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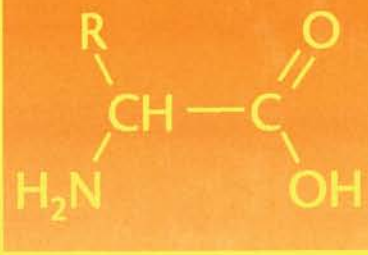

Amino acids, *peptides* and *proteins*

Volume 32

senior reporter J.S. DAVIES

A Specialist Periodical Report

Amino Acids, Peptides and Proteins

Volume 32

A Review of the Literature Published during 1999

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Preface

Once again, the authors in this Volume of Reports, covering the year 1999, have attempted as far as possible to comprehensively cover topics within chapter titles that have long been recognised as pillars of the subject matter under review. Within each chapter there are obviously evolutionary changes over the years, as we now have to reflect that more and more publications are all-encompassing in their subject content. Data on structure, synthesis, biological activity and analogue studies often appear in the same publication; hence some overlap of publications between chapters has become inevitable. Hopefully different viewpoints will have been highlighted as a result.

In last year's volume, we referred to the need to be continually observant of good standards in nomenclature and abbreviations. We understand that IUB-IUPAC are reviewing this area, but while we await publication of their deliberations, we are grateful to Dr. John Jones and to John Wiley & Sons Ltd for permission to include in this volume the short guide on nomenclature and abbreviations which originally appeared in *J. Peptide Science*, 1999, **5**, 465. This article has been reproduced unchanged from the original journal article.

Two major conferences in this research area have taken place while this volume has been in gestation. The 16th American Peptide Symposium in Minneapolis has given rise to *Peptides for the New Millennium*, eds. G.B. Fields, J.P. Tam and G. Barany, Kluwer, Dordrecht, Netherlands, 2000, 829pp. The 26th European Peptide Symposium at Montpellier has given us *Peptides 2000*, eds. Jean Martinez and Jean-Alain Fehrentz, Editions EDK, Paris, 2001, 1055pp, which records the exciting developments presented there. The contents of these books offer an overview of current developments, but the policy adopted by all our Reporters is to allow the work contained in them to mature into full papers before making comment.

This volume has again relied on a group of experienced Reporters, who have laboured hard over many months. Graham Barrett, Donald Elmore, Anand Dutta and Jennifer Littlechild all deserve sincere thanks for their compilations. We understand that this year's will be the last chapter we will receive from Anand Dutta, who has over the years augmented our coverage by accessing a great deal of pharmacological data on peptides, not freely available to many Reporters in this series. We wish Anand well, as he takes on other fields of interest.

Finally the patience and professionalism of the RSC Publications staff have again been instrumental in producing this volume of Reports, which we hope will be a sourcebook and seedcorn for many future activities in this wide and important field.

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Amino Acids, Peptides and Proteins, Volume 32

A Short Guide to Abbreviations and Their Use in Peptide Science

Abbreviations, acronyms and symbolic representations are very much part of the language of peptide science – in conversational communication as much as in its literature. They are not only a convenience, either – they enable the necessary but distracting complexities of long chemical names and technical terms to be pushed into the background so the wood can be seen among the trees. Many of the abbreviations in use are so much in currency that they need no explanation. The main purpose of this editorial is to identify them and free authors from the hitherto tiresome requirement to define them in every paper. Those in the tables that follow – which will be updated from time to time – may in future be used in this Journal without explanation.

All other abbreviations should be defined. Previously published usage should be followed unless it is manifestly clumsy or inappropriate. Where it is necessary to devise new abbreviations and symbols, the general principles behind established examples should be followed. Thus, new amino-acid symbols should be of form Abc, with due thought for possible ambiguities (Dap might be obvious for diaminoproprionic acid, for example, but what about diaminopimelic acid?).

Where alternatives are indicated below, the first is preferred.

Amino Acids

Proteinogenic Amino Acids

Ala	Alanine	A
Arg	Arginine	R
Asn	Asparagine	N
Asp	Aspartic acid	D
Asx	Asn *or* Asp	
Cys	Cysteine	C
Gln	Glutamine	Q
Glu	Glutamic acid	E
Glx	Gln *or* Glu	
Gly	Glycine	G
His	Histidine	H
Ile	Isoleucine	I
Leu	Leucine	L
Lys	Lysine	K

Met	Methionine	M
Phe	Phenylalanine	F
Pro	Proline	P
Ser	Serine	S
Thr	Threonine	T
Trp	Tryptophan	W
Tyr	Tyrosine	Y
Val	Valine	V

Other Amino Acids

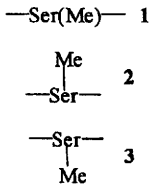
Aad	α-Aminoadipic acid
βAad	β-Aminoadipic acid
Abu	α-Aminobutyric acid
Aib	α-Aminoisobutyric acid; α-methylalanine
βAla	β-Alanine; 3-aminopropionic acid (avoid Bal)
Asu	α-Aminosuberic acid
Aze	Azetidine-2-carboxylic acid
Cha	β-cyclohexylalanine
Cit	Citrulline; 2-amino-5-ureidovaleric acid
Dha	Dehydroalanine (also ΔAla)
Gla	γ-Carboxyglutamic acid
Glp	pyroglutamic acid; 5-oxoproline (also pGlu)
Hph	Homophenylalanine (Hse = homoserine, and so on). Caution is necessary over the use of the use of the prefix homo in relation to α-amino-acid names and the symbols for homo-analogues. When the term first became current, it was applied to analogues in which a side-chain CH_2 extension had been introduced. Thus homoserine has a side-chain CH_2CH_2OH, homoarginine $CH_2CH_2CH_2NHC(=NH)NH_2$, and so on. In such cases, the convention is that a new three-letter symbol for the analogue is derived from the parent, by taking H for homo and combining it with the first two characters of the parental symbol – hence, Hse, Har and so on. Now, however, there is a considerable literature on β-amino acids which are analogues of α-amino acids in which a CH_2 group has been inserted between the α-carbon and carboxyl group. These analogues have also been called homo-analogues, and there are instances for example not only of 'homophenylalanine', $NH_2CH(CH_2CH_2Ph)CO_2H$, abbreviated Hph, but also 'homophenylalanine', $NH_2CH(CH_2Ph)CH_2CO_2H$ abbreviated Hph. Further, members of the analogue class with CH_2 interpolated between the α-carbon and the carboxyl group of the parent α-amino acid structure have been called both 'α-homo'- and 'β-homo'. Clearly great care is essential, and abbreviations for 'homo' analogues ought to be fully defined on every occasion. The term 'β-homo' seems preferable for backbone extension (emphasizing as it does that the residue has become a β-amino

	acid residue), with abbreviated symbolism as illustrated by βHph for $NH_2CH(CH_2Ph)CH_2CO_2H$.
Hyl	δ-Hydroxylysine
Hyp	4-Hydroxyproline
αIle	*allo*-Isoleucine; 2*S*, 3*R* in the L-series
Lan	Lanthionine; *S*-(2-amino-2-carboxyethyl)cysteine
MeAla	*N*-Methylalanine (MeVal = *N*-methylvaline, and so on). This style should not be used for α-methyl residues, for which either a separate unique symbol (such as Aib for α-methylalanine) should be used, or the position of the methyl group should be made explicit as in αMeTyr for α-methyltyrosine.
Nle	Norleucine; α-aminocaproic acid
Orn	Ornithine; 2,5-diaminopentanoic acid
Phg	Phenylglycine; 2-aminophenylacetic acid
Pip	Pipecolic acid; piperidine-s-carboxylic acid
Sar	Sarcosine; *N*-methylglycine
Sta	Statine; (3*S*, 4*S*)-4-amino-3-hydroxy-6-methyl-heptanoic acid
Thi	β-Thienylalanine
Tic	1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid
αThr	*allo*-Threonine; 2*S*, 3*S* in the L-series
Thz	Thiazolidine-4-carboxylic acid, thiaproline
Xaa	Unknown or unspecified (also Aaa)

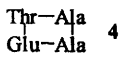
The three-letter symbols should be used in accord with the IUPAC-IUB conventions, which have been published in many places (e.g. *European J. Biochem.* 1984; **138**: 9–37), and which are (May 1999) also available with other relevant documents at: http://www.chem.qnw.ac.uk/iubmb/iubmb.html#03

It would be superfluous to attempt to repeat all the detail which can be found at the above address, and the ramifications are extensive, but a few remarks focussing on common misuses and confusions may assist. The three-letter symbol standing alone represents the unmodified intact amino acid, of the L-configuration unless otherwise stated (but the L-configuration may be indicated if desired for emphasis: e.g. L-Ala). The same three-letter symbol, however, also stands for the corresponding amino acid *residue*. The symbols can thus be used to represent peptides (e.g. AlaAla or Ala-Ala = alanylalanine). When nothing is shown attached to either side of the three-letter symbol it is meant to be understood that the amino group (always understood to be on the left) or carboxyl group is unmodified, but this can be emphasized, so AlaAla = H-AlaAla-OH. Note however that indicating free termini by presenting the terminal group in full is wrong; $NH_2AlaAlaCO_2H$ implies a hydrazino group at one end and an α-keto acid derivative at the other. Representation of a free terminal carboxyl group by writing H on the right is also wrong because that implies a terminal aldehyde.

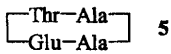
Side chains are understood to be unsubstituted if nothing is shown, but a substituent can be indicated by use of brackets or attachment by a vertical bond up or down. Thus an *O*-methylserine residue could be shown as **1**, **2**, or **3**.



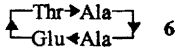
Note that the oxygen atom is not shown: it is contained in the three-letter symbol – showing it, as in Ser(OMe), would imply that a peroxy group was present. Bonds up or down should be used only for indicating side-chain substitution. Confusions may creep in if the three-letter symbols are used thoughtlessly in representations of cyclic peptides. Consider by way of example the hypothetical cyclopeptide threonylalanylalanylglutamic acid. It might be thought that this compound could be economically represented **4**.



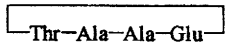
But this is wrong because the left hand vertical bond implies an ester link between the two side chains, and strictly speaking if the right hand vertical bond means anything it means that the two Ala α-carbons are linked by a CH_2CH_2 bridge. This objection could be circumvented by writing the structure as in **5**.



But this is now ambiguous because the convention that the symbols are to be read as having the amino nitrogen to the left cannot be imposed on both lines. The direction of the peptide bond needs to be shown with an arrow pointing from CO to N, as in **6**.



Actually the simplest representation is on one line, as in **7**.



Substituents and Protecting Groups

Ac	Acetyl
Acm	Acetamidomethyl
Adoc	1-Adamantyloxycarbonyl
Alloc	Allyloxycarbonyl
Boc	*t*-Butoxycarbonyl
Bom	π-Benzyloxymethyl
Bpoc	2-(4-Biphenylyl)isopropoxycarbonyl

Btm	Benzylthiomethyl
Bum	π-*t*-Butoxymethyl
Bu^i	*i*-Butyl
Bu^n	*n*-Butyl
Bu^t	*t*-Butyl
Bz	Benzoyl
Bzl	Benzyl (also Bn); Bzl(OMe) = 4-methoxybenzyl and so on
Cha	Cyclohexylammonium salt
Clt	2-Chlorotrityl
Dcha	Dicyclohexylammonium salt
Dde	1-(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl
Ddz	2-(3,5-Dimethoxyphenyl)-isopropoxycarbonyl
Dnp	2,4-Dinitrophenyl
Dpp	Diphenylphosphinyl
Et	Ethyl
Fmoc	9-Fluorenylmethoxycarbonyl
For	Formyl
Mbh	4,4′-Dimethoxydiphenylmethyl, 4,4′-Dimethoxybenzhydryl
Mbs	4-Methoxybenzenesulphonyl
Me	Methyl
Mob	4-Methoxybenzyl
Mtr	2,3,6-Trimethyl,4-methoxybenzenesulphonyl
Nps	2-Nitrophenylsulphenyl
OAll	Allyl ester
OBt	1-Benzotriazolyl ester
OcHx	Cyclohexyl ester
ONp	4-Nitrophenyl ester
OPcp	Pentachlorophenyl ester
OPfp	Pentafluorophenyl ester
OSu	Succinimido ester
OTce	2,2,2-Trichloroethyl ester
OTcp	2,4,5-Trichlorophenyl ester
Tmob	2,4,5-Trimethoxybenzyl
Mtt	4-Methyltrityl
Pac	Phenacyl, $PhCOCH_2$ (care! Pac also = $PhCH_2CO$)
Ph	Phenyl
Pht	Phthaloyl
Scm	Methoxycarbonylsulphenyl
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulphonyl
Pr^i	*i*-Propyl
Pr^n	*n*-Propyl
Tfa	Trifluoroacetyl
Tos	4-Toluenesulphonyl (also Ts)
Troc	2,2,2-Trichloroethoxycarbonyl
Trt	Trityl, triphenylmethyl
Xan	9-Xanthydryl

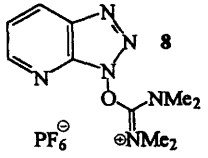
Z	Benzyloxycarbonyl (also Cbz). Z(2Cl) = 2-chlorobenzyloxycarbonyl and so on

Amino Acid Derivatives

DKP	Diketopiperazine
NCA	*N*-Carboxyanhydride
PTH	Phenylthiohydantoin
UNCA	Urethane *N*-carboxyanhydride

Reagents and Solvents

BOP	1-Benzotriazolyloxy-tris-dimethylamino-phosphonium hexafluorophosphate
CDI	Carbonyldiimidazole
DBU	Diazabicyclo[5.4.0]-undec-7-ene
DCCI	Dicyclohexylcarbodiimide (also DCC)
DCHU	Dicyclohexylurea (also DCU)
DCM	Dichloromethane
DEAD	Diethyl azodicarboxylate (DMAD = the dimethyl analogue)
DIPCI	Diisopropylcarbodiimide (also DIC)
DIPEA	Diisopropylethylamine (also DIEA)
DMA	Dimethylacetamide
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMS	Dimethylsulphide
DMSO	Dimethylsulphoxide
DPAA	Diphenylphosphoryl azide
EEDQ	2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
HATU	This is the acronym for the 'uronium' coupling reagent derived from HOAt, which was originally thought to have the structure **8**, the ***H***exafluorophosphate salt of the *O*-(7-***A***zabenzotriazol-lyl)-***T***etramethyl ***U***ronium cation.



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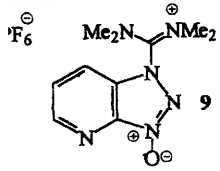
In fact this reagent has the isomeric *N*-oxide structure **9** in the crystalline state, the unwieldy correct name of which does not conform logically with the acronym, but the acronym continues in use.



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	Similarly, the corresponding reagent derived from HOBt has the firmly attached label HBTU (the tetrafluoroborate salt is also used: TBTU), despite the fact that it is not actually a uronium salt.
HMP	Hexamethylphosphoric triamide (also HMPA, HMPTA)
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HOCt	1-Hydroxy-4-ethoxycarbonyl-1,2,3-triazole
NDMBA	*N*,*N*′-Dimethylbarbituric acid
NMM	*N*-Methylmorpholine
PAM	Phenylacetamidomethyl resin
PEG	Polyethylene glycol
PtBOP	1-Benzotriazolyloxy-tris-pyrrolidinophosphonium hexafluorophosphate
SDS	Sodium dodecyl sulphate
TBAF	Tetrabutylammonium fluoride
TBTU	See remarks under HATU above
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TFMSA	Trifluoromethanesulphonic acid
THF	Tetrahydrofuran
WSCI	Water soluble carbodiimide: 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride (also EDC)

Techniques

CD	Circular dichroism
COSY	Correlated spectroscopy
CZE	Capillary zone electrophoresis
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ESR	Electron spin resonance
FAB	Fast atom bombardment
FT	Fourier transform
GLC	Gas liquid chromatography
hplc	High performance liquid chromatography
IR	Infra red
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
nOe	Nuclear Overhauser effect
NOESY	Nuclear Overhauser enhanced spectroscopy
ORD	Optical rotatory dispersion
PAGE	Polyacrylamide gel electrophoresis
RIA	Radioimmunoassay
ROESY	Rotating frame nuclear Overhauser enhanced spectroscopy

RP	Reversed phase
SPPS	Solid phase peptide synthesis
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
TOF	Time of flight
UV	Ultraviolet

Miscellaneous

Ab	Antibody
ACE	Angiotensin-converting enzyme
ACTH	Adrenocorticotropic hormone
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ANP	Atrial natriuretic polypeptide
ATP	Adenosine triphosphate
BK	Bradykinin
BSA	Bovine serum albumin
CCK	Cholecystokinin
DNA	Deoxyribonucleic acid
FSH	Follicle stimulating hormone
GH	Growth hormone
HIV	Human immunodeficiency virus
LHRH	Luteinizing hormone releasing hormone
MAP	Multiple antigen peptide
NPY	Neuropeptide Y
OT	Oxytocin
PTH	Parathyroid hormone
QSAR	Quantitative structure–activity relationship
RNA	Ribonucleic acid
TASP	Template-assembled synthetic protein
TRH	Thyrotropin releasing hormone
VIP	Vasoactive intestinal peptide
VP	Vasopressin

J. H. Jones

1
Amino Acids

BY GRAHAM C. BARRETT

1 Introduction

The literature of 1999 is covered in this chapter, which aims to report and appraise newly-published chemistry of the amino acids, with some biological aspects covered to provide clarification of the chemical content of particular studies. A few references deal with literature appearing a little earlier (from late 1998) and also into the early part of 2000.

Literature citations forming the basis for this chapter have been found through *Chemical Abstracts* (Volume 130, Issue no. 11 to Volume 132, Issue no. 9 inclusive), and from searches of major journals that are favoured by authors of relevant material.

Excessive fragmention by authors and lax refereeing is responsible to a significant extent for the ever-increasing number of references for this chapter. This chapter's policy for dealing with papers reporting obvious results, is to group such papers together without detailed comment on any of them. Conference proceedings are not covered in detail and the patent literature is excluded.

As usual, the carboxylic acid grouping is understood to be implied by the term 'amino acid' for the purposes of this chapter, though interest in boron and phosphorus oxyacid analogues, and also in sulfonic acid analogues, is continuing to grow. Methods applicable for the synthesis of α-aminoalkaneboronic acids, α-aminoalkanesulfonic acids, and α-aminoalkanephosphonic acids and other phosphorus oxyacids are usually extensions of standard methods in the amino carboxylic acid field, and representative examples of syntheses of amino oxyacid analogues are mixed in with corresponding methods for amino carboxylic acids in appropriate locations in this chapter.

2 Textbooks and Reviews

Most of the relevant material under this heading is mentioned in later sections of this chapter. The following sources are listed here where more general topics within amino acid science are reviewed.

Textbooks covering amino acids to a significant extent include protein reviews,[1] plant amino acids,[2,3] peptides,[4] and metabolism.[5]

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Reviews have appeared, of roles for D-aspartic acid in animal tissues,[6] glycine transport systems,[7] biotransformations,[8] PNA,[9] and selenocysteine, the twenty-first coded amino acid.[10] Recommended 1- and 3-letter abbreviations for selenocysteine are U and Sec, respectively[11] (a website, http://www.chem.qmw.ac.uk/iupac/Amino Acid/, is available for all current IUPAC IUB amino acid and peptide nomenclature pronouncements).

Some interesting amino acid papers that do not fall naturally into a section in this chapter are located here. The seventh paper in an idiosyncratic series on orismology (the science of defining words) suggests that the trivial amino acid names have an effect in stimulating research.[12] More important is an unexplained finding that amino acid infusion of a patient during general anaesthesia induces thermogenesis and prevents post-operative hypothermia and shivering, and hospitalization may thereby be shortened.[13]

3 Naturally Occurring Amino Acids

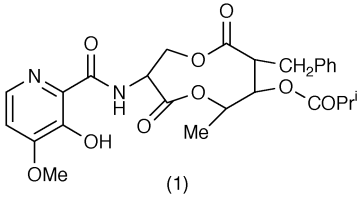
3.1 Occurrence of Known Amino Acids. – This section reports unusual contexts in which known amino acids appear, and these reports can include the most familiar amino acids – glycine as its N-[3-(D-13′-methyltetradecanoyloxy)-15-methylhexadecanoyl] derivative constitutes more than 5% of the lipids of *Cyclobacterium marinus*,[14] and serine appears in UK-2A (1) from *Streptomyces* sp. 517-02.[15] Ethiin (*alias* S-ethyl-L-cysteine sulfoxide) has been found for the first time in alliin.[16] Justiciamide (2), an amide of (2S,4S)-threo-γ-hydroxyglutamic acid found in *Justicia ghiesbreghtiana*, is in the same category of novel derivatives of known amino acids,[17] as is N-acetyl aminomalonic semialdehyde $AcNHCH(CHO)CO_2H$ shown to be the acetyl derivative of the 'lost C_3 fragment' that is a side-product in the biosynthesis of thyroxine (rather than dehydroalanine, as accepted for more than 50 years).[18]

2-Amino-3-cyclopropylbutanoic acid accompanies the known 2-amino-5-chloropent-4-enoic acid in the toxic fungus *Amanita castanopsidis*.[19] (R)-β-DOPA (3) constitutes 2% of the dry weight of the mushroom *Cortinarius violaceus* in the form of its iron(III) complex.[20]

The betaine solorinine (4) previously located in the Canadian lichen *Solorine crocea*, is now shown to be widespread in Pettigeraceae, accompanied in *Pettigera praetextata* by its homologue (NMe_2 instead of NMe_3^+).[21]

Dehydrotryptophan appears in the form of its dioxopiperazine, dipodazine, in *Penicillium dipodomyis* and *Penicillium nalgiovense*.[22] The easy formation of the 2,2′-bi-indole grouping established for the reaction of tryptophan with an aldehyde[23] is seen in the ditryptophan crosslink, a prominent feature of the fascaplysins.[24] Cysteine sulfenic acid occurs in proteins and provides an unusually stable example of this fleeting sulfur functional group.[25]

3.2 New Naturally Occurring Amino Acids. – The claim to have isolated (2,5-dioxo-4-imidazolidinyl)carbamic acid (5) from *Cistanche deserticola Y. C. Ma* requires some reconsideration for the predictable instability of this



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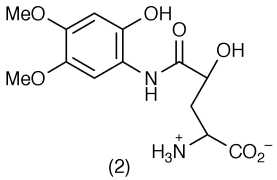

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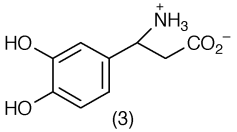

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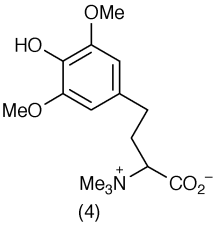

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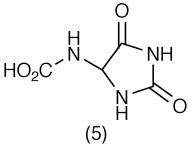

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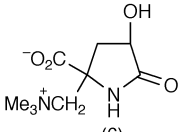

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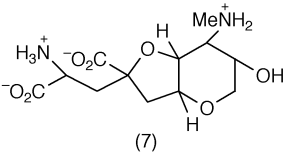

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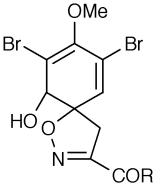

(8) R = $NHCH_2CO(CH_2)_2NHCO_2Me$
(9) R = $NH(CH_2)_2COCH_2NHCO_2Me$

structure (carbamic acids are recognized to be artefacts created during isolation procedures and α-aminoglycine derivatives are easily hydrolysed).[26] Uncertainty should not however surround the claims for dysibetaine (6), a new α,α-disubstituted α-amino acid from the marine sponge *Dysidea herbacea*,[27] and (−)-dysiherbaine (7; see also ref. 268) from the same source.[28] The Caribbean sponge *Plakortis simplex* produces (S)-2-amino-4-ethylpent-4-enoic acid.[29]

Novel bromotyrosine derivatives (8, 9) from the sponge *Aplysina cauliformis* possess cytotoxic properties.[30]

3.3 New Amino Acids from Hydrolysates. – Acylated or amidated versions of new amino acids are covered in this section, whether or not the reported work

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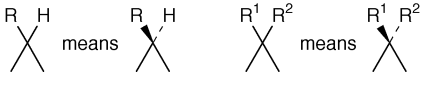
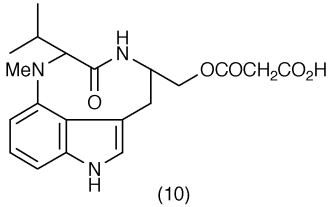
Three-dimensional features of molecules are depicted throughout this Chapter as follows: horizontally-ranged atoms and bonds and ring atoms are to be understood as being in the plane of the paper; substituent atoms and groups attached to these are to be understood to be ABOVE the page if ranged LEFTWARDS and BELOW the page if ranged RIGHTWARDS



actually included hydrolysis of the derivatives to the parent compounds. Peptides and depsipeptides are the usual source of these new amino acids, and polyoxypeptins A and B from a *Streptomyces* sp. (which show potent apoptosis-inducing properties) are notable not only in containing (2S,3R)-3-hydroxy-3-methylproline in the former compound, but other unusual amino acids also (3-hydroxyleucine, N-hydroxyvaline, N-hydroxyalanine, piperazic acid, 5- hydroxyhexahydropiperazine-3-carboxylic acid).[31] The cyclic dipeptide (−)-indolactam (10) from *Streptomyces blastmyceticum* has been characterized.[32]

Higher homologous amino acids are well represented. Five ψ-cyclotheonamides (new cyclic peptides from the marine sponge *Theonella swinhoei*), contain α-ketohomoarginine and vinylogous tyrosine moieties, and are effective as serine protease inhibitors.[33] Zelkovamycin from *Streptomyces* sp. 1454-19 is a cyclic peptide containing several unusual features.[34] Aeshynomate (11) is a derivative of a new γ-amino acid from *Aeshynomene indica* L.;[35] calvine (12) with its 2-epimer (13) derives from the ladybird beetle (*Calvia*);[36] and the 11-membered ring (14) is a component of the alga *Sargassum vachellianum*.[37]

Cyclopentenosine (a new trifunctional crosslinking amino acid from elastin hydrolysates) is a cyclopent-2-en-1-one and αβ,γδ-unsaturated aldehyde, and its imine-enamine tautomers and enantiomers, formed from three allysine residues.[38]



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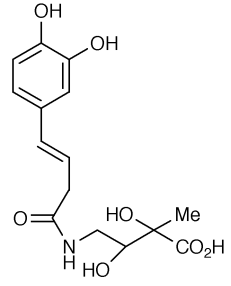

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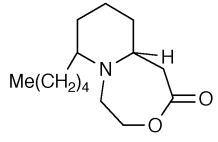
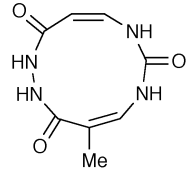


(12)
(13) has the side-chain below the plane of the ring



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4 Chemical Synthesis and Resolution of Amino Acids

Sections 4 and 6.3 of this chapter should be consulted by readers seeking syntheses of particular amino acids, but a considerable degree of cross-referencing has been included to aid searches.

