PROGRESS IN MEDICINAL CHEMISTRY 30

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Preface

In this volume, the medicinal chemistry is reviewed of the rapidly expanding family of retinoids, of promising new semi-synthetic erythromycins and of the multifaceted amidines. Another chapter continues our coverage of inorganic elements which have a medicinal role; this time, recent studies on the various biological roles of manganese are examined. Inhibition of enkephalin-degrading enzymes is reviewed as also are studies of the digitalis recognition site which are expected to lead to the development of more highly selective inotropic drugs.

We thank our industrious authors for presenting a summary of the vast published literature in the above fields. It is hoped that their efforts will be appreciated by newcomers as well as those who wish to be kept up to date with recent work on these six topics. We thank owners of copyright material for granting permission to reproduce extracts from their work, and the Staff of our publishers for their continuing help and encouragement.

August 1992

G.P. Ellis
D.K. Luscombe

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Progress in Medicinal Chemistry – Vol. 30, edited by G.P. Ellis and D.K. Luscombe

1 Recent advances in the chemistry and biology of retinoids

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INTRODUCTION

Retinoids (analogues of vitamin A, retinol) comprise a unique class of anti-tumour agents able to convert a transformed, undifferentiated, neoplastic cell to a normal, terminally differentiated cell phenotype. With the discovery of this cytodifferentiating carcinostatic activity began a surge in the synthesis of new analogues in order to improve the therapeutic efficacy relative to the accepted standard, retinoic acid*, a retinol metabolite and the most potent naturally occuring retinoid. Synthetic efforts were exerted to maximize anti-cancer activity while minimizing the toxic effects exhibited in animals and humans, collectively termed hypervitaminosis A. Over a period spanning more than ten years hundreds of retinoids have been synthesized and methods developed to evaluate their biological and/or therapeutic potential. During this time frame, extensive work has also been done to evaluate the mechanisms of action by which retinoic acid and its synthetic counterparts exert their effects, including the control of cell differentiation and proliferation. Several proteins were found to bind retinoids and were shown to be important in their transport [1]. The fact that a carboxylic acid terminus was necessary for both biological activity and affinity for the cellular retinoic acid-binding protein [2] initially suggested a measure of correlation between activity and the ability of a retinoid to bind to CRABP. Therefore, an important role of this protein in the mechanism of action of retinoids was assumed. The discovery that retinol and retinoic acid were involved in the regulation of gene expression [3,4] led to the hypothesis that retinoids may exert their varied biological effects via regulation of gene transcription through direct binding of a retinoid-receptor complex to DNA [5]. The initial reports [6,7] in December 1987 of the discovery of a human retinoic acid receptor with RA-and DNA-binding properties was only the beginning of a cascade of reports which have provided a greater understanding regarding the possible mechanisms of action of retinoids in development, cell differentiation, and in the conversion of cancerous tissues to a normal physiological state.

Several reviews and books [8–19] have been written during the past decade which describe in detail syntheses, assays for activity, structure–

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*Abbreviations used in this review: APL, acute promyelocytic leukaemia; CEF, chick embryo fibroblasts; CRABP, cellular retinoic acid-binding protein; ODC, ornithine decarboxylase; RA, retinoic acid; RARE, retinoic acid responsive element; RAR, retinoic acid receptor; RXR, retinoid X receptor; TEMPO, 2,2,6,6-tetramethylpiperidine *N*-oxide; TOC, tracheal organ culture; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TTNN, 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2-naphthanoic acid; TTNPB, 4-((*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2- naphthyl)propenyl)benzoic acid.

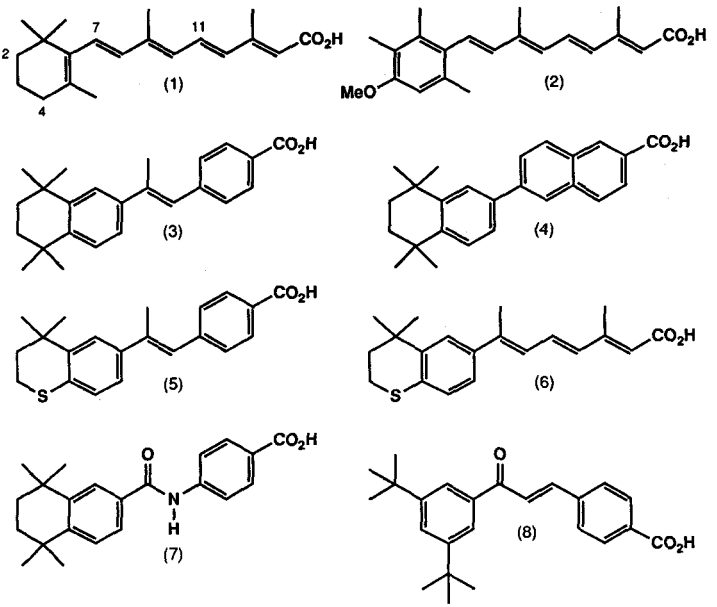
activity relationships, metabolism studies, methods of detection and analysis, and clinical and pharmacokinetic studies. Particular attention must be placed on the recent comprehensive discussions in Chemistry and Biology of Synthetic Retinoids [16], Methods in Enzymology, Volumes 189 and 190 on retinoids [17,18], and Retinoids: 10 Years On [19]. However, the research reports over the last two years have been voluminous, specifically concerning the mechanism of action of retinoids. While considerable work has been exerted in recent years towards the development of novel retinoids with improved potency [16–18,20–32], reports providing insight into the mechanism of action of retinoids have dominated the recent literature. This review will focus on areas of research reported over the last three years (that is, since the publication of the recent books indicated above) although necessary background information will also be given. This review will not cover research in the area of retinal and derivatives and their relation to the visual cycle, which has been well established [33]. However, some recent developments toward a better understanding of the visual cycle have been published [16,34–38]. Although this review focuses on the role of retinoic acid in cell differentiation as it relates to disease, particularly of cancers and the chemoprevention or treatments thereof, much investigational work has been exerted towards determining the role of retinoids, retinoid nuclear receptors, and homeobox genes in development, morphogenesis, and organogenesis [19,39–62].

SYNTHETIC METHODOLOGIES

Hundreds of retinoids have been synthesized over a period spanning almost 15 years. Several firms, research institutes, and academic institutions have dedicated efforts to synthesize retinoids with an improved therapeutic index (ratio of potency to toxicity) relative to the standards, all-*trans*-retinoic acid (1) and 13-*cis*-retinoic acid. The strategies have varied significantly. Two tendencies are observed: (a) to synthesize retinoids with improved anti-cancer activity relative to RA with the expectancy of minimal difference in toxicity relative to RA, and (b) to synthesize retinoids with improved toxicity profiles relative to RA with the expectancy of altered but not diminished anti-cancer activity relative to RA.

To date, a few retinoids have been prepared which have improved activity and/or receptor binding properties (see MECHANISM OF ACTION below) relative to RA as assessed in certain biological assays. Some of these have been shown to be highly toxic relative to RA [63,64]. Compounds (1)–(8) are examples of the structural variety existent among potent

synthetic retinoids. The syntheses of retinoids through 1989 have been reviewed [8,16]. This review will focus on the syntheses of retinoids reported in 1990 and up to the present.



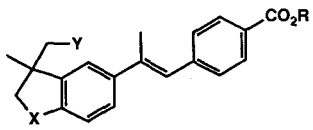
Several retinoids have recently been reported which exhibit similar potency relative to retinoic acid but which may prove to be less toxic than RA and which may exhibit selective anti-cancer activity. These agents belong to the class of retinoids termed heteroarotinoids [27–31,63,65–67]. Heteroarotinoids are heterocyclic analogues of the arotinoids [11,63,68], which are potent aromatic retinoids that include the well-studied compound (3), TTNPB, [4-((*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-propenyl)benzoic acid], and compound (4), TTNN, 6-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-2-naphthanoic acid. The most recent developments in heteroarotinoid research will be addressed below.

Additionally, Shudo and co-workers [20,22–24,69] have recently designed a series of retinoidal benzoic acids with promising anti-cancer activity, especially as indicated by the HL-60 assay. The structure of these aromatic retinoids resemble that of TTNPB but contain a different conjugated group [that is, NHCO, CONH, SO_2NH, COCH=CH, N=N, or N=N(O)] in place of the central propenyl group. Although these compounds have been discussed in a previous review [16], the synthesis and anti-leukaemic activity

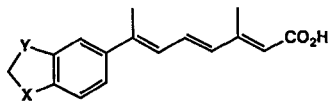
of retinobenzoic acids containing trimethylsilyl or trimethylgermyl groups meta to the central double bond have most recently been reported [24] and will be addressed below.

HETEROAROTINOIDS WITH A FIVE-MEMBERED TERMINAL RING

Aromatic retiniods with a terminal heterocyclic ring have been termed heteroarotinoids [65]. Most heteroarotinoids have an aromatic ring fused to a five or six-membered heterocyclic ring with O, S, or N in the para position relative to the central double bond [27–31,63,65–67]. Although the formation of the heterocyclic ring has generally involved carbocationic intermediates and intramolecular electrophilic aromatic substitution, asymmetric heteroarotinoids (9) and (10) containing a terminal five-membered ring have been prepared utilizing intramolecular free-radical cyclizations [29,31]. These were prepared as mimics of known metabolites of retinoic



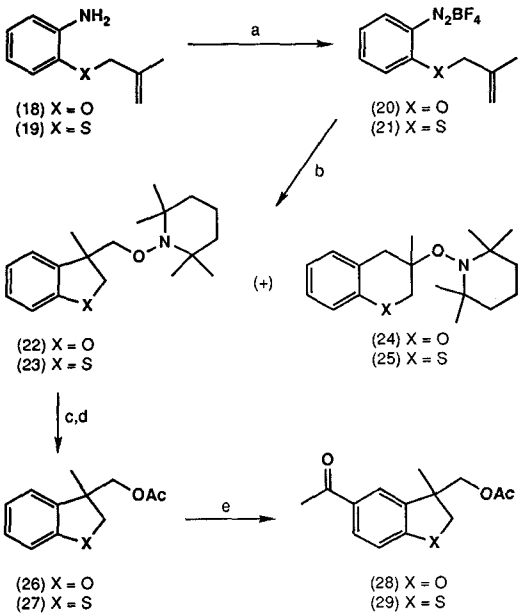
(9) X = O, Y = OH, R = Me
(10) X = S, Y = OH, R = Me
(11) X = O, Y = H, R = Me
(12) X = S, Y = H, R = Me
(13) X = O, Y = R = H
(14) X = S, Y = R = H



(15) X = O, Y = CMe_2
(16) X = S, Y = CMe_2
(17) X = Y = O

acid [70] and may be actual metabolites of the parent non-hydoxylated compounds (13) and (14).

The preparation of heteroarotinoids (9) and (10) (*Schemes 1.1* and *1.2*) [29,31] began with the conversion of the known amines (18) [71] and (19) [72] to the respective fluoroborate diazonium salts (20) and (21), respectively, by standard techniques. Generation of the aromatic free radical was accomplished in acetone in the presence of the stable free radical TEMPO. Cyclization of hetero-substituted aromatic free radicals generally occurs in a regiospecific manner with the formation of an exocyclic primary radical which then couples with a second molar equivalent of TEMPO [73–75]. However, both exo- and endo-cyclic free radicals were generated from salt (21) resulting in the formation of both *O*-substituted nitrogen oxides (23) and (25) [31]. There was no evidence by either TLC or NMR of formation from (20) of substituted pyran (24). Reductive cleavage of the N–O bond in (22) [31,74] and (23) [31] followed by protective acetylation gave acetates (26) and (27), respectively. Friedel-Crafts acylation of these acetates gave

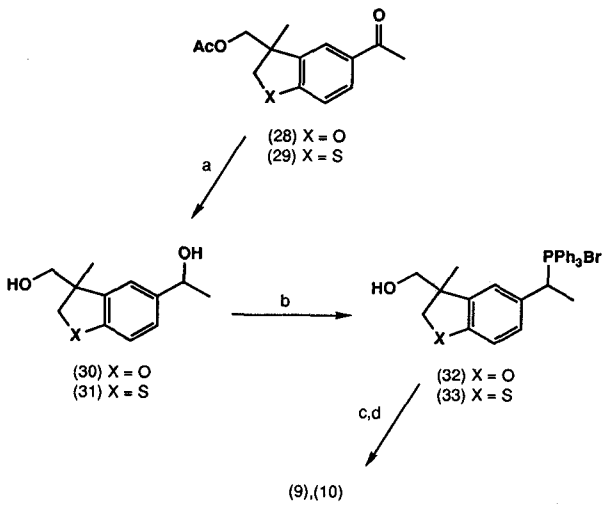


Conditions: (a) HBF_4, $NaNO_2$, H_2O, 0°C; (b) Me_2CO, TEMPO, Δ; (c) Zn, $HOAc/H_2O$, 70°C; (d) C_5H_5N, AcCl, Et_2O/THF; (e) CS_2, $AlCl_3$, AcCl

Scheme 1.1. Preparation of heterocyclic intermediates (28) and (29) [31].

ketones (28) and (29) regeoselectively in high yield. Standard reduction with lithium aluminium hydride gave a diastereomeric mixture of diols (30) and (31), respectively. Interestingly, treatment of these diols with triphenylphosphine hydrobromide gave only the benzylic phosphonium salts (32) and (33), respectively. There was no evidence of formation of primary benzylic salts. Formation of the Wittig reagent and the alkene coupled product proceeded as expected. Detection of both geometric isomers was possible by NMR [29], the *trans* isomers being the major isomers present in the crude products. Purification by chromatography and recrystallization gave the pure *trans* products (9) and (10), respectively, albeit in low yields [31].

In order to evaluate the therapeutic efficacy of heteroarotinoids with reduced hydrocarbon character (and potentially diminished toxicity), other heteroarotinoids containing a five-membered ring have been synthesized [29–32,67]. In view of the high anti-cancer activities of heteroarotinoids (5)



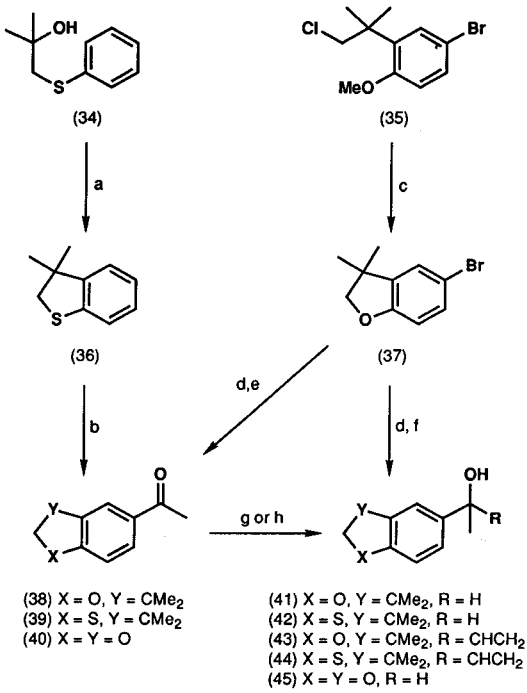
Conditions: (a) $LiAlH_4$, then H_3O^+; (b) MeOH, BrH-PPh_3; (c) nBuLi; (d) -78°C, then 4-$CHOC_6H_4CO_2Me$

Scheme 1.2. Preparation of heteroarotinoids (9) and (10) [29,31].

and (6) [27,28,65,67] and their greatly diminished toxicities relative to the potent arotinoid (3) [63,64], heteroarotinoids (11)–(17) were prepared as outlined in *Schemes 1.3* and *1.4*.

Synthesis of diaryl heteroarotinoids (11) and (12) [27,30,31] began with a Lewis acid-catalyzed cyclization of tertiary alcohol (34) to give dihydrobenzothiophene (36) as the sole isolated product. The chemistry of the ensuing steps was similar to that used to prepare (9) and (10) and other diaryl heteroarotinoids and involved: (a) Friedel–Crafts acylation of a fused aromatic-heterocyclic system, (b) reduction of the resulting ketone to a benzylic carbinol, (c) phosponium salt formation, and finally (d), Wittig coupling to methyl 4-formylbenzoate. The free acids (13) and (14) were obtained by saponification.

Synthesis of substituted octatrienoic acid heteroarotinoids (15) and (16) involved condensation of methyl ketones (38) and (39), respectively, with vinylmagnesium bromide to give benzylic-allylic alcohols (43) and (44). This was followed by phosphorylation to give the respective Wittig salt precursors (48) and (49). Wittig coupling proceeded as previously observed for the chroman and thiochroman analogue [65], with the all-*trans* esters being the major products. Saponification and recrystallization of the



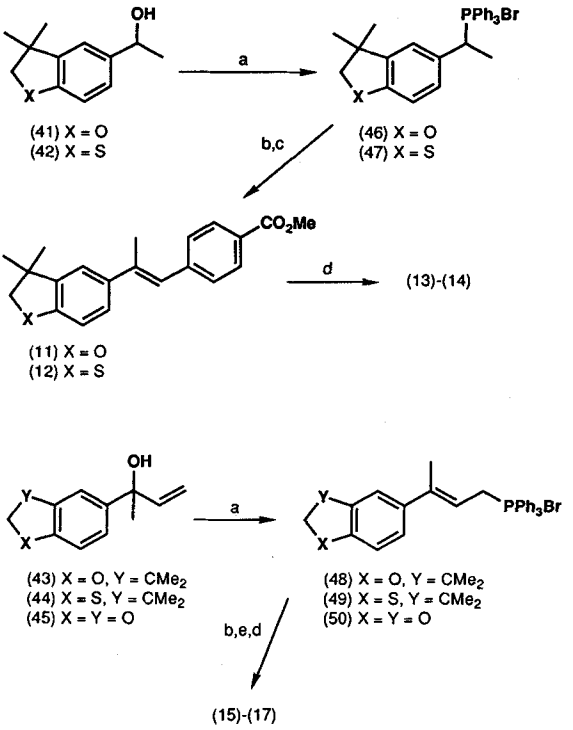
Scheme 1.3. Preparation of heterocyclic intermediates (41)–(45) [27–31].

products gave the pure all-*trans* acids (15) and (16). Heteroarotinoid (17) was similarily prepared starting with ketone (40) and is a dimethyl analogue of pipperetic acid, obtained by base catalyzed hydrolysis of one of the active flavour ingredients in *Piper trichostachyon* [76].

HETEROAROTINOIDS WITH A SIX-MEMBERED TERMINAL RING

7-(Thiochroman-6-yl)-2,4,6-octatrienoates and 4-[2-(thiochroman-6-yl)-propenyl]benzoates

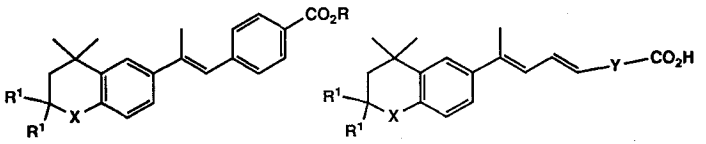
Heteroarotinoids containing a terminal thiochroman system have shown excellent activity [27,28,31,65–67] and toxicity profiles [63,64]. In order to



Conditions: (a) MeOH, BrH-PPh_3; (b) nBuLi, then -78°C; (c) 4-$CHOC_6H_4CO_2Me$; (d) KOH, EtOH, H_2O, then H_3O^+; (e) *trans*-$CHOCMe{=}CHCO_2Et$

Scheme 1.4. Preparation of heteroarotinoids (13)–(17) [27–31].

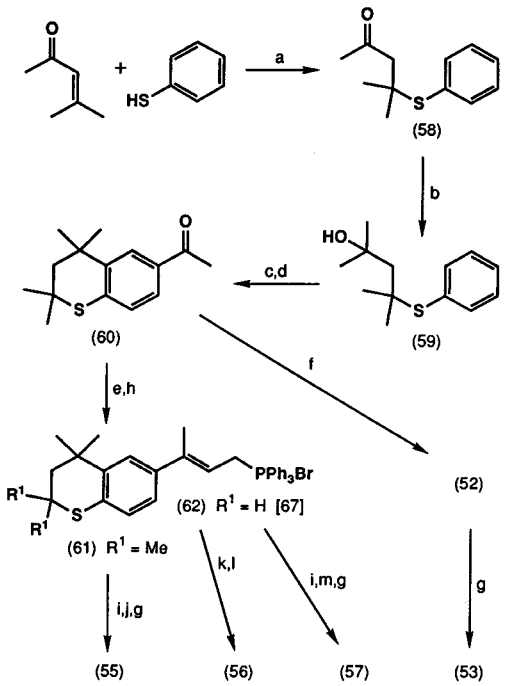
retard oxidation of the sulphur atom and to increase lipophilic bulk near position 4 of the retinoid skeleton, *gem*-dimethyls were introduced adjacent to the sulphur atom.



(5) X = S, R = R^1 = H
(51) X = O, R = R^1 = H
(52) X = S, R = R^1 = Me
(53) X = S, R = H, R^1 = Me

(6) X = S, R^1 = H, Y = CMeCH (*trans*)
(54) X = O, R^1 = H, Y = CMeCH (*trans*)
(55) X = S, R^1 = Me, Y = CMeCH (*trans*)
(56) X = S, R^1 = H, Y = CMeCH (*cis*)
(57) X = S, R^1 = H, Y = cyclopropyl (*trans*)

The resulting heteroarotinoids (52), (53), and (55) were synthesized as shown in *Scheme 1.5* [30]. Michael addition of thiophenol to mesityl oxide gave ketone (58), which, after condensation with methylmagnesium iodide and acidic work-up, gave tertiary alcohol (59). Cyclization using aluminium chloride in carbon disulfide was followed by Friedel–Crafts acylation which gave ketone (60). Coupling of (60) with 4-$MeO_2CC_6H_4CH_2P(O)(OMe)_2$ in the presence of sodium hydride gave ester heteroarotinoid (52) in good yield. Base-catalyzed hydrolysis gave (53). Alternatively, ketone (60) was converted to Wittig reagent precursor (61), which after treatment with butyllithium was coupled with ethyl (*E*)-β-formylcrotonate. The resulting ester was saponified to give all-*trans* trienoate (55).



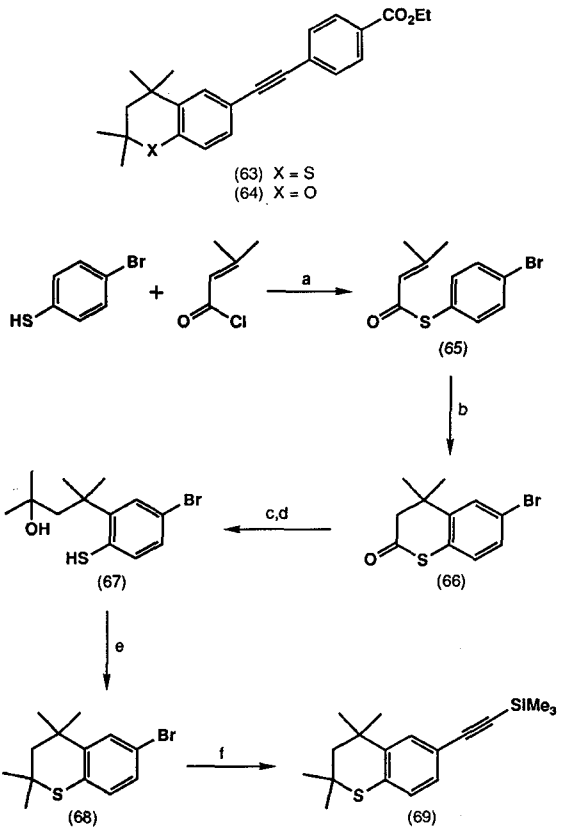
Conditions: (a) Et_3N, $CHCl_3$; (b) MeMgI, then H_3O^+; (c) $AlCl_3$, CS_2; (d) AcCl, $MeNO_2$, $AlCl_3$; (e) CH_2=CHMgBr, then H_3O^+; (f) NaH, 4-$MeO_2CC_6H_4CH_2P(O)(OMe)_2$; (g) KOH, EtOH, H_2O, then H_3O^+; (h) PPh_3•HBr, MeOH; (i) nBuLi, -78°C; (j) (*E*)-CHOCMe=$CHCO_2$Et; (k) 2 KH; (l) 4-hydroxy-3-methylbut-2-enolide; (m) ethyl *trans*-4-formylcyclopropanecarboxylate.

Scheme 1.5. Preparation of heteroarotinoids (52), (53), and (55)–(57) [30].

The 13-*cis*-analogue (56) of (6), was prepared from known [67] phosphonium salt (62) and 4-hydroxy-3-methylbut-3-enolide [77] as indicated in Scheme 1.5. The synthesis of (57), containing a terminal cyclopropyl ring, was effected by Wittig coupling of the ylide from salt (62) with ethyl (*E*)-*β*-formylcyclopropylcarboxylate [78].

4-[(Chroman- and thiochroman-6-yl)ethynyl]benzoates

Heteroarotinoids (63) and (64) containing a central triple bond were prepared for use in the treatment of epithelial disorders [26].

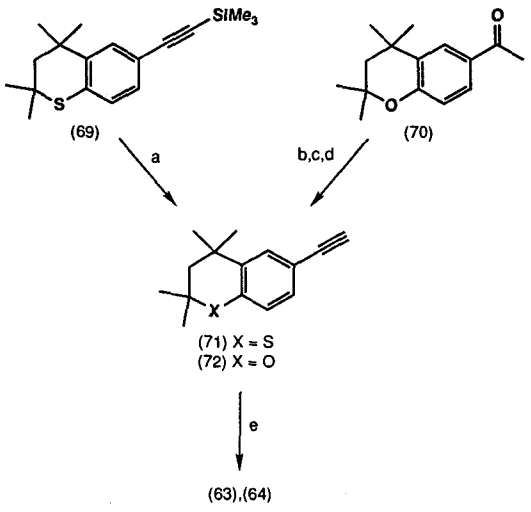


Conditions: (a) 0°C, NaH, THF; (b) $AlCl_3$, CH_2Cl_2; (c) $LiClO_4$-MeMgBr, ether; (d) ice/H_2SO_4; (e) dil. H_2SO_4, Δ; (f) Et_3N, Me_3SiCCH, CuI, $Pd(PPh_3)_2Cl_2$, Δ

Scheme 1.6. Preparation of acetylene intermediate (69) [26].

The chemistry used to prepared these heterocycles (*Schemes 1.6* and *1.7*) is different from that used to prepare the above 2,2,4,4-tetramethylthiochroman analogues (52) and (53): (a) synthesis of the alkynyl retinoids involved ring formation followed by ring opening and then reclosing, and (b) formation of the completed conjugated system did not involve a Wittig reaction but instead, involved coupling of an aryl halide with a substituted ethyne utilizing a paladium catalyst.

The syntheses of (63) begins with base-initiated nucleophilic substitution of 4-bromothiophenol at the carbonyl of dimethylacryloyl chloride. Cyclization of conjugated thioester (65) was catalyzed by aluminium chloride to give 2-oxo-thiochroman-2-one (66). Ring opening of thiochroman (66) utilized methylmagnesium bromide to give tertiary alcohol (67). Intramolecular acid-catalyzed S_N1 substitution gave 6-bromo-tetramethylthiochroman (68). The ensuing coupling reaction to form trimethylsilyl-substituted alkyne (69) was accomplished in a degassed system, under argon, and in a sealed tube at 100°C. Removal of the trimethylsilyl group gave substituted acetylene (71) which, coupled with ethyl 4-iodobenzoate using bis(triphenylphosphine)palladium(II) chloride as catalyst and triethylamine as an acid receptor, gave retinoid (63).



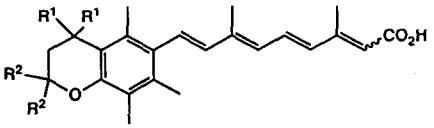
Conditions: (a) KOH; (b) $LiN(i\text{-}Pr)_2$, -78°C, $ClPO(OEt)_2$; (c) RT; (d) $LiN(i\text{-}Pr)_2$/-78°C, then RT; (e) Et_3N, ethyl 4-iodobenzoate, CuI, $Pd(PPh_3)_2Cl_2$

Scheme 1.7. Synthesis of acetylenic heteroarotinoids (63) and (64) [26].

Synthesis of tetramethylchroman analogue (64) was similar to that of thiochroman (63) except that the key ethynyl intermediate (72) was prepared from acetophenone (70). Formation of a phosphoester from the lithium enolate salt of (70) was followed by elimination of the phosphate group to give acetylene (72). Coupling of (72) with ethyl 4-iodobenzoate proceeded as with (71) and gave (64).

9-(Chroman-6-yl)-2,4,6,8-nonatetraenoates

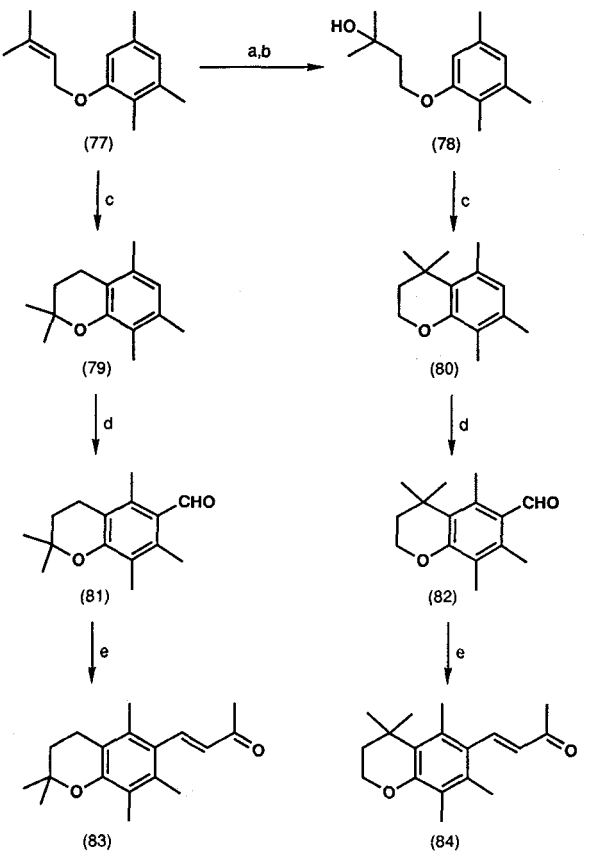
The aromatic ring of most chroman-substituted retinoids replaces C-5, C-6, C-7, C-8 and C-18 of the retinoic acid skeleton. However, the aromatic ring in novel nonatetraenoates (73)–(76) appears to replace the cyclohexenyl ring [C-1–C-6] of the retinoid skeleton as does the aromatic ring in acitretin (2).



(73) R^1 = H, R^2 = Me, *trans*
(74) R^1 = H, R^2 = Me, *cis*
(75) R^1 = Me, R^2 = H, *trans*
(76) R^1 = Me, R^2 = H, *cis*

The synthesis of these nonatetraenoates (*Schemes 1.8* and *1.9*) [21] utilizes low-valent titanium reductive elimination, a methodology which was also used in the stereospecific synthesis of all-*trans* retinoic acid [21]. Because of the high stereospecificity, purification of intermediates prior to the final step was not necessary.

Regioselective placement of a *gem*-dimethyl pair at either the 2,2 or 4,4 position in the chroman ring was accomplished by either acid-catalyzed rearrangement of alkene (77) or by acid-catalyzed ring closure of tertiary alcohol (78), respectively. Formylation and subsequent aldol condensation, cleanly provided α,β-unsaturated ketones (83) and (84), respectively. Condensation of each ketone with ethynylmagnesium chloride provided allyl alcohols (85) and (86), respectively. Activation of the terminal acetylenic carbons in (85) and (86) with ethylmagnesium bromide followed by condensation with either ethyl *(E)*-formylcrotonate or methyl *(Z)*-formylcrotonate gave diols (87),(88) and (89),(90), respectively. Reduction of the esters with DIBAL, protection of the resulting alcohols with trimethylsilyl groups, and reduction with Lindlar catalyst afforded *cis*-alkenes (91),(92) and (93),(94), respectively. Reductive elimination with Ti(0) followed by trimethysilyl deprotection gave the (4E,6E,8E)-nona-



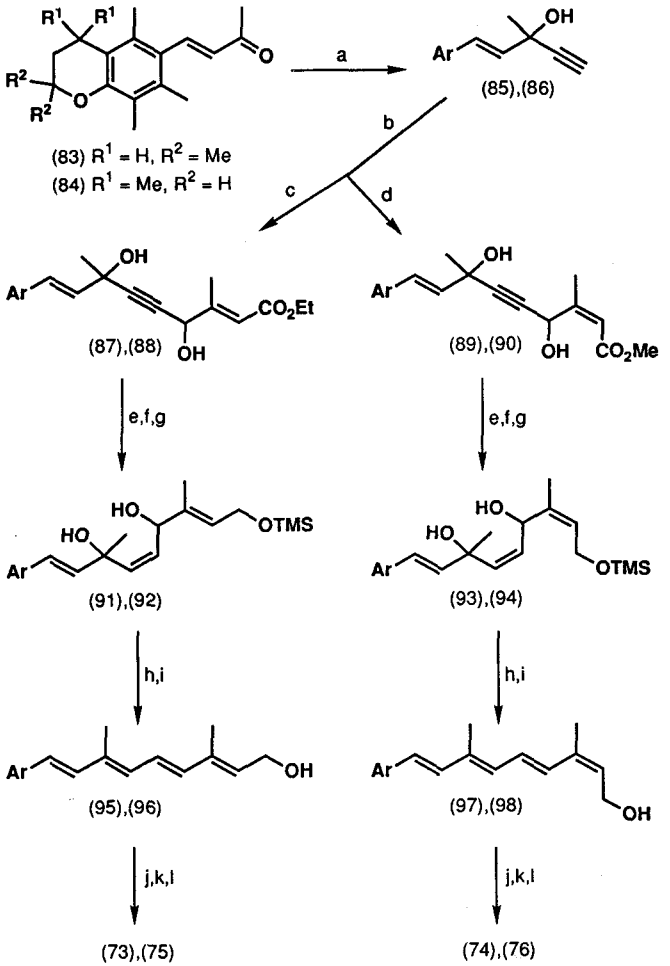
Conditions: (a) mCPBA; (b) $LiAlH_4$, then H_3O^+; (c) H_2SO_4; (d) Cl_2CHOMe, $TiCl_4$; (e) Me_2CO, NaOH

Scheme 1.8. Preparation of heterocyclic intermediates (83) and (84) [21].

tetraenes (95)–(98) in high yield. Oxidation and saponification afforded carboxylic acids (73)–(76) without the need of any intermediate purification starting from allyl alcohols (85) and (86) except for flash chromatography of the immediate ethyl ester precursors to the final carboxylic acids.

TRIMETHYLSILYL AND TRIMETHYLGERMYL RETINOBENZOIC ACIDS

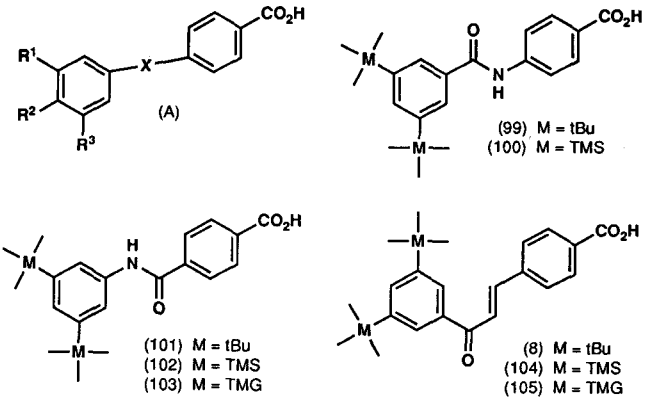
Retinobenzoic acids may be represented by the general structure (A) where



Conditions: (a) HCCMgCl; (b) EtMgBr; (c) *trans*-$CHOCMe{=}CHCO_2Et$; (d) *trans*-$CHOCMe{=}CHCO_2Me$; (e) DIBAL; (f) TMSCl; (g) H_2/Lindlar; (h) $TiCl_3$/LAH (2:1), THF, RT; (i) F-; (j) MnO_2; (k) MnO_2, AgO, NaCN, EtOH; (l) NaOH

Scheme 1.9. Preparation of heteroarotinoids (73)–(76) [21].

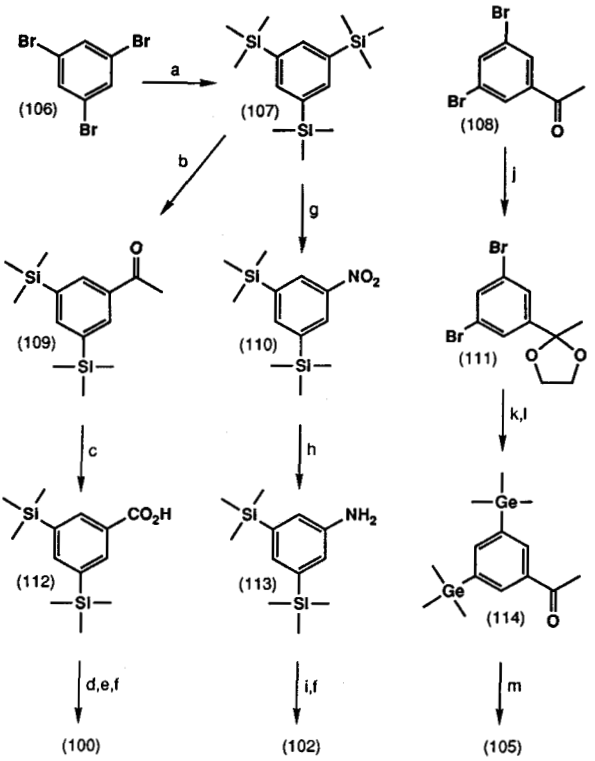
differential carcinostatic activity depends on variation of the spacer group, X [for example, CMe=CH, CONH, NHCO, COCH=CH, etc] [16,20,22–24,69].



The synthesis and biological activity of several trimethylsilyl (R^1, R^2, and/or R^3=TMS) and trimethylgermyl (R^1, R^2, and/or R^3=TMG) analogues have recently been reported [24]. The syntheses of these analogues involved Grignard-like coupling reactions to create C–Si or C–Ge bonds between either heteroatom with an aromatic ring [that is, conversion of 1,3,5-tribromobenzene (106) to (107), and, (111) to (114), *Scheme 1.10*]. With the trimethylsilyl analogues containing a central amide group (99)–(103), placement of the trimethylsilyl groups preceded introduction of the central amino or carboxylic acid group. In the case of the trimethylgermyl-substituted retinoidal chalcones (8), (104), and (105), introduction of the methyl keto group (necessary for the final aldol-coupling step) preceded placement of the trimethylgermyl groups. Acetylation of (107) afforded methyl ketone (109), which was subjected to a haloform reaction to give substituted benzoic acid (112). Regioselective nitration of (107) gave (110), which was hydrogenated to give aniline (113). Keto protection of (108) gave (111). Grignard coupling of (111) with trimethylgermylchloride was followed by keto deprotection to give methyl ketone (114).

The final coupling step involved either an aldol-like condensation of germyl-substituted acetophenone (114) with methyl 4-formylbenzoate or involved condensation of substituted anilines with aroyl chlorides.

Although these and several other [25,32,79–81] active retinoids have been described which contain heteroatoms substituted in rings or along the polyenic side chain, the structural variations possible with hetero-substituted retinoids have been scarcely explored. The potential for specificities of anti-cancer action and of favourable toxicity profiles necessitates further chemical and biological evaluation of this class of compounds.



Conditions: (a) TMSCl, Mg; (b) AcCl, $AlCl_3$, CS_2; (c) $Ca(OCl)_2$, K_2CO_3, KOH, H_2O; (d) $SOCl_2$, benzene; (e) 4-$NH_2C_6H_4CO_2Me$, NEt_3, benzene; (f) H_2O, NaOH, EtOH; (g) fuming HNO_3, Ac_2O; (h) H_2/10% Pd-C; (i) 4-$ClCOC_6H_4CO_2Me$, C_5H_5N, benzene; (j) $HOCH_2CH_2OH$, TsOH, benzene; (k) TMGCl, Mg, THF; (l) C_5H_5N•TsOH, acetone, H_2O; (m) 4-$HCOC_6H_4CO_2Me$, H_2O, KOH, THF, PrOH.

Scheme 1.10. Preparation of trimethylsilyllgermyl retinoidal benzoic acids (100), (102), and (105) [24].

METHODS FOR EVALUATION OF ACTIVITY

Several methods have been developed to assess the potential carcinostatic activity of retinoids. Over 200 cell lines are known to respond to retinoids [82]. Discussions regarding experimental procedures and evaluations and comparisons of many of the methods for assay of activity are contained in a review [83]. Some commonly used assays for retinoid activity, which will be discussed in brief below, include the hamster TOC assay, assay for

inhibition of ODC activity, and the assay of the induction of differentiation of HL-60 leukaemia cells. These three assays (TOC, ODC, and HL-60) have been used to screen hundreds of retinoids and the data obtained has allowed a comprehensive systematic study of structure–activity relationships among retinoids. Most recently, the ability of a retinoid to bind to or activate any or all of the nuclear retinoic acid receptors (RARs) has been an area of increasing study [81,84–88].

The TOC assay [79,83] is a highly sensitive, precise, and reproducible assay of the ability of a retinoid to induce differentiation of stratified squamous keratinizing epithelium from the tracheas of vitamin A deficient hamsters. The tracheas from the vitamin A deficient hamsters are opened and cultured in a serum-free and retinoid-free medium for 3 days. The resulting tracheas, exhibiting squamous metaplasia and containing keratin and keratohyaline granules, are then treated with either retinoid in DMSO (10^{-8} to 10^{-12} M) or only DMSO such that the final concentration of DMSO in the medium does not exceed 0.1%. After 10 days in culture, cross sections (5 μm) are made through the mid-portion of the tracheas, stained, and then examined with a microscope for the presence of keratin and keratohyaline granules. At the concentration tested, analogues are said to be active if neither keratin nor keratohyaline granules are seen, and inactive if both are seen. The ED_{50} is defined as the molarity of retinoid required to reverse keratinization in 50% of the cultures and is usually determined concurrently with that for all-*trans*-retinoic acid.

The ability of a test substance (that is, a retinoid) to inhibit the biosynthesis of the enzyme ODC [80,83] can be readily measured and correlates with the ability of the substance to inhibit skin tumour promotion [89,90]. The decarboxylation of ornithine to putrescine is believed to be a part of the malignant transformation process. 12-*O*-Tetradecanoylphorbol-13-acetate is a potent inducer of ornithine decarboxylase activity. The ability of a retinoid to inhibit TPA-induced ODC activity is therefore a measure of the anti-tumour activity of a retinoid. The method requires the backs of mice to be shaven and the retinoid applied topically prior to TPA treatment. A few hours (for example, 4.5–5 h) after TPA treatment, the mice are sacrificed, the epidermi separated and homogenized, and the resulting mixtures centrifuged. The ODC activity is then determined from the soluble extracts by measurement of the release of $^{14}CO_2$ from radiolabelled ornithine. Percent inhibition of ODC production is then calculated from the the ODC activities (ODC_{ret}) of retinoid treated mice and the ODC activities ($ODC_{control}$) from the mice treated only with TPA (the controls): %Inhibition$=100\times(ODC_{ret} - ODC_{control})/ODC_{control}$. The ID_{50} is sometimes determined and corresponds to the concentration at which 50% of the ODC

activity is inhibited. The experiments are always performed concurrently with assay of the activity of either all-*trans*- or 13-*cis*-retinoic acid.

The ability of a test compound to induce differentiation of HL-60 cells (derived from a patient with acute promyelocytic leukaemia) is also an important assay of retinoid activity [91–93]. HL-60 cells do not produce superoxide anions upon stimulation by agents like TPA. Differentiated HL-60 cells, however, do produce these anions upon similar stimulation. The presence of these anions can be monitored because of their ability to reduce the yellow nitroblue tetrazolium (NBT) to the water insoluble blue-black formazan. Determination of the concentration (ED_{50}) which results in differentiation of 50% of the cells is the measure of the anti-APL activity of the retinoid. Morphological maturation of the cells can also be checked.

The discovery of nuclear RARs has introduced a novel method for evaluating the potential carcinostatic activity of a new retinoid [81,84–88]. This can be done by measuring the ability of a retinoid either to bind to the RARs [84,85,87,88] or to induce activation of reporter genes via an RAR [81,86–88]. The affinity of retinoids for nuclear receptors in HL-60 cells appears to correlate well with anti-APL activity *in vitro* [94,95] as discussed in the next section. In general, a good correlation between retinoid carcinostatic activity and ability to bind to or activate retinoic acid receptors appears to exist.

Several other assays of retinoid activity exist [83] and include evaluations of the ability of a retinoid to inhibit the exponential growth of S91 murine melanoma cells or to induce terminal differentiation of the fully neoplastic F9 teratocarcinoma cells. These and other assays of retinoid activity have been useful but the TOC, ODC, and HL-60 assays have been used most comprehensively in establishing retinoid structure–activity relationships, which are discussed below.

STRUCTURE–ACTIVITY RELATIONSHIPS

Numerous retinoids are now known and these cannot be represented by a single generic structure. Generic stucture types I, II, and III, however, represent the structure of most retinoids synthesized to date (*Figure 1.1*). Compounds represented by structure type I include isomers and closely related analogues of retinoic acid (1), acitretin (2) and its ethyl ester (etretinate), and mono-aromatic heteroarotinoids such as (6), (15)–(17), and (54)–(57). Retinoids type II include substituted naphthalene, stilbene, azobenzene, and diaromatic amides, and other hetero-substituted analogues

containing a terminal carboxylic acid group; for example, compounds (3)–(5), (7), (9)–(14), (51)–(53), (63), (64), and (99)–(103). Structure III includes retinoidal chalcone and flavone carboxylic acids such as (8), (104), and (105). Derivatives may differ in the configurations about the double bonds, ring size, and kind of simple substitution (halogen, alkoxy, epoxy, and alkyl) along the retinoid skeleton. In addition to the heterocyclic substitution indicated in structures I–III, aromatic heterocycles have also been used to replace rings B and E. Tertiary carbons have also been replaced by tertiary silyl or germyl groups [for example, (99)–(105)]. Unless otherwise stated, discussion of structure–activity relationships will utilize the carbon numbering indicated in the drawings for retinoids types I, II, III.

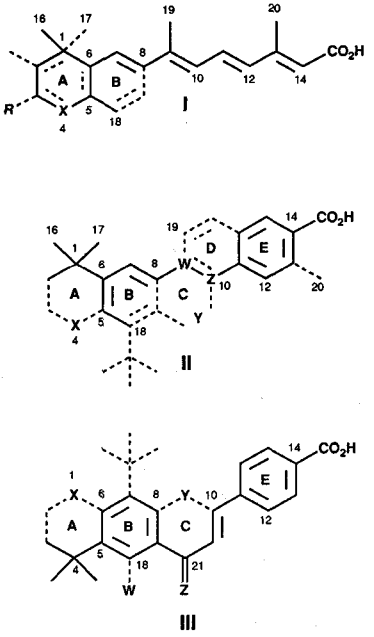


Figure 1.1. General retinoid structures.

The relationship between retinoid structure and retinoid activity has been extensively studied. Because of the large number of geometric isomers and conformers possible with the conjugated retinoid skeleton, a multitude of isomeric and/or 'conformationally-locked' retinoids have been synthesized to determine the shape and size of the binding site of the putative retinoid receptor. Several methods exist for evaluation of retinoid activity. A fair

correlation appears to exist between the structure–activity relationships predicted from one set of data and that obtained using a different bioassay. Comparison, however, of the correlation coefficients (r) obtained from linear regression analysis of the ED_{50}/ID_{50} values from various retinoids examined using different assays of retinoid activity indicated only poor to moderate correlations [79] between data sets. Several factors have been set forth [79,80] to account for this lack of good correlation among the structure–activity results obtained utilizing different assays: (a) differences in the enzymatic activities present in the different culture media (for example, the presence or lack of esterases), (b) differences in the solubilities of the retinoids in culture media versus in the skin, (c) differences in the rates of retinoid transport into the cells, (d) differences in the lengths of time of retinoid exposure to the culture media or within the tissues studied (that is, differences in the lengths of time to which the retinoids are subjected to the retinoid activating or deactivating cellular processes), (e) whether the assay is performed *in vivo* or *in vitro*, (f) differences in the application solvent utilized, and (g) whether or not the cell or tissue system is retinoid-deficient. Also it is apparent that the lack or presence of the CRABPs in certain cell lines can affect the responsiveness of the cells to retinoids [96,97], presumably by regulating the access of retinoids to the nucleus. For example, overexpression of CRABP has been reported to reduce the ability of RA to induce transcription of certain genes [96]. Differential expression of the nuclear retinoic acid receptors (RARs and RXRs, see NUCLEAR RETINOIC ACID RECEPTORS p. 34) amongst the different tissues and cell lines combined with differential retinoid-receptor affinities and inducing capabilities may be another important factor contributing to observed differences in the relative therapeutic efficacy observed for a set of retinoids tested using one biological system compared to that observed in another biosystem. Nevertheless, certain trends are apparent upon comparison of the structures of the various retinoids with the corresponding biological activities [79,80]:

(1) A terminal carboxylic acid group is necessary for maximum activity although occasionally an ethyl or methyl ester group [30] will give similar or enhanced activity.

(2) Isomerization at C-13 results in diminished activity. For example, the ability of all-*trans*-RA to induce differentiation of cells obtained from several patients with acute promyelocytic leukaemia, all-*trans*-RA is 10 times more effective than the 13-*cis* isomer [98].

(3) Maintenance of a planar geometry about C-5–C-6 or C-7–C-8 is not indispensable for activity. For example, replacement of either double bond

with either CH_2CH_2 or a cyclopropyl group does not result in significant loss of activity.

(4) Maintenance of a planar geometry about C-9–C-10 is necessary for activity. For example, replacement of C-9=C-10 with either CH_2CH_2 or a cyclopropyl group results in loss of activity. Replacement of MeC-13=C-14 with a cyclopropyl group also results in a loss of activity [30,67].

(5) For retinoids represented by structure II, transfer of the carboxyl group at C-14 to C-13 results in a loss of activity. Apparently, co-linearity between the C–CO_2H bond and the line running through the centres of both terminal rings A and E is necessary for adequate fit in the receptor ligand-binding site.

(6) For retinoids represented by structure type II, replacement of ring E with a 2,5-substituted furan, thiophene, or pyridine ring [carboxyl at carbon 2 of heterocycle] results in the following order of activity: 1,4-C_6H_4 ~2,5-thiophene > 2,5-pyridine > 2,5-furan (least active).

(7) Maintenance of a cisoid 'restricted conformation' about bonds C-6—C-7 and C-12—C-13 (structures types II and III) can provide activity equal to or better than that exhibited by all-*trans*-retinoic acid in most assays. TTNPB (3), TTNN (4), and (R12) (see *Table 1.1*) are marked by these restrictions and are parents to other type-II retinoids. In the induction of terminal differentiation of HL-60 and U-937 cells, however, removal of the latter conformational restriction results in enhanced activity. The tetramethyl-tetrahydronaphthyl-octatrienoic retinoid (structure type I, X=CMe_2, aromatic ring B) is more potent than TTNPB, especially when either retinoid is administered in combination with cytokines (see POTENTIATIVE AND SYNERGISTIC EFFECTS USING RETINOIDS IN COMBINATION WITH CYTOKINES AND OTHER HORMONES below).

(8) In the naphthalene carboxylic acid series [structure type II, aromatic B, D, and E rings, no C ring], incorporation of a methyl group para to C-6 or at C-19 does not result in a significant loss of activity. However, incorporation of a methyl group at C-10 results in loss of activity.

(9) In the retinoidal stilbene-4-carboxylic acid series (aromatic rings B and E separated by a double bond in structure type II), the following conclusions may be drawn: (a) Transfer of the methyl group at C-9 to C-10 does not result in diminished activity. However, placement of methyl groups at both C-9 and C-10 results in elimination of activity. Apparently, the resulting restricted rotation about C-8–C-9 and C-10–C-11 results in a conformation unsuitable for binding to the ligand binding site of the receptor. (b) The order of activity while changing X is: CMe_2 > CHMe > CH_2 ~ S > =CMe > O > =CH. Apparently, the amount of lipophilic bulk at

positions 1 and 4 and the extent to which that bulk is directed outside the plane of ring A is important for activity. Although the sulphur analogues are usually more potent than their oxygen counterparts [31,63,65,67,79,80], compound (64) containing a chroman ring exhibited greater ODC activity [26] than compound (63) containing a thiochroman ring. However, retinoids (63) and (64) contain adjacent *gem*-dimethyl groups, and because carbon-oxygen bonds are shorter than carbon–sulphur bonds, the *gem*-dimethyl groups of oxygen analogue (64) are closer to position 4 than they are in thioanalogue (63). (c) Isomerization at C(9) results in loss of activity. (d) The bulky lipophylic group meta to the central double bond must remain attached to the ring for maximum activity (that is, (R9) in Table 1.1) is inactive. (e) Replacement of ring B with a 2,4,5-substituted thiophene ring (central double bond attached to carbon 2 of the thiophene ring) maintained comparable or enhanced activity. Similar replacement of ring B in TTNN (4) also retained activity.

(10) Replacement of the central double bond in the most potent stilbene-4-carboxylic acid (TTNPB, (3)) with either CONH or NHCO resulted in activity greater (10-fold in the case of CONH) than either TTNPB or retinoic acid as measured by the HL-60 assay [22,23]. Similar replacement of the central double bond in (5), a heteroarotinoid, eliminated activity as indicated by the TOC assay [79].

(11) For compounds of type II (rings B and E aromatic) possessing a CONH group at positions 9 and 10 and *t*-butyl groups bonded to C-6 and C-18 [23,24], replacement of both *t*-butyl groups with larger trimethylsilyl groups gave enhanced activity [24].

(12) From a study of the retinoidal chalcone-4-carboxylic acids (retinoids type III, rings B and E aromatic, Y=H/H, Z=O), following deductions are drawn: (a) Maximum activity is maintained when a pair of bulky groups (*gem*-dimethyl or *t*-butyl) are bonded to C-5 and C-6 or to C-5 and C-7 [24]. (b) With C-5 and C-6 bridged via the tertiary carbons of $Me_2CCH_2CH_2CMe_2$, activity is improved by addition of hydroxyl groups at both C-8 and C-10. The activity of this diol is reported to be 7.6 times greater than that of RA as determined by the HL-60 assay [20]. The most potent chalcone-like retinoid to date, with a reported HL-60 activity 27 times greater than that of RA, contains an ether group bridging C-8 and C-10 (that is, Y=O) [20]. Although the conformation in $CDCl_3$ of the resulting flavone is reported to be different from that of the diol, the conformation of both in culture or in vivo may be similar. Alternatively, the diol may be converted to the flavone *in vitro* (and possibly *in vivo*) by enzyme catalyzed dehydration. (c) With *meta*-substitution (that is, at C-5 and/or at C-7), the order of reactivity for the *gem*-trimethyl groups is:

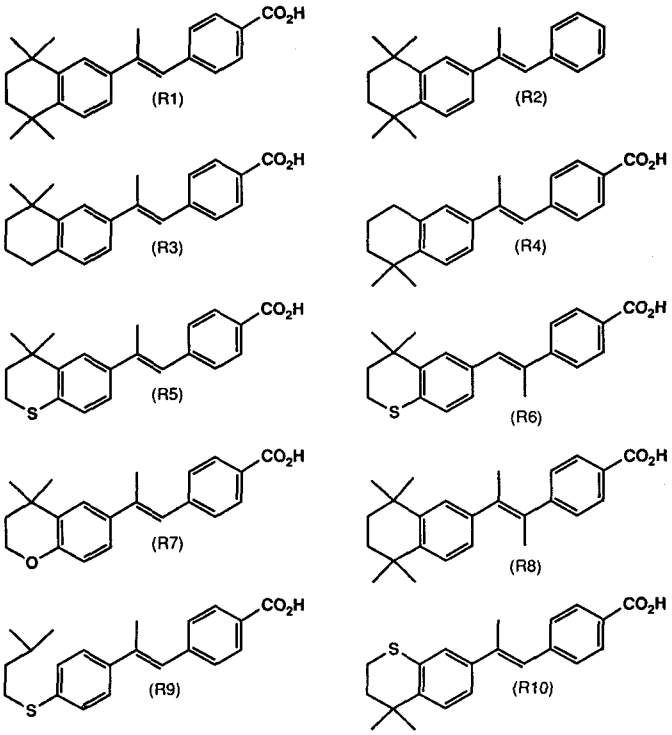
Me_3Ge > Me_3Si > Me_3C. 5,7-Disubstitution gave improved activity over single *meta* substitution, maximum HL-60 activity (10 times that of RA) being obtained with the 5,5-bis(trimethylgermyl) analogue (105) [24]. This again confirms the necessity of having hydrophobic bulk at the terminal opposite the carboxyl group.

(13) Reduction in the size of the terminal A-ring does not result in a significant loss of activity. Replacement of the 4,4-tetramethyl-dihydrothiopyran moiety for a 3,3-dimethyl-dihydrothiophene system did not result in reduced activity as indicated by the ODC assay [30,31]. Although the resulting heteroarotinoid (12) (a methyl ester) gave an ID_{50} 10-fold lower than all-*trans*-RA, the heteroarotinoid was more potent than RA when either retinoid was administered at the higher 34 nm dose. However, toxicity studies (see STRUCTURE–TOXICITY RELATIONSHIPS below) of selected heteroarotinoids indicate that these, especially sulphur heterocycle (6), are less toxic than RA and much less toxic than TTNPB.

The discovery of the nuclear retinoic acid receptors (RARs and RXRs) in humans and the demonstration that these receptors play a central role in regulating gene transcription, has led to an interest in determining whether the activity of retinoids to induce differentiation and to suppress tumour formation can be correlated with the ability of these to bind to or activate the nuclear retinoid receptors. Of particular interest has been the discovery that certain retinoids display high and specific affinity for one of the three RAR types (RARα, RARβ, or RARγ) [81,84,86–88]. This is important considering the fact that these receptor types differentially express themselves among various tissue and cell systems. For example, cells from patients with acute promyelocytic leukaemia (APL) predominantly express RARα and a mutant RARα (*myl*-RARα), both of which are necessary for attaining complete remission (see CANCER AND ABNORMAL EXPRESSIONS OF RETINOIC ACID RECEPTORS p. 36). Interestingly, some of the most potent inducers of terminal differentiation of APL cells, diaromatic amides type II (W–Z = CONH or NHCO), also have high and specific affinities for RARα.

Retinoids which have been examined for their affinities for the nuclear retinoic acid receptors are included in Table 1.1 and are labelled as indicated. There appears to exist a good correlation between the activity of retinoids by standard biological assays and that reflected in the abilities of these retinoids to bind to or activate the nuclear retinoid receptors [81,84,86–88]:

Table 1.1. RETINOIDS STUDIED FOR ABILITY TO ACTIVATE OR BIND TO NUCLEAR RETINOID RECEPTORS

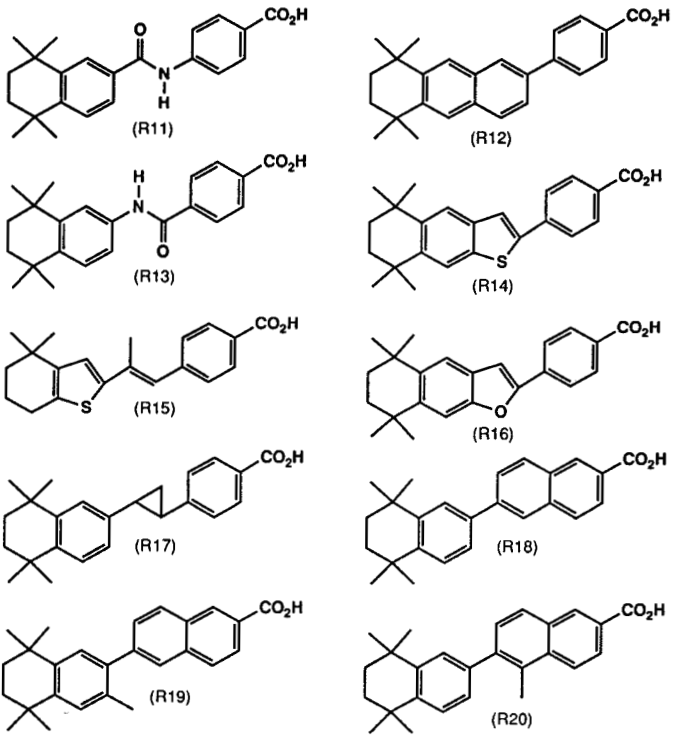


(1) Isomerization at C-13 results in reduction of affinity for both RARα and RARβ [88]. Recently, it was found that all-*trans*-RA was more efficient than 13-*cis*-RA in enhancing the transcription of genes for RARβ and RARγ (presumably via activation of endogenous RARs) in rat lung [99].

(2) The potent retinoids TTNPB (R1), TTNN (R18), and (R12) all bind strongly to nuclear retinoid receptors [81,84,87,88]. TTNN binds poorly, however, with RARα [81,84,88]. (R12) binds with greatest affinity to all three RAR types compared with all other retinoids tested [81,84,88]. Conflicting data on the affinity of TTNPB for RARα and RARβ is apparent [81,88].

(3) Planarity about C-5–C-6 is not indispensable for affinity while it is

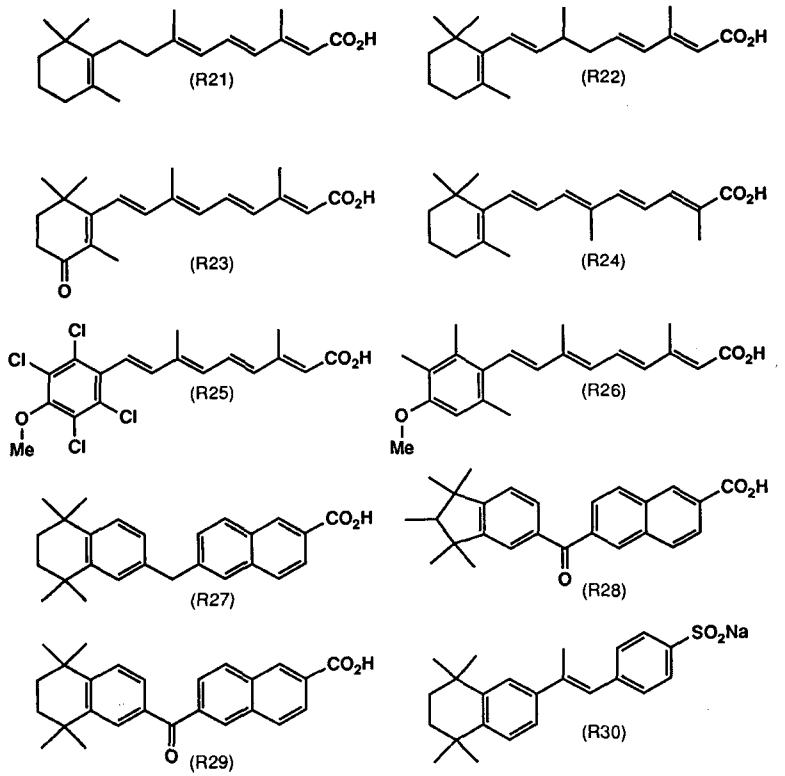
Table 1.1. (Continued) RETINOIDS STUDIED FOR ABILITY TO ACTIVATE OR BIND TO NUCLEAR RETINOID RECEPTORS



necessary about C-9–C-10. (R21) binds to both RARα and RARβ but (R22) has very poor affinity for either receptor [84,88]. (R17) containing a cyclopropyl group in place of C-9=C-10 did not bind to RARα and its affinity for RARβ and RARγ was modest [81].

(4) A terminal carboxylic acid group is necessary for receptor affinity. (R2) was devoid of ability to bind to any receptor and (R30) exhibited RARα and RARβ affinities 10- and 4-fold less, respectively, than that of (R1) [88]. While activation of RARs by retinol has been reported to be much less effective than by retinoic acid, retinol has recently been reported as a potent and rapid (30 min) inducer of transcription of the RARα gene in vitamin A-deficient rats [100]. Possibly, retinoid deficiency sensitizes rat

Table 1.1. (Continued) RETINOIDS STUDIED FOR ABILITY TO ACTIVATE OR BIND TO NUCLEAR RETINOID RECEPTORS



tissue to gene activation by RARs, such that even retinol (a poorer RAR ligand) can rapidly induce RAR-mediated gene transcription.

(5) With the stilbene-like retinoids, maintenance of a bulky lipophilic group at C-4 extending outward and away from the plane of the A-ring is necessary for maximum activity. Therefore, (R7) was poorly bound to RARα and RARγ and its affinity for RARβ was modest [81]. (R23) with a polar oxo group at position 4 had a 20-fold reduction in RAR-affinity compared with all-*trans*-RA [88]. (R3), (R4), (R5), and (R10) all bound strongly with the receptors [81]. Disconnection of one of the bulky groups from ring B (R9) resulted in loss of affinity [81].

(6) Transfer of the methyl group at C-9 in (R5) to C-10 gives (R6) with retained affinity for the nuclear receptors [81]. Placement of methyl groups at both C-9 and C-10 as in (R8) results in elimination of ability to bind to the receptors [81]. Shift of the methyl groups at C-9 and C-13 in RA to C-10 and C-14 gives (R24) which binds poorly to the RARs [84,88].

(7) Although the conformation indicated for (R27)–(R29) may not be that providing activity, these novel retinoids appear to lack a connection between C-8 and C-10 via C-9. (R29) has RARβ and RARγ affinity comparable to RA but binds modestly to RARα. (R28) binds well to RARβ but its affinity for RARα and RARγ was poor and modest, respectively. (R27), lacking the carbonyl group, binds poorly to the RARs. Possibly, the carbonyl group aids in maintaining a planar system such that C-8 and C-10 are proximate.

(8) With analogues of TTNN (R18), the location of a methyl substituent was crucial for activity. (R20) did not bind to any of the receptors while (R19) bound with high and specific affinity for RARβ and RARγ [81].

Several retinoids such as (R19) exhibited specificity in their abilities to bind or activate nuclear receptors. Both heterocyclic retinoids (R14) [81] and (R16) [87] strongly activated RARβ and RARγ (the latter, to a slightly lesser extent); however, activation of RARα by (R14) was only modest and the affinity of (R16) for RARα was also modest. Heterocyclic (R15) also bound strongly to both RARβ and RARγ and poorly to RARα [81]. (R3) and (R5) (a heteroarotinoid) which lack a *gem*-dimethyl group at position 4 showed strongest binding to RARβ and weaker binding for RARα [81]. Retinoids with high and specific affinity for only RARβ or RARγ have not yet been found. However, retinoids with high and specific binding for only RARα have been found. Retinoidal amides (R11) and (R13) bind poorly or modestly with *both* RARβ and RARγ but strongly bind and activate RARα [84,86–88]. It is interesting to note that both of these retinoids are more potent than RA or TTNPB in their abilities to induce terminal differentiation of HL-60 cells which primarily express RARα [94].

Some retinoids [for example, (R25) and (R26)] appear to be strong activators of retinoic acid receptors but do not bind to these receptors [88]. Retinoic acid is only 4 times more potent than (R26) (acitretin) in affinity for RARα and RARβ but the latter does not bind to either receptor. (R25) strongly activates RARβ but activates RARα less efficiently; however, (R25) does not bind to either. Preliminary experiments suggest that these retinoids are transformed *in vitro* to metabolites which *do* bind to RARs [88].

Although it is recommended that screens should be performed in a variety of tissue or cell systems to more adequately profile biological activity,

retinoids with specific activities are important. For example, retinoids with high and specific affinities for *myl*-RARα, a mutant retinoid receptor found in cells from patients with APL and necessary for attaining complete remission [101,102], may exhibit efficient and specific anti-APL activity. Similarily, retinoids with low affinity for CRABP and high affinity for the nuclear retinoid receptors, may demonstrate therapeutic efficiency and specificity in the treatment of human psoriatic skin, which has recently been reported to overexpress CRABP-II [97]. Because maintenance of high RA plasma levels is necessary for preventing relapse in APL patients who attained complete remission [103], retinoids which do not bind to CRABP, such as (R11), may be more effective than RA in the treatment of APL. Evidence suggests that retinoic acid treatment of APL may induce increased CRABP levels and hence reduce available retinoic acid plasma levels [103]. An understanding of the types and levels of retinoid-binding proteins or receptors present in different cell and tissue systems will also be helpful in evaluating the specific therapeutic utility of a given retinoid.

STRUCTURE–TOXICITY RELATIONSHIPS

Toxicity studies of retinoids have involved assessment of teratogenic potential [104,105] and overall toxic effects [63,64] in adult animals upon treatment with retinoids. In general, it appears that the structural features necessary for teratogenic potency overlap with those features required for anti-cancer activity [105]. These requirements may be summarized as follows: (1) An acidic polar terminus is necessary. (2) Conjugation in the three double bonds nearest to the polar terminus must be maintained. Unsaturation of the 7,8 double bond is not indispensable. (3) Substitution of non-polar groups in the terminal ring is required.

However, certain important structural features which maintain or enhance high activity can also result in great reductions in toxicity: (1) Replacement of CMe_2 in the structure of TTNPB with a sulphur atom (that is, (R5)) results in a much improved therapeutic index. Although TTNPB was initially reported to be close to four orders of magnitude more potent than RA, most assays (TOC, ODC, HL-60, and papilloma regression) indicate that the activity of TTNPB ranges from being only slightly more active than RA to only one order of magnitude more active than RA [22,79,80]. However, TTNPB is much more toxic than retinoic acid. One report, based on the relative abilities to induce systems associated with hypervitaminosis-A [11], indicated TTNPB to be 800 times more toxic than RA. In contrast, (R5) is much less toxic than TTNPB [63,64] and maintains

high biological activity [27,63,65] including strong affinity for RARs, especially RARβ [81]. Compound (6), another potent thioarotinoid [28,67], is much less toxic than TTNPB and also less toxic than retinoic acid [64]. (2) Among the heteroarotinoids containing either a sulphur or oxygen atom at position 4, the more potent sulphur analogues are also the least toxic [63,64]. The reason for this trend is not known. Possibly, the lifetime of a thioarotinoid is sufficiently long for exertion of its anti-tumour effects but is metabolized and eliminated without systemic accumulation as the less active and possibly non-toxic sulphone. (3) Although several retinoidal amides such as (R11) and (R13) have been synthesized, few have been studied with regard to their toxicity. (R11), which differs from TTNPB only by replacement of the central propenyl group with CONH, is 3-fold less toxic than RA [88] and more potent than either RA or TTNPB (R1) as determined by the HL-60 assay, by as much as a factor of 7 [22,23]. Therefore, based on HL-60 data and hypervitaminosis-A effects, it can be said that the therapeutic index (activity divided by toxicity) of (R11) for the treatment of acute promyelocytic leukaemia, may be approximately 20 (3×7) times better than RA and approximately 15000 ($800 \times 3 \times 7$) times better than TTNPB. Further study would be required to substantiate this conclusion. These observations suggest that the use of heteroatoms either in the terminal A-ring or within the polyenic side chain may provide improved therapeutic ratios necessary for the future use of retinoids as carcinostatic drugs.

MECHANISMS OF ACTION

In view of the hormone-like properties of retinoic acid (RA), it was postulated early that RA may exert its varied effects by regulating gene expression. While many mechanisms of action have been invoked, the mode of action which is most accepted as underlying these various activities is that RA and its analogues regulate gene transcription [106,107]. The possible mechanisms by which this occurs have become more clear with the discovery of several nuclear retinoid receptors [6,7,95,108–115], specific genes and nucleotide sequences regulated by retinoids [116–135], and the demonstration of the multiple and overlapping interactions of the retinoid receptors, the steroid hormone receptors, responsive genetic elements, and other co-regulatory factors [118,120,121,124,135–139].

The following discoveries led to a common expectancy that CRABP may be a key entity which when bound to RA may directly regulate gene expression: (a) isolation of CRABP in the nucleoplasm [140–144], (b) an

apparent relationship between retinoid activity and retinoid affinity for CRABP [2], and (c) CRABP interacts with nuclear chromatin [140,145] and may mediate the binding of RA to transcriptionally active chromatin [140].

However, certain experiments appeared to raise doubts concerning the possibility of CRABP directly regulating gene transcription or being necessary for RA-induced regulation of gene transcription resulting in a biological response. This protein has been either undetectable or existing in very low concentrations in certain cell lines which differentiate upon RA treatment. Reports indicate a lack of direct correlation between biological activity and affinity for CRABP [146–149]. It has also been demonstrated that ODC inhibition by retinoids is not mediated by CRABP [150]. A DNA-binding domain has also not been identified in CRABP.

In November 1987, reports were independently received from Chambon and co-workers [6] and Evans and co-workers [7] describing human nucleotide sequences which code for proteins containing domains with high amino acid sequence homology compared with both the DNA and ligand-binding domains of the human thyroid and glucocorticoid hormone receptors (hTR and hGR). Interestingly, replacement of the DNA-binding domain of this novel receptor with the DNA-binding domain of either of these hormones, resulted in a hybrid receptor with greatest inducibility by retinoic acid in the activation of transcription of reporter genes whose expression can easily be monitored. The concentrations of RA which produced half-maximal response were in the range consistent with values previously shown to adequately give a half-maximal response in assays for activity (for example, TOC, S91, F9, and HL-60). Cell lines transfected with the gene encoding this receptor increased the capacity for uptake of radiolabelled RA. Thus, a DNA-binding receptor was found, termed RAR for retinoic acid receptor, which was specific for retinoic acid. Since this initial discovery, several nuclear retinoid receptors [95,108–114] and isoforms [151–155] have been characterized. Several nucleotide sequences which respond to RA (retinoic acid response elements, RAREs) have also been identified. Some of the responsive elements have been shown to directly bind to retinoid receptors. Additionally, the regulation of gene transcription by RA has been found to be part of a complex network of interactions involving steroid hormones and other co-regulators. Although other RA-binding proteins (retinol binding protein (RBP), transthretin, and albumin) have been identified, characterized, and shown to be important in transport of retinoids to cells [1], this section of the review will evaluate the role of the cytosolic and nuclear retinoid binding proteins.

CELLULAR RETINOL BINDING PROTEINS

Two cellular retinol binding protein isoforms (CRBP-I and CRBP-II) have been isolated [156]. CRBP is a small protein (MW=15,700) and the gene encoding CRBP has been mapped to chromosome 3 in humans [156]. To date, there is no conclusive evidence that the protein associates directly with genetic material, although CRBP has been isolated from nuclear fractions and in contact with chromatin [157]. CRBP may be a carrier of retinol, the retinol-CRBP complex penetrating the nuclear membrane and delivering retinol to the chromatin. In the developing mouse, tissues expressing high levels of RARβ and RARγ also express high levels of CRBP [158]. Therefore, it has been suggested that a primary function of CRBP is to store and release retinol where high levels of retinoid are needed for RAR-mediated genetic control (as in morphogenetic processes) [158]. Although retinol has generally been shown to be less active than retinoic acid, retinol has recently been shown to rapidly (30 min) induce the transcription of the RARα gene [100]. Therefore, although retinol can be metabolized to the more potent RAR ligand, retinoic acid, it also may itself mediate transcriptional control when retinol concentrations are high. Additionally, CRBP may be involved in regulating retinol metabolism [159]. Recently, it has been shown that CRBP is involved in activating hydrolysis of retinyl esters [160], thus mediating the release of retinol.

Both retinol and retinoic acid are known to be able to induce elevated levels of CRBP mRNA, although induction by RA is more rapid [159]. Recently, RAREs were discovered in the promoter regions of the CRBP-I and CRBP-II genes [132,161]. Induction of the CRBP-I gene appears to be mediated by RARs [132], whereas CRBP-II gene induction is mediated by RXR [161]. Interestingly, RXR mediated induction of the CRBP-II gene expression is suppressed by RAR. Most recently, 9-*cis*-RA was identified as the specific natural ligand for RXRα [162,163] and found to be 40-fold more potent than all-*trans*-RA in the ability to induce transcription of the CRBP gene [163]. This suggests that a feedback mechanism may be involved in which the retinol metabolites all-*trans*-RA and 13-*cis*-RA are themselves involved in regulating CRBP-mediated vitamin A storage, release and/or metabolism.

CELLULAR RETINOIC ACID BINDING PROTEINS

Cellular retinoic acid binding proteins have been found in chickens, bovines, rats, mice, and humans and multiple isoforms identified. CRABP is a small protein (MW ~ 15 kDa) [159] and the gene encoding CRABP has been

mapped to chromosome 3 in humans [164]. Two distinct cellular retinoic acid binding proteins (CRABP-I and CRABP-II) have been identified in both humans and mice [165,166]. Each CRABP isoform shows high amino acid sequence homology between mice (m) and humans (h). These two isoforms appear to have different tissue distributions and possibly different functions. The genes for both hCRABP-II and mCRABP-II were found to be up-regulated by RA treatment; however, the respective concentrations of hCRABP-I and mCRABP-I remained relatively constant.

The RA–CRABP complex has been identified in nuclear fractions and in association with chromatin [140]. There has not been any conclusive evidence that CRABP remains bound to chromatin or that it binds to genetic material. It has not been possible to identify any CRABP amino acid sequence which may function as a DNA-binding domain. It has been postulated that CRABP may act as a shuttle to deliver RA to the chromatin at which time RA complexes with the nuclear retinoic acid receptors (RARs) to regulate gene transcription. However, it has been observed that CRABP and RA can exist in opposing gradients in differentiating tissue [167]. Furthermore, differentiating tissues high in RARβ and RARγ contain low levels of CRABP [158]. This and other data suggest that CRABP may sequester RA and/or steepen the gradients of RA available to the RARs in the nucleoplasm of cells in differentiating tissues [158,167,168].

This hypothesis is also supported by the observation that overexpression of CRABP-I results in reduction of the potency of RA in inducing transcription of several genes [96]. Therefore, while CRBP stores and releases retinol, CRABP sequesters RA [158].

The presence of a carboxyl terminal group is necessary for binding to CRABP and for retinoid activity [2]. However, comparison of other retinoid structural features shows a lack of correlation between retinoid potency and affinity for CRABP. Some potent retinoids do not bind to CRABP but do bind to nuclear retinoid receptors such as the RARs [146–149]. One such retinoid has been reported to be equally or more potent than RA but has lower affinity for RARs than RA [149]. It has been suggested that the reason for this apparent anomaly may be that the potency of RA is reduced because of its ability to bind to endogenous CRABP. It appears, therefore, that one of the functions of CRABP may be to 'regulate the amount of intracellular-active RA and thus control quantitatively the intensity of biological effects [149].'

NUCLEAR RETINOIC ACID RECEPTORS

To date three human and murine retinoic acid receptors (hRARs and mRARs, respectively) have been isolated and characterized [6,7,109–113] and belong to the steroid/thyroid hormone receptor superfamily. These RAR subtypes have been abbreviated as RARα, RARβ (also designated RARϵ), and RARγ and are coded by genes from chromosomes 17, 3, and 12, respectively [169–171]. These differ in their distributions [151,172–178] in the adult and in developing tissues and differ in their RA binding affinities. It is believed, therefore, that they also may serve different functions. Because of the presence of competing retinoid binding proteins such as CRABP, it is difficult to accurately determine dissociation constants of RA–RAR complexes. One group has reported the dissociation constants for RARα, RARβ, and RARγ to be 13, 5.5, and 3.5 nM respectively [87]. In *E.coli*, which lack interfering endogenous CRABP, the dissociation constant of the RARα–RA complex was accurately determined to be 2.1×10^{-10} M [179]. The length of the RARα binding site was recently estimated to be approximately 27–35 Å [180].

RARs [181] have six principal domains, labelled A–F. The A/B domains represent the N-terminal region. Differences in the nucleotide sequences encoding these two regions are important in effecting differential gene trans-activation. The C region represents the DNA-binding domain. The E region functions as the ligand-binding domain. The functions for the D and F regions are less well understood. Great amino acid sequence homology exists among the C regions for the different RAR subtypes. The same is true for the RA-binding E region. Recently, it has been shown that both the C and E regions may also be involved in protein–protein interactions [136].

A second subfamily of DNA-binding retinoid receptors have recently been identified consisting of RXRα, RXRβ, and RXRγ [108,115]. The ligand domains of these novel receptors do not, however, exhibit high amino acid sequence homology to that of the RARs. Interestingly, the ligand which best activated RXR, all-*trans*-retinoic acid, did not bind with high affinity to RXR [108]. Other active retinoids such as TTNPB (3) also did not bind adequately to RXR [108,182]. Therefore, it was postulated that these RXRs possibly represent a distinct RA response pathway relative to the RARs. For example, it was demonstrated that RARs block transcriptional activity induced by the activator-protein-1 (AP-1) complex, whereas RXRs do not [183]. Recently, clues regarding the possible biological role of the RXR retinoid receptors have begun to unfold. It now appears that these retinoid receptors have a central role in integrating the actions of receptors for all-*trans*-RA, thyroid hormone, and vitamin D_3, and possible that of

other hormones. By forming dimers with RARα, the thyroid hormone receptor (TH), and the vitamin D_3 receptor (VDR), RXRα synergistically enhances its own ability to bind to gene responsive elements as well as that of RARα, TR, and VDR [115,184]. RXR may also play an important role in regulating retinol transport and metabolism by activating the expression of the CRBP gene [161]. It was also suggested that these novel receptors may bind with highest affinity to a yet unidentified ligand, possibly a retinoid metabolite. It is now known that 9-*cis*-retinoic acid is the specific natural ligand for RXRα [162,163]. This isomer of RA was shown to be 40-fold more potent than all-*trans*-RA in the ability to induce RXRα transcriptional activity [163]. The dissociation constant for the RXRα–RA(9*cis*) complex has been estimated to range between 9.5 nM [162] and 11.7 nM [163].

Recently, several isoforms have been characterized for each of the three RARs [151–155]. Seven murine cDNA isoforms from the RARα gene were examined [153]. These were generated by differential promoter usage and alternative splicing. In a similar fashion, three cDNA isoforms of the RARβ subtype are known to be generated [154]. Genomic rearrangements involving differential promoter usage were also shown to occur in the generation of RARγ1 and RARγ2 [155]. A total of seven RARγ isoforms have since been identified [181]. Receptor isoforms within a given RAR subtype have distinct tissue distributions [181]. In the mouse, RARα1 shows a relatively ubiquitous expression pattern and particularly high in the skin, while RARα2 is limited primarily to the intestine, lung, and liver and is found in very low concentrations in the brain, muscle, heart, and kidney. While mouse RARβ1 and RARβ3 are abundant in the brain, skin, and lung, RARβ2 mRNA are the only RARβ messages detected in the kidney, heart, liver, and muscle. While RARγ1 is the predominant RARγ isoform expressed in skin, RARγ2 is the predominant RARγ isoform found in adult lung. Therefore, it is reasonable to assume that the various isoforms of each RAR subtype may have different functions in development and differentiation. Interestingly, mRARα2 and not mRARα1 was upregulated by RA treatment [153]. The N-terminal regions among the isoforms in each subtype differ in their amino acid sequences, suggesting that these isoforms may activate different sets of genes.

The multiplicity in the subtypes and isoforms of RARs and the cell-specific differential co-regulation and expression of each subtype/isoform suggests that a single mechanism of action cannot adequately explain all the effects of RA. Similarily, a single mechanism of action cannot adequately explain the varying ability of retinoids to induce differentiation or inhibit proliferation in different tumour or cancer cell lines. It is not totally surprising that retinoids which are not adequately effective in

differentiation of HL-60 cells may exert a strong response in other biological systems: cells from patients with acute promyelocytic leukaemia require the expression of an aberrant RARα for differentiation to a normal phenotype [101,102].

The discovery of subtype specificity in receptor binding of retinoids has led to the suggestion that retinoids may be able to be designed to maximize the response from specific RAR subtypes [81,84]. For example, in the treatment of APL, a retinoid may be designed which maximally and specifically binds to the aberrant but necessary RAR (PML-RARα-) expressed in these patients.

Not only do RARs interact with the promoter regions of genes but they also appear to interact with each other through protein–protein interactions [139]. One report illustrated the importance of multiple and varied protein–RAR interactions in the establishment of cell-type specific RA activity [136]. Antagonism between receptors and even between isoforms of a given RAR subtype has been shown to occur [139]. It appears that the RARs may form homodimers with the same receptor type or heterodimers with other nuclear receptors, including with the thyroid and vitamin D_3 receptors. The formation of a heterodimer between the thyroid receptor T_3 with an RAR has been demonstrated [185]. A retinoic acid receptor termed retinoid-specific binding protein (RSBP) was isolated from HL-60 cells [95]. Its molecular weight was determined to be approximately 95 kDa which is about twice that of other RARs and possibly indicates homodimer formation of RARα present in these cells. Alternatively, RSBP may be the aberrant receptor PML-RARα which has been isolated from cells from patients with APL (see the next section). Hetero- or homo-dimer formation may be necessary for differential gene activation.

