$$

is the sum of products of corresponding elements in $\boldsymbol{x}$ and $\boldsymbol{y}$

Matrix multiplication of a column vector ($\boldsymbol{x}$) by a row vector ($\boldsymbol{z}$)

$$\boldsymbol{Y} := \boldsymbol{x}\boldsymbol{z} \Leftrightarrow y_{ij} := x_i z_j \tag{M7}$$

the element y_{ij} of the matrix $\boldsymbol{Y}$ at the intersection of its ith row and the jth column is the product of the ith element in $\boldsymbol{x}$ and the jth element in $\boldsymbol{z}$ (*Figure 6.31*).

Matrix multiplication of a matrix $\boldsymbol{X}$ by a column vector, $\boldsymbol{z}$, with as many elements as there are columns in $\boldsymbol{X}$

$$\boldsymbol{y} := \boldsymbol{X}z \Leftrightarrow y_i := \sum_{j=1}^{q} x_{ij} z_j \tag{M8}$$

the ith element of the columns vector $\boldsymbol{y}$ is the scalar product of the ith row of $\boldsymbol{X}$ and the column vector z.
Matrix multiplication of a row vector $\boldsymbol{x}$ by a matrix $\boldsymbol{Z}$

$$\boldsymbol{y} := \boldsymbol{x}\boldsymbol{Z} \Leftrightarrow y_j := \sum_{i=1}^{n} x_i z_{ij} \tag{M9}$$

the jth element of the row vector $\boldsymbol{y}$ is the scalar product of $\boldsymbol{x}$ and the jth column of $\boldsymbol{X}$.
Matrix multiplication of a matrix, $\boldsymbol{X}$, by another matrix, $\boldsymbol{Z}$

$$\boldsymbol{Y} := \boldsymbol{X}\boldsymbol{Z} \Leftrightarrow y_{ij} := \sum_{k=1}^{p} x_{ik} z_{kj} \tag{M10}$$

the element y_{ij} of the matrix $\boldsymbol{Y}$ is the scalar product of the ith row of $\boldsymbol{X}$ and the jth column of $\boldsymbol{Z}$. The number of columns in $\boldsymbol{X}$ must therefore be exactly the same as the number of rows in $\boldsymbol{Z}$ (*Figure 6.31*). These rules of matrix multiplication also demonstrate the fact that matrix multiplication is non-commutative, that is, $\boldsymbol{AB} = \boldsymbol{BA}$ is *not* true in general (in particular instances it may, however, be true). The operations (M6), (M7), (M8) and (M9) may all be regarded as special cases of (M10).

APPENDIX C

TABLE FOR APPROXIMATE CRITICAL VALUES OF cvd-sd

No. of objects	*% variance explained by 1st PLS component*			y *is one-dimensional*	
	35%	*45%*	*55%*	*65%*	*75%*
$n = 8$	0.712	0.718	0.724	0.729	0.735
$n = 10$	0.766	0.771	0.777	0.783	0.789
$n = 12$	0.809	0.815	0.821	0.826	0.832
$n = 14$	0.844	0.850	0.855	0.861	0.867
$n = 16$	0.871	0.877	0.882	0.888	0.894
$n = 18$	0.891	0.897	0.902	0.908	0.914
$n = 20$	0.905	0.911	0.917	0.922	0.928
$n = 24$	0.920	0.926	0.932	0.937	0.943
$n = 28$	0.924	0.930	0.936	0.941	0.947
$n = 32$	0.926	0.931	0.937	0.943	0.948
$n = 36$	0.932	0.938	0.944	0.950	0.955
$n = 40$	0.953	0.954	0.956	0.958	0.960