Several reviews of standard syntheses, most of them lacking depth and critical appraisal, have been published: general surveys,[39,40] synthesis of aspartic acid β-semi-aldehyde,[41] uses of β-lactams in syntheses of α- and β-amino acids,[42] synthesis of pipecolic acids and derivatives,[43] synthesis of lipidic amino acids,[44] large-scale synthesis of non-natural amino acids employing enzymes,[45] and syntheses of γ-aminobutyric acid analogues.[46]

Discussion of isotopically-labelled amino acids is distributed throughout this chapter: syntheses of [^{2}H]-,[345,694,847,984] [^{11}C]-,[167,237,374,924] [^{13}C]-,[186,345] [^{15}N]-,[345,353] [^{18}F]-,[236,287,969-972] [^{99m}Tc]-,[932] and [^{128}I]-isotopomers[973] are represented.

Syntheses of phosphorus oxyacids[58,59,71,72,80,142,181,182,218,220,375,407,460,715,721] and sulfur oxyacids[763] are located in sections determined by the underlying functional group chemistry.

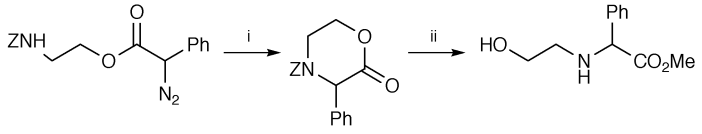
4.1 General Methods for the Synthesis of α-Amino Acids, Including Enantioselective Synthesis. – The various approaches are grouped into conventional categories as in preceding Volumes, and most of the papers are merely listed or given only brief comment where no new methodology is involved.

4.1.1 Amination of Alkanoic Acid Derivatives by Amines and Amine-related Reagents. The standard Gabriel reaction protocol applied to the reaction of fluoroarylamines and methyl α-bromoisovalerate under phase-transfer catalysis conditions yields corresponding N-arylvalines.[47] Another down-to-earth study describes continuous production of glycine from monochloroacetic acid through catalysed ammonolysis.[48] α-Halogeno-α-phenylselenoesters give 1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid esters through Lewis acid-catalysed reaction with N-toluene-p-sulfonyl-β-phenylethylamines.[49]

Further examples of aminolysis by benzylamine of α-halogeno-esters $Br(CH_2)_3CHBrCHRCO_2Et$ exploiting kinetic dynamic resolution (Volume 31, p. 7) achieve diastereoisomeric excesses of 98% (and no less than 85%).[50] Reaction of ammonia with chloroform and an aldehyde [$RCHO \rightarrow H_3N^+CHRCO_2^-$] can be guided to favour one enantiomer when β-cyclodextrin is present.[51]

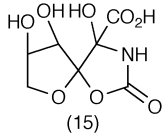
More roundabout, but still simple, amination procedures start with ketones *via* oximes (leading to β-alkoxy-α-amino acids)[52] and insertion of a carbene into an N–H bond (Scheme 1).[53] Diethyl azodicarboxylate as aminating agent for enolates of (S,S)-(+)-pseudoephedrine amides $ArCH_2CONHCHMeCH(OH)Ph$ gives good stereoselectivity.[54]

A review has appeared of amination of silyl enol ethers and glycal derivatives by a nitridomanganese complex.[55] Cyanate as aminating species is featured in conversion of dehydroascorbic acid into (15), an unusual reaction



Reagents: i, $Sc(OTf)_3$, Δ, benzene; ii, H_2–Pd/C, MeOH

Scheme 1



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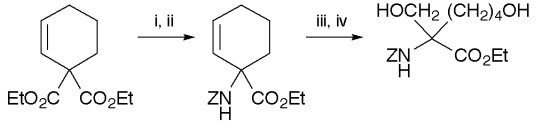
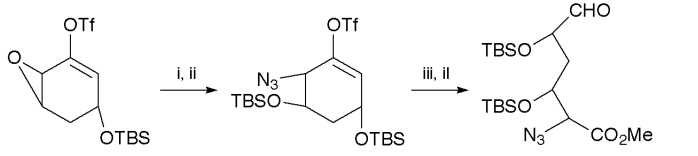


Reagents: i, pig liver esterase; ii, diphenyl phosphoryl azide, NEt_3 then $PhCH_2OH$; iii, O_3/CH_2Cl_2; iv, $NaBH_4$, EtOH

Scheme 2

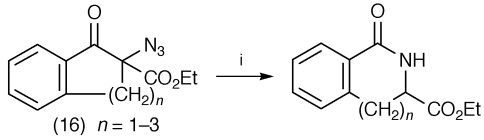
product that releases cyanate when in alkaline solution (this corrects the information in an earlier abstract used to obtain material in Volume 30, p. 5).[56] Prochiral malonates subjected to pig liver esterase-catalysed hydrolysis give half-esters from which an α-hydroxymethyl α-amino acid (*e.g.* the myriocin precursor in Scheme 2) may be obtained using cyanate.[57] Analogous treatment of diisopropyl α-chloroacetoxyphosphonates prepared from aliphatic aldehydes and lipase resolution gives phosphonic acid analogues of coded L-amino acids (valine, leucine, isoleucine, methionine) and α-aminobutyric acid.[58] 1-Amino-2-hydroxypropanephosphonic acid and 1-amino-2-hydroxy-2-phenylethanephosphonic acid have been prepared.[59]

Conversion of methyl α-bromo-esters into corresponding azides *en route* to α-amino acids continues to be a popular approach, radical bromination of carbohydrate C-glycosides giving tetrahydrofuran-based α-amino acids.[60] Preparation of α-azido-esters through epoxide opening (Scheme 3),[61] also applicable to the preparation of α-azidovinyl esters, *e.g.* $Pr^nCH{=}CH(N_3)CO_2Et$ when using diphenyl phosphoroazidate,[62] emphasizes the favoured regioselectivity for the process. α-Azido-β-keto-esters (16 in Scheme 4) undergo Schmidt rearrangement accompanying Bu_3SnH reduction, unusually involving radical intermediates.[63]



Reagents: i, tetramethylguanidinium azide; ii, TBSCl with imidazole; iii, O_3, MeOH then $NaHCO_3$

Scheme 3



(16) $n = 1$–3

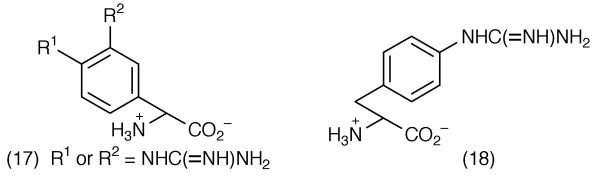
Reagents: i, Bu_3SnH in refluxing benzene

Scheme 4

Asymmetric aminohydroxylation of alkenes gives β-aminoalkanols (*e.g.* the synthesis of the Abbott aminodiol[64]) from which corresponding α-amino acids may be obtained, illustrated in preparations of phenylglycines and phenylalanines (17 and 18 respectively) designed as conformationally restricted L-arginine analogues.[65] The enantioselectivity of the $(DHQ)_2$-AQN aminohydroxylation system is dependent on the structure of the αβ-unsaturated aryl esters which the methodology has been applied.[66] Uses of the reaction have been reviewed.[67]

Aldols from chiral aldehydes and (4-methylphenylthio)nitromethane give oxiranes through oxidation with a metal alkyl peroxide, aminolysis giving α-amino acid thiolesters,[68] also obtainable from N,N-disubstituted 2-amino-alken-2-als $R^1CR^2{=}C(NR^3{}_2)CHO$ through addition of a thiol through an unusual 1,3-shift of the initial 1,2-adduct.[69]

(R)-2-Methylglycidol is the starting point for a synthesis of (S)- and (R)-N-Boc-α-methyl serinal acetonides (Scheme 5), which can be used to prepare (R)-

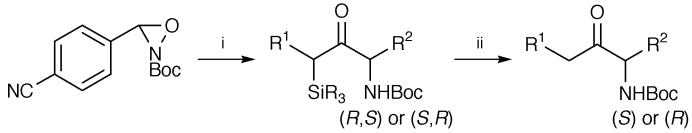


(17) R^1 or R^2 = NHC(=NH)NH_2 (18)

Reagents: i, literature method; ii, see text

Scheme 5

and (S)-α-methyl-α-amino acids respectively without racemization, through Wittig reaction with $Ph_3P^+Me\ Br^-$ and hydrogenation.[70] Related ring-opening syntheses include conversion of 2-methylaziridine-2-phosphonic acid esters into α-amino-α-methylphosphonic acids (including α-methyl-'phosphono-phenylalanine'),[71] and corresponding use of homochiral N-toluene-p-sulfinylaziridine-2-phosphonates,[72] and reductive opening of homochiral substituted aziridine-2-carboxylates (polymethylhydrosiloxane–Pd/C).[73] A route from β-enamino esters to α-amino-β-esters through reaction with ethyl N-[(4-nitrobenzene-sulfonyl)oxy]carbamate is thought to involve an aziridine intermediate.[74] Conversion of N-Boc-oxaziridines into α-aminoketones proceeds with moderate enantiomeric purity through reaction with α-silyl ketones (Scheme 6).[75]

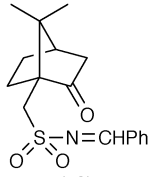


Reagents: i, $R^1CH(SiR_3)COCH_2R^2$, LDA/THF; ii, TBAF, KH_2PO_4, NH_4F, HF/THF

Scheme 6

4.1.2 Carboxylation of Alkylamines and Imines, and Related Methods. Control by the N-protecting group permits (–)-sparteine-catalyzed reaction of $BzlN(SiR_3)CO_2Me$ with EtMeCHLi and carboxylation with CO_2 to give either enantiomer of phenylglycine.[76] Direct asymmetric α-carbalkoxylation of an amine, using an enantiopure carbonate as a chiral CO_2 synthon for ring-opening an achiral zircona-aziridine derived from Cp_2ZrCl_2, exploits the dynamic kinetic resolution principle, and leads to α-amino acid esters in good enantiomeric purity (Volume 29, p. 7).[77]

Reaction of an N-benzylimine with methyl chloroformate gives the corresponding amino acid ester, used for preparation of 9-aminofluorene-9-carboxylic acid[78] and the 4,5-diaza-analogue.[79] Analogous use of a chiral sulfur imine (19 or 20) with a metal phosphite leads to α-amino phosphonic acids.[80] Alkylation at a methylene group adjacent to imine and chiral sulfoxide groupings in $R^1OCH_2C(=NR^2)CH_2S(O)Tol$ offers the opportunity for general α-amino acid synthesis, illustrated for 4-substituted 2-aminoadipic acids.[81]



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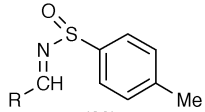
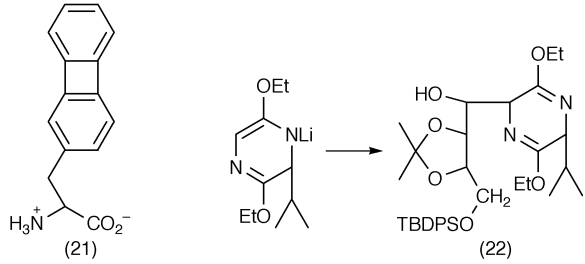


(20)

Alkylation of amines by nitromethane and alkaline permanganate oxidation of the nitromethyl derivative is an indirect carboxylation process that is clearly limited to substrates that can withstand these conditions.[82]

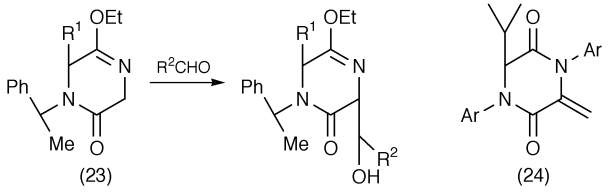
4.1.3 Use of Chiral Synthons in Amino Acid Synthesis. Whereas chiral auxiliaries feature frequently in syntheses of α-amino acids, and are also covered in other sections, some have become identified with routes to amino acids through the names of their creators, and are covered here. Although these synthons are usually glycine derivatives, their use is covered here because papers describing the use of simple glycine derivatives in amino acid synthesis are covered in section 4.1.7.

The standard Schollkopf route employing a cyclized L-valylglycine [an '(R)- or (S)-2-isopropyldiketopiperazine'] or a 3,6-dialkoxy-dihydropiperazine (a 'bislactim ether') derived from it by O-alkylation is illustrated for syntheses of 5-hydroxylysine,[83] 3-(R)- and (S)-carboxyphenyl-(S)-prolines,[84] 2-(3′-alkyl-2′-



(21) (22)

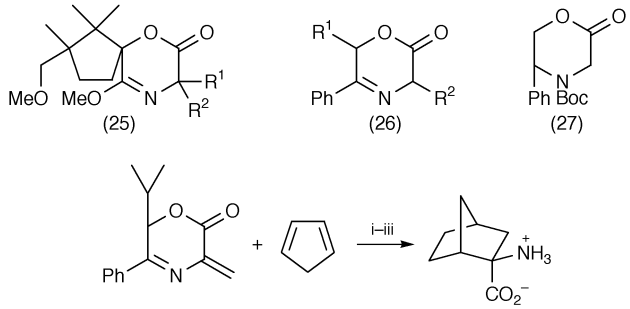
carboxycyclopropyl)glycine,[85] the biphenylene analogue (21) of phenylalanine and its benzocyclobutane analogue,[86] (2S)-2-amino-3-(1H-indol-4-yl) propanoic acid,[87] the β-hydroxy-α-amino acid obtained from (22) using the lithium aza-enolate of the bislactim ether, *en route* to 1-deoxygalactostatin,[88] (2S,3R)-β-hydroxy-3′-isopropenyltyrosine,[89] (−)-sparteine-catalysed aldolization of ethyl 3,6-diethoxy-2,5-dihydropyrazine-2-carboxylate in highly enantioselective fashion,[90] use of a 2-(3-trimethylsilylethyn-1-yl) bislactim ether for substituted tryptophan synthesis.[91] Variants of the process are represented in aldolization of the N-[(S)-2-phenylethyl] synthon (23) to give β-hydroxy-α-amino acids[92] and in conjugate addition of organocuprates to the dehydroalanine homologue (3S)-N,N′-bis(p-methoxybenzyl)-3-isopropyl-6-methylenepiperazine-2,5-dione (24).[93] A particularly interesting use of the latter approach establishes moderate to high diastereoselectivity in addition reactions of carbon radical species.[94] The leaving group of the electrophile used in Schollkopf bislactim ether alkylation affects the diastereoselectivity of the process, with diphenyl phosphate best in this context, compared with tosylate and bromide.[95]



(23) (24)

Further interest shown in 6-substituted piperazine-2,3,5-triones has been rewarded with the finding that alkylation by methyl bromoacetate at C-6 accompanies the expected N-alkylation, so opening up a useful synthesis of α,α-disubstituted amino acids.[96]

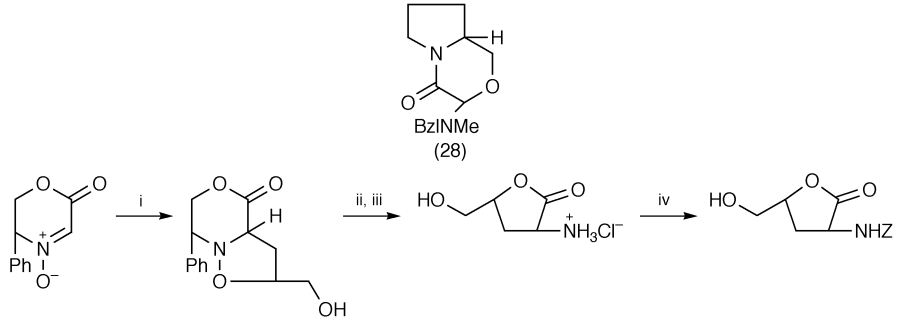
The analogous use of chiral morpholinones continues to develop into novel areas, illustrated with the homochiral synthon (25) used in syntheses of α,α-disubstituted amino acids,[97] and Diels-Alder reactions of a dehydroalanine relative (Scheme 7).[98] (S)-α-Methyl-α-amino acids have been prepared from 3,6-dihydro-2H-1,4-oxazin-2-ones (*i.e.*, 26) through mild phase transfer-catalysed alkylation or allylation.[99] The particular synthon (27) used for preparations of L-[3-^{13}C]phenylalanine and tyrosine (using [α-^{13}C]benzyl bromides made through standard routes from ^{13}CO) is now frequently called Dellaria's oxazinone.[100] L-[α-^{13}C]Aspartic acid has been prepared from the



Reagents: i, MePh, rt for 3 h then 2M HCl, H_2–Pd/C; ii, 6M HCl, 150 °C; iii, propylene oxide, EtOH, reflux

Scheme 7

[2-^{13}C]version of this oxazinone (prepared from phenyl [2-^{13}C]bromoacetate and (S)-2-phenylglycinol).[101] The L-proline-derived synthon (28) offers a synthesis of methyl esters of N-methyl-L-α-amino acids through a conventional alkylation and ring cleavage sequence.[102] (R)-5,6-Dihydro-5-phenyl-1,4-oxazin-2-one N-oxide seems to present a useful entry to a clavalanine synthesis intermediate through reaction with allyl alcohol (Scheme 8).[103]

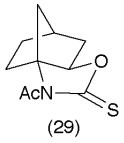


Reagents: i, CH_2=$CHCH_2OH$, $MgBr_2$, $ClCH_2CH_2Cl$; ii, H_2–$Pd(OH)_2$/C; iii, HCl–EtOH; iv, ZCl, aq. $NaHCO_3$

Scheme 8

Oppolzer's camphorsultam is featured in syntheses with glyoxylic acid (ref. 196) and in synthesis of β-amino acids (ref. 427).

Addition of the potassium salt of (R)- or (S)-4-phenyloxazolidin-2-one to monosubstituted nitroalkenes proceeds with good diastereoselectivity.[104] Further applications have been reported for the camphor-derived oxazolinethione (29) that has been advocated for Ti-mediated bromination and aldolization [Ac-→Pr^{i}CH(OH)CHBrCO-] followed by conventional azidolysis and generation of a primary amino group.[105] Extension of the Evans methodology, in which the aldolization step is followed by displacement of the chiral auxiliary to give the corresponding Weinreb amide followed by Mitsunobu inversion, has been illustrated with an efficient synthesis of a D-erythro-β-methylaspartic acid analogue (the 'amino portion' of the β-amino acid ADDA; see ref. 241).[106] A new synthesis of oxazolidin-2-ones uses 1,2-aminoalkanols



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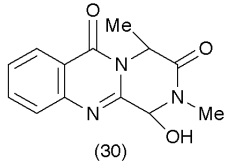

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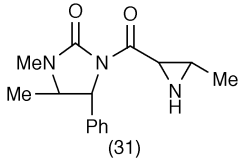
and electrochemically-generated tetraethylammonium peroxydicarbonate, but yields are modest.[107] Further experience has been reported, of applications of N-acyl-5,5-dimethyloxazolidin-2-ones (42) as chiral synthons.[108,250]

(1S)-1-Hydroxy-(4S)-2,4-dimethyl-2,4-dihydro-(1H)-pyrazino[2,1-*b*]quinazoline-3,6-dione (30) is an effective new chiral electrophilic glycine synthon.[109]

4.1.4 Use of Rearrangements Generating a Carbon–Nitrogen Bond. Ring-expansion of chiral N-(α-aminoacyl)aziridine-2-carboximides (31) in highly regio- and stereoselective fashion gives oxazolines from which threonine dipeptides are obtained by mild hydrolysis.[110] This route has been used to prepare (2R,3S)- and (2S,3R)-β-hydroxyphenylalanine from a corresponding carboximide.[111] Uses of the Schmidt rearrangement (ref. 62) and of the Curtius rearrangement (refs. 391, 430, 455, 459, and 794) are covered elsewhere in this chapter.



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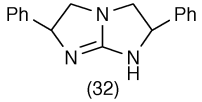
4.1.5 Other Rearrangements. Intramolecular proton transfer after carbonyl group excitation through UV irradiation leads to formation of biradicals from amidoketones. These undergo highly diastereoselective ring closure to give α-amino acids.[112]

4.1.6 Amidocarbonylation and Related Multicomponent Processes. Simple syntheses of particular amino acids are covered later in Section 4.5; marginally less primitive routes are represented by preparations of phenylglycine from benzaldehyde, KOH, NH_4OH, and $CHCl_3$[113] and from glyoxylic acid, MeCN, benzene, acetic anhydride, and H_2SO_4.[114] The former of these studies included β-cyclodextrin in the reagent cocktail but the Abstracts source of this information does not indicate the enantiomeric excess achieved.

Amidocarbonylation, the use of carbon monoxide in conjunction with a nitrile and an aldehyde for the preparation of N-acyl α-amino acids, depends on effective palladium catalysis, and work in Beller's laboratory over many years (Volume 31, p. 13) has achieved good results,[115] an easily-performed Pd/C-catalysed conversion involving a mixture of amide, aldehyde, CO, LiBr, and

1% H_2SO_4.[116] The corresponding preparation of hydantoins by the palladium-catalysed carbonylation of a mixture of an aldehyde and a urea is a new development.[117]

Standard multicomponent approaches are represented in the Strecker synthesis (review, ref. 118) leading from aldehydes to optically-pure α-arylglycines *via* α-aminonitriles when (R)- or (S)-2-amino-2-phenylethanol is used as amine component,[119] and to α-methyl-α-arylglycines from methyl ketones when (R)-phenylglycinol is used.[120] An asymmetric Strecker synthesis of α-substituted and α,γ-disubstituted glutamic acids is based on involvement of (S)-phenylglycinol as esterifying agent for a γ-keto-acid.[121] All four isomers of 1-amino-2-hydroxycyclohexanecarboxylic acid[122] have been prepared analogously using (R)- or (S)-2-phenylethylamine as amine component, leading to 87–98% enantiomeric excesses; a practical observation in this study, that cleavage of a benzyl–nitrogen bond was accomplished by concentrated sulfuric acid, should be worth following up. An asymmetric Strecker synthesis of D-alloisoleucine is based on the easy availability of (S)-2-methyl-1-butanol,[123] and further use of the chiral sulfinimide $TolSONH_2$ (see also ref. 72) has been demonstrated for synthesis of syn- and anti-β-fluoro-α-amino acids.[124] A different approach to the 'asymmetric Strecker synthesis' is the use of a chiral catalyst for mediating the condensation of reactants, exemplified by (32) for the process $PhCH{=}NCHPh_2 \rightarrow PhCH(CN)NHCHPh_2 \rightarrow$ D-phenylglycine,[125] and by a Ti-tripeptide Schiff base complex.[126] Better than 80% yields of aminonitriles with over 99% enantiomeric excess have been achieved in the last-mentioned study. α-Amino aldehydes have been converted into aminonitriles, and these have been proposed for wider use in synthesis as a protected form of their sensitive parents; they can also be put through the standard Strecker reaction to give corresponding α-amino acids.[127]



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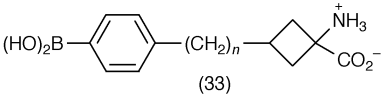
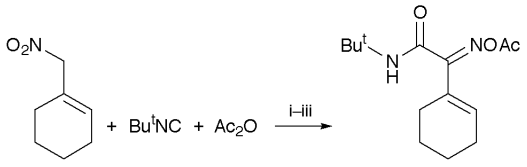


(33)

Synthesis of (33) from the corresponding cyclobutanone illustrates established Bucherer-Bergs methodology.[128] The 'three-component boronic acid Mannich reaction' introduced by Petasis (Volume 31, p. 14), accomplished by mixing an alkenylboronic acid $PhCH{=}CHB(OH)_3$, an amine $BocNHCH_2CH_2NH_2$, and an aldehyde (glyoxylic acid) at room temperature in methanol or dichloromethane, gives $PhCH{=}CHCH(CO_2H)NHCH_2CH_2NH_2$ in 88% yield.[129]

Further development of the Ugi four-component (4CC) condensation is described in synthesis of PNA monomers;[130] in the use of (β-isocyanoethyl) alkyl carbonates $CNCMe_2CHOCO_2R$ so as to lead to N-acyl α-amino acid esters and avoid the troublesome conversion of secondary amide to ester needed in the standard Ugi route;[131] in the use of ethyl glyoxylate,[132] and using an N-Boc-α-amino aldehyde.[133] The first example of a multi-component

condensation using a nitro-compound, an isocyanide and acylating agent (Scheme 9) giving α-oximino-amides, has been reported.[134] An otherwise routine Ugi 4CC uses microwave assistance in an application of solid-phase methodology.[135] An erratum[136] withdraws a claim[137] to have accomplished the first asymmetric Ugi 4CC-synthesis, through use of protected galactosylamine or arabinosylamine and o-isocyanobenzyl alcohol tri n-butylsilyl ether, in view of a prior demonstration by Kunz and Pfrengle (Volume 21, p. 7).