CANCER AND ABNORMAL EXPRESSIONS OF RETINOIC ACID RECEPTORS

Several research groups have discovered that the breakpoint in the chromosomal translocation, t(15;17), present in most patients with APL, occurs within the RARα gene [186–191]. As predicted, APL patients express both aberrant and wild-type RARα mRNA. Interestingly, only APL patients expressing this aberrant mRNA can attain complete remission with RA treatment [101,102]. To attain complete remission, however, it appears that expression of the wild-type RARα is also necessary [192]. After attaining complete remission, expression of the *myl*/RARα oncogene can either no longer be detected or is markedly suppressed [102,193,194]. Recently, several investigators have characterized the mutant RARα protein which results from this rearrangement [195–199]. Because the

translocation involves both the RARα gene on chromosome 17 and the 'promyelocytic leukaemia' gene (*myl* or PML) on chromosome 15, this fusion protein has been referred to as *myl*/RARα or PML/RARα. This aberrant receptor contains the B–F regions of the RARα protein (at the C-terminal) fused to most of the PML protein (at the N-terminal). Interestingly, the wild-type PML protein contains a DNA-binding region of its own. Hence, the fusion protein contains two DNA-binding domains and is predicted to be bifunctional with unique transactivating properties. At least four aberrant PML/RARα receptors, resulting from different chromosomal translocation breakpoints, have been identified with molecular weights of 83 kDa [198], 89 kDa [196], 103 kDa [195], and 106 kDa [197]. The relatively large range of molecular weights represented among these fusion proteins appear to result largely from breakpoints clustered in two different regions of the *myl* gene [199]. The RA-affinity of the 103 kDa receptor was reported to be ten times greater than wild-type RARα [195]. Interestingly, in the absence of RA, the hybrid receptor behaved as a suppressor of gene activation, while in the presence of RA the receptor highly activated gene expression. Although similar results were obtained by Kakizuka et al. [196], de The' et al. [197] demonstrated that use of different reporter genes sometimes resulted in gene suppression in certain cells even in the presence of RA. Further study is required to characterize the natural genes actually regulated by PML-RARα. One of the natural genes regulated by *myl*/RARα may be the RARα gene, which was recently found to be up-regulated by RA in acute promyelocytic leukaemia cells *in vitro* and not in other myeloid leukaemias [200]. These initial findings suggest the following possibilities [195–197]: (a) the PML-RARα gene may represent an oncogene the protein product of which suppresses normal gene regulation by RARs at low physiological concentrations of RA, and (b) that this blockade of normal gene regulation by RARs may be overcome by high doses of RA such as those necessary for attaining complete morphological remission in vivo. Overcoming this blockade may involve the following scenario: (a) RA-induced activation of the *myl*/RARα receptor, (b) up-regulation of the RARα gene by the *myl*/RARα-RA complex, (c) formation of a *myl*/RARα–RARα heterodimer, and finally (d) suppression of the expression of the *myl*/RARα oncogene by the *myl*/RARα–RARα heterodimer. Much work remains to be carried out to establish the molecular basis for the generation and remission of APL and the role played by *myl*/RARα.

Other abnormal expressions of nuclear RA receptors have been observed. Normal epithelial cells (keratinocytes) of the oral cavity typically express RARβ transcripts. Most squamous cell carcinomas (SCC) derived from this

tissue, however, do not express detectable levels of RARβ mRNA, even after RA treatment [201]. Abnormal expressions of the RARβ gene also occurred with high frequency in lung cancer cells [202]. Furthermore, a correlation between RARβ gene expression and keratin 19 gene expression existing in normal keratinocytes was not observed in transformed keratinocytes [201]. Although rearranged transcripts were not observed, these results suggest that disruptions in the normal expression of RAR genes may be involved in neoplastic progression. Sharif and Privalsky [203] recently demonstrated that the ability of the *v-erbA* oncogene product to act in neoplasia best correlated with its ability to repress RAR action. For example, RA-induced growth inhibition of chick embryo fibroblasts (CEFs) involves suppression of activator protein-1 (AP-1) activity by RARα [204]. However, the *v-erbA* oncoprotein abrogates this RA-induced repression, possibly through RAR inactivation through *v-erbA*/RAR dimer formation, resulting in enhanced tumour formation of CEFs [204]. Just as *v-erbA* is a mutant thyroid hormone receptor lacking a thyroid hormone binding domain and confering RA non-responsiveness in chick embryo fibroblasts, so a mutant RAR receptor expressed in RAC65 cells (mutant P19 embryonal carcinoma cells) and lacking an RA-binding domain conferred RA-nonresponsiveness [205,206].

Therefore, it appears that abnormal expressions of retinoic receptors may be involved in neoplastic progressions, and in some cases, these may completely hinder RA responsiveness. In other cases, as in remission of acute promyelocytic leukaemia, abnormal gene regulation by mutant RARs can be overcome by retinoic acid treatment.

GENE REGULATION AND RETINOIC ACID RESPONSE ELEMENTS

In the past decade the expression of several genes has been shown to be regulated by retinoic acid [3,4,116–135,207–214]. Of particular interest are genes, the induction and/or suppression of which are mediated directly by a complex of RA with an endogenous receptor. These genes include: early retinoic acid (ERA-1) gene [116] in F9 cells, transglutaminase gene [84], the gene encoding RARβ [123,125,129], keratin genes [127], complement factor H gene [128], laminin B1 gene [122,131], the rat stromelysin gene [98], the progesterone receptor gene [135], the early growth response gene [209,213] which codes for a transcriptional factor, the alkaline phosphatase gene in P19 teratocarcinoma cells [214], and the interleukin-1β and interleukin-2 genes [134,215].

Direct induction of gene transcription is generally considered to result in rapid and non-delayed production of mRNA and not dependent on

on-going protein synthesis. For some genes, transcriptional regulation is delayed ('late') and is protein-synthesis dependent. For example, the induction of the ornithine decarboxylase gene by RA can be abolished by introduction of protein synthesis inhibitors [216]. Therefore, it has been concluded that the regulation of this gene by RA is not the result of immediate and direct binding of an RA-protein complex with the gene. Other genes the regulation of which is proposed not to be the result of a primary transcriptional event include the genes encoding the hepatocyte nuclear factors HNF-1α and HNF-1β [217], the activator protein-2 (AP-2) gene [218], and the human loricrin gene [208]. Care must be taken in making the assumption that delayed or protein synthesis-dependent regulation of a particular gene cannot involve direct binding of an RA–protein complex with the gene. Recently, it was shown that the 'late' (24–48 h) RA-dependent induction of the laminin B1 gene involves direct binding of a RAR to a RARE in the promoter region of this gene [131]. It appears that this 'late' induction may be the result in part of a weak affinity of the RAR to the RARE. It is also possible that other protein co-regulators interact with the RAR to enhance affinity of the RAR to the RARE and that the 'early' induction of the genes encoding these co-regulators is the initial necessary step.

Only recently have specific nucleotide sequences responsive to retinoic acid been identified in the promoter regions of genes [118–126,128–134]. These sequences are referred to as RAREs. These have been identified among some but not all of the genes whose transcription is directly regulated by RA. For many of the genes whose transcription has been shown to be directly regulated by RA, it has been demonstrated that RARs are involved in this mediation. However, it has been demonstrated that binding of RA to the RAR is not always a pre-requisite for RAR binding to DNA. In fact, binding of RA to the RAR sometimes results in suppression of gene activation, as was shown for the interleukin-2 (IL-2) gene [134]. However, suppression of gene transcription by RARs does not necessarily require binding of the RAR to the gene. For example, repression of transcription activation by the AP-1 complex has been shown to not involve RAR binding to either the rat stromelysin gene or the human collagenase genes [130,183]; instead, as suggested in the suppression of the human collagenase gene, blockage of AP-1 activity may involve protein–protein interactions.

Retinoic acid appears to regulate the activity, transport, storage, and metabolism of members of the vitamin A family by binding to and/or activating RAREs present in genes encoding retinoid-binding proteins or enzymes involved in RA biosynthesis. For example, a mechanism exists for

amplification of the RARβ signal. The RARβ gene contains an RARE in its own promoter [123,125,129] resulting in the auto up-regulation of the gene by its product, RARβ, in F9 teratocarcinoma [219] and S91 murine melanoma cells [220]. A RARE also exists in the RARα2 gene promoter which allows RA-induced up-regulation of RARα2 [153,221]. Like the RARE in the RARβ gene, the RARE in the RARα2 promoter can bind to any of the three RAR subtypes. Retinoic acid regulates the expression of the genes encoding the cellular retinol binding proteins I and II (CRBP-I and CRBP-II) through RAREs present in either CRBP gene [132,161]. The CRBP-I gene is activated by RARs, while the CRBP-II gene is activated by RXRα. However, this induction of CRBP-II activity by RXRα is suppressed by RARα [161]. Interestingly, 9-*cis*-retinoic acid is now known to be the specific natural ligand of RXRα [162,163]. Therefore, it appears that all-*trans* and 9-*cis* RA differentially regulate retinol transport, storage, and/or metabolism by controlling the expression of both cellular retinol binding proteins through different feedback mechanisms. Similarily, production of the cellular retinoic acid binding protein-II (CRABP-II), believed to help establish RA gradients in developing tissue, is activated by RA [165,166]. Aldehyde dehydrogenase-3 (ADH-3) contains a RARE which binds to RARβ and by which RA can activate transcription [222]. This discovery implies that retinoic acid can regulate its own synthesis from retinal. It will be interesting to see whether other auto- and cross-regulatory networks involving retinol, retinal, and retinoic acid are identified.

Several reports have indicated that RA, steroid/thyroid hormones, oncogene and proto-oncogene protein products, vitamin D_3, the activator protein-1 (AP-1) complex, and cAMP derivatives share common genetic response elements and complement each other in synergistic/antagonistic ways [118–121,124,126,130,203,223]. For example, cultured human ecto-cervical epithelial cell differentiation is mediated by the combined direct action of sex steroids, glucocorticoids and retinoids [224,225]. The expression of certain proto-oncogene proteins which bind to RAREs in the human osteocalcin gene results in inhibition of induction by either RA or vitamin D_3 [124]. The oncogene product *v-erbA* has been shown to suppress gene activation by RARs and estrogen receptors [203]. Recently, it was demonstrated that both RA and thyroid hormone T_3 jointly regulate transcription of keratin genes [127]. Similarily, RA-induced activation of the phosphoenolpyruvate carboxykinase (PEPCK) gene involves binding of RARα monomers and RARα homodimers [226]. However, this activity is suppressed in a thyroid hormone-independent manner by TRα, which can form monomers, dimers, and heterodimers with RARα. Alternately, the thyroid hormone and RA cooperate in the induction of genes containing the

palindromic thyroid hormone response element (TREp) through TR-RAR dimer formation [185].

Recently, efforts have been exerted to identify specific nucleotide sequence motifs and the spacings between these motifs which are necessary for specifying differential activation of genes by RA and the related steroid hormones. Several different motifs have been identified in RAREs: stereoaligned repeats of the nucleotide sequence TGACC in the laminin B1 gene [122], a direct repeat of GTTCAC adjacent to a TATA box in the RARβ gene [123,125,129], the AP-1 complex binding motif TGACTCA in the human osteocalcin and rat stromelysin genes [140], an imperfect palindrome of TGACC in the complement factor H gene [128], two direct repeats of GTTCA in the promoter of the RARα2 gene [221], four direct repeats of TGACC in the human oxytocin gene which encodes a neuropeptide (this RARE overlaps with an oestrogen response element) [227], and a direct repeat of GGTCA (antisense of TGACC) in the mCRBP-I gene [132]. The spacing between direct repeats of nucleotide sequence motifs is also important to achieve selective hormonal response [137]. Thus, an increase from 4 to 5 bases between repeats of AGGTCA (antisense of TGACCT) converts a thyroid hormone responsive element (TRE) to a RARE. Alternately, reduction of the spacing to 3 nucleotides produces a vitamin D-responsive element (VDRE). Recently, it has been suggested that separation of the AGGTCA repeats by one nucleotide confers an RXRE (that is, a response element regulated by the RXR retinoid receptor) [161]. Also, the orientation of the motifs is important for selective hormonal response [138]. For example, a direct repeat of the synthetic sequence TCAGGTCA with a three-base pair (3-bp) spacer conferred responsiveness only to RA (via an RAR), whereas a palindromic sequence of the same motif with a 3-bp spacer conferred responsiveness to oestrogen (via the oestrogen receptor). Finally, an inverted palindrome conferred reponsiveness only to the T_3 thyroid hormone (via the T_3 receptor, T_3R). Additionally, multimeric response elements may be important for differential gene activation by receptor dimers or multimers as appears to occur in the gene activation of the CRBP-II gene by either RARα dimers or RXR multimers [161].

POTENTIATIVE AND SYNERGISTIC EFFECTS USING RETINOIDS IN COMBINATION WITH CYTOKINES AND OTHER HORMONES

The biochemical pathways by which retinoids, steroid hormones, and

cytokines affect the state of proliferation and differentiation of cells, appear to overlap. For example, cultured human ectocervical epithelial cell differentiation is mediated by the combined direct action of sex steroids, glucocorticoids and retinoids [224,225]. Retinoid nuclear receptors and nuclear receptors for other hormones (for example, the thyroid hormone receptor) regulate many of the same genes, either through identical responsive elements or through different responsive elements within the same gene promoter. Synergistic and antagonistic interactions can occur among these receptors. For example, expression of the progesterone receptor gene is directly suppressed by RA in the T-47D human breast cancer cell line and involves a nuclear retinoid receptor [135]. Recently, it was found that the retinoid nuclear receptor RXRα can form dimers with either the thyroid hormone receptor (TR) or with the vitamin D_3 hormone receptor, synergistically enhancing the DNA-binding properties of each receptor [115,184]. Interestingly, full activation of the TR/RXR complex was possible only in the presence of both ligands, the thyroid hormone and RA [184]. Retinoic acid also regulates the expression of cytokines such as the transforming growth factors TGFα and TGFβ [228–230] and interleukin-2 (IL-2) [134]. The importance of TGFβ and its interactions with retinoids in determining the state of differentiation in cells is well documented [228,231]. Other cytokines include interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-4 (IL-4), interferon-α (IFNα), interferon-β (IFNβ), interferon-γ (IFNγ), tumour necrosis factor-α (TNFα), granulocyte colony-stimulating factor (G-CSF), and the epidermal growth factor (EGF) [232]. In view of these interactions, it is not surprising that therapies involving RA in combination with any of these cofactors may result in enhanced efficacy.

Combinations of retinoic acid with IFNα, IFNβ, TNF, or G-CSF have been reported to result in synergistic induction of differentiation in the HL-60 acute promyelocytic leukaemia cell line and the U-937 human histiocytic lymphoma cell line (see references in [232]). Until recently [232,233], combinations of retinoids (natural or synthetic) with other cytokines have not been explored.

Bollag and co-workers [232,233] recently demonstrated that specific combinations of certain synthetic retinoids with particular cytokines can result in a dramatically enhanced ability to induce terminal differentiation of HL-60 and U-937 cells relative to that observed by the addition of retinoid alone. Among the cytokines tested (IFNα, IFNβ, IFNγ, IL-1α, IL-1β, IL-2, IL-4, TNFα, G-CSF, TGFβ1, TGFβ2, EGF), IFNγ was found to give the greatest potentiation of retinoid activity in the HL-60 assay, 3- to 8-fold higher greater than that observed utilizing retinoids alone. Significant

potentiation was also effected by retinoids in combination with IFNα, IL-1α, TNFα, and G-CSF. In the absence of retinoid, all cytokines failed to induce differentiation. Similar potentiation effects by retinoids in combination with IFNγ, G-CSF, and TNFα were also observed in the U-937 assay.

Recently, two reports suggested that retinoids in combination with vitamin D_3 may be used in the treatment of neoplastic disorders. Cheng and co-workers [234] showed that RA in combination with 1,25-dihidroxyvitamin D_3 provides additive activity in the induction of human promyelocytic leukaemia (HL-60) cell differentiation and results in synergistic activity in the induction of human megakaryoblastic leukaemia (HIMeg) cell differentiation. Koger and Sutherland [235] demonstrated that the growth inhibitory effects of retinoic acid on T-47D human breast cancer cells were enhanced in a synergistic manner with 1,25-dihidroxyvitamin D_3 or antioestrogen co-treatment.

Great potential appears to exist in the use of retinoids in combination with cytokines, vitamin D3, antioestrogens, and possibly other hormones such as the thyroid hormone. Further studies are necessary to determine whether these preliminary *in vitro* results will translate effectively *in vivo*.

CLINICAL STUDIES

The potential of retinoic acid and other retinoids in the treatment of epithelial disorders and cancer has been the driving force in much of the work with retinoids. Already all-*trans* retinoic acid (Tretinoin) and 13-*cis*-retinoic acid (Isotretinoin/Accutane) are approved in Europe and the United States for clinical use in the treatment of different forms of acne while Acitretin (R26) and its ethyl ester (etretinate) are used in the treatment of psoriasis. The use of retinoids in the treatment of photodamaged skin is also being investigated [19].

Clinical studies in the use of retinoic acid for the treatment or chemoprevention of cancers are becoming more common. Of particular interest has been the use of natural retinoids for the treatment of APL [101,102,193,194,236–241], also called acute non-lymphocytic leukaemia type M3 (ANLL-M3) and French–American–British criteria for M3 leukaemia (FAB M3). The incidence of reports of attainment of complete remissions in patients treated with RA has dramatically increased over the past few years. The following paragraphs describe some of the results obtained to date. Lastly, a brief summary of other studies on the effect of RA in cancer prevention/treatment will be given.

Menger et al. [236] reported complete remission of APL from all 24

patients studied who were treated with all-*trans*-retinoic acid at doses of 45–100 mg/m^2 daily. There were no indications of hypoplasia. Fourteen of the fifteen patients whose marrow suspension cultures were studied showed morphological maturation of the cells in response to all-*trans* retinoic acid. Relapse occurred in 8 of the patients at 2–5 months following discontinuance.

Chomienne and co-workers [238] observed complete remissions in 2 patients treated with RA at 45 mg/m^2 daily over a period of 33 to 62 days, respectively. Remission was maintained for the next 9 months with treatment with methotrexate (12 mg/m^2 daily) and purinethol (100 mg/m^2 daily).

In another study [193] 14 out of 22 patients (16 of which had entered relapse after conventional chemotherapy) attained complete remission with RA treatment (45 mg/m^2 daily) without bone marrow hypoplasia. Complete remission was also accompanied by morphological maturation of the leukaemic cells from the patients. Almost all the patients (20/22) exhibited the t(15;17) chromosomal rearrangement prior to RA treatment. The t(15;17) translocation could no longer be detected in the patients who achieved complete remission. Only 5 patients were still in complete remission 4 to 13 months following RA treatment.

Retinyl palmitate (16,000 IU daily) was found to bring ANLL-M3 patients complete remission within 7–42 days of RA treatment after prior treatment by conventional chemotherapy [239]. This type of RA treatment was termed 'salvage therapy' because of the effect of RA in clearing out residual leukaemia cells by maturation and slowing proliferation. Retinol was the only metabolite in the plasma from the patients. Lo Coco et al. [194] demonstrated complete remission following treatment with RA at 45–50 mg/m^2 daily for 5–8 weeks. Although RARα rearrangements persisted after 2–3 weeks of treatment, these were no longer detectable in the patients after 5–8 weeks of treatment (that is, when the patients achieved complete remission).

Warrell et al. [102] indicated that 9 of the 11 APL patients studied exhibited aberrant expression of RARα. Only these patients attained complete remission with RA treatment (45 mg/m^2 daily) and the expression of aberrant RARα markedly decreased upon attainment of complete remission. Clinical response was associated with maturation of the leukaemic clone.

A study involving 50 APL patients [241] resulted in complete remission for 47 patients when treated with RA (typically, 60–80 mg/m^2 daily). Complete remission was usually maintained with conventional chemotherapy. Relapse occurred in 19 patients after 2 to 25 months of complete

remission. All patients maintained with combination therapy (RA treatment and conventional chemotherapy) entered relapse and were less sensitive to retinoic acid treatment when compared to patients in relapse who had been maintained in complete remission only by conventional chemotherapy. Also, maintenance of complete remission was observed for longer periods of time when conventional chemotherapy was employed upon attainment of initial complete remission as compared to using combination therapy for maintenance of complete remission. The authors do, however, site the clinical study of Sun et al. [242] who reported that alternating RA-treatment with conventional chemotherapy was better than conventional chemotherapy alone for maintenance of complete remission.

Recently, Muindi and co-workers [103] found that a progressive decrease in the retinoic acid plasma levels concurred with continuous RA treatment and may be the cause for relapse in APL patients who attained complete remission despite continued retinoic acid therapy. Despite an increase in the RA dose following relapse, the RA plasma levels did not rise. Cells from these patients did however respond to RA. The authors suggested that this decrease in the RA plasma levels may be the result of a concurrent increase in CRABP levels induced by RA, although further testing is necessary to verify this proposition. If so, retinoids which either do not bind to CRABP or which do not enhance CRABP expression would be needed to maintain complete remission. Retinoids have already been described which do not bind to CRABP and thus may exhibit improved therapeutic profiles relative to RA.

Retinoids have also been studied for their potential in the treatment of oral leucoplakia [243], the prevention of cancers [244,245], and in the treatment of a leukaemia other than APL [246]. In one study, 13-*cis*-retinoic acid was found effective for treatment of oral leucoplakia with 16 out of 24 patients responding positively to the treatment [243]. An average of 63% reduction of skin cancers was observed in 5 patients treated with 13-*cis*-RA (2 mg/kg daily) over a period of 2 years [244]. After discontinuance of therapy, tumour incidence increased 8.5-fold relative to the frequency during treatment. Similarily, 13-*cis*-RA treatment at 50–100 mg m^{-2} day^{-1} was effective in the prevention of second primary tumours in patients with squamous-cell carcinoma of the head and neck [245], although the retinoid was ineffective in preventing recurrences of the original tumour. Recently, the first effective response to oral RA therapy from a patient with a blast crisis of chronic myelogenous leukaemia (CML-BC) was reported [246].

CONCLUSION

The continued and intense unravelling of the role of retinoids in the complex biochemical processes of cell differentiation, proliferation, and in the suppression of aberrant chromosomal expressions suggests great potential exists for the use of retinoids (natural and synthetic) in the treatment of various disorders arising from abnormal cell differentiation. The possibility of continued discovery and design of retinoids with specificity of affinity for the various RAR-subtypes and with altered metabolic and toxicity profiles suggests that the process of retinoid molecular design is not complete. The discovery of the functional relationship between retinoic acid, cytokines, and the steroid hormones provides a stimulus for studies to explore the possibility of combination therapies involving retinoids and the other hormones whose role in gene regulation is being clarified. The recent reports of the prevention and/or remissions of cancers and leukaemias from patients treated with retinoic acid is promising. Studies are needed to further evaluate the potential relationship between cancers and abnormal expressions of the various nuclear retinoic acid receptors. The information gained from such studies may be valuable in the design of novel non-cytotoxic methods of chemotherapy as has recently been found possible in attaining complete remissions of acute promyelocytic leukaemia by treatment with retinoic acid.

ADDENDUM

Several other advances have very recently been reported. With regard to mechanism of retinoid action these include the following findings: (a) 9-*cis* retinoid acid is a more general activator of the nuclear retinoic acid receptors RAR and RXR than is all-*trans*-retinoic acid [247] and (b) induction of CRABP-II transcription is mediated by heterodimers of RAR and RXR [247]. These and other very recent findings (see references cited within ref. [247]), together with those previously mentioned in this review, underscore the apparent central role that RXR and its ligand (9-*cis* retinoic acid) play in the biochemistry not only of members of the vitamin A family but also of vitamin D, of members of the steroid/thyroid hormone family, and of other regulators of transcriptional activity. An important discovery that could lead toward the development of compounds to diminish unwanted side effects during retinoid therapy was the following: a heteroarotinoid [sulphone of retinoid (R5) (see Table 1.1) containing an *n*-heptoxy group *para* to the carbon bearing the *gem*-dimethyls] has recently

been identified as an RARα antagonist and counteracts retinoic acid effects on HL-60 cell differentiation [248]. Finally, an important finding in active cancer therapy were the reports of the use of the combination of 13-*cis*-retinoic acid and interferon α-2a (IFN α-2a) in successful treatments of squamous cell cancers of the cervix and skin [249,250].

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2 Semi-synthetic Derivatives of Erythromycin

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INTRODUCTION

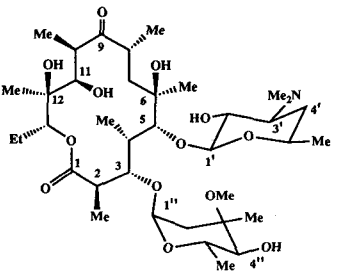
Erythromycin was discovered in 1952 in culture broths of *Streptomyces erythreus*, a microorganism isolated from a soil sample collected in the Philippines [1]. The taxonomy of this organism has now been changed to *Saccharopolyspora erythraea* [2]. Another erythromycin-producing organism, *Aeromicrobium erythreum*, has also been recently reclassified from an *Arthrobacter* species [3]. Erythromycin has been extensively used to treat infections primarily in the respiratory tract, skin and soft tissues, and genital tract and is regarded as one of the safest antibiotics [4–6]. It has become the most widely used member of the macrolide class [7]. Since its discovery, erythromycin has also served as a starting material for the preparation of a wide variety of semi-synthetic derivatives.

The field of macrolide antibiotics has undergone a remarkable renaissance within the last decade [8–11]. New applications have been found for erythromycin itself, while several semi-synthetic entities have been recently approved for sale or are in late stages of clinical trial [12–17]. Meanwhile, new derivatives having improved biological features are still being sought. The heightened interest in macrolides has resulted in the publication of two books and a scientific meeting devoted exclusively to recent developments in the field [18–20]. This review will describe the structures and relevant chemistry of erythromycin and its semi-synthetic derivatives individually, and then compare and contrast them as a group in terms of their biological and medical features.

CHEMISTRY OF ERYTHROMYCIN

STRUCTURE AND PHYSICAL-CHEMICAL DATA

The structure of erythromycin A (1), the principal factor produced during fermentation, was successfully elucidated after an intensive chemical effort [21]. It is composed of a highly substituted, 14-membered lactone named erythronolide A (2), which is glycosidated on the hydroxyl group at C-5 by an aminosugar (β-D-desosamine) and on the hydroxyl group at C-3 by a neutral sugar (α-L-cladinose). X-ray crystallography of erythromycin hydroiodide dihydrate firmly established its stereochemistry [22].



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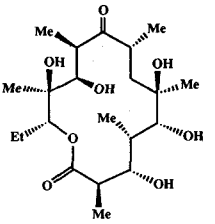

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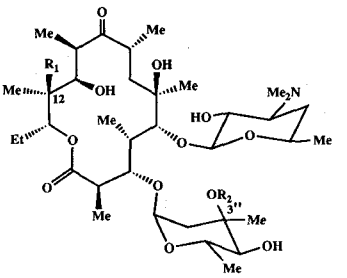
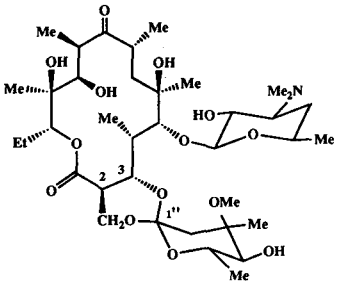
Several minor factors have been isolated from culture broths. The second most abundant factor, erythromycin B (3), is 12-deoxyerythromycin A [23]. Erythromycin C (4) contains mycarose (3-*O*-demethylcladinose) instead of cladinose as the neutral sugar component [24]. Erythromycin D (5) differs from erythromycin A in that both the 12-hydroxyl group is absent and the neutral sugar is mycarose [25].



(3): R_1 = H, R_2 = Me
(4): R_1 = OH, R_2 = H
(5): R_1 = R_2 = H

Erythromycin E (6) possesses an orthoester linkage between the lactone and neutral sugar [26, 27]. It may be formed from erythromycin F (7), in which the methyl group at C-2 is oxidized to a hydroxymethyl group [28, 29]. Additional minor factors are still being isolated from enriched extracts [29,30].

Erythromycin and its derivatives have been extensively studied by NMR. The ^1H and ^{13}C NMR spectra of erythromycin, measured in a range of solvents and by a variety of techniques, have been completely assigned [31–34]. The combination of NMR spectroscopy and X-ray crystallography has been used for conformational analysis, which found good agreement



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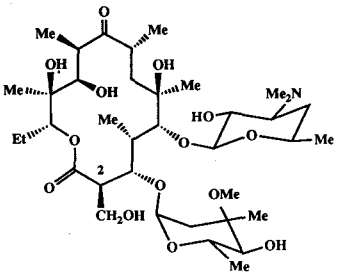


(7)

between conformations in the solution and crystalline states of erythromycin [35]. NMR studies have shown that the major component in solution is the 9-keto tautomer, with both the 6,9- and 9,12-cyclic hemiketals existing as minor components [36]. The goal of these studies is a correlation between molecular structure, conformation, and antimicrobial activity of erythromycin and its derivatives; however, this formidable challenge has not yet been successfully overcome [37].

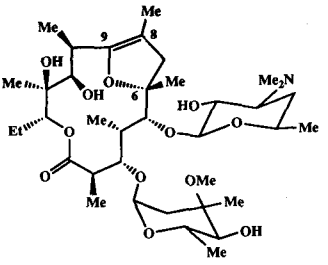
TOTAL SYNTHESIS

Total synthesis of such highly complex structures as erythromycin was originally deemed hopeless [38]. However, after two decades of advances in synthetic reagents, methodology, and strategy, these previously formidable targets yielded to total synthesis, beginning with erythronolide B [39]. This was soon followed by syntheses of erythronolide A [40], 6-deoxyerythronolide B [41], and finally erythromycin A itself [42]. Macrolides are still challenging targets to organic chemists for new approaches and improved routes of total synthesis. Several good summaries of more recent work are available [43–47].

Although recent advances in total synthesis are now approaching feasibility for structure–activity studies, chemical modification of erythromycin is still the most practical route for the synthesis of derivatives, since the starting material is readily available from fermentation sources. All of the commercially available derivatives of erythromycin as well as those in various stages of development are semi-synthetic products. The rationale for synthesis of many of these derivatives has been largely influenced by the mechanism of decomposition of erythromycin.

INTRAMOLECULAR CYCLIZATION

Erythromycin has long been known to decompose under acidic conditions via intramolecular cyclization reactions, initially forming 8,9-anhydroerythromycin-6,9-hemiketal (8), and subsequently, anhydroerythromycin-6,9:9,12-spiroketal (9) [48, 49]. Although formation of (8) and (9) has been generally considered to occur in a sequential manner, more recent kinetic studies have suggested that both compounds can arise independently from erythromycin [50, 51]. Acid-catalyzed decomposition of (9) causes hydrolysis of the acid-labile sugar, cladinose, and further degradation of the lactone ring system [52].



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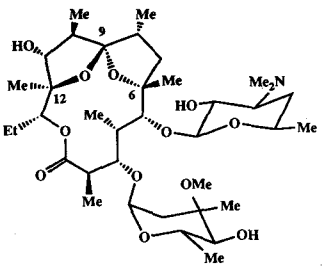


(9)

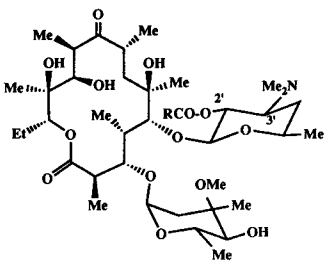
The intramolecularly cyclized products (8) and (9) have substantially reduced antimicrobial activity and are readily formed in the acidic environment of the stomach. Consequently, the need to protect erythromycin from decomposition in the stomach was recognized at a very early period in its development. One approach to preventing degradation is acid-resistant coatings, which permit a capsule or tablet to pass through the stomach intact, subsequently degrading in the more alkaline environment of the upper intestine and releasing the drug at the site of its absorption [4]. This approach is still being widely applied to erythromycin itself, which is presently available from several companies in a variety of enteric-coated tablets and pellets [53]. Protected oral formulations are also necessary to mask the extremely bitter taste that all macrolides possess [54].

FIRST GENERATION SEMI-SYNTHETIC DERIVATIVES

2'-ESTERS AND ACID-ADDITION SALTS

A second approach to protecting erythromycin utilizes certain ester

derivatives which convert the lipophilic molecule into an even more water-insoluble product that is both more resistant to acidic degradation and more palatable than the parent. Many 2′-ester derivatives were synthesized for this purpose soon after the discovery of erythromycin [55, 56]. The 2′-hydroxyl group of desosamine (10) is selectively esterified because the neighbouring 3′-dimethylamino group promotes intramolecular acylation to its adjacent hydroxyl group in the absence of an external base. 2′-Esters are regarded as pro-drugs since they do not bind to bacterial ribosomes [57]. Among the more commonly employed esters are acetate, propionate, ethyl carbonate, and ethyl succinate; a new form of the latter, called Biolid, is being clinically tested [58–60]. 2′-Esters readily hydrolyze to erythromycin, although rates vary for different esters and physiological conditions [61–64]. New esters are still being synthesized and tested for better oral bioavailability and palatability compared to other forms of erythromycin [64–66].



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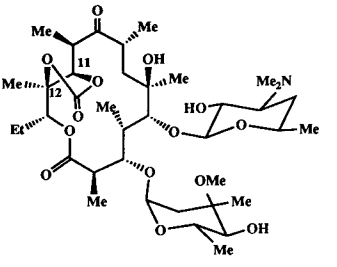


(11)

Another highly water-insoluble moiety which protects erythromycin from acidic decomposition is its acid-addition salt with a highly lipophilic acid. As an example, the stearate salt is a commonly prescribed form of erythromycin [53].

Combinations of a 2′-ester and a lipophilic acid-addition salt have also been extensively employed. Erythromycin estolate (2′-propionate, lauryl sulphate salt) has been widely used due to its better oral absorption than other forms of erythromycin [62, 67, 68]. Similar results have been found with erythromycin acistrate (2′-acetate, stearate salt) [69, 70], which is reported to be less hepatotoxic in animals than other forms of erythromycin [71]. Two salts of 2′-propionylerythromycin,*N*-acetylcysteinate (erythromycin stinoprate) and mercaptosuccinate, are being investigated as combinations of an antimicrobial and mucolytic agent [72–74].

Acid-addition salts with hydrophilic acids are water-soluble forms of erythromycin for intravenous administration. The lactobionate and glucep-

tate salts are most commonly used [53]. Two new water-soluble salts (melibionate and penicillanate) have recently been examined [75]. In another approach, a series of 2′-esters containing either acidic or basic groups on the ester moiety have been synthesized and studied as water-soluble ester pro-drugs of erythromycin [76].

11,12-CARBONATE

In erythromycin-11,12-carbonate (11), the cyclic ester locks the structure of the ring predominantly into the 6,9-hemiketal, with only a minor amount of the 9-keto form present [36, 77–79]. As a consequence, the facile dehydration of the 6,9-hemiketal to the 8,9-anhydro derivative (8) is inhibited. The increased stability results in better potency, pharmacokinetics, and tissue penetration than erythromycin [80, 81]. However, concerns about potential hepatotoxicity have prevented its wider usage [71, 82, 83].

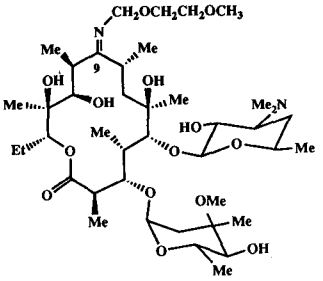
SECOND GENERATION SEMI-SYNTHETIC DERIVATIVES

Another synthetic strategy for partially or completely inhibiting intramolecular cyclization of erythromycin to hemiketal (8) and spiroketal (9) is modification of the functional groups that participate in the cyclization reactions. These groups include the C-9 ketone, C-6 hydroxyl, and C-8 proton in addition to the 11,12-diol discussed above. These approaches have led to a variety of semi-synthetic derivatives of erythromycin, some of which have been recently approved by regulatory agencies or are in late stages of clinical trials [12–17].

ROXITHROMYCIN

Since oximes of erythromycin are less prone to intramolecular cyclization than the parent compound, a structure–activity study was conducted on a series of 9-(*O*-alkyl)oxime derivatives of erythromycin [84]. From this work, roxithromycin, the 9-[*O*-(2-methoxyethoxy)methyl]oxime (12), was selected for further development. The isomer with *E* stereochemistry (oxime substituent syn to C-8 of lactone) is more active than the *Z* isomer [84]. The X-ray crystal structure of roxithromycin reveals a conformation of the lactone similar to that of erythromycin [85]. The similarity was also established by solution NMR studies, which suggested that the orientation of the oxime substituent helped to explain the increased hydrophobicity of roxithromycin and its greater penetration into certain tissues [86, 87].

Monographs and serial reports describing the significant features of roxithromycin have been published [88–92].



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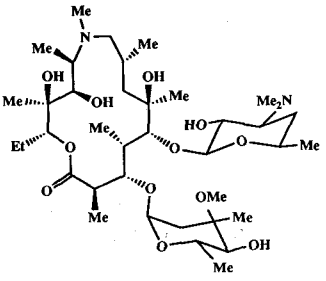


(13)

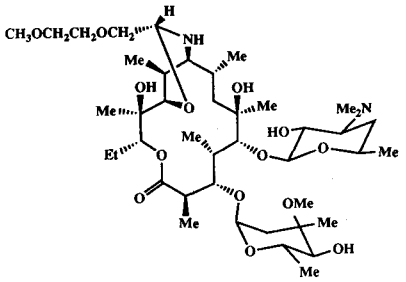
AZITHROMYCIN

Azithromycin (13) is the product from Beckmann rearrangement of erythromycin-9-oxime followed by reduction of the intermediate imino ether and *N*-methylation [93, 94]. Azalide is a term that has been applied to this group of ring-expanded derivatives of erythromycin that contain a second amino group embedded within their 15-membered ring system [93]. Studies of the NMR spectra and X-ray crystal structure of azithromycin and related compounds concluded that their conformation is very similar to erythromycin [93–97]. Consequently, replacement of the ketone in erythromycin by ring expansion and an additional basic, tertiary amino group accounts for many of the differences in activity between azithromycin and erythromycin. A monograph and symposium summaries for azithromycin have recently appeared [10, 98–100].

DIRITHROMYCIN

The product of another approach to replacement of the ketone in erythromycin is dirithromycin (14), a 9-*N*,11-*O*-oxazine derivative of 9(*S*)-amino-9-deoxoerythromycin (9(*S*)-erythromycylamine, 15) [101, 102]. *R* stereochemistry of the substituent at C-2 of the oxazine was established by X-ray crystallography [101]. Condensation of erythromycylamine with 2-methoxyethoxyacetaldehyde initially gives the 2(*S*)-oxazine, which was isolated using diethyl ether as solvent, but it rapidly epimerizes to dirithromycin [103, 104]. A related compound with *S* stereochemistry of the oxazine substituent has also been crystallized [105]. Both epimers readily

hydrolyze *in vivo* to erythromycylamine, an active metabolite [103]. A monograph on dirithromycin is being published [106].



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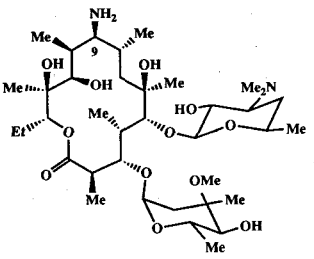
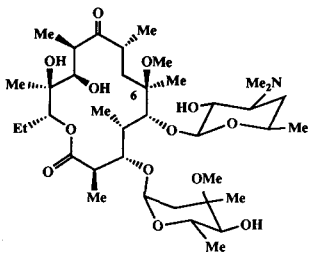


(15)

CLARITHROMYCIN

A different approach to preventing intramolecular cyclization of erythromycin is alkylation of the hydroxyl group at C-6. After synthesis of a series of *O*-alkyl derivatives of erythromycin, clarithromycin (16) was chosen for development [107–109]. X-ray crystal structures and conformational analysis were used to analyze the regioselectivity of *O*-alkylation, which showed that hydrogen bonding from the 12-hydroxyl group enhanced deprotonation of the 11-hydroxyl group [110]. A more selective route to clarithromycin via 6-*O*-methylation of 9-oxime derivatives has been reported [111]. 14(*R*)-hydroxyclarithromycin (17) is an important active metabolite [112, 113], which is most easily prepared by microbial bioconversion with *Mucor circinelloides* [114]. Monographs and summaries of clarithromycin have been published [115, 116].



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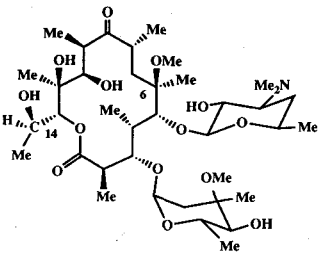
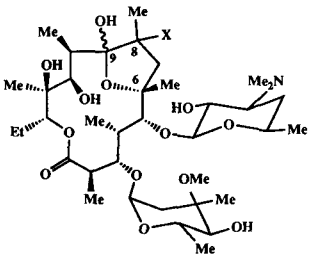


(17)

FLURITHROMYCIN

Replacement of the C-8 proton of erythromycin with a less labile substituent prevents dehydration of erythromycin-6,9-hemiketal (18, X=H) to the 8,9-anhydro derivative (8). Flurithromycin (19) was obtained via mutational biosynthesis by feeding 8(*S*)-fluoroerythronolide A to a biosynthetically-blocked mutant strain of *S. erythraea* [117]. The starting material for bioconversion was prepared by treating 8,9-anhydroerythronolide-6,9-hemiketal with trifluoromethyl hypofluorite [118]. The 2′-ethylsuccinate ester of flurithromycin is now in clinical trial [119].



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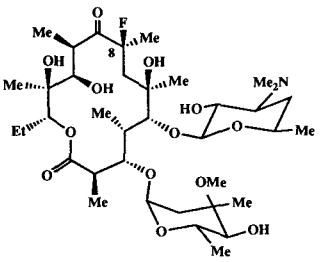
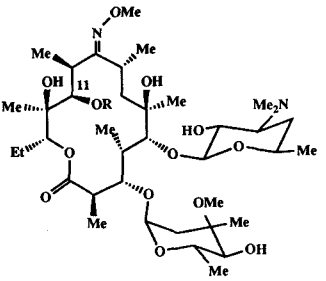


(19)

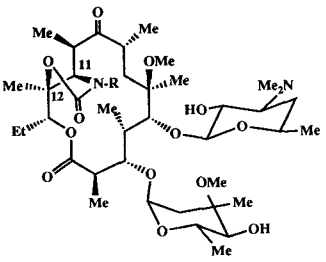
OTHER DERIVATIVES

In addition to those described above, many other semi-synthetic derivatives of erythromycin have been prepared [15, 120], although none has yet progressed to commercialization. ER 42859 (20) was selected for clinical study from a series of 11-*O*-alkylerythromycin-9-oximes; although it



(20)

R = $CH_2OCH_2CH_2NMe_2$



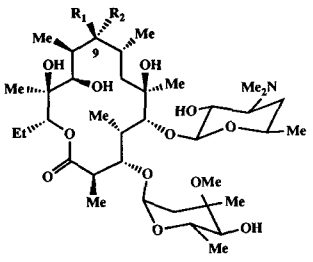
(21)

R = $CH_2CH_2NMe_2$

showed better stability to acid, it produced lower blood levels than erythromycin [121]. X-ray studies showed that its aglycone adopts a conformation different from erythromycin [122]. Synthesis of analogues of erythromycin-11,12-carbonate (11) yielded an active series of cyclic 11-*N*,12-*O*-carbamates, exemplified by A-62514 (21) [123]. Double modifications have also been made, such as erythromycin-9-oxime-11,12-carbonate [124].

Certain 9-*N*-alkyl derivatives of 9(*S*)-erythromycylamine (15) are orally effective against experimental infections and give very high tissue concentrations. From this series, LY281389 (22) was selected for further study [125, 126], but unexpected side-effects halted its development [127]. In contrast to erythromycin, the lactone of (15) interconverts between two conformations [128], and NMR methods to predict conformations of new derivatives have been described [129]. 9-*N*,11-*O*-oxazines of 9-*N*-alkylerythromycylamine epimerize at C-2 of the oxazine analogous to dirithromycin [130], and cyclic adducts of erythromycin-9-imine and aldehydes have been prepared [131]. A series of 9-*N*-alkyl derivatives of the 9(*R*) isomer of erythromycylamine, exemplified by A-69991 (23), exhibited good antibiotic activity [132]. Epimerization of the methyl group at C-2 of the lactone resulted in 2- to 4-fold loss of antibacterial activity [133].

Intramolecular cyclization products (8) and (9) were converted into novel 9,12-epoxy derivatives, such as A-69334 (24), which possessed better pharmacokinetic properties than erythromycin [134]. The structurally related bicyclic macrolide, L53-18A, was found in culture broths of an unidentified *Saccharopolyspora* species [135]. Several 8,9-difluoro-6,9-epoxy derivatives of erythromycin and 8-bromo derivatives of erythronolide B have been prepared [136, 137].



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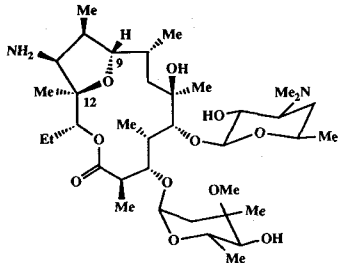


(24)

R_1 = $NHCH_2CH_2CH_3$, R_2 = H
R_1 = H, R_2 = 4,4-dimethylpiperidin-1-yl

Modifications of both saccharide moieties of erythromycin have also been made. Migration of the 3′-dimethylamino substituent to C-2′ has been demonstrated [138]. Acylation of the 4″-hydroxyl group yielded products such as A-63075 (4″-*O*-carbamoylclarithromycin), which minimize gastrointestinal side effects in dogs [139]. Modifications of the 4″-hydroxyl group also increase activity against certain erythromycin-resistant bacteria [140, 141].

DERIVATIVES FROM ALTERED BIOSYNTHESIS

In addition to chemical modifications and microbial bioconversions, derivatives of erythromycin have been obtained by altering the biosynthetic pathways operating in *S. erythraea*. As the tools and knowledge of molecular biology expand, products from this approach are likely to increase. 2-Demethyl analogues of erythromycins A-D (2-norerythromycins) were unexpectedly isolated from mutant strains of *S. erythraea* into which DNA from *Streptomyces antibioticus* (producer of oleandomycin) had been inserted [142]. Targeted disruption of the gene (*eryF*) that codes for 6-deoxyerythronolide B hydroxylase produced a mutant of *S. erythraea* incapable of converting 6-deoxyerythronolide B to erythronolide B [143]. Since the subsequent biosynthetic steps remained operable on the altered substrate, 6-deoxyerythromycin was obtained from fermentation broths of this recombinant strain [143]. Recent developments such as the concept of a modular polyketide synthase and cloning of erythromycin biosynthetic genes will undoubtedly lead to new derivatives from directed genetic engineering [144–147].

BIOLOGICAL AND CLINICAL FEATURES

SPECTRUM OF ANTIMICROBIAL ACTIVITY

Erythromycin is commonly used to treat infections caused by many Gram-positive organisms, *Mycoplasma* species, and certain susceptible Gram-negative and anaerobic bacteria within the respiratory tract, skin and soft tissues, and genital tract [4–6]. The renaissance of erythromycin is partially due to its efficacy against organisms that have emerged as more prominent pathogens, such as *Legionella*, *Chlamydia*, *Ureaplasma*, and *Campylobacter* species. Reports of the *in vitro* activity of semi-synthetic macrolides are now too numerous to cover in this chapter. Several extensive

side-by-side comparisons of many newer derivatives have been performed [148–154]. The monographs and reviews of individual compounds cited above include descriptions of their antimicrobial spectrum; furthermore, some of the earlier literature has been summarized [12, 15].

All of the new semi-synthetic derivatives have excellent activity against bacteria within the traditional spectrum of erythromycin [148–154]. Roxithromycin, clarithromycin and azithromycin show improved activity against *Legionella* pneumonia in guinea pigs [155–158]. They possess good activity *in vitro* against *Chlamydia pneumoniae* (formerly TWAR), although the different methods of evaluation need to be correlated with clinical results [159]. They are also effective against *Chlamydia trachomatis* in a mouse model of chlamydial salpingitis [160, 161]. They possess excellent *in vitro* activity against *Ureaplasma urealyticum* and *Mycoplasma pneumoniae*, but like erythromycin, they are relatively poor against *Mycoplasma hominis* [151, 162]. They inhibit the enteric bacteria, *Campylobacter coli, C. jejuni* and *Helicobacter pylori* (formerly *C. pyloridis*) [163–166], but the possible role of the latter in gastric ulcers and cancer is a subject of substantial current debate [167–170]. Their spectrum of activity also includes *Haemophilus ducreyi,* the causative agent of chancroid [171, 172]. All of the newer macrolides have demonstrated excellent efficacy against traditionally susceptible bacteria in experimental infections in animals; their *in vivo* activity is usually superior to the efficacy of erythromycin [102, 157, 173–177].

Since erythromycin has good activity against most common pathogens of the respiratory tract except *Haemophilus influenzae*, improved activity against this bacterium has long been sought in order to have an antibiotic with broad coverage against respiratory pathogens. Among the macrolides, azithromycin is the most potent *in vitro* against Gram-negative bacteria, including *H. influenzae* [178–180]. Efficacy against this organism in a middle ear infection in gerbils and an acute otitis media model in chinchillas has been demonstrated [174, 181]. The combination of clarithromycin and its metabolite, 14-hydroxyclarithromycin, is more active than erythromycin or clarithromycin alone against *H. influenzae* [182–184].

The promise of the newer macrolides to expand the traditional spectrum of erythromycin is another part of the macrolide renaissance. Clarithromycin, either alone or in combination with other agents, has shown activity against several species of mycobacteria, including *M. avium* complex [185–189], *M. leprae* [190–192], *M. tuberculosis* [193], and other mycobacterial species [194]. Although use of older macrolides against *Cryptosporidium* is controversial, azithromycin has been effective against *C. parvum* in rats [195]. It has also been effective against *Borrelia burgdorferi* in gerbils

[196]. Several newer macrolides have shown activity against *Toxoplasma gondii*, either alone or in combination with agents such as pyrimethamine [197–200]. The combination of a macrolide and a sulphonamide was synergistic against *Pneumocystis carinii* pneumonitis in rats [201]. Azithromycin was active against *Giardia* [202] while roxithromycin had activity against *Treponema* [203] and *Rickettsia* [204]. These recently identified activities are being pursued in clinical trials to determine potential clinical application, while additional activities for these newer macrolides are being sought.

MECHANISM OF ACTION

Macrolide antibiotics are believed to inhibit growth of bacteria by binding to their 50S ribosomal subunits and inhibiting protein synthesis [205]. However, many details of this complex process remain unknown. Proton NMR studies have shown that erythromycin binds through its 9-keto tautomer, but more weakly than expected, suggesting two binding sites with different functions [206–209]. Kinetic studies of macrolide–ribosome complexes indicated efficient binding by both neutral and protonated forms of the macrolide [210]. Attempts to identify ribosomal proteins involved in binding of macrolides have been made by photoaffinity-labelling and affinity chromatography [211–213]. Recent studies have reinforced previous suggestions that erythromycin binds near the site of the peptidyltransferase centre on the ribosome [214]. Although ribosomal binding is a critical feature, other factors such as transport of these lipophilic molecules across bacterial membranes and through biological fluids are equally important in determining overall antimicrobial activity [210, 215].

Although the majority of mechanistic studies have used erythromycin, all other macrolides as well as the lincosamides and streptogramins (collectively known as MLS antibiotics) are believed to operate by a similar mechanism [216]. However, each of the groups possesses slightly different, but overlapping, ribosomal binding domains [217].

DEVELOPMENT OF RESISTANCE

The mechanisms of microbial resistance to antibiotics are generally due to alterations of the antibiotic's target site, enzymatic inactivation of the antibiotic, cell impermeability, reduced cellular uptake, or increased efflux of antibiotic from cells [218]. The occurrence of these mechanisms in macrolide-resistant organisms has been reviewed [219–221]. The most widespread mechanism is modification of the macrolide's ribosomal

binding site due to methylation of a specific adenine residue in 23S rRNA by a methylase that is either inducibly- or constitutively-derived [222, 223]. Since such methylation reduces binding for all macrolides, lincosamides, and streptogramin B, such organisms are cross-resistant and are described as MLS-resistant. Certain deletions in 23S rRNA also lead to erythromycin resistance [224]. In contrast to erythromycin and many of its derivatives which induce MLS-resistance, 16-membered macrolides fail to do so, although both 14- and 16-membered macrolides lack activity against constitutively-resistant organisms [225]. However, several types of semi-synthetic derivatives of erythromycin and clarithromycin have been described which inhibit both types of MLS-resistant bacteria [140, 226, 227].

The inability of most macrolides to penetrate into Gram-negative bacilli is probably responsible for the lack of activity against these organisms, since their ribosomes are generally sensitive [220, 228]. Although reduced uptake of macrolides has been proposed [229–231], an energy-dependent efflux of macrolides and the difficulty of distinguishing between these mechanisms was recently described [232]. Macrolides have been inactivated by esterases [233], 2′-*O*-phosphotransferases [234, 235], and a 2′-*O*-glycosyltransferase [236]. However, several derivatives exhibited greater stability than erythromycin to hydrolysis of the lactone by an *E. coli*-derived esterase [237]. The hypothesis that bacterial resistance genes originated in macrolide-producing organisms has been reviewed [238]. To ilustrate the constant capabilities of bacteria to acquire resistance, strains of *Staphylococcus aureus* exhibiting atypical patterns of macrolide resistance and species of *Campylobacter* possessing mutations in ribosomal protein genes have recently been reported [239–241].

ORAL ABSORPTION AND BIOAVAILABILITY

Macrolides are weakly basic, highly lipophilic substances which are orally absorbed in the upper intestine. Absorption apparently occurs by passive diffusion, since no evidence exists for an active transport system. As discussed above, erythromycin base must be protected from degradation by stomach acid. The rate and extent of absorption depends on parameters such as physicochemical properties of the compound (pKa, lipophilicity), its chemical stability, the chemical entity administered (free base, salt, ester), properties of the dosage form (dissolution rate, tablet, capsule, suspension), pH of the upper intestine, and the patient's gastrointestinal physiology (food, fluid volume) and metabolism (age, underlying disease). General features concerning the absorption of macrolides have been reviewed [242–244].

Erythromycin exhibits low oral bioavailability, low serum concentrations, a short *in vivo* half-life, and a high degree of intra- and inter-subject variability. Crystalline forms of erythromycin were found to be more bioavailable than amorphous forms [245]. The bioavailabilities of dirithromycin and erythromycin acistrate are increased by adding a basic substance such as a carbonate salt to the tablet or capsule [246, 247].

The greater chemical stability imparted to erythromycin by water-insoluble esters and salts or by structural changes in the lactone have already been described for the individual derivatives. These modifications often result in greater oral bioavailability, higher serum and tissue concentrations, and longer plasma and tissue half-lives for the compound compared to erythromycin. As a result of these features, many of the semi-synthetic derivatives are being clinically investigated using lower amounts of drug, less frequent dosage schedules, and shorter duration of therapy relative to erythromycin. The improved pharmacokinetics of the semi-synthetic derivatives may also prove beneficial for eradicating more persistent or localized infections and reducing the side-effects caused by erythromycin. These clinical benefits arising from the improved pharmacokinetics of the newer semi-synthetic derivatives of erythromycin are just as important for the recent resurgence of macrolides as their potentially expanded spectrum of antimicrobial activity.

DISTRIBUTION IN FLUIDS AND TISSUES

Although erythromycin produces relatively low serum concentrations, it achieves higher concentrations of antibiotic in several important tissues such as pulmonary and prostate. It also penetrates effectively into cells and achieves concentrations exceeding that in the surrounding fluids. Each of the newer semi-synthetic derivatives has an individualized pharmacokinetic profile that differs from the others. The monographs and reviews cited above for specific compounds provide numerous details of their individual pharmacokinetic parameters.

As a result of differing rates of hydrolysis, 2′-esters of erythromycin differ from their parent and each other in terms of peak serum concentrations, length of time to achieve that concentration, and area under the concentration vs. time curve (AUC) [63, 69, 248]. Roxithromycin is characterized by relatively high serum concentrations that are several-fold greater than those achieved by erythromycin [244, 249–251]. In contrast, clarithromycin produces relatively moderate serum concentrations, but achieves much higher tissue concentrations than roxithromycin [252, 253]. Azithromycin gives lower serum concentrations, but very high tissue

concentrations which may be as much as 100-fold above serum concentrations [254, 255]. Dirithromycin also produces high tissue concentrations of antibiotic, but low serum concentrations [256].

The need for proper interpretation of the high tissue/serum ratios of macrolides and their clinical relevance have been recently reviewed [257–259]. Numerous studies have been published demonstrating intracellular uptake of macrolides. This phenomenon helps to explain their antimicrobial activity against many pathogens which reside in an intracellular environment, such as species of *Legionella*, *Chlamydia*, *Salmonella*, staphylococci, and mycobacteria. If the compound is bioavailable, high tissue concentrations of antibiotic would permit better eradication of infections localized within that tissue.

Macrolides are now recognized to exert a variety of immunomodulatory effects and act on cells involved in both specific and non-specific host defence functions [260–262]. However, all of the newer macrolides have not been thoroughly investigated, their effects are not always understood, and published experimental results may appear contradictory. All macrolides accumulate to some degree within phagocyctic cells and are believed to concentrate in the lysosomes [263, 264]. As a result of intracellular penetration, they are capable of inhibiting intracellular bacteria, although antibacterial activity does not necessarily correlate with intracellular concentrations [264–266]. Despite high accumulation, azithromycin is inactivated by the acidic intracellular environment to a greater extent than erythromycin or roxithromycin [267]. It has been proposed that azithromycin is transported to sites of infection by the phagocytic cells into which it has penetrated [264, 268]. Its cellular penetration is best explained by passive diffusion and pH partition [269], and its uptake into macrophages has been enhanced by stimulation of the cells with agents such as cytokines [270].

Azithromycin also accumulates in fibroblasts, which are suggested as a possible reservoir of drug within the body [271]. Several studies have suggested effects of macrolides on anti-inflammatory processes [272–275]. However, much more study is required before a thorough understanding of all these effects of macrolides will be achieved.

METABOLISM AND EXCRETION

Macrolides are primarily metabolized in the liver and excreted into bile; metabolism occurs to a lesser extent in the kidneys and lungs with excretion into urine and exhaled breath, respectively [243]. The rate and degree of metabolism depend on factors such as the patient's age, underlying disease

states, and amount of drug administered. This knowledge is important for establishing optimum dosage schedules that maximize efficacy, minimize toxicity, and reduce adverse drug interactions with other medicines that a patient is taking. The metabolism of erythromycin and its semi-synthetic derivatives has been recently reviewed [276].

Among the most common metabolic pathways of 14-membered macrolides are N-demethylation of the aminosugar and hydrolysis of the neutral sugar. Oxidative N-demethylation involving cytochrome P-450 is a major route of erythromycin metabolism [277]. The products of intramolecular cyclization were found in rats after intravenous or intramuscular administration of erythromycin, indicating that they do not occur solely from oral absorption of material produced after degradation by stomach acid [278]. Among the semi-synthetic derivatives, roxithromycin and azithromycin do not undergo extensive metabolism [279, 280]. The metabolism of clarithromycin to its 14(*R*)-hydroxy derivative (17) occurs extensively in humans and monkeys, but not in dogs or rodents. In addition, various combinations of hydroxylation, N-demethylation, and hydrolysis of cladinose were observed [281]. Dirithromycin undergoes facile non-enzymatic hydrolysis of the oxazine ring to yield its active metabolite, erythromycylamine [282].

ADVERSE REACTIONS AND SIDE-EFFECTS

Since erythromycin complexes and inactivates drug oxidizing systems such as cytochrome P-450, it has the potential to alter the metabolism of other drugs. The metabolism and excretion of theophylline, warfarin, carbamazepine, and methylprednisolone are inhibited by erythromycin [283–286]. As a potent antibiotic, it can also affect metabolism by gut micro-organisms of drugs such as digoxin. At least some of the newer derivatives may cause fewer drug interactions and thus may be better tolerated if co-administered with medications for other illnesses [287–289].

Erythromycin is generally regarded as one of the safest antibiotics available. The largest number of side-effects involve gastrointestinal (GI) problems, including nausea, vomiting, diarrhoea, and abdominal pain [290]. In many cases, these GI effects are sufficiently severe to result in cessation of erythromycin therapy. Consequently, a major objective for the semi-synthetic derivatives has been to substantially reduce the incidence and severity of GI effects. An experimental model has been established whereby the effect of derivatives on gut motility in dogs is measured. This model has been used to select derivatives predicted to show fewer GI effects [139]. However, reliance on such a model presupposes that the prokinetic action is

the true and only mechanism by which macrolides cause GI problems, and such correlations with clinical experiences have not yet been proven.

Some of the less frequent side-effects of erythromycin, such as ototoxicity, hepatic injury, and hypersensitivity, have been recently surveyed [291–294]. However, much more clinical experience will be required with the new derivatives before effects such as these can be assessed.

CLINICAL EXPERIENCE

Erythromycin is predominantly used to treat infections caused by susceptible organisms in the respiratory tract, skin and soft tissues, and genital tract [4–6, 295]. Results from some early clinical trials of the new semi-synthetic derivatives have been published within the monographs, summaries and reviews referenced above; however, complete coverage of this rapidly growing body of literature is beyond the scope of this chapter. As a general rule, all of the newer derivatives have successfully treated infections that lie within the traditional therapeutic range of erythromycin itself [16, 296, 297].

The efficacy of some semi-synthetic derivatives against infections that are outside the recognized spectrum of erythromycin has been an especially exciting development. Both clarithromycin and azithromycin have shown activity against *Mycobacterium avium-intracellulare* complex in AIDS patients [298, 299]. The combination of clarithromycin and pyrimethamine gave results comparable to standard regimens against *Toxoplasma* encephalitis in AIDS patients [300]. The use of macrolides against *Cryptosporidium* enteritis is still very controversial; however, positive results were recently reported for the combination of erythromycin and thymostimulin [301]. The combination of roxithromycin and omeprazole eradicated *Helicobacter pylori* from infected patients [302]. A single example of successfully using roxithromycin plus co-trimoxazole against *Borrelia* infection has been published [303]. In addition to expanding the antimicrobial spectrum of macrolides, azithromycin has been used successfully for the treatment of infections with a shorter than usual duration of therapy, presumably due to its higher and more persistent concentrations [304]. Its pharmacokinetic properties also help rationalize its utility for treatment of sexually transmitted diseases with a single dose [305–307]. Additional examples of new and broader uses for macrolides are anticipated as more clinical trials are completed and published.

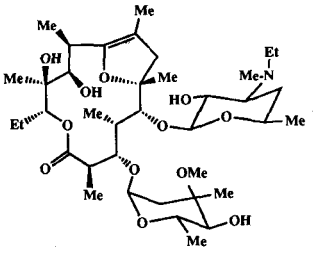
The reported incidence and profile of side-effects observed in early studies of the new derivatives, including fewer gastrointestinal effects than

erythromycin, have been encouraging [16, 297, 308]. However, more clinical experience is needed before this issue can be conclusively settled.

DERIVATIVES FOR NON-ANTI-INFECTIVE APPLICATIONS

During studies to better understand and potentially alleviate the gastrointestinal side-effects of macrolides, erythromycin and oleandomycin were discovered to induce strong muscular contractions in the GI tract of dogs, whereas 16-membered macrolides did not [309]. Clinical studies have subsequently established that erythromycin exerts a variety of effects on the GI tract, such as inducing the migrating motor complex, increasing lower oesophageal sphincter pressure, and stimulating gastric emptying [310, 311]. It has been suggested that erythromycin acts as a non-peptide agonist of motilin, an endogenous peptide responsible for interdigestive GI contractility [311–313]. After finding that cyclic enol ether (8) was 10-fold more potent than erythromycin, extensive structure–activity studies were conducted [314–317], from which EM-523 (25) was selected for clinical development as a GI prokinetic agent [318, 319].

A 12-membered cyclic enol ether (26) has been synthesized by transacylation of (8) or directly from erythromycin [320, 321]. It was initially reported as a minor component in culture broths from which erythromycin had been crystallized [322]. This ring-contracted derivative (26) has also exhibited potent GI prokinetic activity [315, 323].



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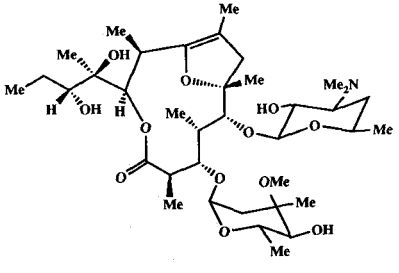

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SUMMARY

Semi-synthetic derivatives of erythromycin have played an important role in antimicrobial chemotherapy. First generation derivatives such as 2′-esters and acid-addition salts significantly improved the chemical stability and

oral bioavailability of erythromycin. A second generation of erythronolide-modified derivatives: roxithromycin, clarithromycin, azithromycin, dirithromycin and flurithromycin, have been synthesized and have exhibited significant improvements in pharmacokinetic and/or microbiological features. In addition, erythromycin itself has expanded its utility as an effective antibiotic against a variety of newly emerged pathogens. As a result of these developments, macrolide antibiotics have enjoyed a resurgence in clinical interest and use during the past half-dozen years, and semi-synthetic derivatives of erythromycin should continue to be important contributors to this macrolide renaissance. Despite these recent successes, other useful niches for macrolide antibiotics will remain unfilled. Consequently, the search for new semi-synthetic derivatives of erythromycin possessing even better antimicrobial properties should be pursued.

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3 Biological Significance of Manganese in Mammalian Systems

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INTRODUCTION

In his classic review of this subject in 1958, George Cotzias began by noting that mangania is the Greek word for magic or voodooism, 'reflecting some reality in the biology of manganese, ... rich in phenomena and lacking in adequate guiding principles' [1]. He further points out that most of the phenomena have been discovered largely by chance, and that several apparently theoretical concepts that are sound *in vitro* fail to predict how this metal behaves in the body. Both these statements still hold true to some extent some 35 years later, but significant advances have been made.

For manganese in mammalian systems (the focus of this review), more information is now available on the sites of action and interaction at the molecular and cellular levels, due to an explosion of new findings within the last decade. Although this has produced more viable hypotheses, manganese exhibits such a multiplicity and complexity of action that no single, unifying concept or model is likely to emerge. Thus, as before, this field remains 'wide open for fruitful investigation [1]'.

Since Cotzias's 1958 review [1], a number of excellent monographs, chapters, and review articles have appeared [2–23]. The period prior to 1984 is perhaps best summarized by the multi-author monograph [18], with regard to concepts of nutrition and transport, interaction with several important enzymes and metabolic processes, and as a biophysical probe. The references in the current review result from a selective search of the

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* The following abbreviations are used in this review: ATP, adenosine-5′-triphosphate; eEF-2, eukaryotic elongation factor-2; EGF, epidermal growth factor; F-2, 6-bisP, fructose-2, 6-bisphosphate; FBPase, fructose-1,6-biphosphatase; GABA, gamma-amino butyric acid; GpNpp, 5′-guanylyl-imidodiphosphate; GS, glutamine synthetase; HDL, high density lipoprotein; IP_3, inositol-1,4,5-triphosphate; MSOX, L-methionine-sulphoximine; NMDA, N-methyl-D-aspartate; NTP, nucleotide triphosphate; OAA, oxalacetic acid; PDE, phosphodiesterase; PEP, phosphoenol phosphate; PEPCK, phosphoenol pyruvate carboxy kinase; PML, polymorphonuclear neutrophils; Quin-2, 2-[(2-bis(carboxymethyl)amino-5-methylphenoxy)methyl]-6-methoxy-8-bis(carboxymethyl)aminoquinoline tetrakis(acetoxymethyl) ester; SOD, superoxide dismutase; src, sarcoma-producing; TCA, tricarboxylic acid; THF, tetrahydrofolate.

literature since 1983. The main purpose is to present an overview of recent findings and concepts, highlighting discoveries that offer interesting avenues for future research, with a bias toward molecular-level mechanisms. Abbreviations used in this review are explained* *(see previous page)*.

MOLECULAR EFFECTS

ENZYMES

First, it is important to distinguish conceptually between 'manganese activated (or inhibited)' and 'manganese containing' enzyme systems – the latter being in the minority, with arginase and manganese superoxide dismutase being the best known examples. Manganese-activated systems are the general rule, typically having tight, rapidly reversible, manganese-specific sites involved with either catalytic or regulatory function. The rapidly reversible nature of Mn(II) binding, plus the fact that Mn(II) is a trace metal existing only at micromolar levels in mammalian tissues, clearly contributes to the 'magical', or 'will-o'-the-wisp' quality noted by Cotzias. The researcher is left with the task of proving that a given enzyme specifically interacts with micromolar Mn(II) in preference to millimolar Mg(II) or Ca(II).

Specific criteria that must be met for enzyme activity to be regulated by Mn(II) have been set forth and discussed by Schramm [7,24]: (1) binding constant(s) of the enzyme for Mn(II) must be at or near the free Mn(II) concentration in the cell compartment where this enzyme exists; (2) Mn(II) binding sites must discriminate strongly for Mn(II) over Mg(II); (3) the amount of intracellular, exchangeable Mn(II) must be equal to or greater than the molarity of target enzyme(s); (4) the intracellular free Mn(II) must respond to altered physiological or hormonal signals.

A surprising number of reports of interaction of Mn(II) with a variety of important enzyme systems, involving activation of either catalysis or regulation, have appeared in the past decade. These systems are summarized in *Table 3.1*, and are striking because of the large number of key enzymes implicated, and also because certain categories or types of chemistry are conspicuous by being under-represented or totally absent.

Oxido-reductases

The limited number of redox enzymes reported to involve Mn(II) or Mn(III) directly in catalysis include catalase, a peroxidase, a hydroxylase, and

Table 3.1. MANGANESE-DEPENDENT ENZYME SYSTEMS

Category			*Enzyme/Substrate*	*Reference*
1	Oxido-Reductases			
	1.1	CHOH donor	isocitrate dehydrogenase	[25]
			6-P-gluconate dehydrogenase	[26]
	1.3	CH–CH donor	(NAD) methylene-THF dHase	[27]
	1.11	H_2O_2 acceptor	peroxidase	[28]
			catalase	[29]
	1.15	O_2^- acceptor	superoxide dismutase	[30,31]
	1.99	(other)	cholesterol-7α-hydroxylase	[32]
			vitamin K epoxidase	[33]
2	Transferases			
	2.1	1-C groups	–	
	2.2	CHO or CO groups	transketolase	[34]
	2.3	Acyl groups	monoacylglycerol acyltransf.	[35]
	2.4	Glycosyl groups	glucosyl, glycosyl	[36,37]
			galactosyl	[38–41]
			glucuronosyl	[42]
			GlcNAc	[43–45]
			NAM, silalyl	[46]
	2.5	Alkyl, Aryl	Met-adenosyl	[47]
			farnesyl	[48]
	2.6	Nitrogenous	–	
	2.7	P-Containing		
		1 OH acceptor	protein kinases	[49–56]
			(Tyr,Ser) protein kinases	[57–67]
			hexokinase	[68,69]
			P-inositol(-4-P) kinase	[70]
		2 nucleotidyl	DNA polymerase	[71–78]
			RNA polymerase	[79]
			reverse transcriptase	[80,81]
	2.8	S-containing	sulpho-	[82,83]
			glutathione	[84]
3	Hydrolases			
	3.1	Esters		
		1 carboxylate	mono-,di-,tri-glycerides	[85,86]
		3 P-monoesters	protein phosphatases	[87–107]
			P-inositol phosphatase	[108]
			5′-nucleosidase	[109]
			FBPase	[110–113]
		4 P-diesters	P-lipase C (inositide)	[114,115]
			cyclic nucleotide PDE	[116]
		11 exo-DNase	DNase (diphtheria toxin)	[117]
		13 exo-RNase	RNase (liver)	[118]
		30 endo-nuclease	endo-nuclease (mito.)	[119–121]

Table 3.1. (continued)

Category		*Enzyme/Substrate*	*Reference*
3.4	Peptide bonds	specific peptidases/proteases	[122–129]
		metallo-protease	[130]
		Leu aminopeptidase	[131]
		prolidase	[132–135]
		other	[136]
3.5	C–N bonds		
	1 linear amides	amino-acylase, amidase	[137–139]
		GlcNAc-6-P deactylase	[140]
	2 cyclic amides	methylene-THF cyclohydrolase	[141]
	3 amidines	arginase	[142–145]
3.6	Acid Anhydrides		
	1 P-containing	ATPase	[146–149]
	2 C-containing	–	
3.8	Halide bonds		
	1 C–X	–	
	2 P–X	Soman hydrolase	[150]
4 Lyases			
4.1	C–C bonds	PEP carboxykinase	[151–157]
4.2	C–O bonds	δ-aminolevulinate dehydratase	[158]
4.6	P–O bonds	adenylate cyclase	[159–168]
		guanylate cyclase	[169,170]
		nucleotide cyclase	[171]
5 Isomerases			
		(none)	
6 Ligases (synthetases)			
6.1	C–O bonds	AA-tRNA synthetases	[172]
6.3	C–N bonds	glutamine synthetase	[173–183]
		γ-Glu-Cys synthetase	[182]
6.4	C–C bonds	pyruvate carboxylase	[184]
		vit. K carboxylase	[33,185,186]
6.5	P-ester bonds	ATP synthase	[187]

manganese superoxide dismutase, all of which utilize one-electron redox reactions. Peroxidase, catalase, and SOD function to destroy or divert potentially damaging radical species and protect the cell from damage, for example, lipid peroxidation. Vitamin K epoxidase is involved in controlling the blood clotting cascade process involving thrombin-mediated hydrolysis of fibrinogen. With the two dehydrogenases cited, Mn(II) appears to play only a regulatory role rather than participating in catalysis. Nonetheless,

these two enzymes occupy key positions in carbohydrate catabolic pathways, at the entry points for the TCA cycle and the pentose-P pathways.

Transferases

A relatively large number have been reported to involve Mn(II) in catalysis. Those recognized for over two decades as dependent upon Mn(II) are the complex glycosyl-transferases involved in biosynthesis of the mucopolysaccharides of connective tissues and growth of long bones, based on manganese deficiency and nutritional studies [18]. Notably absent from the list are transferases involving 1-C or nitrogenous (amino) groups (which typically utilize THF, biotin, and cobalamin cofactors), except for one report of potential Mn(II)-activation with Met-adenosyl transferase [48]. Transferases involving phosphorus-containing substrates are strongly represented, notably protein kinases with varying degrees of specificity (many of which are implicated in abnormal or regulatory function), plus DNA and RNA polymerases (with which Mn(II) can alter the fidelity of replication [71–75]). A few sulpho-transferases involved in biosynthesis of sialic acid groups have also been reported.

Those protein kinases reported to be activated by Mn(II) are key systems, some of which are quite specific in their action. Brockenbrough and Korc [49] report Mn(II) activation of protein phosphorylation in the pancreas as a regulatory mechanism of carbohydrate metabolism [50], potentially through adenylate cyclase. Wente *et al.* [67] have reported specific Mn(II) interactions with a protein-tyrosine kinase derived from the human insulin receptor. Sacks *et al.* [64] reported Tyr-phosphorylation of calmodulin by the insulin receptor kinase. In addition, it appears that protein-Tyr phosphorylation in brain may be manganese-specific, as reflected in the following citations: Ellis *et al.* [60] described a synaptic protein-tyrosine kinase and identified some of its endogenous substrates. Huganir *et al.* [61] reported Mn(II)-specific phosphorylation of the nicotinic acetylcholine receptor by an endogenous Tyr-specific protein kinase. Neer and Lok [62] characterized a Mn(II)-specific pp60v-src-related Tyr-kinase from bovine brain, and Pang *et al.* [63] reported protein-Tyr phosphorylation in synaptic vesicles. Finally, Tsuzuki and Luftig [66] found that the endogenous protein kinase of adenovirus is markedly altered in its specificity by different divalent cations: Mg(II), Co(II), and Mn(II).

Two reports of a manganese-specific brain hexokinase [68,69] are of special interest since as this enzyme is normally considered magnesium-activated. Mn(H) is also reported to activate a phosphoinositide kinase, part

of a P-inositide recycling to IP_3, the key P-inositide of intracellular signalling as a second messenger via calcium-calmodulin. Interestingly, a recent report [65] indicates that non-enzymatic phosphorylation of protein Tyr and Ser residues by ATP is catalyzed by Mn(II) but not Mg(II).

Hydrolases

Within this diverse group of enzymes, those enzymes reported to be manganese-specific are amongst those most important for growth, cell regulation, and metabolic function. These include esterases (primarily of phosphate-containing substrates), peptidases, proteinases, amidases, and hydrolases of ATP and organo-phosphates. For metabolism of second messengers, the breakdown of cyclic-AMP is catalyzed by a cyclic nucleotide phosphodiesterase [116] and a 5'-nucleotidase [109], and the catabolism of IP_3 involves several phosphoinositol phosphatases [108], all manganese-specific systems. Manganese-specific effects on the phospholipase C in brain membranes (coupled with cholinergic- and adrenergic-receptor function) have been described [114,115]. In addition, hydrolysis of DNA and RNA by manganese-specific exo-nucleases from diphtheria toxin and liver plus several mitochondrial endo-nucleases has been reported [117–121]. In the glycolytic pathway in the brain, FBPase exhibits manganese-specific effects on both catalysis [110] and its regulation, particularly with regard to inhibition by F-2,6-bisP [111–113].

The regulation of protein phosphatases is intimately related to that of protein kinases, both being part of an overall cycle that acts as a switching mechanism to 'turn-on' or 'turn-off' certain enzyme activities. Within the past decade, at least 20 publications have described the involvement of Mn(II) with these systems. Most notably, these include the following protein-specific enzymes: phosphorylase phosphatase [87,104], Ca/calmodulin-dependent protein phosphatase [94,99,105–107], and calcineurin phosphatase [91,93,95], each being involved in intracellular signalling and metabolic regulation.

Specific peptidase and protease systems which involve Mn(II) include thrombin limited-proteolysis of prothrombin [122], insulin protease [123], enkephalin-degrading amino-peptidase [124], carnosinase [125,129], kininase [127], and trypsin activation [128]. A metallo(Mn)-protease is involved in the processing of mitochondrial precursor proteins [130]. Several aminopeptidases are also specifically manganese-dependent, namely Leu-aminopeptidase [131] and 'prolidase' or C-terminal proline dipeptidase [132–135]. Other systems that hydrolyze linear and cyclic C–N bonds include various amino-acylases, deacetylases, amidases and methylene-

THF cyclohydrolase. One of the few true manganese-containing enzymes is arginase [142–145], which co-isolates with Mn(II) bound, and is a crucial part of the urea cycle in the liver, breaking the amidine bond of arginine to yield urea and ornithine.

ATPases participate directly in various transport and motile functions. Of significance for this review is a manganese-specific ATPase found in rat brain [146], and Mn(II) which interacts specifically with other membrane-bound ATPases from brain and heart cells, including phospholipid-dependent ATPase activity exhibited by protein kinase C [147–149]. Interestingly, hydrolysis of the cholinesterase-inhibitor Soman (isopropyl methyl-phosphonyl fluoride) is catalyzed by a manganese-dependent enzyme isolated from clonal neuroblastoma cells [150].

Lyases

The number of different lyases reported to interact with or utilize Mn(II) are relatively few. Nevertheless, those that do are important in cell metabolism and regulation. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the reaction:

$$\text{NTP} + \text{oxalacetate} \rightarrow \text{NDP} + \text{PEP} + CO_2,$$

a reaction involving attack of the 2-keto oxygen of OAA on the γ-P of NTP, a process made energetically favourable by the decarboxylation of OAA, leading to PEP rather than pyruvate. This enzyme, catalyzing a key and unique step in the reversal of glycolysis (gluconeogenesis), was proposed some years ago [151] to be activated by Mn(II), but only recently have data become available to verify this hypothesis [152–157].

The other class of lyases that utilize Mn(II) in specific cells or tissues are the adenylate and guanylate cyclases [159–170]. These are used as 'second messengers' from receptors to activate intracellular enzymes involved with carbohydrate metabolism or other functions. These manganese-activated enzymes are found in white and brown adipose tissue [159], the caudate nucleus [160], renal tissue [162], rat brain [161,165,167], and neuroblastoma cells [168]. Mn(II) is further implicated in adenylate cyclase activation by glucagon [163] and the inhibition of forskolin-activated enzyme by GpNpp [164]. Mn(II) interactions are also reported for guanylate cyclase isolated from bovine rod outer segments [169] and from spermatozoan membranes [170]. A managanese-dependent nucleotide cyclase from calf lymphocytes has also been described [171].

Isomerases

No reports of Mn(II)–isomerase interactions have been found.

Ligases (synthetases)

These enzymes catalyze bond-forming reactions which are essential for biosynthesis. They are most often driven by lysis of ATP, bound with a divalent metal ion, most commonly Mg(II). An alanyl-tRNA synthetase has been found to exhibit Mn(II) interactions [172].

One of the most important enzymes in nitrogen metabolism is glutamine synthetase, studied 25 years ago in terms of its interaction with Mg(II), Mn(II), and Co(II) ions [173]. The enzyme present in liver and kidneys is feedback-inhibited by end-product metabolites derived from glutamine–carbamyl-P, Ala, His, Ser, AMP. In contrast, the brain enzyme lacks any response to physiological levels of these compounds [174]. This has led to the hypothesis that brain GS (compartmentalized in glial cells) serves to remove toxic ammonia and the neurotransmitter L-Glu, and may be regulated by brain-specific signals rather than those related to N-metabolism. Evidence that brain GS binds Mn(II) to any significant extent under in vivo conditions is inconclusive. The current data may be summarized as follows: the K_d for Mn(II) is 1-10μM [175–177], which approximates the concentration determined for free Mn(II) in the cytosol using kinetic [179] and ESR methods [unpublished results, this laboratory]. *In vitro,* the enzyme is activated by 1-10μM Mn(II) in the presence of 5mM Mg(II) [175]. Attempts to trap the preferred divalent cation in the active site under *in vivo* conditions were made by breaking bovine brain cells into a buffer solution containing the transition-state and suicide inhibitor, MSOX, plus ATP, leading to the inactive E–MSOXP–ADP complex with an entrapped metal ion. Unfortunately, in these first experiments the external buffer contained no divalent metal ions, that is, there was a large dilution of intra-cellular metals upon cell breakage, resulting in only the most abundant metal ion present, Mg(II), being trapped. This experiment has been repeated with 5μM ^{54}Mn-labelled Mn(II) and 5mM Mg(II) in the external buffer along with MSOX and ATP; under these conditions 30–40% of the MSOX-inactivated enzyme was found to contain bound Mn(II) [unpublished results, this laboratory]. Meister and coworkers [182] have observed that the selective reactivity of various structural analogues of MSOX with GS versus γ-glutamyl-cysteine synthetase [182] is controlled by Mn(II) to a greater extent than by Mg(II). Mn(II) activation of GS from bovine retina has also been reported [183]. Overall, the data indicate that Mn(II) or the

ratio of Mn(II)/Mg(II) [181] probably plays a significant role in the regulation of brain glutamine synthetase. The next challenge is to discover which cell signals alter cytosolic Mn(II) levels, leading in turn to altered enzyme activity.

Scrutton has reviewed the properties of pyruvate carboxylase [184], one of the few systems with an affinity for Mn(II) sufficiently high for it to be called a manganese-containing enzyme. The only carboxylase reported since 1984 to involve Mn(II) has a role in the complex blood-clotting cascade: vitamin K-dependent (glutamate)prothrombin carboxylase (factor Xa), which creates Ca(II) binding sites by formation of γ-carboxy-glutamyl (Gla) side-chain groups on prothrombin, leading its activation to thrombin [185,186].

Finally, Ernster and coworkers [187] report that Mn(II) prevents Ca(II)-inhibition of ATP synthase in brain mitochondria, adding evidence that a disproportionate number of brain enzymes may be manganese rather than magnesium-specific.