REFERENCES

1 S. Hellberg, A multivariate approach to QSAR, Thesis, Univ. Umeå, Sweden (1986).
2 L. Ståhle and U. Ungerstedt, Pharmacol. Biochem. Behav., 24 (1986) 291.
3 U. Ungerstedt, in Measurement of Neurotransmitter Release In Vivo, ed. C.A. Marsden (Wiley, Chichester, 1984) pp. 81–104.
4 J.G.R. Ufkes, B.J. Visser, G. Heuver and C. van der Meer, Eur. J. Pharmacol., 50 (1978) 119.
5 J.G.R. Ufkes, B.J. Visser, G. Heuver, H.J. Wynne and C. van der Meer, Eur. J. Pharmacol., 79 (1982) 155.
6 S. Hellberg, M. Sjöström and S. Wold, Acta Chem. Scand., B 40 (1986) 135.
7 M. Sjöström and S. Wold, J. Mol. Evol., 22 (1985) 272.
8 T.H. Wonnacott and R.J. Wonnacott, Introductory Statistics, 3rd Edn. (Wiley, New York, 1977).
9 S. Siegel, Nonparametric Statistics for the Behavioural Sciences (McGraw-Hill, London, 1956).
10 L. Ståhle and S. Wold, J. Pharmacol. Meth., 16 (1986) 91.
11 K.V. Mardia, J.T. Kent and J.M. Bibby, Multivariate Analysis (Academic Press, New York, 1979).
12 K. Pearson, Phil. Mag., 2 (1901) 559.
13 H. Hotelling, Psychometrika, 1 (1935) 27.
14 H. Wold, in Research Papers in Statistics. Festschrift for J. Neyman, ed. F.N. David (Wiley, New York, 1966) pp. 411–444.
15 D. Morrison, Multivariate Statistical Methods (McGraw-Hill, New York, 1967).
16 A. Christofferson, The One-Component Model with Incomplete Data, Thesis, Univ. Uppsala, Sweden (1970).
17 S. Wold, Pattern Recogn., 8 (1976) 127.
18 S. Wold and M. Sjöström, in Chemometrics, Theory and Application, ed. B.R. Kowalski, Am. Chem. Soc. Symp. Ser., 52 (1977) pp. 243–282.
19 S. Wold, C. Albano, W.J. Dunn, III, K. Esbensen, S. Hellberg, E. Johansson and M. Sjöström, in Pattern Recognition: Finding and Using Regularities in Multivariate Data, eds. H. Martens and H. Russworm Jr. (Applied Science Publ., London, 1983) pp. 147–188.
20 H. Wold, in Systems under Indirect Observation, Vol. II, eds. K.G. Jöreskog and H. Wold (North-Holland Publ. Co., Amsterdam, 1982) pp. 1–54.
21 S. Wold, A. Ruhe, H. Wold and W.J. Dunn, III, SIAM J. Stat. Comput., 5 (1984) 735.
22 I.T. Joliffe, Appl. Statist., 31 (1982) 300.
23 M. Sjöström, S. Wold and B. Söderström, in Pattern Recognition in Practice II, eds. E.S. Gelsema and L.N. Kanal (Elsevier, Amsterdam, 1986) pp. 461–470.
24 L. Ståhle and S. Wold, J. Chemomet., 1 (1987) 185.
25 K. Esbensen and S. Wold, in Proc. Symp. Applied Statistics, Stavanger, Norway (1983) pp. 11–36.
26 S. Wold, P. Geladi, J. Öhman and K. Esbensen, J. Chemomet., 1 (1987) 41.
27 H. Martens, Multivariate Calibration. Thesis, Techn. Univ. of Norway, Trondheim (1985).
28 R.G. Krutchkoff, Technometrics, 9 (1967) 425.
29 G.E.P. Box, W.G. Hunter and J.S. Hunter, Statistics for Experimenters (Wiley, New York, 1978).
30 W. Spendley, G.R. Hext and F.R. Himsworth, Technometrics, 4 (1962) 441.
31 S.N. Deming and S.L. Morgan, Anal. Chem., 45 (1973) 278.

32 C.J.E. Niemegeers and P.A.J. Janssen, Life Sci., 24 (1979) 2201.
33 S. Wold, Technometrics, 20 (1978) 397.
34 M. Stone, J.R. Stat. Soc., B 36 (1974) 111.
35 S. Geisser, J. Am. Stat. Ass., 70 (1975) 320.
36 H.T. Eastment and W.J. Krzanowski Technometrics, 24 (1982) 73.
37 R.G. Miller, Biometrika, 61 (1974) 1.
38 B. Efron, Ann. Stat., 7 (1979) 1.
39 P. Diaconis and B. Efron, Sci. Am., May (1983) 96.
40 B. Efron and C. Stein, Ann. Stat., 9 (1981) 586.
41 R. Bergström and H. Wold, Fix-point Estimation in Theory and Practice (Vandenhoeck and Ruprecht, Göttingen, 1983).
42 G. Stephenson, An Introduction to Matrices, Sets and Groups for Science Students (Dover, New York, 1974).

Index

Cumulative Index of Authors for Volumes 1–25

The volume number, (year of publication) and page number are given in that order

Cumulative Index of Subjects for Volumes 1–25

The volume number, (year of publication) and page number are given in that order

This Page Intentionally Left Blank