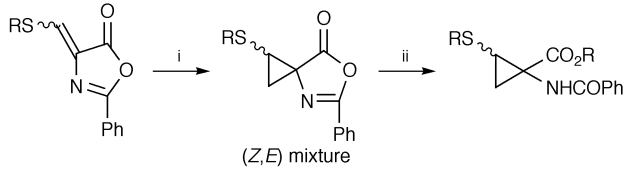


Reagents: i, MePh, NEt_3, 12 h; ii, evaporate; iii, chromatography over SiO_2

Scheme 9

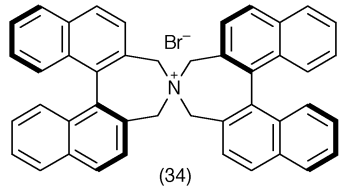
4.1.7 From Glycine Derivatives and from Imines of Glyoxylic Acid Derivatives. Diethyl acetamidomalonate is one of the longest-used glycine synthons, and used in routes to L-azatyrosine (alkylation by 5-hydroxy-2-bromomethylpyridine O-benzenesulfonate, and completed with an enzymic resolution),[138] to aryl-substituted 1,2,3,4-tetrahydroisoquinolin-3-carboxylic acids designed as conformationally restrained phenylalanine analogues (alkylation by α,α-dibromo-4-nitro-o-xylene and routine ensuing steps),[139] and to N-acetyl β-trifluoromethyltryptophan.[140] D- and L-β-(Pyrid-4-yl)alanine have been prepared by this route with resolution through enzymic hydrolysis of the intermediate ethyl 2-acetamido-3-(pyrid-4-yl)propionate.[141]

Equally long in use, the azlactone synthesis employing a 2-substituted oxazol-5(4H)-one as alkylation substrate has served for routes to α-(triphenylphosphanyl)glycine,[142] and 2-alkyl- and 2-arylsulfanyl-1-aminocyclopropanecarboxylic acids (Scheme 10).[143] A standard feature of the azlactone synthesis is the ring-opening step, brought about by simple nucleophiles [ethanolysis of 4-(N,N-dimethylaminomethylidene)oxazolones with NaOEt in EtOH[144]] and continuing efforts are being made to turn this into an enantioselective operation [(−)-cinchonine in MeOH giving (S)-benzoylamino acid methyl esters with 10–33% e.e.,[145] while corresponding preparation of N-benzoylamino acid isopropyl esters using titanium (R,R)-TADDOLates based on the kinetic resolution principle achieves better than 95% e.e. after recrystallization of the products[146]].



Reagents: i, CH_2N_2 (excess), 1–3 h, Et_2O; ii, EtOH, DMAP

Scheme 10



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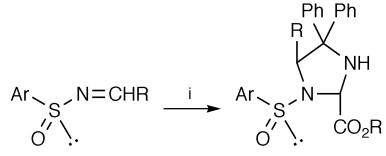
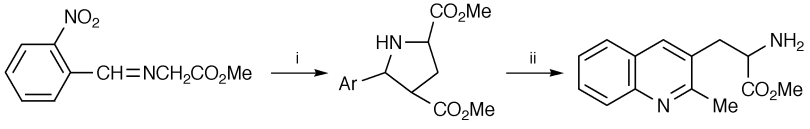
Benzophenone-derived Schiff bases $Ph_2C{=}NCH_2CO_2R$ and N-benzylideneglycines are major contributors to amino acid synthesis and their chiral phase transfer-catalysed alkylation has become one of the most attractive options, especially (−)-cinchonidine-catalysed alkylation of the tert-butyl ester[147] [review ref. 148; work with N-anthracenylmethyl dihydrocinchonidinium bromide (achieving better than 95% e.e.),[149] and similarly enantioselective aldolization;[150] corresponding use of the C_2-symmetric chiral quaternary ammonium salt (34) has been described[151]]. When this procedure is applied to Schiff bases bonded to Wang resin, either enantiomer of the target amino acids can be obtained though e.e. are somewhat modest (51–89%) using cinchonine or tetra-alkylammonium salts of cinchonidine.[152] Amino acid syntheses that do not aspire to enantioselectivity have been described for propargylglycine[153] and its homologues,[154] and dimethylaminomethylidene glycines.[155] Michael addition to acrylates catalyzed by N,N′-bis[(S)-phenylethyl]guanidine leads to no better than 30% e.e.[156] Enolates of these Schiff bases are reactive ambident 1,3-dipoles when O-palladated, participating readily in [2 + 3]cycloaddition reactions leading to proline analogues.[157] Tris(polypyridyl)ruthenium(I) complexes are efficient phase transfer catalysts for alkylation of these glycine Schiff bases.[158] A rhenium tetracarbonyl – glycine ester Schiff base tetrafluoroborate gives an enolate complex after deprotonation, and its substitution behaviour has been explored.[159] Chiral p-tolylsulfinimides yield diastereoisomerically pure N-sulfinyl imidazolidines through cycloaddition to diphenylmethylideneglycine Schiff base enolates (Scheme 11).[160] The equivalent process with o-nitrobenzylideneglycinates is illustrated in a route to β-(quinolin-3-yl)-alanines (Scheme 12).[161]



Reagents: i, $Ph_2C{=}NCH_2CO_2R$ with LDA

Scheme 11

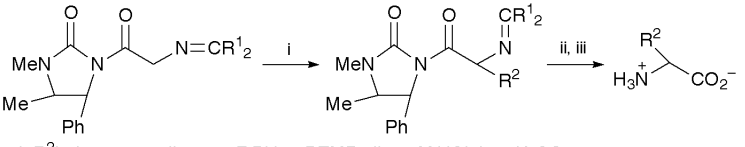


Reagents: i, $MeCOCH{=}CH_2$, DBU, AgOAc in MeCN; ii, H_2–Pd/C

Scheme 12

Alkylation of pyridoxal Schiff bases of amino acid esters, where the pyridoxal grouping carries an ionophoric (Li^+ or Na^+) chiral glyceryl side-chain, shows useful stereoselectivity.[162]

Aza-allyl carbanions formed from N-alkylideneglycinates by lithiation are versatile synthons for the general preparation of α-amino acids,[163] a recent application being the preparation of Z-γ-substituted α,β-dehydroglutamic acids.[164]



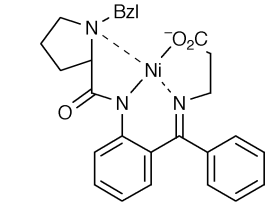
Reagents: i, R^2hal or an acrylic ester/DBU or BEMP; ii, 0·5M HCl then K_2CO_3; iii, LiOH in THF–H_2O then Dowex chromatography

Scheme 13

The alternative approach to asymmetric alkylation of glycine Schiff bases depends on incorporation of a chiral auxiliary, and representative (S)-α-amino acids have been prepared from chiral amides (Scheme 13; R^1 = SMe or Ph)[165] and from (R)-(+)-camphor-based glycine or alanine ester imines (3-bromo-2-fluoropropene as alkylating agent yielding (R)-2-amino-4-fluoropent-4-enoic acid from which 2-amino-4-oxopentanoic acid was obtained by drastic hydrolysis[166]). Preparation of [3-^{11}C]-L-alanine requires a protocol that can be completed within the hour from the moment of generation of [^{11}C]methyl iodide, and benefits from using the well-established glycyl-L-proline Schiff base nickel(II) complex (35).[167] Further results from extensive series of reports of this protocol have been published,[168] and a standard application for the synthesis of (2S,3S)-3-methyl- and 3-trifluoromethyl-pyroglutamic acids[169] and (2S,3S)-3-methyl-3-trifluoromethyl- and (2S,3S,4R)-3-trifluoromethyl-4-methyl-pyroglutamic acids[170] extends the interest of Hruby's group in the synthesis of side-chain methyl homologues of common amino acids. An unusual metallated glycine Schiff base [36; R = H→R = CH(OH)R^1] (see ref. 159) has been used in conventional aldolization followed by mild acid hydrolysis (10% hydrochloric acid) to lead to β-hydroxy-α-amino acids.[171]

N-Acylglycine esters [hippurate esters of *trans*-2-phenylcyclohexanol;[172] N-Boc-, N-Z-, or N-(toluene-p-sulfonyl)glycine tert-butyl esters[173]] must survive deprotonation by a powerful base prior to alkylation, the formation of chelated enolates by use of LiHMDS–$ZnCl_2$ being an effective prelude to allylation in the last-mentioned study, and for alkylation through Michael addition of a chiral alkoxyalkenylcarbene chromium(0) complex in a synthesis of 3-substituted glutamic acids [*e.g.*, 3-(furan-3-yl)-L-glutamic acid from 37; R = (−)-phenylmenthyloxy].[174] R,R-(−)-ψ-Ephedrine-modified glycinamide now has the credential of an *Organic Syntheses* protocol (synthesis of L-allylglycine[175]) and continuing improvements in the use of this synthon, and simplification of the methodology of alkylation, have been established.[176]

α-Heteroatom-substituted glycine derivatives are increasingly popular as



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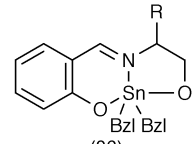

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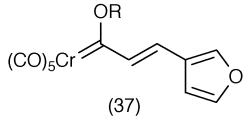

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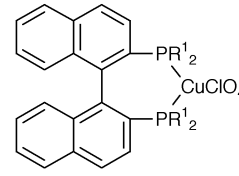
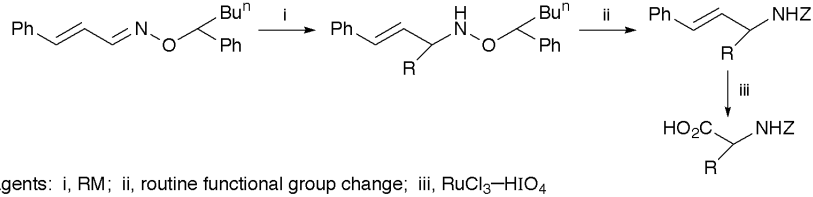


(38)

alkylation substrates; protected α-bromoglycine undergoes alkylation with a nitroalkane anion,[177] and asymmetric alkylation of 2-aza-allyl acetates $Ph_2C{=}NCH(OAc)CO_2R$ with a dialkyl sodiomalonate gives 3-carboxy-L-aspartic acid with better than 93% e.e. with a chiral Pd-catalyst or with (S)-BINAP in MeCN.[178] A similarly effective use of the chiral copper(I) Lewis acid complex (38) in mediating the first examples of asymmetric alkylation of α-alkoxyglycinates has been reported,[179] optimization leading to yields in the 73–93% range and e.e. 70–96%.[180] α-Phosphonoglycine derivatives have been used for the preparation from aldehydes of isoquinoline-3-carboxylates[181] and (E)-pyrrolidin-2-ylideneglycinates[182] (see also refs. 142, 218).

More distant glycine relatives are regularly used for the synthesis of α-amino acids, including azidoacetic acid esters (aldolization illustrated with a synthesis of N-Boc-phenylserines[183]) and (R)-o-(1-phenylbutyl)cinnamaldoxime whose benzylidene moiety serves as a latent carboxy group (Scheme 14).[184] α,β-Unsaturated esters prepared from methyl nitroacetate through Knoevenagel



Reagents: i, RM; ii, routine functional group change; iii, $RuCl_3$–HIO_4

Scheme 14

condensation with aldehydes undergo asymmetric conjugate addition with dialkylzinc reagents,[185] and the doubly [^{13}C]-labelled form of the synthon gives labelled amino acids through routine elaboration of this route.[186] Modest diastereoselectivity is shown when carbohydrate-derived 2-nitropropionates are homologated by $S_{RN}1$ reactions.[187] The nitrone $^{-}O\text{-}N^{+}{\equiv}CCO_2Et$ is admittedly a remote glycine synthon but functions as such in a preparation of

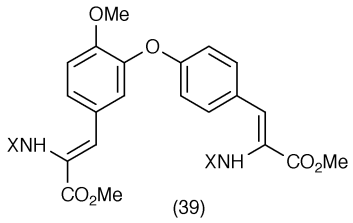
all four stereoisomers of 4-hydroxy-4-methylglutamic acid through cycloaddition to ethyl acrylate followed by *Aspergillus oryzae* protease-catalysed hydrolysis and routine workup.[188] Condensation of cyanoformates $N{\equiv}CCO_2R$ with active methylene compounds has been used in dehydroamino acid synthesis.[189]

Glyoxylic acid and its derivatives give α-carboxyimines $R^1N{=}CR^2CO_2R^3$ that have become increasingly used in α-amino acid synthesis. The usual protocol is *in situ* generation of the imine or the related iminium salt, as in the synthesis of α-aryl-α-amino acid esters from a primary amine, glyoxylate ester, and 1H-benzotriazole,[190] (R)-(−)-thiazolidine-2-carboxylic acid from cysteamine and glyoxylic acid with (2R,3R)-tartaric acid,[191] and similar involvement of a nitroalkane to give β-nitro-α-amino acids.[192] Radical additions to glyoxylate imines have given fascinating results, being effected by O_2–Et_2Zn[193] or Et_3B–RI (to glyoxylic oxime ethers $BzlON{=}CHCO_2Me$ formed from methyl 2-hydroxy-2-methoxyacetate, the hemiacetal of methyl glyoxylate, and benzyloxyamine,[194] also carried out on oxime ethers anchored to a solid phase[195]). Zinc-mediated asymmetric addition of allylic halides to the camphorsultam derivative of glyoxylic acid O-benzyl oxime gives L-azetidine-2-carboxylic acid and its (3R)-phenyl-, naphthyl-, and isopropyl homologues.[196] An alternative use of a standard chiral synthon is seen in a stereoselective Mannich-type reaction of the N-(benzyloxyacetyl)-derivative of the Evans oxazolidinone to $CF_3C(=NZ)CO_2Et$, to give predominantly (91%) the anti-adduct *en route* to D-erythro-β-hydroxy-α-trifluoromethylaspartic acid.[197]

Further results (Volume 31, p. 16) on the ene reaction catalyzed by chiral copper(I) complexes ($CuPF_6$-BINAP) of N-toluene-p-sulfonylimines of glyoxylates with alkenes[198] or allylstannanes[199] have been published, and the asymmetric version of this catalytic aminoalkylation procedure has been reviewed.[200] Furfural can be considered to be a latent form of glyoxylic acid, and the imine formed with (S)-valinol, protected as the O-trimethylsilyl ether, readily undergoes alkylation by organometallic species, the target N-protected amino acid being released by oxidation of the furyl moiety to the carboxy group.[201]

4.1.8 From Dehydro-amino Acid Derivatives. Progress towards effective procedures for the asymmetric hydrogenation of 'α,β-dehydro-α-amino acids', *alias* 2-aminoacrylic acid homologues $R^1R^2C{=}C(NHR^3)CO_2R^4$, continues to depend on catalyst design. Very low enantiomeric excesses result from heterogeneous-catalysed hydrogenation of aminocinnamic acid derivatives in the presence of (−)-cinchonidine or another alkaloid,[202] and for a homochiral bicycloheptanediol-derived phosphine,[203] while 99.9% e.e. has been claimed for a homogeneous-catalysed version of the procedure using protected dehydro-α-amino acids with a water-soluble chiral biphosphinite ligand;[204] a parallel claim for this first water-soluble ligand has appeared, demonstrating a similar performance.[205] Rhodium catalysts carrying a ferrocenyl diaminophosphine ligand,[206] recently-reported rhodium phosphinite complexes,[207] 1,2,5,6-di-isopropylidene-3,4-bis(diphenylphosphino)-D-mannitol[208] and a

closely similar ligand,[209] give almost the same result as does Rh-1,2,5-triphenylphospholane,[210] and 1,2-bis(isopropylmethylphosphino)benzene,[211] while a poly(acrylic acid) supported rhodium(I)/phosphine-catalysed hydrogenation of acetamidocinnamic acid achieves 89% e.e.[212] As in earlier years, there are numerous routine reports on this topic, either repeating existing knowledge or providing modest new results (a new tridentate phosphine ligand gives no better than 70% e.e.[213]). Particular L-amino acids that have been prepared in this way include β-branched allylglycines,[214] thienyl and furyl analogues of phenylalanine,[215] isodityrosines from (39),[216] (S)-2-quinolinylalanine,[217] and bis(glycine)s from (40).[218]



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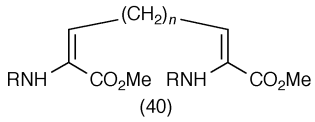


(40)

Dehydro-β-acetamidoalkanols and near relatives give similar results in standard asymmetric hydrogenation protocols.[219]

Enamidophosphonates $AcNHC(=CH_2)P(O)(OMe)_2$ have been investigated as substrates for homogeneous asymmetric hydrogenation, with preliminary results suggesting that phosphorus oxyacids will generally follow the pattern of their carbon analogues as would be expected.[220]

The equivalent asymmetric alkylation through conjugate addition of a Grignard reagent or organocuprate to (S)-2-acetamidoacrylic acid ethoxycarbonyl phenylmethyl ester has been thoroughly investigated.[221] Addition of pyrrole or indole to a chiral 3-alkylidene-dioxopiperazine catalysed by HBr is a useful route to 2-alkyl-tryptophans and pyrrol-2-yl analogues but is troubled by C=C migration,[222] and radical addition (alkylmercury chloride/$NaBH_4$) to polymer-supported 2-acetamidoacrylic acid gives modest yields (49–60%).[223]

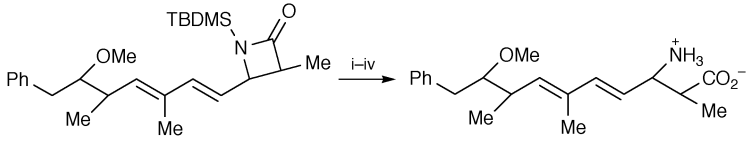
4.2 Synthesis of Protein Amino Acids and other Well-known Naturally Occurring Amino Acids. – The synthesis of coded α-amino acids as targets for trying out new or modified general protocols has been illustrated in the preceding section, and the use of readily available α-amino acids for the synthesis of other amino acids is covered in Section 6.3.Thus, this Section is restricted to (*a*) biotechnological production of coded α-amino acids, and (*b*) synthesis of unusual α-amino acids.

Reviews have appeared covering fermentative production of coded α-amino acids,[224] L-alanine,[225] L-lysine,[226] L-threonine,[227] and D-amino acids,[228] enzymic production of L-threonine and L-allothreonine from 3-substituted 2-oxobutanoic acids using leucine dehydrogenase,[229] D-phenylalanine and D-tyrosine, also from corresponding α-keto acids but by a more roundabout route (glutamate racemase, D-amino acid transferase, glutamate dehydro-

genase, formate dehydrogenase),[230] and L-2-aminobutanoic acid (transamination from threonine or aspartic acid to 2-oxobutanoic acid by recombinant *E. coli* K12).[231] A preparation of (2S,4R)-4-propylglutamic acid from the α-keto acid is efficiently mediated by glutamic oxalacetic transaminase.[232]

Aliphatic coded α-amino acids have featured in several studies, L-isoleucine being produced from *E. coli* engineered to carry a modified threonine deaminase,[233] and similarly from strains of *Corynebacterium glutamicum*.[234] Tyrosine-specific enzymes have been involved in commercial production of L-DOPA (tyrosine phenol-lyase),[235] and 6-[^{18}F]fluoro-L-DOPA (β-tyrosinase with 4-[^{18}F]fluorocatechol and pyruvic acid).[236] The special requirement of rapid reactions is accommodated in preparations of L-[β-^{11}C]-L-DOPA and L-[β-^{11}C]-5-hydroxytryptophan from L-[β-^{11}C]-DL-alanine catalysed by immobilized L-alanine racemase, D-amino acid oxidase, and β-tyrosinase or β-tryptophanase.[237]

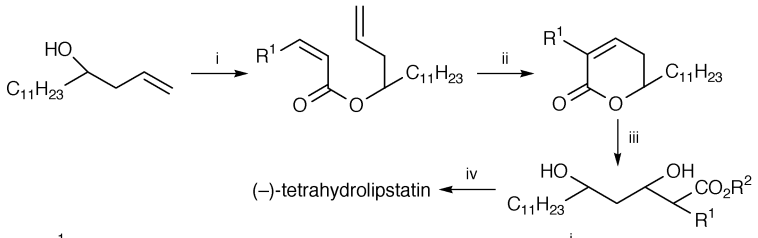
Bacterial hydantoinases and carbamoylases are establishing a prominent role in large scale amino acid production;[238] immobilized *Pseudomonas putida* has been applied for production of D-5-(p-hydroxyphenyl)hydantoin,[239] and recombinant *E. coli* D-hydantoinase can be used to give N-carbamoyl D-(4-hydroxyphenyl)glycine.[240]



Reagents: i, KF, MeOH; ii, Boc_2O; iii, LiOH then HCl, EtOAc; iv, $NH_4^+HCO_2^-$

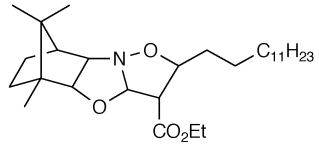
Scheme 15

The more exotic natural amino acids continue to attract novel synthesis methodology, applied to the β-amino acid ADDA (Scheme 15; see also ref. 106),[241] D,L-hypoglycine A [α-amino-β-(methylenecyclopropyl)propionic acid, through ^{i}PrMgBr/Ti(O^{i}Pr)$_4$-mediated addition of ethyl acetate to vinylacetaldehyde diethyl acetal, followed by amination];[242] D,L-coronamic and norcoronamic acids from (E)-methanohomoserine, from which the (1S,2R)-form and allonorcoronamic acids were obtained, though in modest yields;[243] enantiopure aminopolyols and polyoxamic acid derivatives through ring-opening of ethyl *cis*- and *trans*-3-(1′,3′-dioxolan-4′-yl)aziridine-2-carboxylates;[244] (+)-polyoxins J and L from 4-O-tert-butyldiphenylsilyl-2,3-isopropylidene-L-threose [vinylmagnesium bromide followed by Ac_2O/py giving the crucial protected substrate $ROCH_2CH(OPG)CH(OPG)CH(OAc)CH{=}CH_2$ for azidolysis and routine elaboration[245]]; (−)-tetrahydrolipstatin (an N-formyl-L-leucine ester) through olefin metathesis of an acrylate ester (Scheme 16;[246] a differently-conceived synthesis has been reported[247]), and through a [2 + 3]nitrone cycloaddition leading to intermediate (41);[248] the cyclosporin constituent 'MeBmt' [(2S,3R,4R,6E)-3-hydroxy-4-methyl-2-methylamino-oct-6-enoic acid] from a chiral auxiliary acylated by a 2,2-dichlorohex-4-enoyl moiety, treated with Et_3B–$(Me_3Si)_3SiH$,[249] an approach used also with the newly-introduced



Reagents: i, $R^1CH{=}CHCOCl$, Et_3N, DMAP; ii, $(PCy_3)_2Cl_2Ru{=}CHPh$, $Ti(OPr^i)_4$; iii, H_2O_2–NaOH then $(PhSe)_2$, $NaBH_4$; iv, β-lactone formation, coupling with Z–L–Leu–OH, exchange Z for formyl

Scheme 16



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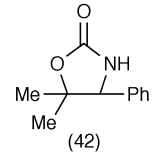

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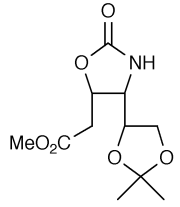

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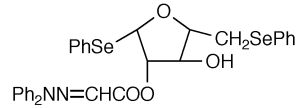
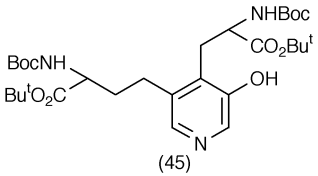


(44)

equivalent Evans-type chiral auxiliary (42) from D-phenylglycine, N-acylated with but-2-enoic acid followed by introduction of the n-heptyl group in excellent e.e. *en route* to aplysillamide B (see also ref. 108).[250] All these amino acids have been synthesis targets in recent years, as have (−)-detoxinine [newly synthesized from L-ascorbic acid *via* (43)[251]], and (+)-furanomycin [prepared from L-xylose, with radical cyclization of (44) as a key step[252]].