NEUROTRANSMITTERS

General effects

Manganese at micromolar concentrations has long been recognized as essential for the proper development and normal function of the nervous system [1,5,8,188]. Recent reports add to the already abundant knowledge of the role of Mn(II) in neurochemistry of the whole organism. A deficiency or an excess of Mn(II) causes severe neurotoxic developmental and functional effects [188–190]. Exposure to abnormal levels of Mn(II) in the developmental stages of life may cause the mature animal to exhibit ataxia or 'startle' responses [191], susceptibility to seizures or epilepsy [192–194]. In these reports most of the effects have been shown to correlate with or interrelate to levels of neurotransmitters or hormones, specific binding to receptors, abnormal diet, or stress [195–198].

Cellular effects

The uptake and release of neurotransmitters is linked to Mn(II) in a number of reports [199–207], some of which consider Mn(II) to be a 'heavy metal' along with Pb(II) or Cd(II). Chandra *et al.* [200], Hussain *et al.* [201], and Lista *et al.* [202] have described manganese-altered ATPase activity, related to the uptake of dopamine and noradrenaline in rat synaptosomes or striatal tissue. Corcoran and Kirshner [203] reported manganese-induced

changes in Ca(II) uptake and catecholamine secretion by cultured adrenal medulla cells while Drapeau and Nachshen [204] described manganese-dependent neurotransmitter release in pre-synaptic nerve endings of rat brain. Mn(II) levels in different regions of neonatal rats have been studied in relation to altered monoamine metabolism [205]. Other workers [206,207] have reported the inter-relationship of manganese-neurotoxicity and treatment with L-DOPA or dopamine.

The action of Mn(II) on receptors and their responses to ligands is becoming increasingly well-documented. Bumgarner *et al.* [208] describe Mn(II) mediation of the A_1-adenosine receptor and adenylate cyclase response to thyroid status in adipocytes while Chambaut-Guerin and Thomopoulos [209] point to Mn(II) participation in forskolin inhibition of cAMP in macrophages. The interaction of Mn(II) with ionic channels that respond to excitory amino acids in hippocampal neurons has been described by Yamamoto and Sato [210]. Vesely [211] reported the Mn(II)-dependent activation of guanylate cyclase by gonadotropin releasing hormone. In terms of intracellular messengers, Mn(II) causes changes in the internal Ca(II) levels and in the Na–Ca exchange fluxes in axons and neuroblastoma cells [212,213], as well as in the incorporation of inositol–P into IP_3 in brain hippocampal cells [213–215].

Other general cellular responses to Mn(II) include a description by Budzik *et al.* [216] of Mn(II) participation in the action of Mullerian-inhibiting substance, and the role of Mn(II) in osteosarcoma cell attachment to fibrinogen and von Willebrand factor, mediated by the fibronectin, vitronectin, and adhesion receptors [217].

Receptors

A summary of those receptors—other than those cited in the previous section—reported since 1984 to exhibit changes or a dependence in their function due to interaction with Mn(II) are presented in *Table 3.2*. The role of Mn(II) occurs at several levels: repeated administration of relatively high Mn(II) results in altered expression and properties of cholinergic and dopaminergic receptors in rat striatal tissue [219,223] and in different forms of the opioid receptor in rat brain [242]. At normal physiological (trace) levels, however, the roles of Mn(II) have been found to: (1) alter the specificity of the receptor binding site [244]; (2) interact with adenylate and guanylate cyclases [231,232,246] as well as specific protein kinases and phosphatases [220,226,228,230] which regulate the activity of the receptor or of intracellular enzymes (see discussion of these enzymes above); (3) stabilize the agonist- versus antagonist-specific form of the receptor

Table 3.2. RECEPTORS REPORTED TO INTERACT WITH Mn(II)

Receptor	*Reference*
Acetylcholine	[218,219]
Adrenergic	[220–222]
Cholinergic	[223,224]
Dopaminergic	[223]
Fibronectin	[225]
Insulin	[226–230]
Muscarinic	[231–234]
Mannose-6-P	[235]
Opioid (δ, κ, μ)	[236–243]
Oxytocin	[244]
Platelet GPIIB/IIIa	[245]
Serotonin	[246]
Tachykinin	[247,248]
Vasopressin	[249]

[234,237,238,247,249], or (4) stabilize specific interactions between receptor subunits [239,245].

Molecular-level effects

Firstly, direct binding with, and interaction of, Mn(II) with L-DOPA [250] allows radical-promoted oxidation of L-DOPA and dopamine [251–253]. This may explain in part the Parkinson-like effects of Mn(II) poisoning [14]. Mn(II) also alters the enzyme-mediated metabolism of myo-inositol in synaptosomes [254]. An intricate interplay between calmodulin and neurotransmitter regulation of neuronal adenylate cyclase, activated by Mn(II), has likewise been reported [255]. Both a novel insulin-stimulated protein kinase from liver [256] and a human EGF receptor (Tyr)protein kinase [257] are Mn(II)-activated. The interaction of human neutrophil guanylate cyclase with the muscarinic cholinergic receptor is regulated by Mn(II) [258]. In the brain, dihydropyridine antagonist binding sites are modified by interactions with metal ions, particularly Mn(II) [259], while phorbol ester binding and protein kinase C activity are inhibited by Mn(II) [260]. Finally, the methylation of calcineurin by protein carboxyl-*O*-methyltransferase and its effect on calmodulin-stimulated phosphatase activity is a managanese-mediated process [261].

REDOX PROCESSES

In addition to the manganese-catalyzed oxidation of catecholamines cited in the previous section [252,253,262], manganese ions participate in radical redox reactions. Amongst these, Mn(II) has been reported to protect against cell damage, while the Mn(II)-Mn(III) redox couple promotes radical (oxidative) damage to cell components. A well-recognized manganese-containing system that protects against superoxide radical damage is mitochondrial manganese-superoxide dismutase [263]. The superoxide radical, despite being a weak oxidant and weak nucleophile, may cause lipid peroxidation and protein oxidations, either directly or via the formation of the more reactive hydroxyl radical [264–266]. Even Mn(II), free in solution, is reported to scavenge radicals produced either by metabolic reactions (especially those involving NAD(P)H) [267–271], chemotherapeutic agents [272], or ionizing radiation [273]. Mn(II), bound to and transported to various tissues by metallothionine, may offer some protection against radical damage [274,275].

Within the past few years, the Stadtman group at NIH has provided an elegant elucidation of the chemistry of Mn(II)-catalyzed radical reactions that lead to catalase-like disproportionation and dismutation of hydrogen peroxide [276–278]. The key complex involves inner-sphere coordination of 3 HCO_3 moieties per Mn(II) ion, which catalyzes the following inter-related redox reactions:

$$O_2^{\cdot-} + \text{Mn(II)} \rightarrow H_2O_2 + \text{Mn(III)} \rightarrow O_2 + \text{Mn(II)}$$
$$O_2^{\cdot-} + \text{Mn(III)} \rightarrow O_2 + \text{Mn(II)}$$

It has also been demonstrated that Mn(II) and Fe(II) catalyze Fenton-like reactions with amino acids, involving inner-sphere bi-dentate coordination of the amino acid, two HCO_3^- moieties per Mn(II), and H_2O_2, leading to radical extraction of the α-H as a primary step, and proceeding through an AA–NHO intermediate, to yield an α-keto acid, an aldehyde, or a carboxylic acid plus carbon dioxide, depending on the conditions and stoichiometry of reactants. Mn(II)- and Fe(II)-promoted oxidative damage to specific residues in proteins as precursor steps that trigger proteolytic degradation and protein turnover have been identified [279] and studied in detail for glutamine synthetase [280,281]. Similar chemistry may explain the Mn(II)-catalyzed oxidation of catecholamines [202,206]. For an overall review of other aspects of Mn(II) chemistry, the reader is referred to the chapter by Reed [282].

DNA, RNA INTERACTIONS

A number of diverse roles for Mn(II) interacting with DNA and RNA have been delineated in recent years. Firstly, Kawanishi and Yamamoto showed that copper and manganese cause site-specific damage in the presence of hydrazine compounds [283,284]. The direct interaction of Mn(II) with DNA has been studied by various biophysical methods, to detect conformational changes and to identify ligand groups in the metal environment [285–287]. Dange *et al.* reported a Mn(II)-dependent ribozyme [288]. In the absence of any enzyme, Mn(II) alone can catalyze pre-biotic DNA synthesis, the oligomerization of nucleotide analogues [289].

Specific roles for Mn(II) in RNA and DNA polymerases plus reverse transcriptase [71–81], in addition to DNase and RNase [117–121] have been cited and discussed above. Additional roles in which the Mn(II)–nucleic acid interaction appears to be important are noteworthy. El Deiry *et al.* [290] discussed the molecular mechanism of mutagenesis by Mn(II) via its interaction with DNA and DNA polymerase I during replication. Zhang-Keck *et al.* [291] reported a role for Mn(II) in specifying the start site for chromatin transcription. Both specificity and fidelity of transcription appear to differ between Mn(II) and other divalent cations [292–296], in addition to altering the kinetics of the reverse transcriptase reaction [297] and the processing and integration of viral DNA ends [298]. Finally, Gross and Kaplansky observed a differential effect of Mn(II) on a translational repressor and the double-stranded RNA-activated inhibitor [299].

ROLE OF MN(II) IN METABOLISM

At normal levels, in deficiency and in excess, Mn(II) exerts specific effects on the metabolism of all major classes of biomolecules [300,301].

Carbohydrates

The effect of manganese deficiency or excess on carbohydrate homeostasis have been studied by Keen, Hurley and coworkers, with some focus on pyruvate carboxylase and PEP carboxykinase [302–304]. Other evidence indicates that Mn(II) has an 'insulin-mimetic' effect, acting to stimulate protein kinases or phosphatases that control enzymes involved in glycolysis, gluconeogenesis, or the hexose mono-P pathway [305–307]. A key role of Mn(II) in mucopolysaccharide metabolism has long been recognized [6].

Lipids

There is an inter-relationship between the levels of Mn(II) present in mammals and choleostasis, since a primary mode of Mn(II) excretion is via the manganese–bilirubin complex [308–312]. This interplay has implications for the prevention of atherosclerosis and other pathologies related to cholesterol [313,314]. Indeed, manganese-deficient rats exhibited altered high-density lipo-protein (HDL) compositions [315], and genetically obese mice which showed abnormal tissue levels of trace metals, responded favourably to dietary supplementation with Mn(II) [316,317]. There is also a Mn(II)-dependence of phosphatidyl-inositol synthesis in various tissues—see also section above [318–320]. Lipid synthesis in myelin- deficient disease states [321] and the transport of choline in erythrocytes [322] exhibit dependence on Mn(II). Likewise, Shidoji *et al.* [323] reported on the interactions between Mn(II) and other divalent ions and retinyl- phosphate, while Zidenberg-Cherr *et al.* [324] indicated a relationship between manganese deficiency, lipid peroxidation (prevented by manganese–superoxide dismutase), and developmental problems.

Proteins

Intake levels of Mn(II) affect the content of pancreatic digestive enzymes [325–329], of free polysomal proteins in the brain [330,331], of brain monoamine oxidase when administered over several generations in mice [332], and of connective tissue proteins [333], as well as the rate of protein turnover in skeletal muscle [334].

Miscellaneous effects

Gutteridge and Bannister [335] found that manganese–superoxide dismutase inhibits the degradation of deoxyribose while Qato and Maines [336] reported that drug metabolism activities in the brain are regulated by Mn(II). Later, Mn(II) was found to induce selective vulnerability in glutathione metabolism and cellular defence mechanisms in rat striatum [337].

CELLULAR EFFECTS

TRANSPORT

Milne *et al.* [338] have recently determined the manganese content of the

cellular components of blood, while Scheuhammer and Cherian had earlier studied the binding of Mn(II) in mammalian plasma [339]. Interactions of Mn(II) and other divalent ions with alpha-lactalbumins have been described by Segawa and Sugai [340], while Lau and Sarkar [341] studied the components in human sera and spinal cord that bind a similar array of divalent metal ions. The uptake of Mn(II) into erythrocytes, accompanied by a thermal transition was investigated by Morariu *et al.* [342]; Gunther and coworkers [343,344] described the induction of a Mn(II)/H(I) and a Mn(II)/Mg(II) antiport in erythrocytes. Merritt *et al.* [345] used Mn(II) to discriminate between Ca(II) influx and mobilization from internal stores in stimulated human neutrophils, while Gelfand and Cheung [346] found that the uni-directional influx versus release of Ca(II) from internal stores may be separated or dissociated in activated lymphocytes.

Mn(II) transport has been studied in several tissues. Schramm and Brandt [347] used hepatocytes to study Mn(II) uptake, efflux and distribution, and found that transport across the plasma membrane involves a facilitated diffusion mechanism, with a number of tight binding sites for Mn(II) in the cell cytoplasm, followed by active transport (probably via the Ca-uniporter system) into mitochondria. The distribution of Mn(II) into cytoplasm and mitochondria was about 40% and 60%, respectively. Kass *et al.* [348] have described the use of Mn(II) to identify and characterize receptor-operated Ca(II) influx in rat hepatocytes. A series of papers on cultured neuronal and glial cells by Tholey, Ledig, Wedler, and coworkers [349–351] have elucidated the dynamics and distribution of Mn(II) in the CNS, using cultured chick and mammalian (rat) cerebral cells. Ashner *et al.* [352] reported similar studies on uptake and efflux of Mn(II) by cultured rat glia, while Valois and Webster [353] described the distribution and retention of Mn(II) in mouse brain *in vivo*. Overall, Mn(II) transport and distribution in chick and rat glial cells are similar, but with some important differences: the apparent K_m for Mn(II) uptake is 0.3μM for rat cells compared to 18μM for chick cells; the intracellular distribution resembles that hepatocytes [347], that is, 30–40% in cytoplasm and 60–70% in mitochondria.

Of particular significance, is the fact that both Ca(II) and K(I) ions (important signalling molecules in the CNS) modulate the rate and/or the extent of Mn(II) uptake in glia to a greater extent than in neurons [349–351]. This is the first molecular level regulatory mechanism reported for cytoplasmic Mn(II)-dependent enzymes, such as glutamine synthetase, in glia. Mn(II) also protects against free radical damage by ethanol metabolism, especially in cultured mammalian glia, to a greater extent than with neurons.[271]

Ernster and coworkers [354] and Gavin *et al.* [355] have investigated

Mn(II) and Ca(II) uptake and efflux kinetics in brain mitochondria. The results indicate that Ca(II) accelerates both Mn(II) uptake and release, but Mn(II) inhibits Ca(II) uptake and release. Na(I)-induced release of Ca(II) was inhibited by Mn(II), but the Na(I)-induced release of Mn(II) was not accelerated by Ca(II). These results suggest that altering the active transport of Mn(II) into the mitochondrial pool—in response to changes in extracellular and cytoplasmic Ca(II), Na(I), and K(I)—may be used to rapidly alter the Mn(II) concentration in the cytoplasm, hence regulating cytoplasmic enzymes that respond to Mn(II). The nature of the Ca(II)/Mn(II) mitochondrial transport system has also been discussed by Allshire *et al.* [356] and Anderson [357] while the transport and exchange of Mn(II) in heart and endothelial cells have been investigated by Hallam *et al.* [358], Jacob *et al* [359], and Rehnberg *et al.* [360].

NERVOUS SYSTEM

The transport of Mn(II) across the blood brain barrier has been investigated by Aschner and Aschner [361], and found to be related to Mn(II) binding to transferrin and iron homeostasis. These authors proposed that manganese neurotoxicity is due primarily to metal accumulation in the mitochondria, with perturbation of the intra-cellular Ca(II) homeostasis [362]. Certainly, our current knowledge of Mn(II) dynamics in the CNS indicates that the rate-limiting steps are movement across the BBB (with relatively more rapid flux into neurons and glia) and slow efflux from mitochondria. The evidence for direct Mn(II) activation of intracellular signalling enzymes (kinases and phosphatases) as well as oxidation of catecholamines, detailed above, indicates that changes in Ca homeostasis cannot be the only effect of elevated manganese in the CNS. Other reviews of the influence and toxic effects of Mn(II) and other metal ions on the nervous system have appeared in the past, some offering excellent historical perspectives to this problem [363–368].

One of the primary effects of Mn(II) deficiency is the increased susceptibility to convulsions or seizures which occurs in both ataxic and non-ataxic animals [369]. More recently, there has been increasing evidence that blood levels of Mn(II) are lower in epileptic than in normal patients [370–375]. One hypothesis for the biochemical basis of this effect in the CNS is that lower Mn(II) concentrations in the glial cytoplasm lowers glutamine synthetase activity, which leads to higher extracellular levels of the excitory L-glutamate. This results in a lower firing threshold for neurons with Glu-activated receptors [177]. Other effects of Mn(II) on the nervous system include changes in the behaviour of cardio-pulmonary nerves [376], effects

on motor neuron membrane potentials [377], and changes in Mn(II) metabolism in isolated presynaptic nerve terminals [378].

HORMONES

One of the best-documented effects of Mn(II) on hormonal levels involves insulin, glucagon and carbohydrate metabolism, the levels of Mn(II) having a direct influence on the biosynthesis and release of these hormones [379–383]. Manganese-deficient rats failed to make sufficient insulin to respond to glucose challenge, and degraded endogenous insulin at a more rapid rate. Mn(II) injections typically induced glucagon release and an increase in blood glucose concentratation [384]. Bond *et al.* [144] observed elevated levels of both Mn(II) and arginase activity in diabetic mouse liver, the latter effect being due to altered enzyme specific activity rather than increased enzyme protein.

Hormonal regulation of Mn(II) metabolism has been reviewed by Failla [385]. Published results since that time include the following: the production of prostaglandins in hepatocytes [386] and regulation of prostacyclin synthesis in aortic endothelial cells [387], hypercorticism and manganese metabolism in brown adipose tissue [388], Mn(II) as a goitrogen [389], involvement in Ca(II) control of prolactin secretion [390,391], release of noradrenaline from the pulmonary artery [392], Mn(II)-dependent activation of guanylate cyclase by gonadotropin releasing hormone [393], rapid changes in oestrogen binding elicited by cGMP and cAMP in human endometrial cells [394], and the effects of Mn(II) on oxytocin analogues towards receptors on myometrial and mammary gland membranes [395].

INTERACTIONS WITH OTHER METALS

Mn(II) and Ca(II) are transported into mitochondria by the same system [396], which alone necessitates some interaction of these two metal ions. However, even at this single site, their interaction is more complex than might be anticipated. The studies of Ernster and coworkers [354] showed that Ca(II) accelerates Mn(II) flux but Mn(II) inhibits Ca(II) flux, and Na(I)-induced release of Ca(II) is inhibited by Mn(II) but the release of Mn(II) is not accelerated by Ca(II). Micromolar concentrations (trace, physiological) of Mn(II) ions modulate Ca(II)-cycling in mitochondria [397], so that Mn(II) levels are elevated in calcium-deficient animals [398]. It has also been observed that Mg(II) and Mn(II) modulate Ca(II) uptake into sarcoplasmic reticulum [399], and conversely, that Mn(II) transport and utilization is altered by Ca(II) and Mg(II) [400]. In smooth muscle, Mn(II)

alters Ca(II) mobilization and the contractile response [401], possibly by blocking the calcium-receptor for calcium reversal [402]. It has also been found that Quin 2 and Mn(II) alter cellular calcium homeostasis in the diabetic pancreas [403]. Furthermore, supraoptic neurons sustain high frequency firing when extracellular Ca(II) is replaced by Mn(II) or other divalent cations [404].

Considering next the interaction of Mn(II) with other transition metal ions, Kies and Harms [405] indicated that Cu(II) absorption is affected by supplemental Mn(II), and Pattison and Cousins [406] found that Mn(II) may alter Zn(II) uptake and exchange in cultured hepatocytes. It has also been found that severe Zn(II) deficiency leads to a redistribution of nine elements, including Mn(II), in rat brain [407]. Wedler and Ley [408] have elucidated the effects of Zn(II) and Cu(II) on the dynamics and distribution of Mn(II) in glial cells: Zn(II) inhibits the initial rate of Mn(II) uptake but enhances the extent of Mn(II) accumulation by mitochondria without altering the rate or extent of Mn(II) efflux, whereas Cu(II) increases both the rate and extent of Mn(II) uptake with no effects on its efflux. These results suggest that Zn(II) and Cu(II), by modulating cytoplasmic Mn(II) levels, might indirectly regulate the activity of Mn-sensitive enzymes. On a nutritional basis, Mn(II) and Zn(II) competitively inhibits the absorption of Fe(III) [409], and chronic exposure to Mn(II) causes changes in the tissue distribution of other essential trace elements such as magnesium, zinc, iron and copper [410].

Another area of importance is related to the heavy metals such as lead, cadmium and mercury. Sarhan *et al.* [411] and Gruden [412] studied the interaction and absorption of Mn(II) and Cd(II) in the intestinal tract, and Kostial *et al.* [413] investigated the effects of a metal ion mixture on the toxico-kinetics of cadmium, mercury and manganese in rats. Using mouse pre-implantation zygotes, Yu and Chan [414] investigated the interaction of manganese, zinc and calcium ions on cadmium toxicity. Shukla and Chandra [415] studied the distribution of lead, manganese and cadmium in various tissues upon concurrent exposure of growing rats while Goering and Klaassen [416] discovered that Mn(II) induces a tolerance to Cd(II) lethality and hepato-toxicity. The effect of Mn(II) on Cd(II) binding to tissue proteins *in vitro* [417] and the influence of dietary Mg(II) and Mn(II), on physiological responses to high dietary Al(III) ions, have also been investigated [418].

MISCELLANEOUS EFFECTS

There is evidence that Mn(II) and Mg(II) exchange on a 1:1 basis in

erythrocytes [419], and that in brush-border receptor-mediated hydrolysis of lactoferrin, the Fe-saturation alters Mn(II) transport and uptake [420]. In liver, the plasma membrane Ca(II)-inflow system has been found to exhibit broad specificity for divalent metal ions [421]. Tissue-specific distribution of Mn(II) may be altered by abnormal states, such as renal failure or transplantation [422], genetic mutations [423], or non-cerebral neoplasms [424]. Certain manganese-specific enzymes are of cellular importance: Mn(II) binding with diphtheria toxin, which catalyzes the inactivation of eEF-2 by ADP-ribosylation [425]; effects of Mn(II) on factor Xa in the blood clotting cascade [426], and the neuromelanogenic/cytotoxic properties of brainstem peroxidase [427].

Manganese-deficiency has been reported to alter mitochondrial structure [428]. Mn(II) inhibits the replenishment of cell Ca(II) required for secretion of histamine by mast cells [429], and to alter the interferon system [430]. Mn(II) also affects the trans-bilayer movement of phosphoglycerides and Ca(II) [431], and influences the progressive motility of human sperm [432]. Mn(II) is likewise implicated in the aggregation of human platelets [433], in fibroblast spreading [434], and cell-substratum adhesion [435].

ORGANISMAL EFFECTS

NUTRITIONAL ASPECTS

This continues to be an active research area, as evidenced by the book on infant nutrition by Chandra [15], and reviews on bioavailability in vivo and *in vitro* [436] and the hormonal regulation of manganese metabolism [437]. Other aspects covered include bioavailability [438], dietary interactions [439], general nutrition [440], and growth rate and metabolism [441].

Since Mn(II) is so essential for prenatal and neonatal development and maturation, much effort has been expended on understanding the mechanism of Mn(II) absorption from milk via the intestinal tract [442–449]. Studies of bio-availability have focused on identifying the manganese-binding proteins in milk, how they interact with the intestinal mucosa, and how these proteins differ in milk obtained from different species and infant formulae. Lactoferrin is the major manganese-binding protein in milk, plus several other managanese proteins, including alpha-lactalbumin. Lonnerdal [450] has identified dietary factors that affect manganese bioavailability.

An understanding of how manganese is transported in the blood to various organs is also required. Kalia *et al.* [451] studied Mn(II) binding to

serum proteins, while Milne *et al.* [452] determined the manganese content of the cellular components of blood. Davidsson *et al.* [453] claim to have identified transferrin as the major manganese-carrier protein in plasma. Others [454] point to a connection between dietary intake during infancy and levels of the metal in plasma and erythrocytes in the mature animal. Evidence that maturational differences in manganese intestinal uptake fail to explain why infant mammals retain the element to a greater extent than adults has been provided by Bell *et al.* [455]. Differences in serum manganese for milk and formula-fed infants have been observed by Craig [456].

Distribution, retention and turnover (excretion) of manganese is controlled by uptake and release rates. These in turn are controlled by the internal binding and transport, being dependent on the cells and organs under investigation. This is a complex process with large differences existing between the same tissues from different species of mammal. Schramm and Brandt [347] have determined the 'manganese economy' of hepatocytes. Adding complexity to the picture, there are also strongly interdependent effects with other nutritional elements such as Ca(II), phosphorus, Cu(II), and Zn(II) [457]. Greger *et al.* [458] examined the relationship between intake, serum concentrations, and urinary excretion of manganese by adult males, while Ballatori *et al.* [459] studied homeostatic control of its excretion. Others [460] found manganese turnover to be affected by excess phosphorus consumption, with calcium-deficient rats exhibiting elevated manganese concentrations in the brain[461]. An overview of its absorption and metabolism in man has been provided by Sandstrom *et al.* [462].

Few tissue or cell types have been subjected to in-depth analysis. Sakurai *et al.* [463] used ESR and neutron activation analysis to determine how Mn(II) and total manganese are partitioned into the organs and subcellular organelles of rats while others [464] have used autoradiography to determine the accumulation and retention of the element in pancreas. Aschner and Aschner [352], Tholey *et al.* [349–351], and Wedler *et al.* [180] have all determined the dynamics and distribution of Mn(II) in neuronal and glial cells of the CNS. Gianutsos *et al.* [465] have shown that the systemic administration of manganese in different forms leads to different levels of accumulation within the CNS. Links between trace metals and immune function in the elderly have also been explored [466].

Strong correlations with the metabolism of bio-molecules and manganese nutrition have been elucidated[467]. Studies by Johnson and coworkers [468,469] showed a connection between both dietary protein and cholesterol on manganese bioavailability, while Wapnir [470] has recently reviewed the area of protein-manganese co-nutrition. Similarly, Holbrook *et al.* [471]

reported that fructose and starch intake levels alter manganese balances in humans.

The effects of long-term manganese deficiencies on bone structure and resorption of bone particles has been investigated by Strause and coworkers [472,473]. Furthermore, dietary levels of the element have been reported to alter the utilization of selenium [474].

DEVELOPMENT AND MATURATION

Manganese at either deficient or abnormally high (toxic) levels can cause serious problems in pre- and post-natal development—effects which have been extensively documented [1,6,8,14,18,25]. Reviews include those by Favier and Ruffieux [475] on physiological variations in serum levels of trace elements, by Hurley *et al.* [476] and Roy and Loh [477] on trace element interactions during development, by Campbell [478] on trace element needs during pregnancy, and by Lee *et al.* [479] on the effects of age and gender on manganese metabolism.

One of the problems first noted as arising from manganese deficiency in animals was abnormal long bone development. This was due to retarded activity of glycosyl-transferases which are essential for skeletal development [6,480]. Developmental and geriatric aspects of this phenomenon have been reported by Bolze *et al.* [481] and Sylvester and Barlow [482]. Changes in otoconia with genetic factors and manganese concentrations have also been reported [483]. Effects of manganese on sexual development, together with reproductive toxicology, were initially noted over 50 years ago [484], and confirmed recently by Webster and Valois [485].

The other major area of manganese involvement in developmental processes is the nervous system [14]. A number of authors [486–488] have provided details on the effects of the metal on brain development. Evidence supports a role for excess Mn(II) in inhibiting neurite extension and formation of neuromuscular synapses [489] and for normal Mn(II) levels to be involved in the development of action potentials in explanted cortical neurons [490]. Collipp *et al.* [491] have reviewed the relationship between manganese in infant formulae and learning disabilities. In the ageing process, some correlation has been found between changes in brain concentrations of Mn(II) and Alzheimer's disease [492].

Since protection against radical damage during the rapid metabolic rates encountered in development and maturation is almost certainly critical, several workers have focused on the subcellular localization and distribution of enzymes that serve this purpose, especially in the brain. Those determined by Del Maestro and McDonald [493,494] include superoxide

dismutase, glutathione peroxidase, and catalase, while Mariucci *et al.* [495] found differential changes between copper/zinc and manganese superoxide dismutase in the developing brain and liver. Abnormal metabolic states in the mother, such as diabetes, alters Mn(II) levels, resulting in retarded foetal growth and congenital malformations [496].

TOXICOLOGY

Since the mid-1980's [497], excellent reviews on the toxicology of manganese have appeared [282,498–506]. Manganese is the tenth most abundant element and has perhaps the largest number of chemically accessible oxidation states (six or more). Manganese can be taken into the human system in various forms and by a variety of routes. The most commonly reported causes of poisoning are miners who breathe dust from manganese-rich ore, welders breathing aerosol from manganese-containing metals, and the ingestion of foods contaminated or overburdened with unusual levels of manganese, such as drinking water, fish and vegetables, from the litho-sphere [507]. The literature is still rife with anecdotal accounts of such instances. Recent use of a manganese anti-knock compound in gasoline, MMT (methyl cyclopentadienyl-manganese-tricarbonyl), has been reported to cause increased problems in heavily air-polluted areas [508–510]. A new addition to the list is a street drug called 'bazooka,' which is cocaine contaminated with manganese-carbonate from free-base preparation methods [511].

The primary acute effects of manganese overload (intoxication) are manifested by dysfunction of the central and peripheral nervous systems [512,513], with symptoms that resemble Parkinson's disease and/or psychosis (schizophrenia): muscular rigidity, tremor, ataxia, flat affect, and hallucinations [14, 514–522] Damage to both white and grey matter of the CNS [523–525], as well as to motor neurons [526], has been documented. Chronic exposure to excess manganese (pre-conception, post-conception, and post-natal), by inhalation or other modes, causes developmental problems [527,528], involving especially the nervous system and muco-polysaccharide synthesis. Further studies on the metabolic fate and distribution of orally administered Mn(II) have been described [529].

The biochemical basis for many of the psychotropic and neurological effects of manganese intoxication are increasingly well-documented in terms of altered neuro-transmitters, receptors, and enzyme activities [530]. Alessio *et al.* [531] have documented the interference of Mn(II) with the neuro-endocrinal system. Research by Bonilla and Prasad [532] and Eriksson *et al.* [533] indicate changes in levels of biogenic amines in discrete

regions of the brain. Kosicka *et al.* [534] found altered glutamate decarboxylase and GABA levels in rats with extrapyramidal syndrome following acute manganese poisoning.

Changes in other organs, cells and organelles due to intoxication are now known, including histochemical changes in the pancreas [535], altered mixed function oxidase (MFO) in hepatic microsomes [536], changes in humoral and cellular immunity [537]—possibly due to chromosomal aberrations [538]—and in natural killer cells [539].

Correlations exist between manganese toxicity and exposure to other toxic substances in the environment, such as ozone [540–542] against which the element offers some protection against radical oxidative damage, or susceptibility to chemically induced cancer [543], cadmium [544,545], other trace elements [546], and alcohol [547]. Lysosomes in the brain have been found to play a role in manganese toxicity [548], and mechanisms for pulmonary clearance of soluble and insoluble forms of the element manganese have been discussed by Drown *et al.* [549].

DISEASE STATES

Fitzgerald and Tierney [550] and Korc [551] have reviewed the roles of trace metals in human disease. A summary of recent citations of disorders or disease states associated with manganese deficiency or poisoning, either as a cause or an effect are presented in *Table 3.3*. The molecular basis for a number of these can be understood from the discussions above regarding the effects of Mn(II) on specific enzymes or metabolic pathways, either in terms of catalysis or mode of regulation. This list reflects the scope and depth of present investigations and the increasing recognition that manganese and other trace metals may be crucially involved in regulating specific metabolic functions.

PHARMACOLOGY

Manganese has been related to the action of pharmacological agents, both in augmenting and antagonizing the effects of these agents. What follows is a summary of recent references on this topic, firstly with regard to specific molecular level effects, and secondly with in reference to specific organs or systems. Araki *et al.* [585] reported that the toxic effects of excess element may be treated in part by mobilizing Mn(II) into the urine by using Ca-EDTA. An interaction of Mn(II) with the insecticide fenitrothione has been investigated [586], and is possibly related to Soman hydrolase [150]. Superoxide has been reported to mediate the toxicity of paraquat in cultured

mammalian cells [587], while the interaction of Mn with lipid metabolism, especially regarding cholesterol, continues to be documented [588,589].

With recent enhanced attention to neurochemistry and receptors, citations on the role of manganese have increased. With human platelets, the influx of Mn(II) and other divalent cations occurs via receptor-mediated channels in the plasma membrane [590,591], although the influx of divalent cations may be independent of receptor stimulation in human endothelial cells [592]. The binding of buprenorphine to opiate receptors is regulated by guanyl nucleotides and Mn(II) [593]. The action of excitory amino acids (Glu, NMDA, Asp) and their antagonists on hippocampal neurons is mediated by Mn(II) [594],as are serotonin receptor binding sites [595]. The interaction of dopamine and Mn(II), possibly through a redox reaction, plays a role in manganese neurotoxicity [596]. Divalent ions such as Ca(II) and Mn(II) have been found to be involved in pore formation in membranes (in many cases mediated by pharmacological agents), which control

Table 3.3. DISEASE STATES RELATED TO MANGANESE DEFICIENCY OR EXCESS

Disease	*Reference*
Acromegaly (human growth hormone deficiency)	[552]
Alcoholism	[553–555]
Amyotrophic lateral sclerosis	[556]
Arteriosclerosis	[557]
Arthritis (rheumatoid)	[558,559]
Cancer	[560]
Catabolic disease	[561]
Diabetes	[562–566]
Down's syndrome	[567]
Epilepsy	[568]
Eye disorders	[569]
Iminodipeptiduria (prolidase deficiency)	[570–572]
Manganese rickets	[573]
Morvan's fibrillary chorea	[574]
Motor neuron disease	[575,576]
Osteoporosis	[577]
Otolith defects	[578]
Perthe's disease	[579]
Phenylketonuria	[580]
Thyroid disease	[581]
Viral hepatitis	[582]
Viral meningitis	[583]
Wilson's disease	[584]

Table 3.4. ORGAN-SPECIFIC PHARMACOLOGY RELATED TO MANGANESE

Organ, System	*Pharmacological Agent, Target*	*Reference*
Blood	$MnCl_2$	[599]
Brain	Mn(II), MPTP, P-450, 2-APB	[600–603]
Hormonal	thyrotropin releasing, pituitary, corticosteroid, bone resorption, progesterone	[604–608]
Heart	Mn(II), diltiazem, verapamil, La(III)	[609–612]
Immune	neutrophils/thapsigargin, natural killer cells/Mn/captopril, SOD	[613–620]
Kidney	atrial natriuretic peptide, diltiazem	[621,622]
Liver	ethanol, aflatoxin, hexobarbitol	[623–625]
Nervous system	Ca-channel blockers, Mn(II)	[626–628]
Pancreas	Ca/beta-cells	[629]
Smooth muscle	isoprenaline, Ca agonists/antagonists, verapamil	[630–633]

exchange of molecules ranging from water [597] to cell-damaging agents such as viruses or toxins [598].

A summary of reported involvement of manganese with the action of pharmacological agents or target cells with specific organs or systems in mammals are presented in *Table 3.4*. In blood, Mn(II) affects its own tissue accumulation by controlling regional blood flow [599]. In brain, the redox metabolism of drugs in the striatum and substantia nigra, and the susceptibility of these regions to drugs and radical damage are altered by manganese [600–602]. Also affected are Glu-sensitive receptors upon the binding of DL-2-aminophosphonobutyrate [603]. Effects related to endocrine/hormonal function include: phorbol ester effects on GH3 pituitary cell adenylate cyclase [605], biphasic effects of Mn(II) on hormone-stimulated bone resorption [607],effects on corticosteroid production in cultured adrenocorticoid cells [606], ionotropic effects of thyrotropin releasing hormone on the myocardium [604], and progesterone stimulation of Ca(II) influx in sperm [608].

In heart function, Mn(II) ions act additively with the anti-arrhythmic drugs verapamil and diltiazem, and with La(III) ions in the control of excitation–contraction coupling [609–612]. In the immune system, thapsigargin has been found to cause Ca(II)-transients and Mn(II) entry into neutrophils [613], and captopril (normally used as an antihypertensive—an inhibitor of angiotensin-inhibiting enzyme) scavenges free radicals by inhibition of PMN (NADP) oxidase [619]. Manganese superoxide dismutase was found to have pharmacokinetic and anti-inflammatory effects

[620] while a number of reports indicate that Mn(II) ions augment natural activity and interferon production, and blocks carcinogenesis by Ni(II) ions [614–618].

In kidney, diltiazem and the atrial natriuretic peptide act together with Mn(II) in controlling haemodynamics [621,622]. In liver, ethanol alters the levels of a number of trace metals, including Mn(II) [623], while Mn(II) itself reduces the effects of aflatoxin and enhances the cytochrome P-450 mediated metabolism of hexabarbitol-type drugs [624,625]. In the nervous system, the effects of ischaemia are modulated by Ca-channel blockers such as Mn(II) [626]. Mn(II) ions have also been reported to modulate or inhibit the transient outward current in cultured sensory neurons or in taste nerve responses [627,628]. In pancreas beta-cells, interactions between the fluxes of Mn(II) and Ca(II) have also been observed [629].

In smooth muscle cells, Mn(II) antagonizes the broncho-dilatation effects of isoproterenol (isoprenaline) [630], and alters the phasic and tonic responses in the vas deferens and the intracellular electrical activity of the muscularis mucosae of the stomach [631,632]. The vasoconstrictor responses of cerebral arteries to noradrenaline, serotonin and potassium were are altered by both verapamil and Mn(II) ions [633].

CONCLUDING REMARKS

Considering the plethora of recent literature citations regarding the roles of manganese in biological systems, it is perhaps useful at this point to summarize those developments that this author considers most significant and to indicate those areas or topics that deserve attention in future research endeavours.

Redox chemistry and ageing; recent findings on the role of Mn(II) in free radical dismutation or disproportionation, with related consequences for cell damage *vs.* protection, seem to indicate potential connections with various disease states of the nervous and circulatory systems. These include Alzheimer's disease, arteriosclerosis, plus the connection between diabetes and damage to the motor and central nervous systems.

Carbohydrate metabolism; the involvement of Mn(II) with key enzymes of glucose storage, mobilization, and catabolic and anabolic metabolism is now well established. Effects at the cell, organ, and whole organism levels are less well understood, especially those related to interaction of Mn(II) with hormonal systems and receptors.

Receptors and receptor action; Mn(II) is clearly implicated in the action and responses of a variety of receptors, but its role at the molecular level is

yet poorly defined. If its role is merely to provide part of the binding site template for a nucleotide triphosphate, then why have so many receptors evolved to utilize Mn(II) rather than the more abundant Mg(II) ions? Does Mn(II) additionally play a structure-stabilizing role for interaction between the signal binding subunit and the G- and adenylate-cyclase subunits?

DNA replication and RNA transcription; in what way, at the molecular level, does Mn(II) alter the specificity and fidelity of catalysis of these processes? Such questions may only be answered by X-ray crystallographic studies or rapid kinetic approaches to elucidate structural details, along with stepwise changes brought about by substitution of Mn(II) for other divalent ions.

Immune and hormone function; the action of Mn(II) in specific phenomena in these areas is as yet poorly defined, with an increasing number of intriguing clues and reports, but clearly also with a real need for more fundamental research at the cellular and molecular levels under chemically defined conditions, for example, with cultured cells.

It is hoped that this review, by gathering up and organizing such a large number of intriguing observations, with various degrees of refinement, offers new starting points and inspirations for future research in this area.

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4 Approach to the Chemotopography of the Digitalis Recognition Matrix in Na^+/K^+-transporting ATPase as a Step in the Rational Design of New Inotropic Steroids

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INTRODUCTION

THERAPEUTIC NEED FOR NEW INOTROPIC DRUGS

The need for new inotropic drugs follows from the prevalence and mortality of congestive heart failure in the developed countries, and from the fact that the cardiac drugs currently available are clearly inadequate to restore health or even to minimize discomfort and disability.

In the USA, more than two million people are afflicted with heart failure, and 400,000 events requiring 900,000 hospitalizations occur every year. The

probability of occurrence of heart failure increases progressively with age. Thus, as the mean age of the population increases in all developed countries, the number of patients with heart failure will rise sharply during the nineties. Once manifest, heart failure is highly lethal with a death rate four-fold to eight-fold greater than that of the general population of the same age [1]. Cardiovascular disease accounts for more than 50 per cent of all deaths. The number of deaths in which congestive heart failure was considered the underlying or contributing cause increased two-fold in the USA between 1955 and 1988 [2]. The total economic cost of cardiovascular disorders in the USA is estimated to be in excess of $60 billion annually [3]. In its chronic form, heart failure produces much private suffering and a large economic burden for the patients, their families, and society.

As recently stated in a review on the therapeutic options in the management of chronic heart failure [4], the combined use of digitalis* and diuretics has for over thirty years been the initial approach to the drug therapy of this disease. These drugs achieved dominance, because physicians had few therapeutic alternatives. During the last decade, many new drugs have been developed and tested in patients with heart failure at a rate of one new agent every month. An overview of the more interesting positive inotropic agents and their putative major mechanisms [5] is illustrated in *Figure 4.1.* Several of the new compounds possess multiple inotropic mechanisms as shown in *Figure 4.2* [6].

In evaluating the place of new drugs in the treatment of chronic heart failure, Packer [4] concluded that the phosphodiesterase inhibitors yielded disappointing results, because their ability to increase cardiac contractility depends on the content of cAMP in the myocardial cell, which declines as heart failure progresses in severity with time. In fact, many well-known positive inotropic drugs, with the exception of digitalis, lose their effectiveness just when they are needed most—in severe heart failure [7]. In addition, reports on increased mortality [8–10] have reduced the initial enthusiasm for the use of the phosphodiesterase inhibitors—'paradise postponed' [11]. The huge investment of money, time, manpower, and resources to develop an orally administrable positive inotropic drug to supplement or replace the digitalis glycosides has generally been disappointing. The prospective randomized milrinone survival evaluation trial, showing that repeated oral administration of milrinone can increase

* Abbreviations used: Digitalis, digitalis-like acting C/D-*cis* and C/D-*trans* steroids including their glycosides; Na^+/K^+-ATPase, Na^+/K^+-transporting adenosine triphosphatase, E.C. 3.6.1.37.

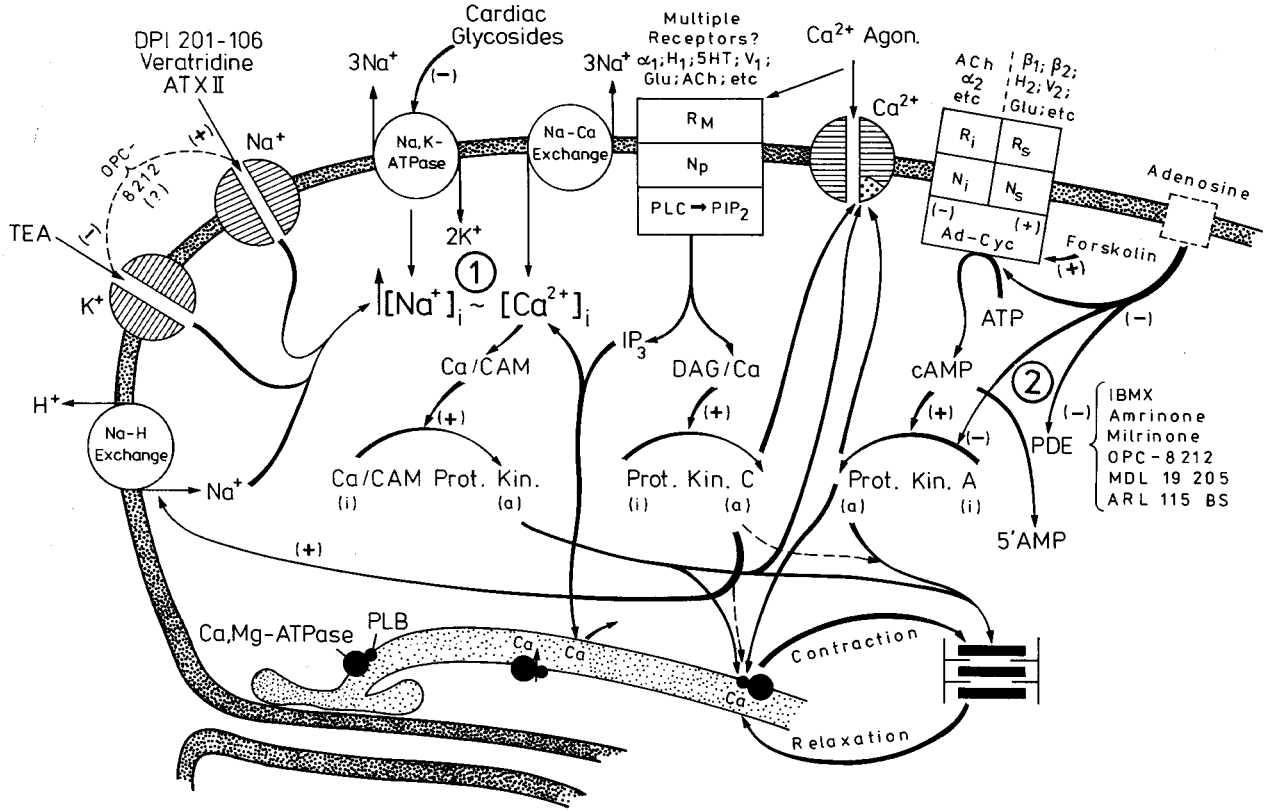


Figure 4.1 Overview of positive inotropic agents and their major mechanisms of action. With regard to the topics of the review, the scheme especially illustrates: (1) the inotropic mechanism affecting the coupled increase of intracellular Na^+ and Ca^{2+} activity produced by inhibition of Na^+/K^+-ATPase, and (2) the inotropic mechanism effecting the elevation of intracellular [cAMP] resulting from inhibition of phosphodiesterase (PDE). Reproduced from [5], where further explanations are given.

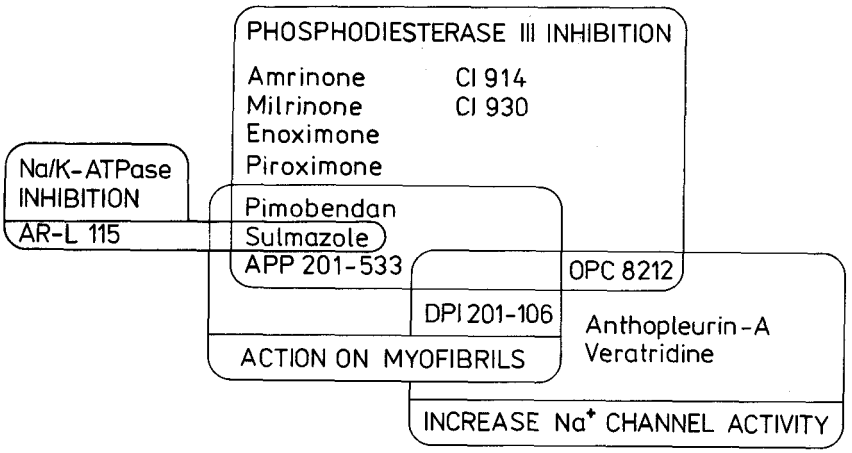


Figure 4.2. Scheme of multiple positive inotropic mechanisms of action for several novel inotropic stimulants. Reproduced from [6].

mortality in heart failure, is having a devastating effect on the further development of this class of non-digitalis positive inotropic drugs [12].

Only digitalis, diuretics, and angiotensin-converting enzyme inhibitors each fulfil some of the criteria of a first-line agent in the treatment of chronic heart failure. However, none of these drugs satisfies all of the desired characteristics, and none can optimally manage the heart failure state when used alone [4]. Moreover, the converting enzyme inhibitors and diuretics show the tendency to impair renal function; cardiac glycosides on the other hand tend to improve renal function. However, digitalis intoxication continues to be one of the most prevalent adverse drug reactions encountered in clinical practice [13]. An evaluation of the long-term strategies in the management of heart failure has most recently led to the conclusion that drugs which potentiate the effects, or increase the activity, of the sympathetic nervous system (phosphodiesterase inhibitors) or the renin–angiotensin system (calcium antagonists) increase cardiovascular morbidity and mortality [14].

In speculating on the outlook for the management of chronic heart failure in the nineties, Kellermann [15] expected for the coming years that every effort must and will be made to further develop and create new compounds. These should not only beneficially affect the haemodynamic and functional impairment of patients with congestive heart failure, but also hopefully contribute to reaching an achievable goal, namely, the prevention of the

clinical manifestation of heart failure. The pharmaceutical industry should not relax its efforts to uncover and study new and improved drugs that can safely restore myocardial contractility [11].

SEARCH FOR NEW INOTROPIC COMPOUNDS BY SCREENING IN ANIMAL MODEL SYSTEMS

The most suitable method for the quantitative evaluation of the positive inotropic effect of a cardioactive steroid is at present the assessment of its influence on the isometric contraction curve, measured in isolated papillary muscles. However, to obtain reliable data for the comparison of the inotropic potency of the various cardioactive steroids, it is important to perform the experiments under identical conditions [16]. Because of the variety of test conditions used, it is not easy or it is even impossible to compare the data reported in the literature [17,18]. A comprehensive survey of the structure–activity relationships found in guinea-pig left atria under identical conditions has recently been presented by R. Thomas and coworkers [19]. Therefore, this aspect will be mentioned here only with regard to the two basic risks for misinterpretation of pharmacological data from animal model systems.

First, all inhibitors of the Na^+/K^+ pump irrespective of their 'microscopic' inhibitory mechanism (see below) produce positive inotropic effects if they do not additionally affect other biochemical systems which preclude the manifestation of the inotropic action [20]. A prominent example concerns the bisguanylhydrazones of 3,20-diketosteroids. The fact that they are structurally quite different from digitalis led to the erroneous conclusion that most of the rules or dogmas dealing with structure–activity relationships in cardioactive steroids had to be modified [21]. However, the steroidal guanylhydrazones have recently been shown by means of the microscopic test outlined below to suppress Na^+/K^+-ATPase activity by a mechanism not involving the occupancy of the digitalis recognition matrix [22].

Second, inhibitors of the Na^+/K^+ pump, even when effective through occupancy of the digitalis recognition matrix, can fail to produce a positive inotropic action, if they additionally affect other biochemical systems precluding the manifestation of their inotropic potency. A recent example is the progestin, chlormadinone acetate. It was shown by LaBella *et al.* [23,24] to produce an inhibition of the Na^+/K^+ pump accompanied by a negative inotropic action. This puzzling effect, however, could be converted to the expected positive inotropic action by glycosidation of chlormadinol acetate, which hinders the penetration of the progestin across the plasma membrane and thus blocks its extragenomic cardiodepressant action [22].

In conclusion, animal model systems, especially heart muscle preparations, are in the end indispensable for the quantitative evaluation of the positive inotropic effect of digitalis-congeneric compounds. They appear, however, to be less suitable for the primary screening of the numerous inhibitors of Na^+/K^+-ATPase, the microscopic mechanism of which is still unknown. The observations obtained under this condition can, of course, lead to a null hypothesis concerning structure–activity relationships.

SCOPE AND OBJECTIVE

One underlying objective of the present review is the deduction of the potential causes for the apparent lack of progress in the search for novel, superior cardiotonic drugs.

In summarizing a review on 'Cardiac Glycosides: Prerequisites for the Development of New Cardiotonic Compounds', Güntert and Linde [25] concluded in 1977 that none of the various structural modifications have, up until then, resulted in a compound with either pharmacological properties superior to the classical cardiac glycosides or with a better therapeutic index. They concluded, however, that the hope that further partially synthetic modifications will realize this goal, is certainly justified. On the other hand, Austel and Kutter [26] in 1978 felt it very risky to strive for new cardiotonics with a greater therapeutic range than realized in the known cardiac glycosides. However, their evaluation was based on erroneously equating the natural representatives with lead structures, which have already been optimized by nature and therefore cannot be improved by synthetic modifications as tried over several decades. Their scepticism, however, was not warranted, because, as the lead structure proper in cardiac glycosides was still unknown at that time, the derivatizations were focussed on the side-chain at C-17 taken to be the pharmacophoric lead. In a handbook chapter entitled 'Chemistry and Structure–Activity Relationships in Cardioactive Steroids', Güntert and Linde again stated in 1981: 'It is hoped eventually to find a lead for the synthesis of specific substances with better cardiotonic activity and less toxicity' [21].

The methodological aims of the present article include the introduction of the basics for the use of Na^+/K^+-ATPase in the search for new inotropic compounds and for the application of the receptor enzyme in primary screening to allow differentiation at the molecular level between digitalis-like inhibitors and non-digitalis-like inhibiting compounds. This will be the first complete presentation of the test system which promises to avoid the misinterpretations previously often encountered .

The primary objective of the contribution is to report fully for the first

time on the interaction between the receptor enzyme Na^+/K^+-ATPase from different origins and numerous C/D-*cis* steroids of the digitalis type and C/D-*trans* steroids of the hormone type, respectively, to allow the definition of their lead structures and the assessment of substituent contributions to their composite potency. Over three decades, this has been made possible through the donation of many rare or even unique compounds by N.K. Abubakirov, H.P. Albrecht, C. Casagrande, K.K. Chen, H. Flasch, E. Haack, D.C. Humber, F.X. Jarreau, F. Kaiser, W. Küssner, K. Meyer, G.R. Pettit, T. Reichstein, J. Renz, H. Ruschig, D. Satoh, H.-J. Schmidt, G. Schubert, F. Sondheimer, A.M. Strosberg, Ch. Tamm, R. Thomas, T.Y.R. Tsai, R. Tschesche, H.-W. Voigtländer, A. von Wartburg, T.R. Watson, J. Wicha, K. Wiesner, and W.W. Zorbach. The given rich fund of about 600 representative compounds has been completed in the laboratory of the authors by targeted partial syntheses of steroid derivatives not available from natural sources.

The ultimate goal of the present endeavour is to unravel the information required to describe the chemotopography of the digitalis recognition matrix. This should eventually allow computer graphics-aided mapping of its three-dimensional structure. As often claimed [27–32], such knowledge could allow the design of novel compounds with selectivity of action.

The presentation of the subjects is in very specific terms. As a consequence, many colleagues will not find their work mentioned here, which should by no means be interpreted as meaning that their contributions are not appreciated. However, to limit this review to a reasonable length, we had to refrain from going into much related detail, which is legitimate also since the pertinent literature has been comprehensively reviewed by Thomas, Gray and Andrews [19].

BASIC PRINCIPLES OF THE USE OF Na^+/K^+-ATPase IN THE SEARCH FOR NEW INOTROPIC COMPOUNDS

Na^+/K^+-ATPase AS THE DIGITALIS RECEPTOR

Knowledge of the function of Na^+/K^+-ATPase as the digitalis receptor comes from two seminal findings. First, the integrating assessment in 1964 of the then available experimental data on the relationship between the contractility of cardiac muscle cells and the movements of K^+, Na^+, and Ca^{2+} across the plasma membrane led to the hypothesis that a transient increase of the intracellular Na^+ concentration, resulting from the digitalis-produced reduction of the Na^+/K^+ pumping capacity of the cell by

inhibition of a portion of the total Na^+/K^+-ATPase, plays the primary role in the control of cardiac force [33,34]. An account of the prehistory of the hypothesis has been published [35]. The indicated interrelationship has often been questioned, but was finally fully corroborated in 1985 [36]. The introduction of Na^+-selective microelectrodes has allowed researchers to directly demonstrate that the intracellular Na^+ activity (a^i_{Na}) and the contractile force are closely correlated during the positive inotropic effect of digitalis at low and high concentrations. The power function of a^i_{Na} has been found to be very steep, so that only small changes in a^i_{Na} are required for a large effect on contractile force. These data have led to the conclusion that the inhibition of Na^+/K^+-ATPase by digitalis at therapeutically active concentrations is causally related to the positive inotropic effect [36]. The mechanism of the impact of a^i_{Na} on the control of intracellular Ca^{2+} activity, the final regulator of contractility, has been illustrated in *Figure 4.1*. Reports on a digitalis-elicited stimulation of the Na^+/K^+ pump associated with a positive inotropic effect [37,38], which are seemingly in conflict with the above hypothesis, have more recently found their reconciling explanation. Digitalis compounds have been shown to inhibit the Na^+/K^+ pump in sympathetic nerve endings and in this way to evoke norepinephrine release in the heart (cf. *Figure 4.3* and [39]), which eventually stimulates both the Na^+/K^+ pump and the contractility of the heart muscle cell [40,41]. The obvious idea that the inotropic digitalis effect results from an inhibition of the Ca^{2+}-pumping systems [42] was not confirmed when studying the Ca^{2+}-transporting ATPases of plasma membrane and endoplasmic reticulum [43–46].

The above presentation of the evidence for the receptor function of Na^+/K^+-ATPase should be completed by mentioning the extensive correlations between the digitalis effects on Na^+/K^+-ATPase or Na^+/K^+ pump and on cardiac contractility observed when varying ionic milieu, exposure time, digitalis concentration, and digitalis structure [41,47–50]. Remarkably, the long-lasting sharp critic of the receptor function of Na^+/K^+-ATPase, G.T. Okita (see Discussion in [51]), has recently reported that digoxin, administered therapeutically in the clinical setting, inhibits the Na^+/K^+ pump in the human heart [52], which is clearly in accordance with its receptor function.

Second, in 1965 the long-time unintelligible, huge species differences in digitalis sensitivity were traced back to the corresponding differences in the affinity of Na^+/K^+-ATPase from cardiac muscle to digitalis glycosides [53]. This was the first indication of the occurrence and functional significance of isoenzymes of Na^+/K^+-ATPase. The original observations have been confirmed and extended by numerous laboratories. So it was suggested that

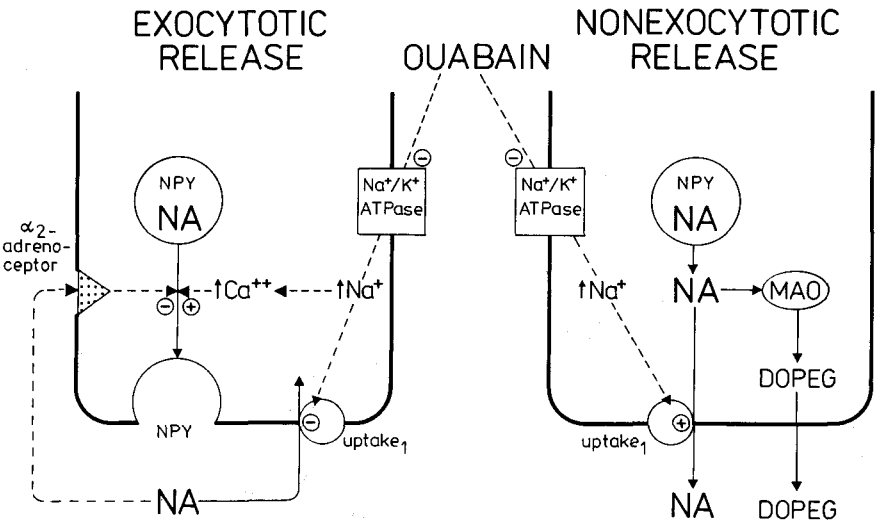


Figure 4.3. Schematic diagram of the mechanism of exocytotic and non-exocytotic release of norepinephrine (NA) from sympathetic nerve endings in the heart, leading to extracellular NA accumulation after inhibition of Na^+/K^+-ATPase by ouabain. Both mechanisms are influenced by an increase in axoplasmic Na^+ activity after Na^+/K^+-ATPase inhibition. Reproduced from [39].

the structural difference between enzymes of different digitalis sensitivity is limited to the digitalis binding domains [54]. The topic will be treated below in greater detail.

MEDICINAL SIGNIFICANCE OF Na^+/K^+-ATPase IN NEURAL TISSUES

The importance of neural digitalis actions summarized below follows from the various functions of Na^+/K^+-ATPase in neural tissues. In neurons and in glial cells, the enzyme maintains the Na^+ and K^+ gradients necessary for impulse conduction and neurotransmitter uptake, respectively [55]. Additionally, Na^+/K^+-ATPase also plays a role in neuronal signalling by modulating synaptic transmission via hyperpolarizing nerve membranes, and it may indirectly regulate the Na^+-gradient-dependent carriers for neurotransmitters [56]. Thus, inhibition of Na^+/K^+-ATPase by digitalis compounds can safely be assumed to play the causal role in triggering both the therapeutic and toxic neural actions of digitalis described by Gillis and Quest [57] as follows. Therapeutic doses activate the parasympathetic and inhibit the sympathetic nervous system. This leads to a decrease in sinus rate of heart contraction, to an antiarrhythmic effect, and to a decrease in

arterial and venous tone. The tissue locations of these actions are the peripheral reflexogenic sites, especially the carotid sinus, the aortic arch baroreceptors, and the cardiopulmonary receptors. High (toxic) doses increase the central sympathetic outflow resulting in ventricular tachyarrhythmias and arterial vasoconstriction. The tissue locations of these actions are sites in the central nervous system which control the sympathetic outflow.

The major determinant of the maximum digitalis dose which can be administered in the digitalis treatment of heart failure, is the development of ventricular arrhythmias. A major factor contributing to the arrhythmogenic action of cardiac glycosides is central neuroexcitation. This has been derived from studies in experimental animals using a variety of techniques (see review [58]).

As shown by Pace and Gillis [58], the exclusion of central autonomic effects results in a significant increase in the dose ratios of toxic to inotropic, and lethal to inotropic doses of digoxin. This permits a larger dose of digoxin to be administered so that a greater inotropic response is elicited. The authors suggested that one of the promising areas of research would be the development of digitalis derivatives, which are excluded from the central nervous system. However, studies with some highly polar semisynthetic digitalis derivatives, which should have a low blood-brain barrier permeability, indicated that this approach may not be fruitful [59,60]. An alternative way to increase the ratio of doses for the therapeutic and toxic digitalis effects could be the design of digitalis derivatives with a more favourable distance between their affinities for the isoenzymes of Na^+/K^+-ATPase in contractile and neural cells. A first endeavour in that direction is presented below.

QUEST FOR STEROIDAL INHIBITORS WHICH DISCRIMINATE BETWEEN ISOENZYMES

The occurrence of isoenzymes of Na/K-ATPase in human tissues has only recently come to light and is correspondingly incomplete. In heart and brain, three isoforms containing the $\alpha 1$, $\alpha 2$, and $\alpha 3$ catalytic subunits have been found to be expressed [61]. It remains to be seen whether all three isoenzymes are located in one cell type or, as is likely [62,63], in more than one. Due to their structural similarity, until now none of the isoenzymes could physically be separated [62,64]. Thus, it has remained an open question whether all three isoenzymes serve equally as targets for the digitalis actions on cardiac muscle and neural tissues.

Experience in the field of isoenzyme analysis has shown that a minor

difference in the three-dimensional structure can cause great differences in their affinities to structurally distinct inhibitors; this may make isoenzyme selectivity possible (cf.[65,66]). Therefore, many types of C/D-*cis* steroids of the digitalis type and of C/D-*trans* steroids of the hormone type have been probed for their potential to differentially inhibit the isoenzymes present in the Na^+/K^+-ATPase preparations from human cardiac muscle and human brain cortex (cf. Tables 4.1–4.5). A Na^+/K^+-ATPase preparation from cardiac muscle of guinea-pig was included in the test series under the well-founded expectation, that in this way, information would be gained on the location and composition of the amino acid stretches forming the binding sites for the steroid nucleus and its side-chains in the digitalis recognition matrix. The underlying reasoning is presented below.

LIMITATIONS AND PROMISES OF THE Na^+/K^+-ATPase SCREENING TEST

As is well known, cardiac glycosides do not produce their effects immediately after administration. Even after intravenous injection, it takes some time until the full effect is felt. This led repeatedly to a discussion of the question as to whether or not it is really unaltered glycoside which acts on the heart. Regarding digitoxin and digoxin, it was shown that they do not require bioactivation, and that their biotransformation instead leads to loss of potency [67]. Clearly, this does not apply to prodrugs. Lanatoside C undergoes deglucosidation and deacetylation by micro-organisms in the intestinal tract, before it becomes absorbed in the form of digoxin [68,69]. Penta-acetyl-gitoxin requires deacetylation by microbial and animal hydrolases before becoming effective [70,71]. Testing the prodrug and its products of biotransformation allows us to decide whether a prodrug has the capacity to serve for drug latentiation as given in penta-acetyl-gitoxin [72,73].

Generally, a targeted strategy to determine the relationship between structure and potency of inhibitors using an isolated receptor enzyme cannot take into account the many pitfalls and complexities of the *in vivo* system arising at different points; for example, the tissue level and the half-time of the inhibitor, the concentration and reserve capacity of the enzyme [34,74], and the residence time of the inhibitor at its binding site. This is stated here only to indicate that the inhibitors characterized in the Na^+/K^+-ATPase test (*Tables 4.1–4.5*) are not necessarily meant to serve as potential drugs.

As reviewed by Sandler and Smith [75], nearly all reversible enzyme inhibitors that have been designed as potential drugs, as well as drugs in current use, are competitive inhibitors. One reason for this is that

competitive inhibitors of an enzyme bear some resemblance to the substrate, since they bind to the same site. This knowledge has provided a starting point in drug design. Digitalis compounds, however, are non-competitive inhibitors of Na^+/K^+-ATPase, and thus do not offer an obvious design aspect. Their 'allosteric' inhibitory character naturally raises the question of what substance do the C/D-*cis* steroids of the digitalis type stemming from herbal sources, structurally mimic in the animal body. This issue has recently been discussed elsewhere [22,76].

Due to the absence of design information, a great number of steroidal compounds had to be tested with the following aims: (i) to define the pharmacophoric lead structure in cardiac glycosides and the functional significance of attached atom groupings, (ii) to probe new structural types, and (iii) to check the 'microscopic' mechanism of inhibitory effectiveness of non-congeneric compounds. One of the fortunate advantages of the Na^+/K^+-ATPase test [72,77] is that it allows this screening to be completed in a short time and at low cost.

APPLICATION OF Na^+/K^+-ATPase IN PRIMARY SCREENING

METHODOLOGICAL ESSENTIALS

As shown elsewhere [78], the determination of structure–activity relationships and receptor kinetics, extrapolatable to man, requires the use of Na^+/K^+-ATPase preparations from human tissues. Autopsy preparations are perfectly suitable when taken within 12 hours post-mortem [78,79]. The enzyme should be only partially purified to avoid the various possible artefacts of intensive purification procedures [80–82]. The interaction of cardiac glycosides and Na^+/K^+-ATPase is controlled by Na^+, K^+, Mg^{2+}, and ATP depending on their combinations and concentrations [41,74,83,84]. The choice of maximized turnover conditions, realized by the presence of appropriate concentrations of the above effectors, should mimic as much as possible the conditions for the development of the *in vivo* digitalis action [85] and avoid a mixture of enzyme conformers differing in their digitalis affinity [83]. Details are given elsewhere [35,72,78,86,87].

MACROSCOPIC SCREENING

This initial test is restricted to the assessment of the equilibrium values of inhibition under conditions which allow calculation of the concentration producing half-maximum inhibition which is the equivalent of the apparent

dissociation constant, K_D', of the complex of the inhibitor with the enzyme. As a suitable measure of the potency of an inhibitor, the K_D'-value will be converted into the apparent Gibbs energy change, $\Delta G^{o'}$, to allow the extrathermodynamic approach to the quantitative analysis of structure–activity relationships [87–89]. This procedure clearly requires that a uniform molecular mechanism underlies the observed inhibitory effectiveness. However, fulfilment of this prerequisite cannot be derived from inhibition studies (therefore named macroscopic screening). As this fact has often been neglected, this aspect of evaluation deserves some consideration.

Digitalis compounds are chemically inert and thus reversible inhibitors of

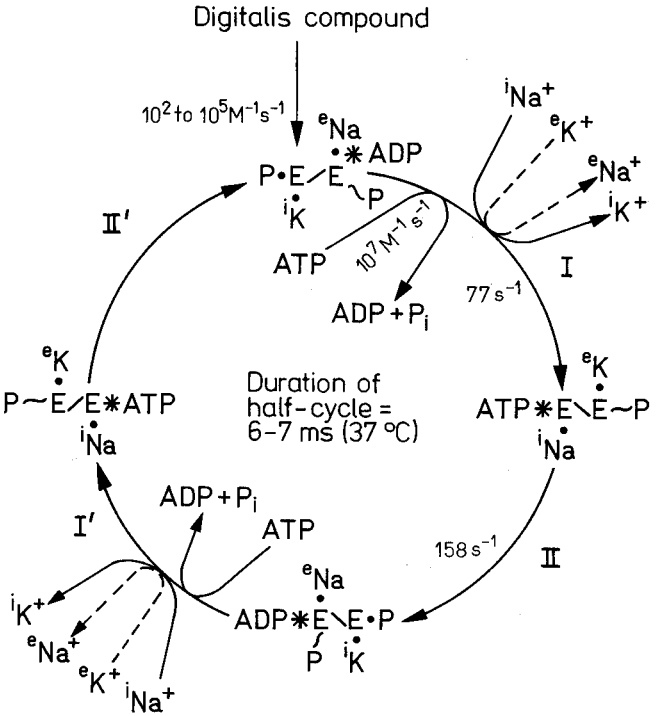


Figure 4.4. Reaction and function scheme of Na^+/K^+-ATPase indicating the large differences in the association rate constants for the formation of the inhibitory complex between various digitalis compounds and the phosphorylated enzyme intermediate on the one hand, and for the formation of the productive complex between ATP and the same intermediate on the other, during full speed operation of the enzyme. The letters E symbolize the two catalytic subunits, and the superscripts indicate the locations of Na^+ and K^+ on the intracellular (i) or extracellular (e) side of the enzyme and of the cell, respectively. Reproduced from [49].

Na^+/K^+-ATPase. The digitalis binding site is intrinsically not pre-existent in the enzyme matrix, but requires the formation of an ATP-generated high-energy conformation of the enzyme. Relaxation of enzyme conformational energy underlies the digitalis-produced inactivation of Na^+/K^+-ATPase [90]. Hence, the interruption of the catalytic cycle by some inhibitory-active ligand at any point (cf. *Figure 4.4*) blocks binding and rebinding of a cardiac glycoside, such as ouabain, to the enzyme.

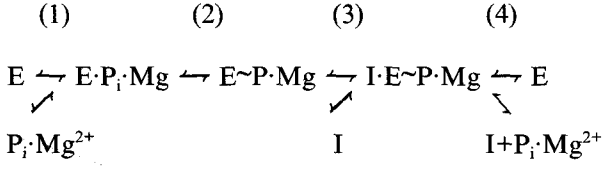
In terms of the often used [^{3}H]-ouabain radioligand potency assay [91,92], this clearly means that an inhibitory ligand effect on [^{3}H]-ouabain binding to the enzyme does not necessarily indicate its competition with ouabain for the digitalis-binding matrix as has often been presumed. In conclusion, all data extracted from the radioligand potency assay, as for example, for prednisolone bisguanylhydrazone [93,94] or chlormadinone acetate [91], should be evaluated with this reservation in mind.

MICROSCOPIC SCREENING

There are only two clear-cut analytical approaches to establish whether an inhibitor, being non-congeneric with digitalis compounds, acts in a digitalis-like manner. Both rely on proof that inhibition follows from occupancy of the digitalis recognition matrix.

The first one exploits the knowledge that some isoenzymes of Na^+/K^+-ATPase exhibit a very distinct pattern of affinity for various C/D-*cis* steroids of the digitalis type. Thus, if an inhibitory-active non-congeneric compound shows the gradation of affinities characteristic for digitalis compounds, then it qualifies as a digitalis-like inhibitor. For instance, this qualification applies to the erythrophleum alkaloid, cassaine, and various C/D-*trans* steroids of the hormone type (listed in *Table 4.5*) but not to the cardioactive compound, progesterone bisguanylhydrazone [22]. The proof, however, is not as easy to produce as required in routine screening.

A second approach is based on the experience that the competent occupancy of the digitalis recognition matrix by any inhibitor (I) of Na/K-ATPase (E), and hence its nature as a mechanistically digitalis-like compound, is proved by its capacity to promote enzyme phosphorylation from orthophosphate [22]:



The progress of this reaction sequence is energetically highly demanding. After binding of Mg^{2+} and P_i to the enzyme, which reduces the entropy of peptide chain mobility [95], Na^+/K^+-ATPase synthesizes, entropically driven [96], the phosphoenzyme E~P with an aspartyl phosphate residue in the catalytic centre [97]. The envelopment of a digitalis-like acting inhibitor by the peptide chain segments of the digitalis recognition cleft triggers a large entropy gain in the inhibitor–phosphoenzyme complex [98], which expresses itself in a stabilization of the phosphoenzyme formed [97]. The outcome is a shift in equilibrium (2) to the right thus elevating the actual phosphoenzyme level measured [97]. A negative response in this phosphorylation promotion test allows discrimination of compounds which do not inhibit Na^+/K^+-ATPase activity through a digitalis-like mechanism, since these do not promote, or even suppress, enzyme phosphorylation (as is the case with progesterone bisguanylhydrazone, Cibacron Blue F3GA, or tamoxifen [22]). In contrast, those which act through a digitalis-like mechanism, promote enzyme phosphorylation in a concentration- and structure-dependent manner (as do cassaine and several C/D-*trans* steroids of the hormone type, including chlormadinol acetate and megestrol acetate [22]). Moreover, a factor from bovine hypothalamus which is not digitalis-like [99], and a digitalis-like factor from human plasma [100] have clearly been differentiated by use of the P_i phosphorylation promotion test.

In conclusion, the microscopic screening emerges as an essential tool to select or reject compounds for modelling the chemotopography of the digitalis recognition matrix and for uncovering the minimal pharmacophoric lead structure in bimolecular recognition studies.

CORRELATIVE ANALYSIS OF THE INFORMATION SETS DERIVED FROM SCREENING

Until further data on the structure of the digitalis recognition matrix in Na^+/K^+-ATPase are available (for the present state of knowledge, see below), information on its chemotopography can only be deduced indirectly by correlating, for a wide selection of compounds, information from the molecular structure with their interaction energies. In a type of 'active analogue approach' [101], a first attempt will be made to derive the minimal structural requirement for specific bimolecular recognition and thus to reveal the demarcation boundaries of the peptide sequences involved, their conformational adaptability, the enveloped form, and the electrostatic surfaces. In the following sections, a chemotopographical interpretation is attempted of those observations which were made with a selected series of digitalis-congeneric C/D-*cis* steroids, and C/D-*trans* steroids of the hormone

type. Analysis starts from the basic assumption that the 'functional' partial structures of the various digitalis-like acting inhibitors of the indicated types interact with like or similar peptide sites, and that any modification of those partial structures does not basically affect the interaction of the others. The underlying concept of additivity has been shown to apply under certain prerequisites [89].

As a rational way to exploit the available information on the correlations which exist between inhibitor structure and inhibitory potency of digitalis derivatives, the extrathermodynamic analysis of the impact of structural variables on the apparent Gibbs energy of interaction with Na^+/K^+-ATPase, $\Delta G^{o'}$, has been performed (details in [88,89]). The extrathermodynamic derivation of the interaction energy-controlling structural parameters is based on the division of the various inhibitor molecules into their variable substructures. An estimate of the contribution of substituents to the observed interaction energy is obtained through determining the $\Delta G^{o'}$ values for the parent and substituted compounds. The differences in the $\Delta G^{o'}$ values of compounds, differing in one substituent, yield the required $\delta\Delta G^{o'}$ values. These are used here as the basis for all evaluations, but are not specifically listed for that purpose because of lack of space.

Interpretation of the $\delta\Delta G^{o'}$ values in terms of the binding site chemotopography is, in principle, admissible since the extrathermodynamic relationships implicitly contain the relation between the macroscopic quantity ($\Delta G^{o'}$) and the microscopic properties (atomic structure, geometry, charge distribution, electric field) [102]. However, any such interpretation has to consider that the observed $\Delta G^{o'}$ value is a composite quantity:

$$\Delta G_{obs} = \Delta G_{int} + \Delta G_D - T\Delta S_{int}$$

where ΔG_{int} = intrinsic Gibbs energy of binding, ΔG_D = unfavourable Gibbs energy from steric and electronic hindrances, and $T\Delta S_{int}$ = intrinsic entropy changes [103]. Since ΔG_D and $T\Delta S_{int}$ may be large in relation to ΔG_{int} the ΔG_{obs} value can be much smaller than ΔG_{int} when the latter is partially used to reduce rotational and translational entropies or to overcome conformational, steric and electrical hindrances. This makes a clear-cut interpretation of the $\delta\Delta G^{o'}$ values more difficult or even impossible. Nevertheless, interpretations believed to be most likely will be offered here as an invitation for rejection or confirmation by computer-aided molecular mapping of the digitalis recognition and binding matrix in the Na^+/K^+-ATPase α-subunit for which the present essay aims to prepare the way.

CONSIDERATION ON THE LOCALITY OF THE DIGITALIS RECOGNITION MATRIX DEDUCED FROM PROTEIN STRUCTURE DISSECTIONS

The search for the locality of the digitalis recognition matrix has intensively been approached through analysis of the connection between digitalis affinity (ouabain taken as a prototype) and the primary structure of Na^+/K^+-ATPase. Studies on various tissue preparations to analyse separately the attack of digitalis compounds on the intra- or extra-cellularly exposed peptide sequences revealed that the digitalis binding matrix is exposed to the extracellular surface of the plasma membrane [104–107]. Affinity labelling with chemically reactive digitalis derivatives demonstrated that the digitalis binding matrix pertains to the N-terminal sequence of the α-subunit of M_r 41,000 [108,109] (cf. *Figure 4.5*).

Comparison of the amino acid sequences in the extracellularly exposed domains of the α-subunit (shown in *Figure 4.5*) between digitalis-insensitive and -sensitive isoenzymes of Na^+/K^+-ATPase clarified that only two amino acids are unique in determining the digitalis affinity. The location of these unique amino acids suggested that the domain between the first and second membrane-penetrating peptide stretch (mostly denoted the H1–H2 junction [113]) profoundly affects the degree of enzyme affinity for ouabain and thus may form at least a part of the ouabain binding site [114,115].

Chimeric constructs reinforced the conclusion that the N-terminal two-thirds of the α-subunit peptide sequence, specifically the H1–H2 junction, include a domain responsible for the variation of ouabain affinity encountered in some isoenzymes of Na^+/K^+-ATPase [116–118]. In more detail, the initial ouabain binding, perhaps through the steroid nucleus, was presumed to occur with the amino acid residues of the H3–H4 junction (which are homologous in all isoenzymes) because all sensitive and less sensitive isoenzymes should allow an initial interaction of ouabain with the digitalis binding matrix. In the case of sensitive isoenzymes, the initial binding was suggested to be stabilized by a conformational change involving the H1–H2 junction [117]. That the steroid moiety of the glycoside interacts with the amino acid residues in the H3–H4 junction, seemed to be supported by the circumstance that considerable homology exists between that sequence and a sequence postulated as being the steroid binding region of the human oestrogen and glucocorticoid receptor [117]. Remarkably, a monoclonal antibody, which binds to the H3–H4 junction, inhibited Na^+/K^+-ATPase activity, but not ouabain binding [119].

Energy transfer experiments with anthroylouabain indicated that a tryptophan residue is located close to the ouabain binding site [120].

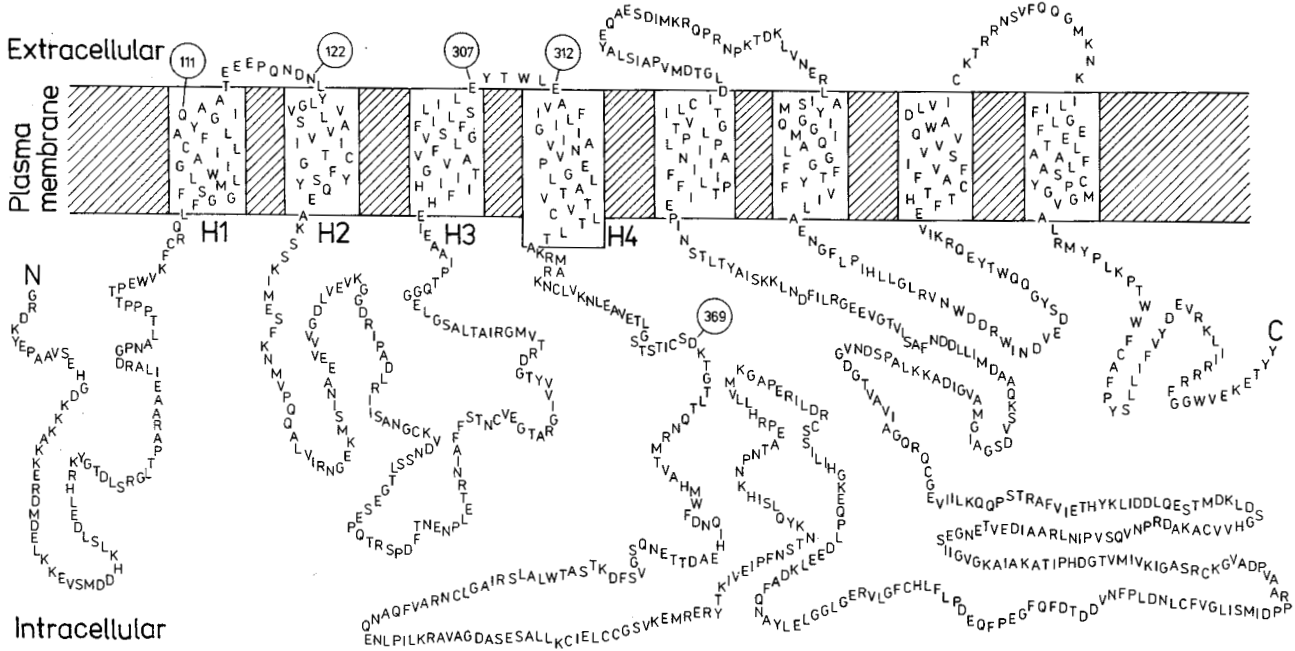


Figure 4.5. Scheme of the primary structure and spatial organization of the α-protomer of Na^+/K^+-ATPase. The amino acid chain penetrates the plasma membrane eight times. The membrane-crossing stretches H1, H2, H3, and H4 carry the extracellularly disposed H1–H2 junction (Q-111 – N-122) and H3–H4 junction (E-307 – E-312), which interplay in building the digitalis recognition and binding matrix. The H4 stretch directly mediates the communication of the matrix with the phosphorylatable aspartyl residue D-369 in the catalytic centre of the enzyme. Reproduced by combining the figures shown in [110] and [111]. The composition and locality of the H1–H2 and H3–H4 junctions are generally accepted, whereas this does not apply for the other extracellular loops [112].

However, as the fluorophore was attached to the rhamnose side-chain, far from both the steroid nucleus and the lactone moiety, the environment monitored by the fluorophore was clearly in the vicinity of the sugar-binding site close to the mouth of the receptor cleft (see below) rather than being in the digitalis recognition matrix itself. This fact was neglected by Thomas *et al.* [19] in their approach to modelling the digitalis binding site, in which they proposed that the digitalis interaction takes place first between the lactone and the tryptophan ring systems.

The result of a site-specific mutagenesis experiment suggested that the carboxyl residue of aspartate 121, which is the penultimate residue in the H1–H2 junction, is involved in the binding interaction between the enzyme and ouabain [121]. The authors considered the possibilities that the carboxylate may be interacting with another extracellular region of Na^+/K^+-ATPase in a manner which helps to maintain the integrity of the binding site or, alternatively, may directly interact with the ouabain molecule, perhaps with its lactone moiety.

Gene constructs have allowed localization of the amino acids that appear to be responsible for the rapid dissociation of ouabain from insensitive isoenzymes [122]. These are charged amino acids that reside on both borders of the H1–H2 junction, where all ouabain-sensitive enzymes have uncharged residues. The authors speculated that the H1–H2 junction may be involved in conformational changes during ouabain binding which, in the case of sensitive isoenzymes, may involve an interaction between the uncharged border residues and the plasma membrane. When charged amino acids are present at these positions, such a stabilizing interaction might be hindered. However, a later systematic structure–function study [123], which examined the effect on ouabain sensitivity of different combinations of charged amino acids at the borders of the H1–H2 junction, revealed that the explanation for the inability of a mutant enzyme to bind ouabain is more complicated than originally thought. The most recent results of mutations, confined to amino acid substitutions within the H3–H4 junction, showed that this region is not involved in isoenzyme differentiation of ouabain affinity [124].

At this point, Lingrel *et al.* [122] have recently stated that a model depicting the residues involved in cardiac glycoside binding to high-affinity isoenzymes of Na^+/K^+-ATPase would be at best speculative. Moreover, they felt that the regions of the enzyme which interact with discrete structural elements of the cardiac glycoside molecule, have yet to be determined. In fact, the interpretations derived from primary sequence variations, site-specific mutations, and chimeric constructs are severely hampered by the knowledge that localized sequence changes can exert

long-range effects on the global behaviour of enzymes including delocalized structural reorganizations, which render topological interpretations difficult [125,126].

In conclusion, identification of the amino acids forming the binding sites for the steroid nucleus, the lactone substituent, and the sugar side-chain in the digitalis recognition matrix requires the disclosure of the complementary information on the connection between variations of digitalis structure and changes of interaction of the derivatives with Na^+/K^+-ATPase isoenzymes of high and low affinity. This route of search, which has been comparatively neglected because of the paucity of suitable data, will be probed in the present review on the basis of a unique body of organized data, which still has not been documented, but only partially discussed in a book chapter [35]. Our endeavour has especially been motivated by the expectation of unravelling along this route the pieces of information required for the computer graphics-aided mapping of the digitalis recognition matrix.

ANALYTICAL USE OF C/D-*CIS* STEROIDS FOR INFORMATION ON THE CHEMOTOPOGRAPHY OF THE DIGITALIS RECOGNITION MATRIX

The present interest in cardiotonic C/D-*cis* steroids emerges here from three research objectives. First, the derivation of the pharmacophoric lead structure in the tripartite cardiac glycosides, which promises to decide upon possibilities for lead structure optimization. Second, paving the way for computer-aided molecular modelling of the digitalis binding cleft in the Na^+/K^+-ATPase protein, which should allow the location of the peptide sequences involved and, fortunately, the identification of the key functional amino acid residues. Third, determination of the degree of specificity of diverse digitalis derivatives in their interaction with isoenzymes of Na^+/K^+-ATPase from different origins. This should prepare the way for studying the separated isoenzymes of the receptor enzyme from human target tissues not as yet available.

CHEMOTOPOGRAPHY OF THE BINDING SITE FOR THE STEROID NUCLEUS

Biological activity of cardiac glycosides has long been known to reside in the aglycone moiety of their tripartite structure. Therein, the lactone side-chain has generally been regarded as the key functional group [127]. Thus, most of the work for further synthetic development of cardiac glycosides has been

directed towards modification of the lactone residue or its replacement by bioisosteric structures [19]. It was not until 1985 that the steroid nucleus of cardiac glycosides was identified as the pharmacophoric lead structure [88,128].

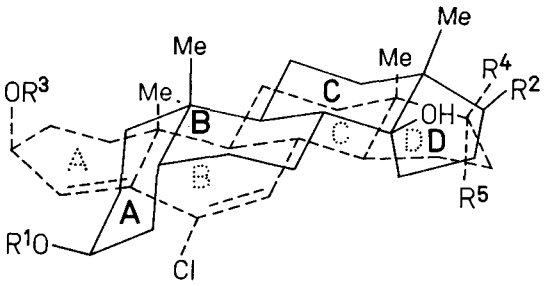


Figure 4.6. Structural formulae of 5β,14β-androstane-3β,14-diol (solid line; R^1 and R^2=H) and chlormadinol acetate (dashed line; R^3=H, R^4=COMe, R^5=OCOMe) indicating its strongly bowed and extended flat shape , respectively. Monosides in R^1 or R^3 positions occupy distinct spatial dispositions.

Specifically, 5β,14β-androstane-3β,14-diol (cf. *Figure 4.6*) was demonstrated [129] to show the same characteristics of interaction with the receptor enzyme as ordinary digitalis compounds judged by the following six criteria: (a) The concentration–inhibition curves with 5β,14β-androstane-3β,14-diol and its 3β-*O*-rhamnoside show the same shape as those with digitoxigenin or its 3β-*O*-rhamnoside. The good fit of the data points with the theoretical curves suggested that with each inhibitor only one class of interaction sites on the enzyme exists; (b) Inhibition of Na^+/K^+-ATPase activity by 5β,14β-androstane-3β,14-diol and its 3β-*O*-rhamnoside is apparently competitive to K^+ at low concentrations of K^+, whereas at higher K^+ concentrations the inhibition becomes more and more non-competitive to K^+; the same response pattern has been described for ouabain [130,131]; (c) The affinity of 5β,14β-androstane-3β,14-diol and its 3β-*O*-rhamnoside to Na^+/K^+-ATPase from human and guinea-pig heart shows the same great differences as found for ordinary digitalis compounds [53]; these were traced back to species differences in the degree of complementarity between the steroid nucleus and its binding site in the digitalis binding matrix [53,87]; (d) 5β,14β-Androstane-3β,14-diol competes with [^{3}H]-digitoxigenin for binding and displaces it from the binding matrix with the same time constant as unlabelled digitoxigenin; (e) The gradation of efficacy in inhibiting Na^+/K^+-ATPase activity and promoting enzyme phosphorylation from orthophosphate with 5β,14β-androstane-3β,14-diol and its glycoside is

similar to that with ouabain or digitoxigenin; (f) The two derivatives, devoid of the lactone side-chain, stabilize the phosphoenzyme formed with ATP in a similar manner to that of digitoxigenin and its 3β-*O*-rhamnoside.

The three-dimensional quality of the interaction between 5β,14β-androstane-3β,14-diol and its binding subsite in the digitalis binding matrix can be judged from a comparison of the measured interaction energy (−22.6 kJ/mol) with its theoretical maximum (−24.6 kJ/mol) calculated as described in [132] from the separate contributions of the component atom groupings. The comparability of the values appears to indicate a rather tight fit of the contacting surfaces. The major portion of the interaction energy may then originate from attractive London dispersion forces which are known for their critical distance sensitivity [133].

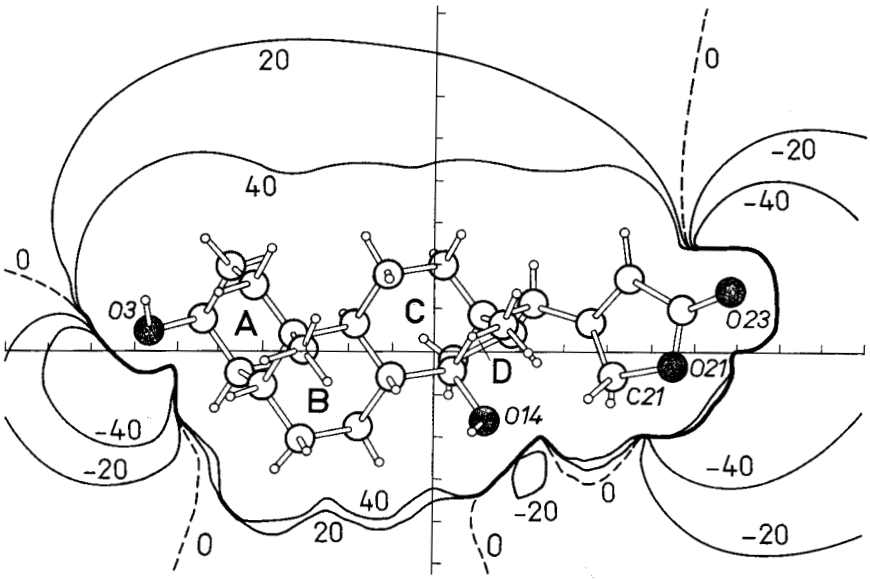


Figure 4.7. Shape of the molecular electrostatic potential in the outer space of digitoxigenin when occupying the probable receptor-bound conformation. Using the co-ordinates derived from X-ray crystal structure analysis, the equipotential energy contours (expressed in kJ/mol) are calculated with the use of an optimized monopol expansion. The energy contours refer to the plane laid across carbon atoms 6, 8, and 9 involved in forming rings B and C. Reproduced from [128].

Calculation of the molecular electrostatic potential distribution reveals that the steroid skeleton is surrounded by a positive field (*Figure 4.7*), the charge of which stems from the CH bonds [134]. The facing amino acid side-chains of the binding subsite may then be supposed to carry negative

Table 4.1. IMPACT OF CHANGES IN THE GEOMETRY OF C/D-*cis* STEROIDS OF THE DIGITALIS TYPE ON THE APPARENT GIBBS ENERGY OF INTERACTION ($-\Delta G^{o'}$) WITH Na^+/K^+-ATPase PREPARATIONS FROM HUMAN HEART (HH), HUMAN BRAIN CORTEX (HB), AND GUINEA-PIG HEART (GH): REPERCUSSIONS ON THE ENERGETIC CONTRIBUTIONS OF THE SIDE-CHAINS AT C-3β AND C-17β. METHODOLOGICAL DETAILS IN [87,88].

No.	*Systematic (trivial) name of inhibitor [Reference to synthetic procedure]*	$-\Delta G^{o'}$ *(kJ/mol)* *HH*	*HB*	*GH*
1	5β,14β-Androstane-3β,14-diol [135]	22.6	21.8	18.7*
2	3β,14-Dihydroxy-5β,14β-androstan-17-one [135]	17.6*		
3	5α,14β-Androstane-3β,14,17β-triol	23.1*	22.0*	23.3*
4	14β-Oestra-1,3,5(10)-triene-3,14,17α-triol	<<21.0*		
5	3β-(α-L-Rhamnosyloxy)-5β,14β-androstan-14-ol [135]	30.8	29.2	21.0*
6	3β-(α-L-Rhamnosyloxy)-5β-androst-14-ene [135]	25.9*	26.7	22.1*
7	3β,14-Dihydroxy-5β,14β-card-20(22)-enolide (digitoxigenin)	43.2	42.0	34.0
8	3β,14-Dihydroxy-5α,14β-card-20(22)-enolide (uzarigenin)	38.1	36.7	29.9
9	3β,14-Dihydroxy-14β-carda-4,20(22)-dienolide (canarigenin)	38.7	39.7	31.6
10	3β,14-Dihydroxy-14β-carda-5,20(22)-dienolide (xysmalogenin)			29.2
11	3β,14-Dihydroxy-5β,14β-carda-16,20(22)-dienolide (16-anhydrogitoxigenin)	22.4*		<17.9*
12	3β,14-Dihydroxy-5β,14α-card-20(22)-enolide			<<17.9*
13	3β-Hydroxy-5β-carda-8(14),20(22)-dienolide			26.1*
14	3β-Acetoxy-5β-carda-14,20(22)-dienolide [136]	28.6*		18.7*
15	3β-Hydroxy-5β-carda-14,16,20(22)-trienolide	25.6*		25.6
16	3β-Hydroxy-5β-carda-8,14,16,20(22)-tetraenolide	23.6*		
17	3β-Hydroxy-5α,14α-card-20(22)-enolide			18.6*
18	3β-Hydroxy-14α-carda-5,20(22)-dienolide			20.0*
19	3β-*O*-(α-L-Rhamnosyl)digitoxigenin	50.4	48.5	41.1
20	3β-*O*-(α-L-Rhamnosyl)uzarigenin		39.4	
21	3β-*O*-(β-D-Glucosyl)digitoxigenin	48.1	46.1	35.6
22	3β-*O*-(β-D-Glucosyl)uzarigenin		29.2	
23	3β-*O*-(β-D-Digitoxosyl)digitoxigenin	49.2	48.0	41.1
24	3β-*O*-(β-D-Digitoxosyl)canarigenin	46.0		38.5
25	3β-*O*-(β-D-Tridigitoxosyl)digitoxigenin (digitoxin)	48.1	46.8	39.9
26	14-Hydroxy-3β-(β-D-tridigitoxosyloxy)-5β,14β-carda-16,20(22)-dienolide(16-anhydrogitoxin)			22.3*
27	3β-(β-D-Tridigitoxosyloxy)-5β-carda-14,16,20(22)-trienolide	28.5	26.8*	
28	3β,14-Dihydroxy-5β,14β-bufa-20,22-dienolide (bufalin)	50.3	48.9	43.0
29	3β,14-Dihydroxy-14β-bufa-4,20,22-trienolide (scillarenin)	49.0		40.2
30	3β-*O*-(α-L-Rhamnosyl)bufalin	55.6	55.9	48.5
31	3β-*O*-(α-L-Rhamnosyl)scillarenin (proscillaridin A)	51.3		46.8
32	17β-(Fur-3′-yl)-5β,14β-androstane-3β,14-diol 3-acetate	40.4	41.1	32.4*
33	17β-(Fur-3′-yl)-5β-androst-14-en-3β-yl acetate	24.0*		
34	17β-(Fur-3′-yl)-5β-androst-8(14)-en-3β-yl acetate	24.1*		

* Extrapolated value owing to limited solubility.

charges. The significance of 5β,14β-androstane-3β,14-diol as the pharmacophoric lead structure in digitalis compounds is marked also by the fact that its strongly bent shape, due to the *cis*-junction of the rings A and B as well as the rings C and D (cf. *Figure 4.6*), is a prerequisite for the development of the maximum interaction energy. The strongly bent shape of the steroid lead implies that the digitalis recognition matrix is primarily a widely open cleft, the closure of which is elicited after slippage of the lead into the cleft and binding to the lining amino acid residues.

Any flattening of the steroid shape by lifting the A-ring or/and the D-ring relative to the B-C plane drops the $\Delta G^{o'}$ value (*Table 4.1*). This is clearly expressed in all cases of flattening of the A/B ring junction through transition from 5β-H to 5α-H (7→9, 23→24, 28→29, 30→31) or a Δ^5 double bond (7→10). Even stronger is the interaction energy decrease in case of all changes in the geometry of ring D through conversion of 14β-OH into 14α-OH (7→12) or through introduction of the double bonds $\Delta^{8(14)}$ (7→13, 32→34), Δ^{14} (5→6, 7→14, 32→33), $\Delta^{14,16}$ (7→15, 25→27), $\Delta^{8,14,16}$ (7→16), and Δ^{16} (7→11, 25→26). The loss of potency may partially be related to the potential of conformational flexibility conferred on the otherwise rigid steroid skeleton by the introduction of the double bonds. This also explains the partially observed reduction of specificity (discrimination between the Na^+/K^+-ATPases from different origins). As expected, lowering of the $\Delta G^{o'}$ value is reinforced by sugar or lactone substituents at C-3β-O or C-17β, respectively. Taken together, the observations indicate that the latter two binding subsites involved dispose of a limited degree of conformational adaptability.

IDENTIFICATION OF THE AMINO ACID SEQUENCE FORMING THE STEROID BINDING SUBSITE IN THE DIGITALIS BINDING MATRIX

The amino acid sequence in the H3–H4 junction has been supposed to form the steroid binding subsite on the basis of some amino acid homology with the putative steroid binding region of the human oestrogen and glucocorticoid receptor [117,137]. This interpretation, however, is at variance with three findings: (a) A monoclonal antibody, which binds to the H3–H4 junction, does not suppress ouabain binding [119,138] so that this junction does not appear to be the steroid binding subsite, the competent occupancy of which elicits the closure of the digitalis binding cleft (see above); (b) The amino acids of the H3–H4 junction are homologous in the digitalis-sensitive and -insensitive isoenzymes of Na^+/K^+-ATPase [114,117]; (c) Systematic mutational amino acid substitutions in the H3–H4 junction do not change

ouabain sensitivity [124]. Thus, this region is not involved in digitalis affinity differentiation.

The amino acid sequence in the H1–H2 junction, especially the two border residues, have been documented to be the determinant of the differences in digitalis sensitivity known for Na^+/K^+-ATPase preparations from human, pig, and sheep origin on the one hand, or from rodent origin on the other [61,64,113,115–119,121–124,138]. For any digitalis-type inhibitor, the steroid lead, 5β,14β-androstane, is the determinant for sensing the difference in the digitalis affinity of Na^+/K^+-ATPase isoenzymes. This is shown in *Table 4.1* for the isoenzymes from cardiac muscle of man and guinea-pig. Moreover, perusal of the data in *Table 4.2* and *Table 4.3* shows that the attachment of a lactone and sugar side-chain on the steroid lead does not alter the defined role of the steroid nucleus, such that these substituents evidently are not involved in sensing the isoenzyme distinctions in digitalis sensitivity.

As affinity is a function of complementarity [139], the inescapable conclusion appears to be that the amino acids of the H1–H2 junction form the steroid binding subsite. Specifically, the negatively charged chain of the three glutamic acid residues 115–117 (*Figure 4.5*) may constitute some binding partners of the positively charged surface of the steroid skeleton (*Figure 4.7*).

CHEMOTOPOGRAPHY OF THE BINDING SITE FOR SIDE-CHAINS AT C-17

The lactone moiety at C-17β of cardiotonic steroids has long been believed to be responsible for the inotropic activity [140] by eliciting the inhibitory conformation change of the receptor enzyme [127]. Indeed, it is the structural feature most distinct from hormonal steroids. Moreover, the interaction energy contribution of the butenolide or pentadienolide side-chain (−20.5 or −27.6 kJ/mol, respectively) to the composite interaction energy of cardiotonic steroids is almost as great or even greater than that of the steroid lead (−22.7 kJ/mol) [88,89,128]. Understandably, during the past five decades several attempts have been made to ascribe the characteristic actions of digitalis compounds to their lactone moiety by studying the properties of simple α,β-unsaturated lactones [140–152]. In the course of these endeavours, the triggering of positive inotropy or its absence was traced back to peroxide generation and Michael addition reactivity of the lactones [140,141,144,146–150] or to their lack of such reactivity [142,143,145,150,152]. No Michael addition reactions were observed with cardenolides [150], nor apparently with a number of analogues in which the butenolide moiety was replaced by cyclic Michael acceptor systems [151].

The mentioned differences in chemical reactivity appeared to be related to the absence or presence of bulky substituents at the C=C double bond.

Even the most elaborate imitation of cardenolide structure, realized in 3-(*trans*-2-hydroxycyclohexylmethyl)but-2-enolide, which has all the structural elements considered to be essential for the cardenolide action (i.e. the butenolide at 'C-17' and the hydroxyl at 'C-14') failed to elicit inotropy [152]. This finding together with the above-mentioned data leads to the conclusion that separate lactone structures have no pre-existing binding site in the digitalis binding matrix of the receptor protein. As a partial structure in cardenolides, the considerable contribution of the lactone moiety to the composite interaction energy [88,89,128] appears then to require initially the shaping of its binding site through a conformation change induced by the steroid moiety [88,90].

The long- and medium-range attractive forces between the digitalis binding domain and cardenolides or bufadienolides may be viewed as being controlled by the molecular electrostatic potential of the digitalis compounds. In digitoxigenin (*Figure 4.7*), the most evident feature is the essentially dipolar character of the molecule. The nodal line separates the circumscribed negative potential well surrounding the butenolide side-chain and the extended positive region surrounding the steroid nucleus.

The distribution of electrostatic potential fields between receptor and ligand may be assumed to orientate the approaching digitalis molecule towards the small mouth of the receptor-binding cleft thus helping to pull the molecule, with the lactone ring ahead, into the cleft [35,128]. The receptor potential field may hence provide an 'entropy sink' by reducing the translational and rotational entropy of the attacking inhibitor.

The deduced preselection of the dipolar species in the electric field of the receptor may be assumed to enormously accelerate the diffusional approach of dipolar ligands [153]. The resulting formation of a diffusional encounter complex (*Figure 4.8*) represents the initial step in molecular recognition and selection which is followed by a sequence of consecutive steps, during which more and more substructures make contact with the subsites of the receptor cleft, which eventually may fully encompass an inhibitor molecule when being complementary.

The goodness of the fit between digitoxigenin or bufalin (carrying a butenolide or pentadienolide substituent at C-17β, respectively) and the binding subsites for steroid nucleus plus lactone side-chain emerges from the close similarity of the measured interaction energies, −43.2 or −50.3 kJ/mol (cf. (10) and (11) in *Table 4.2*), to the theoretical maximum, −52.7 or −55.1 kJ/mol, respectively, calculated by the method described by Andrews *et al.* [132].

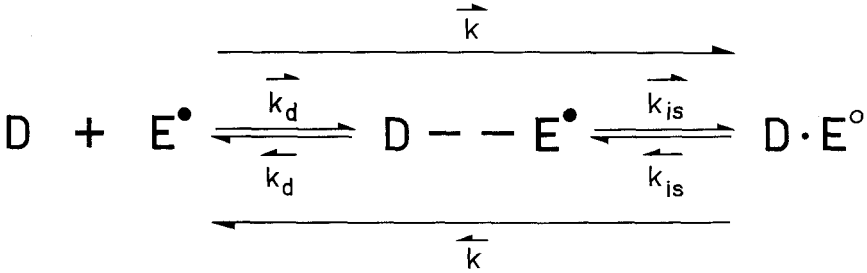


Figure 4.8. Scheme of the two steps in the reversible formation of the inhibited digitalis complex, in which D is digitalis, E° the enzyme in the receptive state, $D\text{–}E^{\circ}$ the uninhibited diffusive complex, $D{\cdot}E^{\circ}$ the inhibited isomerized complex, $k_d^{\rightarrow}$ and $k_d^{\leftarrow}$ diffusion rate constants, $k_{is}^{\rightarrow}$ and $k_{is}^{\leftarrow}$ isomerization rate constants, and $k^{\rightarrow}$ and $k^{\leftarrow}$ the observed composite forward or reverse rate constants. Compared with the diffusional rate constant of slowly diffusing reactants, lying near 10^9 $M^{-1}{\cdot}s^{-1}$, the $k^{\rightarrow}$ values were found by 4 to 6 orders of ten smaller, and the $k^{\leftarrow}$ values by 5 to 7 orders smaller [90], but close to the isomerization constant characterizing the conformational change in enzyme–substrate interactions ranging from 10^2 s^{-1} to 10^4 s^{-1}. A slow rate of the formation of the inhibited complex $D{\cdot}E^{\circ}$ appears to result partly also from the rare formation of a productive diffusive complex with the enzyme in the receptive intermediary state, which traps the digitalis molecule in the appropriate rotational state and spatial disposition upon the binding matrix to isomerize into the inhibited complex.

One of the first steps in this process appears to be hydrogen bonding. The capacity of the carbonyl oxygen in the lactone moiety to serve as hydrogen bond acceptor was originally deduced from physicochemical model studies [163,164]. This is now well supported by the description of deep negative potential walls around the carbonyl and bridge oxygens (*Figure 4.7*) and of the marked electronegativity of both oxygens [35,165]. Recent calculations have shown that such oxygens can accept up to two hydrogen bonds which would increase the directionality of bond formation [166]. The special role of the lactone moiety in hydrogen bonding emerges also from the findings (cf. *Table 4.2*) that (with the exception of OH: 1→2, OCOMe: 1→3, or COMe: 1→4) all substituents at C-17β (1→5...11) increase the interaction energy, but that the highest increments are provided by the butenolide (1→10) and pentadienolide rings (1→11), which hence demarcate best the chemotopography of the lactone binding subsite.

The energetic contribution of the pentadienolide ring is but partially replaced by a pyridine (11→7) or a pyridazine ring (11→8), although their nitrogen atoms can likewise serve as hydrogen bond acceptors due to their electronegativity and well-suited potential minima [165,167]. However, the conversion of the pyridyl into a pyridonyl substituent (7→12) reduces the

Table 4.2. IMPACT OF CHANGES IN THE SIDE-CHAIN AT C-17 OF C/D-*cis* STEROIDS OF THE DIGITALIS TYPE ON THE APPARENT GIBBS ENERGY OF INTERACTION ($\Delta G^{o'}$) WITH NA^+/K^+-ATPase PREPARATIONS FROM HUMAN HEART (HH), HUMAN BRAIN (HB), AND GUINEA-PIG HEART (GH): ENERGETIC CONSEQUENCES OF VARIATIONS IN CONSTITUTION, CONFIGURATION, AND CONFORMATION

No.	*Systematic (trivial) name of inhibitor [reference to synthetic procedure]*	$-\Delta G^{o'}$ *(kJ/mol)* *HH*	*HB*	*GH*
1	5β,14β-Androstane-3β,14-diol [135]	22.7	21.8	18.7*
2	5β,14β-Androstane-3β,14,17β-triol	22.2*	22.2*	15.6*
3	5β,14β-Androstane-3β,14,17β-triol 17-acetate [135]	21.6*	20.7*	16.4*
4	3β,14-Dihydroxy-5β,14β-pregnan-20-one		21.0*	
5	17β-(*N*-Methylmaleimid-3′-yl)-5β,14β-androstane-3β,14,16β-triol 3,16-diacetate	24.8*		
6	17β-(Fur-3′-yl)-5β,14β-androstane-3β,14-diol 3-acetate [136]	40.4	41.1	32.4*
7	17β-(Pyrid-3′-yl)-14β-androst-4-ene-3β,14-diol [154]	40.9	40.5	34.8
8	17β-(Pyridazin-4′-yl)-5β,14β-androstane-3β,14-diol 3-acetate	41.9	39.9	
9	17β-(2′,5′-Dihydro-2′-oxothien-4′-yl)-5β,14β-androstane-3β,14-diol 3-acetate	43.0	41.1	
10	3β,14-Dihydroxy-5β,14β-card-20(22)-enolide (digitoxigenin)	43.2	42.0	34.0
11	3β,14-Dihydroxy-5β,14β-bufa-20,22-dienolide (bufalin)	50.3	48.9	43.0
12	17β-(Pyrid-2′-on-3′-yl)-14β-androst-4-ene-3β,14-diol	<<17.9*	<<21.1*	
13	3β,14-Dihydroxy-24-nor-5β,14β-chol-20(22)-ene-21,23-lactone	30.9		
14	3β-(β-D-Glucosyloxy)-14-hydroxy-24-nor-5β,14β-chol-20(22)-ene-21,23-lactone (actodigin)	34.1	34.0	22.5
15	(23*S*)-23-Methylactodigin	34.6	32.3	23.1*
16	(23*R*)-23-Methylactodigin	38.3	36.4	26.1*
17	(20*R*)-3β,14-Dihydroxy-21-nor-5β,14β-chol-22-ene-24,20-lactone [155]	28.6	29.4	22.1*
18	(20*S*)-3β,14-Dihydroxy-21-nor-5β,14β-chol-22-ene-24,20-lactone [155]	23.9*	23.3*	25.5*
19	(20*S*)-20,22-Dihydrodigitoxigenin	33.5	32.2	24.2*
20	(20*R*)-20,22-Dihydrodigitoxigenin	31.3	30.4	22.9*
21	(21*R*)-21-Fluoro-3β,14,16β-trihydroxy-5β,14β-card-20(22)-enolide ((21*R*)-21-fluorogitoxigenin)		35.7	
22	(21*S*)-21-Fluorogitoxigenin		31.6	
23	(21*R*)-21-Methyldigitoxigenin 3-acetate [156]	32.7	33.1	27.0*
24	(21*S*)-21-Methyldigitoxigenin 3-acetate [156]	27.8	28.2	22.4*
25	22-Methyldigitoxigenin 3-acetate [157]	39.2	36.2	30.5
26	22-Methyl-3β-*O*-(β-D-tridigitoxosyl)-digitoxigenin (22-methyldigitoxin)	45.1	41.8	35.6
27	12β,14-Dihydroxy-22-methyl-3β-(β-D-tridigitoxosyloxy)-5β,14β-card-20(22)-enolide (22-methyldigoxin)	42.2	40.9	33.3

Table 4.2. (continued)

No.	*Systematic (trivial) name of inhibitor [reference to synthetic procedure]*	$-\Delta G^{o'}$ *(kJ/mol)* HH	HB	GH
28	22-Hydroxydigitoxigenin 3-acetate [158]	38.4	37.7	32.4
29	22-Hydroxydigitoxigenin 3,22-diacetate [158]	40.4	39.3	32.0
30	22-Hydroxydigitoxigenin 3-acetate 22-propionate [158]	39.9	38.7	31.0
31	22-Methoxydigitoxigenin 3-acetate [158]	31.7	30.0	24.8
32	22-Allyldigitoxigenin [159]	28.0	27.4	
33	22-Propyldigitoxigenin [159]	25.8	24.9	
34	(21*R*)-3β-Acetoxy-16β,24-epoxy-14-hydroxy-21-isopropyl- 5β,14β-card-20(22)-enolide			24.2*
35	(*E*)-3β-Acetoxy-14-hydroxy-5β,14β-card-17(20)-enolide			21.1*
36	(*Z*)-3β-Acetoxy-14-hydroxy-5β,14β-card-17(20)-enolide			19.8*
37	(21*R*)-3β-Acetoxy-14,21-epoxy-5β,14β-card-20(22)-enolide [136]	25.9*	23.8*	<<17.9*
38	3β-Acetoxy-14-hydroxy-5β,14β-pregn-16-ene-21,16-lactone [160]			<<17.9*
39	3β-Acetoxy-14-hydroxy-5β,14β-pregn-17(20)-ene-21,16β-lactone [160]			<<17.9*
40	(*E*)-Methyl 3β,14-dihydroxy-24-nor-5β,14β-chol-20(22)-en-23-oate [172]			33.3
41	3-(3β,14-Dihydroxy-5β,14β-androstan-17β-yl-methyl)but- 2-en-4-olide [174]			23.2*
42	17α-Digitoxigenin [161; 175]			<<17.9*
43	3β,14-Dihydroxy-5β,14β,17α-cardanolide			20.3*
44	3β-Acetoxy-14,17β-dihydroxy-5β,14β,17α-card-20(22)-enolide [162]			20.0*
45	3β,16β-Diacetoxy-14,17β-dihydroxy-5β,14β,17α-card-20(22)-enolide			<<17.9*
46	3β-(β-D-Cymarosyloxy)-5,14-dihydroxy-19-oxo-5β,14β,17α-card-20(22)-enolide (allocymarin)			<20.2*

* Extrapolated value owing to limited solubility.

interaction energy even below that of the steroid lead (1→12), probably indicating a strong repelling effect of unpaired hydrogen bonding partners.

The strong distance and angular dependence of the energy from hydrogen bonding [166] may help to account for the great changes of Gibbs energy contribution arising from the butenolide moiety, when the spatial disposition of the carbonyl oxygen is altered (cf. *Figure 4.9*), for example, through turning its attachment to C-17β of the steroid nucleus from the C-20-position (as in digitoxigenin: 10 in *Table 4.2*) to the C-22-position (as in actodigin genin or congeners: 13...16 in *Table 4.2*) or the C-21-position (as in [20*R*]- or [20*S*]-3β,14-dihydroxy-21-nor-5β,14β-chol-22-en-24,20-lactone: 17 and 18 in *Table 4.2*).

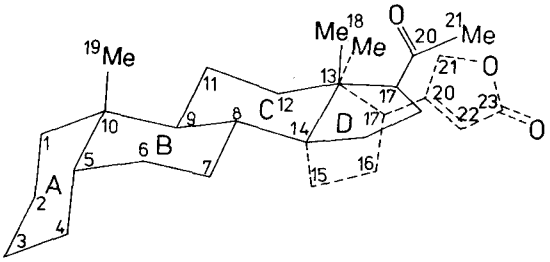


*Figure 4.9. Impact of the transition from C/D-*cis *junction (dashed line) to C/D-*trans *junction (solid line) on the spatial disposition of the side-chain at C-17.*

In this order, the contribution of the butenolide group to the composite interaction energy, expressed in kJ/mol, drops from −20.5 to −8.2, −5.9, or −1.2, respectively (cf. *Table 4.2*). This stepwise decrease may be caused partially by increasingly unfavourable changes of hydrogen bond length and angle. However, the observed maximum loss of −19.3 kJ/mol is much higher than is accountable from the annihilation of one hydrogen bond, the interaction energy of which may range between −2.1 and −7.5 kJ/mol [168].** The difference could partially be explained by a simultaneous change of the carbonyl oxygen from a paired into an unpaired hydrogen bond acceptor, which could reduce the binding energy by 2.1 – 6.3 kJ/mol [169].

The electrostatic properties of the bridge oxygen in the lactone moiety and the general hydrogen bonding characteristics mentioned above suggest that it may also be effective as hydrogen bond acceptor (cf. *Table 4.2*). This seems to be supported by the finding that the transformation of the butenolide moiety of digitoxigenin into a furan group (10→6) lowers the interaction energy contribution by only 2.8 kJ/mol [88,89], which numerically corresponds approximately to the annihilation of one hydrogen bond. Remarkably, the replacement of the bridge oxygen by NH considerably weakens the inhibitory efficacy [151]. On the other hand, a sulphur bridge in exchange for the oxygen bridge (10→9) does not alter the interaction energy contribution of the side-chain [89], as sulphur can likewise serve as hydrogen bond acceptor (cf. [168]).

** The range of observed (not the intrinsic) values may be accounted for by differences in the gain of entropy on the accompanying release of protein-bound and ligand-bound water into bulk solution, through which hydrogen bond formation is an energetically favourable process [169].

Reduction of the C-20 – C-22 double bond in the butenolide substituent, accompanied by bending of the ring plane about that bond and thus by changes of the spatial disposition of the ring oxygens, decreases the interaction energy contribution by −9.7 kJ/mol (20*S* isomer) or −11.9 kJ/mol (20*R* isomer) (see *Table 4.2*: 10→19 or 10→20). The interaction energy losses may result partly from transforming paired hydrogen bond accepting oxygens into unpaired ones, and partly from steric hindrances.

In-depth interpretation of the relation between the hydrogen bond stereochemistry and energy of interaction with the lactone binding site requires consideration of the conformational freedom of the cardenolides. The torsional energy barrier for the rotation of the butenolide side-chain about the C-17 – C-20 bond appears to be low compared with the energy of interaction between cardenolides and the receptor [170,171]. Hence, the close proximity of the cardenolide molecule to the asymmetric potential field, determining the stereospecificity of the digitalis binding site, must express itself in the conformation assumed by the cardenolide molecule in the receptor-bound state [170].

Bulkier substituents at C-21 or C-22 of the butenolide ring can be assumed to favour its occupation of the 14,22- or 14,21-conformation, respectively, in which the C-22 proton or the C-21 protons, respectively, are placed in potential energy minima near C-14β-OH [170]. At C-21, a methyl group (23 and 24 in *Table 4.2*) strongly depresses the interaction energy, whereas at C-22 a methyl group (25...27), acetoxy group (29), or propionyloxy group (30) only reduces the $\Delta G^{o'}$ value a little. These differential responses favour the conclusion that the 14,21-conformation is preferred in the receptor cleft. Cardenolide derivatives, in which the butenolide moiety and the steroid lead are covalently immobilized in different geometric dispositions, show a very much reduced interaction energy (*Table 4.2*: 34...39). To yield unequivocal information, rigid analogues would be required that are as active as the most potent parent compound [35]. At the present stage of exploration, the data suggest that the development of high interaction energies requires a certain degree of conformational freedom so that the digitalis compounds can assume within the allowed range the most favourable 14,21-conformational position in the receptor-bound state.

Replacement of the butenolide or pentadienolide moiety of cardenolides or bufadienolides by open side-chains with an electron-rich heteroatom and a conjugated C=C bond may at best contribute similarly high interaction energies (*Table 4.2*: 40, and [88,89,128,172]). The energetic contribution of a MeCHOH– side-chain at C-17β of 14-amino-3β-rhamnosyloxy-5β,14β-pregnan-20-ol is with 20α-ol by −11.9 kJ/mol greater than with 20β-ol [173].

This geometric dependence points to the possibility that only 20α-ol is involved in favourable hydrogen bonding possibly donating one hydrogen bond or accepting two hydrogen bonds (cf. [166]).

The significance of hydrogen bonding in the bimolecular recognition between digitalis-like inhibitors and Na^+/K^+-ATPase appears to be now generally accepted [19,128,165,171]. It may well be that all active representatives have their hydrogen bond accepting moieties pointing in the same direction, whereas less active compounds have their hydrogen bonding group pointing away from the putative hydrogen donor amino acid [171]. Hence, the spatial disposition of the hydrogen accepting atoms may allow the nature of the hydrogen donating amino acid side-chain to be identified.

Thomas *et al.* [19] suggested that, besides the hydrogen bond, an ion-dipole interaction is involved in the binding of the lactone moiety to its receptor site. The focus of it was supposed to be a fractional positive charge at C-20. However, in CNDO calculations Bohl and Süssmilch [165] found only small positive or negative charges at C-20 of the butenolide or pentadienolide ring, and concluded that a significant electronic contribution of this atom to the binding of cardenolides and bufadienolides appears questionable. On the other hand, by molecular orbital calculations using butadiene as a transferable model, Scrocco and Tomasi [134] showed that the C=C group is characterized by a large zone of strong positive charge lying in the 'butenolide plane' (cf. Figure 3 and Figure 10 in [134]). This charge may thus be involved in a two-point attachment of the lactone side-chain to its site.

This interpretation appears to be complemented by the findings that the hydroxy carbonic acid of digitoxigenin, formed by saponification of the lactone moiety, and the similar derivative with $-CH=CH-COO^-$ at C-17β are virtually inactive [19]. This may be assumed to result from the presence of a repellant negative charge in the lactone binding site, which could represent the counter ion in the two-point attachment suggested by Thomas *et al.* [19].

The size and shape of the lactone binding site appears to be essentially delineated by the butenolide and pentadienolide side-chains at C-17β since they evolve the highest interaction energy contributions (*Table 4.2*). This interpretation may also apply to open-chain substituents at C-17β such as the methyl β-steroidal crotonates (40 in *Table 4.2*) which, through rotation about the C-22 – C-23 bond, can assume a butenolide-like shape and thus contribute a similarly high interaction energy [172]. However, α,β-unsaturated esters with primary alcohols of increasing chain length show an increasing loss of potency [19].

Changes in the spatial orientation of the butenolide side-chain resulting

from insertion of a methylene group between C-17 and C-20 (41 in *Table 4.2*) [174], or from displacement of the butenolide residue from C-17β- to C-17α-position (42..46 in *Table 4.2*) [175], all reduce virtually to zero the interaction energy increments of the side-chains in these cardenolide derivatives. Apparently, this is the outcome of increasing difficulties in their long-range recognition by Na^+/K^+-ATPase and in their capacity to slip into the binding cleft and to form the diffusional encounter complex (cf. *Figure 4.8*).

IDENTIFICATION OF THE HYDROGEN-DONATING AMINO ACID INVOLVED IN LACTONE GROUP BINDING

As derived above, the steroid binding subsite is located in the H1–H2 junction including the glutamic acid residues 115–117 (cf. *Figure 4.5*). This clearly points to the location of the lactone group at C-17β of the steroid skeleton in the appropriate neighbourhood. All the evidence presented in the last paragraph favour the conclusion that the hydrogen bond-accepting oxygen atoms play a prominent role in the binding of the lactone moiety to its subsite. The postulated two hydrogens could be donated by the amide portion of glutamine 119 or asparagine 122. Due to the involvement of an ionic partner, this linkage may be assessed to be a particularly strong hydrogen bond [134], as required according to the interaction energy contribution by the lactone group. The strong positive charge calculated as being localized in the butenolide plane could have its countercharge in the negatively charged aspartate residue 121, which has been shown to be intimately involved in ouabain binding [121].

CHEMOTOPOGRAPHY OF THE BINDING SITE FOR SUGAR SIDE-CHAINS AT C-3β-OH

The significance of the carbohydrate moiety for the biological efficacy of cardiac glycosides has been widely documented, but the separate rare sugars forming the chain have as such not been reported to develop a comparable action. Specifically, rhamnose and digitoxose alone do not appear to bind to Na^+/K^+-ATPase, since they fail to inhibit enzymatic activity and to suppress glycoside binding [50]. Apparently, the sugar-binding site does not pre-exist, but is formed in the process of cardiac glycoside binding. The molecular modelling of the sugar chain's contribution to the effective recognition by the digitalis binding site of Na^+/K^+-ATPase requires first the decision as to which part of the saccharide chain is directly involved.

Analysis of interaction energies (1...7 in *Table 4.3*) shows that the usual

Table 4.3. IMPACT OF CHANGES IN THE SIDE-CHAIN AT C-3 OF C/D-*cis* STEROIDS OF THE DIGITALIS TYPE ON THE APPARENT GIBBS ENERGY OF INTERACTION ($\Delta G^{o'}$) WITH NA/K-ATPase PREPARATIONS FROM HUMAN HEART (HH), HUMAN BRAIN CORTEX (HB), AND GUINEA-PIG HEART (GH): ENERGETIC CONSEQUENCES OF VARIATIONS IN THE STRUCTURE OF AGLYCONE OR THE COMPOSITION, CONFIGURATION, CONNECTIVITY, AND LENGTH OF THE SIDE-CHAIN.

No.	*Systematic (trivial) name of inhibitor*	$-\Delta G^{o'}$ *(kJ/mol)*		
		HH	*HB*	*GH*
1	3β,14-Dihydroxy-5β,14β-card-20(22)-enolide (digitoxigenin)	43.2	42.0	34.0
2	3β-*O*-(β-D-Digitoxosyl)digitoxigenin	49.2	48.0	41.1
3	3β-*O*-(β-D-Tridigitoxosyl)digitoxigenin (digitoxin)	48.1	46.8	39.9
4	3β,12β,14-Trihydroxy-5β,14β-card-20(22)-enolide (digoxigenin)	39.7	37.9	29.0
5	3β-*O*-(β-D-Digitoxosyl)digoxigenin			39.2
6	3β-*O*-(β-D-Didigitoxosyl)digoxigenin			35.9
7	3β-*O*-(β-D-Tridigitoxosyl)digoxigenin (digoxin)	46.6	43.0	37.2
8	3β-*O*-(α-D-Rhamnosyl)digitoxigenin			30.5
9	Digitoxigenin 3-tetrahydropyranyl ether			35.6
10	3β-*O*-(β-D-Glucosyl)digitoxigenin	48.1	46.1	35.6
11	3β-*O*-(α-L-Arabinofuranosyl)digitoxigenin	46.1	43.9	36.4
12	3β-*O*-(α-D-Digitoxosyl)digitoxigenin			36.6
13	3β-*O*-(β-D-Rhamnosyl)digitoxigenin			37.1
14	3β-*O*-(D-Ribosyl)digitoxigenin			37.2
15	3β-*O*-(α-L-Thevetosyl)digitoxigenin (neriifolin)			39.8
16	3β-*O*-(3′-*O*-Methyl-α-L-rhamnosyl)digitoxigenin	48.3	49.7	40.7
17	3β-*O*-(α-L-Rhamnosyl)digitoxigenin (evomonoside)	50.4	48.5	41.1
18	3β-*O*-(β-D-Cymarosyl)digitoxigenin			41.6
19	3β-*O*-(4′-Dehydro-2′,3′-*O*-isopropylidene-α-L-rhamnosyl)digitoxigenin	47.0	46.1	41.6
20	3β-*O*-(2′,3′-*O*-Isopropylidene-α-L-rhamnosyl)-digitoxigenin	48.1	47.5	41.8
21	3β-*O*-(4′ξ-Amino-4′-deoxy-α-L-rhamnosyl)-digitoxigenin	52.2	50.5	45.8
22	3β,5,14-Trihydroxy-19-oxo-5β,14β-card-20(22)-enolide (strophanthidin)			30.7
23	3β-*O*-(D-Ribosyl)strophanthidin			30.8
24	3β-*O*-(β-D-Rhamnosyl)strophanthidin			32.7
25	3β-*O*-(β-D-Cymarosyl)strophanthidin (cymarin)			33.8
26	3β-*O*-(β-D-Digitoxosyl)strophanthidin (helveticoside)			34.8
27	3β-*O*-(α-D-Rhamnosyl)strophanthidin			35.4
28	3β-*O*-(α-L-Rhamnosyl)strophanthidin (convallatoxin)			36.8
29	3β-*O*-(α-D-Mannosyl)strophanthidin			29.6
30	Strophanthidin 3-tetrahydropyranyl ether			29.4
31	3β-*O*-(α-L-Mannosyl)strophanthidin			31.0

Table 4.3. (continued)

No.	*Systematic (trivial) name of inhibitor*	$-\Delta G^{o'}$ *(kJ/mol)*		
		HH	*HB*	*GH*
32	1β,3β,5,11α,14,19-Hexahydroxy-5β,14β-card-20(22)-enolide (ouabagenin)	37.6	34.4	32.1
33	3β-*O*-(α-L-Rhamnosyl)ouabagenin (ouabain)	44.9	44.4	36.2
34	3α-Methyldigitoxigenin	36.5	35.6	28.2
35	3β-*O*-(β-D-Glucosyl)-3α-methyl-digitoxigenin	36.7	36.3	29.0
36	3β-O-(α-L-Rhamnosyl)-3α-methyl-digitoxigenin	37.2	35.0	27.3*
37	(21*S*)-21-Methyldigitoxigenin 3-acetate	27.8	28.2	22.4*
38	(21*S*)-3β-*O*-(β-D-Glucosyl)-21-methyldigitoxigenin	33.7	33.2	23.3*
39	(21*R*)-21-Methyldigitoxigenin 3-acetate	32.7	33.1	27.0*
40	(21*R*)-3β-*O*-(β-D-Glucosyl)-21-methyldigitoxigenin	37.1	36.6	27.8

*Extrapolated value owing to limited solubility

order of decreasing $\Delta G^{o'}$ values is as follows: aglycone-monosaccharide > aglycone-disaccharide > aglycone-trisaccharide >>> aglycone. This gradation indicates that only the proximate sugar provides atom groupings for an attractive interaction with the sugar binding site. The decrease of the $\Delta G^{o'}$ values by the additional sugars may result from the increase of rotational and translational entropies.

In line with the above reasoning, studies with digitoxigenin-monodigitoxoside, digitoxigenin-didigitoxoside, and digitoxigenin-tridigitoxoside, which carried a chemically reactive substituent at 4′-OH, 4″-OH, or 4‴-OH, respectively, showed that the catalytic peptide of Na^+/K^+-ATPase is nearly exclusively labelled with the 4′-derivative and decreasingly less labelled with the 4″- and 4‴-derivatives because of the increasing distances from the steroid nucleus [176].

Taken together, the findings suggest that: (a) the terminal and middle digitoxose remain outside the digitalis recognition matrix; (b) the sugar next to the steroid nucleus is positioned near the mouth of a cleft, which becomes formed from the matrix and is closed in the process of glycoside binding; (c) the lactone side-chain is positioned at the bottom of the cleft; (d) the distance between the carbonyl oxygen of the lactone and C4′ of the proximate sugar, about 19Å in 5βH-cardenolides, indicates the length of the cleft [88,128,177].

The following attempts to derive information on the chemotopography of the sugar binding site will hence be confined to the impact of monosides on the interaction energy (*Table 4.3*). The paucity of available data including

but small structural variations restricts modelling attempts to rather narrow limits.

Interpretation of the $\Delta G^{o'}$ values is made more difficult by the two degrees of rotational freedom about the glycosidic bonds which conformational energy calculations suggest exist [171,178]. The conformational flexibility of the sugar residue may help to account for the finding that both β-D- and α-L-sugars with C1 or 1C conformation, respectively, may increase the interaction energy. Actually, some attractive force between the carbohydrate substituent and the sugar binding subsite of Na^+/K^+-ATPase appears to override existing rotational energy barriers and thus to govern the spatial disposition of the sugar hydroxyls. Thus, in the various hexoses, the C-4′ and bridge oxygens seem to border similar fragments of the sugar binding subsite in the receptor cleft [35].

The quantitative effect of monoglycosidation at C-3β-OH depends not only on the sugar structure but also on the origin of the enzyme used for testing. With the enzyme preparation from guinea-pig cardiac muscle and digitoxigenin as aglycone, α-D-rhamnose decreases the interaction energy by 3.5 kJ/mol, whereas all other monoside substituents increase it. The rising order of $-\delta\Delta G^{o'}$ values (in kJ/mol) is: 1.6 for tetrahydropyran (9) and for β-D-glucose (10), 2.4 for α-L-arabinofuranose (11), 2.6 for α-D-digitoxose (12), 3.1 for β-D-rhamnose (13), 3.2 for D-ribose (14), 5.8 for α-L-thevetose (15), 6.7 for 3′-*O*-methyl-α-L-rhamnose (16), 7.1 for α-L-rhamnose (17) and β-D-digitoxose (2), 7.6 for β-D-cymarose (18) and 4′-dehydro-2′,3′-isopropylidene-α-L-rhamnose (19), 7.8 for 2′,3′-*O*-isopropylidene-α-L-rhamnose (20), and 11.8 for 4′-amino-4′-deoxy-α-L-rhamnose (21).

With the enzyme preparation from human cardiac muscle and digitoxigenin as aglycone, the order of $-\delta\Delta G^{o'}$ increments is, as far as estimated, in parts distinctly different: 2.9 for α-L-arabinofuranose (11), 3.8 for 4′-dehydro-2′,3′-isopropylidene-α-L-rhamnose (19) and 2′,3′-*O*-isopropylidene-α-L-rhamnose (20), 4.9 for β-D-glucose (10), 5.1 for 3′-*O*-methyl-α-L-rhamnose (16), 6.0 for β-D-digitoxose (2), 7.2 for α-L-rhamnose (17), and 9.0 for 4′-amino-4′-deoxy-α-L-rhamnose (21).

A comparison of the $-\delta\Delta G^{o'}$ values reveals that β-D-glucose and 2′,3′-isopropylidene-α-L-rhamnose effect quite different interaction energy increments with the isoenzymes from guinea-pig and man. This indicates some species differences in the chemotopography of lining amino acid residues in the sugar binding subsite. On the other hand, α-L-rhamnose and its 4′-amino-4′-deoxy derivative provide the highest interaction energy increments with both isoenzymes. In context with the whole set of the above data, this invariance appears to mean that the sugar-binding subsite typically interacts best with 4′-OH or 4′-NH_2 in α-L-rhamnose by providing

acceptor groups for hydrogen bond donating amino acid chains. This view is supported by the additional findings reported below.

The impact of glycosidation at 3β-OH of strophanthidin (22) on the interaction energy with the isoenzymes from guinea-pig cardiac muscle is somewhat modified by the presence of 5β-OH and 19-CHO. This is shown by the following order of increasing $-\delta\Delta G^{o'}$ values (in kJ/mol): 0.1 for D-ribose (23), 2.0 for β-D-rhamnose (24), 3.1 for β-D-cymarose (25), 4.1 for β-D-digitoxose (26), 4.7 for α-D-rhamnose (27), and 6.1 for α-L-rhamnose (28). Remarkably, the interaction energy becomes depressed through glycosidation by 1.1 for α-D-mannose (29), by 1.6 for tetrahydropyran (30), and by 5.6 for α-L-mannose (31). The presence of a methyl group in 3α-position of digitoxigenin annuls the usual strong increase of the $\Delta G^{o'}$ value by glycosidation at 3β-OH with β-D-glucose (34→35) or α-L-rhamnose (34→36). Apparently, neighbouring substituents change the conformational freedom of the sugar and its spatial orientation to the sugar binding subsite. Methyl groups on C-21 of the butenolide moiety, however, do not influence the interaction energy contribution of 3β-OH glycosidation (cf. 1→10 with 37→38 and 39→40).In conclusion, all highly potent cardenolide monosaccharides have α-L-rhamnose or its 4′-amino-4′-deoxy derivative as the sugar component. Since NH_2 serves better than OH at C-4′ in generating the high interaction energy, a hydrogen bond accepting reaction with an acid proton appears to underlie the maximum efficacy. Because this involves an acid partner, that linkage would be a particularly strong hydrogen bond [134]. The presence of a carboxyl side-chain in the sugar subsite is suggested by the finding that, through electrostatic repulsion, acid substituents at 3β-OH of cardenolides as glucuronide, sulphate [48], or dicarbonic acid esters [19] decrease or eliminate the activity of the parent compound.

IDENTIFICATION OF THE AMINO ACID SEQUENCE FORMING THE MONOSIDE BINDING SUBSITE

An energy transfer experiment with anthroylouabain, in which the rhamnose side-chain of ouabain was esterified at C-3′-OH with anthracene-9-carboxylic acid, indicated that a tryptophan residue is located near the mouth of the cardiac glycoside binding cleft [120]. A tryptophan residue is not present in the H1–H2 junction, but is a constituent of the H3–H4 junction, i.e. Trp 310 (cf. *Figure 4.5*). This means that the monoside binding subsite in the cleft is formed by the H3–H4 amino acid sequence which includes the glutamic acid residue 307 and the tyrosine residue 308. Both carry acid protons [179] able to be donated to the hydrogen bond-accepting 4′-OH or 4′-NH_2 in the monoside substituent of cardiac glycosides as

deduced above. If the proposed alignment of the steroid nucleus and the lactone side-chain in the H1–H2 junction, and the alignment of the monoside substituent in the H3–H4 junction apply, it follows that the receptor cleft closure implies the folding of the H1–H2 junction over the H3–H4 junction to form a three-dimensional assembly enveloping a tripartite digitalis compound.

The intrinsic inhibitory activity of 5β,14β-androstane-3β,14-diol, the pharmacophoric lead structure in cardiac glycosides, is not different from unity [88,129], although it cannot occupy the lactone and the monoside binding subsites in the H1–H2 or H3–H4 junctions, respectively. This indicates that the steroid lead alone by occupying its subsite triggers its folding over the H3–H4 junction. Only the latter directly communicates through the membrane-penetrating H4 amino acid sequence with the sequence which forms the catalytic centre and carries the phosphorylatable aspartyl residue 369 (cf. *Figure 4.5*). Taken together, these interconnections mean that the steroid lead-triggered cleft closure is associated with the change of catalytic centre geometry, which underlies the drastic reduction of ATP affinity [180], and hence represents the microscopic mechanism of Na^+/K^+-ATPase inhibition. These primary events may induce the formation of large aggregates of enzyme molecules [181].

TOPOGRAPHICAL CONNECTION BETWEEN THE BINDING SITES FOR THE STEROID LEAD, THE LACTONE, AND MONOSIDE SUBSTITUENTS

As derived in the foregoing sections, the steroid lead triggers the closure of the recognition cleft in Na^+/K^+-ATPase and thus induces the formation of the lactone and sugar binding sites. This link holds firmly true only for the cardiac glycosides with 5β,14β-androstane-3β,14-diol as the pharmacophoric lead structure. Due to the snug fit with the juxtapositioned peptide sequences, the rigid skeleton of 5β,14β-androstane-3β,14-diol determines the spatial disposition of the 17β-lactone and 3β-*O*-substituents in the cleft. This structural connection explains the finding that the interaction Gibbs energy of the tripartite digitalis glycosides is the sum of the energetic contributions of the substructures [88,89,128]. The strict additivity requires that the three binding subsites are formed by tightly coupled peptide sequences. An interesting analogy from organic-chemical studies on molecular recognition is offered by the co-operative response displayed by binding sites in the formation of organic host-guest complexes portrayed in *Figure 4.10*.

A Free-Wilson analysis of 95 digitalis-like inhibitory-acting compounds concerning the relationship between structure and interaction energy [89]

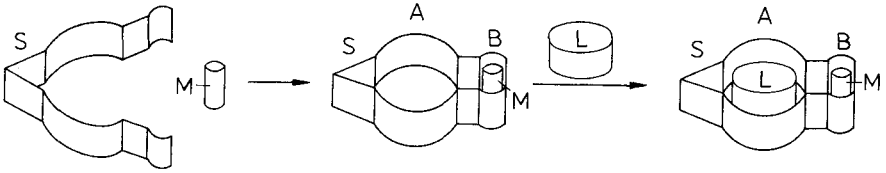


Figure 4.10. Schematic representation of the co-operativity displayed by binding sites in the formation of organic host-guest complexes. S=hinge, M=metal ion, A=apolar binding site, B=polar binding site, L=lipophilic guest molecule. Reproduced from [182].

revealed that the additivity model no longer applies when the *cis*-junctions of rings A/B or C/D are altered (see *Table 4.1*). The bulky substituents at C-3β-*O* or C-17β are then more or less shifted from the sugar or lactone binding subsites, respectively (cf. *Figure 4.6* and *Figure 4.9*), and thus cannot produce their usual interaction energy contribution, but may even diminish down to zero the composite interaction energy [88,89,128]. When a diffusional encounter complex is still formed, the inactivity of those digitalis derivatives will simply result from a conflict between the side-chain and the 'receptor essential volume' (for definition see [101]).

The weakening or even loss of potency in such derivatives of C/D-*trans* steroids has led to the tacit opinion that C/D-*trans* steroids as such cannot elicit inotropic activity and serve as potential cardiac drug candidates. Most remarkably, however, certain steroid representatives with *trans* junction of the rings C and D have recently been demonstrated to evolve similar or even higher interaction energies with human Na^+/K^+-ATPase compared with their C/D-*cis* counterparts [88]. This discovery has prompted studies on the impact of glycosidation on the biological activity of C/D-*trans* steroids in view of their possible suitability to serve as a novel type of pharmacophoric lead structure [22,183,184]. This is evaluated below.

ADDITIONAL CHEMOTOPOGRAPHICAL INFORMATION ON THE STEROID BINDING SUBSITE DERIVED FROM THE INTERACTION ENERGY CHANGES BY SMALL SUBSTITUENTS ON THE STEROID SKELETON

The above-offered interpretation that the interaction of 5β,14β-androstane-3β,14-diol involves a snug fit with its binding subsite has to be qualified by consideration of the impact of various small substituents on the interaction energy (*Table 4.4*). Modifications at C-3 deserve special interest in view of the fact that 3β-OH carries the pharmacologically important sugar component. Surprisingly, most modifications in this place bring about

Table 4.4. IMPACT OF SMALL SUBSTITUENTS AT DIVERSE POSITIONS OF C/D-*cis* STEROIDS OF THE DIGITALIS TYPE ON THE APPARENT GIBBS ENERGY OF INTERACTION ($\Delta G^{o'}$) WITH NA^+/K^+-ATPase PREPARATIONS FROM HUMAN HEART (HH), HUMAN BRAIN CORTEX (HB), AND GUINEA-PIG HEART (GH): ENERGETIC CONSEQUENCES OF OH ESTERIFICATION.

No.	*Systematic (trivial) name of inhibitor [reference to synthetic procedure]*	$-\Delta G^{o'}$ *(kJ/mol)* *HH*	*HB*	*GH*
1	3β,14-Dihydroxy-5β,14β-card-20(22)-enolide (digitoxigenin)	43.2	42.0	34.0
2	3-Deoxydigitoxigenin			32.5
3	3-Dehydrodigitoxigenin			31.9
4	3β,12β,14-Trihydroxy-5β,14β-card-20(22)-enolide (digoxigenin)	39.6	37.9	29.0
5	3-Dehydrodigoxigenin			28.5
6	Digitoxigenin 3-acetate	42.5	41.9	34.4
7	3β,14,16β-Trihydroxy-5β,14β-card-20(22)-enolide (gitoxigenin)	37.4	35.7	27.7
8	Gitoxigenin 3-acetate			30.0
9	Digitoxigenin 3-nitrate			30.7
10	Digitoxigenin 3-benzoate			26.7
11	3β-Amino-3-deoxydigitoxigenin	41.6	39.9	38.1
12	3β,14-Dihydroxy-5α,14β-card-20(22)-enolide (uzarigenin)	38.1	36.7	29.9
13	3β-Amino-3-deoxyuzarigenin		38.0	35.8
14	3β,14-Dihydroxy-5β,14β-bufa-20,22-dienolide (bufalin)	50.3	48.9	43.0
15	3β-Amino-3-deoxybufalin	48.5	47.9	43.1
16	3β-Acetamido-3-deoxydigitoxigenin	42.5		36.9
17	3-Deoxy-3β-hydroxyethylaminodigitoxigenin			36.9
18	3-Deoxy-3β-(n-propanesulphonamido)digitoxigenin	46.1	44.2	39.9
19	3-Epidigitoxigenin	32.5	30.5	26.9
20	3-Epidigoxigenin			20.9*
21	3-Epibufalin			32.3
22	3β,14-Dihydroxy-14β-carda-4,20(22)-dienolide (canarigenin)			33.3
23	3-Epicanarigenin			25.2
24	3-Episarmentogenin			27.7
25	3-Dehydrosarmentogenin			30.6
26	11-Dehydro-3-episarmentogenin			19.8*
27	11-Dehydro-3-episarmentogenin 3-acetate			31.0
28	12-Dehydro-3-epidigoxigenin			<<23.9*
29	12-Dehydro-3-epidigoxigenin 3-acetate			35.4
30	3α-Amino-3-deoxybufalin			24.4
31	3α-Acetamido-3-deoxydigitoxigenin	34.8	30.5	
32	3β,11α,14-Trihydroxy-5β,14β-card-20(22)-enolide (sarmentogenin)	41.1		31.6
33	12-Epidigoxigenin			28.8
34	11-Episarmentogenin			27.0
35	16-Epigitoxigenin	31.0	29.4	26.5*

Table 4.4. (continued)

No.	Systematic (trivial) name of inhibitor [reference to synthetic procedure]	$-\Delta G^{\circ\prime}$ (kJ/mol) HH	HB	GH
36	7β-Hydroxydigitoxigenin			26.1*
37	15β-Hydroxydigitoxigenin			24.3
38	17α-Hydroxydigitoxigenin 3-acetate [162]			22.7*
39	15α-Hydroxydigitoxigenin			16.9*
40	3β,14,16β-Trihydroxy-5β,14β-bufa-20,22-dienolide (deacetylbufotalin)	44.8		38.7
41	3β,11α,14-Trihydroxy-5β,14β-bufa-20,22-dienolide (gamabufotalin)	48.5		38.0
42	16-Epidiginatigenin			17.3*
43	3β-Acetoxy-12β,14,17α-trihydroxy-5β,14β-card-20(22)-enolide			23.1*
44	3β,12β,14,16β-Tetrahydroxy-5β,14β-card-20(22)-enolide (diginatigenin)			25.8*
45	3β-*O*-(β-D-Tridigitoxosyl)digitoxigenin (digitoxin)	48.1	46.8	39.9
46	3β-*O*-(β-D-Tridigitoxosyl)digoxigenin (digoxin)	46.6	43.0	37.2
47	3β-*O*-(β-D-Tridigitoxosyl)gitoxigenin (gitoxin)	44.3	41.3	35.4
48	16-Epigitoxin	36.7	35.2	29.0
49	17α-Hydroxydigitoxin			28.5
50	16-Epidiginatin			27.1
51	3β-*O*-(β-D-Tridigitoxosyl)diginatigenin (diginatin)			31.4
52	Digoxigenin 12-acetate			25.8
53	Digoxigenin 12-nitrate			28.8
54	Digoxin 12-acetate			28.6
55	17α-Acetoxydigitoxin			20.9*
56	3β,14-Dihydroxy-15-oxo-5β,14β-card-20(22)-enolide (15-oxodigitoxigenin)			20.9*
57	3β,5,14-Trihydroxy-19-oxo-5β,14β-card-20(22)-enolide (strophanthidin)			30.7
58	3β,5,14,19-Tetrahydroxy-5β,14β-card-20(22)-enolide (strophanthidol)			34.8
59	3β,5,14-Trihydroxy-19-oxo-5β,14β-bufa-20,22-dienolide (hellebrigenin)			37.6
60	3β,5,14,19-Tetrahydroxy-5β,14β-bufa-20,22-dienolide (hellebrigenol)			39.1
61	3β-*O*-(β-D-Cymarosyl)strophanthidin (cymarin)			33.8
62	3β-*O*-(β-D-Cymarosyl)strophanthidol (cymarol)			38.0
63	Strophanthidol 19-nitrate			27.2*
64	3β-Hydroxy-15-oxo-5β,14β-card-20(22)-enolide			28.3
65	Digitoxigenin 3,14-diacetate			<18.1*
66	14β-Amino-14-deoxydigitoxigenin 3-acetate		28.8	
67	14β-Azido-14-deoxydigitoxigenin 3-acetate [185]		26.9	
68	(20*R*)-3β-(α-L-rhamnosyloxy)-5β,14β-pregnane-14,20-diol		34.3	
69	(20*R*)-14-Amino-3β-(α-L-rhamnosyloxy)-5β,14β-pregnan-20-ol	45.2	45.1	37.4

Table 4.4. (continued)

No.	*Systematic (trivial) name of inhibitor [reference to synthetic procedure]*	$-\Delta G^{o'}$ *(kJ/mol)* *HH*	*HB*	*GH*
70	Gitoxin 16-formate			41.1
71	Gitoxigenin 16-acetate			36.5
72	Gitoxin 16-acetate	43.4		37.4
73	3β-*O*-(α-L-Oleandrosyl)gitoxigenin (deacetyloleandrin)			36.2
74	16β-Acetoxy-14-hydroxy-3β-(α-L-oleandrosyl)-5β,14β-card-20(22)-enolide (oleandrin)	47.3	46.5	42.1
75	Deacetylcinobufagin			30.0
76	16β-Acetoxy-14,15β-epoxy-3β-hydroxy-5β,14β-bufa-20,22-dienolide (cinobufagin)			38.1
77	Gitoxigenin 16-nitrate	47.7	48.7	41.3
78	Gitoxin 16-nitrate			40.0
79	16-Epigitoxigenin 16-acetate			27.8
80	16-Epigitoxin 16-acetate	42.1		33.3
81	16-Epigitoxigenin 16-nitrate			40.9
82	16-Epigitoxin 16-nitrate	47.9	46.6	39.6
83	16-Epigitoxin 16-azidoacetate			28.4
84	16-Epigitoxin 16-aminoacetate			25.3

* Extrapolated value owing to limited solubility.

either small changes or reductions of the interaction energy as a perusal of *Table 4.4* shows.

As exemplified by the impact of structural changes in various aglycones, elimination (1→2) or dehydrogenation (1→3, 4→5) of 3β-OH results in only a small reduction of the $\Delta G^{o'}$ value. Apparently, 3β-OH as such does not play an essential role in the binding of an aglycone to the steroid binding subsite. Esterification of 3β-OH with acetic acid (1→6, 7→8) results in little change, whereas esterification with nitric acid (1→9) and still more with benzoic acid (1→10) significantly reduces the interaction energy. Replacement of 3β-OH by $-NH_2$ (1→11, 12→13, 14→15), by –NH–CO–Me (1→16), $-NH-(CH_2)_2-OH$ (1→17), or $-NH-SO_2-(CH_2)_2-Me$ (1→18) mostly increases but a little the $\Delta G^{o'}$ value, thus confirming the above conclusion.

In all types of aglycones, the conversion of 3β-OH to 3α-OH (1→19, 4→20, 14→21, 22→23) produces a strong reduction of the interaction energy, which becomes diminished by dehydrogenation to 3-keto (19→3, 20→5, 24→25) or even eliminated by esterification (26→27, 28→29). The latter effect appears to be based on the transformation of an unpaired hydrogen bonding candidate into a strong hydrogen bond-forming residue.

Other substituents in 3α-position as an amino (30) or acetamido (31) group depress the $\Delta G^{o'}$ value to a similar level to that found with 3α-OH, so that the same repelling mechanism could underlie the decrease.

Hydroxyl groups at the steroid skeleton are known to determine the type of hormonal action. Thus, the impact of hydroxyl groups in cardiac steroid action demands some consideration. Disappointingly, hydroxylation always reduces the energy of interaction of digitalis compounds with the Na^+/K^+-ATPase preparations from human cardiac muscle and brain cortex as well as from guinea-pig cardiac muscle as numerically differentiated in the following, based on data in *Table 4.4*.

The extent of potency loss differs considerably depending on the location and spatial disposition of the OH groups. Most evaluations are based on the data assembled with the guinea-pig enzyme but, as far as controlled, the basic conclusions also apply to human enzymes. Hydroxylation of digitoxigenin diminishes the $\Delta G^{o'}$ values differently. The numerical values of the decreases (in kJ/mol) span a wide range: 2.4 for 11α-OH (32), 5.0 for 12β-OH (4), 5.2 for 12α-OH (33), 6.3 for 16β-OH (7), 7.0 for 11β-OH (34), 7.5 for 16α-OH (35), 7.9 for 7β-OH (36), 9.7 for 15β-OH (37), 11.3 for 17α-OH (38), and 17.1 for 15α-OH (39). As far as checked, similar reductions of interaction energy are found with other aglycones such as bufalin (14→40, 14→41), digoxigenin (4→42, 4→43), and gitoxigenin (7→44), as well as with glycosides such as digitoxin (45→46, 45→47, 45→48, 45→49) and digoxin (46→50, 46→51).

The detrimental effect of hydroxylation appears to be based on reduction of the positive steroid surface charge and on steric hindrance, at least when esterification produces a further reduction of interaction energy as found for 12β-OH (4→52, 4→53, 46→54) and 17α-OH (49→55). This interpretation seems to be at variance with the findings that dehydrogenation of 11α-OH (24→26) and 15β-OH (37→56) effects a further decrease in $\Delta G^{o'}$. However, this derivatization is associated with some deformation of the rings C or D, which possibly determines the outcome. Reduction of an aldehyde group at C-10 (57→58, 59→60, 61→62) significantly increases the interaction energy, but this effect becomes more than reversed by esterification (58→63).

Most remarkably, 14β-hydroxylation (64→56) reduces the interaction energy by 7.4 kJ/mol. On the other hand, acetylation of 14β-OH (6→65) or replacement of 14β-OH by 14β-NH_2 (6→66) or 14β-N_3 (6→67) depresses the $\Delta G^{o'}$ value by 5.6 or 7.5 kJ/mol, respectively, but do not eliminate the potency. Apparently, 14β-OH is not an indispensable structural feature in digitalis compounds. In line with this reasoning is the finding that in (20*R*)-3β-(α-L-rhamnosyloxy)-5β,14β-pregnane-14,20-diol, replacement of

14β-OH by 14β-NH_2 increases the interaction energy by −10.8 kJ/mol (68→69). The difference in the response by a cardenolide or a pregnane should result from the distinction between the features of the side-chains at carbon 17β, namely bulky-inflexible versus small-flexible. The detrimental energetic effect of hydroxylation, seen in all positions mentioned, suggests a snug fit of the interacting surfaces of the steroid skeleton and steroid binding subsite. This view, however, does not apply in the immediate neighbourhood of C-16. Thus, the detrimental effect of 16β-OH becomes eliminated or even overcompensated by esterification with formic acid (47→70), acetic acid (7→71, 47→72, 73→74, 75→76), or nitric acid (7→77, 47→78). A similar favourable effect has been seen with 16α-OH by esterification with acetic acid (35→79, 48→80) or nitric acid (35→81, 48→82), although in this case the effect appears to be somewhat smaller. Surprisingly, esterification of 16α-OH with azidoacetic acid (48→83) and aminoacetic acid (48→84) does not improve or may even weaken the potency, possibly due to an increasing conflict with the receptor essential volume.

The following conclusions appear to appropriately describe the present state of information: (a) hydroxyl functions at the steroid skeleton do not discriminate between the isoenzymes from human cardiac muscle and brain cortex (see below); (b) the lead structure proper in digitalis compounds is not 5β,14β-androstane-3β,14-diol, but 5β,14β-androstane.

CHEMOTOPOGRAPHICAL INFORMATION DERIVED FROM THERMODYNAMIC PARAMETERS OF DIGITALIS INTERACTION WITH Na^+/K^+-ATPase

With changes of digitalis structure, the values for $\Delta G^{o'}$, $\Delta H^{o'}$, and $\Delta S^{o'}$ (observed with hog cardiac Na^+/K^+-ATPase, which behaves similarly to the human enzyme) vary by as much as −50 kJ/mol, 250 kJ/mol, and 950 J/mol per K, respectively [49,90]. The observed interconnection and the magnitude of these thermodynamic qualities indicate that the thermodynamic denominator of the inhibitory action of digitalis compounds is a relaxation of the conformational energy of the enzyme protein, which has been built up through phosphorylation with ATP. Removal of the lactone and/or sugar side-chain considerably reduces the entropy gain showing that the measured $\Delta S^{o'}$ value results from the interaction of all parts of the tripartite structure with the appropriate subsites. A large entropy gain in the enzyme protein, found irrespective of enzyme origin and ligation, appears to be a common characteristic of the inhibitory action of all digitalis derivatives.

The movement of large protein domains involves a negligible entropy gain [186]. Hence, the formation and closing of the digitalis binding cleft

cannot essentially contribute to the large entropy and enthalpy changes observed, but rather appears to act as a 'switching device' in eliciting the peptide chain rearrangement underlying enzyme inactivation. The digitalis binding matrix, exposed on the external surface of the plasma membrane, and the catalytic centre exposed on the cytoplasmic side, are about 74Å apart, but connected by the H4 membrane-crossing amino acid sequence (cf. *Figure 4.5*). Along this pathway, the digitalis-elicited relaxation of conformational energy appears to be transmitted to the catalytic centre thus effecting here a decrease of ATP affinity [180] and suppression of enzyme phosphorylation with ATP [131,187]. These biochemical correlates explain the loss of the enzyme's capacity to change into a state of high conformational energy as required for its function.

Although the molecular mechanism underlying the inhibitory action of digitalis compounds appears to be basically quite uniform, the Na^+/K^+-ATPase nevertheless emerges also from the thermodynamic analysis as a protein, the digitalis binding matrix of which can, within certain limits, adapt to the moulding interaction with digitalis derivatives of widely different structures. The observed great range of values observed for the equilibrium and activation qualities [49,90] implies a corresponding degree of conformational plasticity which renders more difficult a straightforward three-dimensional modelling of the digitalis alignment in the binding cleft on the basis of the complementarity postulate as initially approached [72,188]. In other words, the results of computer-aided molecular mapping of the digitalis binding matrix, achieved by means of the active analogue approach, are primarily valid for its chemotopography after receptor cleft closure.

As shown elsewhere [35,90], the thermodynamic activation and equilibrium parameters of the interaction between digitalis compounds and Na^+/K^+-ATPase have an essential or even a dominant impact on the development, strength, and decline of their pharmacological actions. Consequently, the determination of the kinetic receptor parameters provides a powerful tool in the targeted research on lead structure optimization to be touched upon in the final section.

APPROACH TO THE CHEMOTOPOLOGICAL DIFFERENCE BETWEEN THE DIGITALIS BINDING MATRIX OF Na^+/K^+-ATPase PREPARATIONS FROM HUMAN CARDIAC MUSCLE AND HUMAN BRAIN CORTEX

As stated by Fersht [189], there are two components of biological specificity: the thermodynamic-equilibrium binding through simple intermolecular interactions and kinetic-control of complex formation through rates. The

major determinant has been suggested as being thermodynamic, but a comparison of the equilibrium Gibbs energies of the interaction between 76 steroid derivatives with Na^+/K^+-ATPase preparations obtained from human cardiac muscle and human brain cortex was rather disappointing [87].

The comparative analysis included the impact of seven major structural modifications (cf. *Tables 4.1–4.4*): (a) flattening of the A/B-ring plane through conversion of A/B-*cis* into A/B-*trans* junction, introduction of the Δ^4 double bond, or aromatization of ring A; (b) flattening of C/D-ring plane through introduction of the Δ^{14} double bond; (c) structural variations at C-3 through introduction of a methyl group in 3α-position, esterification of C-3β-OH, glycosidation at C-3β-OH with various sugars or sugar derivatives and replacement of C-3β-OH by C-3β-(n-propylsulphonamide) or C-3β-NH_2; (d) introduction of hydroxyl groups at very different positions in the steroid skeleton; (e) esterification of C-16β-OH with acetic or nitric acid; (f) substitution of C-17β-H by various side-chains differing in electron density distribution, van der Waals geometry, and energetically preferred rotational disposition; (g) change of the spatial orientation of the butenolide ring at C-17β through introduction of the Δ^{16} double bond.

These structural modifications in parallel produced decreases or increases of the energy of interaction of the derivatives with the cardiac and cerebral enzyme preparations. Indeed, all derivatives showed on average a 1.5-fold higher affinity to the cardiac enzyme preparation. In any single determination, however, this affinity difference lay within experimental error, which, at first attempt [87], rendered difficult the assignment of that medicinally important distinction to an individual substructural feature of the various inhibitors. There are three reasons for this difficulty: (a) It has only recently become known [61], that the two enzyme preparations were composed of a mixture of three isoenzymes of unknown digitalis affinity; (b) Thermodynamic equilibrium studies cannot differentiate isoenzymes when they differ in their digitalis affinity by only one order of magnitude (or less), and when they are present in about equal proportions [190]. The differential affinity factor of 1.5 observed here could, for example, indicate the presence of two isoenzyme populations differing by a factor of ten in their digitalis affinity, with the enzyme preparation from cardiac muscle containing 80%, and the preparation from brain cortex 20%, of the higher affinity species. These considerations clearly call for studies of the isolated isoenzymes from known cellular origin, which are not yet accessible; (c) The impact of the various structural modifications on the observed $\Delta G^{o'}$ values is not as easy to quantify, since the $\Delta G^{o'}$ value represents the interaction energy for the entire molecule, not just the interaction energy of a selected substructure. In

the light of the knowledge that the steroid nucleus is the pharmacophoric lead, it appears almost safe to suppose that a proportion of isoenzymes in human cardiac muscle and brain cortex differs in the chemotopography of the steroid subsite in the digitalis-binding matrix, though much less than with the isoenzymes from cardiac muscle of man and guinea-pig (*Tables 4.1–4.4*).

CONCLUSIONS

(a) Determination of the thermodynamic equilibrium parameters for the interaction between diverse steroidal inhibitors of the C/D-*cis* digitalis type and Na^+/K^+-ATPase preparations from human cardiac muscle and brain cortex, consisting of a mixture of isoenzymes of unknown proportion and properties, does not allow the elucidation of structural features of the digitalis-binding matrix possibly endowed with isoenzyme specificity. (b) Comparison of the differences of the $\Delta G^{o'}$ values characterizing the interaction of diverse digitalis derivatives with Na^+/K^+-ATPase from cardiac muscle of man and guinea-pig leads to the conclusion that the sensor of species-dependent digitalis affinity is the geometry of the steroid nucleus reflecting the geometry of the steroid binding subsite in the digitalis recognition matrix. This knowledge, taken together with data from protein dissection studies, has paved the way for locating of the binding subsites for the tripartite cardiac glycosides, which is required for computer-aided modelling. (c) Identification of the steroid nucleus 5β,14β-androstane (with C/D-*cis* junction) as the pharmacophoric lead structure in cardiac glycosides suggests the possibility that the seeming failure of all previous attempts to the synthetic further development of the natural representatives by variation of the side-chain at C-17 (formerly thought to play a key functional role) has been based on the preservation of the lead structure in that synthetic work. This prompted the initiation of an evaluation of C/D-*trans* steroids of the hormone type as a first step towards lead structure optimization reviewed below.

ANALYTICAL USE OF C/D-*TRANS* STEROIDS FOR INFORMATION ON THE CHEMOTOPOGRAPHY OF THE DIGITALIS RECOGNITION MATRIX

Our topical interest in hormonal C/D-*trans* steroids has originated from three research objectives. First, the potential connection of C/D-*trans* steroids with endogenous digitalis. In 1953, Szent-Györgyi [191] suggested

that endogenous, digitalis-like acting steroids exist in normal blood serum. Of 22 steroids, only deoxycorticosterone and progesterone were found to exert such an action, but their efficacy was too low to account for the activity of the serum. Several recently published studies [192–195] arrived at the conclusion that the endogenous digitalis is a steroid and of adrenal origin. The reports, however, that endogenous digitalis is indistinguishable from or identical with the C/D-*cis* steroids digoxin [196] or ouabain [197–199], respectively, have not been confirmed [195]. In a recent review entitled 'Endogenous Digitalis: Reality or Myth?' [200], one former protagonist of the cardenolide nature of endogenous digitalis stated that 'the notion that genuine cardenolides are produced in the mammalian body should still be regarded with caution, although the corresponding findings definitely warrant further research'. Most recently, the equating of ouabain with endogenous digitalis [197] and the concept of a hormonal function of the putative endogenous digitalis [201] have been called into question for methodological and conceptual reasons, respectively [202]. Nevertheless, certain pregnanes, as far as investigated are truly digitalis-like acting compounds, i.e., they are inhibitory-effective through occupancy of the digitalis binding matrix of Na^+/K^+-ATPase [22]. Their efficacy is rather low so that they can be considered only as models for the chemical nature of endogenous digitalis [22,76].

Second, the biochemical mechanism underlying the transient positive-and lasting negative-inotropic actions of various C/D-*trans* steroids. Specifically, such dual responses or only cardiac depression were seen with deoxycorticosterone acetate [203], spirolactone [203], aldosterone [204], progesterone [205], canrenone [206], canrenoate [207], and chlormadinone acetate [23,24]. The absence of a positive inotropic action was especially surprising in the case of those steroids which were known to be effective inhibitors of both the Na^+/K^+ pump (canrenone [208,209], spironolactone [208], chlormadinol acetate [23]) and the Na^+/K^+-ATPase (spirolactone [210], canrenone [211,212], progesterone [210], chlormadinone acetate [24,213]). This dichotomy between the actions on contractility and the receptor enzyme has led to doubts on the receptor nature of Na^+/K^+-ATPase [23] and, alternatively, to the notion that the cardiac depression results from some unknown mechanism which overrides any positive effects on contractility that should result from inhibition of the Na^+/K^+ pump [24]. The more specific hypothesis [183] that the negative-inotropic action of the above C/D-*trans* steroids of the hormone type results from intracellular non-genomic actions (defined in [214]) has proved correct. Glycosidation of chlormadinol acetate, expected to suppress its permeation across the plasma membrane, but still to allow its interaction with the extracellularly exposed

digitalis binding matrix, did actually transform the depressant-active aglycon into a derivative of lasting positive-inotropic action [183]. This experience favours the generalization that C/D-*trans* steroids of the hormone type are able to elicit positive inotropism like C/D-*cis* steroids of the digitalis type. This has prompted the synthesis of a series of C/D-*trans* pregnane glycosides as presented in *Table 4.5*.

Third, inquiry about the pharmacophoric lead structure in progestins and check of structure–activity relations to provide further information on the chemotopography of the digitalis binding matrix. This approach has only recently been started so that the conclusions offered below are of tentative significance. The available experimental data are listed in *Table 4.5*.

CHEMOTOPOGRAPHY OF THE BINDING SITE FOR THE STEROID NUCLEUS

Although relatively sparse, the available data as listed in *Table 4.5*, allow the search for the nature of the lead structure in C/D-*trans* steroids of the hormone type being the minimum structure with highest potency. Unfortunately, the covered range of efficacies is rather small, which limits in-depth interpretation.

The oestrogens (1...3) show some interaction energy. Their low potency is not caused by the absence of an OH group at C-14 or by the C/D-*trans* junction as revealed by a comparison with their congeners carrying that hydroxy group and possessing C/D-*cis* junction (4,5). The transition from oestrogens to androgens (6,7) and corticosteroids (11,12,13) indicates that their efficacy is mostly still lower than that of the oestrogens. Remarkably, the potency of progesterone (9) is similar to that of oestradiol-17β (1).

The steroids characterized by a hydroxy group at C-3 and a Δ^5 double bond (14...20) belong (except 14 and 15) to the compounds of medium potency. The synthetic derivatives with a methyl group at C-6 (21...28) mostly show a higher interaction energy, though this is not simply caused by the substituent, as is revealed by the similarity of the $\Delta G^{o'}$ values for compounds with (22) and without (19) the methyl group. The combination with ring unsaturations and the two side-chains at C-17 appears to be more important. The replacement of C-6-methyl by C-6-chloro (26→29, 28→30) results in a further increase of interaction energy.

Surprisingly, the representatives with the simplest, saturated steroid skeleton (31...35) do not fall much behind the potency of the group just considered. Of the minimum structure steroids, 5α,14α-androstane (36) and 5β,14α-androstane (37), the latter shows the higher interaction energy and thus by definition is the pharmacophoric lead structure in C/D-*trans* steroids.