Recognition of the importance of polyfunctional protein crosslinks (+)-pyridinoline and its deoxy homologue has stimulated further exploration of routes for their synthesis (see Volume 30, p. 30), from Boc-L-glutamic acid α-tert-butyl ester *via* tert-butyl (2S)-2-(Boc-amino)-4-(2-oxiranyl)butanoate,[253] *via* (45),[254] or from tert-butyl (2S)-2-(Boc-amino)-6-aminohexanoate.[255] A route to the 3-hydroxypyridinium salt, (+)-deoxypyridinoline, starts from the pyridine (46) that is conveniently obtained from Vitamin B_6;[256] an alternative



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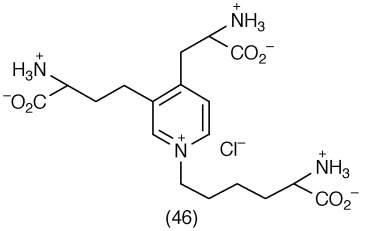
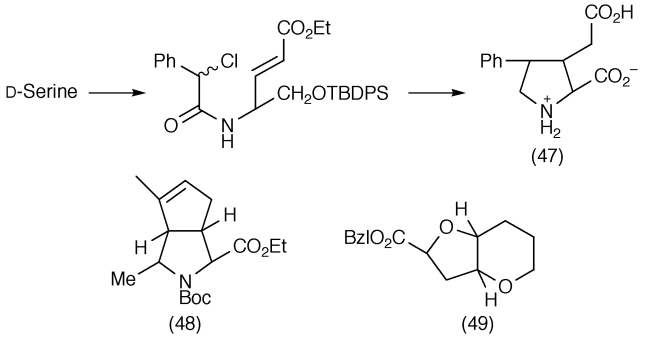


(46)

biomimetic synthesis uses protected L-lysine and L-glutamic acid.[257] A synthesis of the pyridinium crosslink, pentosidine, from tert-butyl (2S)-2-(Boc-amino)-6-iodohexanoate and an N^{δ}-(imidazopyridyl)ornithine, has been described (see ref. 939).[258]

Further kainoid synthesis routes have been established (for a review see ref. 259), mostly continuing to address the main problems of setting up appropriate stereochemical parameters of the three substituents on the tetrahydropyrrole framework in an ever more efficient manner. (−)-α-Kainic acid arises from titanium-mediated diene metallabicyclization of $PhOCH_2CR^1{=}CHCH_2NBzl\text{-}CHR^2CH{=}CH_2$ (prepared from an L-serine-derived aldehyde; see Section 6.3),[260] and from L-pyroglutamic acid *via* ketyl radical cyclization on to an enecarbamate so as to deliver the C-4 substituent.[261] D-Serine provides a starting point for a synthesis of phenyl allokainoid (47) employing a radical cyclization,[262] and addition of 3-trimethylsilylcyclopentene and to a phenylaziridine ensures correct relative stereochemistry in a synthesis of racemic phenylkainic acid.[263] A related route from L-pyroglutamic acid to 5α- and 5β-substituted kainic acids involves stereoselective nucleophilic substitution of the N-acyliminium ion of (48) by organocopper reagents.[264]



(+)-α-alloKainic acid has featured as the target in routes from a D-serine-derived alkynylenone, reaction with Et_3Al being followed by palladium-catalysed allylic carbonate reductive transposition,[265] and from L-serine by $Rh_2(OAc)_4$-catalysed CH insertion of an α-diazoacetamide tethered to (S)-4-(buten-3-yl)-2,2-dimethyl-1,3-oxazolidine.[266]

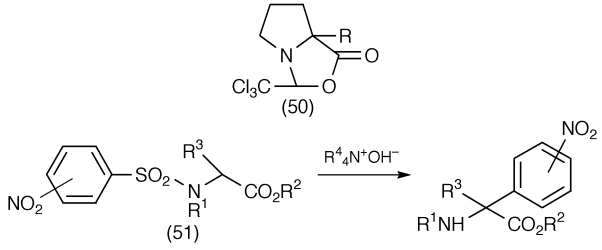
4-Arylkainic acids can be obtained by a highly stereoselective Michael addition reaction of dimethyl 2-oxoglutarate with a nitrostyrene, followed by reduction of the nitro-group, deoxygenation, and epimerization.[267]

Analogues of the neuroexcitatory amino acid dysiherbaine (7), lacking hydroxy and N-methyl groups, have been synthesized from the Garner aldehyde and the lithium enolate of ester (49).[268]

4.3 Synthesis of α-Alkyl α-Amino Acids – The particular interest in α-methyl analogues of the coded L-amino acids has extended to more general types of structure under this heading (see also refs. 96, 99). The classical synthesis

routes (hydantoin and Bucherer-Bergs syntheses) have given good service for preparing racemic forms of these derivatives. These methods are less successful for the preparation of enantioselective modifications of α-alkyl α-amino acids, and uses of modifications of the chiral synthons and chiral auxiliaries that have already been covered in this chapter (Section 4.1.3) provide the main strategy. Alkylation of chiral 1,4-benzodiazepin-2,5-diones formed from N-methylisatoic anhydride and (S)-phenylethylamine,[269] benzylation of Schiff bases of alanine esters catalysed by (R)-2-hydroxy-2′-amino-1,1′-binaphthyl (up to 68% e.e.)[270] or by sodium (R,R)-TADDOLate.[271]

Alkylation of α-amino acid derivatives provides a more direct route to α-alkyl homologues, but usually requires substantial activation of the α-carbon [homologation of 2-(trichloromethyl)oxazolidinone (50; R=H→R = alkyl)];[272] or other special characteristics as with N-alkyl N-(o- or p-nitrophenyl)sulfonyl-amino acid esters (51) which undergo intramolecular arylation through a N–C rearrangement, though not the Stevens-type route previously assigned to the process.[273] The lithium enolate of methyl N-Boc-O-TBDPS-hydroxyprolinate undergoes alkylation by an alkyl halide in good yield only when excess HMPA is used (10 eq.), and stereoselectivity depends on the reagent and the N-protecting group.[274] S_N1 Nucleophilic cleavage of cyclic sulfamidates derived from an α-alkyl serine should be a versatile new general approach to α-alkyl-α-amino acids.[275]



4.4 Synthesis of α-Amino Acids Carrying Alkyl Side-chains, and Cyclic Analogues. – The synthesis of 'non-natural α-amino acids', most of which are designed either for their potential physiological activity or for use in peptide synthesis, is covered in this section if general synthesis methods are used for their preparation. Examples synthesized from readily available amino acids are mostly covered later in Section 6.3.

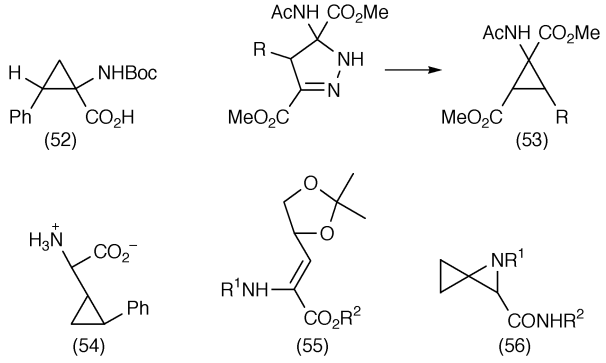
The long-running interest in amino acids with side-chains carrying a cycloalkyl moiety is based on their potential as conformationally-constrained versions of physiologically-active amino acids. Of the many options available, cyclopropyl analogues of coded α-amino acids continue to attract attention. These compounds have their own trivial names ['2,3-methanophenylalanines' (52) and three stereoisomers, have been prepared by well-established routes and separated by chromatography over polysaccharide-derived chiral stationary phases[276]].

Conformationally-constrained analogues of phenylalanine, tyrosine, trypto-

phan, and histidine have been reviewed.[277] The (1S,2S)-cyclopropane precursors of these compounds have been prepared by palladium(0)-catalysed alkylation and S_N1 cyclization of 1,4-dichlorobut-2-ene using deprotonated α-substituted alkanenitriles, d.e.s from 88–100% having been achieved.[278] A traditional route has been developed to constrained aspartic acids (53), involving ring-contraction of 4,5-dihydro-1H-pyrazoles in boiling DMF with loss of N_2,[279] and another familar concept is represented in $KOBu^t$ mediated cyclization of substituted β-chloroethyl aminoacetonitriles, *e.g.* $ClCH_2CMe_2CH(NH_2)CN$.[280] Routes such as that to (+)-R-1-amino-2,2-difluorocyclopropane-1-carboxylic acid through cyclopropanation of $CH_2{=}C(CH_2OAc)_2$ and lipase-catalysed desymmetrization, and routine ensuing steps, involve good stereochemical control.[281]

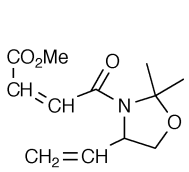
Homologous cyclopropylglycines ['3,4-methanophenylalanines' – (54) and near relatives] are also of considerable interest as mimetics of natural neuroactive amino acids, (2R,1′S,2′R,3′S)-2-(2′-carboxy-3′-phenylcyclopropyl)glycine being a potent antagonist for the metabotropic glutamate receptor,[282] synthesized by standard methods such as that leading to (2R,1′R,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine [reaction of ethyl (dimethylsulfuranylidene) acetate with (55) prepared from (S)-glyceraldehyde].[283]

Azaspiropentanecarboxamides (56) prepared from methyl 2-chloro-2-cyclopropylidene acetate and a primary amine followed by $NaH–NEt_3$ cyclization,[284] and racemic bicyclopropylidenyl- and methylenespiropentyl-substituted alanines prepared from the corresponding substituted methanols (with I_2) as alkylating agents towards ethyl N-benzylideneglycine,[285] represent a novel alternative type of peptide mimetic.



(2S,1′R,2′S,3′S)-2-(2′,3′-Dicarboxycyclobutyl)glycine and its (2S,1′R,2′R,3′S)-isomer have been prepared from 3-azabicyclo[3.1.1]heptan-2-ones that result from intramolecular photocycloaddition of (57).[286] Simpler cyclobutanes, 1-amino-3-fluorocyclobutane-1-carboxylic acid and its [^{18}F] isotopomer have been prepared for brain tumour imaging through positron emission tomography.[287]

Novel bicyclic glutamic acid analogues (58) and (59) have been prepared from cyclohexane-1,4-dicarboxylic acids through conventional alicylic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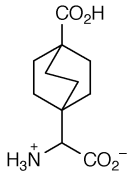
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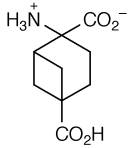
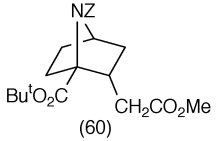

(59)

methodology and use of the Corey-Link amino acid synthesis [$CO_2Me \rightarrow CHO \rightarrow CH(OH)CCl_3 \rightarrow CH(NH_3^+)CO_2^-$].[288] Substituted 1-amino-2-hydroxycyclohexane-1-carboxylic acids are accessible from 4-chloromethyleneoxazol-5(4H)-ones through $EtAlCl_2$-mediated cycloaddition to butadienes followed by replacement of Cl by OH.[289]

Synthesis of new proline analogues, a prominent interest over the years because of the importance of post-translationally modified natural products, and of excitatory amino acids (kainoids and related compounds), continues with 1-amino-(2R,4R)-4-aminopyrrolidine-2,4-dicarboxylic acid,[290] other 4-substituted prolines [from (R)-BocNHCH(CH_2OH)$CHSO_2Ph$ with (2R)-2,3-isopropylideneglyceraldehyde;[291] 3-substituted prolines by $ZnBr_2$ cyclization of enolates of alkyl N-but-3-enyl-N-(S)-phenylethylglycinates[292]], (2S,3R,4R)-3,4-dihydroxyproline[293] and diastereoisomers prepared through lengthy routes from D-ribonolactone[294] and from D-gulonolactone.[295] Numerous bicyclic prolines have been prepared by conventional cycloaddition processes: the glutamic acid analogue (60) *via* the pyrrolidine (61) from L-serine,[296] (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylic acid and the 2-thia-analogue as potent Group II metabotropic glutamate receptor agonists,[297] azabicycloheptanes [(62) and its enantiomer],[298] intramolecular aziridine–allylsilane cyclization to give (63),[299] [3+2]cycloaddition of (−)-8-phenylmenthol-derived Fischer carbene complexes with diazomethane derivatives to give Δ^2-pyrazolinecarbenes as precursors (64) to 5-azaprolines,[300] $TiCl_4$-mediated addition of 3-vinylindoles to the iminium ion precursor $MeOCH_2N(CO_2Et)CH(CO_2Et)_2$ giving 3-indolylprolines,[301] and the spiro-diamino acid (65), useful as a template for combinatorial chemistry.[302] Competitive intramolecular substitution occurring in a route to a cyclopropylglycine gives the bicyclic proline [(66) in Scheme 17] as side-product.[303]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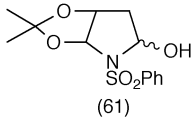
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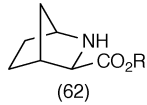
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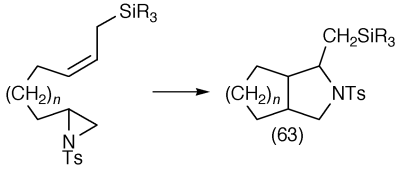
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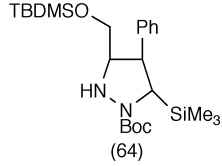
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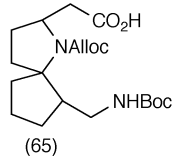
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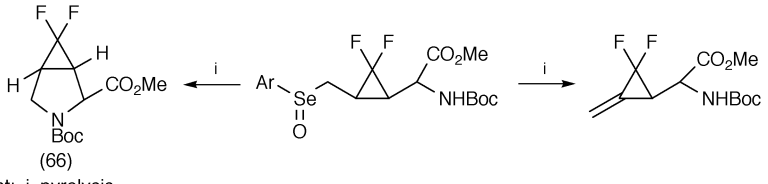
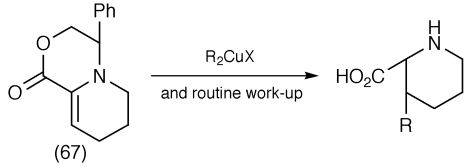


Reagent: i, pyrolysis

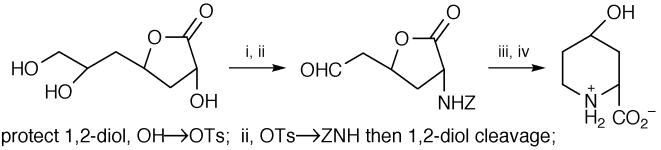
Scheme 17



3-Alkylpipecolic acids have been prepared by an extension of the homochiral morpholinone methodology (Section 4.1.3) to (67).[304]

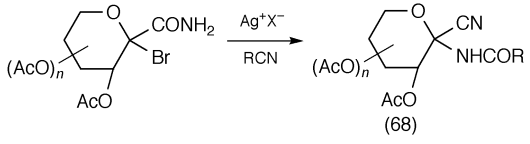
Opportunistic syntheses of unusual amino acids from alkaloids over the years are now extended to quincorine and quincoridine, oxidation giving the corresponding bicyclic aminodicarboxylic acid.[305] Another non-general example is provided in Birch reduction of N-Boc-pyrrole-2- and 3-carboxylates[306] and analogous amides.[307] 3,4-Dehydro-proline analogues and β-prolines are formed, with good diastereoselectivity when homochiral esters were employed, and when a chiral acid was used for protonation at the quenching stage of the process. Electroreduction of pyridine-dicarboxylic acids gives dihydro- and tetrahydro-analogues.[308]

Advances in enantioselective synthesis of pipecolic acid analogues have been recorded for (2R,4S)-4-hydroxypipecolic acid and its (2S,4R)-isomer (Scheme 18),[309] and for both enantiomers of *cis*-6-(hydroxymethyl)-pipecolic acid and its *cis*,*cis*-4-hydroxy-analogue.[310]



Reagents: i, protect 1,2-diol, OH⟶OTs; ii, OTs⟶ZNH then 1,2-diol cleavage;
iii, CHO⟶ CH_2OMs then OH^-; iv, H_2—Pd/C

Scheme 18



Anomeric amino acid derivatives have been prepared from C-(1-bromo-1-deoxy-D-glycopyranosyl)formamides *via* 1-cyano-analogues (68).[311]

A routine preparation of 7-phenylazo-1,2,3,4-tetrahydroisoquinoline-3-car-

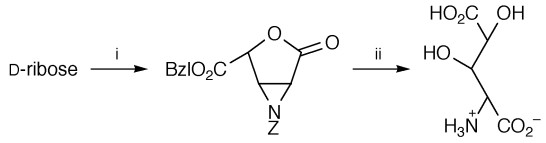
boxylic acid has been published,[312] and the Ph_4PCl-mediated Heck reaction is now becoming a regular means of using bromoarenes to prepare complex amino acids, illustrated with 1,2,3,4,5,6-hexahydro-3-benzazocine-2-carboxylic acid and 2,3,4,5,6,7-hexahydro-1H-3-benzazonine-2-carboxylic acid.[313]

4.5 Models for Prebiotic Synthesis of Amino Acids. – The feedback from evolving theories which informs new thinking has been evident over the years (for reviews of current ideas see refs. 314–317). There is an increasing volume of work on this topic, some of which extends traditional studies (synthesis of amino acids from a $CO/N_2/H_2O$ mixture at 1 atm pressure under 1–2 KeV X-irradiation;[318] or in a magnetoplasma dynamic arc jet[319]). Many of the reports are for or against a new idea, as with a role for thermophiles,[320] opposition to the claimed reduction of CO_2 by the $FeS\text{-}H_2S/FeS_2$ redox couple that is required by simple amino acid-forming reactions (the reducing power of this couple decreases drastically with rising temperature, so undersea hydrothermal vents seem to be an unlikely prebiotic source for amino acids),[321] and continuing support for stereoselective UV photolysis of interstellar dust by circularly-polarized synchrotron radiation from neutron stars.[322] The excess of the L-enantiomer for some amino acid constituents of the Murchison meteorite (ref. 1119) is considered to support the last-mentioned controversial hypothesis. Interstellar dust as the basis of UV photochemical amino acid production has been supported.[323] This paper describes millimeter array spectroscopic observation of glycine in the dense cloud from which Sagittarius B2 is forming, and provides a puzzle because the gas-phase chemistry associated with amino acid production is considered to be unlikely in dense clouds. But UV photolysis of interstellar ice grains is more likely, and therefore asteroids and comets are ruled out as prebiotic delivery vehicles to Earth for amino acids.

Maintaining a plausible scenario for terrestrial prebiotic amino acid synthesis calls for consideration of mineral surfaces as likely catalysts, and the topic has been reviewed.[324]

4.6 Synthesis of α-(ω-Halogenoalkyl)-α-Amino Acids. – The standard synthesis methods have been applied to compounds under this heading, such as D,L-α-aminoperfluoroalkanoic acids $R(CH_2)_{n-1}CH(NH_3^+)CO_2^-$ ($R = C_6F_{13}$, C_8F_{17}; $n = 3$, 10),[325] and α-bis(fluoromethyl)glycine.[326] Similar applications are described in refs. 124, 281, 303. Direct fluorination of a protected pyroglutaminol leading to 4,4-difluoro-L-glutamic acid using N-fluorobenzenesulfonimide is unusually simple.[327]

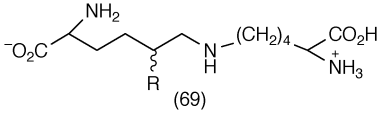
4.7 Synthesis of α-(ω-Hydroxyalkyl)-α-Amino Acids. – Numerous examples of compounds of this structural class have been prepared by routine methods (Section 4.1, see also refs. 66, 68, 92, 105, 171, 877). More unusual synthesis routes are represented: leading to the four stereoisomers of β-hydroxyhistidine;[328] D- and L-cycloserine derivatives prepared by solid-phase methodology;[329] and a route from D-ribose to (3S,4S)-dihydroxy-L-glutamic acid (Scheme 19).[330]



Reagents: i, literature method; ii, $BF_3.Et_2O/PhCH_2OH$; iii, H_2–$Pd(OH)_2/C$

Scheme 19

4.8 Synthesis of N-Substituted α-Amino Acids. – The conversion of primary amines into N-substituted versions is covered in relation to amino acids in Section 6.3, while side-chains carrying nitrogen functional groups are collected here. Crosslinking of proteins through a secondary amine is represented in lysinonorleucine (69; R = H) and its 5-hydroxy analogue (69; R = OH), for which a conventional synthesis has been reported.[331]



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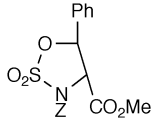
The substantial topic of protein nucleic acids (PNAs) continues to expand, based on the availability by synthesis of N-(β-purinyl and -pyrimidinyl) alanines (reviews: refs. 9, 332).

4.9 Synthesis of α-Amino Acids Carrying Unsaturated Aliphatic Side-chains. – α,β-Unsaturated α-amino acids are accessible through DBU-mediated elimination from sulfamidites (70) with $SOCl_2$ in CH_2Cl_2 to give *cis*-alkenes,[333] and through cobalt hexacarbonyl-mediated acylation of an alkyne $RC{\equiv}CCO_2H$ and Curtius development of the carboxy group into NHZ and ceric ammonium nitrate oxidation, which unexpectedly provides a 3-substituted N-alkoxycarbonyl-2,3-dehydro-aspartic acid anhydride.[334] The azlactone synthesis with 4-methylcyclohexanone followed by resolution (reaction with L-phenylalanine cyclohexylamide and separation of the diastereoisomeric dipeptides) gives an α,β-dehydroamino acid that owes its optical activity to the cyclohexyl chiral centre.[335]



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Further conventional elimination procedures are represented in a synthesis of β,γ-dehydro-L-valine from γ-(phenylselenyl)-L-isoleucine[336] and in new examples of rearrangements of allyl glycinates to allylglycines [$R^1NHCH_2CO_2$-$CH_2CH{=}CHR^2 \rightarrow R^1NHCH(CHR^2CH{=}CH_2)CO_2H$] with stereochemical control

through the presence of R^1 = L-α-aminoacyl.[337] Separation of isomers of 2-amino-3-methylpent-4-enoic acid prepared in this way, using L-aminoacylase and L-amino acid oxidase, provides the (2S,3R)-diastereoisomer, hydrogenation completing an efficient route to L-alloisoleucine.[338] A nitrosoketene from Meldrum's acid has been used in a synthesis of allylglycine and cyclopentenylglycine through [1,3]cycloaddition of the derived cyclic nitrone to alkenes.[339]

Unsaturated homologues of α-aminopimelic acid $HO_2CC(=CH_2)$-$(CH_2)_3CH(NH_3^+)CO_2^-$ and $HO_2CCH=CH(CH_2)_2CH(NH_3^+)CO_2^-$ have been prepared for use as reversible inhibitors of meso-diaminopimelic acid D-dehydrogenase, from aspartic and glutamic acids *via* side-chain aldehydes, by an S_H2' allylstannane coupling [$MeO_2CC(=CH_2)CH_2SnPh_3$ + $I(CH_2)_2CH$-$(NHR^1)CO_2R^2$] and a Wittig synthesis, respectively.[340]

4.10 Synthesis of α-Amino Acids with Aromatic or Heteroaromatic Groupings in Side-chains. – This remains an active topic because of the opportunities offered by aryl and heteroaryl moieties for synthetic modifications, giving access to isotopically-labelled amino acids and analogues of physiologically active substrates.

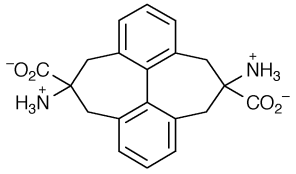
Further reviews commemorating last year's vancomycin syntheses (Volume 31, p. 32) have appeared;[341,342] one covers the synthesis of the amino acid building blocks,[343] and the other describes the route used by Boger[344] (see also ref. 877).