Table 4.5. APPARENT GIBBS ENERGIES OF INTERACTION ($\Delta G^{o'}$) BETWEEN DIVERSE C/D-*trans* STEROIDS OF THE HORMONE TYPE AND NA^+/K^+-ATPase PREPARATIONS FROM HUMAN HEART (HH), HUMAN BRAIN CORTEX (HB), AND GUINEA-PIG HEART (GH): IMPACT OF CHANGES IN STEROID SKELETON AND SIDE-CHAINS AT C-3 AND C-17

No.	*Systematic (trivial) name of inhibitor*	$-\Delta G^{o'}$ *(kJ/mol)*		
		HH	*HB*	*GH*
1	Oestra-1,3,5(10)-triene-3,17β-diol (oestradiol-17β)		21.5*	
2	Oestra-1,3,5(10)-triene-3,17α-diol (oestradiol-17α)		22.7*	
3	3-Hydroxyoestra-1,3,5(10)-trien-17-one (oestrone)		21.1*	
4	Oestra-1,3,5(10)-triene-3,14,17β-triol	20.9*		
5	14β-Oestra-1,3,5(10)-triene-3,14,17β-triol	17.3*		
6	3α-Hydroxy-5α-androstan-17-one (androsterone)		<<21.0*	
7	17β-Hydroxyandrost-4-en-3-one (testosterone)		<<25.1*	
8	Androst-4-ene-3,17-dione		22.6*	
9	Pregn-4-ene-3,20-dione (progesterone)	23.3	22.5	20.9*
10	17α-Pregn-4-ene-3,20-dione	20.9*		
11	11β,17α,21-Trihydroxy-pregn-4-ene-3,20-dione (cortisol)		<<14.9*	20.0*
12	21-Hydroxypregn-4-ene-3,20-dione (deoxycorticosterone)		21.4*	
13	11β,17α,21-Trihydroxy-pregna-1,4-diene-3,20-dione (prednisolone)	15.8*	17.8*	
14	3β-Hydroxyandrost-5-en-17-one		20.3*	
15	Androst-5-ene-3β,17α-diol		18.8*	
16	17-Oxoandrost-5-en-3α-yl acetate		23.3*	
17	3α-Hydroxyandrost-5-en-17-one		23.8*	
18	17α-Pregn-5-en-3β-ol	<<21.0*	21.0*	
19	17α-Acetoxy-3β-hydroxypregn-5-en-20-one	24.5*	22.6*	
20	(20*S*)-Pregn-5-ene-3β,20-diol		25.2*	
21	17α-Acetoxy-6α-methyl-5β-pregnane-3,20-dione		21.8*	
22	17α-Acetoxy-3β-hydroxy-6-methyl-pregn-5-en-20-one	24.2*	23.3*	
23	17α-Acetoxy-3α-hydroxy-6α-methyl-5β-pregnan-20-one		24.0*	
24	17α-Acetoxy-6α-methylpregn-4-ene-3,20-dione (medroxyprogesterone acetate)		25.2*	
25	17α-Acetoxy-3β-hydroxy-6α-methyl-5β-pregnan-20-one		27.1*	
26	17α-Acetoxy-3β-hydroxy-6-methyl-pregna-4,6-dien-20-one		27.6	
27	17α-Acetoxy-3β-hydroxy-6α-methyl-pregn-4-en-20-one		28.0	
28	17α-Acetoxy-6-methylpregna-4,6-diene-3,20-dione (megestrol acetate)	30.4	28.5	26.9*
29	17α-Acetoxy-6-chloro-3β-hydroxypregna-4,6-dien-20-one (chlormadinol acetate)	32.0	30.6	22.5*
30	17α-Acetoxy-6-chloropregna-4,6-diene-3,20-dione (chlormadinone acetate)		31.4	
31	3β-Hydroxy-5α-pregnan-20-one		<<23.9*	
32	5β-Pregnane-3,20-dione		22.4*	
33	3β-Hydroxy-5β-pregnan-20-one		23.8*	
34	3α-Hydroxy-5β-pregnan-20-one		24.3*	

Table 4.5. (continued)

No.	Systematic (trivial) name of inhibitor	$-\Delta G^{o'}$ (kJ/mol) HH	HB	GH
35	5β-Androstane-3β,17α-diol		23.4*	
36	5α-Androstane	21.3*		
37	5β-Androstane	23.3*		
38	17α-Acetoxy-6α-methyl-3α-(α-L-rhamnosyloxy)-5β-pregnan-20-one		24.3*	
39	3α-(α-L-Rhamnosyloxy)-5β-pregnan-20-one		24.8*	
40	3β-(α-L-Rhamnosyloxy)-5α-pregnan-20-one		27.8*	
41	17α-Acetoxy-6-methyl-3β-(α-L-rhamnosyloxy)pregna-4,6-dien-20-one		29.0*	
42	3β-*O*-(α-L-Rhamnosyl)chlormadinol acetate	26.6*	25.3*	
43	3β-*O*-(β-D-Glucosyl)chlormadinol acetate	26.5	25.9*	
44	3β-*O*-(α-L-Arabinofuranosyl)chlormadinol acetate	27.2*	24.1*	
45	3β-(α-L-Rhamnosyloxy)-17α-pregn-5-en-20-one		22.9*	
46	17α-Acetoxy-3β-(α-L-rhamnosyloxy)pregn-5-en-20-one	29.9	28.0	23.8*
47	17α-Acetoxy-6-methyl-3β-(α-L-rhamnosyloxy)pregn-5-en- 20-one	31.4	28.5	23.8*
48	17α-Acetoxy-6α-methyl-3β-(α-L-rhamnosyloxy)-5β-pregnan-20-one		33.1	
49	3β-(α-L-Rhamnosyloxy)-5β-pregnan-20-one		30.6	

* Extrapolated value owing to limited solubility.

As far as hitherto studied, the representatives of the C/D-*trans* series (9, 19, 22, 28, 29) develop, like those of the C/D-*cis* series, a greater affinity with the enzyme from human cardiac muscle than with the enzyme from human brain cortex, and a much higher interaction energy than with the enzyme from guinea-pig cardiac muscle. All C/D-*trans* steroids studied promote, as known for C/D-*cis* steroids, the phosphorylation of the enzyme with orthophosphate in the presence of Mg^{2+} [22,129]. Judged by the two most distinctive criteria (i.e., enzyme origin responsiveness and phosphoenzyme stabilization [129]), the C/D-*trans* steroid representatives probed can be classified as truly digitalis-like acting, i.e., the digitalis binding matrix occupying inhibitors of Na^+/K^+-ATPase.

CHEMOTOPOGRAPHY OF THE BINDING SITE FOR SIDE-CHAINS AT C-17

The hitherto accumulated data yield only limited information although still enough to bring out the most essential reason for the large difference between the C/D-*cis* steroids of the digitalis type and the C/D-trans steroids

of the hormone type as to their interaction energy with the digitalis binding matrix.

Remarkably, the translocation of the MeCO– side-chain from 17β- to 17α-position (9→10) results in a rather small loss of interaction energy. This indicates that the C-20-carbonyl does not play such a key role as hydrogen bond acceptor as does the C-23-carbonyl in cardenolides. The replacement of an oxo-group at C-17 by a MeCHOH– substituent in β-position (14→20), or by 17β-COMe and 17α-*O*-COMe substituents (14→19) does but slightly increase the $\Delta G^{o'}$ value. However, the bulkier and less flexible butenolide side-chain at C-17β depresses the interaction energy quite dramatically in consequence of the conversion of C/D-*cis* into C/D-*trans* junction (see *Table 4.1*: 7→12) and thus demarcates the receptor essential volume (for definition see [101]) in that region. Generally, the side-chains at C-17 of the checked steroids do not reveal chemically reactive amino acid atom groupings in their neighbourhood.

In conclusion, the major if not sole reason for the comparatively low affinity of C/D-*trans* steroids of the hormone type to the digitalis-binding matrix is the absence of great interaction energy contributions from the side-chain at C-17. This is in contrast to the lactone substituents at C-17β in the C/D-cis steroids of the digitalis type.

CHEMOTOPOGRAPHY OF THE BINDING SITE FOR A SUGAR SIDE-CHAIN AT C-3

The present exploration confines itself to test the possibility of whether 3β-*O*-glycosidation increases the interaction energy with Na^+/K^+-ATPase to the same extent as found with C/D-*cis* steroids (cardenolides and bufadienolides). To this end, various progesterone derivatives were used as the steroid moiety, and α-L-rhamnose was chosen as the sugar component, because this monosaccharide is able to replace isoenergetically the trisaccharide chain of digitalis glycosides [87,88]. The results, as presented in *Table 4.5*, show that the energetic impact of C-3-*O*-rhamnosylation is very much a function of A/B ring configuration in the aglycon [184]. No increment in interaction energy is obtained by 3α-*O*-rhamnosylation of A/B-*cis* steroids (23→38, 34→39) and by 3β-*O*-rhamnosylation of an A/B-*trans* steroid (31→40). Remarkably, only a small increment (26→41) or even a considerable reduction (29→42..44) of the $\Delta G^{o'}$ value results from 3β-*O*-rhamnosylation of the highly flexible $\Delta^{4,6}$ steroids, which thus appear to adopt in the binding matrix an A/B-flat conformation similar to the A/B-flat configuration of A/B-*trans* steroids (see above). On the other hand, the Δ^5-steroids appear to adopt an A/B-bent conformation, since their 3β-*O*-rhamnosylation yields a similarly great $\Delta G^{o'}$ increase (19→46, 22→47)

as typical for the A/B-*cis* steroids (25→48, 33→49) and for the A/B-*cis*,C/D-*cis* steroids (cf. *Table 4.3*).

In conclusion, in C/D-*trans* steroids the impact of A/B-bent or -flat configurations on the outcome of 3β-*O*-glycosidation is essentially like that in C/D-*cis* steroids. Hence, the sugar binding matrix appears to be the same for steroid glycosides of both hormone and digitalis type.

PRELIMINARY CONCLUSIONS

(a) The C/D-trans pregnane derivatives fulfil the criteria to be judged as digitalis-like acting inhibitors of Na^+/K^+-ATPase, in which 5β,14α-androstane is the lead structure. (b) The interaction energies of 5β,14β-androstane-3β,14-diol and 5β,14α-androstane are much alike. Thus, the digitalis binding matrix displays a remarkable adaptability to moulding by the D-ring of both steroid types. This parallels the property of steroid hormone receptors in so far as the association between the receptor and the D-ring end of the steroids either does not occur or is far less stereospecific than the association between the receptor and the A-ring region [215]. (c) The low potency of the pregnanes is based on the fact that their characteristic side-chain at C-17β does not contribute to the interaction energy. (d) The presence of a lactone residue at C-17β in C/D-*trans* steroids, in contrast to its impact in C/D-*cis* steroids, results in no increase in interaction energy, and can even nullify their potency; this indicates a conflict with the receptor essential volume. (e) The introduction of a rhamnose residue in C-3β-OH decreases or increases the interaction energy of the aglycone depending on the flat or bowed shape of the A/B-ring junction of the aglycones. In the case of double bonds in rings A and/or B, which introduce a potential of conformational flexibility, the shape of the A/B ring junction will finally be formed in the binding cleft by exploiting some of the interaction energy. (f) The maximum of the interaction energy increment contributed by the rhamnosylation in C/D-*trans* steroids is as great as in C/D-*cis* steroids. Apparently, the binding of the rhamnosyl residue occurs in both steroid classes to the same subsite in the digitalis binding matrix. (g) As documented elsewhere [183], glycosidation transforms the progestin, chlormadinol acetate, which is known for its strong negative inotropic action [23,24,183], into a positive-inotropically acting glycoside via suppression of plasma membrane permeation and elimination of hormonal effects. In anaesthetized cats, chlormadinol acetate glycosides produce a long-lasting positive inotropic response. In total, its circulation parameters are all more favourable than those of digitoxin when studied under the same conditions [183]. In particular, 3β-*O*-(α-L-arabinofurano-

syl)chlormadinol acetate does not evoke arrhythmias, which in the case of digitoxin, precede the death of the animals. This distinction might possibly be due to a more or less favourable discrimination between Na^+/K^+-ATPase isoenzymes in contractile and neural cells, respectively. In any event, the result of the initial pharmacological evaluation of the above compounds favours a broader assessment of glycosides of C/D-*trans* steroids of the hormone type.

GENERAL CONCLUSIONS

REASSESSMENT OF STUDIES WITH PARTIALLY SYNTHETIC DIGITALIS DERIVATIVES

The Free–Wilson analysis of the inhibitory action of 95 digitalis-like steroids on Na^+/K^+-ATPase from human cardiac muscle allowed to predict that 3β-(4′ξ-amino-4′-deoxy-α-L-oleandrosyloxy)-14-hydroxy-16α-nitroxy-5β,14β-bufa-20,22-dienolide would surpass the inhibitory potency of known most active natural and synthetic digitalis compounds by two orders of magnitude [89]. However, the objective of endeavours towards a partially synthetic further development of the therapeutically used cardiac glycosides is not the design of derivatives with maximum potency, but the realization of compounds outstanding in superior therapeutic qualities. This goal has so far been missed possibly because the synthetic compounds generally exhibited a reduced potency which discouraged in-depth pharmacological evaluation.

Such thinking neglects the point that potency does not necessarily parallel selectivity of action which is obviously the major determinant of therapeutic usefulness. Any mechanism for specificity is known to involve both attractive and repulsive interaction forces [216]. Specifically, in bimolecular recognition, unfavourable steric interactions or unpaired charged donors or acceptors can provide far higher specificity than favourable uncharged hydrogen bondings [168,217]. In conclusion, among the numerous partially synthetic digitalis derivatives, there may have been several with improved therapeutic range, which have never undergone a qualified pharmacological evaluation or been tested in man. Nevertheless, a few digitalis derivatives have been reported to be superior to the natural parent compounds in pharmacological studies. Thus, the dihydro-derivatives of ouabain, digoxin, and digitoxin were found in the failing heart-lung preparation as well as in anaesthetized dogs, to have a greater margin of safety as judged by the

higher ratio of doses for the first inotropic effect and ventricular fibrillation [218].

Actodigin (3β-glucosyloxy-14-hydroxy-24-nor-5β,14β-chol-20(22)-ene-21,23-lactone) and its (23*R*)-23-methyl derivative (14 and 16 in *Table 4.2*) have been demonstrated to possess a significantly greater margin of safety than the dihydro-glycosides or ouabain, respectively [219]. Compared to ouabain, actodigin in equi-inotropic concentrations caused significantly less electrophysiological toxicity [220]. The 3β-*O*-glucoside of (21*R*)-21-methyldigitoxigenin (40 in *Table 4.3*) has been compared with digitoxin and reported to have a dramatically reduced toxicity as measured by the ratios of both the irregularity dose to the minimal therapeutic dose and the lethal dose to the minimal therapeutic dose [221]. 3β-*O*-Glucosyl-3α-methyldigitoxigenin (35 in *Table 4.3*) was compared with digitoxin on isolated atria from guinea-pig hearts and shown to produce a two-fold higher maximum increase of the contractile force, and to cover in the dose–response curve a larger range before toxic signs terminated the inotropic response. Comparable results were obtained in heart–lung preparations and intact animals [222].

LND 623 (14-amino-3β-rhamnosyloxy-5β,14β-pregnan-20β-ol; 69 in *Table 4.4*) has been tested in 5 animal models and shown to elicit a significantly greater increase in maximum contractile force than either ouabain or digoxin, though it is active in the same concentration range [223]. Comparative studies using Na^+/K^+-ATPase preparations from rat and dog cardiac muscle indicated that LND 623 exerts its more potent inotropic activity via a selective inhibition of the Na^+/K^+-ATPase isoenzymes of high affinity for cardiac glycosides; this selectivity was not detected with ouabain [224].

Digitalis compounds with an oxygen function at C-16 deserve special consideration, because they have been evaluated in the clinical setting. 16β-Formyloxydigitoxin (gitaloxin) is the main active constituent in 'Gitalin' (Verodigen^R) [225]. This drug was found to be superior to other digitalis preparations including digitoxin and digoxin [226–228]. Batterman *et al.* [227] stated that gitalin possesses a greater therapeutic range and affords a greater degree of safety than any other digitalis preparation. The especially favourable therapeutic profile of 16β-formyloxydigitoxin could be based on the fact that the central nervous toxicity of 16β-hydroxyaglycones and 16β-hydroxydigitoxin is much weaker than that of the congeners without oxygen function at C-16 [229–231]. More recently, 16β-acetoxydigitoxin, the active metabolite of the prodrug penta-acetylgitoxin (Pengitoxin WHO) [71] has been found to be exceptionally well tolerated in the treatment of cardiac failure [232–234].

Most recently, 16α-hydroxydigitoxin (16-epigitoxin; 48 in *Table 4.4*) has been shown to have a greater therapeutic range than naturally occurring glycosides [235,236]. Its wider therapeutic range appears to be based on the weaker effect on the specialized Purkinje conduction fibres in cardiac muscle [237,238]. The authors concluded that the glycoside-binding areas of Na^+/K^+-ATPase in neural and contractile cells differ in conformation and possibly in structure.

Taken together, all findings could plausibly be explained by supposing that the various partially synthetic digitalis derivatives discriminate in parts more favourably than the natural compounds between the isoenzymes in contractile and neural cells. The common denominator for the special properties of the considered derivatives appears to be their potential for distinctly rapid formation and dissociation of competent complexes with certain receptor isoenzymes. Thus, some relation between reactivity and selectivity, to be considered below, could underly their special properties.

MAJOR INSIGHTS FOR COMPUTER-AIDED MOLECULAR MAPPING OF THE DIGITALIS BINDING MATRIX

The evaluation of equilibrium Gibbs energies of interaction between many sorts of C/D-*cis* and C/D-*trans* steroids of the digitalis and hormone type, respectively, and Na^+/K^+-ATPase preparations from human cardiac muscle and brain cortex as well as from guinea-pig cardiac muscle (see *Tables 4.1–4.5*), leads to the following tentative conclusions (cf. *Figure 4.5*): (a) The steroid moiety and the side-chain at C-17 bind to the H1–H2 junction, whereas the sugar side-chain at C-3 binds to the H3–H4 junction of the catalytic protomer of Na^+/K^+-ATPase. (b) Both junctions represent the digitalis recognition matrix and together form initially a cleft 19Å deep. (c) Triggered by binding of the steroid component, the cleft becomes closed to envelop the tripartite inhibitor. (d) Cleft closure is accompanied by interruption of the catalytic cycle via communication between the H3–H4 junction and the catalytic centre through the H4 membrane-crossing amino acid stretch. (e) The superposition on top of one another of the van der Waals's surfaces of the best fitting inhibitors, identified by the greatest $\Delta G^{o'}$ values, yields then the functional receptor shape ('Gestalt') after cleft closure. (f) Superposition of the van der Waals surfaces of badly fitting inhibitors, recognized by small $\Delta G^{o'}$ values, on the surface of the fitting receptor shape demarcates the locality of the receptor essential structural fragments, for the occupancy of which inhibitor structural fragments compete. (g) Modelling of the tertiary structure of the digitalis recognition and binding site from the amino acid sequences in the H1–H2 and H3–H4

junctions could be alleviated by comparison with the probably similar tertiary structures of steroid hormone receptors when available. Actually, there is a striking homology in the primary structure of a large number of receptor classes [239]. Most recently, Raynaud and colleagues [240] constructed a model for the determination of the three-dimensional spatial distribution of the functions in the hormone-binding domain. Their co-ordinated model of the steroid-binding domain comprises hydrophobic clefts of about 20Å by 10Å or 17Å by 6Å. Due to flexibility, the cleft can accomodate the steroid hormone size and shape [240], and show tolerance of steric bulk in the binding process [241]. In the steroid hormone receptor superfamily, a structurally relatively invariant region is the C-terminal steroid binding domain; the functional consequence of this sequence conservation is cross-binding of ligands [242]. Thus, digitoxin (but not digoxin) binds to the oestrogen receptor and acts like an oestrogen causing breast growth in elderly men [243]. (h) Knowledge of the tertiary structure of the digitalis binding matrix will finally allow examination of the above proposals on the amino acid residues which could participate in the interaction with the steroid nucleus and its side-chains at C-3 and C-17.

PERSPECTIVE

PERFECTING THE PRIMARY SCREENING BY USE OF Na^+/K^+-ATPase

One of the major obstacles in making full use of Na^+/K^+-ATPase in primary screening is the fact that the enzyme preparations from human cardiac muscle and brain cortex consist of a mixture of isoenzymes [61], the cellular origins, the proportions, and the properties of which are still unknown. The physical separation of pure isoenzymes appears at present to be inaccessible. In the meantime, several ways out of this dilemma may be tried. These include the use of enzyme preparations from kidney or skeletal muscle, which are known to express only the $\alpha 1$ isoenzyme or preferentially the $\alpha 2$ isoenzyme, respectively [61].

For enzyme preparations from human cardiac muscle and neural tissues, various kinds of functional separation could be probed, such as differential sensitivity to inhibition by Ca^{2+} [244,245], hydrolytic enzymes [246,247], and various reagents [248,249]. However, a perfect, generally applicable solution to the problem is not yet at hand. A more rewarding approach could be the functional differentiation by changing the concentration and proportion of the ligands of Na^+/K^+-ATPase which determine differently the molecular turnover of the isoenzymes [248,250].

The most promising and most direct, method to diagnose the presence of distinctly digitalis-sensitive isoenzymes in an enzyme preparation would be to determine the rates of formation and dissociation of the complexes between Na^+/K^+-ATPase and steroidal inhibitors as a function of their structure. In fact, whenever the thermodynamic equilibrium parameters fail to differentiate, the association and dissociation rates could help towards a distinction, as differential rates are inherently related to distinct specificities. This interconnection, known as the reactivity–selectivity principle from physicochemical studies [251,252], is likewise valid for the dynamics of molecular recognition in biological systems [153,253]. A hypothetical

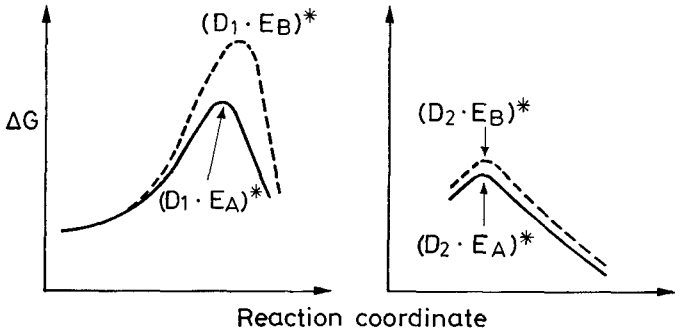


Figure 4.11. Hypothetical reactivity–selectivity profile for the interaction of a weakly reactive digitalis compound (D_1) or a strongly reactive digitalis compound (D_2) with Na^+/K^+-ATPase isoenzymes E_A or E_B, which differ in the chemotopography of the digitalis binding matrix. Selectivity is shown to be a reflection of the differential height of the activation Gibbs energy barrier, which is equivalent to the association rate constant. In other words, the inhibitor D_1 will preferentially bind to the isoenzyme E_A, whereas inhibitor D_2 will equally bind to both isoenzymes, E_A and E_B (adapted from [251]).

example for the differential recognition of isoenzymes by differently reactive digitalis compounds is presented in *Figure 4.11.*

Actually, the determination of the 'receptor-kinetics' (pharmacodynamics) of a digitalis compound (*Table 4.6*) can be indispensable for the understanding of its *in vivo* behaviour as shown for the above-mentioned 16α-hydroxy-digitoxin [51,254]. Naturally, knowledge of the dynamics of interaction will also make possible the more profound interpretation of the thermodynamic equilibrium data given in *Tables 4.1–4.5.*

Table 4.6. DIFFERENTIAL EFFECT OF DERIVATIZATION OF DIGITOXIN ON THE EQUILIBRIUM CONSTANT K_D' AND THE RATE CONSTANTS k_1' AND k_{-1}' FOR THE INHIBITORY EFFECT ON NA^+/K^+-ATPase ACTIVITY FROM HUMAN CARDIAC MUSCLE

Glycoside	k_1' [$min^{-1} \cdot \mu M^{-1}$]	k_{-1}' [min^{-1}]	K_D' [μM]
Digitoxin	9.0	0.043	0.005
22-Methyldigitoxin	6.1	0.091	0.015
12β-Hydroxydigitoxin	3.0	0.045	0.015
16β-Hydroxydigitoxin	2.4	0.059	0.025
16α-Hydroxydigitoxin	0.2	0.080	0.420
Ouabain	1.5	0.028	0.019

OPTIMIZATION OF THE LEAD STRUCTURE 5β,14α-ANDROSTANE

The rise to this challenge appears to be possible as a result of the evaluation and utilization of the data presented in *Tables 4.1–4.5*. This procedure will certainly be combined with the exploitation of the concepts and methods in computer-assisted rational drug design as recently reviewed [255]. The receptor structure-based strategies appear to be most promising [256–259]. For this multidisciplinary, cyclic approach, a variety of computer programs are available, ranging from interactive designs with computer graphs to automated database searching as referred to in [259].

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5 Amidines and guanidines in medicinal chemistry

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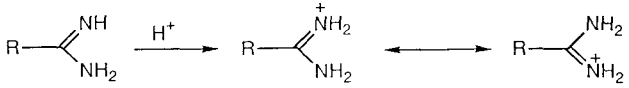
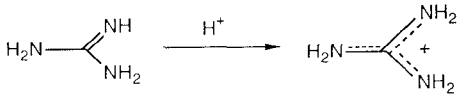
INTRODUCTION

Although one of us has been interested in the preparation and properties of amidines and guanidines for most of the last decade [1,2] we were surprised to discover how many useful drugs carry one of these groups and even more at the range of activities shown by them. By now the alert reader will have glanced at the contents pages and will probably be similarly impressed. In preparing this review we have made use of many reference books and on-line data bases. We hope to have included all the important compounds which have been tested and reported by early 1992. We are grateful to Messrs B. Wagner and M. Burke for help with the computer based searches. Many writers, particularly pharmacologists, classify all the title compounds as amidines with guanidines considered as aminoamidines. Although there is some justification for this, as chemists we see differences between the two groups and believe they should be kept separate, as we have done here. The interesting property of the two related groups lies in their high basicity which follows from their ability to share the positive charge of the

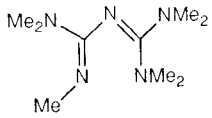


Scheme 5.1.



Scheme 5.2.

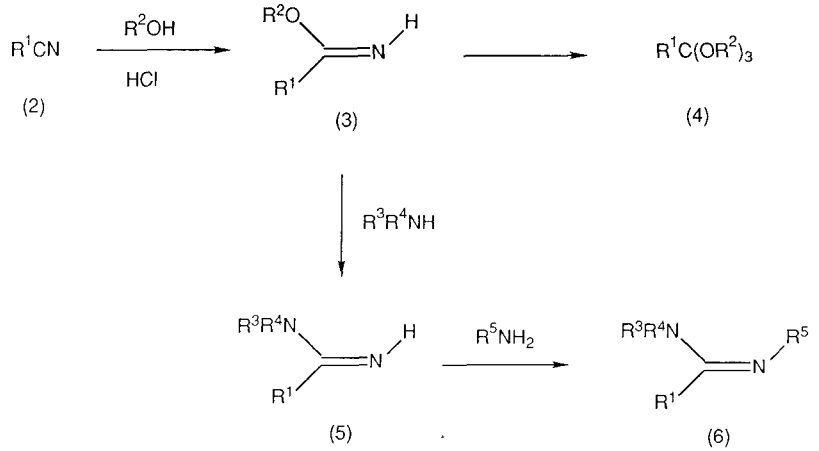
protonated derivatives over the two nitrogen atoms of an amidine, *Scheme 5.1.*, or the three nitrogens of a guanidine, *Scheme 5.2.* Guanidine is reported to have a pK_a of 13.6, tetramethylguanidine (a useful basic catalyst) also of 13.6 and pentamethylguanidine of 15.6 [3]. Even more remarkable is heptamethylbiguanide (1) which has a pK_a of 17.1, making it a stronger base than sodium ethoxide! [3]. Several important drugs are



(1)

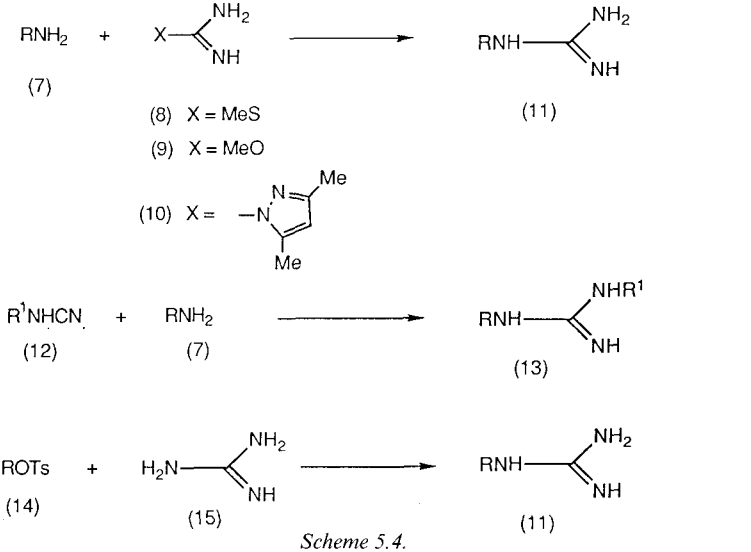
derived from biguanide and are included in the review. However, almost all the amidines and guanidines with pharmacological activity are not strong bases (rare exceptions are the essential amino acid arginine, streptomycin and capreomycin). These nuclei are very sensitive to substitution so that any electron-withdrawing group bonded to one of the nitrogens dramatically reduces the basic strength [1]. For example, phenylguanidine has a pK_a of 10.77 whilst that of acetylguanidine is 8.33. Strong electron withdrawers produce essentially neutral molecules, e.g., cyanoguanidine and nitroguanidine have pK_a values of −0.4 and −0.93 respectively [4].

The drugs are classified under their main physiological activities and within each group are arranged roughly in order of increasing molecular complexity. Of the general synthetic methods, the classical Pinner reaction was developed to provide a route from a nitrile (2) via an imidate (3) to an orthoester (4). Alternatively, the imidate can be treated with ammonia or a primary or secondary amine to give an amidine (5) which under more forcing conditions can be converted into the substituted amidine (6), (*Scheme 5.3.*) [5].



Scheme 5.3.

Many guanidines are prepared by one of the methods of *Scheme 5.4.* Nucleophilic attack by an amine (7) on an amidine derivative such as (8), (9) or (10) gives a substituted guanidine (11). The use of an *S*-methylisothiourea (8) is known as the Rathke reaction and usually gives high yields. An amine can add to a cyanamide (12) to give a disubstituted guanidine (13) and finally, guanidine (15) (or a substituted guanidine) can react with a sulphonic ester (14) to give the substituted guanidine (11).

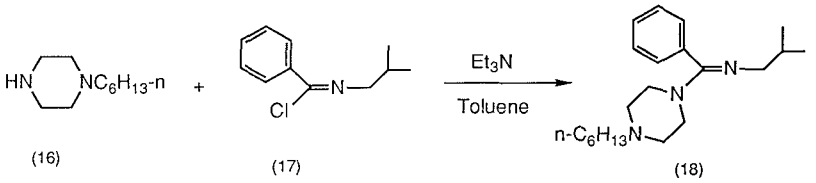


Scheme 5.4.

CARDIOVASCULAR DRUGS

BUCAINIDE

Reaction of hexylpiperazine (16) with the iminochloride (17) in the presence of triethylamine in toluene yielded bucainide (18), (*Scheme 5.5.*) [6]. Bucainide is rapidly absorbed and acts as a hypoglycaemic and as an antiarrhythmic agent [7].

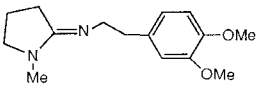


(16) (17) (18)

Scheme 5.5.

MIXIDINE

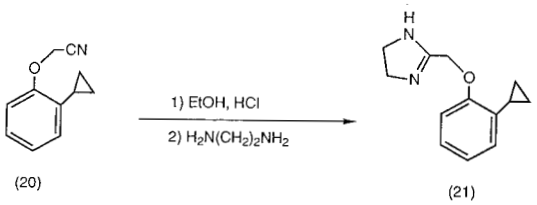
1-Methyl-2-ethoxypyrrolidinium fluoroborate and 2-(3,4-dimethoxyphenyl)ethylamine reacted together to give mixidine (19) [8]. Mixidine causes a slowing and strengthening of the heart rate through a direct effect on the atrial sinus [9–11].



(19)

CIRAZOLINE

Reaction of 2-cyclopropylphenol with chloroacetonitrile in the presence of potassium carbonate gave the ether (20). The Pinner reaction converted this to the imidazoline cirazoline (21) (*Scheme 5.6.*). It can also be made from 2-cyclopropylphenol and 2-chloromethylimidazoline using sodium ethoxide in refluxing ethanol [12]. Cirazoline is an α_1-adrenergic agonist, but also shows α_2-adrenergic antagonist action. These actions cause vasoconstriction in the nasal mucosa and it has been evaluated as a decongestant [13].

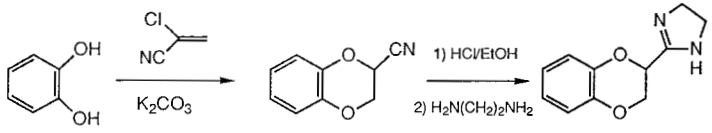


(20) (21)

Scheme 5.6.

IDAZOXAN

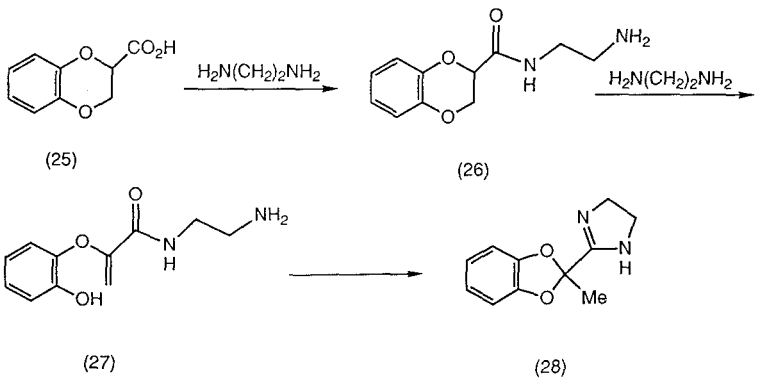
Catechol (22) was treated with 2-chloroacrylonitrile in basic conditions; Michael addition to one OH group followed by a Williamson's reaction onto the other gave the nitrile (23) which was elaborated on to idazoxan (24) (*Scheme 5.7.*) [14, 15]. It is claimed to be one of the most potent and selective α_2-adrenergic receptor antagonists [16–19]. Idazoxan shows unusual differential binding to α_2-adrenoreceptors of different species [20]



(22) (23) (24)

Scheme 5.7.

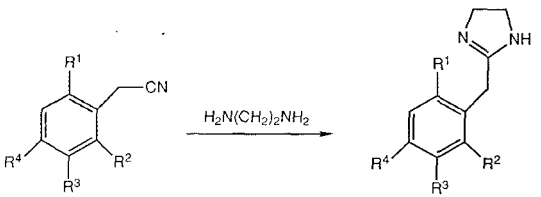
An early claim to have prepared structure (24) from the acid (25) turned out to be a mistake [21]. The benzodioxanecarboxylic acid (25) was treated with an excess of ethylenediamine and the product isolated by vacuum distillation. The first formed amide (26) suffered base catalysed ring opening on heating to give the olefin (27) which reclosed to the benzodioxole (28), a formula isomer of idazoxan, (*Scheme 5.8.*) [15, 22]. This ring opening is general for benzodioxans [23]. Compound (28) has antihypertensive activity [16, 21]:



Scheme 5.8.

TOLAZOLINE, DOMAZOLINE, XYLOMETAZOLINE AND OXYMETAZOLINE

Simple 2-benzylimidazolines (30–33) were prepared by condensation of the appropriate phenylacetonitrile (29) with ethylenediamine either at elevated temperatures or in the presence of catalytic amounts of carbon disulphide (*Scheme 5.9.*). Tolazoline was also prepared in high yield by Neef's method [24]. Tolazoline (30) [25, 26] is a vasodilator but, domazoline (31) [27], xylometazoline (32) [28] and oxymetazoline (33) [29] are all vasoconstrictors. Oxymetazoline was shown to be an agonist for both pre- and post-synaptic α_2-adrenergic receptors [30]. Tolazoline can be used in animals to give rapid arousal from anaesthesia, for example it is reported to reverse the effect of xylazine with ketamide in wolves [31], white tailed deer [32] and elephants [33], in each case it was fully effective and caused no distress to the animals. It is useful in neonates such as lambs and calves to treat persistent foetal circulation syndrome (persistant pulmonary hypertension) [34] and is recommended for the same condition in human newborns [35, 36]. Oxymetazoline found use in dentistry as a gingival retraction agent [38].



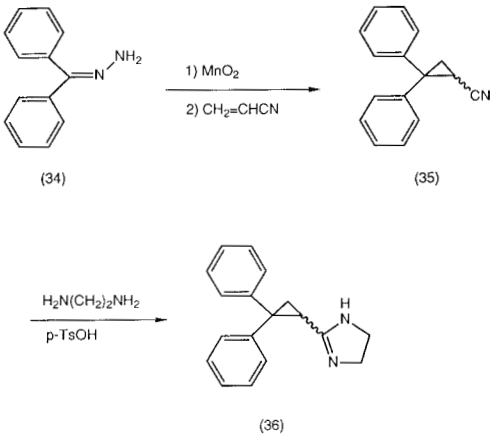
(29)

(30) $R^1 = R^2 = R^3 = R^4 = H$
(31) $R^1 = R^3 = OMe$, $R^2 = R^4 = Me$
(32) $R^1 = R^2 = Me$, $R^3 = H$, $R^4 = {}^tBu$
(33) $R^1 = R^2 = Me$, $R^3 = OH$, $R^4 = {}^tBu$

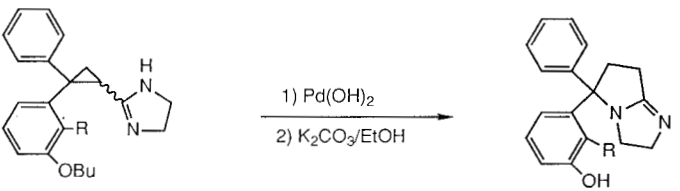
Scheme 5.9.

CIFENLINE

Cifenline (cibenzoline, 36) is a Class I antiarrhythmic agent synthesized as shown in Scheme 5.10. [39]. Benzophenone hydrazone (34) was diazotised and treated with manganese dioxide to give the carbene which inserted into the olefin link of acrylonitrile to give the trisubstituted cyclopropane (35). Ethylenediamine in the presence of toluene sulphonic acid then gave the imidazoline (36) in a high yield. ^{14}C-labelled cifenline partially metabolized to the derived imidazole and several hydroxylated derivatives which were excreted as conjugates [40]. Two of these were isolated, hydrolyzed, and assigned structures (39) and (40) on the basis of NMR studies. The structures were confirmed by synthesis via the base catalysed ring expansions of the cyclopropanes (37) and (38) (*Scheme 5.11.*) [41].



Scheme 5.10.



(37) R = H

(38) R = MeO

(39) R = H

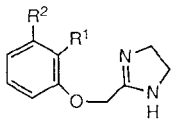
(40) R = MeO

Scheme 5.11.

Cifenline treated atrial fibrillation successfully in some patients [42] and is an effective therapy for chronic ventricular arrhythmias [43,44]. Its long half life *in vivo* allowed only twice a day dosage. This produced good patient compliance when it controlled ventricular arrhythmias throughout a two-year trial [45]. Another trial showed cifenline to be slightly superior to quinidine for patients with premature ventricular depolarisations [46].

FENMETAZOLE AND FENOXAZOLINE

The phenoxymethyl imidazoline derivatives fenmetazole (41) [47,48] and fenoxazoline (42) [49,50] were obtained by the action of ethylenediamine on the appropriate nitrile or imidate. Both drugs have analgesic and antidepressant actions [51].

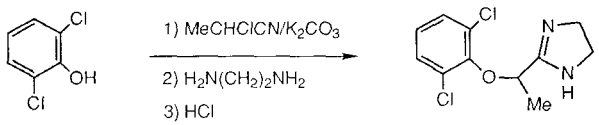


(41) $R^1 = R^2 = Cl$

(42) $R^1 = {}^iPr$, $R^2 = H$

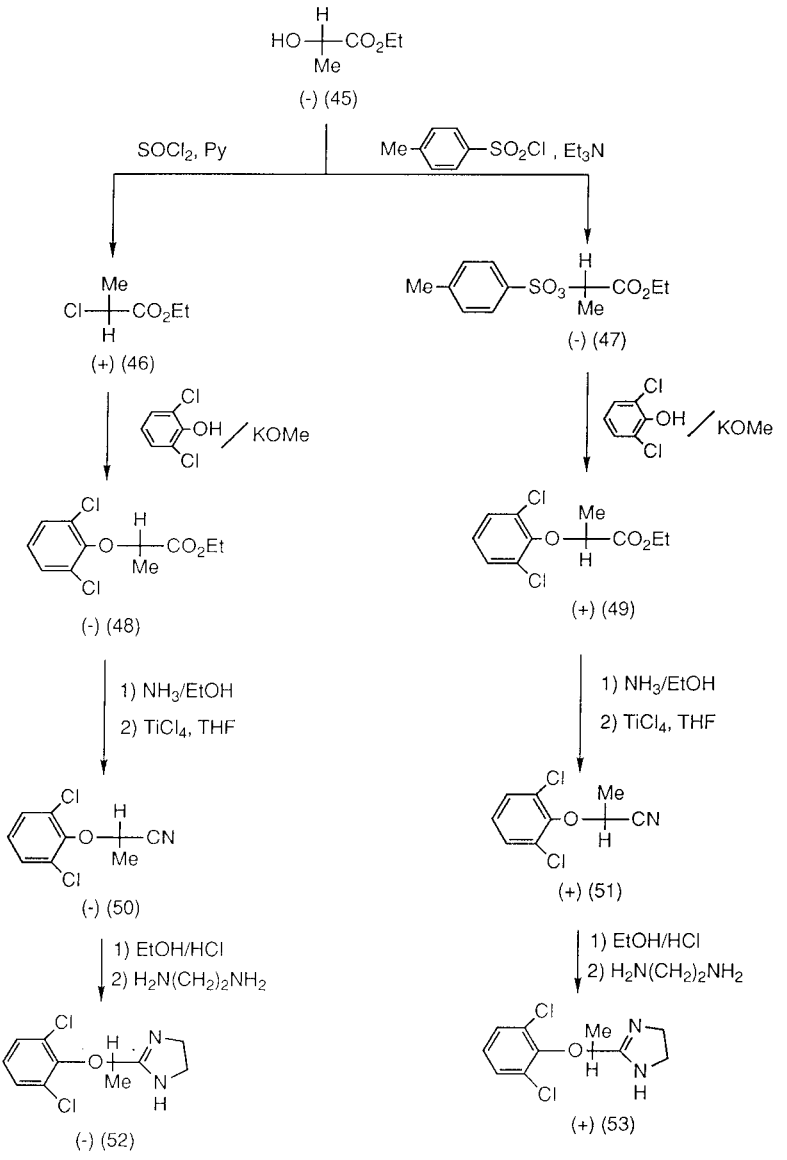
LOFEXIDINE, DEXLOFEXIDINE AND LEVLOFEXIDINE

The racemic phenoxymethylimidazoline lofexidine (44) is unusual among the imidazolines in having α_2-adrenoreceptor agonist activity; it is used as an antihypertensive and is similar in potency to clonidine (133) [52]. The route of preparation is shown in *Scheme 5.12*. Resolution of (44) with (−)-dibenzoyltartaric acid gave the enantiomers levlofexidine [(−)-lofexidine] (52) and dexlofexidine [(+)-lofexidine] (53) [53].



(43) (44)

Scheme 5.12.

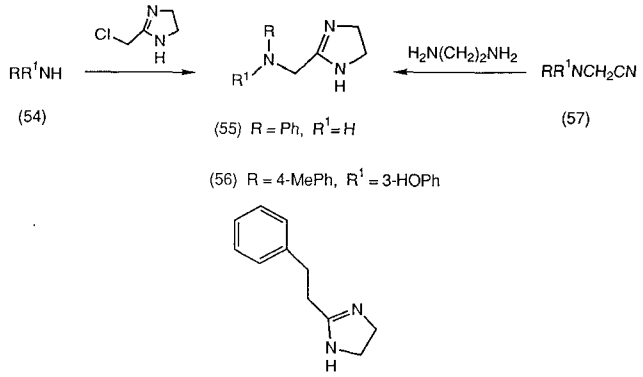

(-) (45)
SOCl2, Py
Me-SO2Cl , Et3N
(+) (46)
(-) (47)
KOMe
(-) (48)
(+) (49)
1) NH3/EtOH
2) TiCl4, THF
(-) (50)
(+) (51)
1) EtOH/HCl
2) H2N(CH2)2NH2
(-) (52)
(+) (53)

Scheme 5.13.

Stereospecific syntheses of the two enantiomers both started from the readily available ethyl L(−)-lactate (45). Reaction with thionyl chloride in pyridine gave an S_N2 inversion of configuration to the 2-chloro ester (46), but esterification of the lactate with tosyl chloride gave the reactive sulphonic ester (47) with retention of configuration in a mechanism which does not touch the chiral carbon. Displacement of the chlorine in (46) or the tosyl group in (47) in conventional Williamson's etherifications proceeded in both cases with inversion to give (48) and (49) respectively. The final steps only involved the ester group and proceeded with complete retention of configuration. The esters were converted to the nitriles (50) and (51) by ethanolic ammonia followed by titanium tetrachloride and the nitriles were taken on to the imidates (EtOH–HCl) before reaction with ethylenediamine to give the (−)-(52) and (+)-(53) modifications of lofexidine, (*Scheme 5.13.*). Levlofexidine (52) is a potent antihypertensive, 10 times more active than dexlofexidine (53) [53 – 55]. A careful recent study with achiral and chiral lanthanide shift reagents allowed the optical purity of lofexidine to be determined by NMR [56].

PHENAMAZOLINE AND PHENTOLAMINE

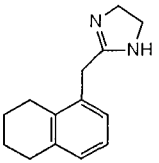
The aryl aminoalkylimidazolines (55) and (56) were prepared by either of the routes shown in *Scheme 5.14.* Phenamazoline (55) [57,58] is a vasoconstrictor, but its carbon analogue (58) is a vasodilator. Phentolamine (56) [59] is a non-selective adrenergic blocking agent [26,60,61] now used in conjunction with papaverine and prostaglandin E1 for the diagnosis and treatment of impotence [62, 63].



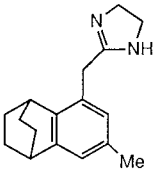
Scheme 5.14.

TEFAZOLINE, METRAFAZOLINE AND TETRAHYDROZOLINE

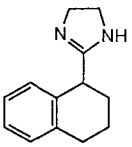
Nitriles and imidates were also the starting materials for the preparation of the tetrahydronaphthalenes tefazoline (59) [64, 65], metrafazoline (60) [66] and tetrahydrozoline (61) [67, 68]. Tetrahydrozoline is useful in dental medicine as a gingival retraction agent [38].



(59)



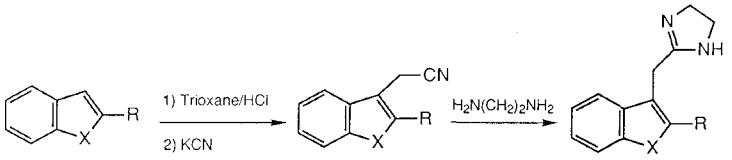
(60)



(61)

COUMAZOLINE AND METIZOLINE

The periferal vasoconstrictors coumazolone (64) [69] and metizoline (65) [70] were synthesized by chloromethylation of the appropriate heterocycle (62) and reaction with potassium cyanide followed by treatment of the nitrile so obtained (63) or its derived imidate with ethylenediamine, (*Scheme 5.15.*).

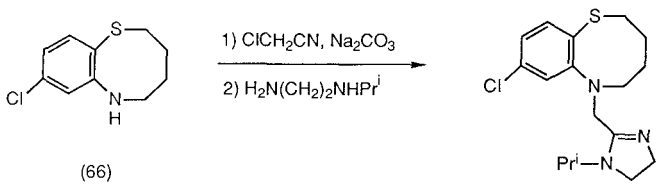


(62) (63) (64) X = O, R = Et (65) X = S, R = Me

Scheme 5.15.

DAZOLICINE

Reaction of the benzothiazocine (66) with chloroacetonitrile followed by cyclization with *N*-isopropylethylenediamine furnished dazolicine (67) (*Scheme 5.16.*) [71]. This is a potent antiarrhythmic drug, claimed from clinical trials to have a low incidence of side-effects [72].

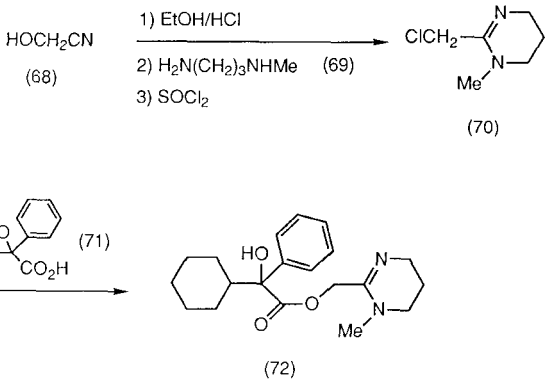


(66) (67)

Scheme 5.16.

OXYPHENCYCLIMINE

The Pinner reaction of glycolonitrile (68) with the propylenediamine (69) followed by treatment with thionyl chloride gave the chloromethylpyrimidine (70). Horenstein-Pählicke esterification of the glycolic acid (71) with (70) in the presence of potassium iodide gave oxyphencyclimine (72) (*Scheme 5.17.*) [73, 74] which possesses anticholinergic effects. The R(+) enantiomer has 29 times the potency of the S(-) form as a muscarinic receptor inhibitor [75].

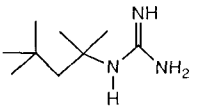


Scheme 5.17.

GUANOCTINE

This simple alkyl guanidine (73) could not be made from the primary amine and S-methylisothiourea, presumably because of the steric hinderance, but

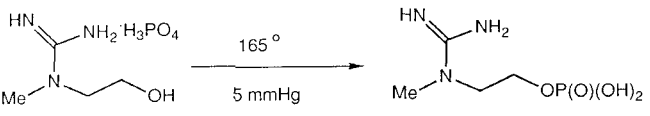
addition to cyanamide succeeded [76]. Guanoctine has adrenergic neuronal blocking properties.



(73)

CREATINOLFOSFATE

Creatinol-*O*-phosphate can be prepared from partially hydrolysed phosphoryl chloride and creatinol hydrobromide at room temperature [77]. Alternatively, thermal dehydration of creatinol phosphate (74) under reduced pressure gives creatinolfosfate (75) (*Scheme 5.18.*) [77, 78]. A commercial procedure employs polyphosphoric acid in this method [79]. The drug is stable in aqueous solution at room temperature, but its isomer creatinol-*N*-phosphate hydrolyses rapidly to creatinol [77]. Creatinolfosfate has anti-ischaemic and antiarrhythmic properties and gives an improved ionic balance and a stronger heart beat [80]. It is useful in the clinical treatment of cardiac insufficiency and rhythm and conduction disturbances [81].

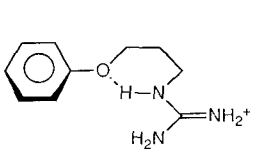


(74) (75)

Scheme 5.18.

GUANOCLOR AND GUANOXYFEN

Rathke reaction of the appropriate primary amine gave guanoxyfen (76) [82] and the appropriate hydrazine guanoclor (77) [83]. In the latter case, guanylation occurred exclusively on the terminal nitrogen. The antihypertensive activities of the two compounds were mediated through adrenergic blocking actions. It was suggested that strong intramolecular hydrogen bonds in the salts, as shown, gave the active conformations [84].



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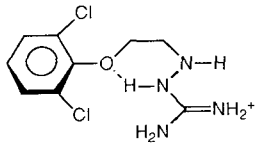
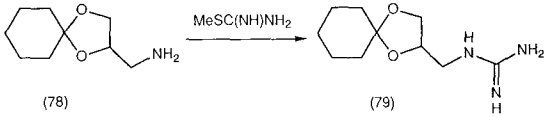


(77)

GUANADREL

The 1-chloropropane-2,3-diol ketal of cyclohexanone was subjected to Gabriel amination to give (78) which was converted to the valuable antihypertensive guanadrel (79) by the Rathke reaction (*Scheme 5.19.*) [85]. This is a peripherally active adrenergic neurone blocker comparable to guanethidine or methyldopa, but is claimed to avoid their side-effects [86, 87]. It is effective and well-tolerated for treatment of hypertension in the elderly [88].

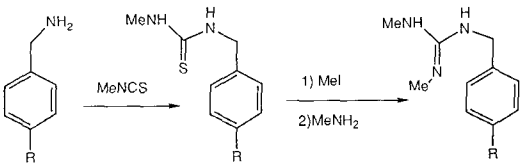


(78) (79)

Scheme 5.19.

BETHANIDINE AND MEOBENTINE

Benzylamines (80) were treated with methyl isothiocyanate to give the thioureas (81) which were *S*-alkylated and exposed to methylamine to give dimethylbenzylguanidines (*Scheme 5.20.*). Bethanidine (82) [89] was used to treat cardiac arrythmias, but its hypotensive effect was too powerful. Meobentine (83) [90] possessed similar antiarrhythmic and antifibrillatory



(80) (81) (82) R = H

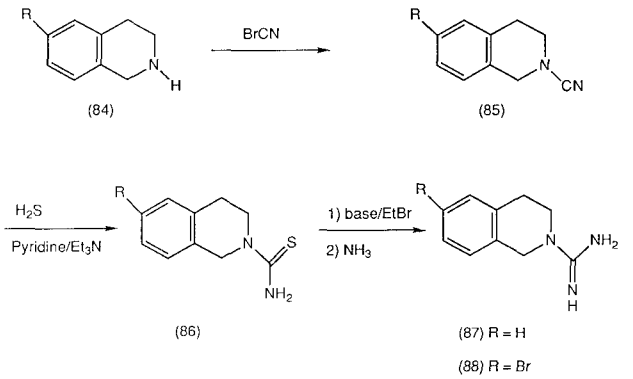
(83) R = OMe

Scheme 5.20.

activity, but less sympathetic blocking action giving little or no reduction in blood pressure [91,92]. The mechanism of the antifibrillatory action of these two drugs has been discussed [93,94].

DEBRISOQUIN AND GUANISOQUIN

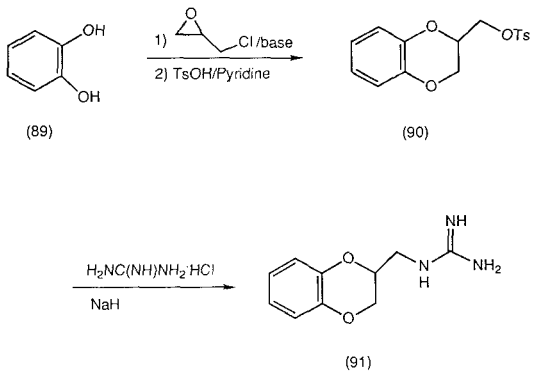
Guanidine derivatives of tetrahydroisoquinoline are generally prepared by the route shown in *Scheme 5.21.* The von Braun reaction converts the secondary amine (84) into the cyanamide (85) and hydrogen sulphide addition in pyridine-triethylamine gives the thiourea (86). Alkylation with bromoethane and treatment with ammonia complete the preparation of debrisoquin (87) and guanisoquin (88) [95]. Recently, direct reaction of (85) with anhydrous ammonia to give (87) has been achieved [96]. The two guanidines are antihypertensives of which debrisoquin is the more useful. It appears to act in part by inhibition of neuronal monoamine oxidases [97].



Scheme 5.21.

GUANOXAN

Treatment of catechol (89) with an excess of epichlorhydrin in a basic medium followed by tosylation gave the benzodioxane (90) which reacted with guanidine in the presence of sodium hydride to give guanoxan (91) in good yield, (*Scheme 5.22.*) [98,99]. It is an antihypertensive acting primarily by preventing the release of noradrenaline from adrenergic nerve endings. Like guanethidine, it depletes peripheral neurones of their stores of noradrenaline, but in contrast to guanethidine it also depletes noradrenaline in the hypothalamus [99–102].

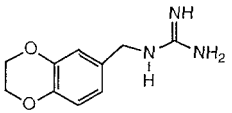


Scheme 5.22.

Guanoxan formed salts with many optically active acids, but could not be resolved in this way because they were too insoluble to recrystallize. However, its hydrolysis product, 2-aminomethylbenzodioxane, was resolved and converted to (+)-(91) and (−)-(91) the former of which carried most of the α-adrenoreceptor blocking activity although the isomers were equipotent as adrenergic neurone blockers [103].

GUABENXAN

The appropriate primary amine and *S*-methylisothiourea give guabenxan (92) [104]. This is an antihypertensive which appears to have particular value in treating adrenaline- and tyramine-induced hypertension [105].

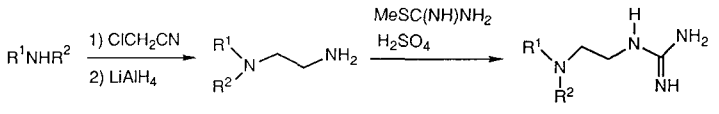


(92)

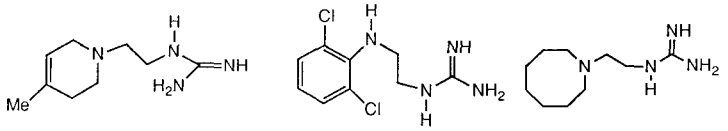
GUANACLINE, GUANCLOFINE AND GUANETHIDINE

A primary or secondary amine (93) treated with chloroacetonitrile followed by reduction gives a diamine (94) which undergoes the Rathke reaction preferentially at the primary amine group to give a β-aminoguanidine (95). In this way were prepared guanacline (96) [106], guanclofine (97) [107] and guanethidine (98) (*Scheme 5.23.*) [108–110]. All are antihypertensives; the

most useful is guanethidine which has a long duration of action and is effective in the treatment of severe hypertensive states. However, the dosage is difficult to regulate so in clinical use it has largely been replaced by newer drugs [111].



(93) (94) (95)

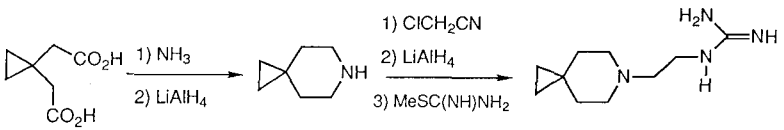


(96) (97) (98)

Scheme 5.23.

SPIRGETINE

Treatment of cyclopropane-1,1-diacetic acid (99) with ammonia to give the imide followed by reduction with lithium aluminium hydride gave the spiropiperidine (100). Alkylation with chloroacetonitrile, reduction, and the Rathke reaction gave spirgetine (101) (*Scheme 5.24.*). The product was claimed to have antihypertensive action with a duration twice that of guanethidine [112].

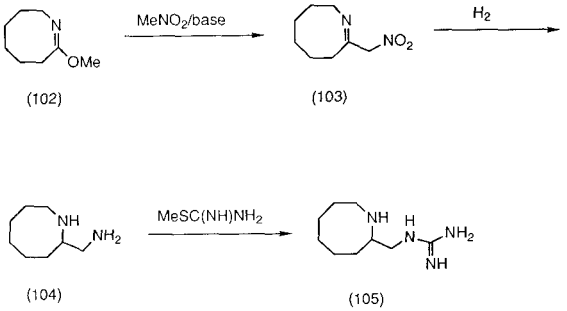


(99) (100) (101)

Scheme 5.24.

GUANAZODINE

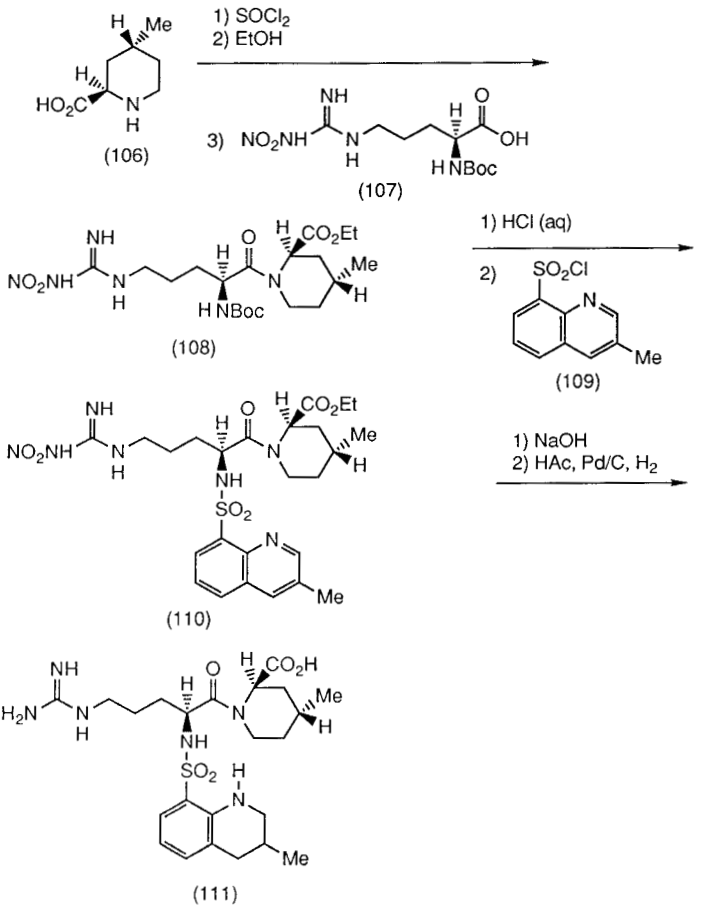
Base-catalysed attack of nitromethane on the imidate (102) gave the derivative (103) which was hydrogenated to the diamine (104). Finally, treatment with *S*-methylisothiourea gave guanazodine (105) (*Scheme 5.25.*) [113]. The actions are similar to those of guanethidine, but this drug is less potent [114].



Scheme 5.25

ARGATROBAN

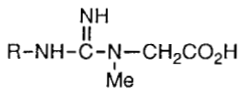
Fractional distillation of a *cis/trans* mixture of ethyl 4-methyl-2-piperidine-carboxylate gave the *trans* racemate which was hydrolysed with aqueous hydrochloric acid and resolved with L-tartaric acid to give the single stereoisomer (106). Esterification and reaction with protected L-arginine (107) furnished (108) which was selectively deprotected and treated with the quinolinesulphonyl chloride (109) to give (110). Basic hydrolysis and hydrogenation in an acidic solvent provided argatroban (111) (*Scheme 5.26.*) [115,116]. This compound is an antithrombotic agent. The potency is 15000 times that of its enantiomer [115, 117]. It showed promise during clinical haemodialysis even in the absence of antithrombin III [118] and has been recommended for post cardiovascular surgery to treat disseminated intravascular coagulation [119]. A preliminary trial suggested that it is a safe and effective treatment for progressive cerebral thrombosis [120].



Scheme 5.26.

PHOSPHOCREATINE AND PHOSPHOCREATININE

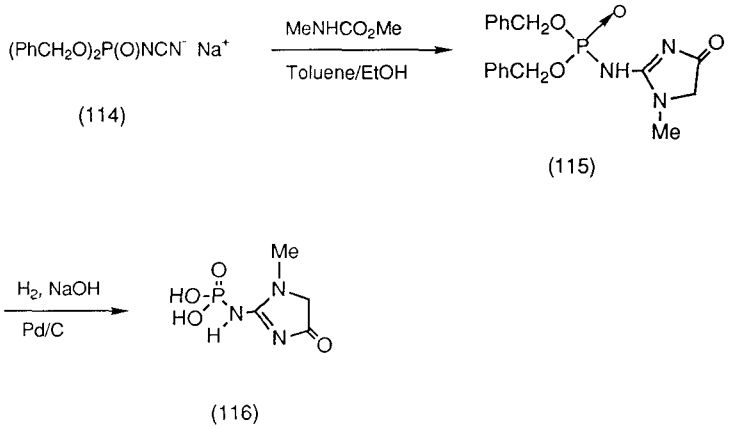
The naturally occuring phosphocreatine (113) may be prepared from creatine (112) with phosphorus oxychloride in alkaline solution [121].



(112) R = H

(113) R = $(HO)_2PO$

Dibenzyl phosphite was treated with sulphuryl chloride in toluene followed by cyanamide in aqueous alkali to give the salt (114). This was condensed with sarcosine methyl ester to give the imidazolinone (115) which after hydrogenolysis gave phosphocreatinine (116) (*Scheme 5.27.*) [122].



(114)

(115)

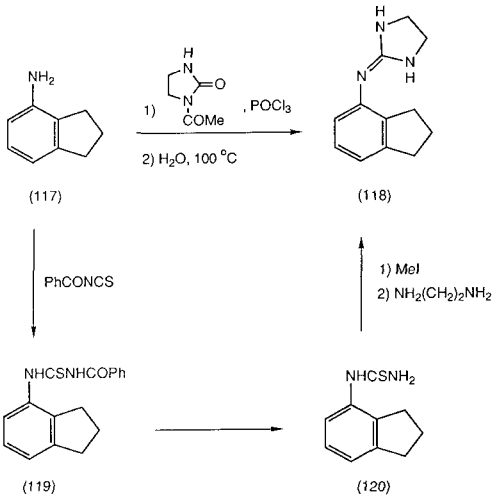
(116)

Scheme 5.27.

Phosphocreatine is an effective treatment for acute myocardial infarction and greatly reduces ventricular tachycardia paroxysms [123]. It has been prepared in cardioplegic solutions for use during heart valve replacement operations where it significantly reduced ischaemic damage [124]. Phosphocreatinine, although less well investigated, also has a beneficial effect on the ischaemic heart [125,126].

INDANAZOLINE

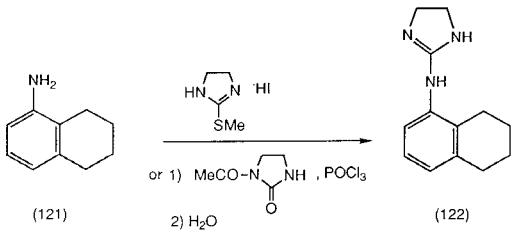
4-Aminoindane (117) was smoothly converted into indanazoline (118) by either of the routes of *Scheme 5.28.* The reaction with 1-acetylimidazolidone and phosphorus oxychloride was followed by an aqueous deacetylation [127]. The drug is a potent vasoconstrictor [128] used as a safe and effective nasal decongestant, recommended to help relieve the symptoms of the common cold [129,130].



Scheme 5.28.

TRAMAZOLINE

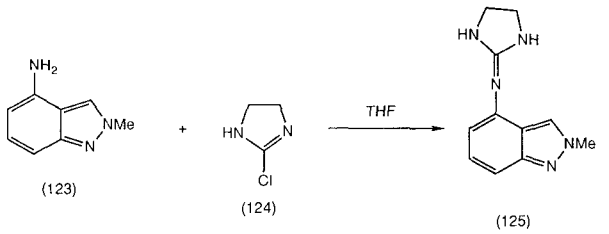
1-Aminotetrahydronaphthalene (121) was converted to tramazoline (122) by either of the routes of *Scheme 5.29*. Although the reaction with 2-methylmercaptoimidazoline gave low yields, the reaction with 1-acetylimidazolidone followed by deacetylation was high yielding [131,132]. Tramazoline is an adrenergic agent which produces a marked and protracted vasoconstriction on mucosal preparations [133]. Its pharmacology and therapeutics have been reviewed; it is an effective nasal spray [134].



Scheme 5.29.

INDANIDINE

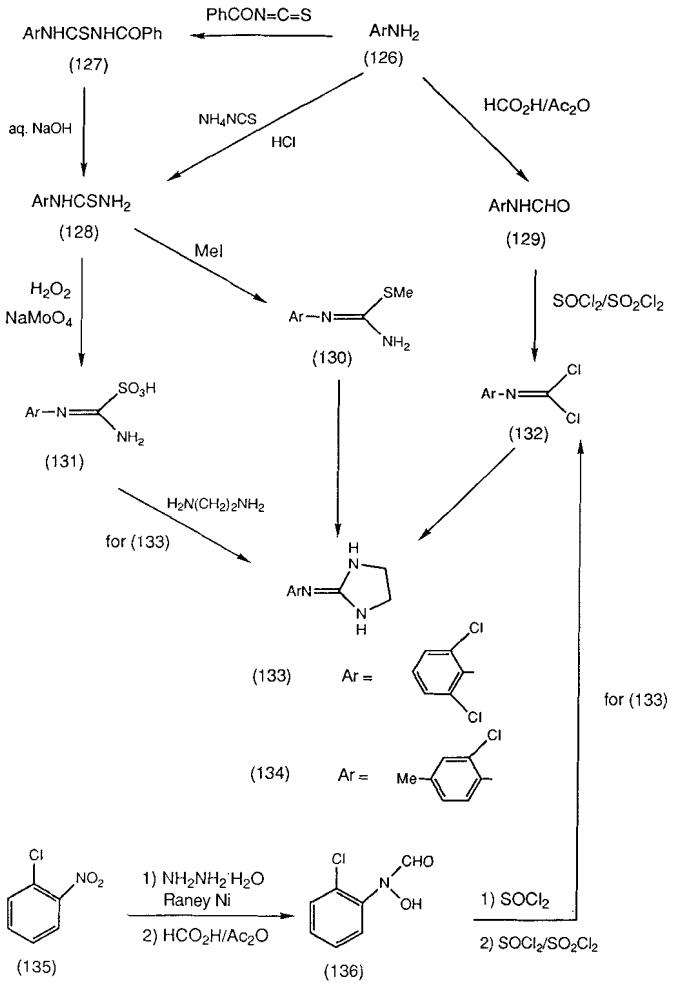
Exposure of 4-amino-2-methylindazole (123) to 2-chloroimidazoline (124) in tetrahydrofuran gave indanidine (125) (*Scheme 5.30.*) [135]. The drug was synthesized in the search for clonidine-like hypotensives, but failed to show any such activity [136]. In broader animal tests it acted as an α_1-adrenoceptor agonist and led to the discovery of a new sub-type of this receptor [137,138]. This was confirmed; there was no centrally or peripherally mediated hypotensive activity and very low α_2-adrenoceptor affinity [139,140].



Scheme 5.30.

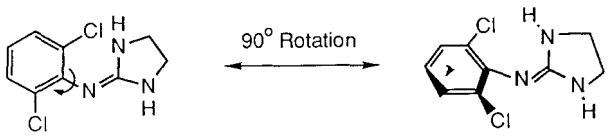
CLONIDINE AND TOLONIDINE

Clonidine (133) and tolonidine (134) were synthesized by both methods of *Scheme 5.31.* In the conventional procedure, the primary arylamine (126) reacted with an acidified solution of ammonium thiocyanate to give the thiourea (128). However, the two step route via the benzoyl thiourea (127) gave higher yields. *S*-Methylation and treatment with ethylenediamine then gave the active moleules. [141-143]. Alternatively, the mixed formic acetic anhydride was used to prepare the formamide (129) which was converted to the dichloroimine (132) before exposure to the diamine [143]. Two new procedures were recently reported for the preparation of clonidine. The thiourea (128) was oxidized to the sulphonic acid (131) which also reacted with ethylenediamine [144]. 2-Chloronitrobenzene (135) was partially reduced by hydrazine–Raney nickel to a hydroxylamine which was formylated to (136). The other ortho position was chlorinated ($SOCl_2$) before more vigorous chlorination (SO_2Cl_2) gave the dichloroimine (132). This provided an inexpensive route to clonidine [145].



Scheme 5.31.

Clonidine, tolonidine and other imidazolidine drugs exist in solution predominately in the imino forms. NMR studies suggested the orthogonal (133b) rather than the planar (133a) conformation [146–148]. Clonidine is 80% protonated at physiological pH and CNDO/2 calculations indicated an interplanar angle of about 40° [149,150]. The experimental pKa's of clonidine and tolonidine are 8.05 and 9.41 respectively while those from a comparative molecular field analysis were 7.94 and 9.21 [151].

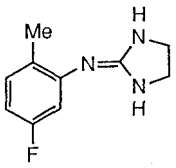


(133a) (133b)

Clonidine was developed as a nasal decongestant, but its marked antihypertensive properties rapidly attracted attention. It is widely used clinically (Catapress®). Low doses give effective control of blood pressure in both supine and upright positions. There is no orthostatic hypotension after rest or exercise and a low incidence of side-effects is experienced [152]. Clonidine has been reviewed and deduced to be indicated for all grades of essential and secondary hypertension [153,154]. A recently developed transdermal preparation gives good control of blood pressure for seven days [155]. It also seems to have useful effects in reducing the craving associated with the withdrawal syndrome. It is an agonist for both pre- and post-synaptic α_2-receptors [30] and the anxiety and irritability of tobacco withdrawal may be associated with this [156]. Similarly, it suppresses nervous system hyperactivity during alcohol withdrawal and may be helpful in the management of opiate withdrawal [157, 158]. The actions of tolonidine in lowering blood pressure and heart rate are similar to those of clonidine, but it is less potent [159–161]. Clonidine, tolonidine and flutonidine (137) lowered the blood pressure and decreased the heart rate of cats and dogs, but also increased blood glucose and showed sedative actions [162]. These drugs were shown to produce sympathetic inhibition by action on a central nervous system α-adrenergic mechanism [163].

FLUTONIDINE

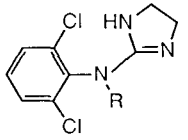
Flutonidine (137) can be synthesized by any of the general methods for guanidines mentioned in the introduction [164]. It is a hypotensive agent, less potent than clonidine but with very much reduced CNS effects [165], it also causes sedation [166].



(137)

ALINIDINE AND PICLONIDINE

Alkylation of clonidine (133) with allyl bromide gave alinidine (138) [167] and with 2-chlorotetrahydropyran piclonidine (139) [168]. Preliminary clinical trials of alinidine demonstrated a bradycardic effect mediated through direct action on the atrial sinus [167]. The slow and economic heart rate was thought to be desirable in the management of coronary heart disease [169]. Piclonidine is less potent than clonidine, but it does not evoke hypertensive episodes or cause sedation [168,170,171].



(138) R = CH_2=$CHCH_2$

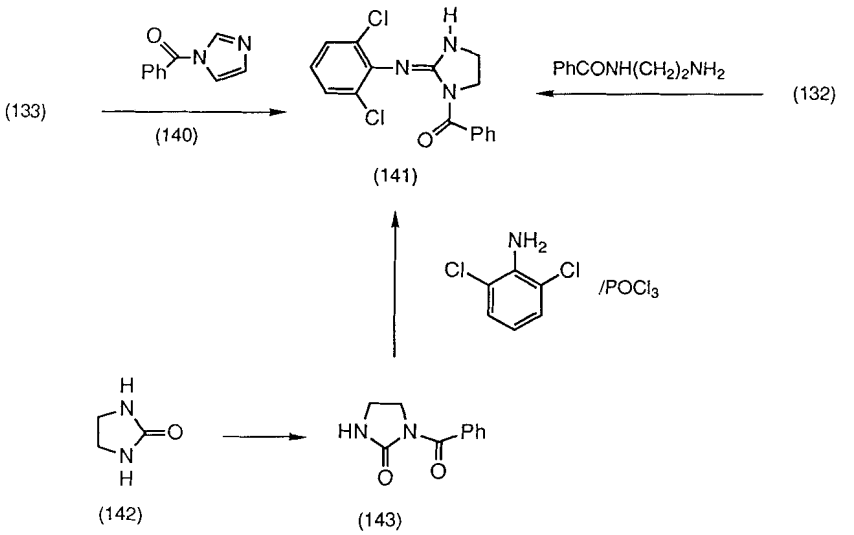
(139) R = 

BENCLONIDINE

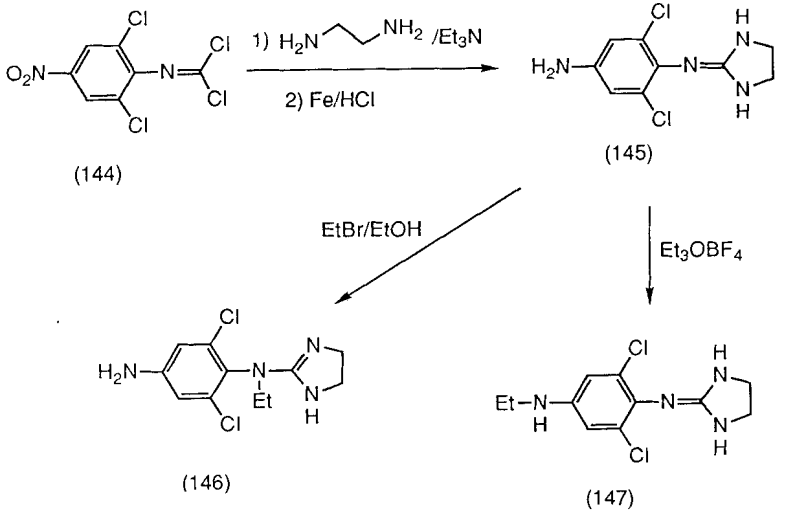
Direct acylation of clonidine (133) with benzoylimidazole (140) gives benclonidine (141) [172]. Alternative methods are condensation of the dichloroimine (132) with benzoyl ethylenediamine or benzoylation of imidazolidinone (142) followed by condensation with dichloroaniline in the presence of phosphoryl chloride [173] (*Scheme 5.32.*). Benclonidine is similar in its antihypertensive effects to clonidine, but is less potent.

APRACLONIDINE

The dichloroimine (144) was prepared in a similar way to (132). Exposure to ethylenediamine formed the imidazolidine ring and metal/acid reduction of the nitro group then gave apraclonidine (145) (*Scheme 5.33.*) [174,175], a drug of comparable activity to clonidine. Alkylations of apraclonidine were found to follow the hard and soft acid and base principle. Bromoethane, a soft reagent, reacted at the 'soft' imino nitrogen to give (146) whilst triethyloxonium tetrafluoroborate alkylated the 'hard' amino group to give (147) [174].

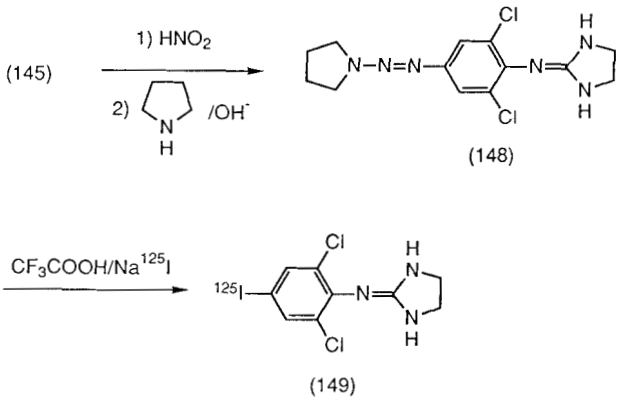


Scheme 5.32.



Scheme 5.33

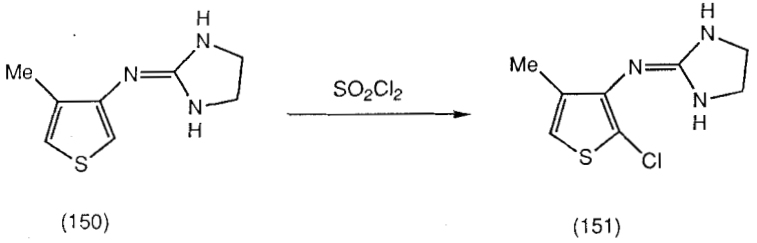
Apraclonidine (*p*-aminoclonidine) was found to lower the intraocular pressure in normal patients and was effective in preventing the acute rise in intraocular pressure which follows argon laser iridotomy [176,177]. The drug was converted to radioiodinated *p*-iodoclonidine (149) by diazotization, coupling with pyrrolidine and decomposition of the resulting triazene (148) with radioactive sodium iodide in trifluoroacetic acid (*Scheme 5.34.*) [175]. The product is a good probe for the α_2-adrenergic receptor.



Scheme 5.34.

TIAMENIDINE

This thiophene derivative was prepared from the isothiourea (130, Ar = 3-methylthiophen-4-yl) and ethylenediamine to give intermediate (150) which was chlorinated with sulphuryl chloride to give tiamenidine (151) (*Scheme 5.35.*) [178]. X-ray crystallography and quantum mechanical calculations showed that the conjugated imino form is predominant in the

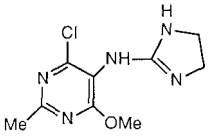


Scheme 5.35.

neutral molecule [179]. Tiamenidine is an antihypertensive, less potent than clonidine, but with an improved therapeutic ratio [180]. A single dose was found to lower the blood pressure significantly during clinical trials [181–183]. Rebound hypertension has been noted following sudden withdrawal [184, 185].

MOXONIDINE

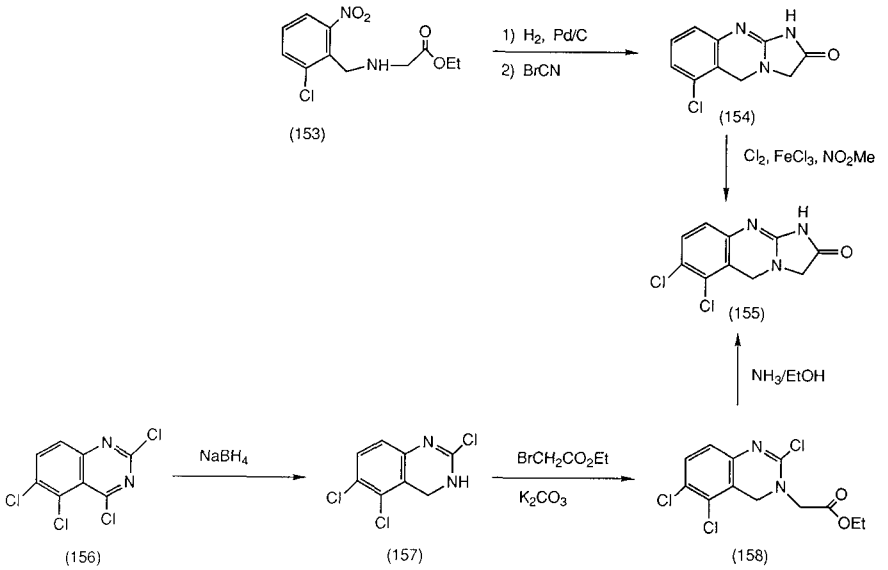
This antihypertensive (152) [186] was synthesized from 3-amino-4-chloro-2-methoxy-6-methylpyrimidine and 1-acetylimidazolidinone followed by hydrolysis as for indanazoline (118) and tramazoline (122) [187, 188]. It is a highly selective full agonist to central α_2-adrenergic receptors [189] which is reliable and safe in long term treatment of hypertension [190]. In a comparative trial, it proved as effective as clonidine against mild to moderate essential hypertension [191].



(152)

ANAGRELIDE

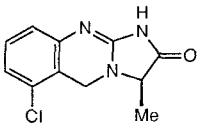
Compound (153) was prepared from the appropriately substituted benzyl chloride and ethyl glycine, the nitro group reduced and the diamine treated with cyanogen bromide to give the tricyclic (154). Chlorination gave anagrelide (155) [192–194]. An alternative route devised by a Japanese group began with the selective reduction of 2,4,5,6-tetrachloroquinazoline (156) to the amidine (157) which, with ethyl bromoacetate, gave the ester (158). This was heated with ethanolic ammonia in a sealed tube to provide anagrelide in 78% yield (*Scheme 5.36.*) [195]. It is a potent inhibitor of ADP and of immune complex induced platelet aggregation [196,197]. The reduction of platelet count in human patients was thought to be an advantage in treatment of polycythemia rubra vera or idiopathic thrombocytosis [198–200]. It may also decrease metastasis formation [201]. In recent trials it gave a good control of thrombocythemia in chronic myeloproliferative diseases and several related conditions [202–204].



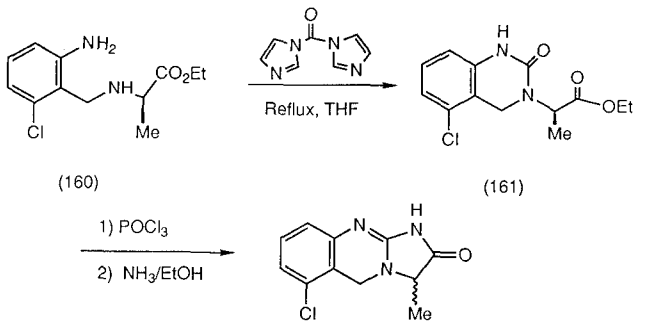
Scheme 5.36.

QUAZINONE

Substitution of ethyl D-alanine for the ethyl glycine of *Scheme 5.36* led to optically pure quazinone (159). If the intermediate (160) was treated with carbonyl di-imidazole and the resulting urea (161) cyclized with phosphorus oxychloride and ammonia, the racemic product (162) resulted (*Scheme 5.37.*) [205]. Quazinone is an inhibitor of platelet aggregation; its enantiomer is less active [206].