Synthesis of ^{13}C-, ^{15}N-, ^{2}H-isotopomers of L-phenylalanine and L-tyrosine in any chosen combination of labelling atoms in various positions in the molecule calls for construction of the aromatic moiety from 1,6-disubstituted hexatrienes and application of standard amino acid syntheis protocols.[345] 4-Substituted phenylglycines continue to provide attractive synthesis targets for pharmacological studies, *e.g.* (R,S)-4-phosphonophenylglycine as a potent and selective Group III metabotropic glutamate receptor agonist, reached through routine methods.[346] Phenylalanines of similar potential include 4-(carboxymethyl)- and 4-(carboxydifluoromethyl)-,[347] p-porphyrinyl-,[348]

Other modified phenylalanines reported, are: β-hydroxy-β-(fluoronitrophenyl)alanines,[349] the biphenyl-based bisamino acids (71) and (72),[350] (2S,3R)-β-methyltyrosine (tyrosine phenol lyase in a notable application to a non-natural substrate).[351]

Tryptophan synthase can be used analogously, for preparations from L-serine of furano- and selenophenyl- analogues of tryptophans (73–75),[352] while standard chemical synthesis leads to racemic α-[^{15}N]-tryptophan (from [^{15}N]-glycine *via* the hydantoin, condensed with indole-3-aldehyde and Al-Ni/H_2O reduction of the resulting dehydrotryptophan)[353] and an analogous preparation of dihydrotryptophan.[354]

A standard ibotenic acid synthesis modified to allow N^{α}-alkyl derivatives of this isoxazolylglycine to be prepared[355] has given samples for testing for metabotropic glutamate receptor activity. Thiazole, imidazole, and oxazole-containing amino acids[356] and 'biheterocyclic' amino acids have been built from protected α-azidoglycine and homologues by [1,3]cycloadditions.[357]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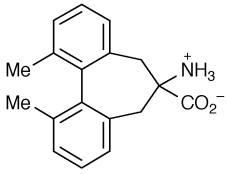
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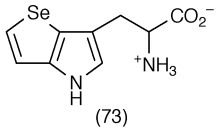
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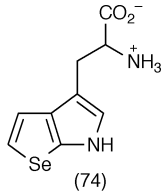
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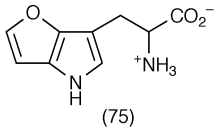
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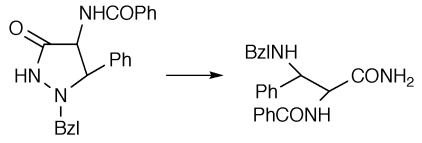
Numerous β-(heteroaryl)alanines have been prepared (see also refs. 138, 139, 160), often intended as analogues of common amino acids [4′-phospho-2′-furyl)-L-alanine as an N^{im}-phosphohistidine mimic;[358] N-benzoyl-(2R,3R)-3-phenyl-3-(pyrazol-1-yl)-L-alanine,[359] D,L- and L-β-(6,7-dimethoxy-4-coumaryl)alanine[360]] but also including natural products [pyrimidin-4-yl substituted amino acids, one of which is L-lathyrine, prepared from amidines and alkynyl ketones[361]]. Michael addition of heterocyclic nucleophiles to a protected dehydroalanine gives β-(1,2,4-triazol-1-yl)alanine and others of the same type.[362] Standard methods for this class of amino acid are illustrated in condensation of 2-Boc-amino-5-bromopentanoic acid with imidazoles and 1,2,4-triazoles[363] and Lewis acid-catalysed condensation of the β-alanylzinc synthon $BocNHCH(CO_2H)CH_2ZnI$ with an aryl iodide (for the preparation of C-glycosylated tyrosines).[364]

4.11 Synthesis of α-Amino Acids Carrying Amino Groups, and Related Nitrogen Functional Groups, in Aliphatic Side-chains. – Most of the current examples under this heading have been prepared through standard protocols, aldolization of $(MeS)_2C{=}NCMe(COR)CO_2Et$ with an α-metallated ethyl isocyanoacetate leading to syn,syn- and syn,anti-ONN′-protected 2,4-diamino-3-hydroxyglutaric acids.[365] A similar reaction of RCH=NTs and ethyl isocyanoacetate catalysed by Me_2SAuCl with a chiral ferrocenylphosphine gives (4R,5R)- and (4S,5S)-imidazol-2-ines, from which corresponding homochiral 2,3-diaminoalkanoic acids were obtained by hydrolysis and 2,3-diaminoalkanols through reduction.[366] The Garner aldehyde approach to tri-amino acids (-CHO→-$CH_2NRCH_2CH_2NHBoc$) was found to be too cumbersome in comparison with a conventional sequence *via* asparagine and diaminopropanoic acid.[367]

(4R,5R)-4-Benzoylamino-5-phenyl-3-pyrazolidinone (76) has provided a family of 3-alkylaminophenylalanines[368] and heteroaryl analogues[369] through condensation with carbonyl compounds followed by Raney nickel reduction.

Conformationally-constrained arginine analogues, $H_2NC(=NH)NHCH_2$-$CH{=}CHCH(NH_2)CO_2H$ (E- and Z-isomers) and the N-(n-propyl) and keto



(76)

homologues (C=O in place of CH_2), and (m-guanidinophenyl)glycine, have been prepared.[370]

A long-running project is reported on, describing syntheses of the tertiary amine and quaternary ammonium analogues of S-adenosylmethionine (NMe and N^+Me_2 in place of SMe^+).[371]

4.12 Synthesis of α-Amino Acids Carrying Boron Functional Groups in Side-chains. – The long-studied o-carboranylalanine [3-{1,2-dicarba-*closo*-dodecaboran(12)-1-yl}-2-aminopropanoic acid] spontaneously fragments to *nido*-carboranylalanine containing the dodecahydro-7,8-dicarba-nido-undecaborate(1−) cage with loss of a boron atom.[372]

4.13 Synthesis of α-Amino Acids Carrying Silicon Functional Groups in Side-chains. – A novel vinylsilane-containing amino acid has been prepared for use in a conventional pipecolic acid synthesis involving N-acyliminium ion cyclization.[373]

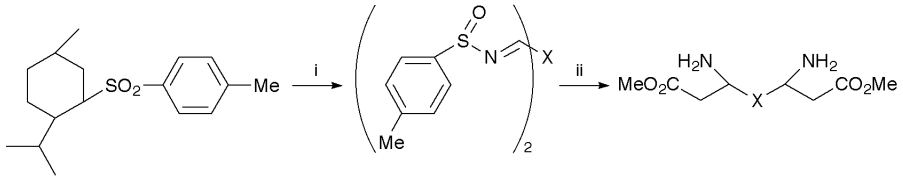
4.14 Synthesis of α-Amino Acids Carrying Phosphorus Functional Groups in Side-chains. – Main examples under this heading are covered elsewhere in this chapter (Section 4.10; *e.g.* ref. 346) reflecting the importance of phenylalanines carrying phosphorus functional groups in the aryl moiety, and uses of phosphonoglycines in synthesis (refs. 142, 181, 182, 218, 721).

4.15 Synthesis of α-Amino Acids Carrying Sulfur-, Selenium-, or Tellurium-containing Side-chains. – [^{11}C-Methyl]methionine is available within 15 minutes from $^{11}CH_3SH$ and O-acetyl-L-homocysteine, through efficient catalysis by γ-cyano-α-aminobutyric acid synthase.[374] The phosphinic acid analogue of methionine has been described[375] (see also ref. 143).

S-Neopentyl cysteic and homocysteic acids have been prepared to provide isosteric sulfonate analogues of aspartic and glutamic acids, respectively.[376]

4.16 Synthesis of β-Amino Acids and Higher Homologous Amino Acids. – Reviews of preparations of β-amino acids and β-lactams through addition of lithium amides to α,β-unsaturated carbonyl compounds,[377] of enantioselective synthesis of β-amino acids,[378] and of statines[379] have been published. β-Amino acid synthesis has been reviewed from a chemical process perspective.[380]

Several synthesis strategies that are standard in the α-amino acid series are also routine for higher homologues, particularly the newer amination methods,



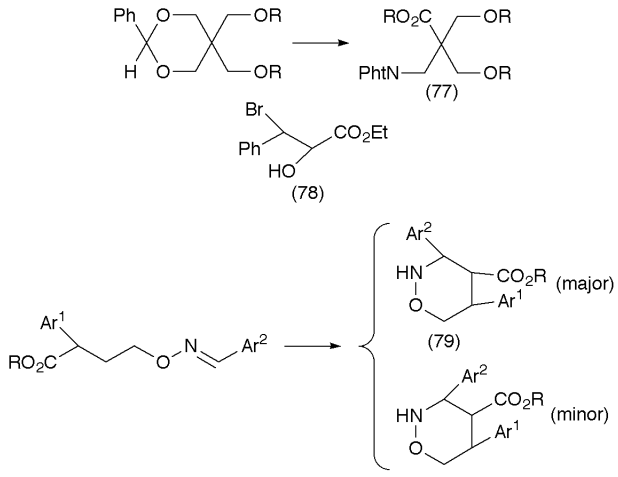
Reagents: i, $LiN(SiMe_3)_2$, OHC—X—CHO; ii, $MeOC(=CH_2)ONa$ then H_2O

Scheme 20

an unusual example featuring bis-imines (Scheme 20).[381] Amination of methyl nicotinylacetate with (S)-α-phenylethylamine gives enantiomerically-enriched (S)-3-amino-3-(pyrid-3-yl)propanoate after work-up.[382] More conventional examples involve enantioselective addition of (S)-α-phenylethylamine and other chiral amines to (E)-$PhCOCH{=}CHCO_2Et$,[383] a lithium (R)-(α-methylbenzyl)allylamide to isopropyl (E,E)-hepta-2,5-dienoate *en route* to the highly-functionalized β-amino acid constituent of sperabillins B and D,[384] hydrazoic acid to α,β-unsaturated imides catalysed by chiral (salen)Al(III) complexes,[385] sulfinylimines (formed from chiral 2-methylpropanesulfinamide with a carbonyl compound) to lithium or titanium enolates[386] and ytterbium(III)-catalysed addition of toluene-p-sulfinylimines to lithium (α-carboxyvinyl)cuprates,[387] N-acyloxyiminium ions (formed from nitrones with acyl halides) to chiral enolates,[388] nitrones to achiral N-crotonyl-1,3-oxazolidin-2-ones catalysed by chiral ytterbium(III) complexes.[389] Amination can be effected *via* β-nitro-acid derivatives, as in a route to enantiomerically-pure alkyl *cis*- and *trans*-2-aminocyclohexanecarboxylates starting from Diels-Alder adducts from nitroalkenes and 2-aminodienes.[390] The conformationally-constrained β-amino acid (−)-(1R,2S)-2-aminocyclobutane-1-carboxylic acid has been prepared from *cis*-cyclobutane-1,2-carboxylic acid anhydride through pig liver esterase-catalysed hydrolysis and Curtius rearrangement of the resulting half-ester.[391] Condensation of aldimines with silyl enolates catalysed by a Zr-(R,R)-bis(naphthol)methane complex gives substituted β-amino acid esters with high e.e.[392]

No attempt at asymmetric bias is involved in the addition of an imidoyl chloride to a lithium ester enolate to give fluorinated β-enaminoesters, ZnI_2/$NaBH_4$ reduction giving syn-β-amino-β-(fluoroalkyl)-α-methylalkanoate esters,[393] or in addition of metallated 2-alkyloxazolines, -thiazolines and imidazolines to alkanenitriles [het-CH^-R^1 Li^+ + $R^2CN \rightarrow NH_2CR^2{=}CR^1$-het] to give precursors of β-enamino acids.[394] Development of one of the functional groups of a malononitrile into a carboxy group *via* an oxazoline, while the other nitrile becomes an aminomethyl group,[395] and an alternative approach to the same substrate,[396] is a variation on this theme. β-Phthalimido-α,α-disubstituted alkanoic acids (77) have been prepared from O-benzylidene-pentaerythritol.[397] Poly(aniline)-supported cobalt(I) acetate catalyses the condensation of methyl acetoacetate, an aldehyde, and acetonitrile followed by reduction (synthesis of β-aryl homo-isothreonines).[398]

Gabriel syntheses have led to (S)-N-benzoyl 3-phenylisoserine from the



bromo compound (78),[399] and to the racemate from benzaldehyde, ethyl chloroacetate and ammonia (*via* trans ethyl 3-phenylglycidate),[400] and to (3S)-amino-(2R)-methylbutanoic acid through amination of a bromolactonization product formed using S-(−)-N-methoxypyrrolidinecarboxamide as chiral auxiliary.[401] Substitution reactions leading to methyl 3-aryl-3-(piperidin-1-yl) propionates[402] and 3-aryl-3-hydroxylaminopropionates,[403] and addition of Reformatzky reagents to aldimines, have been reported.[404]

Ring opening of *trans*-3-substituted aziridine-2-carboxylic acids has been established as an efficient route to anti-α-substituted-β-amino acids, and the route can include *Candida antarctica* lipase resolution.[405] SmI_2-Mediated cleavage of aziridines simplifies their use in β-amino acid synthesis.[406] Ring opening of (R)-diethyl oxiranephosphonate by benzylamine and hydrogenolysis gives (R)-2-amino-1-hydroxyethanephosphonic acid.[407] An O-alkyl oxime has been used to give a 9:1-mixture of 1,2-oxazine (79) and its diastereoisomer, ring-opening and recyclization giving the substituted β-proline ABT-627.[408] Other β-proline syntheses are initiated by [3+2]cycloaddition of N-tosylimines to 2-alkynoates and allenoates,[409] Pd-mediated addition of propargylamines to Michael acceptors,[410] and $ZnCl_2$-mediated asymmetric Michael-type annulation of the (R)-phenylethylamine enaminoesters $MeO_2CCH{=}C(NR)CH_2CH_2CH{=}CO_2Me$.[411] Dihydroxylated β-pipecolic acids have been prepared from the readily-available Dieckmann adduct 3-ethoxycarbonylpiperidin-4-one, chloromethyl ethers reacting with the derived dianion and effecting 5-alkoxymethylation, opening up a new route to azasugars.[412] A less flexible route to β-pipecolic acids is based on diastereoface-selective asymmetric addition to chiral 1,4-dihydropyridines derived from nicotinic acid amides.[413]

Further preparations of α-substituted-β-amino acids include hydroxyalkyl compounds $R^1CH_2NHCH_2CH(CO_2Me)CH(OH)R$[414] and $TsNHCH_2C(OH)(CO_2Me)CH(OH)R$[415] prepared from Baylis-Hillman adducts (see also ref. 416), while anti-α-hydroxy-α-alkyl-β-amino acids are available through alkyl-

ation of *trans*-oxazoline-5-carboxylic acids (formed by iodocyclization of alkyl 3-benzoylaminoalkanoates[417]) followed by ring-opening and resolution using penicillin G acylase.[418]

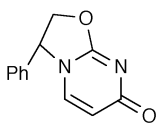
The trichloroacetimidate rearrangement applied to PhCH=CHCH(OH)-CH_2OH gives N-benzoyl-(2R,3S)-phenylisoserine methyl ester after development of functional groups.[419] An unusual rhodium(II)-induced decarboxylative rearrangement of diazoalkyl urethanes $TsNHCO_2CHRC(N_2)CO_2Et$ gives enamines ($TsNHCR{=}CHCO_2Et$ or its isomer).[420]

Chiral synthons leading to β-amino acids are similar to those used for α-amino acid asymmetric synthesis; the (S)-phenylglycinol-derived heterocycle (80) undergoes alkylation with organocopper reagents[421] and its near-relative (-$CR^1R^2CH_2$- in place of -CH=CH-) gives α-methyl-β-amino acids through enolate alkylation with electrophiles.[422] The related tetrahydropyrimidinone (81) prepared from L-asparagine is a convenient source of α-dialkyl-β-amino acids (R = H→R = alkyl; further alkylation can be effected),[423] and its methoxytetrahydropyrimidine analogue has been used for a synthesis of α-alkylaspartic acids.[424] Manipulation of this synthon into lithium enaminates (lithiated dihydropyrimidines) gives a substrate that readily undergoes electrophilic substitution to give α-branched β-amino acid esters.[425] Enantioselective α-alkylation of acyclic lithium amide enolates is facilitated by a novel chiral pentamine ligand.[426]

Camphorsultam derivatives of oxime ethers, *i.e.* N-(β-oximino)acyl derivatives of the Oppolzer auxiliary, undergo addition of alkyl radicals to give α,β-dialkyl-β-amino acids.[427] An N-acyl chiral ephedrine-derived imidazolidinone, another auxiliary that is familiar through its use for the asymmetric synthesis of α-amino acids, has been applied to β-amino acid synthesis, through addition of $PhCH{=}NSO_2Tol$ to its titanium enolate [RCH_2CON-Imid→PhCH(NHTs)CHRCO-Imid],[428] and titanium[429] and sodium[430] enolates of chiral N-acyloxazolidinone imides have been applied similarly, giving modest (60%) d.e., the former in reaction with α-alkoxyamines (*e.g.*, 2-ethoxypiperidines), the latter in reaction with tert-butyl bromoacetate to give β-substituted β-amino acids through application of the Curtius rearrangement protocol.

Highly enantioselective hydrogenation of (E)-β-acylaminoacrylates to give β-amino acids has been achieved using standard homogeneous catalysis protocols (Rh/MeDuPhos),[431] and amination [(R)-(+)-N-benzyl-α-methylbenzylamine/BuLi] of the equivalent substrate (a substituted cinnamic acid) has been used for synthesis of the β-tyrosine moiety of C-1027.[432] These β-amino acid precursors are available from sulfonyl imines and activated bisaminals.[433]

Synthesis of β-amino acids starting from α-amino acids is a continuously developing approach, and could even be described as over-developed in areas that have been well researched already (*e.g.*, Arndt-Eistert homologation of α-amino acid derivatives *via* N-protected α-aminoacyldiazomethanes[434]). α-Amino acids are used to prepare UNCAs (Volume 29, p. 72, Volume 31, p. 55) that have been used in a preparation of β-amino-α-hydroxy acids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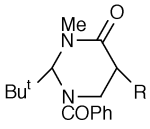
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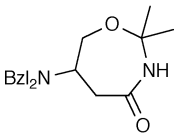
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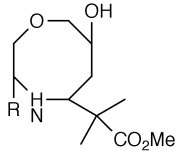
(norstatines) *via* the ketoacetylenic homologue.[435] A different homologation ($CO_2H \rightarrow CH_2OH \rightarrow CH_2CN$ *etc.*) has been used to prepare homophenylalanine.[436] L-Aspartic acid is a readily-available β-amino acid whose α-carboxy-group is adaptable to suit certain synthesis objectives, as for (2S,3R)-3-Z-amino-4-phenyl-2-hydroxybutanoic acid [prepared *via* (4S,5R)-2-benzyloxy-5-phenyloxazoline-4-acetate] as a constituent of (−)-bestatin[437] and the 6-amino-oxazepin-4-one (82), intended for use as a conformationally-restricted β-amino acid.[438] Chiral β-amino alcohols (originating in L-α-amino acids) are the starting point for the uneventful preparation of cyclic β-amino acids (83).[439] Direct α-hydroxylation of an N-protected (3S)-amino-alkanoic acid as its metal enolate provides a diastereoisomer mixture,[440] and homophenylalanines are conveniently prepared from β-amidozinc reagents $IZnCH_2CH(NHBoc)CH_2CO_2Me$ through coupling with an aryl iodide (in DMF to suppress a β-elimination side-reaction), and substitution of the Zn–Cu analogue by allylic halides.[441] *trans*-Cinnamyl alcohol has been elaborated into (S,S)-2-aminomethylcyclopropane-1-carboxylic acid through conventional functional group manipulations.[442]

The γ-amino acid family includes several members that are important for their physiological properties, and the most effective general synthesis strategies can be classified into different addition processes [aldimines to cinnamates to give 4-amino-3,4-diarylbutanoic acids,[443] allylamines with methyl chloroformate mediated by BuLi-(−)-sparteine to give (S)-2-substituted 4-aminobutanoic acids or ring-opening of equivalent β-lactams to give the (R)-enantiomer,[444] (S)-N-Boc-α-aminoaldehydes to triphenylphosphoranes $Ph_3P{=}CR^2CO_2Et$ to give $BocNHCHR^1CH{=}CR^2CO_2Et$[445] and a similar route to (Z)- and (E)-4-amino-2-(trifluoromethyl)-but-2-enoic acid from N,N-bis-Boc-glycinal and ethyl 2,2-dichloro-3,3,3-trifluoropropionate using Reformatzky conditions followed by reductive elimination[446]].

Special cases are also represented, N-allyl α-bromoamides leading to 3-aza-2-oxo-bicyclo[3.1.0]hexanes that give *cis*-2,3-methanoGABAs by reductive ring-opening (Li-NH_3).[447] Chain extension by C-acylation of Meldrum's acid by an N-protected amino acid activated with isopropenyl chloroformate leads to γ- and δ-amino-β-keto-esters [*e.g.* $RNHCH(CO_2R^1)CH_2COCH_2CO_2R^2$].[448]

Oxazolidinones are prepared from α-amino acids (*e.g.* 84), homologated using a Wittig reaction ($>C{=}O \rightarrow >C{=}CHCO_2Et$) in a diastereoselective synthesis of (3S,4S)- and (3R,4S)-4-methylamino-3-hydroxy-5-phenylpentanoic acid (N-methyl-AHPPA), a constituent of the cyclic depsipeptide hapalosin and of statine;[449] a different synthesis approach starts from (2R,3R)-2,3-epoxy-4-phenylbutan-1-ol.[450] Glycine crotyl ester $CF_3CONHCH_2CO_2CH_2CH{=}CHMe$ subjected to (−)-quinine-catalysed Claisen rearrange-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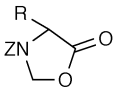
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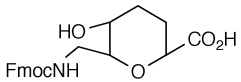
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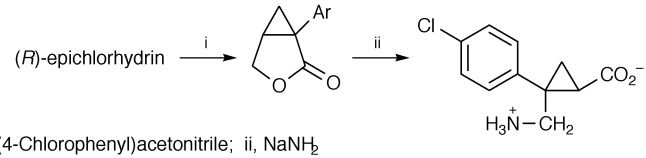
ment (Volume 31, p. 32) and two-carbon homologation ($CO_2H \rightarrow COCH_2CO_2Et$) gives isostatine,[451] and homologation of the Weinreb amide of N-Boc-L-leucine ($CO_2H \rightarrow COC{\equiv}CSiMe_3$) followed by borane reduction mediated by a chiral oxazaborolidine leads to statine; the route can be adapted to provide norstatine.[452] Synthesis of all four stereoisomers of 4-amino-3-hydroxy-2-methylpentanoic acid [one of which is a constituent of bleomycin A_2 and the (2R,3S,4S)-isomer is present in the marine toxin janolusimide] depends on crotylboration of N-Boc-L- or D-alaninal as the crucial step.[453] N-Z-α-(p-Tolyl)thio-trifluoroalaninal, (R)- or (S)-ZNHCMe[S(p-MeC_6H_4)]CHO, starts an aldolization route to syn-γ-amino-γ-trifluoromethyl-β-hydroxybutyric acid, as an alternative to 'non-oxidative Pummerer rearrangement' employing an α-lithiosulfoxide as a chiral hydroxyalkyl equivalent.[454] Simpler homochiral γ-amino-β-hydroxybutyric acids (such as the γ-benzyl homologue that is present in hapalosin) have been prepared through aldolization of (4S)-benzyl-3-(3-phenylpropanoyl)-1,3-oxazolidin-2-one with acrolein followed by Curtius rearrangement and generation of the carboxy group by oxidation of the C=C grouping.[455]

Development of methods for the synthesis of pyrrolidin-2-ones has had a long history, but asymmetric synthesis has been studied only relatively recently, with new examples illustrating the options available [*e.g.* (S)-malic acid→(S)-$HOCH_2CH(OH)CO_2Me$→(S)-4-hydroxypyrrolidin-2-one;[456] N-(3-ethoxycarbonylprop-2-enyl)-N-methoxycarbonylacetyl-(S)-phenylethylamine converted into the pyrrolidin-2-one or into the tetrahydropyridine (the direction of cyclization being controlled by the reaction conditions) *en route* to diastereoisomers of 2-aminomethylcyclobutane-1-carboxylic acid;[457] (−)-sparteine-catalysed addition of an alkyl carbamate-derived cuprate to an allenic ester $Bu^tOCON(CH_2R)_2$ + $R^1CH{=}C{=}CHCO_2Et$[458]]. The equivalent (S)-3-hydroxy-γ-butyrolactone has been used as starting material for (R)-4-amino-3-hydroxybutanoic acid (GABOB) and (R)-3-hydroxy-4-trimethylaminobutanoic acid [(R)-carnitine] *via* a 4-cyanobutanoate ester prepared as source of the 4-aminobutyronitrile through Curtius rearrangement.[459] The classical carnitine synthesis from (R)-(−)-epichlorhydrin has been adapted to provide phosphocarnitine.[460] 3-Trimethylammonio-2-hydroxycyclohexanecarboxylic acid stereoisomers have been synthesized as conformationally constrained carnitine analogues.[461] Other familiar γ-amino acids or their analogues have been synthesized: GABA, by use of immobilized *E. coli* fed on waste from L-glutamic acid production,[462] (R)-(−)-baclofen through [2 + 2]cycloaddition of 4-chlorostyrene to dichloroketen and ensuing functional group development,[463] and baclofen analogues (Scheme 21).[464] Pentafluorophenyl 4-(Fmoc-



Reagents: i, (4-Chlorophenyl)acetonitrile; ii, $NaNH_2$

Scheme 21

amino)-N-methylpyrrole-2-carboxylate[465] is representative of a class of γ-amino acids not reviewed exhaustively here, but mention of them is appropriate since such compounds are used in syntheses of peptide mimetics.

Homologation of baclofen to 5-amino-4-(p-chlorophenyl)-pentanoic acid and preparation of the 3-aryl isomer by ring-opening of the corresponding piperidinone has been reported,[466] indicative of conventional access to δ-amino acids. N-Protected α-aminoaldehydes continue to provide the most popular starting materials for this class of amino acid, In-mediated coupling with an alkyl 2-bromomethylacrylate giving mainly syn-homoallyl alcohols without racemization *en route* to aminoalkyl-substituted α-methylene-γ-butyrolactones[467] that act as substrates for C- and O-nucleophiles (*e.g.* cyanide delivered by trimethylsilyl cyanide[468]). An extraordinary double alkylation of an α,β-unsaturated imine with a keten silyl acetal and allyltributyl stannane, giving a δ-amino acid derivative $R^1NHCH(CH_2CH{=}CH_2)CH_2CHR^2CMe_2$-$CO_2Et$, has been reported.[469] Other standard synthesis protocols have provided substituted 5-(Z-amino)pentanals $ZNHCH(^iPr)COCH_2CH(CH_2Ph)$-CHO,[470] δ-aminolaevulinic acid,[471] and partially deoxygenated aminogluconic acids (85 and its isomers).[472] Homologation of α-amino acids into δ-amino acids *via* ketosulfones is a standard protocol (*e.g.*, ref. 786). 6-Amino-2-substituted hexanoic acids have been prepared from lysine *via* the triflate of 6-amino-2-hydroxyhexanoic acid,[473] and an excellent new synthesis of galantinic acid starts with L-serine, employing an oxazolidine-based strategy with chain elongation steps that are familiar through the many applications in synthesis of the Garner aldehyde (Section 6.3).[474]

4.17 Resolution of D,L-Amino Acids. – Classical procedures based on separation of enantiomers or diastereoisomers by crystallization, and amplification of enantiomer ratios by asymmetric transformations, continue to be applied. The former category is illustrated in the phenomenon of preferential crystallization of one enantiomer from stirred D,L-glutamic acid containing small amounts of L- or D-lysine (leading to 10% e.e. for crops of crystals produced in the first 30 min but 0% thereafter[475]) and of (R)- or (S)-1,4-thiazane-3-carboxylic acid from S-(2-chloroethyl)-D,L-cysteine.[476] Esters formed from D,L-bromo-acids and (R)- or (S)-3-hydroxy-4,4-dimethyl-1-phenylpyrrolidin-2-one under dynamic kinetic resolution, ensuing Gabriel synthesis with phthalimide giving corresponding phthaloylamino acids (resolution of D,L-phthaloylamino acids with these esters is also described).[477] Resolution of α-aminonitriles through asymmetric transformation using (R)-mandelic acid–amygdalin exploits the Dimroth principle.[478]

A useful practical demonstration that sensitive N-protected α-heteroatom-substituted glycines can be resolved by fractional crystallization of their (+)-(1S,2S)-2-amino-1-phenylpropane-1,3-diol salts or (+)- or (−)-menthol esters has been reported for N-protected D,L-2-alkoxyglycines.[479]

Enzyme-mediated resolutions are applicable to near relatives as well as to the common α-amino acids, and there are further indications of the scope for broadened specificities accompanying modified reaction conditions. Uses of esterases in this context have been reviewed,[480] and enantioselective hydrolysis of methyl D,L-phenylalaninate by pancreatin in toluene-water mixtures[481] and of dimethyl D,L-2-aminosuberate by papain in aqueous DMF or by subtilisin in acetonitrile with minimum water content[482] indicate the general approach. Increasing interest in the use of readily-available alcalase is being shown, with hydrolysis of familiar amino acid esters under physiological conditions[483] and, specifically, methyl D,L-phenylalaninate.[484] Carboxypeptidase A acting on the N-trifluoroacetyl derivative of β-methyl-D,L-tryptophan[485] and the reverse process with D,L-valine undergoing enantioselective acetylation mediated by immobilized L-aminoacylase[486] (see also ref. 338) are further examples of classical methods, as are uses of penicillin G acylase (refs. 20, 418, 736), lipases (refs. 58, 405) and chymotrypsin (ref. 138). Enantioselective conversion of the D-enantiomer of a D,L-hydantoin into the corresponding N-carbamoyl-D-amino acid[487] is now regularly used, also conveniently operated with an immobilized form of D-hydantoinase.[488] A new approach illustrated with the resolution of D,L-threo-β-[4-(methylthio)phenyl]serine is based on the use of D-threonine aldolase from an *Arthrobacter* sp.[489]

Chromatographic resolution has continued to develop into more efficient versions. N-Boc- and -Z-D,L-α-amino acids can be resolved by elution over polysaccharide-based chiral stationary phases (CSPs),[490] N-(3,5-dinitrobenzoyl)-D,L-α-amino acid esters over homochiral phenylurea derivatives,[491] dansylamino acids over immobilized bovine serum albumin[492] and human serum albumin,[493] N-Boc-D,L-amino acids[494] and N-methylamino acids[495] over a teicoplanin-based CSP, amino acid esters[496] and N-protected amino acids[497] over immobilized α-chymotrypsin. Some of these studies use an underivatized amino acid, always D,L-tryptophan (a particularly convenient test species as used in the classical demonstration of enantiomer discrimination by natural homochiral species such as cellulose) with immobilized bovine serum albumin,[498,499] and bovine serum albumin membranes.[500] β-Cyclodextrin and its heptakis(3-O-methyl) derivative,[501] and L-tryptophanamide covalently bonded to β-cyclodextrin,[502] are examples of CSPs in one of the most active research categories; β-cyclodextrin-bonded CSPs are more effective for enantiomer resolution of N-benzoylamino acids compared with other common derivatives.[503] Synthetic CSPs are ever more sophisticated in concept, with ruthenium-porphyrin complexes carrying (S)- or (R)-α-methoxy-α-(trifluoromethyl)phenylacetyl residues on each side of the porphyrin plane,[504] α-(acetamidopyridyl)binaphthalenes bridged by but-2-yne-1,4-diyl- or 1,4- xylylene moieties,[505] quinine immobilized on 3-mercaptopropyl-silica gel (greater chiral discrimination compared with the N-Boc-quinine analogue),[506] and Crownpak

CR(+)®, an ODS matrix coated with a chiral crown ether applicable to resolution of hydrophobic amino acids.[507] The best known approach with CSPs based on dinitrophenyl derivatives has been extended to N-acylated L-proline anilides.[508] Separate enantiomers of D,L-N-(2,4-dinitrophenyl)amino acids assemble in chloroform–water mixtures containing a lipophilic 2′-deoxyguanosine derivative.[509]

The use of polymeric CSPs that have been imprinted by a homochiral additive during their preparation has broadened considerably, with membranes imprinted with protected L,L,L-tripeptides showing enhanced recognition of the L-enantiomer during adsorption of N^{α}-acetyl-D,L-tryptophan.[510] Corresponding adsorbents have been prepared from octadecyltrichlorosilane and indium and tin oxides[511] and sugar acrylates imprinted with Z-L-aspartic acid (and recognising the imprint),[512] imprinted acrylamide–methacrylic acid–vinylpyridine copolymers,[513] crosslinked poly(alkene)s (imprinting with L-phenylalanine leads to good chiral discrimination),[514] poly(acrylate)s (imprinting with Boc-L-phenylalanine and recognizing the imprint when adenine and 2-aminopyridine are incorporated in the polymer).[515] Further studies from other pioneers in this area (Volume 31, p. 44) employ common poly(alkene) polymers studied after imprinting with Boc-L-tryptophan,[516] and D-phenylalanine.[517] Nylon-6 imprinted with L-glutamine was found to show enhanced adsorption for the imprint in comparison with its enantiomer,[518] and cellulose acetate membranes imprinted with Z-D-glutamic acid allowed the D-enantiomer to permeate preferentially when presented with D,L-glutamic acid.[519] Poly(aniline) imprinted with (R)-camphorsulfonic acid adsorbs L-phenylalanine but not its enantiomer.[520]

The explanation for the enantiomeric imbalance in the amino acids originating in living organisms on Planet Earth has settled down to a few distinct categories of speculation, each with a considerable volume of literature.[521] Prebiotic (and current) delivery of extraterrestrial amino acids that have undergone enantioselective photodecomposition having been subjected to circularly-polarized infrared radiation is a favoured theory,[522] and radiolysis and radioracemization considered to have been verified in some laboratories for solid D- or L-leucine has been extended to representative oligo(L-leucine)s and poly(L-leucine)s.[523] Calculations of the parity-violating energy shift for L-valine support its small energy advantage relative to its enantiomer, thus giving more credence to both the electroweak energy theory and the Salam phase-transition theory as the basis for the predominance of the L-enantiomers of the α-amino acids.[524]

5 Physico-chemical Studies of Amino Acids

5.1 X-Ray Crystal Analysis of Amino Acids and Their Derivatives. – Crystal structure analysis data have been reported for amino acids [hexagonal L-cystine (electron density determination),[525] D,L-cysteine,[526] L-asparagine hydrate, D,L-glutamic acid hydrate, D,L-serine, and L-threonine (fast diffraction

using synchrotron radiation giving charge density distribution),[527] D,L-histidine,[528] 6-fluoroDOPA monohydrate[529]], amino acid salts [mono-L-alaninium nitrate,[530] mono-β-alaninium nitrate,[531] L-argininium diphenylacetate,[532] L-cysteinium L-tartrate monohydrate[533]], 1:1-amino acid–amino acid pairs [L-valine or L-leucine co-crystallized with D-2-aminobutanoic acid, D-2-aminopentanoic acid, or D-methionine,[534] L-isoleucine co-crystallized with each of seven D-amino acids,[535] D-norleucine co-crystallized with each of a number of L-amino acids[536]], derivatized amino acids [ethyl N-acetyl-L-tyrosinate (charge density study),[537] methyl D-phenylglycinate perchlorate–18-crown-6 complex,[538] N-benzenesulfonyl-D-glutamic acid,[539] N-(cytosinyl)-L-tyrosine,[540] N-[3-(cytosin-1-yl)propionyl]-L-isoleucine,[541] methyl N-ferrocenylglycinate,[542] 2-(toluene-p-sulfonylamino)- and -methylamino-butanoic acid fluorides and the corresponding 2-methylpropanoic acid derivatives,[543] N-trityl- and N-(9-phenylfluorenyl)-N-carbonylamino acid anhydrides (NCAs)[544]], and N-Boc-β-alanine N′-methylamide.[545]

5.2 Nuclear Magnetic Resonance Spectrometry. – Conventional studies cover ^{1}H-NMR of solid tyrosine derivatives (alone and mixed with L-leucinamide),[546] and N-acetyl-D-aspartic acid[547] and other amino acids assessed by ^{1}H-NMR *in vivo*.[548] N-Acetyl α-amino acid esters[549] and N-acyl (4R,2S)-4-amino-2,4-dimethylbutanoic acid,[550] N-(p-tolyl)- and N,N-dimethylglycines,[551] α-tert-butyl β-benzyl N-(p-chlorobiphenylsulfonyl)-3-allylaspartate,[552] and kainic acid[553] have yielded ^{1}H-NMR data, used to assess intramolecular hydrogen bonding, relative stereochemistry, and conformational equilibria.

Determination of enantiomeric excess data for amino acids has been reported for weakly acidic solutions containing the lanthanum(III) N,N,N′,N′-tetrakis(2-pyridinylmethyl)-(R)-propylenediamine complex[554] and a similar approach using (R)-(+)-[{Pd(η^5-C_5H_5)Fe(η^5-C_5H_3CMe=NAr)}(μ-Cl)]$_2$.[555] ^{1}H-NMRdata for (R)- or (S)-N-Boc-phenylglycyl derivatives of α-substituted primary amines $R^1R^2CHNH_2$ can be used for the same objective, also the reverse approach in which an amine of known absolute configuration provides the amides from amino acids whose enantiomeric excess values are required.[556]

Complete assignments of ^{13}C-NMR resonances have been deduced for N-(alkanoyl)- and N-(3-oxoalkanoyl)-L-homoserine lactones.[557]

Decay of ^{1}H-^{15}N-NMR 2-spin order data for L-tryptophan provides information on exchange kinetics for indole protons with water,[558] while more fundamental instrumental aspects leading to resolution enhancement have been established through ^{15}N-NMR of [per-^{15}N]L-arginine hydrochloride.[559]

^{31}P-NMR monitoring of aminoacylation of 5′-adenosine monophosphate by amino acids using standard condensation reagents illustrates one of the simplest uses of routine NMR spectroscopy.[560]

^{77}Se-NMR data for ^{77}Se-enriched L,L-selenocystine[561] and D,L-selenomethionine[562] have been determined.

5.3 Optical Rotatory Dispersion and Circular Dichroism. – Current options for deducing structural information from CD data are well shown for

L-histidine, at the membrane–water interface with phosphatidylcholine. The sign of the Cotton effect developed in the imidazole chromophore has been interpreted in terms of structure of the ion-pair that forms at the interface.[563]

CD data obtained with more conventional systems, [Pd(dmba)(acac)] complexes [dmba = 2-{(dimethylamino)methyl}phenyl-Cl],[564] Mo, Rh, or Ru complexes $[M_2(O_2CR)_4]^{n+}\ X^{n-}$ (molybdenum complexes give two Cotton effects in the 300–400 nm region; the other complexes give features at wavelengths up to 600 nm),[565] and copper(II) complexes of N,N-dialkylamino acids,[566] have been used in traditional applications of CD for the assignment of absolute configuration to amino acids. The last-mentioned study is notable for having demonstrated the acquisition of this information with only microgram quantities of an amino acid.

5.4 Mass Spectrometry. – As discussed in the adjacent sections, spectroscopic instrumentation continues to be applied to amino acids and their derivatives in both routine and pioneering contexts. MS study of underivatized amino acids currently falls in both these contexts, acknowledging the tailoring of ion sources to the special needs of amino acid studies. Glycine cations are accompanied by anions as a result of mild ionization, shown in a study aimed at correlating experimental data with molecular orbital calculations of bond cleavage.[567] Collision neutralization of the α-glycine cation leads to the corresponding radical which is stable on the microsecond time scale.[568] Features in the MS of sodiated and caesiated glycine and arginine indicate that sodium ions are solvated by both amino and carboxy groups, but caesium ions are solvated only by the carboxy group.[569] A long-standing problem, the differentiation of leucine from isoleucine by MS, succumbs to standard electrospray ionization MS when the respective $[M-H]^-$ ions, *m/z* 130, are separated on the basis of asymmetric waveform ion mobility, to allow quantitation of a mixture of one equivalent of either amino acid in the presence of 625 equivalents of the other.[570] Photoionization of ion beam-desorbed amino acids using femtosecond laser pulses at 195 nm and 260 nm has been studied, leading to decarboxylated ions at the shorter wavelength (except for tyrosine and tryptophan, from which the side-chain cation is produced by α-cleavage).[571] Chemical ionization MS of amino acids using dimethyl ether as reagent has been reviewed;[572] use of 2-methoxyethanol generates $[M+H]^+$, $[M+13]^+$, $[M+27]^+$, and $[M+77]^+$ from amino acids, with the first and last of these being the most abundant.[573]

Unashamedly routine applications of MS to amino acid analysis have appeared for homocysteine (after stable isotope dilution with $[^2H_8]$homocystine),[574] and mono- and dihydroxypipecolic acids in plant samples (negative ion MS–MS).[575]

MS spectra of amino acid derivatives are determined either to support analytical studies, or for extending the scope of newly-developing instrumental techniques. In the former category, MALDI-TOF data have been secured for N-acetylcysteine–1,4-dihydronaphthalene adducts,[576] MALDI-PSD and electrospray data for thionopeptides (thermal chain cleavage at the thioamide

residue),[577] electrospray ionization mass spectra for PTHs[578] and N-terminal analysis of peptides through PTH generation from PTC-peptides.[579] Valine and its naphthylamide, nitroanilide, N-dansyl, and PTH derivatives have been studied by laser-desorption MS, from which the benefits of derivatization for reliable analysis are demonstrated.[580] Estimation of the (N^{ε}-trimethyl)lysine content of human serum by MS using a salt of the methyl ester suffers interference from homoarginine due to coincident relative molecular mass, a problem that is avoided by derivatization with acetylacetone or by acetylation.[581] L-Amino acid methyl ester salts are incorporated better than their enantiomers into matrices of D-mannose, D-galactose, or D-glucose, and SIMS data for the resulting complexes can be used for optical purity determination of partly racemic amino acids.[582] Chiral recognition is established through FABMS of chiral crown ether complexes[583] and spiroacetal polyether complexes[584] of amino acid derivatives. These astonishing results are matched by MS of 1:1-amino acid–β-cyclodextrin complexes, determined after conventional electrospray ionization.[585] Collision-induced dissociation spectra of protonated trimers of amino acids formed by electrospray ionization in the presence of Boc-L- or D-phenylalanine, Boc-L-proline or O-benzyl Boc-L-serine, have been interpreted in terms of chiral recognition.[586]

Some of the mild ionization methods are applied to samples evaporated on to metal surfaces, and Li and Na binding energies of N-acetylamino acids have been determined to provide insight into factors affecting release of ions into the gas phase.[587]

5.5 Other Spectroscopic Studies of Amino Acids. – This, something of a catch-all section for spectroscopic data of amino acids not covered in preceding sections, has expanded in recent volumes because of simplified instrumentation for some classical and newer techniques. Fourier transform infrared data for glycine[588] and valine[589] in an argon matrix have proved to be a convenient means of demonstrating the proportions of the three predominant conformers in the former case and their interconversion through UV irradiation, and in the latter case the first observation of the non-ionized tautomer. Low temperature IR study of D,L-serine has been reported,[590] and familiar laboratory IR spectroscopic protocols have been applied to arginine and its derivatives,[591] to L-alanine,[592] and to the establishment of dimers in CCl_4 solutions of Boc-L-phenylalanine.[593]

IR-Raman spectroscopic studies of D,L-histidinium dinitrate and L-histidinium sulfamate,[594] L-asparagine monohydrate,[595] complexes of N-benzoyl-L- and D-leucine with β-cyclodextrin,[596] and L-tryptophan in KBr,[597] represent standard data-gathering applications, while surface-enhanced Raman scattering data for lysine adsorbed on gold colloid,[598] α,ω-diamino acids adsorbed on gold and silver surfaces,[599] and representative amino acids adsorbed on an electrochemically-roughened silver surface[600] provide information on structural and conformational aspects.

Microwave spectra of [^{18}O]-glycine[601] and alaninamide isotopomers[602] have been interpreted in terms of distributions of conformations.

ESR spectroscopy, the indispensible support of studies of mechanism in radical chemistry, has revealed attack by the hydroxyl radical at the α-carbon atom of glycine and side-chain H-abstraction with other amino acids,[603] and formation of transient radicals from histidine through reaction with hydroxyl radicals generated in the titanium(III)–H_2O_2 system.[604] Autoxidation of methyl 4-(N-hydroxyamino)-N-toluene-p-sulfonyl-L-prolinate has been shown to generate aminoxy radicals.[605] ESR monitoring of X-irradiated L-alanine reveals the formation of the well-known deamination product $MeC{\cdot}HCO_2H$ at room temperature but at higher temperatures another stable radical tends to predominate.[606]

Vibronic spectra of tyrosine and tryptophan in helium droplets at 0.38 K (determination of electron energy levels)[607] and electron diffraction of gaseous L-alanine (rotational constants and evidence for the adoption of the neutral tautomeric form) have been reported.[608]

5.6 Physico-chemical Studies of Amino Acids. – Sub-sections introduced recently for this chapter continue to provide a rational grouping of topics in this category. Some areas are relevant to an understanding of certain roles of amino acids in living systems, while other topics are routine. The development of the Amino Acid Index (a database for various physicochemical and biochemical properties of amino acids and pairs of amino acids) has been described.[609]

5.6.1 Measurements for Amino Acid Solutions. Solutions of familiar α-amino acids have featured in studies in which measurements are made leading to apparent molar volumes[610-615] (including viscosity-B coefficients,[610,613] and compressibility data[614-616]) standard molar enthalpies of solution and dilution,[617] enthalpic interaction[618,619] and pairwise enthalpic interaction coefficients[620] and activity coefficients.[621] ω-Amino acids are featured in a study in which apparent molar volumes have been determined.[622] Equivalent conductance data for amino acid mixtures in water have been interpreted to reveal side-chain interactions in certain amino acid pairs.[623]

Traditional study of the solubility of common aliphatic amino acids in aqueous $NaNO_3$ or KNO_3 shows no sign of coming to an end.[624] Solubility of one amino acid of a pair in aqueous media increases as the concentration of the other increases,[625] an observation that does not stand up to scrutiny in all combinations of L-cystine, L-tyrosine, L-leucine, or glycine with another amino acid.[626] Factors governing the solubility of amino acids in cationic reversed micelles have been investigated.[627] Of practical value is attainment of 0.2–3M solutions of amino acids in a water-free aprotic system [DMF, tertiary base, CF_3CO_2Na, $Ba(ClO_4)_2$, $Ca(ClO_4)_2$, $NaClO_4$, BaI_2, or $Ca(NO_3)_2$].[628]

Ion-exchange equilibria for amino acids in conventional separation systems have been the subject of thermodynamic modelling[629] and data for equilibria involving amino acids with a liquid sulfonic acid ion exchange medium have been obtained.[630]

Dissociation constants of amino acids have been determined for solutions in

aqueous isopropanol[631] and in aqueous dioxan,[632] and their protonation constants in aqueous dioxan.[633] An assessment has been made of the variation in the values for L-leucine as a function of ionic strength.[634]

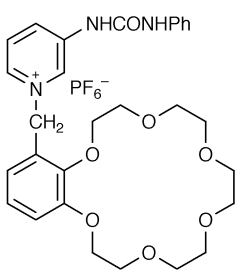
N-Hexadecyl Z-L-phenylalaninamides show remarkable gel-forming properties in organic solvents.[635]

5.6.2 Measurements for Solid Amino Acids. Finely-dispersed L-alanine undergoes a phase transition at 170 K as revealed by phonon echo signal data.[636] Combustion energy data determinations for 13 amino acids have been reported.[637]

5.6.3 Amino Acid Adsorption and Transport Phenomena. Partition of amino acids between immiscible organic and aqueous phases[638] has practical importance in various contexts, *e.g.* in continuous resolution of D,L-isoleucine by countercurrent fractional extraction using an enantioselective two-phase system.[639] A long-running study (Volume 30, p. 47) of the distribution of L-phenylalanine in aqueous di(2-ethylhexyl)phosphoric acid–octane[640] has developed into a study of transport of this amino acid,[641] L-histidine,[642] and L-glutamic acid[643] through corresponding emulsion membranes.

In a study that models an aspect of *in vivo* cellular behaviour, partition coefficients have been measured for amino acids in an aqueous two-phase system developed from dextran, poly(ethylene/glycol), and water.[644] Transport of amino acids through membranes is the other major interest in this category, again with an understanding of *in vivo* systems as the objective, and (−)-menthol- and (−)-nopol-derived mono- and dialkyl phosphates, phosphites, and phosphinites have been established to act as carriers of aromatic amino acids through supported liquid membranes but showing only low or moderate enantioselectivity.[645] A similar study using heteropolysiloxane membranes carrying chiral complexants has demonstrated facilitated transport of the L-enantiomer from D,L-phenylalanine through a pH gradient.[646] Studies of the effects of pH on interfacial transport of amino acids through a cation exchange resin have been reviewed.[647]

5.6.4 Host–Guest Studies with Amino Acids. Refinement of the understanding of the design of cage structures that act as receptors for amino acids follows from the establishment of a widening range of efficient host–guest systems, and there is no lack of new examples in the recent literature. The familiar structural types are not neglected, and recent studies cover 18-crown-6 in water–1,2-dichloroethane[648] and dibenzo-18-crown-6[649] as the means of switching amino acids into the organic phase. Substituted analogues (86)[650] and (87)[651] achieve the same result with neutral aqueous solutions and chloroform, by presenting attachment points for carboxylate and protonated amino groups of amino acids in their zwitterionic form. 5-(2-Carboxyphenyl)-10,15,20-triphenylporphyrins carrying homochiral substituents show selective recognition of amino acid esters,[652] and water-soluble porphyrins that act in this way with amino acids seem particularly promising.[653] Microcalorimetric studies show



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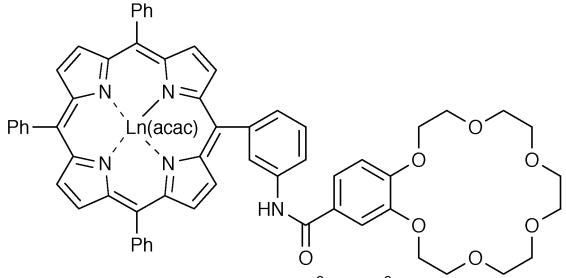
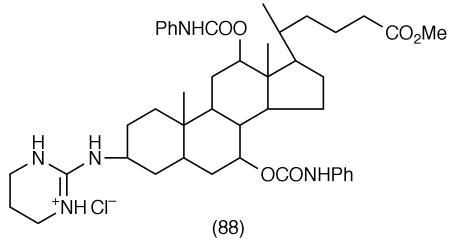


(87) Ln = Er^{3+} or Gd^{3+}



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efficient binding of p-sulfonatocalix[*n*]arenes ($n = 4,6,8$) to lysine and arginine in water,[654] supported by ^{1}H-NMR titration experiments,[655] with rigid peptidocalix[4]arenes showing improved binding characteristics for amino acids[656] and glycyl- and histidyl-calix[4]arenes showing useful complexation of cobalt(II) ions.[657] 5-(Guanidiniocarbonyl)-N-ethylpyrrole-2-carboxamide shows a propensity to bind α-(N-acetylamino) acids in 40% aqueous dimethyl sulfoxide,[658] and guanidinium-substituted cholic acid hosts (88) mediate the extraction of α-(N-acylamino) acids into chloroform from water, showing e.e. approaching 80%.[659] Weak complex formation in water between adenine and non-polar aliphatic amino acids, and stronger binding of polar and aromatic amino acids, is revealed in a thermometric study.[660]

Poly(vinyl alcohol) membranes substituted with β-cyclodextrin have been prepared, showing moderately enantioselective permeation by α-amino acids (improved to 25.4% e.e. with D,L-tryptophan after O-acetylation of the membrane).[661] An erratum[662] draws attention to misleading spectroscopic data concealing the use of impure samples of mono-[6-(m-toluidinyl)-6-deoxy]-β-cyclodextrin (Volume 30, p. 50) in host–guest studies, developed further by the same research group for organoselenium-modified β-cyclodextrins carrying an aromatic grouping.[663] Comparison of the relative effectiveness of L- and D-dansyl-L-leucine-modified β- and γ-cyclodextrins as hosts for amino acids and their derivatives,[664] and chiral recognition properties towards dansylamino acids of a β-cyclodextrin capped by an L-alanyl-crown(3)-L-alanine,[665] have been reported.