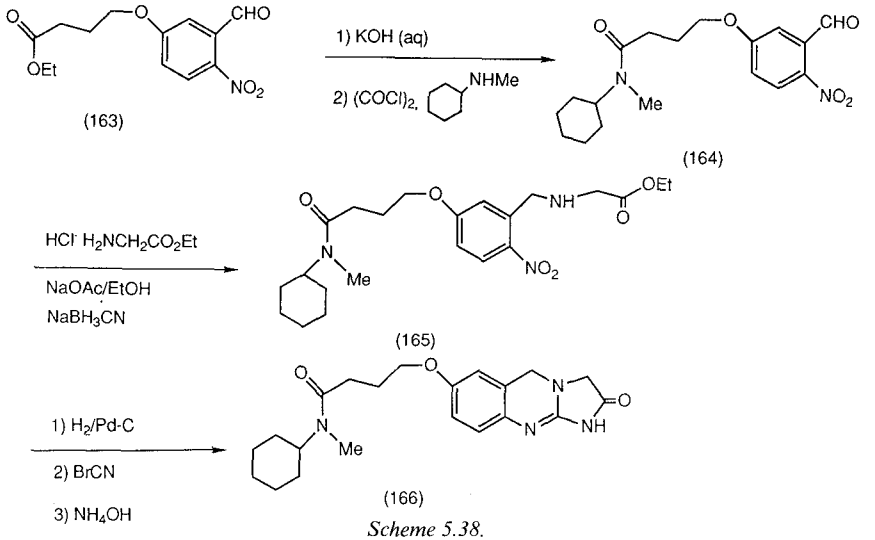
(159)



Scheme 5.37.

LIXAZINONE

Alkylation of 5-hydroxy-2-nitrobenzaldehyde with ethyl 4-bromobutyrate gave the ester (163) which was hydrolysed and converted to the amide (164). Reductive alkylation of the aldehyde group using glycine ethyl ester and sodium cyanoborohydride gave the nitro ester (165) which was hydrogenated and cyclized to lixazinone (166) (*Scheme 5.38.*) [207–210].

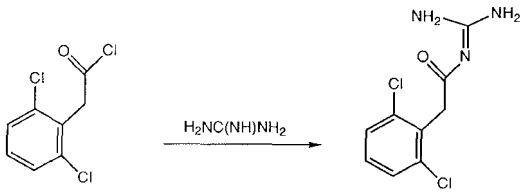


Scheme 5.38.

The drug, a structural combination of cilostamide [211] and anagrelide (155), is a potent and selective inhibitor of cyclic AMP phosphodiesterase. The acyl guanidine unit is both weakly basic (pK_a 3.5) and weakly acidic (pK_a 11.3), but the solubility of the compound is too low for it to be physiologically useful [209, 212].

GUANFACINE

The dichlorophenacyl chloride (167) reacted with guanidine base to give guanfacine (168) (*Scheme 5.39.*). Esters of 2,6-dichlorophenylacetic acid will also react with free guanidine in this way. Alternatively, the acid chloride (167) can be treated with *S*-methylisothiourea to give the *N*-acyl-*S*-methylisothiourea which on ammonolysis is elaborated on to guanfacine [213]. The drug is a centrally acting antihypertensive comparable in potency with clonidine and methyldopa and is well tolerated. There are marked reductions in both systolic and diastolic blood pressure [214–217]. It is often used as an $\alpha^{+}{}_2$-adrenergic agonist in conjunction with a thiazide diuretic, but alone it is an alternative to clonidine or guanabenz [218]. It is an effective initial treatment of mild to moderate essential hypertension and causes less drowsiness than similar drugs [219]. Guanfacine has particular value for its long duration of action, it is rapidly absorbed from the gut and elimination is slow, once a day dosage is recommended [155, 220, 221].

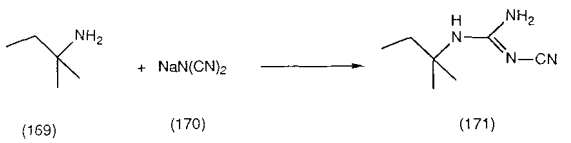


(167) (168)

Scheme 5.39.

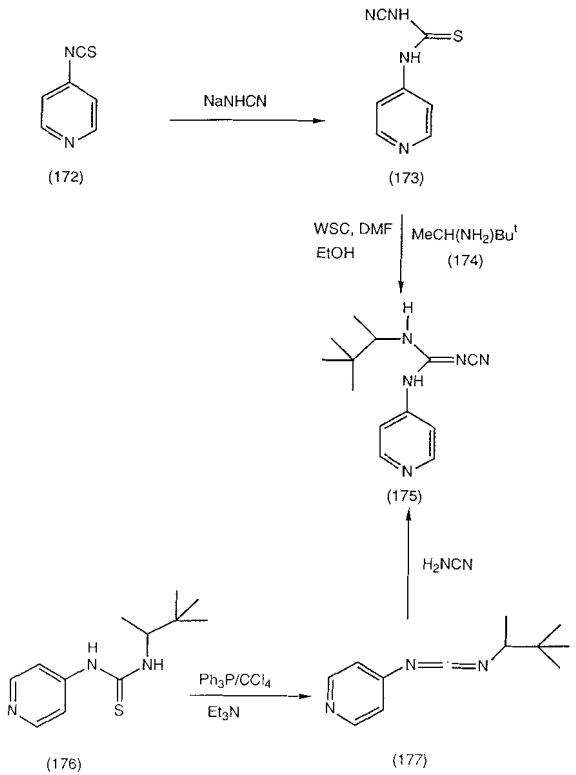
GUANCYDINE AND PINACIDIL

Guancydine (171) was prepared from neopentylamine (169) and sodium dicyanamide (170) (*Scheme 5.40.*) [222]. The preparation of pinacidil (175) by the Rathke reaction gave low yields and required the safe removal of noxious methyl mercaptan. Instead, sodium cyanamide added to 4-pyridylisothiocyanate (172) to give the substituted thiourea (173). In a



Scheme 5.40.

remarkable reaction, this condensed with the amine (174) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride (water soluble carbodi-imide) to give pinacidil in 78% yield after only 30 mins. at room temperature [223] (*Scheme 5.41.*). The compound was previously made by the addition of cyanamide to the di-imide (177) [224].

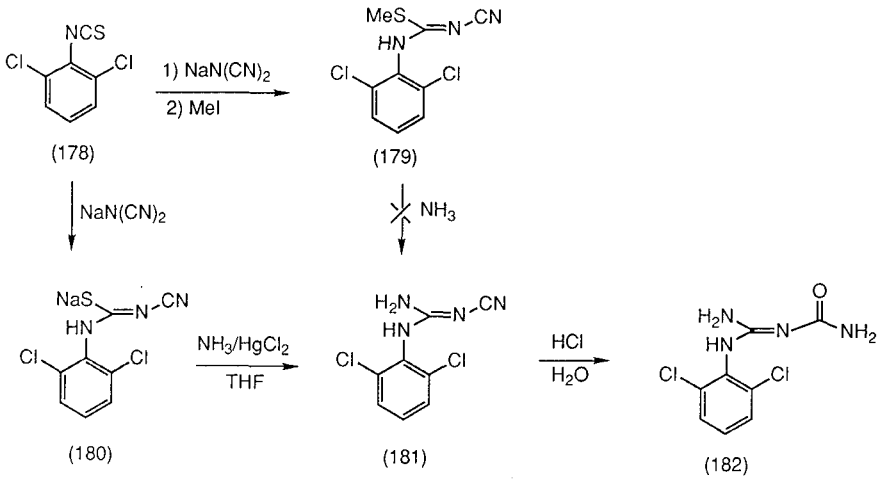


Scheme 5.41.

Guancydine is an antihypertensive [222, 225]; the effect appears to be due to relaxation of peripheral vascular smooth muscles [224]. Pinacidil proved to be 150 times more potent in the hypertensive rat [224], and to have a similar mechanism of action. The fall in blood pressure is accompanied by an increase in heart rate and cardiac output [226, 227]. Pinacidil shows a new mechanism of action as a potassium channel opener [228–230].

BICLODIL

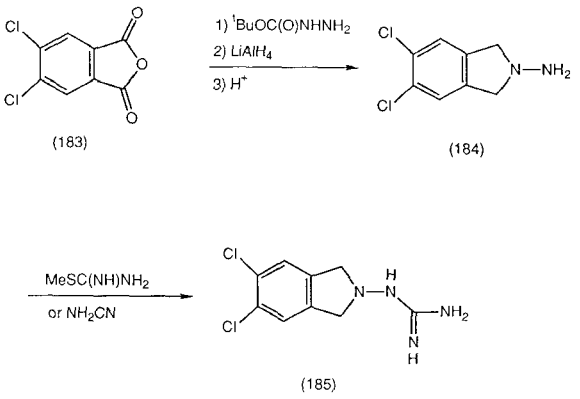
The isothiocyanate (178) was converted to the isothiourea (179), but on treatment with ammonia the nitrile reacted to give a guanidine without replacement of the methylthio group. It was suggested that steric hindrance from the *ortho*-chlorines was responsible for this unexpected reactivity. However, the crude salt (180) readily gave (181) when treated with ammonia in the presence of mercuric chloride. Hydrolysis then afforded biclodil (182) (*Scheme 5.42.*) [231]. The compound has been examined as a vasodilator useful in congestive heart failure [232], but is also used to treat coccidiosis [233].



Scheme 5.42.

AGANODINE

The dichlorophthalic anhydride (183) was converted to the phthalimide with t-butyl carbazate. Lithium aluminium hydride reduced the ring carbonyl groups, but the urethane carbonyl was protected by the bulky alkyl group; hydrolysis then gave the 2-aminoisoindoline (184). Rathke reaction or treatment with cyanamide gave aganodine (185) (*Scheme 5.43.*) [234]. Aganodine is a very selective α_2-adrenergic receptor agonist; it does not cross the blood-brain barrier, but acts peripherally to give a sustained increase in arterial blood pressure [181,235]



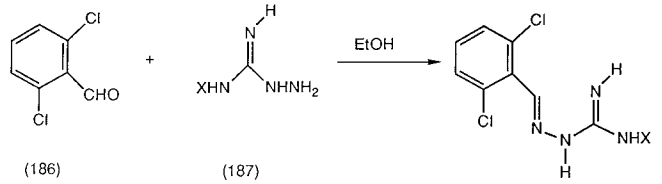
(183) (184) (185)

Scheme 5.43.

GUANABENZ AND GUANOXABENZ

Generally, benzylidineaminoguanidines and benzylideneaminohydroxyguanidines were best obtained by condensation of a benzaldehyde, for example (186) with the appropriate aminoguanidine e.g. (187) in a protic solvent such as ethanol (*Scheme 5.44.*). Alternatively, the hydroxy derivatives were prepared by displacement of the methylthio group of the benzaldehyde methylisothiosemicarbazone with hydroxylamine [236,237]. Guanabenz (188) and guanoxabenz (189) have both central and peripheral α_2-adrenoceptor mediated antihypertensive actions [30,238–240]. However, the vascular relaxation effects of guanabenz are mediated by a different mechanism, possibly it is a potassium channel opener or it may effect calcium mobilization [241]. Recent results suggest that guanabenz and clonidine may be of long term benefit in congestive heart failure, but more work will be needed to confirm this [242]. Guanabenz does not cause

reactive fluid retention as many sympatholytics do [155]. A recent study suggested that guanoxabenz may have some value in distinguishing between subgroups of the α_2 receptor [243].



(186) (187)

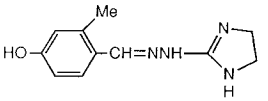
(188) X = H

(189) X = OH

Scheme 5.44.

IDRALFIDINE

Idralfidine (190) was prepared by simple condensation between the appropriate aldehyde and 2-hydrazinoimidazoline [244]. This drug is a hydrophilic antihypertensive developed for the treatment of glaucoma. Clonidine and related compounds are too potent for this; they are difficult to control. Idralfidine has one hundredth the potency of clonidine [245].



(190)

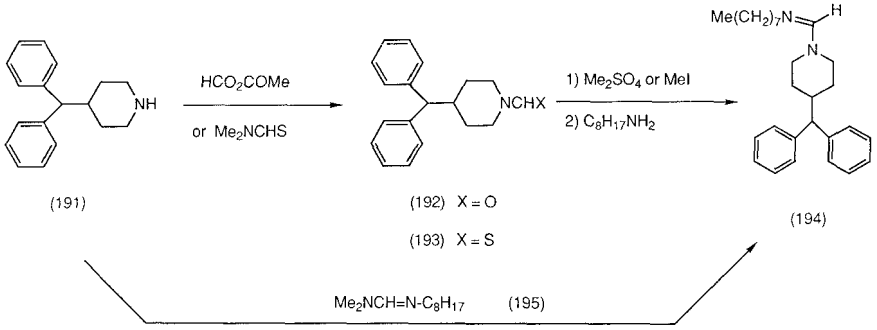
ANTIHISTAMINES

Four histamine H_2 blocking agents were licenced for clinical use in the U.K. and the U.S.A. by 1990. Cimetidine and ranitidine are by far the most widely used and are about equally effective. Less information is available for famotidine and nizatidine, but they seem to be comparable with the earlier drugs when administered in the correct doses. The four compounds can be used to treat gastric and duodenal ulcers, gastro-oesophageal reflux and hypersecretory states [246]. They are particularly useful in the management of Zollinger-Ellison syndrome. This condition is caused by a gastrin secreting tumour, usually of the pancreas, which leads to a marked gastric hypersecretion. In most cases severe ulceration of the stomach or

duodenum results [247]. Clearly, famotidine, nizatidine and other H_2 inhibitors not yet licenced (see below) stand ready to replace the pioneering drugs should either fall from grace [248]. The history of the discovery of the histamine H_2 receptors and details of a number of drugs which act through them has recently been published [249].

FENOCTIMINE

The piperidine (191) was converted to the formamide (192) with acetic formic mixed anhydride or to the thioformamide (193) with *N,N*-dimethylthioformamide. Compound (192) was *O*-methylated with dimethyl sulphate or (193) was *S*-methylated with iodomethane and either intermediate exposed to octylamine to give fenoctimine (194) (*Scheme 5.45.*) [250, 251]. Alternatively, octylamine was converted to N^1,N^1-dimethyl-N^2-octylformamidine (195) with dimethylformamide-dimethyl sulphate. Reaction of this amidine with the piperidine (191) gave fenoctimine in improved yield [252]. The drug is more potent than cimetidine in suppressing gastric acid secretion in animals and clinically shows marked inhibition of food-stimulated acid secretion [250,253,254].

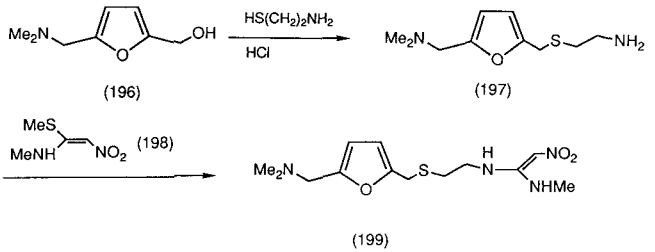


Scheme 5.45.

RANITIDINE

Replacement of the hydroxy group of the furanmethanol (196) with cysteamine readily took place in the presence of hydrogen chloride. The resulting primary amine (197) was warmed with the synthon (198) to give ranitidine (199) (*Scheme 5.46.*) [255]. This was introduced as Zantac in 1981 (U.K.) and in 1983 (U.S.A.) and is difficult to distinguish from cimetidine in

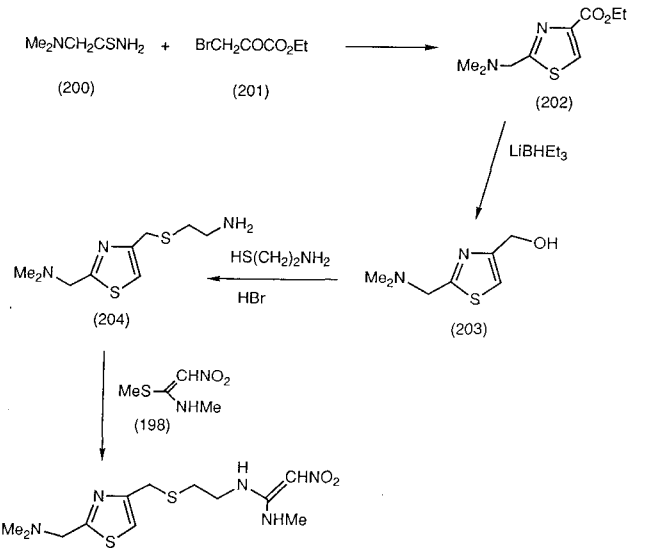
terms of safety and efficacy [256]. Ranitidine has about three times the potency of cimetidine, but is otherwise very similar in its effects. It promotes the healing of gastric and duodenal ulcers and is also used to maintain the patient after recovery. It also relieves reflux oesophagitis and is used to prevent or treat damage from ulcerogenic drugs [257].



Scheme 5.46.

NIZATIDINE

Dimethylaminothioacetamide (200) and ethyl bromopyruvate (201) condensed to give the thiazole (202) which was reduced with lithium triethylborohydride to the alcohol (203). This condensed with 2-aminoethanethiol in 48% hydrobromic acid to the aminosulphide (204) which with the amine (198) gave nizatidine (205) (*Scheme 5.47.*) [258, 259].

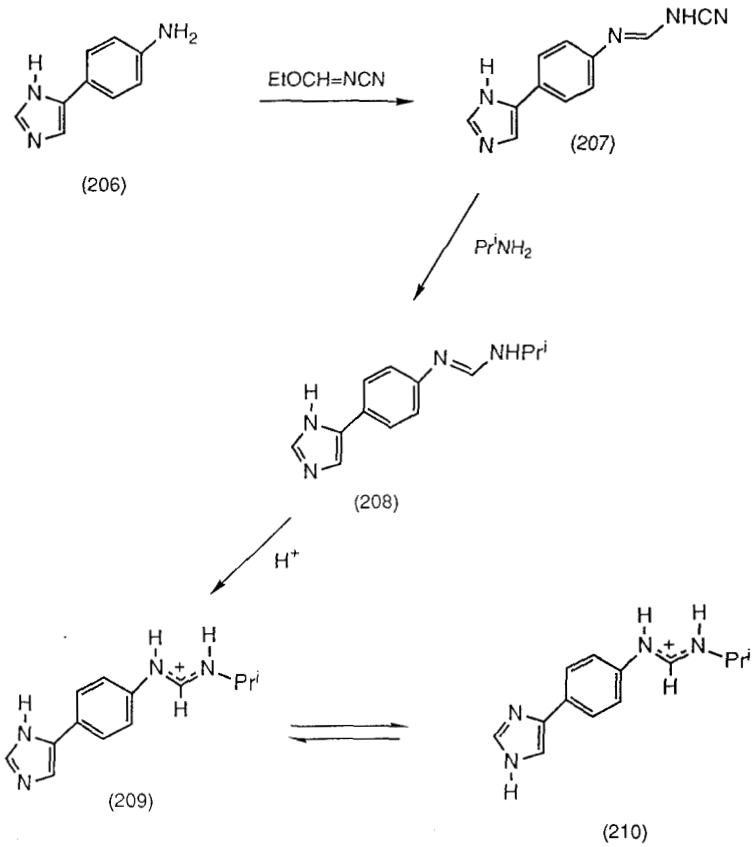


Scheme 5.47.

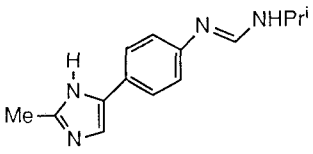
Nizatidine was launched in 1987 (U.K.) and is the least studied of the clinically available antisecretory agents. It is orally active and about four times the potency of, but less antiandrogenic than, cimetidine [260,261]. Nizatidine is effective taken once a day at bedtime, but it reduces the heart rate which could be of concern in the elderly [262, 263].

MIFENTIDINE AND BISFENTIDINE

The amidinonitrile (207) was prepared from the amine (206) and treated with isoproplyamine in water. The loss of a cyanamide unit gave mifentidine (208) as a precipitate (*Scheme 5.48.*) [264,265]. Bisfentidine (211) was prepared similarly [266].



Scheme 5.48.

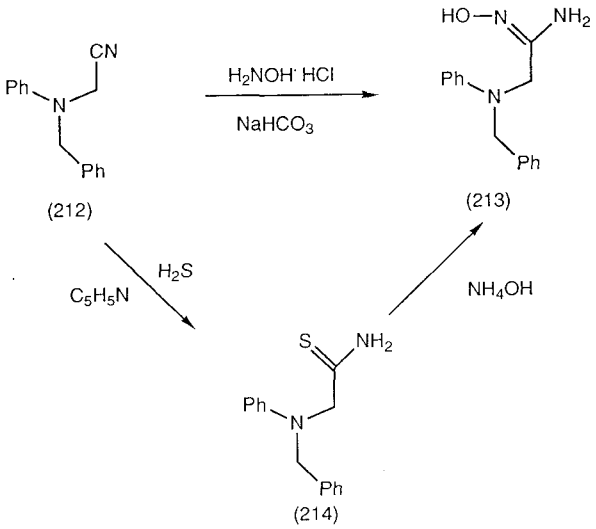


(211)

The amidine system of mifentidine has a pKa of 8.88 and the imidazole ring a pKa of 5.58 [265]. The drug has been shown to exist (95.3%) as the monoprotonated formamidine (209⇌210) at physiological pH [267]. MNDO semiempirical SCF-MO calculations indicate that this monocation is the pharmacologically active species [268]. Clinical studies have shown it to be a more potent histamine H_2 blocker than ranitidine [265]. One clinical trial failed to distinguish it from ranitidine for treatment of duodenal ulcers [269, 270] and it did well in a preliminary trial with gastric ulcer patients [271]. Bisfentidine was about 3 times the potency of ranitidine, and although less investigated is probably very similar to mifentidine in its activity [266].

CETOXIME

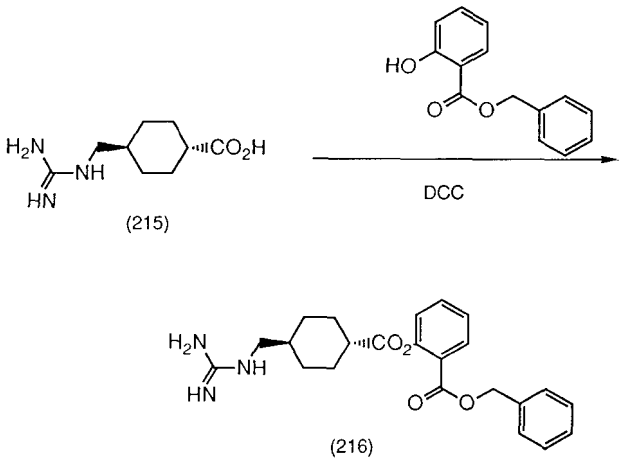
A series of amidoximes including cetoxime (213) was patented as long acting antihistamines. The aminonitrile (212) reacted directly with hydroxylamine in ethanol to give (213) or could first be converted into the thioamide (214) (*Scheme 5.49.*) [272].



Scheme 5.49.

BENEXATE

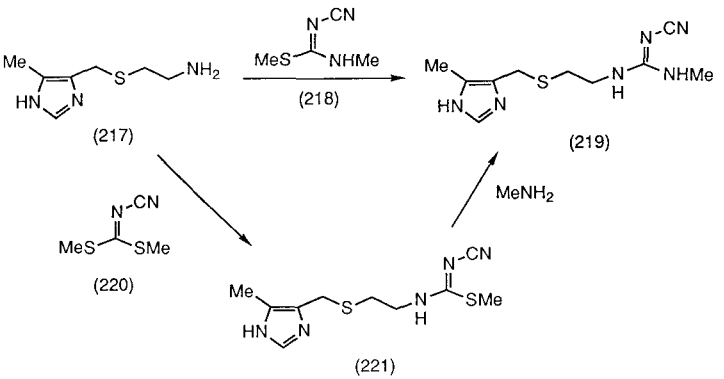
The reaction of *trans*-4-aminomethylcyclohexanecarboxylic acid with methyl isothiourea disulphate gave the guanidine (215). The acid group was esterified with benzyl salicylate in the presence of dicyclohexylcarbodi-imide to give benexate (216) (*Scheme 5.50.*) [273, 274]. Benexate is an antiulcer drug about as effective as cimetidine in the rat and it has both antisecretory and cytoprotective effects [275–277]. It has inhibitory effects on proteases such as trypsin, chymotrypsin, thrombin and urokinase which give it anti-inflammatory and antiallergic properties [274].



Scheme 5.50.

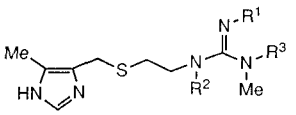
CIMETIDINE

The primary amine (217) underwent the Rathke reaction with the isothiourea (218) to give cimetidine (219). A better yield was obtained with dimethyl cyanodithioimido-carbonate (220) followed by methylamine, *Scheme 5.51* [278, 279]. Hydrolysis of cimetidine (219) gives the guanylurea (222) slowly at room temperature but the guanidine (223) at elevated temperature [278]. A first report indicated that nitrosation gave only (224), but this was ammended when (225) was discovered as a minor product. The rate of nitrosation decreases markedly as the pH increases [280, 281]. The solution conformation of cimetidine has been investigated [278]. The MNDO calculations indicate it has a high molecular flexibility with respect



Scheme 5.51.

to the cyanoguanidine group and the preferred folded and half-folded conformations present the biologically advantageous spacial ordering [282].



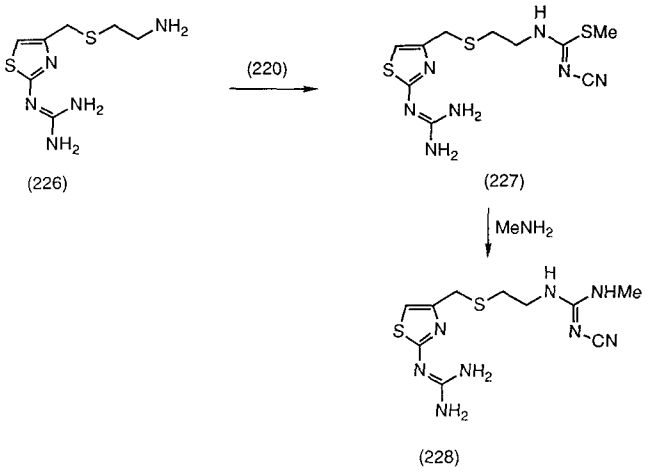
(222) $R^1 = H_2N\text{-}CO\text{-}$, $R^2 = R^3 = H$
(223) $R^1 = R^2 = R^3 = H$
(224) $R^1 = CN$, $R^2 = H$, $R^3 = NO$
(225) $R^1 = CN$, $R^2 = NO$, $R^3 = H$

Cimetidine was the first of the histamine H_2 inhibitors to be licenced (Tagamet, U.K. 1976, U.S.A. 1977) for the treatment of gastric and duodenal ulcers and it dramatically reduced the number of cases where surgery was needed. It became the drug with the highest world sales, but was recently overtaken by ranitidine [283]. Both the pharmacology [284,285] and clinical use [286] have been widely investigated. Both cimetidine and ranitidine are safe and effective and seem to be of equal value in the clinic. They give high rates of healing by the endoscopic test, but suffer the disadvantage that patients relapse unless kept on maintenance therapy indefinitely [287].

TIOTIDINE

The 2-guanidinylthiazole (226, see *Scheme 5.56*) with dimethyl *N*-cyanodithioimido carbonate (220) gave (227) which with methylamine was

converted to tiotidine (228) (*Scheme 5.52.*) [288]. The use of dimethyl or diethyl N-cyanoimidocarbonate, the oxygen equivalent of (220), was also successful in this sequence and was adopted for the manufacture of tiotidine to avoid the production of the environmentally unacceptable methanethiol (or, in classical language, the toxic and odourful methyl mercaptan) [289].



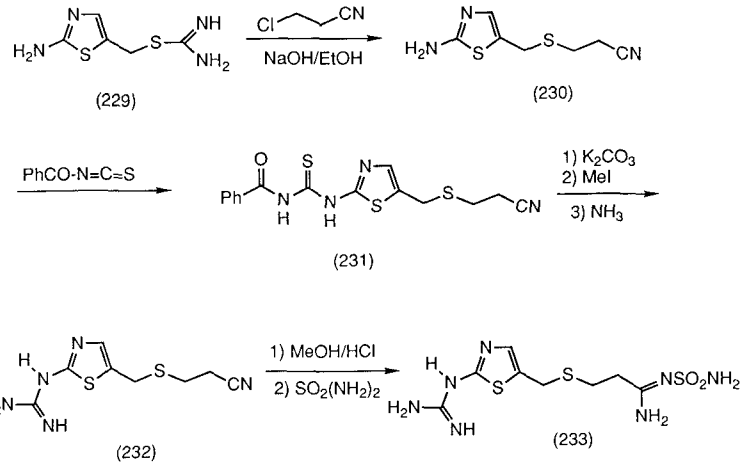
Scheme 5.52.

Tiotidine was shown to be 10 times more potent as an H_2-blocker than cimetidine and was an orally effective antisecretory agent in man [290, 291]. Careful tautomeric analysis showed that the arylimino tautomer shown is favoured over the nonconjugated form by a substantial factor [292]. However, the recognition of characteristic gastric neoplasms at a late stage in its chronic toxicity testing prevented its clinical adoption [708]. Tritiated tiotidine was found to be useful in labelling the histamine H_2 receptor site in guinea pig cerebral cortex membranes [293] and recently revealed the presence of such receptors in guinea-pig left atrium [294].

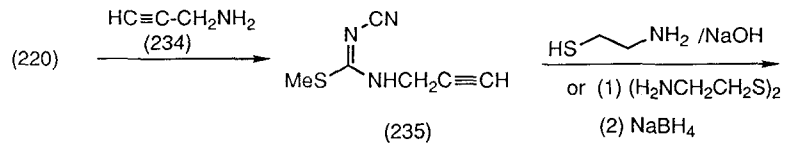
FAMOTIDINE

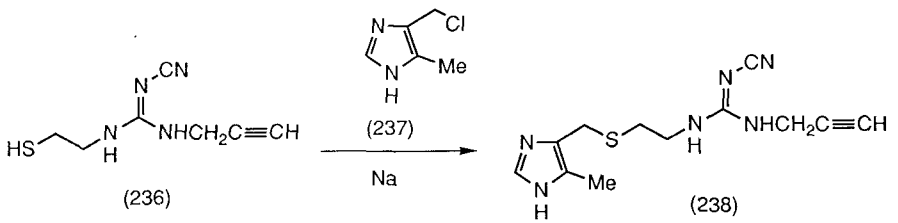
S-(2-Aminothiazol-5-ylmethyl)isothiourea (229) reacted with 3-chloropropionitrile in basic conditions to give the nitrile (230) which with benzoyl isothiocyanate gave the adduct (231). Basic hydrolysis, *S*-methylation and ammonolysis afforded the guanidine (232) which underwent the Pinner reaction with sulphamide to give famotidine (233) (*Scheme 5.53.*) [295]. The

drug is a histamine H_2-receptor antagonist, up to 50 times more potent than cimetidine in reversing histamine-induced gastric acid secretion and diamprit induced hypertension [296,297] and 8 times ranitidine's potency [298]. It is well tolerated and does not interfere with the elimination of antipyrine or show antiandrogenic activity as does cimetidine [299, 300].



Scheme 5.53.

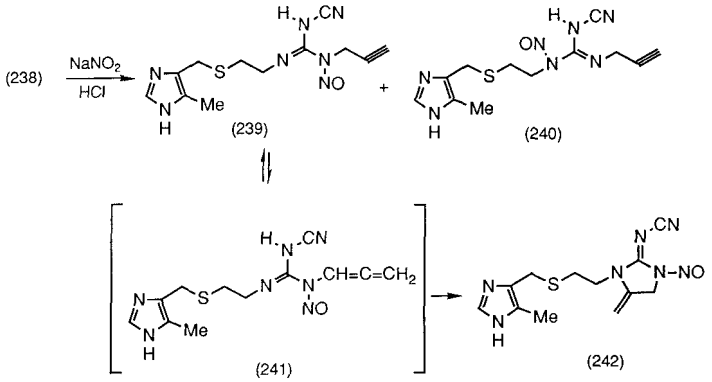




Scheme 5.54.

ETINTIDINE

Replacement of one of the methylmercapto groups in (220) with propargylamine (234) gave the *S*-methylisothiourea (235). The second methylmercapto group was attacked by cysteamine to give the guanidine (236) which was treated with sodium followed by the chloromethylimidazole (237) to give etintidine (238) [301]. The intermediate (236) could also be generated by reaction of cystamine with (235) and reductive cleavage of the S-S bond with sodium borohydride (*Scheme 5.54.*) [302].

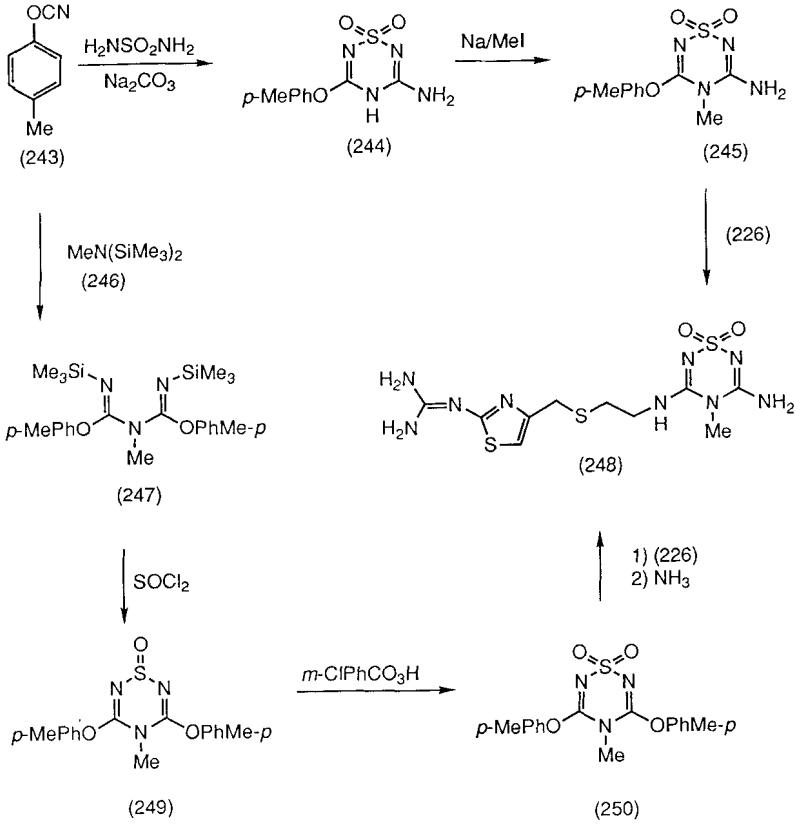


Etintidine was compared with cimetidine for susceptibility to *in vitro* nitrosation [281]. With excess sodium nitrite it gave the nitrosamines (239) and (240) and the imidazole (242). The latter was formed from (239) via the allene (241) under neutral or basic conditions. Both drugs were nitrosated more rapidly at pH 1 than at pH 3 and cimetidine reacted considerably faster than etintidine. With a nitrite concentration 150-500 times that expected in human gastric juice, the nitrosation of etintidine was just detectable. Etintidine is a potent H_2 receptor competitive antagonist with a low afinity for H_1, cholinergic or β-adrenergic receptors [303]. Although it could have value in the treatment of peptic ulcers, early trials suggested that it was not clearly better than cimetidine, ranitidine or tiotidine [304,305]. Interest continues, however, and it was more recently reported to have about twice the potency of cimetidine and to be superior at suppressing meal-stimulated gastric acid secretion [287,306].

TUVATIDINE

Tolyl cyanate (243) reacts with sulphamide to give the thiatriazine dioxide (244) which is methylated at the 4-position to give (245) and the toloxy

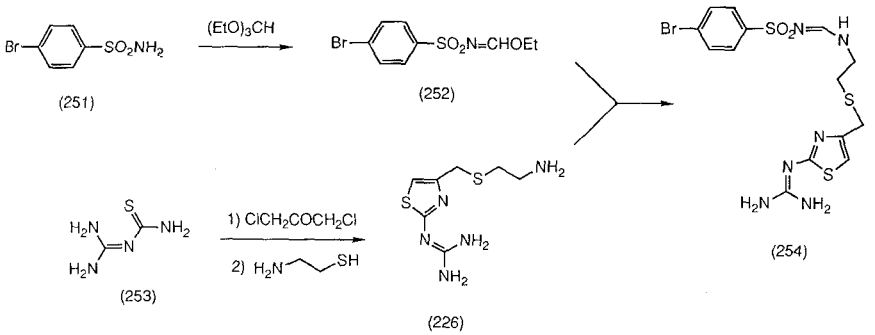
group substituted by (226) to give tuvatidine (248) [307]. Alternatively, (243) can react with bis(trimethylsilyl)methylamine (246) over a long period of time to give (247) which is cyclized with thionyl chloride to the *S*-oxide (249). Peracid oxidation to (250) followed by replacement of the toloxy groups with (226) and ammonia gave tuvatidine (*Scheme 5.55.*) [307]. This H_2 antagonist showed gastric antisecretory activity at a higher potency than cimetidine or ranitidine in the rat, guinea-pig and dog and it is longer lasting. It is devoid of histamine H_1, muscarinic or adrenergic activity [308,309]



Scheme 5.55.

EBROTIDINE

4-Bromobenzenesulphonamide (251) was treated with triethyl orthoformate to give the formimidate (252). Amidinothiourea (253) was cyclized with 1,3-dichloroacetone followed by treatment with 2-aminoethanethiol to give the thiazole derivative (226). The convergent synthesis was completed by reaction between (252) and (226) to give ebrotidine (254). The new compound was as active as cimetidine as an inhibitor of gastric acid secretion (*Scheme 5.56.*) [288, 310].



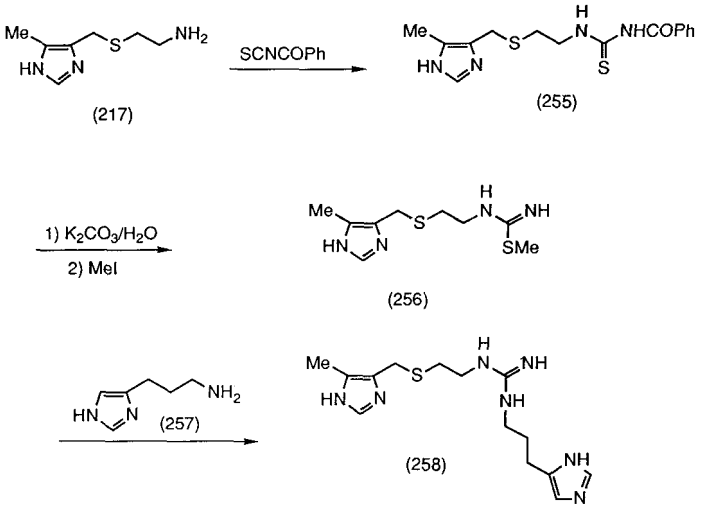
Scheme 5.56.

IMPROMIDINE AND SOPROMIDINE

The primary amine (217) was heated with benzoyl isothiocyanate to give the benzoyl thiourea (255). Base-catalysed hydrolysis and methylation gave the expected isourea (256) which with 3-(4-imidazolyl)propylamine (257) gave impromidine (258) [311] (*Scheme 5.57.*).

This first synthesis was reported to be troublesome in the isolation and purification of the product [312]. The preferred method is illustrated in *Scheme 5.58.* Reaction of the simple amine (257) with the imidocarbonate (259) formed the *O*-phenylisourea (260) which was cyclized in base to the imidazodiazepine (261). Ring opening with primary amine (217) and hydrolysis completed the synthesis [312, 313].

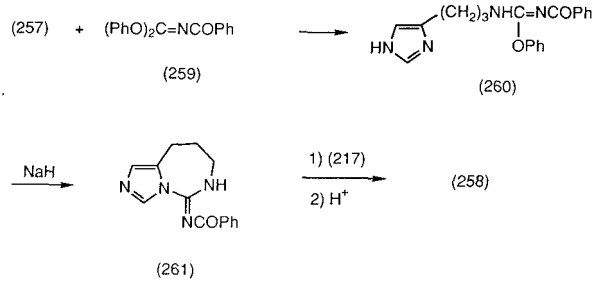
Impromidine is a selective H_2 agonist used as a diagnostic aid; the potency varies between 9 and 100 times histamine depending on the test system [312, 314,315]. In humans, it produces maximal gastric acid secretion, an effect inhibited by cimetidine [316]. It is a potent inhibitor of histamine methyl transferase and diamine oxidase [315]. The two imidazole units and the



Scheme 5.57.

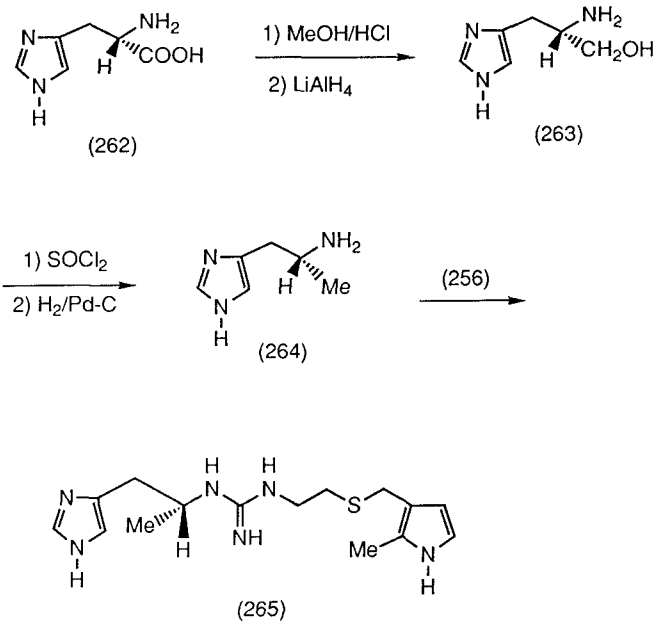
guanidine system give macroscopic pKa values of 6.41, 7.26 and 11.6, so the monocation (guanidine group protonated) is the dominant species (65%) at physiological pH, a further 33% is a mixture of the two dications where the guanidine and one imidazole are protonated and about 2% is the trication [312].

The chiral structural isomer sopromidine (265) was prepared from L-histidine (262) (*Scheme 5.59.*). The amino acid was esterified and reduced to the chiral alcohol (263) which was converted to the alkyl chloride and hydrogenated to the amine (264), enantiomerically pure (*R*)(−)-α-methylhistamine. Reaction with the isothiourea (256) then gave (265) [317–320].



Scheme 5.58.

Sopromidine has 7.4 times the potency of histamine on the atrium, but the (*S*)-enantiomer (prepared from D-histidine) is a competitive H_2 antagonist with about a quarter the potency of metiamide. It is remarkable that this high stereoselectivity is shown by a receptor for the non-chiral histamine [317,318].

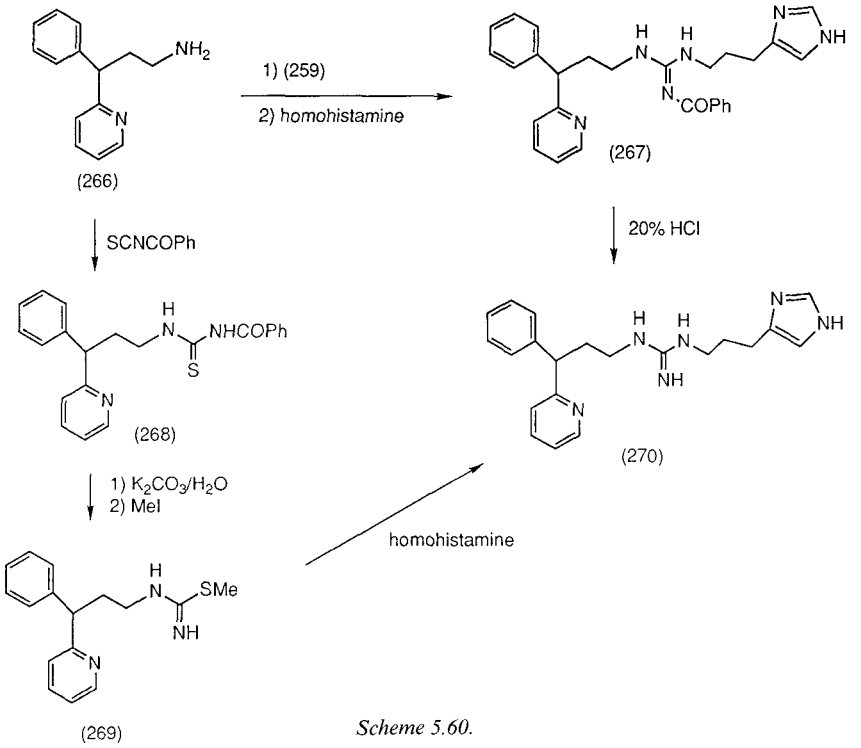


Scheme 5.59.

ARPROMIDINE

The pheniramine-like amine (266) was treated with diphenyl N-benzoylcarbonimidate (259) and the resulting benzoylisourea refluxed with homohistamine in acetonitrile or pyridine to give the benzoylguanidine (267) which could be hydrolysed to arpromidine (270) in 20% hydrochloric acid. An alternative route which avoids the strong acid uses reaction with benzoylisothiocyanate to give the benzoylurea (268) which undergoes mild alkaline hydrolysis and *S*-methylation before reaction with homohistamine (*Scheme 5.60.*) [321]. This and a number of derivatives were prepared in the hope that the replacement of the cimetidine moiety of impromidine with the

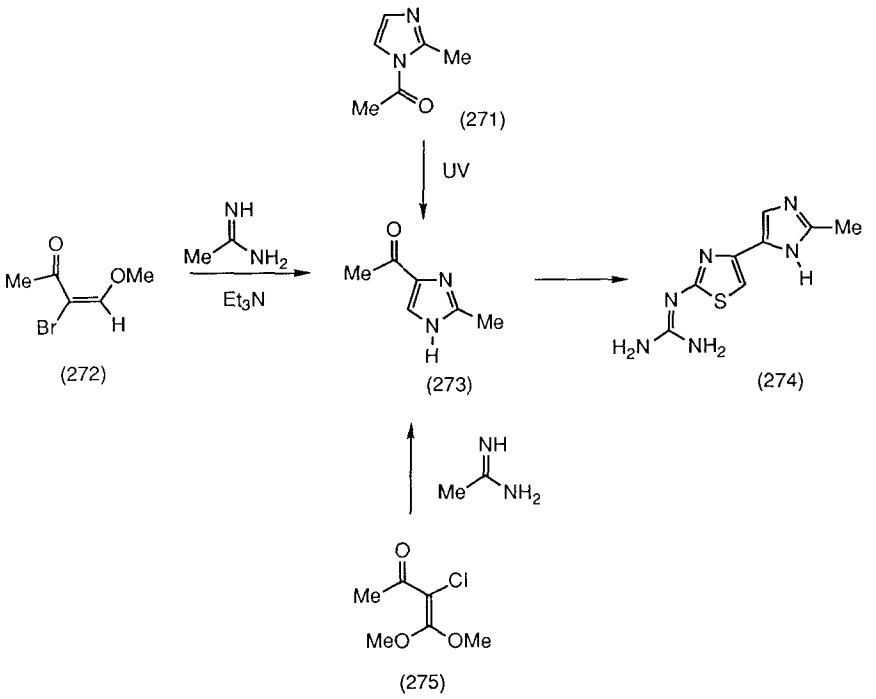
more lipophilic, H_2-nonspecific pheniramine moiety would give more potent H_2 agonists. Arpromidine has about 100 times the activity of histamine at the H_2 receptor of the guinea pig atrium with H_1-antihistaminic activity similar to pheniramine [321].



Scheme 5.60.

ZALTIDINE

2-Bromoacetoacetaldehyde was methylated with dimethyl sulphate to give the enol ether (272). Treatment with an acetamidine salt in the presence of triethylamine or sodium acetate gave 2-methyl-4-acetylimidazole (273) which was brominated at the acetyl methyl group and exposed to amidinothiourea to form zaltidine (274) [322–324]. The intermediate imidazole (273) can also be generated by photolysis of 1-acetyl-2-methylimidazole (271) [323,325], or from 3-chloro-4,4-dimethoxy-3-buten-2-one (275) (*Scheme 5.61*) [326].

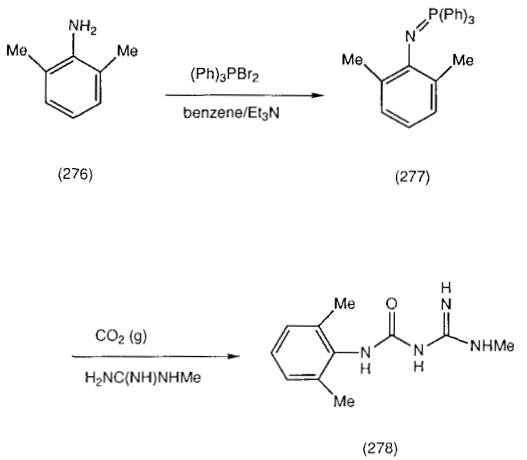


Scheme 5.61.

Zaltidine is a potent histamine H_2-receptor antagonist both on isolated atria and on gastric acid secretion. Its suitability for treatment of stomach and duodenal ulcers has been suggested [322,327–329]. It is effective with a long duration of action in human patients [330]. Zaltidine also has potential for use in the treatment of rheumatoid arthritis [331, 332].

LIDAMIDINE

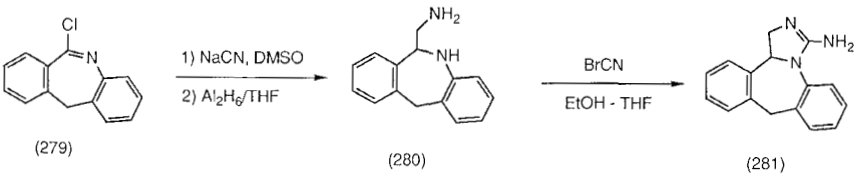
In the usual method of preparation of N-substituted amidinoureas from an isocyanate and a substituted guanidine, phase transfer conditions were found to improve the yields [333–335]. The interesting alternative route of *Scheme 5.62* gave a high yield of lidamidine (278) from the phosphazine (277) treated with carbon dioxide and methylguanidine [336]. Lidamidine is a well-tolerated antidiarrhoeal agent and has beneficial effects in gastroduodenal motor dysfunction [337–340]. Its *N*-demethyl metabolite (WHR1049) turns out to be equally effective and longer lasting than lidamidine [341].



Scheme 5.62.

EPINASTINE

The chloroimine (279) gave the nitrile which was reduced with aluminium hydride to the diamine (280). The action of cyanogen bromide then gave epinastine (281) (*Scheme 5.63.*) [342] which is a peripherally acting antihistamine. It is a highly selective histamine H_1 receptor antagonist (H_1:H_2 receptor affinity ratio 400:1), but is devoid of CNS sedative or anticholinergic effects [343–345]. It was assumed from its physical constants, including a remarkably high pK_a (11.2), and its hydrogen bonding ability that it cannot cross the blood/brain barrier; no CNS effects were seen in clinical trials [342].

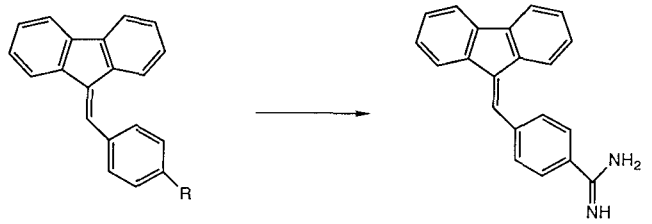


Scheme 5.63.

ANTI-INFLAMMATORY AGENTS

PARANYLINE

The bromobenzalfluorene (282) gave the nitrile (283) with cuprous cyanide and a Pinner reaction then gave the amidine paranyline (284), *Scheme 5.64*. Excess ethanol was needed to ensure a good yield of the intermediate ethyl imidate hydrochloride. Paranyline has anti-inflammatory and antifungal actions [346,347].



(282) R = Br

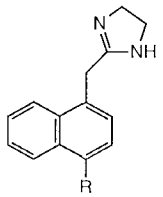
(283) R = CN

(284)

Scheme 5.64.

NAPHAZOLINE AND CLONAZOLINE

Naphazoline (285) [25] and clonazoline (286) [348] were made from the appropriate imidate and nitrile respectively by reaction with ethylenediamine. Clonazoline is about four times more potent than naphazoline as a vasoconstrictor, but both compounds are used to treat inflammatory conditions of the conjuntival and nasal mucosa.

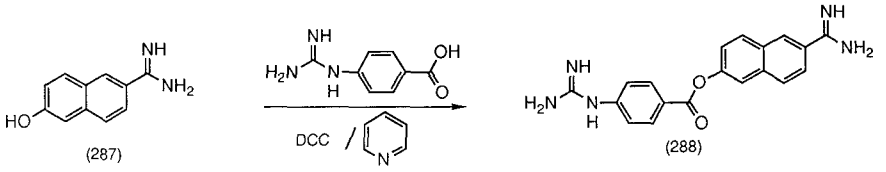


(285) R = H

(286) R = Cl

NAFAMOSTAT

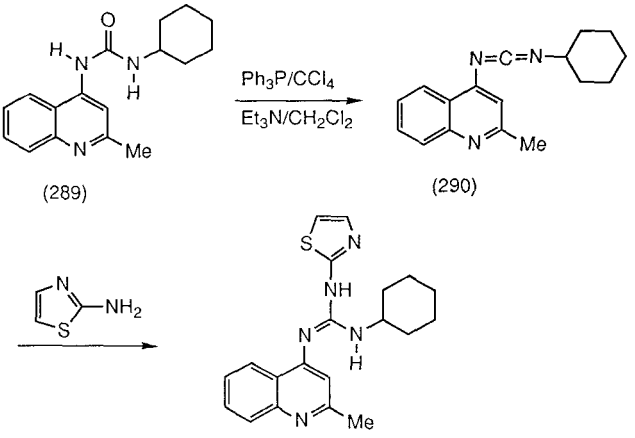
Cyanation of 6-bromo-2-naphthol with cuprous cyanide followed by the Pinner reaction gave amidine (287) which was esterified with 4-guanidinobenzoic acid to yield nafamostat (288) (*Scheme 5.65.*) [349,350]. Nafamostat was shown to be an effective inhibitor of a number of enzymes [351, 352]. It acted in this way in acute pancreatitis in the rat to reduce mortality in a dose dependent manner. *In vitro* it was 10 to 100 times more potent than gabexate [353]. Nafamostat is an inhibitor of synthetic protease and of classical and alternate pathways of complement activation [354]. Complement activation during cardiopulmonary by-pass operations produces anaphylatoxins which lead to severe complications. Nafamostat inhibits this activation [355]. Added to ointments containing human epidermal growth factor it accelerated the healing of wounds due to its protease inhibitor action [356]. Applied as an ointment to the insulin injection site it reduced degradation and increased the hypoglycaemic action [357].



Scheme 5.65.

TIMEGADINE

The urea (289) was dehydrated in the presence of triphenylphosphine, carbon tetrachloride and triethylamine to give the carbodi-imide (290) and addition of 2-aminothiazole precipitated timegadine (291). This has a powerful anti-inflammatory action, but no hypotension or other biological activity could be detected (*Scheme 5.66.*) [358].



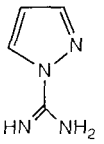
(289) (290)

(291)

Scheme 5.66.

PRAXADINE

The Knorr pyrazole synthesis with 1,1,3,3-tetraethoxypropane and aminoguanidine readily gave praxadine (292) which is an anti-inflammatory and analgesic agent [359, 360].



(292)

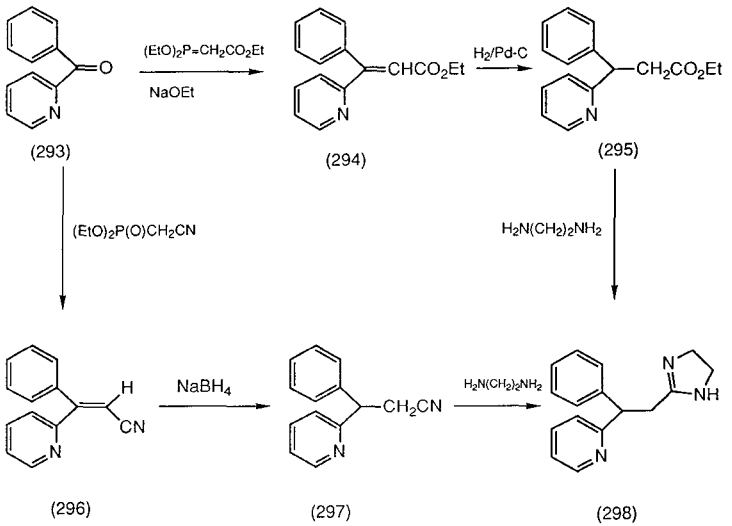
ANTIDIABETIC DRUGS

Antidiabetic drugs have been discussed in ref. 360a.

MIDAGLIZOLE

Phenyl 2-pyridyl ketone (293) underwent Wittig-Horner reactions to give the unsaturated ester (294) and the acrylonitrile (296). However, neither

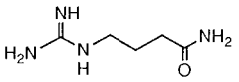
product could be induced to react with ethylenediamine even at high temperature and attempts to convert the nitrile to its imidate were equally unsuccessful. Catalytic hydrogenation of (294) gave the more reactive saturated ester (295) which was easily converted into midaglizole (298). The olefinic bond of (296) could be reduced with sodium borohydride to give (297) which also was an intermediate for midaglizole (*Scheme 5.67.*). This drug has a potent inhibitory effect on adrenaline-induced human blood platelet aggregation [361, 362] and showed high orally effective hypoglycaemic activity; it was hailed as a valuable new antidiabetes agent [362,363], but it also shows anti-asthmatic activity [364]. A member of a new class of selective α_2-adrenergic receptor antagonists [363,365,366], it turned out to exert its hypoglycaemic action by its effect on the α_2-receptors of the islets of Langerhans in stimulating insulin secretion [362, 367].



Scheme 5.67.

TIFORMIN

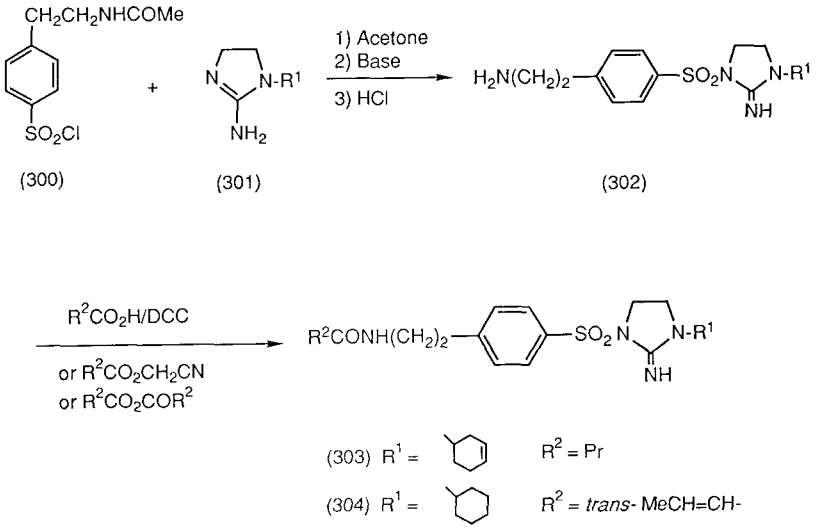
The Rathke reaction of 4-aminobutyramide readily gave tiformin (299) [368], claimed to be an antihyperglycaemic agent.



(299)

GLIBUTIMINE AND CGP 11112

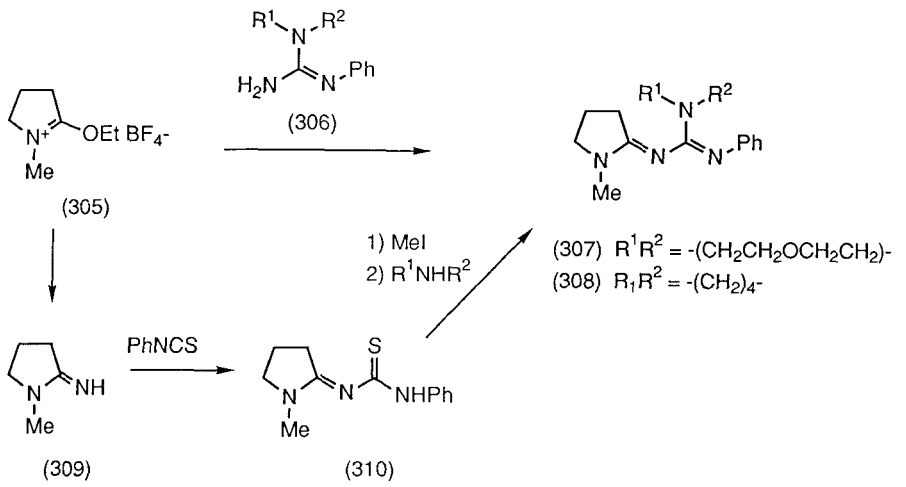
The sulphonyl chloride (300) condensed with an aminoimidazoline (301) to give, after deprotection, an imino derivative (302). Acylation with an acid, ester or anhydride gave glibutimine (303) or CGP 11112 (304) (*Scheme 5.68.*) [369, 370]. Like the sulphonyl ureas, of which these are imino derivatives, the drugs are oral hypoglycaemics. CGP 11112 is 20 times more potent than tolbutamide, but comparable to phenformin (315) [370,371]. The X-ray structure shows a hydrogen bond between the imine hydrogen and the sulphonyl oxygen [372].



Scheme 5.68.

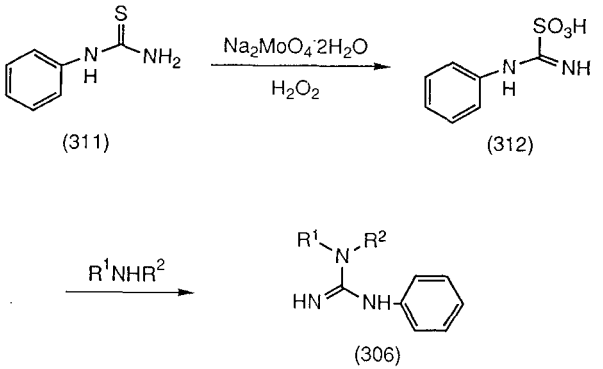
LINOGLIRIDE AND PIROGLIRIDE

The lactam salt (305) is readily available from 1-methyl-2-pyrrolidinone and triethyloxonium tetrafluroborate. On treatment with a substituted guanidine (306) it gave linogliride (307) or pirogliride (308). Alternatively, the iminopyrrolidine (309) and phenyl isothiocyanate gave the thiourea (310) which was converted to the required drugs via *S*-methylation and amination (*Scheme 5.69.*) [373]. Recently an elegant preparartion of guanidines like (306) was announced.



Scheme 5.69.

A thiourea such as (311) was oxidized with sodium molybdate and hydrogen peroxide to an amidine sulphonic acid (312). The sulphonic acid group was replaced by an amine to give the guanidine (*Scheme 5.70.*) [374]. The avoidance of methyl mercaptan as a by-product was a considerable advantage.



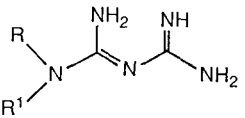
Scheme 5.70.

Linogliride and pirogliride are oral hypoglycaemic agents which differ in their mechanism of action from the biguanides and sulphonylureas. Both are potent enhancers of glucose-induced insulin secretion [375,376].

Linogliride was 2 to 8 times more potent than pirogliride in inducing hypoglycaemia in non-diabetic animals [376] and stimulated insulin release from the rat pancreas [377]. It significantly lowers both fasting and post prandial glucose levels in non-insulin-dependent diabetics [378] and was recently shown to decrease ATP sensitive potassium channel activity which may explain the antidiabetic activity [379].

BUFORMIN, METFORMIN AND PHENFORMIN

Buformin (313), metformin (314) and phenformin (315) are biguanides which are prepared by fusion of equimolar amounts of the appropriate amine hydrochloride and cyanoguanidine at elevated temperatures [380, 381]. It was pointed out that phenformin exists with the biguanide double bonds conjugated, a condition which was assumed to hold for most biguanides despite the fact that they are usually drawn unconjugated. Dissociation constant determination established that the compound is monoprotonated at physiological pH [382]. All three drugs are hypoglycaemics. Phenformin has also been examined as a treatment for schizophrenia [383, 384]. Severe lactic acidosis led to the withdrawal of phenformin in several countries [385], but this is only rarely a complication with metformin [386].



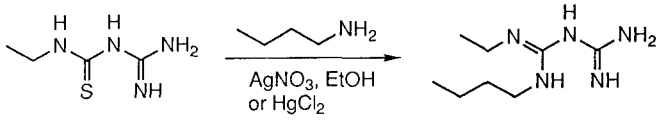
(313) R = Bu, R^1 = H

(314) R = R^1 = Me

(315) R = $PhCH_2CH_2$, R^1 = H

ETOFORMIN

The guanylthiourea (316) reacted with butylamine in the presence of silver nitrate or mercuric chloride at room temperature to give the biguanide etoformin (317) (*Scheme 5.71.*) [387]. The compound was claimed to be an antidiabetic agent with a better therapeutic ratio than phenformin or buformin.

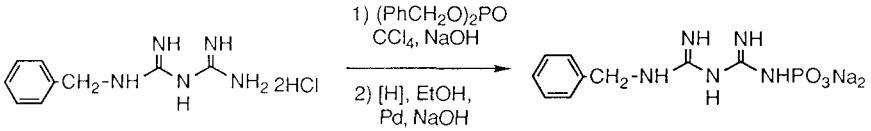


(316) (317)

Scheme 5.71.

BENFOSFORMIN

The biguanide (318) as its hydrochloride was condensed with dibenzyl phosphite in carbon tetrachloride containing sodium hydroxide followed by hydrogenolysis to give benfosformin (319) (*Scheme 5.72.*) [388]. Benfosformin has an antidiabetic action about equipotent with phenformin and 2 to 3 times the potency of metformin [389].



(318) (319)

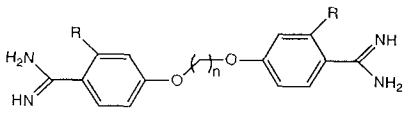
Scheme 5.72.

ANTIBACTERIAL AND ANTIFUNGAL DRUGS

PROPAMIDINE, PENTAMIDINE, HEXAMIDINE AND DIBROMOPROPAMIDINE

The dinitriles for the preparations of (320) to (323), were best obtained from the appropriate dibromoalkane and the sodium 4-cyanophenate. In the subsequent Pinner reaction it was found that an excess of ethanol was needed to get good yields of the ethyl imidate salt and a large excess of alcoholic ammonia ensured efficient conversion to the amidine since the imidate base reacts preferably with ammonia, but any traces of the salt tend to react with solvent to give the triethyl orthoester [390–392]. 4-Bromophenol reacted with $K^{14}CN$ in the presence of copper(II) sulphate and sodium bicarbonate to give radioactive 4-hydroxybenzonitrile which was converted by the above procedure to pentamidine labelled at the

amidine carbon atoms [393]. The report described a one-step exchange reaction between pentamidine and tritiated water in the presence of a homogeneous rhodium(III) chloride catalyst which gave the drug regiospecifically labelled at the positions ortho to the amidine groups.

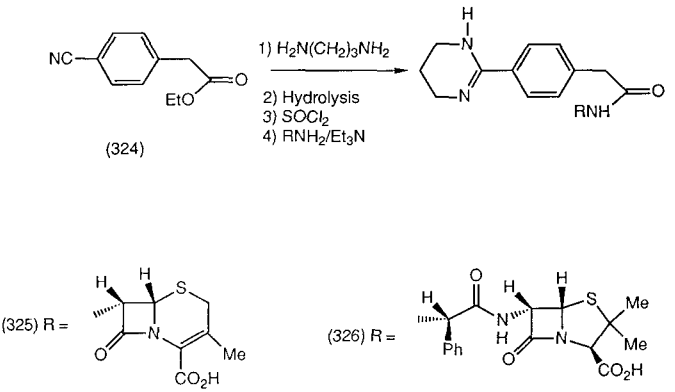


(320) R = H, n = 3
(321) R = H, n = 5
(322) R = H, n = 6
(323) R = Br, n = 3

Propamidine (320) is used for its antiprotozoal and antiamoebic properties. It has also been shown to have a rapid bacteriostatic action by inhibition of protein synthesis and a slower bactericidal action [394]. Pentamidine (321) has found use as an antiprotozoal agent against *Trypanosoma* [391] and is valuable against antimony resistant *Leismania* species [395]. An early, but detailed review of these activities was published by Schoenbach and Greenspan [396]. It is the recommended treatment for pneumonia caused by the opportunistic *Pneumocystis carinii* in AIDS patients [397–402]. It has proved effective against bovine babesiasis (redwater) [403]. It inhibits replication of *Toxoplasma gondii* (a protozoan parasite of humans and other mamals) in cell cultures [404]. Alone it has good antistaphylococcal activity [405] and in combination with ketoconazole or itraconazole, it is used to treat fungal infections with *Candida albicans* [406]. Hexamidine (322) [407] and dibromopropamidine (323) [408] are topical antiseptics sometimes used as preservatives in ointments.

CEFROTIL AND ROTAMICILLIN

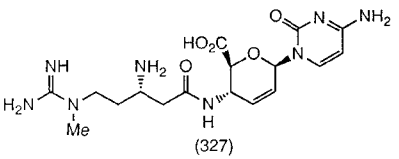
The nitrile (324) was treated with propylenediamine to form the tetrahydropyrimidine system and then the ester was hydrolysed, converted to the acid chloride and reacted with the appropriate amine to give either cefrotil (325) [409] or rotamicillin (326) [410], both of which are antibiotics (*Scheme 5.74.*).



Scheme 5.74.

BLASTICIDIN S

Blasticidin S (327) is an antifungal agent used against rice blast disease in Japan. It was isolated in 1958 from *Streptomyces griseochromogenes* [411] and the structure and absolute stereochemistry were elucidated by chemical means [412–415] and confirmed by X-ray spectroscopy [416,417]. The biosynthesis has also been determined [418,419].

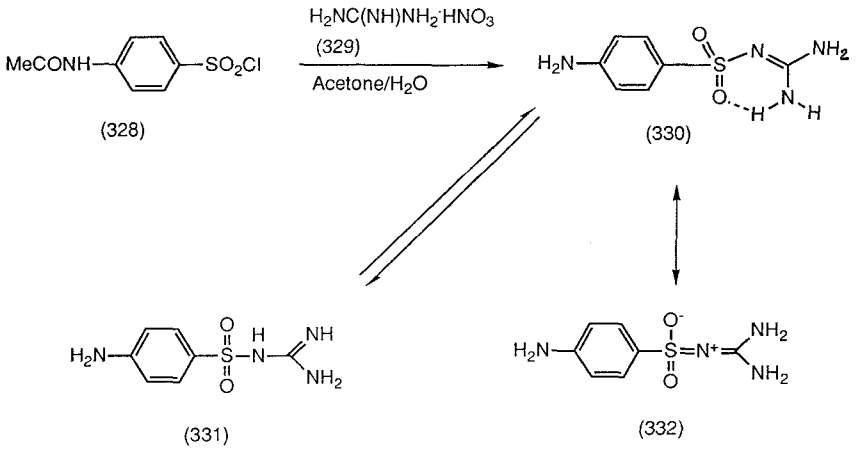


(327)

SULPHAGUANIDINE

Sulphaguanidine (330) was prepared by condensing acetylsulphanilyl chloride (328) with guanidine nitrate (329) in aqueous acetone containing sodium hydroxide [420–422] (*Scheme 5.75.*). It is unusual among the sulpha drugs in being insoluble in aqueous alkali. Infrared spectra [423], proton [424] and ^{15}N NMR spectra [425] and X-ray crystallography [426] reveal that the drug exists as the conjugated tautomer (330) both in solution and the solid state rather than (331) which had been used for many years. The

low acidity and basicity were explained by the important contribution from the canonical structure (332) [425].

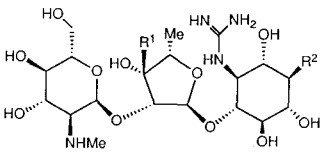


Scheme 5.75.

Sulphaguanidine is one of a series of antibacterials which came to prominence during and immediately after World War II. It is unusual among sulpha drugs in that large quantities can be ingested without the development of high blood levels and consequent toxic reactions. Because of this poor absorption it can be used safely to treat intestinal infections. It has been used extensively in veterinary medicine, especially against coccidiosis in chickens.

STREPTOMYCIN AND DIHYDROSTREPTOMYCIN

Streptomycin (333) is prepared by fermentation of *Streptomyces griseus* [427] although its total synthesis has been achieved [428]. It is a broad-spectrum antibiotic; one of the first to be discovered. It has particular value against tuberculosis, but is used to treat several other infections as well [429]. Dihydrostreptomycin (334), also an antibiotic, can be obtained by hydrogenation of streptomycin or by fermentation [430–432]. The total synthesis was achieved by a Japanese group [433–436]. The mechanism of action of streptomycin and dihydrostreptomycin has been reviewed [437, 438].



(333) $R^1 = CHO$, $R^2 = HNC(NH_2)NH$

(334) $R^1 = CH_2OH$, $R^2 = HNC(NH_2)NH$

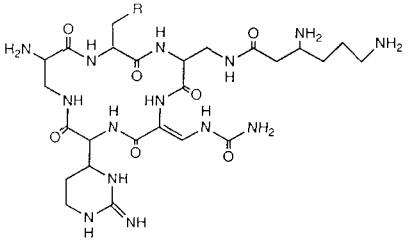
(335) $R^1 = CH_2OH$, $R^2 = OCONH_2$

BLUENSOMYCIN

Bluensomycin (335) is an antibiotic isolated in 1962 from cultures of *Streptomyces verticillus* [439]. The structural elucidation follows from the publications of several groups [440–442]. The pK_a is reported to be 7.53 [440].

CAPREOMYCINS

The capreomycins were first isolated in 1960 from *Streptomyces capreolus* [443]. The structures of capreomycin IA (336) and IB (337) were first proposed in 1971 [444], but were revised in 1976 by Shiba *et al.* who also achieved the total synthesis [445–449]. The natural mixture, which includes the minor components capreomycins IIA and IIB, is used as an antituberculosis agent. The drug is ineffective orally, but by the subcutaneous route it was a good treatment for mouse and bovine tuberculosis including a streptomycin resistant strain [450].

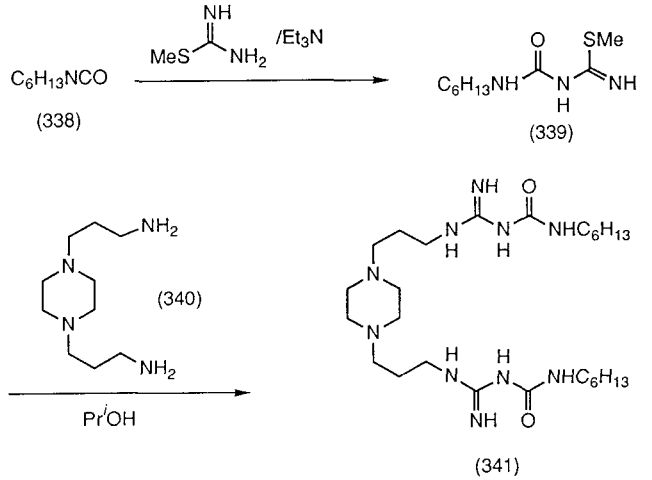


(336) R = OH

(337) R = H

IPEXIDINE

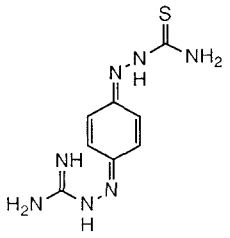
Hexyl isocyanate (338) reacted with *S*-methylisothiouronium iodide under basic conditions to give the isothiobiuret (339) which with the piperazine derivative (340) gave ipexidine (341), [451] (*Scheme 5.76.*). The drug is an anticaries, antigingivitis and antiplaque agent, similar in potency and mechanism of action to chlorhexidine [452,453].



Scheme 5.76.

AMBAZONE

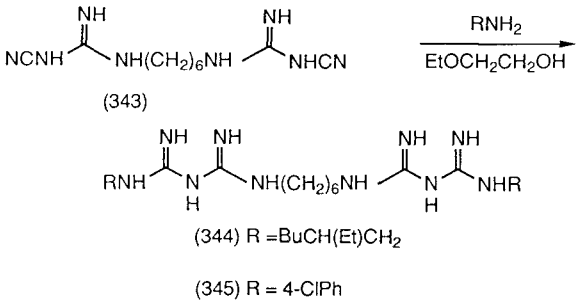
One carbonyl group of *p*-benzoquinone condensed with aminoguanidine nitrate and subsequently the second condensed with thiosemicarbazide to produce ambazone (342) [454]. This simple derivative has antibacterial action [455].



(342)

ALEXIDINE AND CHLORHEXIDINE

The reaction of 1,6-hexanediamine with two equivalents of sodium dicyanamide gave the biscyanoguanidine (343) which reacted with two equivalents of 2-ethylhexylamine to give alexidine (344) or of 4-chloroaniline to give chlorhexidine (345) (*Scheme 5.77.*) [456,457].

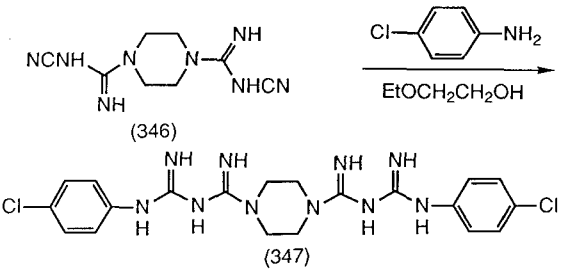


Scheme 5.77.

Both bisdiguanides, alexidine [458, 459] and chlorhexidine [456,457, 460–462] are antibacterials used in mouth washes to control the formation of dental plaque. Chlorhexidine is safe and effective for use in oral hygine [463], for example in the prevention or treatment of gingivitis [464]. The mechanism of its action against *Clostridium perfringens* is to disrupt the cell membranes and cause leakage of cytoplasmic material [465].

PICLOXYDINE

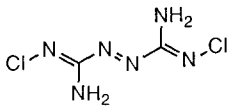
Piperazine hydrochloride was heated with sodium dicyanimide in butanol to give the bis cyanoguanidine (346) which was treated with 4-chloroaniline hydrochloride in 2-ethoxyethanol to give picloxyidine (347) (*Scheme 5.78.*) [466,467]. Picloxydine has high antibacterial activity against a wide variety of gram negative and gram positive organisms [466].



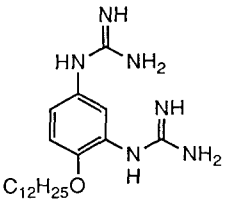
Scheme 5.78.

CHLOROAZODINE

Guanidine nitrate was added to sodium acetate solution and treated with sodium hypochlorite to give chloroazodine (348) [468]. The low dipole moment (1.95 D in dioxane) and the UV absorption (λ_{max} 315, ϵ 14 700) suggest that this antibacterial drug exists predominately in the all *trans* form in solution [469].



(348)



(349)

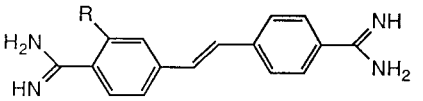
LAUROGUADINE

Lauroguadine (349) which showed 'remarkable antibacterial activity' was made from the appropriate *m*-phenylenediamine and cyanamide [470,471].

ANTIPROTOZAL AND OTHER ANTIPARASITIC DRUGS

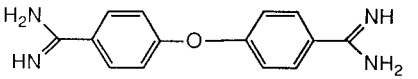
STILBAMIDINE, HYDROXYSTILBAMIDINE AND PHENAMIDINE

Stilbamidine (350) and hydroxystilbamidine (351) were prepared in the same way as (320) to (323) from dicyanostilbene and hydroxydicyanostilbene respectively. Phenamidine (352) was also prepared by a Pinner reaction following an initial etherification between 4-hydroxybenzonitrile and 4-bromobenzonitrile [391]. Stilbamidine and pentamidine (321) have hypoglycaemic actions and were evaluated as antidiabetic agents. Their trypanocidal activity depends on the fact that trypanosomes require high blood sugar levels in order to reproduce [391,396,472,473]. Phenamidine is effective against bovine babesiasis [403], and is the treatment of choice for *Babesia gibsoni* infections of dogs [474].



(350) R = H

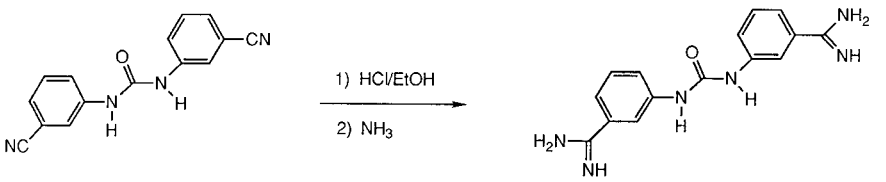
(351) R = OH



(352)

AMICARBALIDE

Phosgene and 3-aminobenzonitrile reacted to give the urea (353), which was treated with ethanolic hydrogen chloride followed by ammonia to give amicarbalide (354) (*Scheme 5.79.*). The di-isethionate salt of this diamidine is used to treat *Babesia divergens* infections of cattle [391, 475].



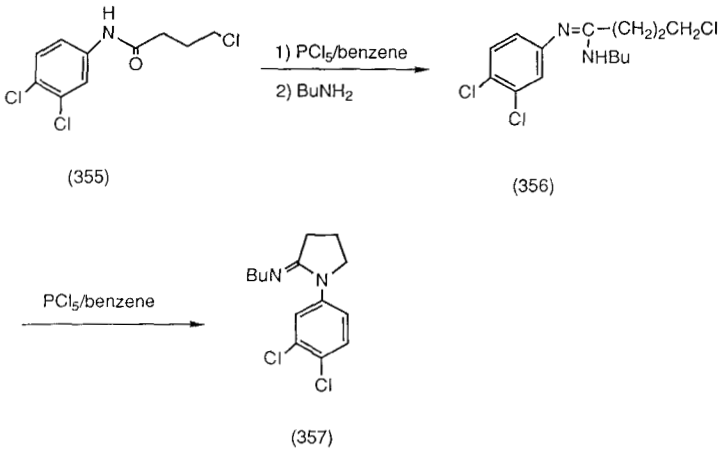
(353) (354)

Scheme 5.79.

CLENPIRIN

The amide (355) was converted to the chloroimine with phosphorus pentachloride before exposure to butylamine to give the amidine (356). A

second treatment with phosphorus pentachloride induced the ring closure to clenpirin (357) (*Scheme 5.80.*) [476]. Clenpirin is used to treat infections of tropical cattle with the tick *Boophilus microplus* including strains resistant to other compounds [477–479].

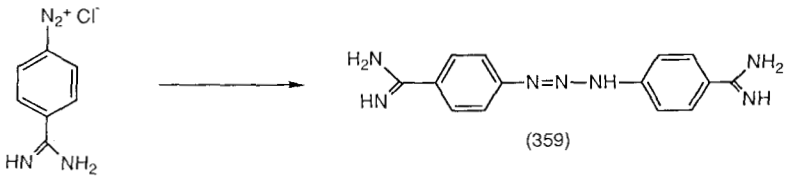


(355) (356) (357)

Scheme 5.80.

DIMINAZENE

The diazonium salt of 4-amidinoaniline (358) on self condensation gave diminazene (359) (*Scheme 5.81.*) [480, 481]. It is used to treat bovine trypanosomiasis in Africa and has had clinical trials in man. It is of particular value against strains of trypanosomes which have become resistant to the phenanthridine and aminoquinaldine classes of trypanocides [482, 483]. Diminazene, usually formulated as its diaceturate (Berenil), also shows babesidal activity [484].

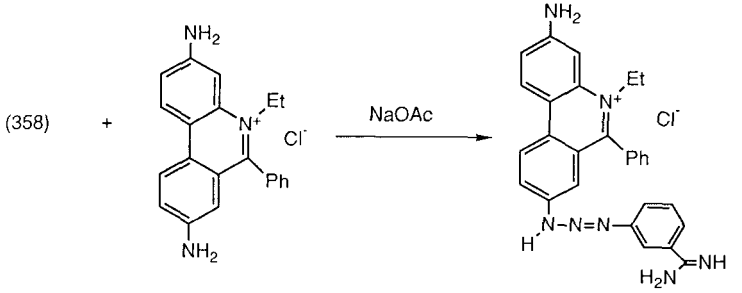


(358) (359)

Scheme 5.81.

ISOMETAMIDIUM

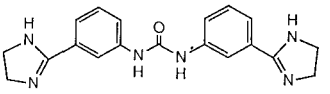
Under carefully controlled conditions the diazonium salt (358) was coupled with homidium chloride (360) to give the trypanocide isometamidium (361) (*Scheme 5.82.*) [485, 486]. Isometamidium chloride is exceptionally potent against *Trypanosoma congolense* [482]. Its pharmacology has been reviewed [487].



Scheme 5.82.