The foregoing host types have become well-established by now, and newer ideas are coming forward. Thus, Z-L-alanine and titanium n-butoxide adsorbed on TiO_2 gel give multilayered structures which participate in cycles of solvent extraction and selective binding of the L-enantiomer from solutions

of Z-D,L-alanine (similar examples of 'molecular imprinting' of adsorbents are dealt with in Section 4.17).[666] 'Carbosilane' dendrimers [a 1,3,5-benzenetriamide core substituted at nitrogen with tri(tri-alkylsilylalkyl)silylpropyl groupings] form 1:1-complexes with Fmoc-amino acids in $CHCl_3$, with structure-dependent association constants.[667]

5.7 Molecular Orbital Calculations for Amino Acids. – Calculations for amino acids and their derivatives follow objectives that are familiar from literature coverage in most of the preceding volumes of this series, with occasional extensions into novel areas. For amino acids, outcomes from MO studies of physical properties are: steric and electrostatic properties,[668] solvation parameters derived from atomic radii for constituents of side-chains,[669] densities of aqueous amino acid solutions,[670] absolute proton affinities,[671] charge distribution and molecular electrostatic potentials,[672] conformations (L-alanine[673] and tryptophan[674] in water, side-chain modified L-phenylalanine derivatives,[675] intramolecular interactions of side-chain groupings with the carboxylate anion in arginine[676]), gas-phase tautomerization of sarcosine,[677] spectroscopic data (near-edge X-ray absorption fine structure for cysteine,[678] NMR spectrum of histidine,[679] pH-dependent fluorescence decay of tyrosine and tryptophan[680]). Reactions of amino acids for which experimental data are compared with MO calculations are: high-temperature $^2H_2$–hydroxy-L-proline isotopic exchange,[681] stability and decomposition of stable glycine radicals[682] and L-alanine radicals,[683] and cationized arginine radicals $Arg{\cdot}M^+$ (M = alkali metal ion).[684]

Amino acid derivatives given similar attention are: N-acetylproline N′-methylamide (*cis–trans* isomerism[685]), N-formyl-L-proline N′-methylamide,[686] N-acetylalanine N′-methylamide,[687-689] N-acetyl-L-leucinamide (hydration parameters for comparison with structure determined by neutron scattering),[690] and betaine (crystal structure).[691] Association constants and related parameters have been computed for L-α-amino acid–β-cyclodextrin complexes.[692]

6 Chemical Studies of Amino Acids

6.1 Racemization. – Topics of interest under this heading continue to be researched further, falling mainly into distinct areas: laboratory studies of links between structural features and tendency to racemise; exploitation of racemization kinetics for fossil dating. Protein hydrolysis involves racemization of serine, an unlikely explanation having been advanced[693] that the D-enantiomer is more readily decomposed in the presence of the L-isomer. Further knowledge of the neglected amino acid racemase from *Pseudomonas putida* demonstrates its ineffectiveness with aromatic and acidic amino acids, allowing $^1H - {}^2H$ exchange with retention of configuration for L-phenylalanine and (S)-phenylglycine in ^2H_2O.[694]

The credibility of fossil dating through determination of D:L-ratios of

indigenous amino acids has suffered considerably because corrections to racemization kinetics cannot be computed for catalytic effects of other consituents in the fossil. Amino acids resident in samples for 10^5 – 10^6 y are totally racemized, and the dating methodology for much younger samples employs those amino acids that are most rapidly racemized. It is these for which new data have been obtained. Thus, aspartic acid racemization applied to bone dating needs to take account of a rapid initial phase which seems to be due to structural changes in the protein (L-asparagine→L-aspartic acid→L-cyclic imide→D-aspartic acid). Although the calculated L:D values are borne out experimentally for the aspartic acid content of proteins at 95–140 °C, the model fails for dentin at 37 °C because the tendency towards cyclic imide formation is conformation dependent and is particularly difficult for this protein and for collagen.[695] For L-isoleucine, whose racemization rate (α-chiral centre) is subject to catalytic effects of unknown origin, it has been suggested that the very slow racemization at the β-chiral centre would be a better basis for dating of fossils.[696] In other words, the method should be restricted to much older fossils than those that have been subjected to the technique recently; this proposal, however, overlooks the fact that the inevitable structural change at the α-chiral centre will affect the kinetics of the racemization process at the β-chiral centre.

Conference reports (ref. 521) include reviews on amino acid racemization and original papers, *e.g.* aspartic acid racemization data for dentin from cave bear fossils, which places the lifetime of the creatures in a wide range of the Pleistocene era.[697]

6.2 General Reactions of Amino Acids. – *6.2.1 Thermal Stability of Amino Acids.* Thermal degradation of amino acids requires investigation, not only for its obvious importance in food science, but also so that problems that arise in amino acid sampling for analysis may be understood. Controversy has arisen over claims that amino acids can be sublimed unchanged (Volume 31, p. 53), since there have been many reports over the years of self-condensation and other changes to amino acids at elevated temperatures. Amino acids on silica gel at 230–250 °C give piperazin-2,5-diones, hexahydroimidazo[1,2-*a*]-pyrazin-3,6-diones and hexahydroimidazo[1,2-*a*]imidazo[1,2-*d*]pyrazin-3,8-diones.[698] Loss of serine and threonine is complete after samples are held for 4 h at 120 °C or for 7 min at 300 °C, leading to pyrazines among other products.[699] Differential thermal analysis and thermogravimetry have been used to study the thermal degradation of α-, β-, and γ-aminobutyric acids and threonine.[700]

6.2.2 Reactions at the Amino Group. The literature on oxidation of amino acids by familiar oxidants continues to be voluminous and too routine to cover here; the policy of previous volumes, to restrict discussion to careful studies with novel oxidant systems, is illustrated by choosing to mention a study of deamination and subsequent decarboxylation of glycine by gold(IV) species, leading to gold(0), glyoxylic acid and ammonium formate.[701] This relatively

standard outcome of oxidation is implicit in the reaction of amino acids with ninhydrin at elevated temperatures, a process which is accelerated in cationic micelles.[702]

Other familiar deamination reactions, aspartate transaminase-mediated equilibration of L-aspartic acid and 2-oxoglutaric acid with L-glutamic acid and oxaloacetic acid (determination of reaction constants),[703] non-stereoselective conversion of α-amino acids into α-hydroxy acids by *Clostridium butyricum*,[704] and inversion of configuration of L-alanine by NAD^+/L-alanine dehydrogenase oxidation, electrochemical regeneration of NAD^+ and reductive amination of pyruvate at the mercury cathode,[705] have been the subject of quantitative studies. The last-mentioned study demonstrates the importance of optimized experimental conditions in making the overall process viable, through circumventing the unfavourable thermodynamics of certain electrochemical steps.

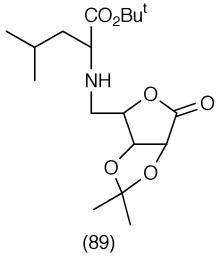
Reductive deamination of α-aminocarbonyl compounds by SmI_2 in THF-HMPA together with a proton source gives the expected result with methyl phenylalaninate, but an unusual outcome in the ring-opening of methyl N-benzylprolinate to give $BzlNH(CH_2)_4CO_2Me$,[706] also seen in reaction of iodine with (diacetoxyiodo)benzene or iodosylbenzene that leads to decarboxylation of pivaloylproline to give $RCONH(CH_2)_3CHO$ through an intermediate N-acyliminum ion.[707]

A permanent feature of this section because of its fascination and importance, studies of the mechanism of the Maillard reaction and its products, continues to reveal surprising new aspects. Thus, L-alanine–pentose or hexose mixtures generate pyrazinium radicals *en route* to conventional Maillard products.[708] Analysis by GLC-MS suggest that the formation of branched-chain alkyl-substituted pyrazines from such mixtures proceeds *via* a Strecker aldehyde.[709] As would be expected, glycosylated amino acids are mild reducing agents and their role in reducing nitrite to nitric oxide under anaerobic conditions[710] may give them an important physiological role. Fully-protected glucosylamino acids formed by Mitsunobu coupling of N-(o-nitrobenzenesulfonyl)amino acids with 2,3,4,6-tetra-O-acetyl-D-glucose, and their Amadori rearrangement products, have been described.[711]

Schiff bases formed between cinnamaldehyde and an L-amino acid ester are equally well viewed as homochiral azadienes, and their complexes with $Fe(CO)_5$ catalyse formation of 4-methoxycyclohexa-1,3-diene-$Fe(CO)_3$ with modest preponderance of the (R)-enantiomer.[712] Schiff bases formed between amino acid esters and a diaryl ketone are readily converted into 1,2-diaryl-2,2-dichloroaziridines through addition of dichlorocarbene,[713] and N-aziridination (solid phase-tethered amino acid reacted with α-bromoacrylates) has been simplified.[714] Azadienes $R^1R^2C{=}CHN{=}CHP(O)(OEt)_3$ can be converted into aziridinephosphonates with diazomethane.[715] Other reactions that involve *in situ* Schiff base formation include monoalkylation [N-ethylation through reaction with acetaldehyde and $NaBH(OAc)_3$,[716] and N-methylation using hexafluoroacetone-protected amino acids[717]]. N-(β-Boc-Aminoalkyl)ation of α-amino acid esters using N-protected α-aminoaldehydes[718] and analogous

N-(β-Fmoc-aminoalkyl)ation with the equivalent α-amino acid S-ethyl thioesters[719] have been developed; in the former case, this step was followed by N-acylation with (thymin-1-yl)acetic acid and related nucleobase moieties, using TBTU as condensing reagent to give four new PNA monomers (see also Section 4.8). In another study, new PNA monomers, one carrying an N-(pyreneacetyl) grouping[720] and others carrying homologated glycine moieties,[721] have been prepared. N-(2,4-Diethoxycarbonylbuta-1,3-dienyl)amino acid esters are formed using ethyl propynoate as reactant.[722] The conversion of amino acids into silapiperidines through reaction with $Ph_2Si(CH_2CH_2OTs)_2$ offers a new selectively-removeable N-protecting group.[723] Diborane-iodine reduction of solid-phase-tethered N-α-acylamino acids gives secondary amines (tethered α-imino acids).[724]

N-Methylation *via* oxazolidinones obtained from α-amino acids, through treatment with $Na(CN)BH_3/TMSCl$[725] is a standard protocol; straightforward amino acid derivatization operations such as these are covered in a new textbook.[726] N-Alkylation of tert-butyl L-leucinate with a pentose triflate gave the derivative (89) which was developed into the 3-aminopiperidin-2-one (90) intended as a seryl-leucine surrogate.[727] The triflate of ethyl (S)-lactate reacts with ethyl L-alaninate in refluxing nitromethane to give the homochiral C_3-triisopropylamine $N(CHMeCO_2Et)_3$.[728] Mannich reactions of amino acids with 3-phenoxychromones have been described.[729]



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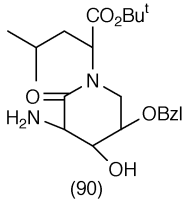

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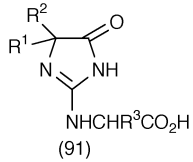

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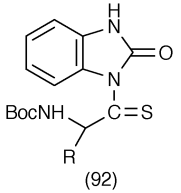
Cyclization of bis(chloromethyl)phenol-formaldehyde tetramers bonded through nitrogen to amino acid methyl esters gives chiral concave calix[*n*]-arenes capable of molecular recognition favouring one enantiomer of a chiral ammonium salt.[730] N-Arylation of amino acids using electron-deficient aryl fluorides is a classical operation that has been extended to 4(6)-mono- and difluoropyrimidinylation through use of 2,4,6-trifluoropyrimidine as reagent.[731] N-(Imidazol-5-on)yl derivatives (91) have been described.[732]

N-Acylation of amino acids serves a range of purposes, particularly the need for reversible N-protection for applications in synthesis. Improved methodology for well-established groupings in this category are: N-formyl [introduced into ethyl N-(ethyl phosphonomethyl)glycinate with triethyl orthoformate],[733] N-trifluoroacetyl (introduced using N-trifluoroacetylsuccinimide),[734] N-acetyl (cleaved with α-chymotrypsin in acetone–alcohol mixtures),[735] N-phenylacetyl (introduction into β-amino acid esters using penicillin G acylase; see also Section 4.17),[736] N-tert-butoxycarbonyl (cleaved with $AlCl_3$;[737] preparation of

tert-butyl N-Boc-S-trityl-L-cysteinate[738]), and N-benzyloxycarbonyl (clean removal using zinc powder in neutral aqueous conditions[739]).

The tri-isopropylsilyloxycarbonyl ('Tsoc') grouping has been advocated for N-protection; it is labile to fluoride ion, so is orthogonal to Boc, Z, and Fmoc in the context of peptide synthesis.[740] N-(Propargyloxycarbonyl)amino acids, originally reported in 1994,[741] are stable to TFA but are cleaved by $Co_2(CO)_8$ in TFA,[742] and are readily cleaved by benzyltriethylammonium tetrathiomolybdate.[743] An N-Z- or N-Boc-sulfonamide $CF_3SO_2NR(p\text{-}CF_3\text{-}C_6H_4)$, R = Z or Boc, is an effective alkoxycarbonylation reagent.[744] The N-[(E)-2-(methylsulfonyl)-3-phenyl-2-propenyloxycarbonyl] (Mspoc) group (introduced using Mspoc-ONSu) is less prone to premature deblocking during peptide synthesis compared with previously-advocated Bspoc and Bsmoc groupings.[745]

N-Acetylation and N-phenylacetylation of PNA monomers has been described, for preparation of corresponding derivatives of the classical DNA mimics.[746] N-Acylation and thioacylation procedures relevant to amino acid analysis are: N-[(S)-(O-acetyl)lactoyl]ation to determine D:L-ratios,[747] N-[β-(Boc-aminoalkyl)thioacyl]ation using (92),[748] phenylthiocarbamoylation[749] and preparation of analogous fluorescent Edman derivatives using 7-[(N,N-dimethylamino)sulfonyl]-2,1,3-benzoxadiazol-4-yl isothiocyanate[750] and (93; R = SO_2Me or SO_2Ph).[751]



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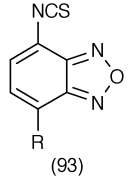

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Allylic carbonates $R^1CH{=}CR^2CH_2OCO_2Et$, carbon monoxide, and $PdCl_2$/dppb react with amino acid esters to give β,γ-unsaturated amides $R^1CH{=}CR^2CH_2CONHR^3$.[752] A classical acylation procedure that is unusual in the amino acid context employs an α-bromoketene (prepared from 3-aryl-2,2-dicyano-oxirane, Li_2NNiBr_4, and Et_3N) leading to N-(α-aryl-α-bromoacetyl)amino acid esters.[753] Other N-acyl derivatives reported in the recent literature are N-(o-carboxybenzoyl)-[754] and N-(o-aminobenzoyl)-[755] L-α-amino acids (the latter showing UV fluorescence properties useful in analysis), and N-acyl-N-hydroxy-L-phenylalanine derivatives,[756] the last-mentioned showing promise as carboxypeptidase A inhibitors. Debenzoylation of N-benzoylamino acid derivatives through N-tert-butyloxycarbonylation followed by $Mg(OMe)_2$ in MeOH at room temperature has been established within a Paclitaxel synthesis.[757]

Photoreactive N-(6-azido-1-oxoindan-4-onyl)amino acids have been prepared for molecular probe studies, for the identification of putative receptors and binding proteins in plants.[758]

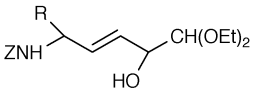
Conversion of amino acids into ureas through reaction of derived isocyanates with amino acid esters,[759] and through a solid phase-tethered isocyanato-

acid with simple amines,[760] provides starting materials for potentially effective pharmaceuticals. Decarbamoylation of simple ureas $H_2NCONHCHRCO_2H$ occurs using nitrogen peroxide (or its equivalent; a mixture of nitric oxide and oxygen) in water.[761]

6.2.3 Reactions at the Carboxy Group. Like the preceding section, papers in this category describe a similar mixture of improvements to well-established procedures, as well as novel procedures. The carboxy group of an N-protected amino acid may be displaced by arylation (*e.g.* N-tosylalanine→Ph_2CHMe with benzene and conc H_2SO_4; Volume 31, p. 62)[762] and by a sulfonic acid group [(R)-N-ethoxycarbonyl-D,L-norleucine→2-aminohexanesulfonic acid].[763] Hypotaurine partially disproportionates into taurine, 2-aminoethyl-2-aminoethanethiolsulfonate, and ethanolamine as its solution in hydrochloric acid is evaporated.[764]

Esterification (tert-butyl esters prepared using a di-tert-butyl dicarbonate;[765] methyl esters prepared using a strong acid ion exchange resin suspended in methanol,[766] 9-fluorenylmethyl esters prepared using 9-fluorenylmethylchloroformate;[767] aryl esters from aryl 4-nitrobenzenesulfonates[768]), reduction (CO_2H→CH_2OH using $NaBH_4$-$NiCl_2$ or MoO_3 in water,[769] using $NaBH_4$-cyanuric chloride,[770] *via* pentachlorophenyl esters using $NaBH_4$-I_2 in THF,[771] *via* oxazolidinones using $NaBH_4$,[772] *via* the BuLi–DIBALH 'ate complex' for highly hindered α,α-dialkyl-α-amino acid esters, or using $LiAlH_4$ with persilylated α-benzylhistidine[773]), and further elaboration of the CH_2OH group (→CH_2I→$CH_2CH{=}CH_2$) to convert L-norvaline into the substrate for a Grubbs' ring-closing metathesis synthesis of (S)-(+)-coniine have been reported.[774] Classical Grignard addition to protected serines (CO_2H→CPh_2OH) yields ligands for zinc reagents that have been developed for aldehyde elaborations.[775] Acid chloride preparations [Fmoc-amino acids with bis(trichloromethyl) carbonate[776]] have been studied. N-Protected β-aminoalkanols provide the starting point for the preparation of N-(β-aminoalkyl)amino acid derivatives through Mitsunobu coupling with N-Pmc-amino acid esters.[777]

Partial reduction to give N-protected L-α-aminoaldehydes has become a standard starting point for general organic synthesis, now that initial difficulties in methodology and retaining optical stability of the products have been overcome. An undergraduate exercise proposed to fill five 8-hour laboratory periods starts from L-amino acids and proceeds *via* Z-L-α-aminoaldehydes to products (94) and (95).[778] N-Protected β-amino aldehydes can be prepared



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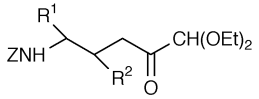

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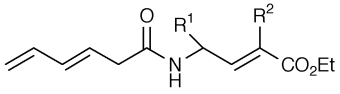


(96)

from α,β-amino acids ($NaBH_4$ reduction of unsymmetrical anhydride, then MnO_2 oxidation) and thence to δ-amino-α,β-unsaturated alkanoates through Wittig homologation.[779]

N-Boc-α-Amino aldehydes (ref. 127) have been converted into (E)-methoxyalkenes BocNHCHRCH=CHOMe and onwards to α-phenylseleno-β-amino aldehydes that can be transformed into epoxides and aziridinecarboxylic acids;[780] into the Diels-Alder substrate (96) through an obvious series of reactions;[781] and two-carbon elongation of an L-serine ester after conversion into an N-tritylaziridinecarboxylate, involving Claisen condensation with the enolate of an alkyl acetate to give the γ-amino-β-ketoester $R^1NHCHR^2COCH_2CO_2R^3$ that opens up synthesis of trisubstituted E-alkenes and N-allylamines.[782] Allylation of protected L-tyrosinal gives a mixture of syn- and anti-2-amino alcohols developed further into β-turn mimetics.[783] Further elaboration of 2,3-epoxyalkanols produced by transformation of the carboxy group of a Boc-amino acid gives 1-cyano-2,3-diols by reaction with diethylaluminium cyanide.[784] A route to erythro-(N-protected α-aminoalkyl)-epoxide that differs from the usual elaboration of an amino acid has been illustrated using $PhCH_2CH(OH)C{\equiv}CTMS$ for preparation of the phenylalanine-related compound used in the production of Saquinavir.[785]

The generation of a ketosulfone by reaction of a protected L-tyrosine ester with the dilithio anion of methyl phenyl sulfone requires two equivalents of reagent.[786] Formation of an ylide [$CO_2H{\rightarrow}COC(=PPh_3)CN$] from a protected L-phenylalanine by coupling to $Ph_3P{=}CCN$ opens up a route to α-keto-amides and peptidic α-hydroxy-amides found in bacterial secondary metabolites phebestin, probestin and bestatin.[787] α-Ketophosphonates can be obtained by Arbuzov reaction of a protected amino acid chloride with triethyl phosphite.[788]

α-Aminoketones can be formed *via* Weinreb amides [$CO_2H{\rightarrow}CONMe(OMe){\rightarrow}COCH_2CH_2Ph$][789] and morpholides;[790] α-(N,N-dibenzylamino) ketones are substrates for stereoselective conversion into tertiary alcohols through non-chelation controlled Grignard type reactions[791] and stereoselective reductive amination.[792] The latter process gives 1,3-diaminoalkanes when applied to β-aminoketones prepared from Weinreb amides of N-protected β-amino acids;[793] an alternative route to these involves Curtius rearrangement [$BocNHCHRCH_2CO_2H{\rightarrow}BocNHCHRCH_2NHCO(Nsu)$].[794] Conversion of N-Boc-β-alanine into N-[3-(N-Boc-amino)thiopropanoyl]phthalimide and thence to ethyl 3-aminodithiopropanoate has been developed using standard thionation protocols.[795] C_2-Symmetrical enantiopure β,β′-diaminoalkyl sulfides have been prepared through a lengthy route starting from α-amino acids.[796]

N-α-(Boc-Amino)acylsilanes $BocNHCHRCOSiMe_2Ph$ offer valuable three-carbon elongation opportunities, those from phenylalanine and isoleucine giving statines through aldol addition and conversion into N-protected β-aminoalkanols.[797] Condensation of $TMSCH_2MgCl/CeCl_3$ with ethyl (R)-β-amino-β-phenylpropionate gives homologue $PhCH(NR^1R^2)CH_2C(=CH_2)CH_2TMS$.[798]

Enantioselective amino acid ester hydrolysis data have been accumulating for many years, and added to by finding up to threefold differentiation between N-protected D- and L-phenylalanine p-nitrophenyl esters in the

presence of (+)-tubocurarine,[799] and similar mediation by chiral metallomicelles [lipophilic copper(II) complexes][800] and N-benzyloxycarbonyl-L-Phe-L-His-L-Leu-OH[801] in the hydrolysis of N-dodecanoyl D- and L-phenylalanine p-nitrophenyl esters.