IMIDOCARB

Imidocarb (362) was obtained by reaction of 3-(2-imidazolin-2-yl)aniline dihydrochloride with phosgene and sodium acetate [488]. Imidocarb is a valuable drug against *Babesia ovis* in sheep. None of the parasitic organisms could be detected after 6 days of treatment [489]. The drug proved to be more effective than tetracycline in the treatment of ehrlichiosis in dogs with the further advantage that any concurrent babesiosis was also controlled [490]. Ehrlichiosis is a widely occurring rickettsial disease caused by *Ehrlichia canis*.

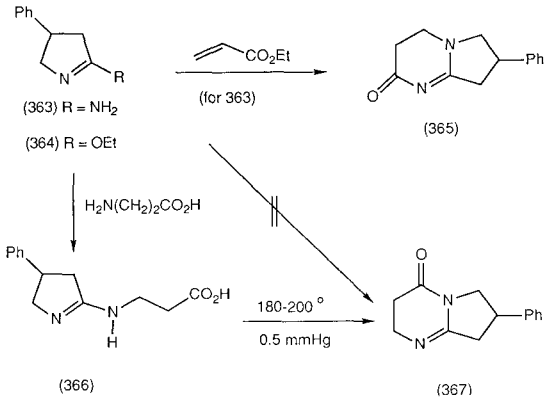


(362)

ROFELODINE

The aminodihydropyrrole (363), reacted with ethyl acrylate to give the conjugated pyrrolopyrimidone (365) exclusively. To get the non-conjugated

rofelodine, the imidate (364) was first reacted with β-alanine to give the acid (366). Ring closure was effected on distillation; the distillate crystallized to give the required product in good yield, (*Scheme 5.83.*). Rofelodine inhibits reserpine-induced ptosis in the cat [491].



Scheme 5.83.

AMITRAZ

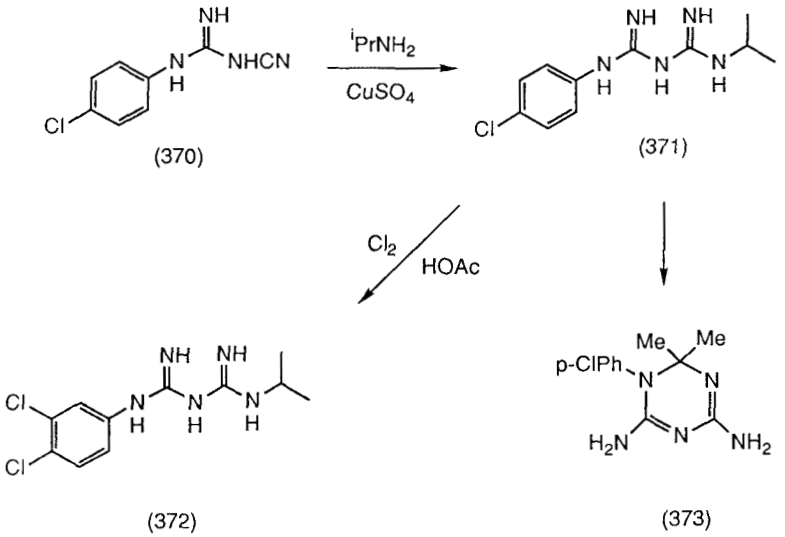
Self-condensation of the formamidine (368) in xylene with a catalytic quantity of 4-toluenesulphonic acid gave amitraz (369) (*Scheme 5.84.*) [492]. Dimethyl carbamoyl chloride and ethyl trifluoroacetate are also catalysts for this reaction. A one-pot condensation of 2,4-dimethylaniline, triethyl orthoformate and methylformamide with or without zinc chloride was reported to give amitraz in high yield [493, 494]. The drug is used as an insecticide and acaricide particularly against several species of cattle ticks in South Africa [495, 496] and is very effective in the treatment of refractory *Demodecosis canis* (mange) in the dog [497].

(368) (369)

Scheme 5.84.

CHLORGUANIDE, CHLORPROGUANIL AND CYCLOGUANIL

Diazotized 4-chloroaniline was coupled with dicyandiamide to give the cyanoguanidine (370) which reacted with isopropylamine under cupric sulphate catalysis to give chlorguanide (proguanil, 371) [498]. Direct chlorination of (371) gave chlorproguanil (372) (*Scheme 5.85.*) [499–501]. Both drugs have high antimalarial activity [499], however, chlorguanide is metabolised to the more active form cycloguanil [502]. Cycloguanil (373) was simply prepared by the fusion of equimolar amounts of 4-chloroaniline, dicyandiamide and acetone in the presence of a strong acid [503–505]. It is an antimalarial in its own right. Chlorguanide shows advantages over chloroquine and quinacrine both as a prophylactic and a curative agent [506].

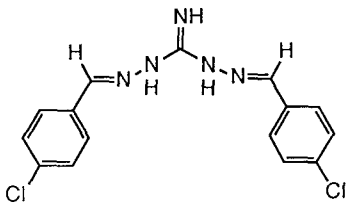


Scheme 5.85.

ROBENIDINE

The *bis*-hydrazone robenidine (374) was simply prepared by condensation of 4-chlorobenzaldehyde with diaminoguanidine in acidic alcohol [507]. The same reaction on the oxime of 4-chlorobenzaldehyde gave a good yield of (374) [508]. Robenidine was found to be highly effective in preventing chicken coccidiosis from any of eight *Eimeria* species [509]. It completely

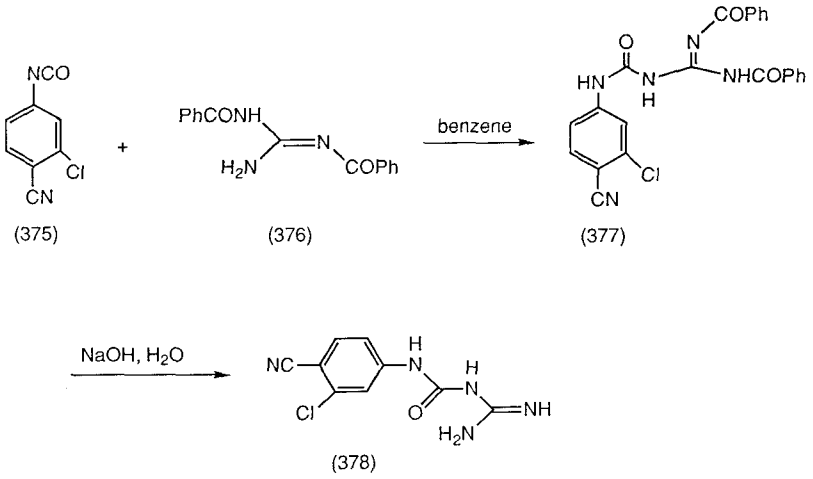
controlled infection by a strain of *Eimeria tenella* which was resistant to the other drugs available [510].



(374)

CLOGUANAMIL

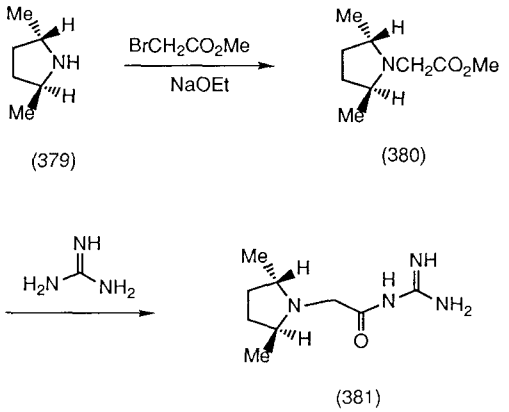
Amidinoureas can be prepared by the addition of guanidine to isocyanates, but yields are generally low because guanidine has a very low solubility in aprotic solvents. An alternative route to cloguanamil (378) was developed in which the readily soluble N^1,N^2-dibenzoylguanidine (376) and the isocyanate (375) formed the adduct (377) in benzene. Alkaline hydrolysis then gave cloguanamil in good yield and high purity [511] (*Scheme 5.86.*). The drug is an antiprotozoal agent, particularly useful against malaria parasites.



Scheme 5.86.

ROLGAMIDINE

Trans-2,5-dimethyl-3-pyrroline (379) was alkylated with methyl bromoacetate to give (380) which with guanidine gave rolgamidine (381) (*Scheme 5.87.*) [512]. This is an antidiarrhoeal agent.

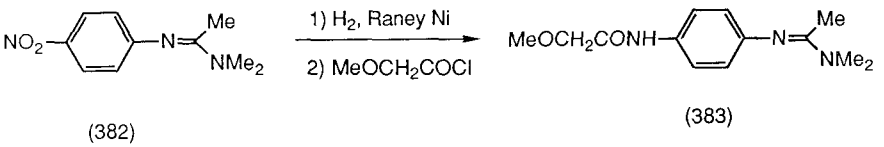


Scheme 5.87.

ANTHELMINTICS

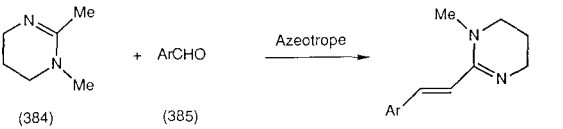
AMIDANTEL

Reduction of the nitro group of (382) followed by amidation with methoxyacetyl chloride afforded amidantel (383) (*Scheme 5.88.*) [513]. This anthelmintic successfully treated nematode, filaria and cestode infections in rodents and showed high efficacy against hookworms and round worms in dogs [513].



Scheme 5.88.

Not surprisingly, cinnamonitrile failed to react cleanly with diamines. However, the 1,2-dimethyltetrahydropyrimidine (384) condensed with aromatic aldehydes (385), with azeotropic water removal, to give the derivatives pyrantel (386), morantel (387) and oxantel (388) [514–516] (*Scheme 5.89.*). Formate esters were found to be effective water scavengers in large scale production [517].



(386) Ar = 2-thienyl

(387) Ar = 3-methyl-2-thienyl

(388) Ar = 3-hydroxyphenyl

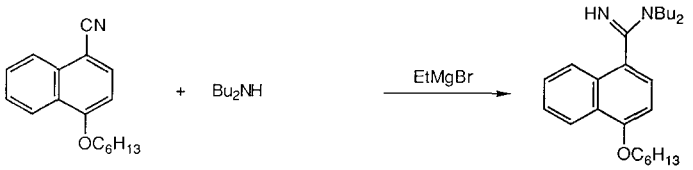
Scheme 5.89.

The three compounds are all anthelmintic agents in human and veterinary medicine. Pyrantel is a highly effective broad spectrum nematocide, but is of no value against adult whipworm [514]. Oxantel, however, is useful in the treatment of adult whipworm infection [515]. Pyrantel is an effective anthelmintic with both prophylactic and therapeutic effects in mice and dogs [518,519] and in sheep [520,521]. *Toxocara vitulorum* is transmitted from buffalo cows to their calves in the milk. Pyrantel and febantel (402) are good treatments against both immature and adult parasites [522]. Pyrantel and oxantel/pyrantel mixtures were efficatious in treating children infected with the tropical organisms *Ascaris lumbricoides*, *Trichuris richiura* and *Necator americanus* [523]. Morantel is given to cattle as a general anthelmintic. Its very low absorption from the gut means that it does not have to be withdrawn before milking or slaughter for meat [524]. It is very effective against *Haemonchus contortus* and *Trichostrongylus columbriformis* in goats [525] and has broad spectrum activity against gastrointestinal nematodes, lung worms and some tissue nematodes in most species of domestic animals, but resistance development has been noted in some organisms [526–528]. Strategies for avoiding resistance build-up by rotating drugs have been suggested [529–531]. There is evidence that pyrantel and morantel may act by acetylcholine receptor inhibition in the CNS of the parasite [532]. The *trans* isomers (386) and (387) isomerize to the *cis* forms when their solutions are exposed to sunlight [514], but (388) does not show

this reaction. Maximum light absorption for (386) is at 312 nm and for (387) at 318 nm, but (388) does not absorb significantly above 290 nm, the cutoff point for sunlight filtered by the atmosphere [533].

BUNAMIDINE

Bunamidine (391) was the best of a series of 4-alkoxynaphthamidines for use in veterinary medicine against a variety of cestodes (tape worms). The failure of the Pinner reaction on the naphthonitrile (389) was presumed to be due to deactivation by the alkoxy group and steric hinderance from the peri proton. However, treatment with the anion from dibutylamine (390) was successful [534, 535] (*Scheme 5.90.*). Bunamidine is widely used against all the important tape worms of dogs and cats [524].

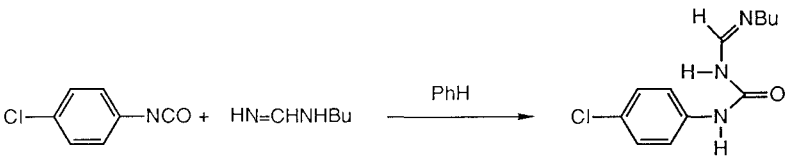


(389) (390) (391)

Scheme 5.90.

CARBANTAL

The anthelmintic carbantal (394) was prepared for use in veterinary medicine by addition of butylformamidine (393) to the isocyanate (392) [536].



(392) (393) (394)

Scheme 5.91.

NO2
NHCO2Me
N=
SMe
(395)
+
H2N
SO3H
(396)
NaOH
MeOH
NO2
NHCO2Me
N=
HN
SO3H
(397)
NO2
N=PPh3
(398)
+
S
MeO2CNH
N
H
SO3H
(399)
PhH, reflux

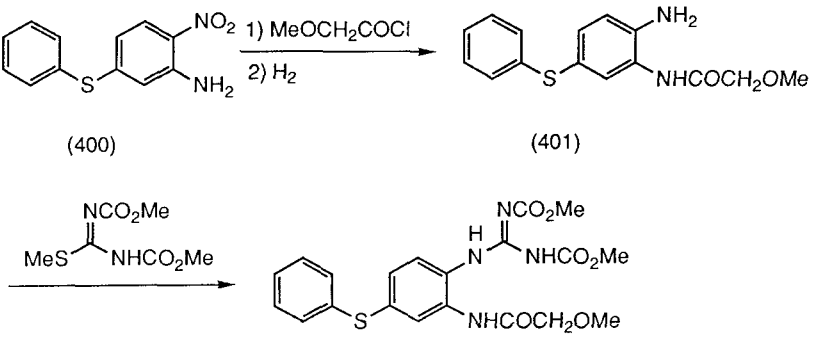
Scheme 5.92.

NETOBIMIN

The Rathke reaction of the isothiourea (395) with taurine (396) gave netobimin (397) [537]. A new guanidine synthesis employs the phosphimine (398) in reaction with the appropriate thiourea (399) which gives the drug in at least 90% yields (*Scheme 5.92.*) [538]. Netobimin is an anthelmintic; it was effective against pre-adult forms of some *Trichinella* species in mice, but less effective against larvae or adults [539]. It is a useful treatment of goats infected with the lungworm *Muellerius capillaris* or with digestive tract strongyles [540] and against liver flukes (*Fasciola hepatica*) in sheep [541]. The efficacy against nematodes in yearling heifers was 67.4% [542]. The anthelmintic activity depends on the rate of metabolic oxidation to albendazole sulphoxide and on to albendazole sulphone [543,544].

FEBANTEL

2-Nitro-5-phenylthioaniline (400) was acylated with methoxyacetyl chloride followed by hydrogenation to give the amine (401) which underwent the Rathke reaction with S-methyl bismethoxycarbonylisothiourea to give the trisubstituted guanidine febantel (402) (*Scheme 5.93.*) [545,546]. The drug is an anthelmintic useful in many branches of veterinary medicine. In sheep and cattle a single dose gave almost complete elimination of intestinal nematodes and of dictyocaulus [547]. Febantel is a broad spectrum anthelmintic in horses and showed negligible toxicity at 40 times the recommended dose [548]. A single dose was fully effective against several species of nematodes in sheep [549,550].

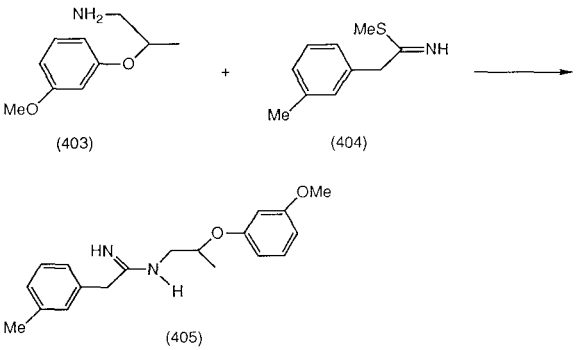


Scheme 5.93.

CENTRAL NERVOUS SYSTEM DRUGS

XYLAMIDINE

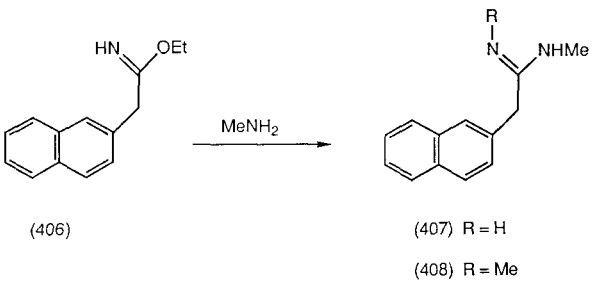
A Pinner reaction between the primary amine (403) and the thioimidate (404) (from the thioamide and iodomethane) gave xylamidine (405) (*Scheme 5.94.*). Base catalysed reaction between 2-chloropropionitrile and 3-methoxyphenol followed by lithium aluminium hydride reduction provided the amine (403). Xylamidine is a powerful $5HT_2$ receptor antagonist, and has some value in pharmacological research [551].



Scheme 5.94.

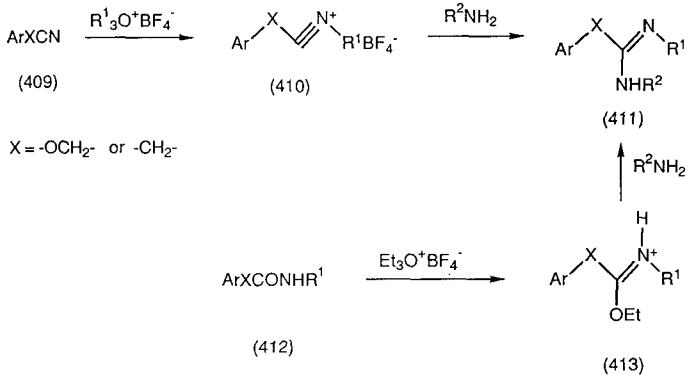
NAPACTADINE

The Pinner intermediate (406) was exposed to an excess of methylamine in warm ethanol to give (407) very rapidly. Introduction of the second methylamino group to give napactadine (408) was achieved by keeping the reaction at 50–55°C for a prolonged time, (*Scheme 5.95.*) [552].



Scheme 5.95.

Other ways to prepare this drug and similar compounds are summarized in *Scheme 5.96* [553]. Arylalkyl nitriles (409) reacted with trimethyloxonium tetrafluoroborate in nitromethane to form the methylnitrilium salts (410). Treatment with methylamine then gave the dimethyl amidines (411) (R^1, R^2 = Me). The diethyl derivative (411, R^1, R^2 = Et) was obtained with triethyloxonium fluoroborate and ethylamine. Unsymmetrical dialkylamidines were prepared from amides (412) with triethyloxonium fluoroborate to give the salts (413) which with the appropriate primary amine gave the amidines (411). These compounds were successfully developed as antidepressants based on fenmetazole (41) when the dialkyl amidine unit proved to be a bioisostere for the imidazoline group [553].



Scheme 5.96.

XILOBAM

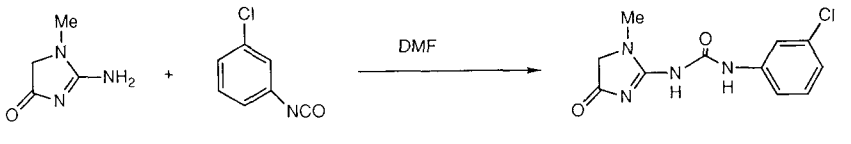
1-Methyl-2-pyrrolidinone was *O*-alkylated with triethyloxonium tetrafluoroborate and the resulting enol ether exposed to ammonia to give the amidine (414). Treatment with 2,6-dimethylphenyl isocyanate (415) gave xilobam (416) in high yield, (*Scheme 5.97.*) [554,555]. It is a potent skeletal muscle relaxant, but has much lower activity of the anticonvulsant, sedative or hypnotic type and no anti-anxiety properties [554, 556].

(414) (415) (416)

Scheme 5.97.

FENOBAM

Creatinine (417) and 3-chlorophenyl isocyanate (418) reacted in dimethylformamide to give the adduct fenobam (419) (*Scheme 5.98.*) [557, 558]. This is an anxiolytic agent which produces less sedation and drowsiness than the benzodiazepines [559–561].

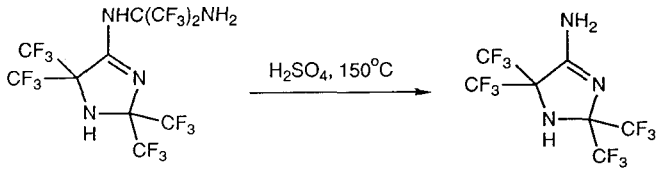


(417) (418) (419)

Scheme 5.98.

MIDAFLUR

Condensation of hexafluoroacetone imine with sodium cyanide in dimethylformamide at −30°C gave the adduct (420) which was pyrolyzed or hydrolyzed in concentrated sulphuric acid at 150°C to give midaflur (421) (*Scheme 5.99.*) [562]. This compound is remarkably stable; it is not decomposed by heating to 500°C, and is resistant to acid and oxygen at high temperatures [563]. Midaflur is more potent than several better known psychosedative drugs with anticonvulsant and tranquillizing activities [564]. It is a muscle relaxant with low toxicity [565–567].



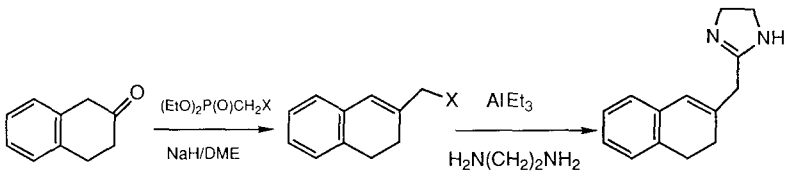
(420) (421)

Scheme 5.99.

NAPAMEZOLE

2-Tetralone (422) under standard Wadsworth-Emmons conditions gave the nitrile (423) or the ester (424). Either derivative with ethylenediamine and triethyl aluminium in toluene afforded napamezole (425) (*Scheme 5.100.*)

[568]. This is an α_2-adrenoreceptor antagonist having structural features common to the α-adrenergic agent idazoxan (24) and the α_1-adrenoreceptor agonist naphazoline (285). The combination of α_2-adrenoreceptor antagonist and serotonin-selective reuptake blocking activities led to napamezole being tested as an antidepressant [568].



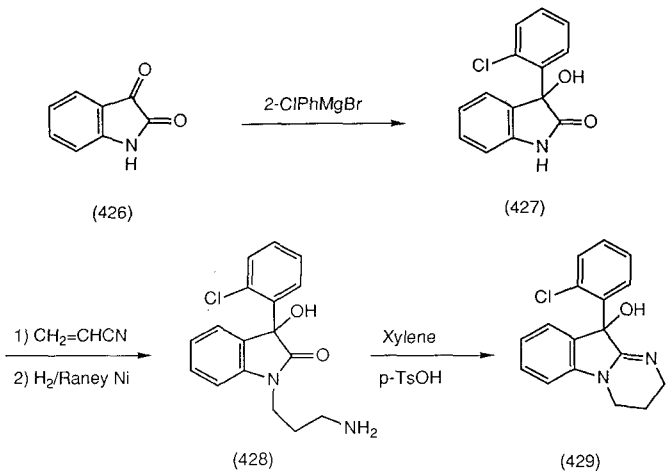
(422) (423) X = CN (425)

(424) X = CO_2Et

Scheme 5.100.

CICLAZINDOL

2-Chlorophenylmagnesium bromide attacked only the ketonic carbonyl group of isatin (426) to give the oxindole (427). Michael addition to acrylonitrile followed by reduction gave the saturated primary amine (428) which in refluxing xylene containing 4-toluene sulphonic acid cyclized to ciclazindol (429) (*Scheme 5.101.*). The intermediate (428) can also be

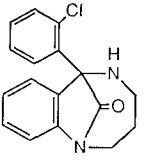


(426) (427)

(428) (429)

Scheme 5.101.

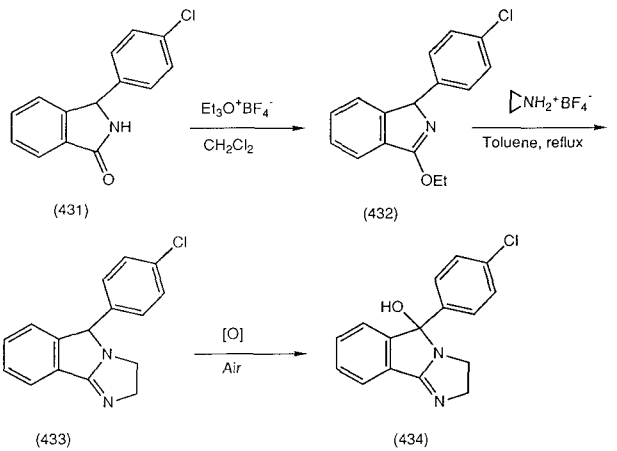
prepared by the reaction of (427) with *N*-bromopropylphthalimide and hydrolysis [569]. The bridged isomer (430) is a by-product formed during the routine preparation of ciclazindol. It arises from a rearrangement of the drug under the acidic conditions [570]. Ciclazindol is an antihyperglycaemic agent which appears to act by reducing appetite and weight [571]. It increased the resting metabolic rate without any central nervous system stimulation [572]. It is also an antidepressive with activity similar to the tricyclic antidepressives, but with some advantages. In addition, clinical trials suggested that it may have some value as an anorectic [573].



(430)

MAZINDOL

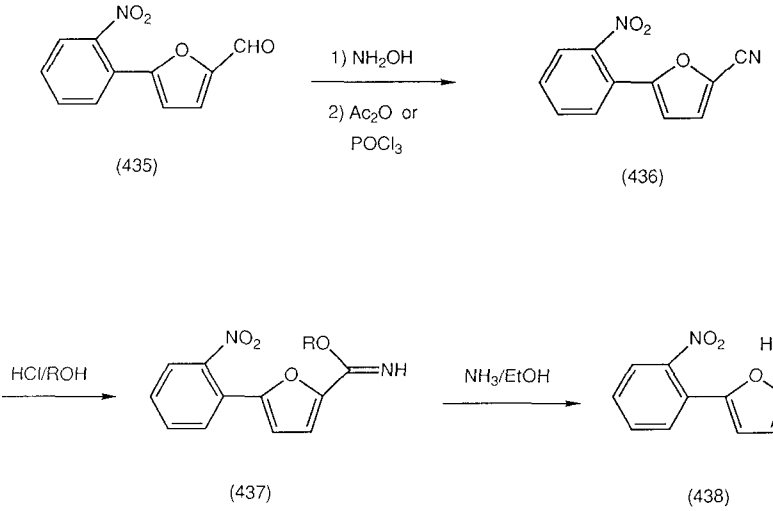
The phthalimidine (431) was treated with triethyloxonium tetrafluroborate to give the imidate (432) which with an ethyleneimine salt gave the tricyclic amidine (433). When (433) was exposed to the air for a prolonged period it was oxidized to mazindol (434) (*Scheme 5.102.*). The patent claimed that mazindol has both CNS stimulant and appetite depressant activities [574]. It has been closely studied as an anoretic [575] and it increased both the resting metabolic rate and CNS activity [572]. Mazindol gave significantly increased glucose utilization in the rat brain [576].



Scheme 5.102.

NITRAFUDAM

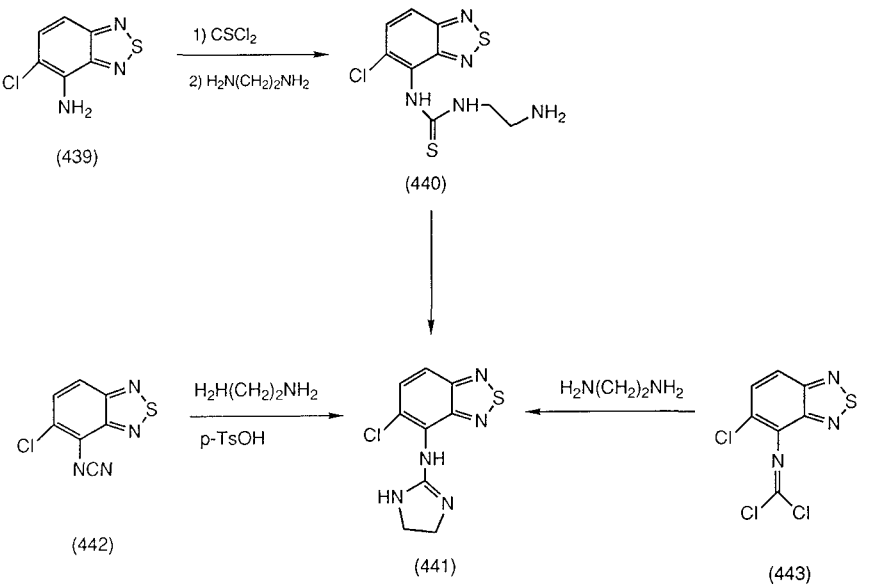
2-Nitroaniline was diazotized and coupled with furfural in the presence of cupric chloride to give the aldehyde (435). Dehydration of the oxime under standard conditions gave the nitrile (436) which was treated with hydrogen chloride in methanol or ethanol (or methanolic sodium methoxide) to give the imidate (437) followed by ammonia, ammonium chloride or ammonium acetate in ethanol to give nitrafudam (438) (*Scheme 5.103.*). This drug is a member of a class of antidepressants which avoids the anticholinergic, antihistaminic and cardiovascular side-effects of the tricyclic antidepressants [577].



Scheme 5.103.

TIZANIDINE

4-Amino-5-chloro-2,1,3-benzothiadiazole (439) was treated with thiophosgene followed by ethylenediamine to give the thiourea (440) which cyclized to tizanidine (441) on refluxing with potassium hydroxide and lead acetate in methanol [578]. Condensation of the cyanamide (442) or the dichloroimine (443) with ethylenediamine provided alternative routes to this drug (*Scheme 5.104.*) [579, 580].

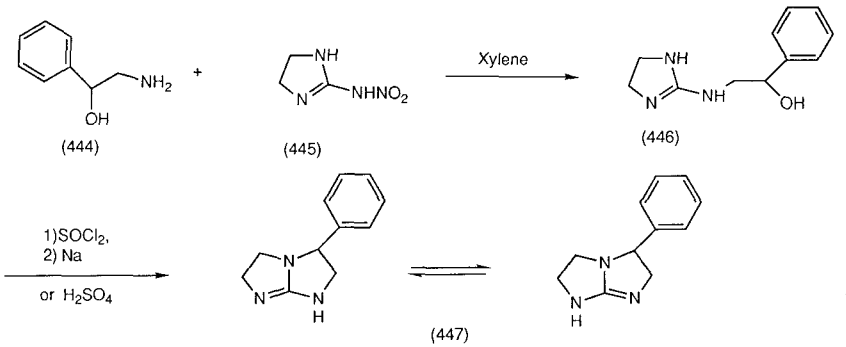


Scheme 5.104.

Tizanidine is an α_2-adrenergic agonist [581] which is a good and well tolerated muscle relaxant used to treat muscular spasms and spastic conditions [582–587]. Its action on adrenoceptors and its antinociceptive effect are well studied; it depresses polysynaptic excitation of spinal motor neurones [588–591]. Recent clinical trials demonstrated the value of tizanidine in the treatment of tension headaches [592] and lower back pain [593].

IMAFEN AND DEXIMAFEN

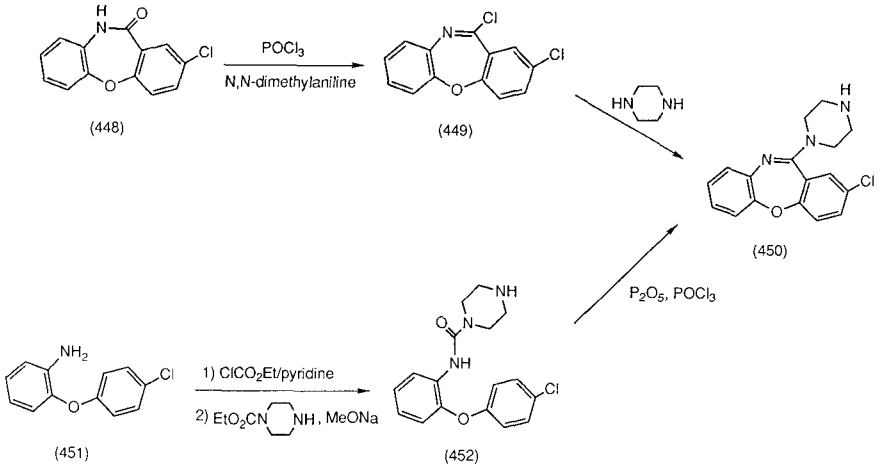
The phenylethanolamine (444) reacted with the nitramine (445) in xylene to give the guanidine (446). Conversion to the chloroalkane followed by treatment with sodium or direct exposure of (446) to concentrated sulphuric acid gave imafen (447) (*Scheme 5.105.*). Resolution with (+)-tartaric acid revealed that deximafen is the more active isomer [594–597]. The drugs have both been used as antidepressants [598].



Scheme 5.105.

AMOXAPINE

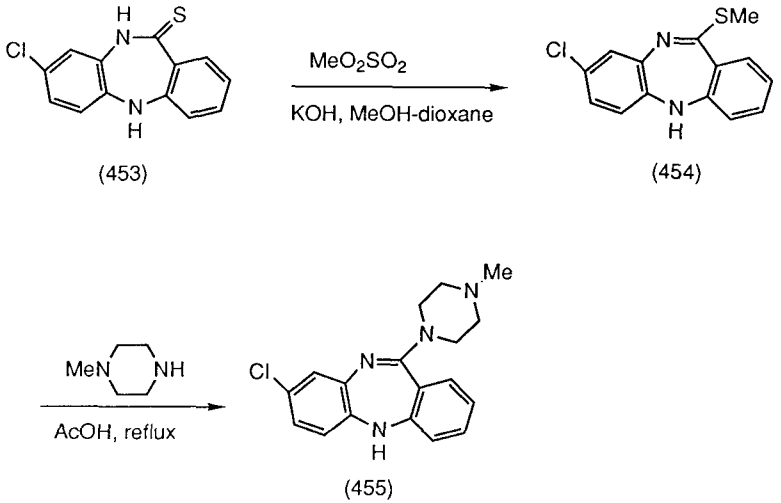
The lactam (448) was converted to the imidochloride (449) which was treated with piperazine to give amoxapine (450) (*Scheme 5.106.*) [599]. Alternatively, the amino ether (451) was acetylated with ethyl chloroform and the product treated with 1-ethoxycarbonylpiperazine to give the urea (452) which underwent the Bischler-Napieralski cyclization to give (450) (*Scheme 5.106.*) [600]. Amoxapine is an antidepressant with rapid onset of action, valuable in the treatment of psychotic depression [601–607]. The chemistry and physicochemical properties of the drug have been reviewed [608,609].



Scheme 5.106.

CLOZAPINE

The thiolactam (453) was converted with dimethyl sulphate to the imidothioether (454) which on a prolonged reflux with 1-methylpiperazine gave clozapine (455) in good yield (*Scheme 5.107.*) [610]. The Bischler-Napieralski procedure was reported to give a poor yield [611].

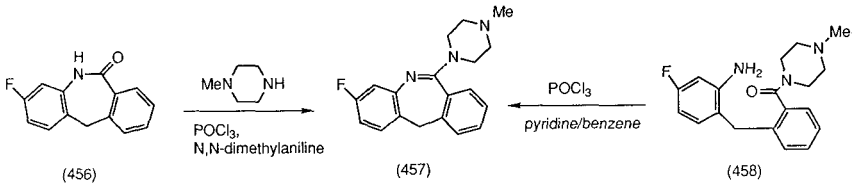


Scheme 5.107.

Clozapine has found use in the treatment of refractory cases of schizophrenia, but an apparent increased risk of agranulocytosis may preclude its use until other antipsychotics have been tried [612]. Nevertheless, it is regarded as the first member of a new class of drugs for mental illness which avoid several side-effects associated with traditional remedies [613, 614]. However, recent studies have clearly shown that it has unusual pharmacological and clinical properties so it has been the subject of many reports and reviews [615,616,617].

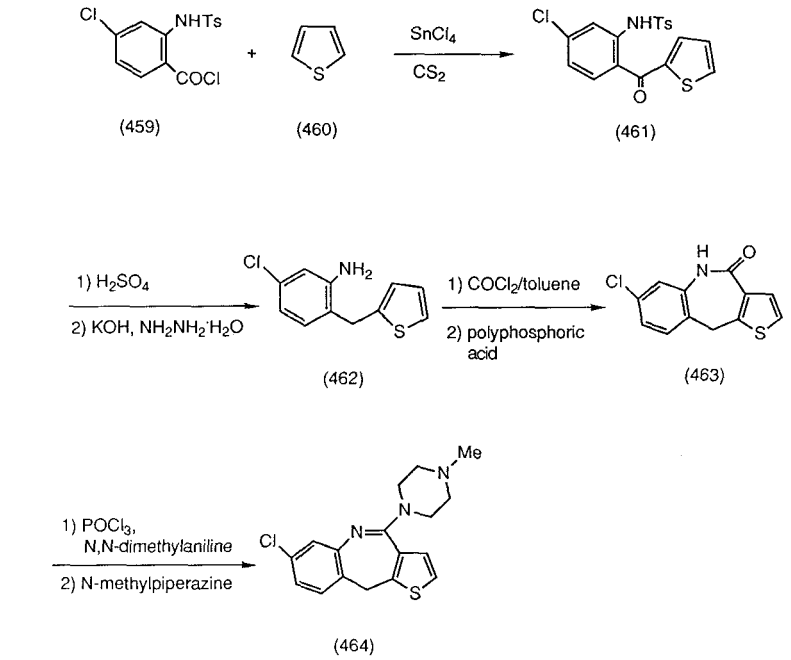
FLUPERLAPINE

Fluperlapine (457) was synthesized from the amide (456) in a similar way to amoxapine (450) and clozapine (455). An alternative was the intramolecular dehydration of the amide (458) (*Scheme 5.108.*) [618–621].



Scheme 5.108.

Fluperlapine shows potent central antiserotonin activity [622]. The pharmacological profile is similar to that of clozapine with additional antidepressant properties [623]. It has a good antipsychotic effect and alleviated the apathetic and depressive syndromes in a trial with schizophrenic patients [624,625]. The drug is beneficial in the treatment of patients vulnerable to neurological side-effects [626].



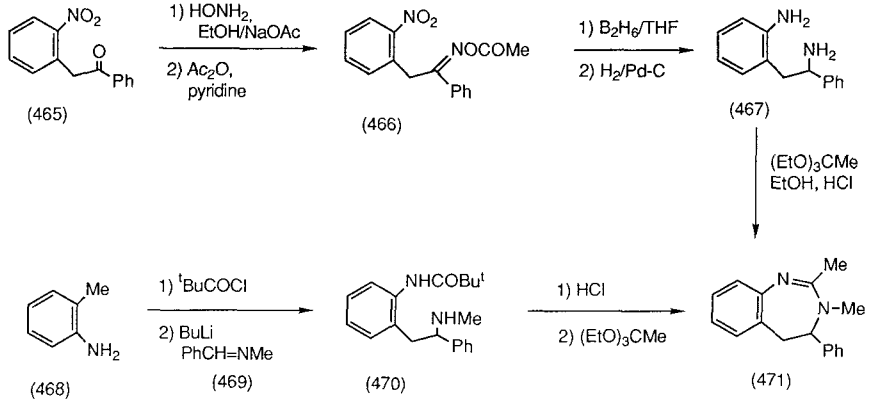
Scheme 5.109.

TILOZEPINE

Friedel-Crafts reaction of the benzoyl chloride (459) with thiophene (460) gave the ketone (461) which was deprotected and reduced to the amine (462). Phosgene reacted with the primary amine to give the isocyanate which cyclized under the influence of polyphosphoric acid. The resulting lactam (463) was converted to tilozepine by phosphorus oxychloride followed by 1-methylpiperazine (*Scheme 5.109.*) [627]. Tilozepine showed good clozapine-like antischizophrenic action in a clinical trial, but epileptiform seizures in a few patients caused the investigation to be terminated [627].

DAZEPINIL

The nitroketone (465) was converted to the oxime acetate (466) which was treated with diborane to reduce the imine group followed by hydrogen to reduce the nitro group and give the diamine (467). The diamine condensed with triethyl orthoacetate to yield dazepinil (471) (*Scheme 5.110.*) [628]. An improved route began with o-toluidine (468) which was *N*-protected to allow the directed metallation of the methyl group. Treatment with the Schiff's base (469) followed by deprotection and exposure to triethyl orthoacetate gave dazepinil in improved yield [629]. This benzodiazepine has antidepressant properties.

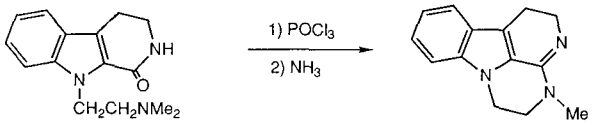


Scheme 5.110.

METRALINDOLE

Refluxing the carboline (472) with phosphorus oxychloride followed by

neutralization with ammonia gave metralindole (473) (*Scheme 5.111.*) [630, 631]. Metralindole is an antidepressant, clinically available in some countries [632,633].

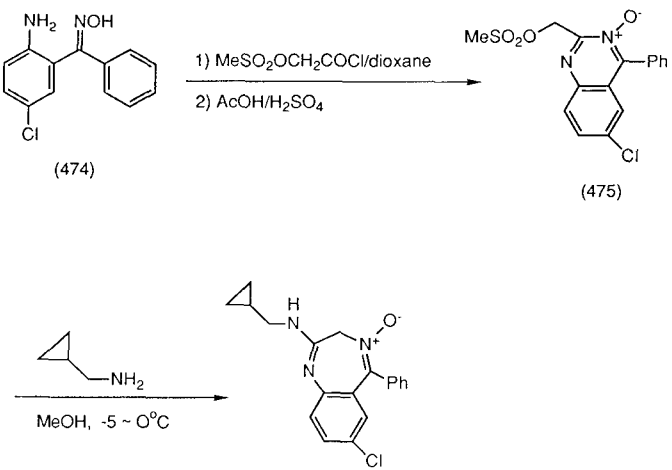


(472) (473)

Scheme 5.111.

CYPRAZEPAM

Treatment of the oxime (474) with mesyloxyacetyl chloride gave the quinazoline oxide (475). The good leaving group of (475) was displaced by the appropriate primary amine and the resultant quinazoline oxide underwent rearrangement to give the seven membered ring sedative cyprazepam (476) (*Scheme 5.112.*) [634,635].



(474) (475)

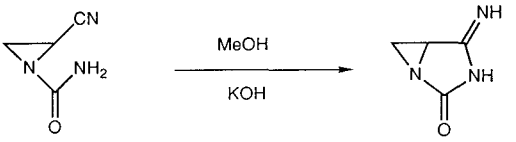
(476)

Scheme 5.112.

ANTINEOPLASTIC AND ANTIVIRAL DRUGS

IMEXON

The substituted aziridine (477) was cyclised in methanol containing a catalytic amount of potassium hydroxide to imexon (478) (*Scheme 5.113.*) [636], which is an antitumour agent acting mainly on the T-cells [637].



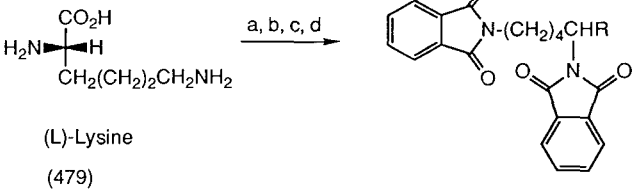
(477) (478)

Scheme 5.113.

SPERGUALIN AND DEOXYSPERGUALIN

The antibiotic spergualin was discovered in culture filtrates of *Bacillus laterosporus* BMG162-aF2 and its structure determined to be (−)-(15*S*)-1-amino-19-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione (494); the C-11 stereochemistry was undefined [638, 639]. The total synthesis was reported by a Japanese group [640]. Arndt-Eistert homologation of L-lysine (479) via steps a to e, *Scheme 5.114.*, gave the diamino acid (484). Selective ω-amino protection of (484) with a benzyloxycarbonyl group gave (485) which was deaminated with sodium nitrite to (486) with retention of configuration. Esterification and ammonolysis gave (487) which was deprotected to (488) and treated with 2-methyl-1-nitroisourea [641] to give the intermediate (489).

N-Protection of 3-amino-1-propanol followed by tosylation gave (490) (*Scheme 5.115.*). Treatment with N^1-benzyloxycarbonyl-1,4-diaminobutane in the presence of lithium bromide followed by a second butoxycarbonyl protection gave (491) which was debenzylated by hydrogenolysis and treated with 2,2-diethoxyacetic acid, 1-hydroxybenzotriazole and dicyclohexylcarbodi-imide to give (492). Careful acid hydrolysis gave the diamino diol (493).



(L)-Lysine

(479)

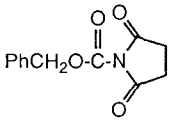
(480) R = CO_2H
(481) R = COCl
(482) R = $COCHN_2$
(483) R = CH_2CO_2Me

e, f, g, h, i, j → $R^1NH(CH_2)_4CR^2HCH_2COR^3$

(484) R^1 = H, R^2 = NH_2, R^3 = OH
(485) R^1 = Cbz, R^2 = NH_2, R^3 = OH
(486) R^1 = Cbz, R^2 = OH, R^3 = OH
(487) R^1 = Cbz, R^2 = OH, R^3 = NH_2
(488) R^1 = H, R^2 = OH, R^3 = NH_2
(489) R^1 = $(H_2N)_2C{=}N$, R^2 = OH, R^3 = NH_2

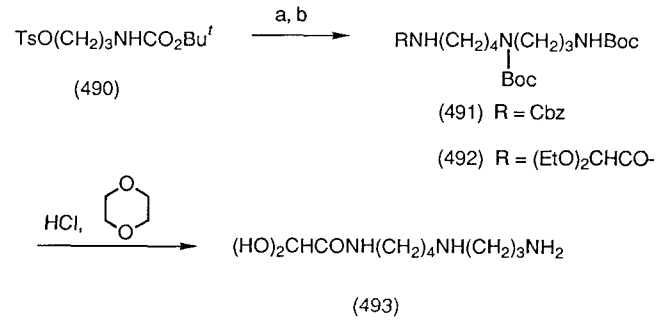
Cbz = $PhCH_2OCO$-

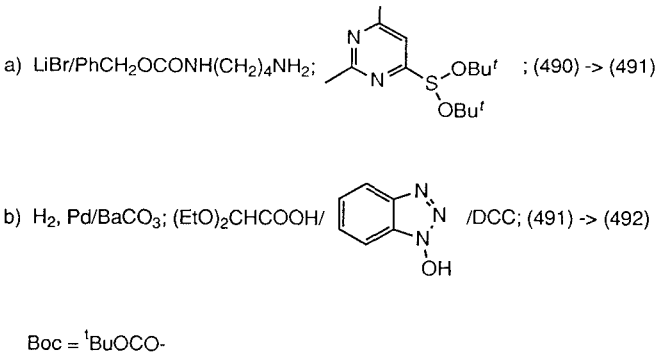
a) *Phthalic anhydride*, (479) -> (480)
b) $(COCl)_2$, (480) -> (481)
c) CH_2N_2, (481) -> (482)
d) MeOH/$PhCO_2Ag$, (482) -> (483)
e) NH_2NH_2, (483) -> (484)

f)  /Pyr/Et_3N, (484) -> (485)

g) $NaNO_2$/HAc, (485) -> (486)
h) CH_2N_2; NH_3/MeOH, (486) -> (487)
i) Pd/C, (487) -> (488)
j) MeNH-CO-NHNO; Pd/C, (488) -> (489)

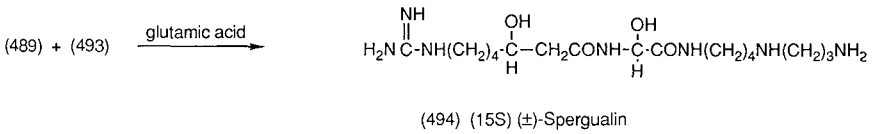
Scheme 5.114.





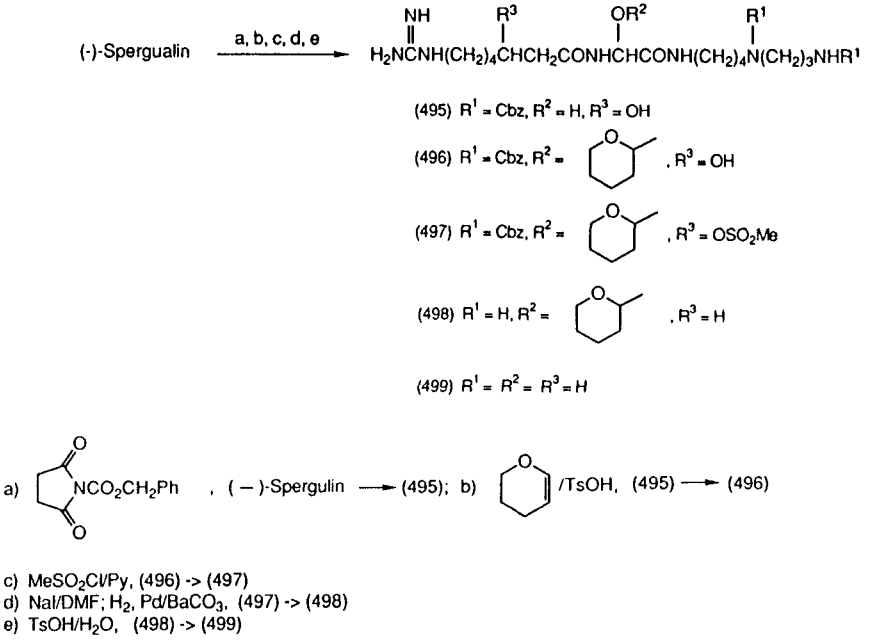
Scheme 5.115.

In the presence of glutamic acid, the amide (489) condensed with the acetal (493) (presumably via the free aldehyde) to give racemic spergualin (494) which was separated by HPLC to give (−)-spergualin identical with the natural product, (*Scheme 5.116.*).



Scheme 5.116.

(−)-15-Deoxyspergualin was prepared from (−)-spergualin, (*Scheme 5.117.*) [642]. Protection of the primary and secondary amino groups gave (495) and selective protection of the C-11 hydroxy group with 3,4-dihydro-2*H*-pyran gave the key intermediate (496). This was mesylated to (497), treated with sodium iodide, and hydrogenated to the 15-deoxy derivative (498) which was hydrolysed to (−)-15-deoxyspergualin (499). The stereochemistry at C-11 was retained during this process.



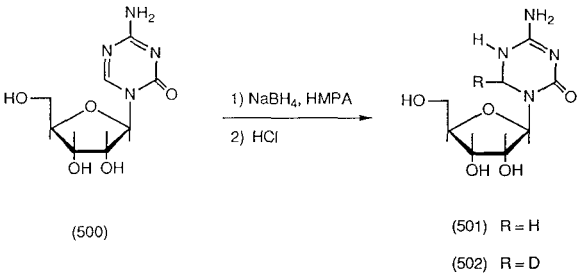
Scheme 5.117.

Both compounds are antitumour antibiotics, spergualin was active against several cell lines and also showed dose dependent immunosuppressive activity [643, 644]. Spergualin is cytotoxic to several cultured cell lines, but is inhibited by aminoguanidine (a monoamine oxidase inhibitor) and sometimes enhanced by added amine oxidase. It was suggested that the oxidized form may be the active drug [645]. Of the two drugs and a series of analogues, deoxyspergualin was the most potent against mouse leukaemia L-1210 and about eight times the potency of spergualin [642,646]. Although originally described as an antibiotic, deoxyspergualin is now being vigorously evaluated as an immunosuppressive. It is effective in treating

accelerated kidney rejection [647] and prolongs allogenic graft survival in kidney, pancreas, liver and heart transplantation [648]. Deoxyspergualin suppresses antibody formation, has an anti-proliferative effect in preventing xenograft rejection [649, 650] and is immunosuppressive on both monocytes and lymphocytes although the most marked effect is on the latter [651].

DIHYDRO-5-AZACYTIDINE

5-Azacytidine (500) was reduced with sodium borohydride in hexamethyl phosphoramide followed by acidification to dihydro-5-azacytidine (501). The deuterated derivative (502) was made similarly from sodium borodeuteride, (*Scheme 5.118.*) [652, 653]. 5-Azacytidine (500) is an antitumour agent, effective against human acute myeloblastic leukaemia and acute lymphoblastic leukaemia. However, it is unstable in aqueous solution, so the stable dihydro derivative (501) was made. This is 30 × less potent than (500), but has the same spectrum of activity; however it has a superior therapeutic index and is better able to cross the blood-brain barrier. It may be a prodrug for 5-azacytidine [654–658].

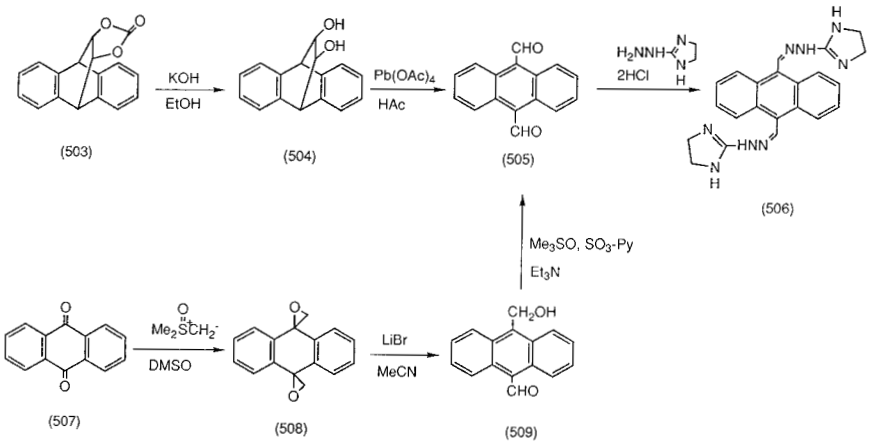


Scheme 5.118.

BISANTRENE

The Diels-Alder addition of vinylene carbonate to anthracene gave the adduct (503) which was hydrolysed to the diol (504) followed by mild oxidation to the dialdehyde (505). Simple condensation with 2-hydrazinoimidazoline gave bisantrene (506) [659–661]. An alternative, high yielding approach to the dialdehyde began with dioxiration of anthraquinone (507) with dimethylsulphonium methylide to give (508) which was rearranged to the alcoholic aldehyde (509). Oxidation with sulphur trioxide in dimethyl sulphoxide gave the dialdehyde (505) (*Scheme 5.119.*) [662–664].

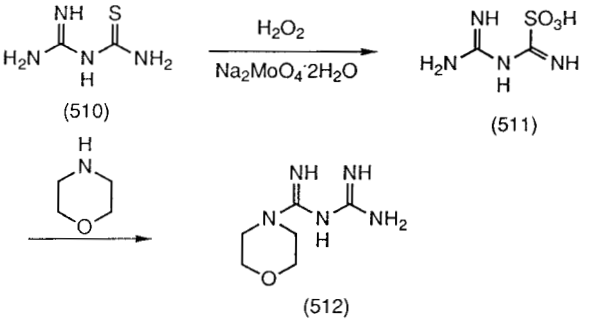
Bisantrene is a DNA intercalator and has been shown to inhibit the growth of several tumours including leukaemias and melanomas [660,665]. It is valuable in the treatment of metastatic breast cancer [666]. The drug is surprisingly stable, with a half life in monkeys of up to six days [660].



Scheme 5.119.

MOROXYDINE

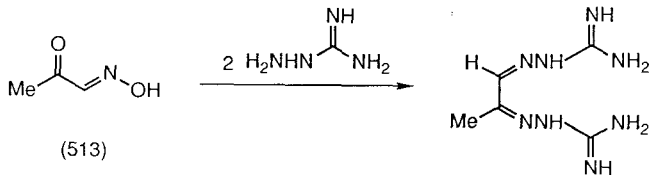
Moroxydine (512) was first synthesized by introducing morpholine to dicyanodiamidine [667], but an improved method is the oxidation of amidinothiourea (510) with hydrogen peroxide and sodium molybdate dihydrate to give (511) followed by reaction with morpholine [374] (*Scheme 5.120.*). This avoids the evolution of methyl mercaptan. The drug has antibiotic properties and some action as an influenza suppressant [668].



Scheme 5.120.

MITOGUAZONE

Pyruvaldehyde condensed with aminoguanidine to give mitoguazone (514) [669–671]. ^{14}C-Labelled *S*-methylisothiouronium sulphate was used to prepare labelled aminoguanidine and subsequently labelled mitoguazone [671]. An alternative is the oxidation of acetone with butyl nitrite to give methylglyoxal 1-oxime (513) which then reacts with aminoguanidine [672] (*Scheme 5.121.*). Mitoguazone is an antineoplastic drug which is very effective against acute myelocytic leukaemia and was the first compound to give a significant remission of this condition [669,673,674]; however, this was achieved with one dose a day which produced an unacceptably high toxicity [675]. Trials in patients with hard cancers showed some positive results at the one dose a week level, but the toxicity was still too severe [675].

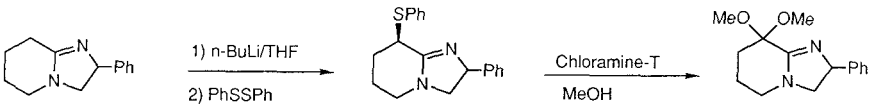


(513) (514)

Scheme 5.121.

OXAMISOLE

Lithiation of the pyridoimidazoline (515) with butyl lithium and a quench with diphenyl disulphide gave (516) which with chloramine-T in methanol gave oxamisole (517) (*Scheme 5.122.*) [676]. This ketal has been investigated recently as a T-cell immunorestorative agent [677, 678]. It increased the production of interleukins in mouse cells and has some antiviral activity against murine hepatitis [679, 680].



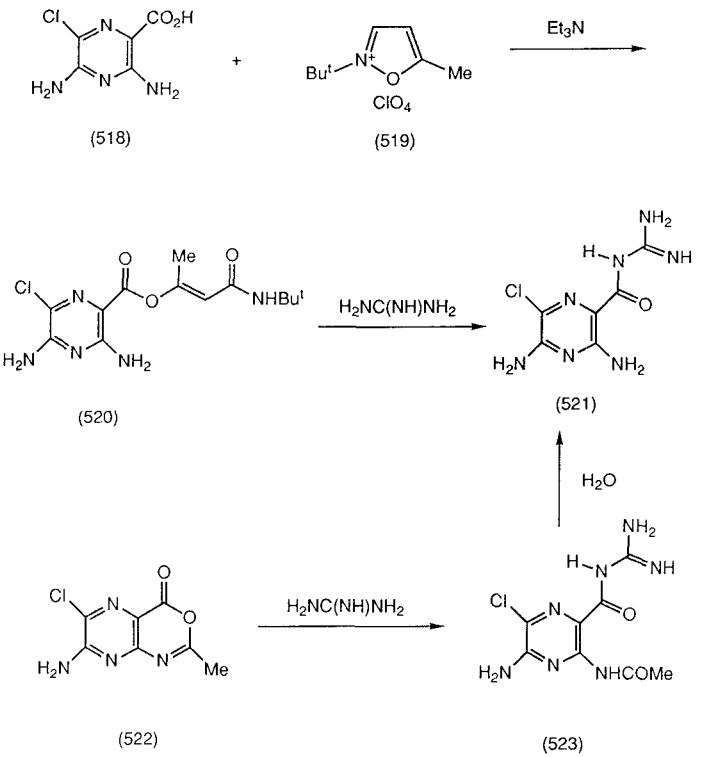
(515) (516) (517)

Scheme 5.122.

MISCELLANEOUS DRUGS

AMILORIDE

Although amiloride (521) can be made from the appropriate methyl ester and guanidine base [674], the procedure of *Scheme 5.123* requires milder conditions and gives excellent yields [681, 682]. The highly reactive enol ester (520) is prepared from the acid (518) and 2-*t*-butyl-5-methyl-isoxazolium perchlorate (Woodward's reagent L, 519) and treated with guanidine base to give amiloride. Alternatively, the pyrazinooxazinone (522) reacted with guanidine to give the derivative (523), the acyl group of which could be selectively hydrolysed to give (521) [683–685]. Amiloride is described as a potassium-sparing diuretic, it blocks the sodium channels in the distal tubules of the nephron which prevents sodium reabsorption and interfers with the normal sodium/potassium exchange pump [686,687].

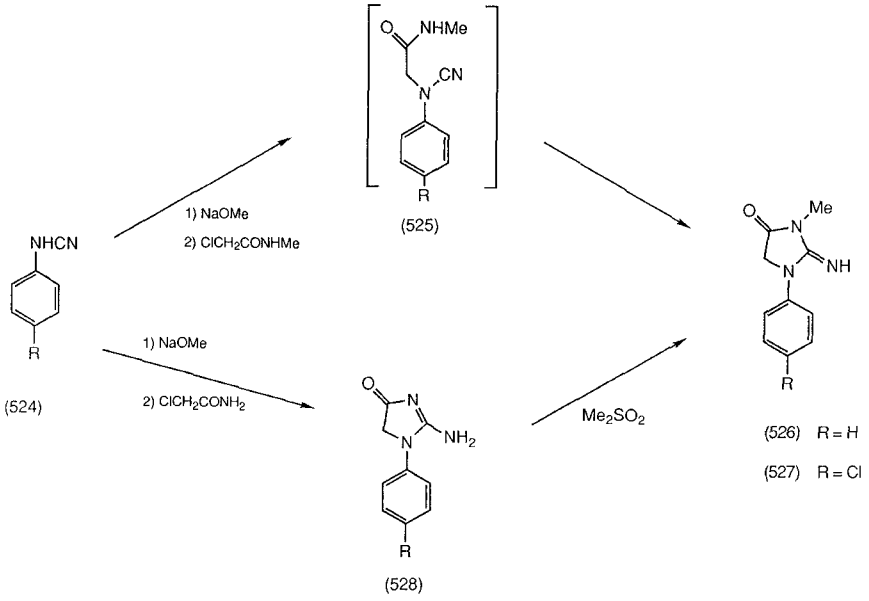


(518) (519) (520) (521) (522) (523)

Scheme 5.123.

AZOLIMINE AND CLAZOLIMINE

Sodium methoxide-catalysed alkylation of a phenylcyanamide (524, R = H or Cl) with *N*-methylchloroacetamide afforded the intermediate (525) which cyclised during the prolonged reaction to give azolimine (526) or clazolimine (527) (*Scheme 5.124.*). Cyclization with chloracetamide and methylation of the imidazolidone (528) with dimethyl sulphate provided an alternative route [688]. Both drugs are diuretics with the advantage that there is little potassium loss associated with their use [688]. In animal studies, azolimine antagonized the effects of mineralocorticoids on renal electrolyte excretion. Its greater efficiency in the presence of these steroids distinguishes it from noncompetitive mineralocorticoid antagonists such as amiloride and triamterene. It also improved sodium-potassium ratios when used in combination with several classical diuretics [689].



Scheme 5.124.

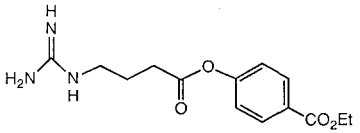
PENTIGETIDE

This pentapeptide (asp-ser-asp-pro-arg) was prepared by the Merrifield solid phase method [690]. Pentigetide has the same amino acid sequence as

a unique region of the ϵ-chain of immunoglobulin E. It was shown to block a standard Prausnitz-Küstner reaction [691, 692]. Clinical trials suggested it as a safe new treatment for allergies [693]; it was effective as a nasal spray against allergic rhinitis [694].

GABEXATE

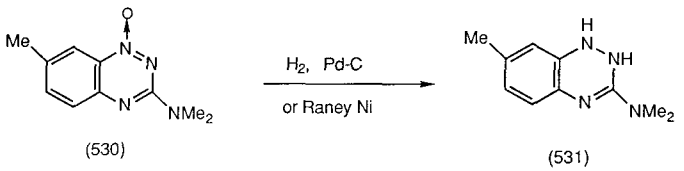
Gabexate (529), a proteinase enzyme inhibitor, is simply prepared by esterification of guanidinocaproic acid with ethyl 4-hydroxybenzoate [695–697]. It has proved a valuable addition to wound healing ointments [356]. Such ointments were recently applied over the injection sites of subcutaneous insulin injections. They reduced insulin degradation and increased its hypoglycaemic effect [357]. In a preliminary trial, it appeared to be useful in suppressing the progress of acute pancreatitis induced by deoxycholic acid [698]. It blocks the coagulation cascade and is better than heparin for the disseminated intravascular coagulation which sometimes follows hepatic resection [699,700].



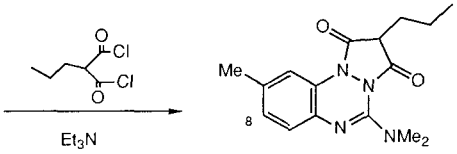
(529)

APAZONE

The benzotriazine-1-oxide (530) [701] was hydrogenated to the 1,2-dihydrobenzotriazine (531) which reacted with propylmalonyl chloride in the presence of triethylamine (or sodium and diethyl 2-propylmalonate) to give apazone (azapropazone, 532) (*Scheme 5.125.*) [702–704]. Although the dioxopyrazolidine ring and the exocyclic amine group are readily hydrolysed or oxidized, the main metabolite was found to be the 8-hydroxy derivative [705]. Apazone has anti-inflammatory activity comparable to phenylbutazone, mefenamic acid and flufenamic acid. It also shows antinociceptive and weak antipyretic activity [706].



(530) (531)



(532)

Scheme 5.125.

CONCLUSION

This summary of the physiologically active amidine and guanidine compounds clearly establishes the biological versatility of these related chemical groups. Many of the compounds have given temporarry excitement to their discoverers and then been dropped – the commonest experience in medicinal chemistry. However, there are important, clinically used, medicines in all the major categories of therapeutic action discussed in the review.

As the manuscript was being finalized, a summary of exciting new work on the role of nitric oxide (NO) as a neurotransmitter was published [707]. This simple and very unstable compound is not only a neurotransmitter in the brain, but acts in the dilation of blood vessels and activates leucocytes to attack tumour cells, fungi and bacteria. In addition, nitric oxide can inhibit blood coagulation by preventing platelet aggregation and appears to be the regulator of the male erection (cf phentolamine). No doubt other functions will be discovered in the future, but the interesting finding for the present review is that the NO is formed by nitric oxide synthetase from arginine –the only essential amino acid which carries a guanidine group. Since most of the active guanidine drugs have at least one unsubstituted =NH or NH_2 group it is interesting to speculate that they will be shown to act by giving nitric oxide as well.

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6 Inhibitors of Enkephalin-degrading Enzymes as Potential Therapeutic Agents

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INTRODUCTION

Endogenous opioids have been associated with the modulation of a large number of physiological processes. In accord with this it has been demonstrated that several opioid receptor subtypes exist, namely, mu (μ), delta (δ), kappa (κ), epsilon (ϵ) and sigma (σ) which are presumed to be involved in distinct pharmacological responses [1]. This, taken together with multiplicity of probable and established physiological roles of the endogenous opioids, has made therapeutic applications of opioid modulating drugs difficult.

The hypothalamus contains high densities of enkephalinergic neurones [2,3] whilst the pituitary contains only small amounts of enkephalins. A variety of hormonal effects have been identified which are probably mediated through hypothalamic mechanisms. Involvement of the opioids has been suggested in the withdrawal syndromes after chronic morphine treatment [4], alcohol dependence [5] and the pathophysiology of schizophrenia [6]. The enzyme enkephalinase (membrane metallopeptidase 3.4.24.11) is activated by ethanol [7] and high densities of opioid receptors are found in the emotion-modulating centres of the limbic system [8]. An opioid element has been demonstrated in epilepsy [9], learning and retention [10]. Opioids depress cardiovascular and respiratory systems [11] and influence body temperature mechanisms [12]. A role for the enkephalins in gastric motility and secretion has also been suggested [13].

MODE OF TERMINATION OF ENKEPHALINERGIC SIGNALS

It has now been widely accepted that the transient nature of the pharmacological actions of the enkephalins is due to their rapid hydrolysis at the enkephalinergic synapse in a manner similar to the deactivation of acetylcholine by acetylcholinesterase. It has been shown that a certain degree of re-uptake or internalisation of the enkephalins by brain synaptosomes does occur [14,15]; however, this is not thought to be a major mechanism of termination of the enkephalinergic signal. Evidence for the premise that the enkephalinergic signal is terminated mainly by enzymic degradation has been based on the findings that endogenous enkephalins

are rapidly deactivated *in vivo* [16] and that inhibitors of enkephalin degrading enzymes increase endogenous levels of the enkephalins [17]. There are, however, several peptidases in the central nervous system (CNS)* capable of catalysing the hydrolysis of the enkephalins and this review is concerned with the structure–activity relationships and therapeutic potential of agents capable of inhibiting these peptidases.

ENKEPHALINS

DISCOVERY, DISTRIBUTION AND FUNCTION

Two pentapeptides, [Leu]^5-enkephalin (H–Tyr^1–Gly^2–Gly^3–Phe^4–Leu^5–OH) and [Met]^5-enkephalin (H–Tyr^1–Gly^2–Gly^3–Phe^4–Met^5–OH), with potent opiate agonist activity were first isolated from pig brain by Hughes *et al.* [18]. Since the isolation of the enkephalins, a number of other endogenous opioids, with opioid receptor agonist activity, have been identified [19]. The endogenous opioid, β-endorphin (β-LPH$_{61-91}$, or C-fragment) was found to contain a fragment corresponding to [Met]-enkephalin and was initially proposed as the precursor of the enkephalins [19]. However, this biosynthetic relationship is unlikely since the distributions in the brain of [Met]-enkephalin and beta-endorphin have been found to be very different.

The adrenal gland has been shown to contain high levels of enkephalins and a precursor peptide pro-enkephalin [20]. The structure of pro-enkephalin has been characterized from the adrenal gland mRNA and found to contain four repeating units of the [Met]-enkephalin sequence and one [Leu]-enkephalin sequence; this ratio closely resembles that for the pentapeptides in the brain.

The distribution of the enkephalins generally parallels that of opioid receptors, the highest concentration being in the hypothalamus, corpus striatum and nucleus accumbens [20,21].

Structure–activity relationships for the pentapeptides have been extensively investigated *in vitro* in the mouse vas deferens and guinea-pig ileum [21]. These studies have revealed the critical importance of the amino acid sequence and pentapeptide chain length for affinity and pharmacological

* Other abbreviations used in this review: ACE, angiotensin converting enzyme; ANP, atrial natriuretic peptide; DADL, (D-Ala^2-D-Leu)-enkephalin; DAP, dipeptidylaminopeptidase; GABA, gamma-aminobutyric acid; PTC-amino acid, phenylthiocarbamyl-amino acid.

activity of the enkephalins. In this context, tripeptides, such as Tyr–Gly–Gly are totally devoid of pharmacological activity. The activity profile of tetrapeptides is largely dependent on their amino acid composition and sequence. Tetrapeptides, lacking the terminal leucine or methionine residues retain binding affinity but show little physiological activity. Removal of glycine in positions 2 or 3 results in almost complete loss of activity whereas removal of N-terminal tyrosine results in total loss of activity. Replacement of the pentapeptide Phe^4 with Tyr, D-Phe or a cyclohexyl analogue of phenylalanine results in a significant loss of activity. In general, increasing the length of the pentapeptides by addition of amino acids to either the C-terminal or N-terminal results in a loss of activity but there are exceptions. The peptides α-endorphin (β-LPH$_{61-76}$) and β-endorphin (β-LPH$_{61-91}$) have been found to exhibit opioid properties and the activity shown by these longer peptides is probably due to their ability to form appropiate conformations resembling that of the enkephalins as well as their resistance to enzyme degradation [21,22]. Overall, studies indicate the importance of the N-terminal tyrosine, the side-chain substituent in position 4 and the distance between the aromatic rings of tyrosine and phenylalanine for effective binding and pharmacological activity in the pentapeptides.

Both [Met]^5-enkephalin and [Leu]^5-enkephalin inhibit opioid receptor binding with affinity comparable with that of morphine. However, in marked contrast to morphine, analgesia after either intracerebroventricular or intravenous (high dose) administration of [Met]^5-enkephalin was found to be of short duration and was only detected in selected tests [21]. This indicated that the enkephalins were rapidly inactivated both in the brain and blood and led to the development of enkephalin analogues resistant to degradation. Coy *et al.* [23] substituted D-amino acids into the peptide chain, an approach which had already proved successful for stabilizing analogues of luteinizing hormone-releasing hormone (LH-RH) and somatostatin. The most potent analogues of [Met]-enkephalin were [D-Ala^2]-enkephalin and [D-Ala^2]-enkephalinamide. Substitution by other D-amino acids, such as [D-Tyr^1], [D-Leu^2], [D-Ala^3], [D-Phe^4] and [D-Met^5], gave analogues with very weak activity. The [D-Ala^2]-enkephalinamide analogue was more potent than the [D-Ala^2]-enkephalin analogue suggesting that [D-Ala^2]-enkephalinamide is resistant to degradation at the *C*-terminus. Other developments have involved N-methylation, removal of the terminal carboxyl group, and insertion of hydrophilic residues in the *C*-terminal amino acid residue (see review [24]). Enkephalin analogues induce effective, long-lasting analgesia in rodents [25] indicating that the transient action of the enkephalins is due to rapid enzymic degradation.

The enkephalin analogue FK 33-824 (Tyr-D-Ala-Gly-MePhe-Met-(O)^5-

ol) is resistant to degradation by enkephalinase, angiotensin converting enzyme (ACE), and aminopeptidases and shows greater potency than analogues resistant to aminopeptidase alone [26]. In clinical trials, FK 33-824 exhibited side-effects such as an oppressive feeling of 'heaviness' of the limbs and facial flushing which limited its usefulness [27,28].

ENKEPHALIN-DEGRADING ENZYMES AND THEIR INHIBITORS

Identification of a specific 'neuropeptidase' responsible for the deactivation of the enkephalins has proved difficult, due to the large number of enzymes for which the enkephalins act as substrates and the broad specificity of those enzymes. A number of enzymes, which act at different sites of the pentapeptides, have been implicated in the role of enkephalin-neuropeptidase (see Figure 6.1).

Aminopeptidases. These enzymes cleave the Tyr^1–Gly^2 amide bond of the enkephalins releasing Tyr; they include soluble aminopeptidase (EC 3.4.11.11; aminoacylpeptide hydrolase), aminopeptidase M11 (puromycin-sensitive aminopeptidase) and aminopeptidase M (EC 3.4.11.2; aminopeptidase N, puromycin-insensitive aminopeptidase).

Enkephalinase (EC 3.4.24.11). (membrane metalloendopeptidase, MEP; neutral endopeptidase, NEP 24.11; enkephalinase A; enkephalin-dipeptidylcarboxypeptidase; neutral proteinase from kidney brush border; atriopeptidase; CD10/CALLA/common acute lymphoblastic leukaemia antigen). This cleaves the enkephalin pentapeptides at the Gly^3–Phe^4 bond.

Angiotension Converting Enzyme (ACE) (EC 3.4.15.1). (Kinase II; dipeptidylpeptidase A). This cleaves the Gly^3–Phe^4 amide bond of the pentapeptides releasing the fragment Tyr–Gly–Gly.

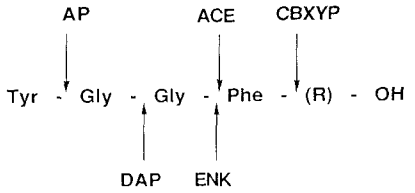


Figure 6.1. Diagramatic representation of the peptidases involved in the metabolism of the enkephalins. AP (aminopeptidases), DAP (dipeptidylaminopeptidase), ACE (angiotensin converting enzyme), ENK (enkephalinase) and CBXYP (non-specific carboxypeptidases). R represents methionine in [Met]-enkephalin or leucine in [Leu]-enkephalin.

Dipeptidylaminopeptidase (DAP) (EC 3.4.14.4). (enkephalinase B; dipeptidylaminopeptidase III). This cleaves the Gly^2–Gly^3 amide bond of the pentapeptides releasing the fragment Tyr–Gly.

ASSESSMENT OF ENKEPHALIN-DEGRADING ENZYMES AS ENKEPHALIN NEUROPEPTIDASES

In order to unambiguously identify the neuropeptidase(s) responsible for inactivation of endogenous enkephalins, certain criteria were proposed by Schwartz *et al.* [26], which all had to be met for an enzyme to be accepted as the enkephalin-degrading neuropeptidase. Enkephalinase activity in mouse striatum progressively increases on chronic treatment with morphine. Although the maximal increase in enkephalinase activity is only by about 20–25%, this effect is significant since other enkephalinase-hydrolysing enzymes do not show such changes under similar conditions [29,30].

In washed brain slices, an *in vitro* model for the evaluation of effects of membrane-bound enzymes on enkephalin hydrolysis, the main hydrolysis products have been identified as Tyr and Tyr–Gly–Gly. The formation of Tyr–Gly–Gly is dependent on the action of enkephalinase and is reduced in the presence of thiorphan (an enkephalinase inhibitor). In the presence of bestatin (a general aminopeptidase inhibitor), the formation of Tyr is reduced; Tyr is also reduced, to a lesser extent, in the presence of puromycin (an aminopeptidase M11 inhibitor). In the presence of thiorphan the formation of Tyr increases whereas in the presence of bestatin there is an increase in formation of Tyr–Gly–Gly. The level of Tyr–Gly in this model is low and is unaffected by either thiorphan or bestatin indicating that the action of DAP is unimportant. Recovery of endogenous enkephalins released by depolarisation of brain slices is enhanced in the presence of thiorphan or bestatin and is complete when both inhibitors are present. This does not occur in the case of puromycin or captopril (an ACE inhibitor) [31].

In vivo, both bestatin and thiorphan display antinociceptive properties in behavioural tests which are additive when the two inhibitors are co-administered [26,32]. Puromycin does not display similar activity. Taken together, these data strongly indicate that enkephalinase and puromycin insensitive aminopeptidase (aminopeptidase M) are involved in the metabolism of endogenous enkephalins.

The biological activities of enkephalinase and aminopeptidase inhibitors have been found to be very weak when they are administered alone. However, enkephalinase inhibitors potentiate the effects of enkephalin analogues which are resistant to degradation by the aminopeptidases such

as [D-Ala^2-Met^5]-enkephalin, indicating that both enkephalinase and aminopeptidase inhibition is necessary for the detection of behavioural effects of the enkephalins [25,33]. This view has been confirmed by the greater effect of the combination inhibitor kelatorphan (see p. 361) potentiating the analgesic effects of [Met]^5-enkephalin than that produced by co-administration of bestatin and thiorphan [34]. *In vitro*, kelatorphan inhibits enkephalinase, DAP and aminopeptidase M [35]. The higher potency of kelatorphan compared with the combined potency of thiorphan and bestatin may suggest a possible role for DAP in enkephalin degradation. However, another combined inhibitor phelorphan (see p. 361), which inhibits enkephalinase, DAP and soluble enkephalin-degrading aminopeptidase, is less effective than thiorphan in protecting endogenously released [Leu]^5-enkephalin [36].

Both enkephalinase and membrane-bound aminopeptidases, particularly aminopeptidase M, are strong candidates as brain enkephalin-degrading neuropeptidases and fulfil the criteria laid down for this role [26]. The significance of aminopeptidase M11 has been insufficiently investigated for the enzyme to be disregarded as an enkephalin neuropeptidase. The role of DAP appears to be minor, at least in the CNS, although a peripheral role may be indicated [37]. ACE is unlikely to be involved in deactivation of endogenously released enkephalin.

AMINOPEPTIDASES

The distribution of aminopeptidases does not generally correspond to that of the enkephalins. However, membrane-bound enkephalin-degrading aminopeptidases have been detected on neuronal and glial cell membranes and an opioid receptor-associated aminopeptidase has been isolated indicating a synaptic role in degradation of the enkephalins [38]. The distribution of membrane-bound aminopeptidase activity in regions of the rodent brain does not correlate with either enkephalin levels or opiate receptors, suggesting that this enzyme activity is not restricted to enkephalinergic synapes.

Tyr was identified as the amino acid released together with Gly–Gly–Phe–Met in initial *in vitro* studies on the metabolism of enkephalins by brain tissue. This led to the conclusion that the first step of enkephalin metabolism consisted of the cleavage of the Tyr^1–Gly^2 amide bond by aminopeptidases [26]. Enkephalin-hydrolysing activity has been detected both in the soluble [39] and particulate fractions [40] of cerebral preparations, the specific activity being the highest in soluble fractions. The membrane-bound aminopeptidase can be differentiated from the soluble form in terms of

inhibition by EDTA and leucyl-β-naphthylamide indicating the existence of two separate enzymes [26].

The role of aminopeptidases in the metabolism of exogenous and endogenous enkephalins *in vitro* and *in vivo* has been investigated using the aminopeptidase inhibitors bestatin and puromycin [31]. Giros *et al.* [41] have characterized three membrane-bound aminopeptidases from rat brain, designated M1, M and M11 that are sensitive to inhibition by bestatin. Aminopeptidase M1 displays very low affinity for enkephalins and was considered unlikely to be an enkephalin-neuropeptidase. Aminopeptidase M showed a low sensitivity towards puromycin [42], whereas, aminopeptidase M11 was highly sensitive to inhibition by both puromycin and bestatin. In order to establish whether both aminopeptidase M and M11 were responsible for the inactivation of endogenous enkephalins, the effects of bestatin, puromycin and anti-aminopeptidase M antibodies were investigated on [^{3}H]-enkephalin hydrolysis by semipurified aminopeptidases, brain membranes and brain slices [41]. It was concluded that the puromycin-sensitive aminopeptidase (M11) appeared to predominantly participate in the hydrolysis of exogenous enkephalins whereas puromycin-insensitive aminopeptidase (M) was predominant in endogenous enkephalin metabolism. These data confirmed the earlier observation that the recovery of (Met)^5-enkephalin from brain slices was significant in the presence of bestatin and thiorphan (an enkephalinase inhibitor) but not in the presence of puromycin [31,32]. Although the data suggest aminopeptidase M as the important aminopeptidase in inactivation of endogenous enkephalins, in the absence of highly selective aminopeptidase M inhibitors, the situation *in vivo* remains to be confirmed.

Leucine aminopeptidase (EC 3.4.11.1)

A major part of the work on the structural requirements for binding of inhibitors to the active site of either aminopeptidase M or aminopeptidase M11 has been conducted using bestatin (see *Figure 6.3A*) as the model inhibitor and interpreted from the known structure–activity relationships of bestatin analogues towards leucine-aminopeptidase [43].

A hypothetical model of the binding of bestatin to leucine-aminopeptidase was first proposed by Nishizawa *et al.* [43] (*Figure 6.2B*). Four functional groups present in bestatin are considered to be essential for efficient interaction with the active site of leucine-aminopeptidase; the C-1 substituent, a free *N*-terminal amino group, an alcohol group in the C-2 position and a free *C*-terminal carboxyl group.

N-Terminal amino acid residue substituent. The inhibitory activity of bestatin-related compounds where the benzyl group of bestatin was removed or substituted by an alkyl or a phenyl group was reduced whereas a cyclohexylmethyl substituent (hexahydrobestatin) increased inhibitory potency. *p*-Nitrobestatin was found to be 5 times more potent than bestatin whereas *p*-aminobestatin was less potent. The *o*- and *p*-chlorobestatin had decreased and increased inhibitory activity, respectively. (2*S*,3*R*)-*p*-Methylbestatin was found to be the most potent inhibitor of the many bestatin analogues examined.

In the bestatin analogues examined the (*R*)-configuration at C-1 (as opposed to the (*S*)-configuration in the natural substrate) was important for enzyme inhibitory activity but stereochemical requirements for other chiral centres are not very stringent.

N-Terminal amino group. The *N*-benzyloxycarbonyl derivative of the *N*-terminal amino group in bestatin and its corresponding amide displayed only weak inhibitory activity suggesting that a free *N*-terminal amino group was essential for interaction with the active site of the enzyme.

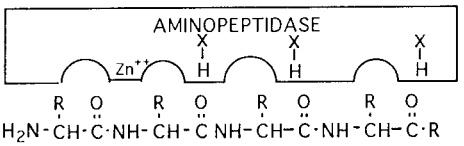
C-Terminal carboxyl group. In bestatin-related analogues where the L-leucine moiety was substituted by other alpha- (α), as well as beta- (β), gamma- (γ), and epsilon- (ϵ) amino acid residues, the strongest inhibitory activity was shown by the compound containing the α-amino acid moiety. The involvement of the free carboxyl group of bestatin in binding to the enzyme was indicated by the weak inhibitory potency of decarboxylated bestatin and a leucyl-glycine derivative.

Aminopeptidase M (EC 3.4.11.2)

A comparative study of the structure–activity requirements for inhibitors of leucine aminopeptidase and aminopeptidase M has been conducted [44] using bestatin and amastatin (see *Figure 6.3B*) as model inhibitors. It was found that amastatin (a tetrapeptide-type) was 100-fold more potent as an inhibitor of aminopeptidase M than bestatin (a dipeptide-type) and that for amastatin analogues, a peptide larger than a di- or tri-peptide was required for maximal inhibition of aminopeptidase M, whereas inhibition of leucine aminopeptidase was more effective with dipeptide derivatives. The isoamyl amide derivative of amastatin was a potent inhibitor showing that a free *C*-terminal carboxyl group was not essential for good inhibition in tri- or tetra-peptide derivatives.

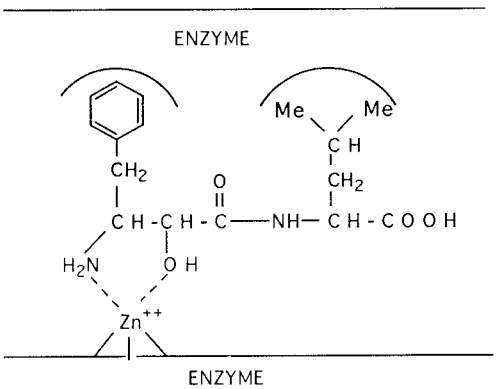
Evaluation of steady state velocities for hydrolysis of enkephalins by aminopeptidase M, in the presence of amastatin, indicated that amastatin

A



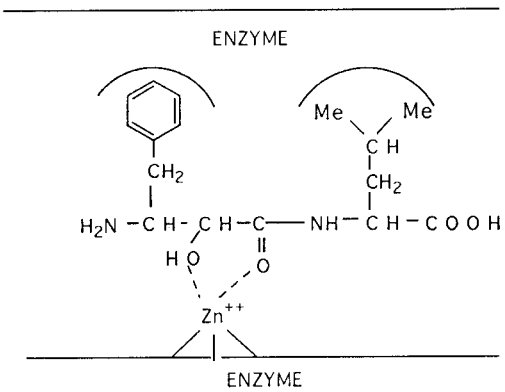
Hypothetical model of the binding of enkephalins to aminopeptidase.

B



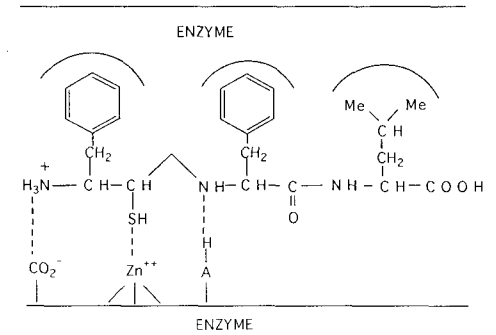
Hypothetical model of the binding of bestatin to leucine aminopeptidase (EC 3.4.11.1) [43].

C



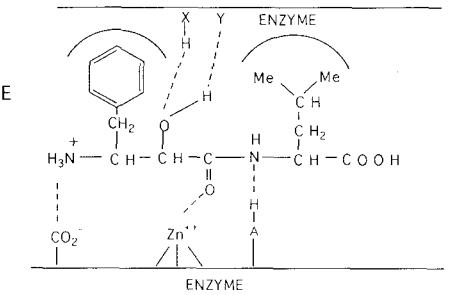
Hypothetical model of the binding of bestatin acting as a bidentate ligand for the active zinc site [45].

D



Hypothetical model of binding of a tripeptide derivative of diamino thiol inhibitor to the aminopeptidase active site [48].

E



Hypothetical model of bestatin binding to the active site of aminopeptidase [48].

Figure 6.2. Hypothetical models of interaction between substrate or inhibitor with the active site of aminopeptidase.

was a slow binding inhibitor with maximal inhibition being reached after 30 min. It was proposed that the slow binding process was due to a conformational change that transformed the initially formed enzyme-inhibitor complex into a new, more tightly bound complex [44]. Further investigation using analogues of amastatin and bestatin indicated that the binding of the hydroxyl group to the active site of the enzyme occurred in the formation of the initial enzyme-inhibitor complex, rather than later in the formation of the tightly bound complex.

Interaction of bestatin and amastatin with the Zn^{2+} present at the active site of aminopeptidase M is visualised [43] as occurring through the amino-and hydroxyl ligands of leucine aminopeptidase (*Figure 6.2B*). An

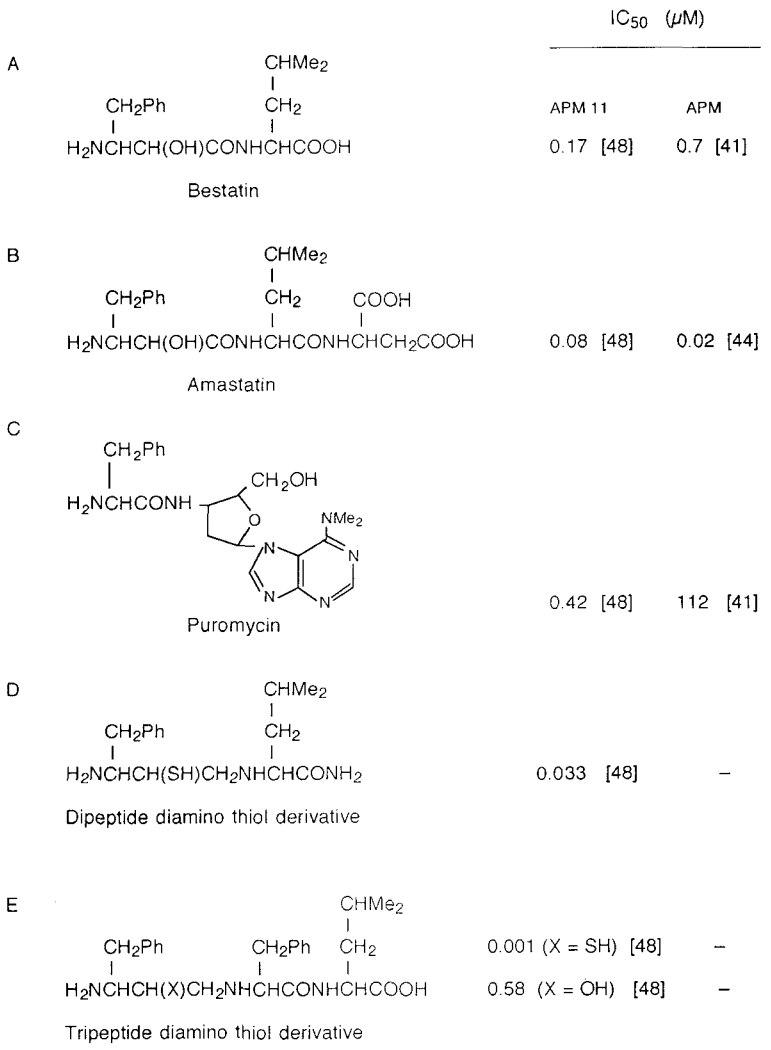


Figure 6.3. Inhibitors of enkephalin-degrading aminopeptidases.

alternative model for the interaction between bestatin and the Zn^{2+} has been proposed [45] (*Figure 6.2C*) and is based on the known bidentate interaction between the Zn^{2+} at the active site of thermolysin and hydroxamate inhibitors, where both the carbonyl group and the 2(*S*)-hydroxyl groups act as ligands.

The specificity of inhibitors towards the active site of aminopeptidase M has been investigated using small non-peptide inhibitors with a positively charged free amino group and the P_1 side-chain of varying size and hydrophobicity [46]. Generally, compounds with aliphatic side-chains in the P_1 position were more potent than compounds with cyclic residues. Linear chains were preferred to branched chains (see *Table 6.1*). This suggests the S_1 subsite of aminopeptidase M is not very large but is likely to be deep. Thiol was the most efficient group for interaction with Zn^{2+} at the catalytic site of aminopeptidase M in this series of compounds. The lowest activity was found with phosphonate and carboxylate groups (see *Table 6.1*). A drastic reduction in inhibitory potency is observed in N-substituted derivatives suggesting the presence of a glutamate (or aspartate) in the active site which is not easily accessible (see *Table 6.1*)

Bergin and Clapp [47] have described ethyl D-cysteinate as a potent competitive inhibitor ($K_i = 3.5 \times 10^{-7}$M) of aminopeptidase M. D-Cysteine and ethyl L-cysteine inhibit more than two orders of magnitude less effectively. Inhibition studies on a series of n-alkyl esters of D-cysteine reveal an optimum at the n-butyl ester ($K_i = 1.8 \times 10^{-7}$M). Cytosolic leucine aminopeptidase is not significantly inhibited by ethyl D-cysteinate.

Aminopeptidase M11

The structure–activity requirements for the inhibition of aminopeptidase

Table 6.1. STRUCTURE–ACTIVITY RELATIONSHIPS FOR PHENYLALANINE DERIVATIVES AS INHIBITORS OF AMINOPEPTIDASE M

Effect on S_1 subsite [46]		*Effect of Zn^{2+} binding ligand* [46]		*Effect of substituents on N-terminal amino group* [46]		
CH_2R + | $H_3NCH\ CH_2SH$		CH_2Ph + | $H_3N\ CH\ CH_2\ X$		CH_2R^1 + | $H_2N\ CH\ CH_2SH$ | R^2		
R	*IC_{50} (nM)*	*X*	*IC_{50} (μM)*	*R^1*	*R^2*	*IC_{50} (μM)*
Ph	30	SH	0.03	$CHMe_2$	H	0.02
Cyclohexyl	45	CONHOH	75	$CHMe_2$	Me	40
SMe	11	PO_3H_2	500	Ph	H	0.03
$CHMe_2$	22	CO_2H	800	Ph	CH_2Ph	160
S(O)Me	20					

M11 have been investigated [48] using amino thiol inhibitors (see *Figure 6.3D*).

N-Terminal asymmetric centre. Although both bestatin and amastatin have a D-configuration at the *N*-terminal chiral centre, with the diamino thiol inhibitors the L(*S*) isomer showed a greater enzyme inhibitory potency than the corresponding D(*R*)-isomer by a factor of 200.

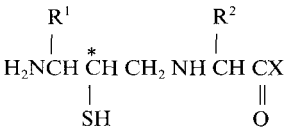
C-Terminal carboxylic acid function. Compounds containing a free C-terminal carboxylic acid function were found to have a much reduced inhibitory potency when compared with their C-terminal amide derivatives [48] (see *Table 6.2*).

Length of peptide-type backbone. Aminopeptidase M11 inhibitory activity is enhanced markedly by addition of a phenylalanine residue to the chain (see *Figure 6.3E*). This indicates that aminopeptidase M11 contains an extended active site which favours the binding of a tripeptide or possibly a longer peptide.

Zinc binding ligand. Generally the amino thiol tripeptide derivatives were found to be more potent inhibitors than their alcohol counterparts, a 600-fold decrease in enzyme inhibitory potency being observed for this change (see *Figure 6.3E*).

N-Terminal function. Removal of the *N*-terminal function and its associated benzyl group led to a marked decrease in aminopeptidase M11

Table 6.2. EFFECT OF A FREE C-TERMINAL CARBOXYLIC ACID GROUP ON INHIBITORY POTENCY FOR AMINOPEPTIDASE [48]



R^1	R^2	*	X	IC_{50} (μM)
CH_2Ph	Leu	A	OH	4.4
CH_2Ph	Leu	B	OH	0.22
CH_2Ph	Leu	A, B	NH_2	0.033
CH_2CHMe_2	Leu	A, B	NH_2	0.034

*A and B indicate stereoisomers whose configurations are unknown.

inhibitory potency indicating the importance of the free amino group and the benzyl moiety in binding to the active site of the enzyme.

From the data obtained, Gorden *et al.* [48] proposed a model for the binding of the tripeptide amino thiol inhibitors to the active site of aminopeptidase (*Figure 6.2D*). The model proposes that the sulphydryl moiety directly interacts with the Zn^{2+} at the active site and the amino group binds to a specific recognition site on the enzyme, which may allow proper alignment of the substrate with the active site. It has been suggested that such an amino group recognition site may be an ionized carboxyl group which could interact with both protonated and uncharged forms of the amino group.

Thiobestatin was synthesized to investigate whether bestatin itself interacted with the aminopeptidase M11 active site in the same manner as the amino thiol derivatives [48]. The replacement of the hydroxyl in bestatin with a sulphydryl function had little effect on inhibitory potency suggesting that the mode of binding of bestatin and thiobestatin to aminopeptidase involved a mechanism other than the interaction of Zn^{2+} with the hydroxyl or sulphydryl group. An alternative model for the binding of bestatin to the enzyme active site, based on the transition state analogue concept, has been proposed (*Figure 6.2E*) where the C-2 hydroxyl group serves as a mimic of the incoming nucleophile (oxygen of tetrahedral hydrolysis intermediate) rather than binding to Zn^{2+}. An alternative model proposed chelation to Zn^{2+} by the amino and carbonyl groups of bestatin.

The structural requirements for binding to the active sites of aminopeptidase M and aminopeptidase M11 appear to be very similar. The important requirements being a free *N*-terminal amino group, a benzyl or larger substituent at C-1 for interaction with the S_1 hydrophobic site and a zinc binding ligand on C-2. Stereochemically, the requirement appears to be for the (*S*)-form at C-1 for amino thiol inhibitors, but the (*R*)-form for both bestatin and amastatin. In contrast to the requirements by leucine aminopeptidase, inhibitors containing a tri- or tetra-peptide type of backbone are more effective than the dipeptide derivatives and a free *C*-terminal carboxylic acid function is not essential for good inhibitory potency and amide derivatives are more potent inhibitors. It is probable that bestatin and amino thiol type of inhibitors interact with the aminopeptidase active site in different ways. This is because substitution of the Zn^{2+} ligand at the C-2 hydroxyl by thiol in the amino thiol inhibitors led to a significant increase in aminopeptidase M11 inhibitory potency, whereas a similar conversion in bestatin had little effect.

ENKEPHALINASE (EC 3.4.24.11)

Enkephalinase activity was first characterized as a high-affinity component of the total enkephalin-hydrolysing activity in mouse striatal membranes during a course of experiments designed to identify ^{3}H-[Leu^{5}]-enkephalin binding sites in washed particulate fractions [29]. In addition, a second site which had parallel distribution but was pharmacologically distinguishable from the opiate receptor was identified. It was observed that while intact ^{3}H-enkephalin remained bound to the opiate receptor, the other site was bound by ^{3}H-peptide fragments. On evaluation, using Porapak column chromatography, ^{3}H-enkephalin hydrolysis was found to proceed in a biphasic manner. A fraction, representing 20–25% of the total peptidase activity, corresponded to high affinity activity and produced the tripeptide Tyr–Gly–Gly as the hydrolysis product. These data suggested the presence of a high affinity enzyme involved in the metabolism of the enkephalins. The formation of Tyr–Gly–Gly from cleavage of the Gly^{3}–Phe^{4} amide bond of ^{3}H-[Leu]- and ^{3}H-[Met]-enkephalin by enkephalinase has now been established [49,50].

Distribution

Enkephalinase distribution is wide-spread in the brain and peripheral organs and soluble forms are present in human plasma and cerebrospinal fluid. In the central nervous system, its distribution largely parallels regional distribution of the enkephalins and opioid receptors (striatum > cortex > cerebellum) and it is localized in the particulate fraction of brain homogenates [26,50] in a non-uniform fashion in mice, rats and humans. In the cortex or the striatum, opioid receptors and the enzyme display a parallel subcellular distribution pattern. Both enkephalinase and opioid receptors are present on the membranes from nerve-terminal regions, suggesting their involvement in synaptic transmission.

In rodents, the highest activity is found in the striatum and the lowest in the cerebellum, with a 3-fold difference between the two regions. In humans, the enkephalinase distribution parallels that of the enkephalins, rather than the opioid receptors, with the highest levels in the globus pallidus and substantia nigra [51]. Lesions, by injection of kainic acid (a cell-body toxin) into rat striatum, lead to a significant decrease in opioid receptor binding as well as enkephalinase activity suggesting that both membrane components are present on post-synaptic neurons. A similar decrease in opioid receptors and enkephalinase activity was observed on specific lesioning of the nigro-striatal dopaminergic neurons suggesting that dopaminergic neurons

possess enkephalinase and opioid recognition sites [51]. Enkephalinase has been detected in peripheral organs such as lung, testes, thyroid, salivary glands, kidney and adrenals [52,53] and has recently also been detected in synovial fluid from arthritic joints [54].

Assay of enkephalinase

Enkephalinase activity has not only been found in the brain but also in peripheral tissues [52,53,55] using radiometric procedures. An early methodology relied on the use of [^{3}H]-Tyr–Gly–Gly as the degradation product [29,56]. Although reliable, a disadvantage of such an approach was that it was necessary to employ inhibitors of other enkephalin degrading peptidases in order to render the assay more enkephalinase-specific. In this respect, the comparatively aminopeptidase-resistant analogues [^{3}H]-(D-Ala^2, Leu^5)-enkephalin or [^{3}H]-D-Ala^2, Met^5)-enkephalinamide may be used as substrates and the resulting cleaved fragment [^{3}H]-Tyr–D-Ala^2–Gly may be separated using polystyrene bead chromatography [51,57]. Other substrates such as glutaryl–Ala–Ala–Phe–naphthylamide [60] or succinyl–Ala–Ala–Phe–aminomethylcoumarin [58,59,60] have also been employed although they rely on the release of an absorbing or fluorescent moiety following the addition of aminopeptidases.

Ontogenetic development

Striatal levels of [Met]^5-enkephalin exhibit a 4–5-fold increase between birth and adulthood in rats [30]. The density of opiate receptors and striatal enkephalinase activity (measured by formation of Tyr–Gly–Gly) increase by about 2–fold between birth and adult-hood but the pattern of development appears to be different. The enkephalinase activity is significantly higher than in adults by days 15–21 of development whereas this is not the case for opiate receptor binding. The enkephalinase activity appears to reach adult level by day 15, possibly reflecting an early development of enzyme activity during brain maturation. The properties of the enzyme do not appear to change as its K_m for [Leu]^5-enkephalin at 21 days is not significantly different from that obtained for the adult enzyme.

Purification and physicochemical properties

A major part of the initial work on enkephalinase has been carried out on the crude enzyme obtained from the particulate fraction of mouse and rat striatal preparations. Partial purification of enkephalinase from rat and

rabbit brain was initially carried out by solubilization of the enzyme from the particulate fraction using detergents such as Triton-X-100 to release the enzyme, which is an intergral membrane glycoprotein [26]. Further purification has been carried out using techniques such as ion-exchange chromatography, gel filtration, affinity chromatography and immunoadsorption [61,62]. The optimal pH toward a variety of subtrates is pH 7.0, and the purified enzyme appears to be stable at 4°C for several months. Enkephalinase enzyme activity is completely inhibited by chelating agents such as EDTA and activity is restored by divalent metals such as Zn^{2+} and Co^{2+}, suggesting that enkephalinase is a zinc-metallopeptidase [63].

Purified enkephalinase has a K_m of around 20 μM and V_{max} of 80 nmol/min/unit enzyme [33]. The K_m values reported for enkephalinase vary according to the source of the enzyme, the level of purification and the substrate used [64–68].

Substrate specificity and structure–activity relationships

Enkephalinase is not only involved in deactivation of the enkephalins but also in degradation of other peptides and hormones including Substance P, cholecystokinin, bradykinin, chemotactic peptide and atrial natriuretic factor (ANP) [67,69–72]. Peptides such as the dynorphins and β-endorphin are not hydrolysed efficiently [73]. The specificity of the enzyme has been indirectly characterized by establishing the inhibitory potency of various peptides towards hydrolysis of ^{3}H[Leu]^5-enkephalin [26].

A model of the enkephalinase binding site has been developed, based on information from the earlier development of inhibitors of related zinc metallopeptidases such as carboxypeptidase A, angiotensin converting enzyme and thermolysin [74]. The manner in which competitive inhibitors bind at the active site of carboxypeptidase has largely been derived from X-ray crystallographic studies of carboxypeptidase A and thermolysin which suggest close similarities between the active sites of various zinc-metallopeptidases. The amino acid sequence of enkephalinase (749 amino acids) has been deduced from its complementary DNA [75]. It consists of a 27-amino acid-residue cytoplasmic domain, a 13-amino acid-residue hydrophobic domain and a large extracellular domain containing the active site. Comparison with the primary structure of thermolysin TLN (316 amino acids) has shown that except for two highly homologous short regions, the sequences of these two enzymes are different. However, most of the residues involved in the catalytic sites of both enzymes are similar [76]. The three zinc-coordinating residues of enkephalinase have been identified as His-583, His-587 and Glu-646 corresponding to His-142,

His-146 and Glu-166 in thermolysin, respectively [77]. Glu-584 has been identified as the residue involved in the acid-base catalytic mechanism occurring at the active site. Comparison of enkephalinase with thermolysin shows that the two His residues align perfectly with the catalytic Glu residue although in enkephalinase the coordinating residue Glu-646 is located at a greater distance than the equivalent Glu-166 in thermolysin. This is in accord with the greater size of enkephalinase (749 amino acid residues) compared with thermolysin (316 amino acid residues) [77]. These studies suggest that the geometry of the zinc-binding site and catalytic mechanisms of enkephalinase and thermolysin are very similar. The hypothetical model of the active site of enkephalinase proposed by Roques *et al.* [74] (*Figure 6.4*) assumes the enzyme to be a metalloenzyme with zinc at its active site where the S_1' site accommodates the Phe^4 aromatic ring of the substrate and the S_2' site accepts either the terminal methionine or leucine. Other proposed sites of interaction between the active site and substrate include a positively charged arginine residue to bind with the *C*-terminal ionized carboxyl and a hydrogen donor group to bind the terminal amide (peptide) linkage. The Gly^3–Phe^4 peptide bond of the substrate is considered to be cleaved via the addition of water.

The optimal amino acid requirements for the S_1' and S_2' sites, zinc-complexing ligands of varying affinity together with the importance of the other binding sites have been determined [78,79] using enkephalinase

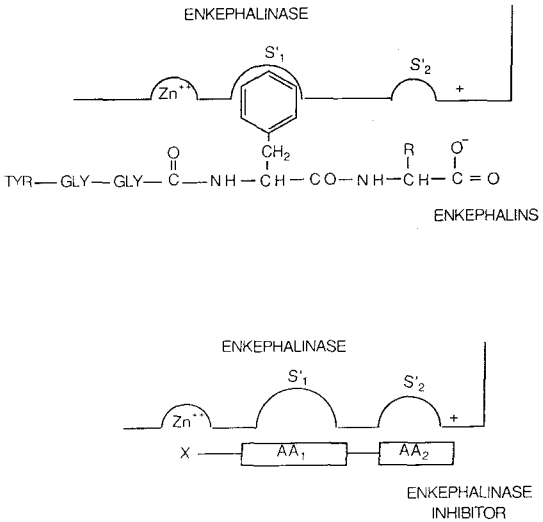


Figure 6.4. Hypothetical model of the active site of enkephalinase (EC 3.4.24.11) [74].

inhibitors with a general structure X–AA_1–AA_2 where X is the zinc complexing ligand and AA_1 and AA_2 are different amino acid types of structure corresponding to P_1' and P_2' positions, respectively (see *Figure 6.4*) [33].

S_1' Subsite. Enkephalinase inhibition is maximized when the C-2 side-chain of AA_1 is hydrophobic, optimal activity being observed for benzyl, although cyclohexyl and biphenyl moieties are also acceptable, indicating that the S_1' subsite of enkephalinase can accommodate large groups [33,79,80]. Introduction of a methyl-, methoxy- or amino-substituent on the phenyl ring does not affect inhibitory potency. In contrast, nitro- and dimethylamino-substituents reduce potency. The position of the substituent is also important since *p*-nitro substitution leads to a 100-fold reduction in potency whereas *o*-nitro derivatives only show an 18-fold decrease [81,82].

The presence of a C-2 substituent is essential for good enkephalinase inhibition and is more important than the absolute configuration of the compound. However, the (*S*)-isomer exhibits greater potency as an enkephalinase inhibitor [79,81] although the (*R*)- and (*S*)-isomers interact with the enzyme with the same order of potency. This indicates flexibility within the region of the active site containing the S_1' subsite and zinc.

S_2' Subsite. The general requirements for the S_2' subsite in enkephalinase do not appear to be stringent as determined by Llorens *et al.* [78] using the dipeptides Phe–Y or Tyr–Y. Compounds without a side-chain (Phe–Gly, Tyr–Gly) or substitution on the alpha carbon with a methyl group (Phe–Ala, Tyr–Ala), an aromatic ring or a large hydrophobic residue all showed good inhibitory activity. The (*S*)-isomer is the preferred isomer at this subsite. Inhibitory potency was reduced by 100-fold for charged amino acids such as Phe–Asp, Tyr–Arg, Tyr–Lys. Enhancement of enkephalinase inhibitory activity occurs when AA_2 is β-alanine or γ-aminobutyric acid whereas a loss of activity occurs when it is proline [80]. This is in contrast to requirements for the S_2' subsite of ACE where L-proline derivatives are the most potent inhibitors [79,83].

C-Terminal carboxylic acid. The presence of a free terminal carboxylate in the P_2' amino acid residue in a substrate increases the interaction with enkephalinase. Esterification or amidification of the *C*-terminal group of $[Met]^5$-enkephalin leads to a 6- and 30-fold decrease in binding [78], respectively. Replacement by a hydroxymethyl also leads to a drastic loss of activity [73,79]. This feature is also apparent in enkephalinase inhibitors [79,84] although the differences are smaller (*Table 6.3*).

This interaction is considered to be due to the ionized carboxyl group and the protonated Arg-102 present at the active site. On this basis, inhibitors

Table 6.3. IMPORTANCE OF A FREE TERMINAL CARBOXYLIC ACID GROUP ON THE INHIBITORY POTENCY OF ENKEPHALINASE

$$\begin{array}{c} CH_2Ph \\ | \\ HSCH_2CONHCHRX \\ * \end{array}$$

R	*X*	*	*Enk* IC_{50} (μM)	*Ref.*
$-CH_2CHMe_2$	CO_2H (L)		0.019	79
$-CH_2CHMe_2$	CH_2OH (L)		145	79
$-CH_2CHMe_2$	$CONH_2$ (L)	A	0.049	79
		B	0.640	
H	CO_2H		0.004	84
H	COOMe		0.017	84
H	$CONH_2$		0.020	84
H	$CONHCH_2Ar$		0.030	84

*A and B indicate stereoisomers whose configurations are unknown.

containing a sulpho instead of a carboxyl group on the *C*-terminal amino acid residue have been designed (see *Table 6.4*) [81]. They show greater potency and selectivity for enkephalinase in comparison to ACE, the *m*-aminobenzenesulphonic derivative exhibiting the highest inhibitory potency and selectivity. Phenolic derivatives have also shown fairly good inhibitory activity [85], however, potency is enhanced in compounds containing cycloleucine in the P_1 position [86].

Table 6.4. EFFECT OF SUBSTITUTION OF CARBOXYL BY SULPHONIC ACID GROUP ON INHIBITORY POTENCY OF THIORPHAN ANALOGUES FOR ENKEPHALINASE [81]

$$\begin{array}{c} CH_2Ph \\ | \\ HSCH_2CHCONHR^3 \end{array}$$

R^3	IC_{50} (*nM*)
CH_2CO_2H	2.6
CH_2SO_3H	2.2
C_6H_4-3-SO_3H	0.27
C_6H_4-4-SO_3H	1.7
C_6H_4-2-SO_3H	140

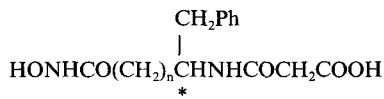
Zinc-complexing ligand. Amino acids or short peptides containing terminal zinc liganding groups such as thiol, carboxyl, phosphoramidate or hydroxamate are potent inhibitors of enkephalinase [33,73]. The structures of the inhibitor–zinc complexes formed have been determined indirectly by X-ray crystallography using inhibitors of the related enzyme thermolysin [87,88]. The structure of a complex of thermolysin and the inhibitor 2-benzyl-3-mercaptopropanoyl-L-alanylglycinamide has been used as a model for thiol-inhibitor-zinc complexes of other metalloenzymes. The structure of the complex shows that the thiol inhibitor binds to thermolysin with sulphur (presumably in an anionic form) tetrahedrally coordinated to zinc with displacement of a water molecule bound to the enzyme. Tetrahedral coordination to the zinc has also been observed [87] with the carboxylate group and phosphinyl group of the inhibitors L-benzylsuccinic acid and phosphoramidon, respectively. The structure of the thermolysin–hydroxamic acid inhibitor complex differs from the previously observed complexes in that the hydroxamate moiety forms a bidentate complex with the zinc through the carbonyl oxygen and the hydroxyl group, so that a penta-coordinate complex is formed between three ligands from the protein and two from the hydroxamate group.

The position of the zinc-binding group in inhibitors seems critical from studies on the hydroxamic acids, the optimal potency being obtained in compounds in which the zinc-binding ligand is separated by a single carbon from the chiral centre of the AA_1 residue (see *Table 6.5*) [73,89].

Influence of the peptide bond between AA_1 and AA_2 residues. *N*-methylation of the peptide link in the dipeptides Phe–Gly, Phe–Ala or Phe–Leu, leads to a 100-fold reduction in inhibitory activity indicating that the amide group is hydrogen bonded to the active site of the enzyme [78]. Support for this now comes from the observation that a retro-peptide bond in Phe–Ala reduces inhibitory activity 10-fold. Whereas there is only a modest loss of inhibitory activity in (*R*)-thiorphan (thiol analogue of the dipeptide Tyr–Gly), there is a 100-fold loss in activity in the (*S*)-isomer of retrothiorphan (see *Table 6.6*) [90].

Both (*R*) and (*S*) isomers of thiorphan exhibit a high affinity for enkephalinase. This indicates that the major interactions between the enzyme and the inhibitor complex (thiol group, phenyl ring and the H-bond involving the amide group) are satisfied by the stereochemistry of both isomers. The inversion of the amide bond, as in retrothiorphan, requires a conformation of the inhibitor, such that only the (*R*) isomer is able to interact satisfactorily to form the enzyme–inhibitor complex, the inhibitory potency of the (*S*) isomer being reduced nearly a 100-fold. It appears that

Table 6.5. IMPORTANCE OF THE POSITION OF THE ZINC-BINDING LIGAND, RELATIVE TO THE CHIRAL CENTRE AND ITS EFFECT ON ENKEPHALINASE INHIBITORY ACTIVITY [89]

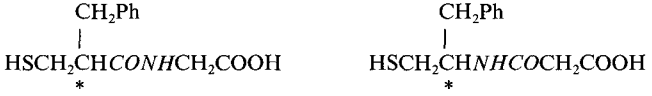


n	*	*Enk* IC_{50} *(nM)*
0	R	3350 ± 500
0	S	2500 ± 250
1	R	0.5 ± 0.05
1	S	2.5 ± 0.5

(*R*)-retrothiorphan is able to adopt a conformation which resembles that of either (*R*)- or (*S*)-thiorphan.

Retro-inversion of hydroxamate enkephalinase inhibitors [89] leads to a 5-fold difference in potency between the (*R*) and (*S*) isomer compared with the 100-fold difference obtained with retrothiorphan. This difference between the thiol and hydroxamate inhibitors may be explained in terms of the geometrical parameters existing in their inhibitor–zinc complexes since in the thermolysin–thiol inhibitor complex, the zinc is tetra-coordinated [87], whereas in the thermolysin–hydroxamic acid inhibitor complex it is penta-coordinated [88]. The conformational space accessible to the thiol function is less than that accessible to the hydroxamate function, as there is only one degree of freedom in retrothiorphan (a rotation around the alpha

Table 6.6. EFFECT OF RETRO-INVERSION ON THE ENKEPHALINASE INHIBITORY ACTIVITY OF THIORPHAN AND RETROTHIORPHAN [90]



*	*Enk* IC_{50} *(nM)*	*	*Enk* IC_{50} *(nM)*
R	1.60 ± 0.4	*R*	2.30 ± 0.7
S	1.90 ± 0.4	*S*	210.0 ± 10

carbon–CH_2 bond) whereas there are two degrees of freedom in hydroxamic acid inhibitors (rotation around the alpha carbon–CH_2 and CH_2–CO bonds). In this context the hydroxamate group of (*S*)-beta-amino acid can ligand almost as strongly as that of the (*R*)-beta-amino acid [89].

Enkephalinase inhibitors

Enkephalinase inhibitors have been developed from early studies which utilized simple dipeptides as probes for identifying the structural requirements of the active site of the enzyme [78] together with information evolved from the development of ACE inhibitors. The different enkephalinase inhibitors have been classified according to the zinc binding ligand in the inhibitor molecule namely: sulphydryl, carboxyalkyl and phosphoryl [33,71,91] (see *Figure 6.5*).

Sulphydryl-based inibitors. Development of the first potent enkephalinase inhibitor, thiorphan (see *Figure 6.5B*), was based on the use of the sulphydryl group as the zinc-binding ligand inserted into dipeptides shown to be optimal for binding with the active site of enkephalinase [74]. This established the sulphydryl group as an efficient zinc complexing ligand for enkephalinase. However, although thiorphan showed potent inhibitory activity for enkephalinase (K_i = 4.7 nM), it was also found to be a relatively efficient inhibitor of ACE (K_i = 150 nM) [74] as was also found for other sulphydryl containing dipeptide inhibitors [79].

Retrothiorphan, developed to improve selectivity towards enkephalinase [90], inhibits enkephalinase with K_i 6–10 nM but does not inhibit ACE at a concentration of 10 μM. Substitution of glycine by aminoheptanoic acid in thiorphan (SQ 29072) resulted in increased selectivity for enkephalinase (IC_{50} 26 nM) with reduced potency [71,72].

Carboxyalkyl-based inhibitors. A series of carboxyalkyl enkephalinase inhibitors derived from the dipeptide Phe–Leu have been developed (see *figure 6.5A*) [92]. Substitution of the sulphydryl group of thiorphan by a carboxyl group gave an enkephalinase inhibitor which also exhibited ACE-inhibitory activity but shortening of the alkyl chain by a methylene group gave a selective enkephalinase inhibitor (IC_{50} 0.7 μM) which did not possess appreciable ACE-inhibitory activity (IC_{50} >1000 μM).

N-Carboxyalkyl-based enkephalinase inhibitors were developed [80,93] from concepts similar to those used in the development of *N*-carboxyalkyl ACE inhibitors [94]. They found compounds with two aromatic amino acid residues, occupying the S_1 and S_1' subsites, to be more effective enkephalinase inhibitors [80,93]. In these compounds while small aliphatic amino

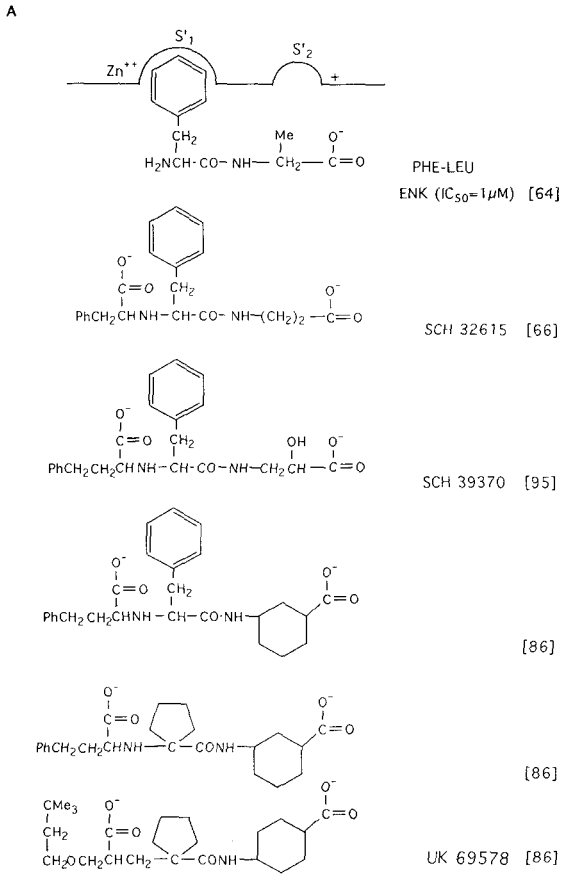
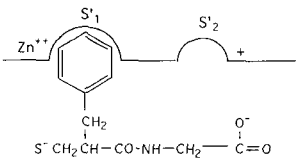


Figure 6.5. Diagramatic representation of enkephalinase inhibitors, classified according to their zinc-binding ligand and represented as interacting with the proposed binding sites of enkephalinase. A, carboxyalkyl; B, sulphydryl; C, phosphoryl.

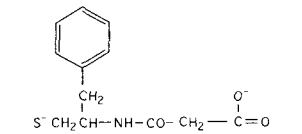
acids in AA_2 were effective for binding to the S_2' subsite, activity was enhanced considerably by the presence of a β-alanine or γ-aminobutyric acid (GABA) in this position. These compounds exhibited approximately a 5000-fold selectivity for enkephalinase compared with ACE and a 500-fold selectivity compared with thermolysin as determined from their inhibitory potency ratios, respectively. It was proposed that the *N*-carboxyalkyl group served to bind the zinc and that the β-alanine residue was a critical

B

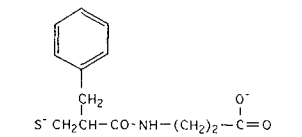


THIORPHAN

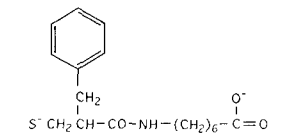
ENK(IC_{50}=2nM) [74]



RETROTHIORPHAN [90]



S 228603 [129]



S 229072 [70]

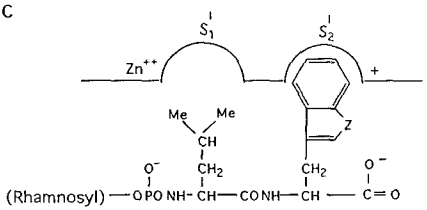
Figure 6.5. (cont.)

component in determining selectivity for enkephalinase, since significant ACE inhibitory activity was observed when alanine was present.

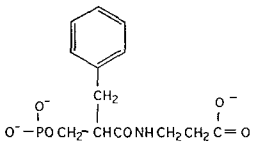
Carboxyalkyl dipeptide inhibitors such as SCH 39370 were developed to investigate the atrial natriuretic peptide (ANP)-potentiating effects of enkephalinase inhibitors [95]. Recently, more conformationally constrained molecules of AA_2 based on GABA have been developed (see *Figure 6.5A*) [86]. The *cis*-3-aminocyclohexane carboxylic acid analogue ($K_i = 2.6 \times 10^{-7}$ M) gives good inhibitory potency which is enhanced by replacement of the P_1 residue, phenylalanine, with cycloleucine ($K_i = 1.5 \times 10^{-7}$ M). UK 69578 ($K_i = 2.8 \times 10^{-8}$ M) was the most potent compound of the series with the (+)-enantiomer ($K_i = 1.4 \times 10^{-8}$) showing 30-fold increase over the corresponding (−)-enantiomer ($K_i = 4.7 \times 10^{-7}$).

Phosphoryl-based inhibitors. These were designed on the proposed similarity between thermolysin and enkephalinase. The thermolysin inhibitor phosphoramidon is a potent enkephalinase inhibitor but is non-specific

C



PHOSPHORAMIDON
ENK(IC_{50}=2nM)



PHOSPHONIC ACID DERIVATIVE
ENK(IC_{50}=20nM)

Figure 6.5. (cont.)

since it inhibits both enkephalinase and thermolysin. Other phosphorus-containing complexing ligands in a peptide skeleton were examined by Altstein *et al.* [96] in an attempt to develop selective enkephalinase inhibitors; the most potent compound described, phosphoryl-Leu-Phe, was an effective enkephalinase inhibitor (IC_{50} = 1 μM). A phosphonic acid dipeptide enkephalinase inhibitor (*Figure 6.5C*), containing a β-alanine residue, has been developed [33] which is a selective enkephalinase inhibitor (IC_{50} = 20 nM) with virtually no ACE inhibitory activity (IC_{50} > 100 μM).

ANGIOTENSIN CONVERTING ENZYME (EC 3.4.15.1)

Angiotensin converting enzyme cleaves a dipeptide from the decapeptide angiotensin I from the carboxyl terminal to form the octapeptide angiotensin II and also hydrolyses the nonapeptide bradykinin. It has been detected in the central nervous system [97] and purified ACE is capable of hydrolysing the enkephalins at the Gly^3–Phe^4 amide bond [98]. Early reports of the ACE inhibitor captopril potentiating the effects of enkephalins *in vitro* [99] and potentiation of morphine analgesia *in vivo* [100] suggested an involvement of ACE in the *in vivo* metabolism of the enkephalins. However,

different distribution and *in vitro* substrate specificity of ACE and enkephalinase [101], together with a report [102] suggesting that the *in vitro* effects of captopril are due to a non-specific anti-oxidant effect (arising from the sulphydryl group in captopril protecting $[Met]^5$-enkephalin from conversion to a less active metabolite), indicate that it is unlikely that ACE plays a role in enkephalin metabolism in vivo [103]. However, it is important to consider the structural requirements of the active site of the enzyme to elucidate the differences between ACE and enkephalinase in the design of specific enkephalinase inhibitors.

A model of the active site of ACE, based on the similarity between ACE and carboxypeptidase A has been proposed (*Figure 6.6*) [83]. The active site consists of a positively charged residue which binds the negatively charged *C*-terminal carboxyl group of the substrate, a hydrogen bonding site which binds to the terminal non-scissile peptide bond (C=O) and a Zn^{2+} moiety

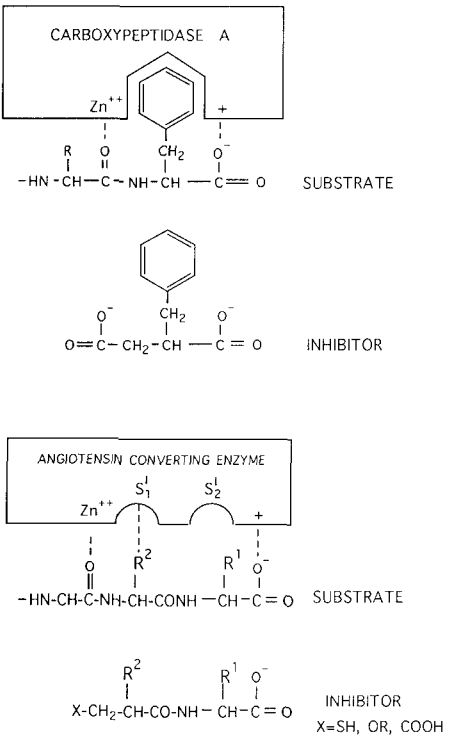


Figure 6.6. Diagramatic model of the active site of carboxypeptidase A and hypothetical active site of ACE proposed by Cushman et al. [83].

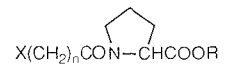
which forms a complex with the scissile amide bond of the peptide substrate. It was assumed that as ACE did not show specificity for *C*-terminal hydrophobic amino acids, its active site was unlikely to have a hydrophobic pocket corresponding to that of carboxypeptidase A. Two sites, having affinity for the R^1 and R^2 side-chains of the two terminal amino acid residues of the substrate are included in the model and its general validity has since been confirmed by structure–activity studies [83,79,104,105].

Importance of the R^1 substituent. Studies using succinyl derivatives possessing different *C*-terminal amino acids. It was shown that succinyl-L-proline was the most potent inhibitor [83]. Other studies have indicated that *C*-terminal aromatic amino acid and leucine residues are also acceptable, but are less efficient than the proline residue for ACE inhibition [79]. It has been suggested that the superiority of L-proline is due to its rigid structure which may lock the carboxyl group into a favourable conformation for interaction with the positively charged residue at the active site of the enzyme.

Zinc binding ligand. Replacement of the carboxyl group of succinyL-L-proline by a sulphydryl function. This produced an inhibitor which was 1650-fold more potent than succinyL-L-proline (see *Table 6.7*). Efficient binding of substrate to the active site requires an optimum acyl chain length of n = 2 (*Table 6.7*) which allows for the most favourable alignment of the zinc-complexing ligand without interfering with the binding of the amide carbonyl and the proline carboxyl group at their respective binding sites [81,85,106].

R^2 substituents. The presence of a methyl substituent at R^2 (see *Figure 6.6*) increases the inhibitory potency of D-succinyL-L-proline [83]. The benzyl

Table 6.7. EFFECTS OF THE ZINC BINDING LIGAND AND THE ACYL CHAIN LENGTH ON THE INHIBITORY POTENCY OF SOME ACE INHIBITORS [83]



X	*n*	*ACE IC_{50} (μM)*
COOH	2	330
SH	2	0.20
SH	1	1.1
SH	3	9.7

group is also a suitable R^2 substituent, but replacement of the phenyl ring by cyclohexyl results in loss of potency [105].

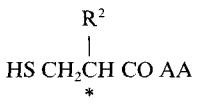
Hydrophobic interaction of C-2 substituents may contribute to increased binding, however, the main contribution to increased potency is likely to be due to the restriction in conformation introduced at the chiral centre [83]. It has been reported [106] that an R^2 substituent such as methyl is not required for binding, but its presence adds to inhibitory potency. Comparison of inhibitory potencies of perindoprilat, enalaprilat and captopril (*Figure 6.7*) with their respective C-9 demethylated derivatives [107] has shown this to be true for inhibitors with proline (a relatively small ring) at the *C*-terminal of the molecule. In compounds such as perindoprilat, the larger perhydroindole group appears to be sufficient to fill the S_1'–S_2' pockets and give the right orientation to the remaining part of the molecule for interaction with the enzyme. Methyl at the C-9 position does not add to inhibitory potency.

In thiol inhibitors with R^2 substituents, the (*S*)-isomer shows higher inhibitory potency than the (*R*)-isomer [105] (*Table 6.8*). Perindoprilat (IC_{50} (*S*) = 1.5 nM, (*R*) = 1100 nM), enalaprilat (IC_{50} (*S*) = 1.2 nM, (*R*) = 2500 nM) and captopril (IC_{50} (*S*) = 23 nM, (*R*) = 1.5×10^6 nM) show a similar preference for the (*R*)-isomer [107].

C-Terminal carboxyl group. Esters of 3-mercaptopropanoyl-L-proline are 100–200 fold less potent than their carboxylic acid derivatives (see *Table 6.9*), indicating enhanced binding by a free *C*-terminal carboxyl group.

Importance of the amide bond. The results show that *N*-methylation of mercaptoalkanoyl inhibitors [83] does not significantly decrease inhibitory potency, indicating that hydrogen bonding does not play a crucial role in the

Table 6.8. EFFECTS OF R^2 SUBSTITUTION AND THE STEREOCHEMICAL CONFIGURATION OF C-2 ON ACE INHIBITORY ACTIVITY [79]



AA	R^2	*	*ACE* IC_{50} (μM)
Pro	H	-	0.2
Pro	Me	*D*	0.023
Pro	Me	*L*	2.4
Leu	CH_2Ph	*S*	0.037
Leu	CH_2Ph	*R*	0.048

binding of the substrate [105]. These data are in agreement with the good affinity exhibited by ACE inhibitors bearing proline. Furthermore, analogues which either lack the amide bond or in which the amide group is shifted have a significantly reduced inhibitory potency compared to the parent compound [83] indicating that the presence of an amide bond in the correct position is critical for the binding of substrate.

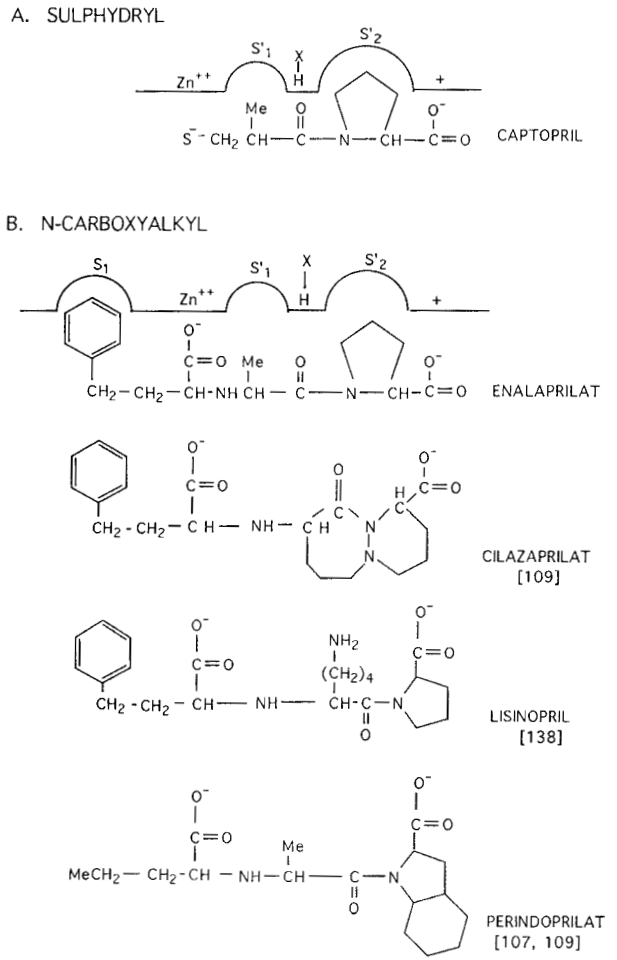
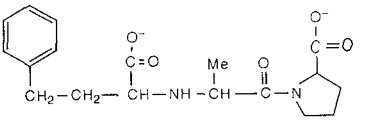


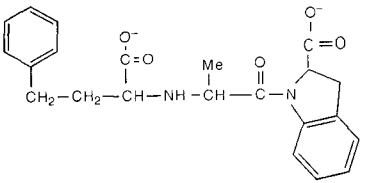
Figure 6.7. Diagramatic representation of ACE inhibitors classified according to their zinc-binding ligand and represented as interacting with proposed active binding sites of ACE.

ACE inhibitors

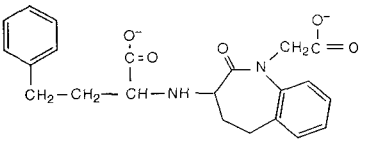
Captopril was the first orally active, clinically useful, potent ACE inhibitor which was designed on the basis of the predicted similarity between the active sites of ACE and carboxypeptidase [83]. Investigation of alternative zinc-binding functions to replace the thiol group with optimization of the orientations of the *C*-terminal carboxyl and amide carbonyl for binding to the enzyme led to the development of a new class of thiol-free ACE inhibitors such as enalaprilat, with enhanced activity and binding specificity (see *Figure 6.7*) [94,108]. Introduction of bulky hydrophobic groups at the *C*-terminal to further enhance the enzyme-inhibitor interaction have led to the development of cilazaprilat and benazeprilat [109,110] (see *Figure 6.7*).



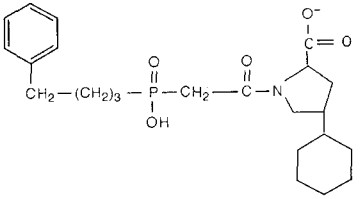
RAMIPRILAT
[107, 109]



QUINAPRILAT
[109]



BENAZEPRILAT
[110]



FOSINOPRILAT
[111]

Figure 6.7 (cont.)

Fosinoprilat [111] (see *Figure 6.7*) differs from other ACE inhibitors in that it has a compensatory dual route of elimination via the liver and kidneys, and may be of benefit in patients with diminished hepatic or renal function. The rank order of ACE inhibitory potency *in vitro* has been identified as quinaprilat = benazeprilat > perindoprilat > lisinopril > enalaprilat > fosinoprilat [111].

With the exception of captopril and lisinopril, ACE inhibitors are not well absorbed orally and are administered as esters (prodrugs enalapril, cilazapril, etc.) which undergo hydrolysis *in vivo* to release the active drugs [110].

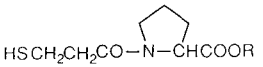
DIPEPTIDYLAMINOPEPTIDASE (DAP; EC 3.4.14.4)

Dipeptidylaminopeptidase, which produces the dipeptide Tyr–Gly on hydrolysis of enkephalins, was first reported to be present in rat brain [112]. The enkephalin-hydrolysing aminopeptidase and DAP activity in monkey brain was separated by Hazato *et al.* [113] who showed that DAP activity was unaffected by either bestatin or puromycin but was inhibited in the presence of metal chelators. This indicated that DAP was a metalloenzyme. DAP was originally reported to be a membrane-bound enzyme since it was found in the membrane fraction of rat brain homogenate [112], but further studies with the enzyme isolated from calf-brain striatum showed that DAP is not membrane-bound, but was present in the membrane fractions due to occlusion in vesicle-like structures [65].

The affinity of DAP for $[Leu]^5$-enkephalin was found to be comparable to that of enkephalinase (EC 3.4.24.11). However, the ability of the two enzymes to react with inhibitors such as thiorphan (enkephalinase inhibitor) differed considerably [65,66]. The inhibitory potency of di-, tri- and tetra-peptides was investigated [114] so as to characterize the active site of DAP. Generally, a decrease in the length of the peptide resulted in a reduction in affinity for the enzyme. However, affinity of the pentapeptide substrate, appears to lie largely in the two carboxyl-terminal amino acids, since removal of the Tyr–Gly–Gly moiety only leads to a 3-fold decrease in affinity for the remaining dipeptide. The highest inhibitory activity was found in dipeptides containing bulky side-chains on both amino acid residues although dipeptides with charged side-chains also have inhibitory activity with the negatively charged Glu-residue dipeptides being more effective than positively charged Arg-residue.

On the basis of these data a model of the active site of DAP has been proposed (see *Figure 6.8*). In this model, the active site consists of two hydrophobic pockets large enough to incorporate the phenylalanine or

Table 6.9. EFFECTS OF ESTERIFICATION OF THE *C*-TERMINAL CARBOXYL ON ACE INHIBITORY ACTIVITY [83]



R	*ACE* IC_{50} *(μM)*
H	0.2
Et	17.0
CMe_3	30.0

leucine side-chains, a positively charged group to bind the ionized carboxyl group of the substrate and a catalytic site containing Zn^{2+}. Evidence for the hydrophobic character of the S_1' and S_2' subsites was obtained from the observation that X–Gly dipeptides were less active as inhibitors than X–Ala and X–Val and a similar pattern was given by the dipeptides Gly–X, Ala–X and Val–X. In other words, the glycine residue was too small to interact with either of the hydrophobic subsites. The stereospecific requirements of the S_1' and S_2' subsites do not appear to be stringent although the L,L-dipeptide was preferred. The presence of a free carboxyl group at the *C*-terminal appears to be essential for effective inhibition since amidification or esterification resulted in a loss of inhibitory activity for example

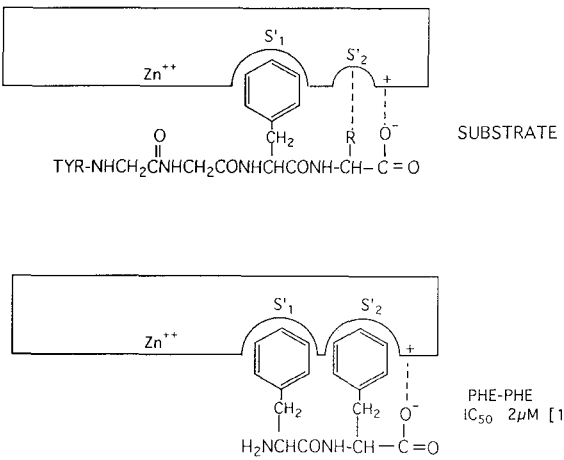


Figure 6.8. Schematic representation of interactions between the hypothetical model of dipeptidylaminopeptidase active site and substrate or inhibitor.

Phe–Phe, IC_{50} = 2 μM; Phe–Phe–NH_2, IC_{50} = 60 μM; Phe–Phe–O–t But, IC_{50} = 95 μM.

The dipeptide Phe–Phe is a selective inhibitor of DAP [114] and has been used as a basis for the development of more potent combined inhibitors of enkephalin-degrading enzymes [36].

COMBINED INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES

Hydroxamic acid derivatives have been reported to inhibit thermolysin, enkephalinase and enkephalin-degrading aminopeptidases. Benzyloxycarbonyl amino acid hydroxamates (*Figure 6.9A*) were reported to be potent inhibitors of both enkephalinase and soluble enkephalin-degrading aminopeptidase. Other hydroxamic acid derivatives also act as inhibitors of enkephalinase and membrane-bound enkephalin degrading aminopeptidase [68] (*Figure 6.9B*).

The first combined inhibitor of enkephalinase, DAP and aminopeptidase

		IC_{50} (nM)				
		ENK	AP	APM	DAP	Ref.
A	$HONHCOCH(CH_2Ph)NHCOOCH_2Ph$	200	1500[a]	—	—	[115]
B	$HONHCOCH(CH_2CHMe_2)CONHCH(Me)CONHCH_2CONH_2$	3.0	950	—	—	[68]
C	$HONHCOCH_2CH(CH_2Ph)CONHCH(Me)COOH$ (R) (S) Kelatorphan	1.7	—	350	0.9	[38]
D	$HONHCOCH_2CH(CH_2Ph)CONHCH(CH_2Ph)COOH$	3.0	—	22.5	0.46	[116]
E	$HSCH_2CONHCH(CH_2Ph)CONHCH(CH_2Ph)COOH$ Phelorphan	20	13000[b]	—	350	[36]

Figure 6.9. Combined inhibitors of enkephalin degrading enzymes. ENK (enkephalinase). AP (aminopeptidases), APM, (aminopeptidase M), DAP (dipeptidylaminopeptidase). D,R,S (stereochemical configuration), [a](membrane-bound aminopeptidase). [b](soluble aminopeptidase).

M, kelatorphan (*Figure 6.9C*) [35] was based on a modified Phe–Ala dipeptide and exhibited an IC_{50} in the nanomolar range for enkephalinase and DAP and in the micromolar range for aminopeptidase M [89]. In order to improve the affinity of kelatorphan for aminopeptidase M without affecting its activity for enkephalinase and DAP, different modifications were introduced into the *C*-terminal moiety [116]. Addition of a β-alanine residue in the P_2' position did not significantly affect the potency for enkephalinase or DAP. However, progressive lengthening of the chain decreased inhibitory activity for the two enzymes, with a marked effect on enkephalinase inhibitory potency. The effect of increasing the chain-length on aminopeptidase M inhibitory potency differed in that the potency increased for β-alanine (2 methylene spacer) or β-aminovaleric acid (a 4 methylene spacer), but remained unaffected when the P_2' residue contained 3 methylene groups. *N*-Methylation of the amide bond of a kelatorphan analogue (*Figure 6.9D*) significantly decreased inhibitory potency for enkephalinase (IC_{50} = 150 nM) but did not influence the potency for DAP (IC_{50} = 2.3 nM) or aminopeptidase M (IC_{50} = 120 nM) [116]. The inhibitory potency for enkephalinase was unaffected by the absolute configuration of the *C*-terminal-substituted β-amino acid but this appeared to modulate affinity for both aminopeptidase M and DAP. A preference for a benzyl group rather than a methyl group, in the beta-position of the β-alanine residue was seen for inhibition of both aminopeptidase M and DAP (see *Figure 6.9D*).

A different approach [36], based on a model of the active site of DAP and the dipeptide inhibitor Phe–Phe, was used in the development of the inhibitor phelorphan (*Figure 6.9E*). This is a potent inhibitor of enkephalinase, with a moderate effect on DAP and a weak effect on soluble enkephalin-hydrolysing aminopeptidase (AP). The distance between the amino acid side-chain interacting with the S_1' subsite and the zinc binding ligand is critical for inhibition of DAP and enkephalinase; increasing this distance in phelorphan by a single carbon dramatically decreased the inhibitory potency for the two enzymes, whereas affinity for aminopeptidase remained unaffected. The importance of the interaction of the inhibitor with both S_1' and S_2' subsites for effective inhibition of DAP was indicated by the inhibitory profile of the phelorphan derivative XAMS0 1999, where glycine is the P_2' residue. XAMS0 1999 inhibits enkephalinase with a potency comparable to that of phelorphan but does not inhibit DAP or aminopeptidase M.

Retrokelatorphan is equipotent with kelatorphan towards enkephalinase, but is significantly less potent towards DAP and aminopeptidase M (*Figure 6.10A*) [89]. Extension of the malonyl function in the P_2' position to give a

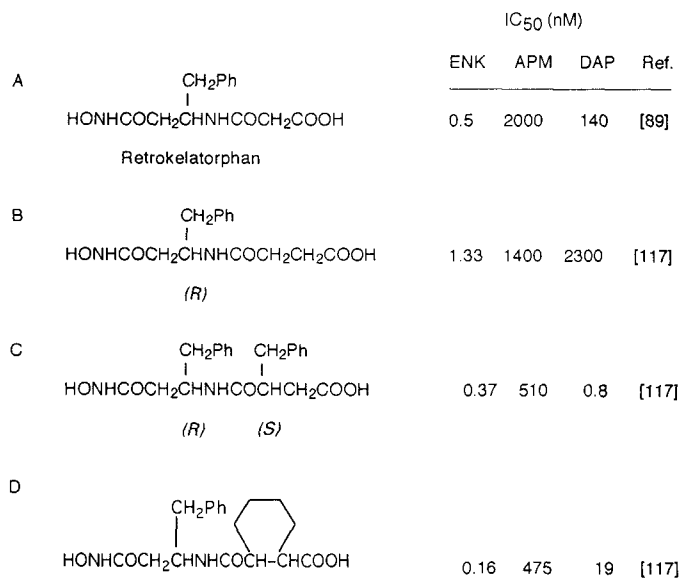


Figure 6.10. Inhibitory potencies of retrokelatorphan and its derivatives. ENK (enkephalinase), APM (aminopeptidase M), DAP (dipeptidylaminopeptidase).

succinyl function did not improve inhibitory potency towards any of the three enzymes [117] (*Figure 6.10B*). However, substitution of a benzyl group in the alpha position relative to the retroamide bond (*Figure 6.10C*) gave significant improvement in potency towards DAP, and to a lesser extent aminopeptidase M. A similar improvement in inhibitory potency was obtained by bridging the 2-carbon chain of the terminal amino acid with a cyclohexyl group (*Figure 6.10*D).

Some 2-substituted-(2′-aminophenyl)-4-thioxohydantoic acids (*o*-amino-PTC-amino acids) are weak inhibitors of puromycin-insensitive, bestatin-sensitive aminopeptidase (possibly aminopeptidase M). The compounds exhibited antinociceptive activity when administered by the intracerebroventricular (icv) route alone (IC_{50} = 0.04–0.87 μM per animal) and showed a striking prolongation of the antinociceptive action of (D-Ala^2-D-Leu^5)-enkephalin (DADL) when co-administered (icv) [118]. The most potent compound, *o*-amino-PTC-proline had a greater effect alone (icv) in the mouse-tail immersion test than thiorphan and also potentiated the action of DADL. In the mouse abdominal constriction test, when administered icv, the glycine, leucine and proline analogues had IC_{50} values of 120 μg, 252 μg

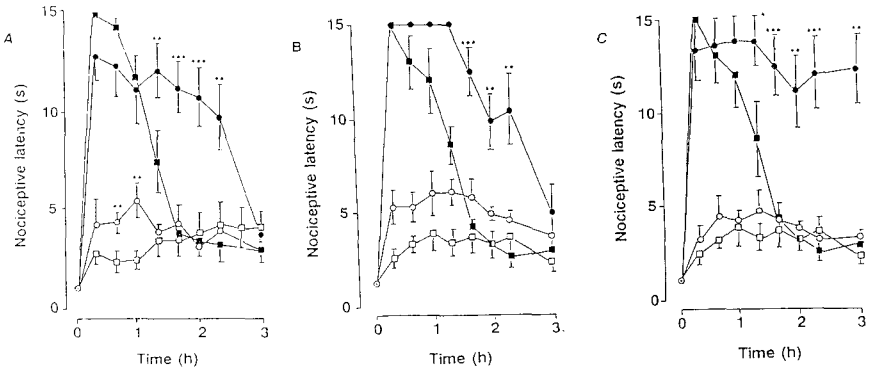
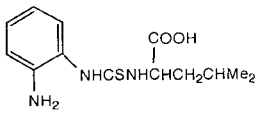


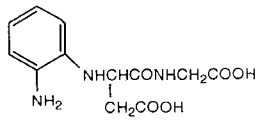
Figure 6.11. Effects of (A) ○-amino-PTC-glycine, (B) o-amino-PTC-leucine, (C) o-amino-PTC-proline on the analgesic activity of DADL in the mouse tail immersion test. -△--o-amino-PTC-glycine (100 μg, i.c.v.); ■-DADL (1 μg, i.c.v.); -●- combination of o-amino-PTC-glycine (100 μg, i.c.v.); and DADL (1 μg, i.c.v.); -□- -saline vehicle (10 μl, i.c.v.). (Reprinted with permission from Z. Yu et al. (1989) J. Enzyme Inhib. 3, 103–117.

and 11.4 μg per animal, respectively and all were more potent than thiorphan (265 μg per animal) (*Figure 6.11*).

The use of the *o*-amino-PTC-amino acid structure (1) in modelling studies using molecular graphics and superimposition on thiorphan as a reference structure, led to the design of 4-carboxymethylamino-4-oxo-3-(2′-amino-



(1)



(2)

Scheme 6.1

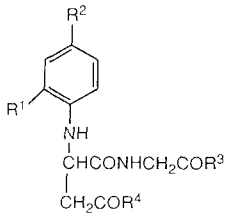
phenylamino)butanoic acid (2) as a potential inhibitor of enkephalinase and aminopeptidase..

Synthesis of (2) was unsuccessful but other aryl-substituted and -unsubstituted compounds were screened for enkephalinase and aminopeptidase inhibitory activity (see *Table 6.10*) [82].

4-Carboxymethylamino-4-oxo-3-(4′-aminophenylamino)butanoic acid (3) and the corresponding unsubstituted analogue (6) are fairly potent inhibitors of enkephalinase with weak inhibitory potency towards aminopeptidase M11. The compounds (4) and (5) represent the first combined inhibitors of enkephalinase and aminopeptidase M11, however, aminopeptidase M11 is not considered to be of primary importance in degradation of endogenous enkephalins [41].

A range of penicillins have been examined as competitive reversible inhibitors of enkephalinase [119,120]. Carfecillin (K_i = 0.18 μM) was the most potent inhibitor in the series, whereas cloxacillin (K_i = 27.5 μM), ampicillin (K_i = 41 μM), nafcillin (K_i = 59 μM) and carbenicillin (K_i = 158 μM) had moderate potency and benzylpenicillin (K_i = 885 μM), mezlocillin (K_i = 437 μM) and azlocillin (K_i = 556 μM) were weak inhibitors. Structure–activity relationships within the series have been rationalized

Table 6.10. *IN VITRO* ENKEPHALINASE (EC 3.4.24.11, ENK) AND AMINOPEPTIDASE MII (APMII) INHIBITORY ACTIVITY OF SOME BUTANDIOIC ACID DERIVATIVES [82].



					IC_{50} (μM)†	
COMPOUND	R^1	R^2	R^3	R^4	*Enk**	*APMII*
3	H	NH_2	OH	OH	0.243 ± 0.03	32 ± 13.1
4	H	NH_2	OEt	OH	0.140 ± 0.014	15 ± 1.61
5	H	H	OEt	OH	0.39 ± 0.015	65 ± 0.13
6	H	H	OH	OH	0.195 ± 0.002	75 ± 20

*Equivalent to K_i under the assay conditions

†Mean ± (S.E.M.) for three determinations carried out at 40 nM ^{3}H(Leu) enkephalin concentration.

from a consideration of molecular graphics analysis of the match between receptor binding groups with thiorphan as well as log P values.

PRODRUGS

Although several potent inhibitors of enkephalin-degrading enzyme inhibitors, such as thiorphan and kelatorphan, are known they are not orally active as antinociceptive agents and therefore are not suitable as potential clinical agents for analgesic use. Bioavailability seems to be the overriding factor in the production of an antinociceptive effect *in vivo*. Acetorphan [121] (see *Figure 6.12*), although 1000-fold less potent as an inhibitor than thiorphan *in vitro* becomes nearly as potent as thiorphan when pre-incubated with cerebral membranes due to conversion to thiorphan by hydrolysis of the thio- and benzyl-ester. Acetorphan is a parenterally-active inhibitor and penetrates the blood-brain barrier due to its lipophilicity and generates thiorphan in the brain (i.e. it is a prodrug of thiorphan). Similarly, SCH 34826 a lipophilic ester of the N-carboxyalkyl enkephalinase inhibitor SCH 32615 [93] (see *Figure 6.12*), is orally active due to ready absorption in the gastro-intestinal tract and hydrolysis in the brain to the active drug. The majority of enkephalin-degrading enzyme inhibitors under development as potential therapeutic agents are prodrugs.

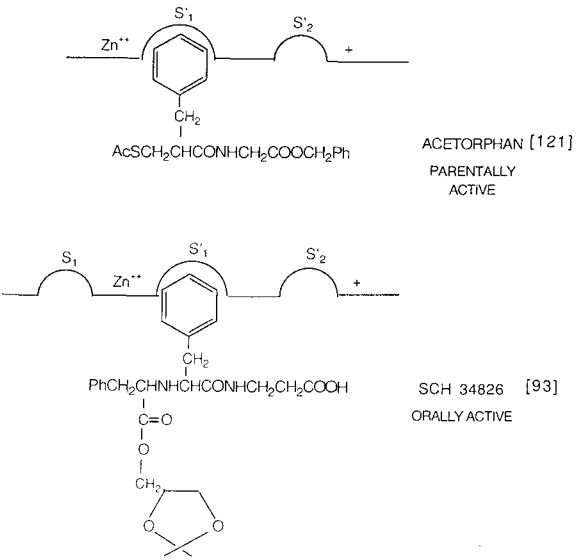


Figure 6.12. Prodrugs.

INHIBITORS AS POTENTIAL THERAPEUTIC AGENTS

The pharmacology of enkephalin-degrading enzyme inhibitors should parallel that of the enkephalins if inhibitors increase the level of endogenous enkephalins. However, other peptides and hormones such as atrial natriuretic peptide and Substance P are cleaved by enkephalinase (membrane metalloendopeptidase). Enkephalinase inhibitors may thus have a potential therapeutic role in areas which are unrelated to the actions of the enkephalins.

ANALGESIA

Antinociceptive effects of degradation-resistant enkephalin analogues, such as DADL, and enzyme inhibitors have been widely investigated. When enkephalin-degrading enzyme inhibitors are administered to an unperturbed animal, there is generally no observable effect [33]. This lack of reaction may be explained in terms of endogenous enkephalinergic tone since when the enkephalinergic system is inactive, there is no substrate (enkephalin) to potentiate and hence no effect to be observed. Nociceptive tests with enkephalin-degrading enzyme inhibitors are commonly carried out using the mouse abdominal constriction test and low temperature mouse hot-plate test. The mouse tail immersion test is generally not used as the majority of enkephalinase inhibitors including thiorphan [122] and SCH 34826 [93] have been reported to be inactive in this test. It has been suggested that under the conditions of this test, the enkephalin-containing endogenous pain suppression system is inactive hence the effects of enkephalinase inhibition are not detected. In contrast, in nociceptive tests where the noxious stimuli are prolonged, as in the mouse abdominal constriction and low temperature hot-plate tests, the endogenous opioid system is activated, thus enabling enkephalinase inhibitors to exhibit antinociceptive effects [33]. Antinoceptive tests have been classified into two groups [123] by determination of the antinociceptive activity of thiorphan and bestatin (when administered alone and in combination): the threshold dosages of morphine and the pronociceptive activity of naloxone, in a variety of classical screening tests. In the writhing, hot-plate jump and vocalization tests, both thiorphan and bestatin exhibited significant antinociceptive activity, particularly when administered together. This was naloxone- reversible and naloxone itself produced a significant pronociceptive effect when administered alone. The most sensitive tests for the analgesic effects of morphine were the writhing and hot-plate jump tests. In the tail withdrawal, hot-plate licking and tail flick tests, thiorphan and

bestatin were ineffective and naloxone failed to produce a pronociceptive effect. These tests were also less sensitive to the analgesic effects of morphine. This indicates that the analgesic effects of enkephalinase inhibitors may be mediated by neuronal pathways which are activated by triggering factors such as noxious stimuli or stress.

The majority of the *in vivo* effects of enkephalin-degrading enzyme inhibitors have been investigated either by co-administration with an enkephalin analogue or by introduction of an external stress factor to activate the endogenous enkephalinergic system.

Some enkephalin degrading enzyme inhibitors which exhibit potent enzyme inhibitory activity *in vitro*, are only active in nociceptive tests in vivo, when administered icv. A discrepancy exists, for some compounds, between enzyme inhibitory potency *in vitro* and antinociceptive effects observed *in vivo*. For example both the (*R*) and (*S*) enantiomers of thiorphan exhibit similar potency *in vitro*, yet the antinociceptive effect obtained in vivo is greater for the (*R*)-isomer than the (*S*)-isomer [124]. Similarly, the retrokelatorphan derivative (*Fig 6.10B*) [117], exhibits inhibitory potency for enkephalinase, DAP and aminopeptidase M, in a range similar to that of kelatorphan [125]. However, the antinociceptive effects of retrohydroxamates are significantly weaker than kelatorphan, an effect which cannot be explained in terms of metabolic stability as retro-inversion of an amide bond tends to protect it from hydrolysis.

4-Carboxymethylamino-4-oxo-3-(4′-aminophenylamino)butanoic acid (3), its ethyl ester (4) and corresponding unsubstituted-aryl analogues (6) and (5) (see *Table 6.10*) are fairly potent inhibitors of enkephalinase (K_i 0.14–0.39 μM) with inibitory potency (K_i 15–75 μM) towards aminopeptidase M11 [82]. In the mouse abdominal constriction test, the esters (4) and (5) showed systemic antinociceptive activity with ED_{50} values of 62 and 81 mg/kg, respectively. In the mouse tail immersion test, both (4) and (5) exhibited antinociceptive activity when administered icv. The results from the mouse abdominal constriction test for compounds (4) and (5) indicated the same rank order of potency as their *in vitro* inhibitory potency for enkephalinase and aminopeptidase M11. Another notable observation is that these compounds also exhibited the same rank order in their antinociceptive effects when administered icv alone in the mouse tail immersion test. This direct effect has not been reported for other more potent enkephalin-degrading enzyme inhibitors. Compound (4) uniquely exhibited antinociceptive activity when administered subcutaneously in the mouse tail immersion test, an effect which is only partially reversible by naltrexone. This result is in contrast to that for compound (5), which displayed only one third and one quarter of the potency of enkephalinase and aminopeptidase

M11 when compared to (4), yet was inactive when administered subcutaneously in this test.

These data raise several questions regarding the effect of these compounds on endogenous opioid-mediated analgesia. These compounds are less potent enkephalinase inhibitors than thiorphan [122]. Both (4) and (5) display antinociceptive activity when administered alone in the tail immersion test, in which it has been proposed that the endogenous enkephalinergic system is inactive or devoid of tone [33].

It is not possible from the data available to predict the minimum level of *in vitro* enkephalinase inhibitory potency required in an agent to produce satisfactory oral antinociceptive effect (assuming satisfactory absorption on oral administration, stability to first pass liver metabolism and good penetration of the blood-brain barrier). A value of K_i in the low nM region might not be essential for effective oral antinociceptive activity provided the bioavailability of the compound is good and a K_i value in the high nM region may well be adequate.

Chronic intermittent administration of acetorphan does not lead to development of withdrawal symptoms in mice either when the drug is withdrawn or when challenged with naloxone [121]. This offers an advantage for enkephalin-degrading enzyme inhibitors over narcotic analgesics. However, chronic cerebral infusion of thiorphan in rats induces tolerance to locomotor and antinociceptive effects of acetorphan indicating that intermittent administration of enkephalin-degrading enzyme inhibitors might be preferable [126].

ANTIDEPRESSANT EFFECT

The opioids may play a mediating role in the hypoalgesic and mood-elevating effects of antidepressants such as the tricyclics and iprindole. Evidence is based on the effects of enkephalin-degrading enzyme inhibitors in mice using experimental models for depression (forced swimming test, mouse despair test) [121,127]. Bestatin and thiorphan do not have any effect on immobility when administered alone but enhance effects of imipramine and iprindole which are also ineffective at low doses when given alone. These effects are naloxone-reversible. Acetorphan also elicits an antidepressant effect in the mouse despair test and affects turnover indices of 5-HT and noradrenaline [121]. These data suggest a possible role for enkephalin-degrading enzyme inhibitors as adjuncts to antidepressant drugs, which would allow lower doses of antidepressants to be used which in turn may lead to a reduction in unwanted effects such as sedation.

CARDIOVASCULAR EFFECTS

Atrial natriuretic peptide (ANP) has multiple central and peripheral actions which are opposite to those induced by angiotensin II and vasopressin [69,70,128].

Atrial natriuretic peptide (ANP) a cyclic 28-residue polypeptide hormone, produced mainly in the heart by myocytes as a precursor (proANP, 126-residues), stimulates diuresis, natriuresis, vasodilation and suppresses the renin–angiotensin–aldosterone system. It is released into the circulation by atrial distension due to plasma volume expansion and has a short duration of action due to inactivation by a clearance receptor ANPc, which is biologically inactive and is thought to internalize bound ANP for lysosomal degradation by enkephalinase (membrane metalloendopeptidase) degradation in the kidney. Enkephalinase (present in the brush border of the kidney) cleaves the Cys^{7}–Phe^{8} bond of the peptide, opening the cysteine linked ring which is important for biological activity and also cleaves the Ser^{25}–Phe^{26} bond [70,71,95,117].

The profile of ANP activity suggests a potential therapeutic role in the treatment of hypertension and congestive heart failure. However, the usefulness of ANP itself is limited by its rapid inactivation, and enkephalinase inhibitors have been assessed as potential therapeutic agents [70,71,86,129] by prolonging the action of ANP.

Evidence from animal models suggests that enkephalinase inhibitors have little ANP-like activity in normotensive rats, although the actions of exogenous ANP are prolonged. This inability to affect endogenous ANP levels may be due to low levels of ANP normally occurring in the circulation and the two distinct mechanisms for its deactivation, one of which involves enkephalinase. It has been suggested that at low concentrations ANP is preferentially deactivated by internalization via ANPc receptors while enzymic degradation by enkephalinase only occurs at higher concentrations [71].

The enkephalinase inhibitors, candoxatril (UK 79 300), the indanyl ester (pro-drug) of the (+)-isomer of candoxatrilat (UK 69 578) (see *Figure 6.5*) [130,131] and sinorphan ((*S*)-acetorphan) (see *Figure 6.12*) [132] are undergoing evaluation in humans for hypertension and congestive heart failure. The cardiovascular effects of SCH 34826 (see *Figure 6.12*) [133] have also been assessed.

Glycopril and alatriopril, the diester prodrugs of glycoprilat (Enk, K_i (*S*) = 5.6 nM, (*R*) = 12 nM; ACE, K_i (*S*) = 6.5 nM, (*R*) = 420 nM) and alatrioprilat (Enk, K_i (*S,S*) = 5.1 nM, (*R,S*) = 13.7 nM; ACE, K_i (*S,S*) = 9.8

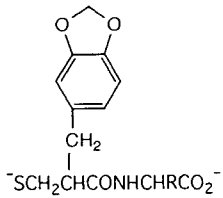
nM, (*R,S*) = 215 nM) (see *Figure 6.13*) [134] are also undergoing assessment as potential therapeutic agents.

EFFECTS ON THE INFLAMMATORY PROCESS

Substance P and related neuropeptides induce neurogenic inflammatory responses in the respiratory tract such as cough, mucus secretion, smooth muscle contraction, plasma extravasation and neutrophil adhesion [135].

Evidence for enkephalinase (neutral endopeptidase) as the modulating enzyme in neurogenic inflammation has been obtained from potentiation of effects of substance P by enkephalinase inhibitors. A model for the role of enkephalinase in the respiratory tract has been proposed [135,136] which suggests that enkephalinase exists on the surfaces of target cells for neuropeptides with the active site of the enzyme on the outside of the cell facing interstitial space. Neuropeptides such as substance P diffuse through the tissues to the receptor where enkephalinase and the receptor compete for the peptide. Enkephalinase does not significantly affect the affinity of the peptide for the receptor but regulates binding by limiting the concentration of peptide available to the receptor therefore under normal circumstances the neurogenic response is mild and protective.

Endogenous neurogenic inflammatory responses are exaggerated by cigarette smoke, viral infection and the industrial pollutant toluene di-isocyanate. They are also potentiated by enkephalinase inhibitors thiorphan and phosphoramidon but are unaffected by the ACE (kininase II) inhibitor captopril. When Substance P is administered intravenously both phosphoramidon and captopril potentiate the bronchomotor responses. This effect has been attributed to the distribution of enkephalinase and ACE in lung tissue. Enkephalinase exists in high concentrations on epithelial and smooth muscle cells where the concentration of ACE is low, whereas the concentration of ACE is high on pulmonary endothelial cells. Substance P has been shown to be inactivated by enkephalinase in the smooth muscle



GLYCOPRILAT, R = H
ALATRIOPRILAT, R = Me

Figure 6.13. Combined inhibitors of enkephalinase and angiotensin converting enzyme [134].

and epithelium of the airway tissue and in the pulmonary endothelium by ACE [137]. Aerosolized human recombinant enkephalinase inhibits cough produced by both endogenous and exogenous neuropeptides [138].

This evidence suggests that enkephalinase inhibitors may produce adverse effects similar to ACE inhibitors such as angioneurotic oedema and persistent cough which have been linked to suppression of bradykinin deactivation [139,140,141].

Substance P also plays a role in neurogenic inflammation of experimental arthritis and can induce the release of IL-1, tumour necrosis factor, and IL-6 from monocytes [72]. Enkephalinase has been detected in the synovial fluid from arthritic joints [54] suggesting that it may play a balancing role in the pro-inflammatory process of various neuropeptides and cytokines in the joint.

CD10 Cell surface antigen (originally identified as common acute lymphoblastic leukaemia antigen, CALLA) has been identified as being virtually identical to enkephalinase [142]. Met-enkephalin mediates naloxone-reversible changes in cell shape, migration and aggregation of both invertebrate haemocytes and human neutrophils [72]. In the presence of enkephalinase inhibitors such as phosphoramidon and thiorphan, the concentration of Met-enkephalin required to induce morphological changes in human polymorphonuclear leukocytes is reduced, whereas the presence of captopril has no effect. This indicates that opioid neuropeptides play an important role in mediating inflammatory responses and that their action is modulated by enkephalinase.

Activated helper T cells are also a source of immunoreactive Met-enkephalins. Up to 0.5% of mRNA in T cells encodes pre-proenkephalin so that enkephalins may behave as cytokines [143]. The immunoregulatory peptide, thymic humoral factor 2 (THF) augments T cell function which leads to increased production of interleukin-2 (IL-2) [144]. THF is an octapeptide Leu–Glu–Asp–Gly–Pro–Lys–Phe– Leu, and is inactivated by enkephalinase which hydrolyses the Lys^6–Phe^7 bond. The immunomodulator effects of THF have been utilized in clinical conditions associated with impaired immune response.

SUMMARY

A limited number of enzymes such as membrane metalloendopeptidase (enkephalinase) and angiotensin converting enzyme appear to be involved in deactivation and modulation of circulatory regulatory peptides. Peptides such as the enkephalins are also involved in a large number of physiological

processes. This multiplicity of physiological roles has made it difficult to establish the therapeutic role of enkephalin-degrading enzyme inhibitors.

Other factors such as difficulty in quantification and thus measurement of processes involved in pain and mental illness have also hindered the process of establishing any therapeutic role of enkephalin-degrading enzyme inhibitors in these conditions. However, they have proved to be useful pharmacological 'tools'. The most likely therapeutic role at present appears to be in the treatment of cardiovascular disorders.

As a 'profile' of pharmacological actions of enkephalin-degrading enzymes emerges, it is becoming apparent that bioavailability rather than a high degree of specificity or inhibitory potency may be the most important factor. This may be used to an advantage in future developments by the use of less specific or combined inhibitors in the form of prodrugs, designed to be active at specific sites such as the central nervous system.

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