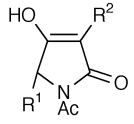
Ammonolysis of methyl D,L-phenylglycinate in tert-butanol at 40 °C catalysed by Novozym 435 (*i.e.*, *Candida antarctica* lipase B) leads to D-phenylglycinamide in 78% e.e. at 46% conversion, pyridoxal-mediated racemization of the unconverted ester contributing to an efficient protocol (but only if operated at −20 °C, when the amide racemises much more slowly than the ester).[802] Not surprisingly, ammonium chloride with an N-protected amino acid and base gives primary amides through a peptide synthesis protocol.[803] A reaction mixture containing an N-protected amino acid, isobutyl chloroformate, and an amine gives amides through a kinetically-controlled process; the disdain with which experienced peptide chemists would dismiss such a protocol is unwarranted since N-tert-butyloxycarbonylation is discovered to be insignificant.[804] Solid phase synthesis of Fmoc-amino acid amides [reductive amination of tethered 4-formyl-3,5-dimethoxyphenoxyvaleric acid (HCO-link-P→R^1NHCH-link-P→R^2CONHR^1CH-link-P→R^2CONHR^1 by conventional protocols] is applicable also to the preparation of sulfonamides[805] (it is presumably suitable for phosphorus acid amides, too). HMDS-Promoted amidation of Boc-L-alanine requires drastic conditions (110 °C) but gives mono-acyl derivatives with di-amines.[806] Condensation of arylalkylamines with N-Z-L-phenylalanine carbamoylmethyl ester is effected by the use of α-chymotrypsin in acetonitrile with low water content,[807] and tyrosinase-mediated cleavage of N-protected amino acid phenylhydrazides offers a novel C-protection strategy.[808] Hydroxylaminolysis of N-protected amino acids leading to N-protected α-aminohydroxamic acids is best accomplished *via* oxazolidinones,[809] for which a simple preparation driven by microwave irradiation has been developed.[810]

6.2.4 Reactions at Both Amino and Carboxy Groups. Heterocyclic synthesis using α-amino acids has been reviewed[811] with special reference to aziridine-2-carboxylic acid and 3-aminoazetidin-2-ones.[812] Standard applications have been illustrated in recent papers, with new details: N-acetyltetramic acids (97) prepared through condensation of N-acetyl-L-α-amino acid N′-hydroxysuccinimidyl esters with malonate anions are only partially racemized;[813] ammonium formate serves as condensing agent for conversion of α-[N,N-di(carboxymethyl)amino] acids into 3,5-dioxopiperazinoalkanecarboxylic acids;[814] α-(N-acylamino) acids attached to a solid phase reduced and then converted into 1,6-disubstituted 2,3-diketodihydropiperazines.[815] Oxazolidinone formation from α-amino acids and trifluoroacetone, a useful one-step protection strategy for both amino and carboxy groups, gives an intermediate iminium species (98) when conducted with N-chloromethylamino acids (prepared from the amino acid, formaldehyde, and $SOCl_2$; [1,3]cycloaddition with alkenes verifies the nature of the intermediate).[816] Dieckmann reaction of adducts of (R)-β-amino esters with methyl acrylate, and hydrogenation of the

resulting enol ethers, gives 2,4,5-trisubstituted piperidines with high diastereoselectivity.[817]

Condensation of tartaric acid with N-benzylaminoalkanols prepared from L-α-amino acids leads to 3-aza-6,8-dioxabicyclo[3.2.1]octane-7-carboxylic acids,[818] and amino acid phenylhydrazides yield hexahydro-1,2,4-triazin-6-ones in aqueous formaldehyde.[819] A longer pathway starting from L-proline leading to the saturated bicyclic system (99) *via* (S)-2-hydroxymethyl-N-Boc-pyrrolidine provides a chiral catalyst for Baylis-Hillman reaction of aldehydes with vinyl ketones.[820]

'N-Carboxyanhydrides' (NCAs; *alias* oxazolidin-2,5-diones), well known for their propensity to polymerise to give oligo- and poly(α-amino acid)s, and for acting as Friedel-Crafts acylating agents towards arenes in the presence of $AlCl_3$,[821] can be formed in less than one hour at room temperature from solid N-carbamoylamino acids (Volume 29, p. 72) in an atmosphere of nitric oxide and oxygen in proportions 4:1.[822] Thiohydantoins are released in the newly-revived C-terminal peptide sequence determination protocol, and standards have been prepared from N-protected amino acids, dicyclohexylcarbodi-imide, and trimethylsilyl isothiocyanate (see also Volume 30, p. 60).[823]



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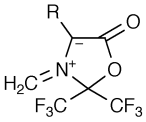

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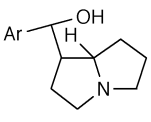

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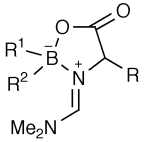

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Oxazaborolidinones (100) can be obtained as a single diastereoisomer by crystallization-induced asymmetric transformation; the stereogenic boron atom resists equilibration on the time-scale of enolate alkylation with iodomethane and other common electrophiles.[824] These heterocycles, derived from homochiral α-amino acids, are employed as chiral catalysts for aldol reactions,[825] and for asymmetric borane reduction of cyclic meso-imides.[826]

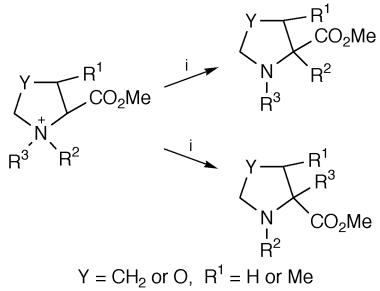
The formation of peptides from amino acid mixtures is becoming a major topic of research, particularly in the context of prebiotic protein synthesis from partly-resolved amino acids (for a review see ref. 827). Most of these studies involve an insoluble inorganic medium as catalyst; clays promote the formation of glycine oligomers but lack the ability to perform similarly with alanine,[828] and clays are therefore established to offer an alternative to salt-induced self-condensation (Volume 31, p. 63) of amino acids.[829] However, oligomerization of glycine has been demonstrated in a flow reactor that simulates a submarine hydrothermal vent but lacks any condensation reagent or metal ion or template catalyst (such as a clay or other mineral).[830] Carbonyl di-imidazole is a condensation reagent that causes oligomerization of L-glutamic acid in water, but not of γ-carboxy-L-glutamic acid, which is oligomerized by magnesium salts and hydroxylapatite.[831]

6.2.5 Reactions at the α-Carbon Atom of α- and β-Amino Acids. Papers under this heading are mostly collected under applications of α-amino acid alkylation

in synthesis (Sections 4.1.5, 4.5, 6.3). Conventional α-alkylation of homochiral β-(pyrrolidin- and piperidin-2-yl)acetates is highly diastereoselective,[832] and α-hydroxylation of β-benzoylamino esters through iodination of the anion (NaHMDS) followed by hydrolysis depends on the intermediacy of a phenyloxazoline.[833] α-Thiocyanation of enamino esters is achieved using 4-chloro-5H-1,2,3-dithiazol-5-one.[834]

6.3 Specific Reactions of Amino Acids. – This section collects papers that deal with structural changes to side-chains of common amino acids, through reactions that often also involve amino or carboxy groups. Procedures in this category can be applied to proteins for modification of side-chains to assist amino acid analysis procedures.[835] A 'Practical Approach' monograph includes protocols for side-chain modifications to several coded L-α-amino acids.[836]

Saturated aliphatic side-chains show a limited range of reactions that result in their functionalization. Oxidation with 3,3-dimethyldioxirane leads mainly to O-insertion into C–H bonds (notably, in the side-chain in preference to the α-CH bond),[837] sodium m-chloroperbenzoate and O_2 can convert benzylic methylene of 1-aminoindane-1-carboxylic acid derivatives to C=O,[838] and proline 3- and 4-hydroxylases mediate the regio- and stereospecific hydroxylation of L-2-azetidinecarboxylate, 3,4-dehydro-L-proline, and L-pipecolic acid.[839] Halogenation is particularly useful since it can open up further synthesis opportunities, such as conversion of protected 4-bromoglutamic acid into heteroatom substitution products, *e.g.* 4-mercaptoglutamic acid.[840] Cyclopropane formation from methyl (S)-N-phthaloyl 4-bromoleucinate *via* the α-methoxyamide that is generated with $NaBH_4$–MeOH to give the protected '2,3-methanovaline',[841] and of '3,4-methano-L-glutamic acid', *alias* L-2-carboxy(2-carboxycyclopropyl)glycine, prepared from the 3,4-dehydro-amino acid orthoester and diazomethane has been reported.[842] Completely stereospecific [1,2]- or [2,3]-shifts occur with ylides generated from N,N-dialkylproline or -threonine derivatives by treatment with Bu^tOK (Scheme 22).[843]

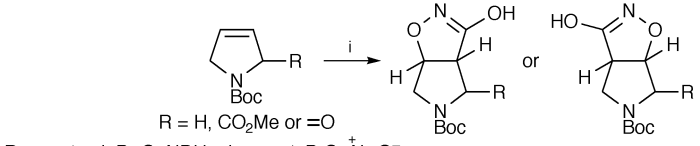


Reagent: i, $KOBu^t$

Scheme 22

α,β-Dehydroalanine prepared on a solid phase exhibits standard Diels-Alder addition behaviour.[844] Methyl N-cinnamyl-N-Z-L-vinylglycinate undergoes sensitized intramolecular [2+2]photoaddition to azabicyloheptanes,[845] and N-

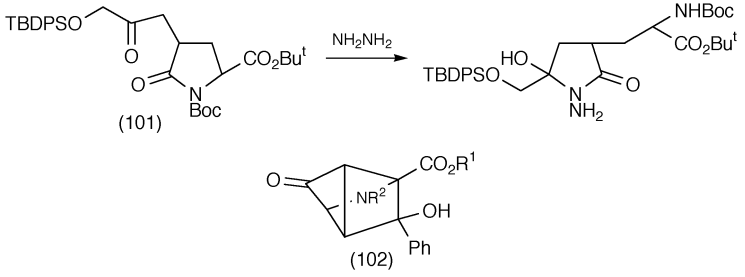
ethynyl-L-allylglycine gives highly functionalized prolines through an intramolecular Pauson-Khand reaction.[846] 3,4-Dehydro-L-proline is one of the most readily accessible alkene analogues of common L-α-amino acids, and is the starting point for preparation of a version of L-proline in which all methylene groups are stereoselectively labelled with ^2H (catalytic deuteriation, RuO_4 oxidation to the labelled pyroglutamate, then syn-selective deuteriation of the derived aminal with Et_3Si^2H-BF_3.Et_2O).[847] Cycloaddition of bromonitrile oxide to Δ^3-pyrrolines gives the bicyclic isoxazolinylprolines (Scheme 23),[848,849] one of these studies covering a broad range of substrates (ref. 848) and the other study (ref. 849) proposing the products from dehydroproline as kainate receptor agonists. 1,2-Didehydroproline benzyl ester N-oxide is a source of isoxazolidine and isoxazoline analogues of proline through cycloaddition to alkenes and alkynes respectively.[850]



Scheme 23

Propargylglycine ethyl ester has been subjected to Rh_2{(2S)-nepy}$_4$-catalysed cyclopropenation to give ethyl 2-aminomethylcycloprop-2-ene-1-carboxylate as destined for testing as GABA analogues.[851]

A keto group in an amino acid side-chain activates neighbouring structures towards attack, as in 'ring-switching' conversion of the pyroglutamate analogue (101) into a β-substituted L-alanine.[852] As a consequence of photoactivation, different types of structural change can ensue, as with phenyl ketones derived from L-4-oxoproline giving (102).[853] Baeyer-Villiger oxidation

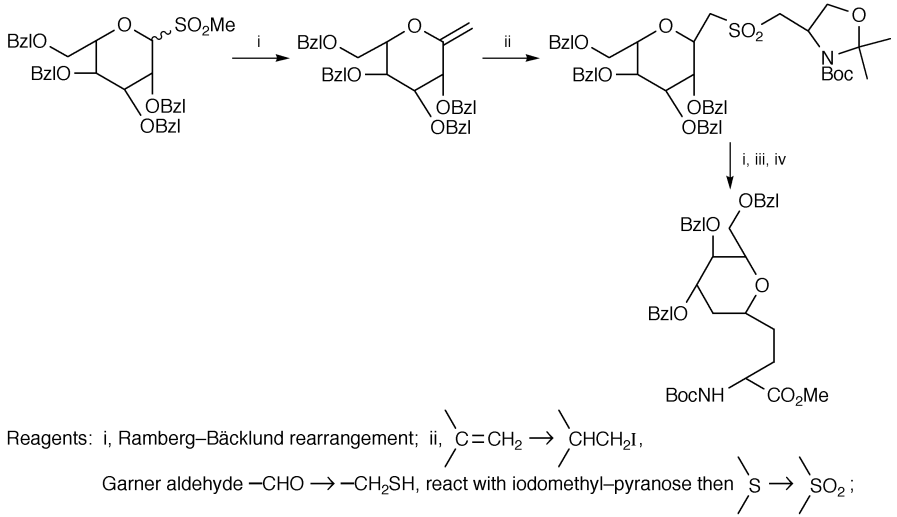


[m-chloroperbenzoic acid catalysed by copper(II) acetate] of this proline derivative gives L-aspartic acid, and prior alkylation adjacent to the keto-group of the substrate delivers β-substituted aspartic acids.[854] tert-Butyl N-Z-4-oxoprolinate undergoes reductive amination with amino acid esters to give (4S)-4-alkylaminoprolines, from which 3-oxo-1,4-diazabicyclo[2.2.1]heptanes have been obtained by cyclization.[855] *trans*-3-Alkylprolines have been prepared by aldolization of the enolate of N-(9-fluoren-9-yl)-4-oxoproline and routine

steps to complete the process.[856] Full reduction of the keto-group in the corresponding aryl ketones using $Et_3SiH/TiCl_4$ establishes syntheses of N-protected 2-amino-4-arylbutanoic acid and 2-amino-5-arylpentanoic acid.[857]

Hydroxyalkyl side-chains generate a profusion of synthesis opportunities, and reviews have appeared of serine derivatives[858] and of N-tritylserine and allothreonine derivatives.[859] O-Glycosylation can be effected through Michael addition of protected serines and threonines to D-galactals,[860] O-prenylation *via* methyl N-Z-aziridinecarboxylate,[861] and O-cyclohexylation *via* the cyclohexenyl ether.[862] Other straightforward manipulations lead to γ-carboxy-D,L-glutamic acid (dehydration to give methyl N-tetrachlorophthaloyl dehydroalaninate, used for Michael addition to a dialkyl malonate),[863] 2,3-disubstituted glutamic acid derivatives through 1,4-addition of the lithium salt of L-threonine-derived 2-phenyl-4-methyloxazoline-5-carboxylate ester to Z-α,β-unsaturated esters,[864] (S)-3,4-dehydroproline (from O-allyl-D-serine),[865] and biomimetic conversion into 4-bromotryptophan (D,L-serine, 4-bromoindole, and *Aspergillus* acylase).[866] Mitsunobu processing of β-hydroxy-α-amino acids to give β-substituted α,β-di-amino acids benefits from the use of cyclic orthoester protection of the carboxy group.[867] L-Serine initiates a route to 3-amino-2-phenylpiperidines including a Substance P antagonist,[868] and D-serine has been used for synthesis of oxazolidinylpiperidines that are starting materials for the preparation of azasugars.[869]

Further uses have been reported for the Garner aldehyde (see also refs. 260, 268, 367), in which the amino group and side-chain function of L- or D-serine are mutually protected through cyclization, and the carboxy group is reduced to aldehyde. An improved synthesis has been published (88% overall yield in four steps)[870] by a group which has developed uses of the (R)-synthon for preparation of C-glycosyl-serines and α-asparagines (Scheme 24)[871] and cross-

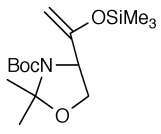


Reagents: i, Ramberg–Bäcklund rearrangement; ii, $\rangle C{=}CH_2 \rightarrow \rangle CHCH_2I$,
Garner aldehyde $-CHO \rightarrow -CH_2SH$, react with iodomethyl–pyranose then $\rangle S \rightarrow \rangle SO_2$;
iii, $TsNHNH_2/NaOAc/85\,°C$; iv, Jones oxidation then CH_2N_2

Scheme 24

coupling of the derived organoborane (side-chain = $CH_2CH_2BR_2$) with vinyl and aryl halides to give novel α-amino acids.[872] A synthesis of β-(tri-O-benzyl-2-deoxygalactopyranosyl)-D-alanine has used the Garner aldehyde with the Wittig reagent of the monosaccharide,[873] an approach used to give α,β-unsaturated ketones (-CHO→-CH=CHCOMe) which after hydrogenation and alkylidenecarbene formation [-CH_2CH_2COMe→-CH_2CH_2C(=C:)Me] and 1,5-C-H insertion gave the isomeric spirocyclopentene from which the '2,5-methanoleucine' derivative could be obtained.[874] An extended route to the manzamine tetracyclic system starts with side-chain aldolization of a Garner aldehyde [-CHO→-(2-ketopiperidin-3-yl)-CH(OH)-], the serine moiety being eventually incorporated into the synthesis target.[875]

The α-methylserine-based Garner aldehyde is illustrated in Scheme 5.[70] The L-threonine-based Garner aldehyde has been converted into the new homo-alanine carbanion equivalent (103) whose use in α-linked C-glycosyl amino acid synthesis (*i.e.*, synthesis of methylene isosteres of O-glycosylserines) has been demonstrated.[876] Uses for preparation of syn- and anti-β-hydroxy-α-amino acids that are constituents of vancomycin involve (R)- and (S)-Garner aldehydes in stereocontrolled arylation.[877] Simple exploration of the chemistry of these synthons gives useful results, such as cyanohydrin formation from HCN in pentan-2-ol with complete stereoselectivity,[878] and fluorination of the N-Boc-Garner aldehyde by diaminosulfur tetrafluoride leading to the extraordinary product (104).[879] Standard reactions allow replacement of aldehyde by novel functional groups, providing the corresponding alkynone (-CHO→-C≡CCOR)[880] as well as the other changes that initiate the applications described in the other papers in this section. Synthesis of sphingosines involves extension of the Garner aldehyde side-chain to $COCH{=}CHC_{13}H_{27}$, and $Zn(BH_4)_2$ reduction,[881] and an equivalent route to the same target has been described.[882]



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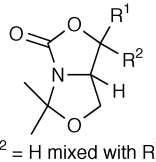
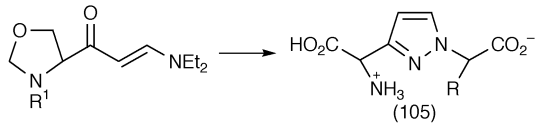


(104) R^1 = F, R^2 = H mixed with R^1 = H, R^2 = F

A related synthon prepared from L-serine, (S)-(+)-4-(2-oxazolidonyl)methyl triphenylphosphinyl iodide, has been engaged in Wittig syntheses with aldehydes to give alkenes, from which β,γ-unsaturated amino alcohols were prepared.[883] L-4-Oxaproline is a little-used synthon, shown to co-operate in some of the typical functional group changes described above (-CO_2H→-COC≡CTMS *via* the Weinreb amide) to give 1,2-dihydropyrazolyl diacid derivatives (105) by condensation with hydrazino acids $H_2NNHCHRCO_2H$.[884] Routine work as far as the procedures are concerned, but leading to interesting and important synthesis targets, has involved (2S,3R)-threonine [synthesis of enantiomerically-pure piperazine derivatives *via* (106)],[885] L-homoserine [synthesis of novel PNAs, N-Fmoc-δ-amino acids with an ether linkage in the main



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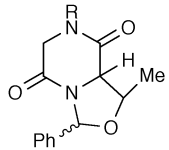

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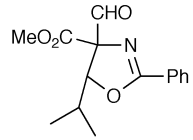
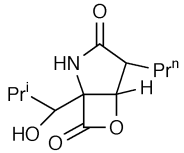


(107)

chain and one of the four nucleobases on a side-chain],[886] and a total synthesis of (+)-lactacystin from (2R,3S)-hydroxyleucine (Volume 31, p. 20) through anti-crotylation of the oxazoline (107).[887] (2S,3S)-N,N-Dibenzyl-hydroxyleucine is liable to cyclize to the protected 3-amino-β-lactone when its carboxy group is activated.[888] A potent analogue (PS-519; 108) of clasto-lactacystin β-lactone has been prepared through a doubly-diastereoselective aldol condensation of oxazoline and aldehyde.[889]

Aspartic and glutamic acids offer a wide range of uses in synthesis, usually aimed at, or proceeding by way of, saturated heterocyclic derivatives. Protected aspartic acid has been used to prepare enantiopure 6-alkylpipecolic acid,[890] N-protected 3- and 4-substituted aminopyrrolidinones,[891] side-chain aryl ketones of aspartimides (from N-acylaspartic anhydrides through Friedel-Crafts arylation) for 1,6-photocyclization to piperidin-2-ones,[892] α-methyl-L-aspartic acid by methylation (MeI-LDA) of the useful synthon (109), prepared from asparagine.[893] D-Aspartic acid is the starting point for a synthesis of allophenylnorstatine, a crucial step being stereoselective hydroxylation of (110).[894] Dianions formed from N-protected dialkyl aspartates undergo 1,2-asymmetric induction during quenching with an electrophile, with preference for the anti-product, but this can be reversed if bulky alkyl groups esters are used.[895] β,β-Dimethylation of dialkyl N-(9-phenylfluorenyl)-D-aspartates and ensuing functional group manipulations provides corresponding new β,β-dimethyl-α-amino acids.[896] Aldolization of the enolate to di-isopropyl squarate followed by easy decarboxylation promoted by the strongly electron-withdrawing squaryl group gives the novel α-amino diacids (111).[897]



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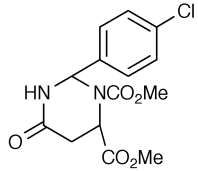

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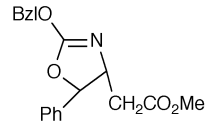

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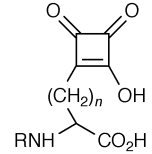
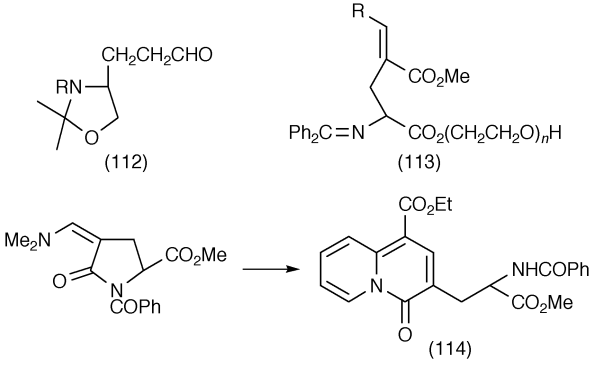


(111)

Novel glycosylated amino acids have been prepared from α-tert-butyl N-Fmoc-aspartate through DCCI-DMAP coupling to C-6 of the glycoside,[898]

and corresponding 2-deoxy-2-fluoroglycosylaspartate and serinates have been described.[899]

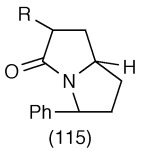
Homologues of the Garner aldehyde can be prepared from any α-amino acid after first reducing the α-carboxy group to CH_2OH, and this has led to (112) by applying standard procedures to a γ-alkyl L-glutamate.[900] Side-chain extension leading to (113; R = H) can be carried further by application of the Heck reaction (*e.g.*, giving 113, R = Ph, using PhI), the poly(ethylene glycol) ester group acting as phase transfer catalyst as well as polymer support for the reactant.[901] Hofmann rearrangement of N^{α}-protected L-glutamine esters to give corresponding N^{α}-protected (2S)-aminobutanoates is a long-known process but its electrochemical variant in trifluoroethanol–MeCN is novel.[902] Side-chain Weinreb amides of solid phase-tethered glutamic acid derivatives have been converted into aldehydes and thence to homologated esters (-CHO→-CH=$CHCO_2R$),[903] and dicyclohexylcarbodi-imide coupling of Z-L-glutamic acid with tryptamine gives the imide, to start a route to indolo[2,3-*a*]-quinolizidines.[904]



Uses of L-pyroglutamic acid in synthesis are well-appreciated (review, see ref. 905). Condensation of its 4-(dimethylamino)methylene derivative with ethyl (pyrid-2-yl)acetate opens up a family of new β-(heteroaryl)-L-alanines (114).[906] C-4-Alkylation can be achieved with O,C-dilithio anions,[907] and N-ethyl N-(*trans*-2-butenoyl)-4,4-dimethylpyroglutamate provides a useful substrate for the study of asymmetric Michael addition reactions.[908] A synthesis of kainic acid starts with L-pyroglutamic acid.[264] Deuteriation of N-Boc-3,4-dehydro-pyroglutamic acid tert-butyl ester gives the (2S,3S,4S)-[3,4-$^2H_2$]isotopomer of this increasingly-used synthon; ring cleavage gives the labelled 2-Boc-amino-5-iodopentanoate, displacement by cyanide ion and reduction providing a route to (2S,3S,4S)-[3,4-$^2H_2$]lysine.[909] Conformationally constrained lysine, ornithine and alanine have been synthesized from pyroglutamic acid *via* the well-established pyroglutaminol derivative (115).[910] Cyclopropanation of the synthon and manipulation of the product has given modified glutamates and arginines,[911] and constrained homoglutamic acids have been prepared by alkylation of the 'Thottathil bicyclic lactam' (115, with saturated lactam ring).[912] The unsaturated 2-amino-adipic acid homologue

(116) has shown similar potential in synthesis, *e.g.* 1,4-addition on treatment with R_2CuLiI_2 and routine work-up giving 2-amino-4-substituted adipic acids.[913]

(S)-Pyroglutaminol derivative (117) has led to a 5-hydroxylated pyrrolidinone, which is structurally related to natural products, *e.g.*, epolactaene and lactacystin,[914] and (S)-pyroglutaminylzinc iodide is suitable for homologation ($\text{-}CH_2ZnI \rightarrow \text{-}CH_2C{\equiv}CCH_2SiMe_3$) for a new synthesis of (−)-epibatidine.[915] The 3,4-epoxide of (S)-pyroglutaminol has been used in a synthesis of (2S,3S,4R)-3,4-dihydroxyglutamic acid.[916] (S)-1-Benzyl-2-hydroxypyrrolidine derived from pyroglutamic acid has been used to prepare (2S,3S)-3-hydroxy-2-phenylpiperidines.[917]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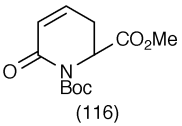
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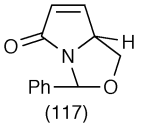
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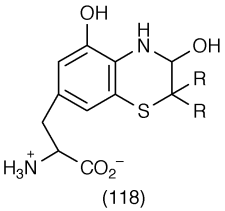

(118)

Reactions at the thiol group of N,C-protected cysteine, leading to djenkolic acid [a consequence of deprotection of the S-dimethylphosphinothioyl derivative using $(Bu_4N)F$],[918] L-felinine (addition to 3-methylcrotonaldehyde, $NaBH_4$ reduction),[919] S-(dihydroxyphenyl)ation and oxidation of the resulting S-cysteinyl-DOPA *via* the 3-hydroxy-3,4-dihydro-1,4-benzothiazine to give (118),[920] S-iminothioethers as intermediates in a mild amidine synthesis (RCN/NH_3/N-acetylcysteine),[921] and formation of (4R)-thiazolidine-2,4-dicarboxylic acid as a mixture of (2R,4R)- and (2S,4R)-diastereoisomers through condensation of L-cysteine with glyoxylic acid in aqueous ethanoic acid at 30 °C,[922] have been described. More routine work deals with preparation of N-Boc-S-alkyl-L-cysteines[923] and S-[^{11}C]methylation.[924] Interest in the last-mentioned preparation lies in practical details for rapid working, involving reactions on C_{18}-Sep-Pak in this case; solid-phase synthesis of 1,4-benzothiazepin-5-ones from resin-bound cysteine with 2-fluoro-5-nitrobenzoic acid is completed with routine steps.[925] S-(Allyloxycarbonylmethyl)ation can be reversed by Pd-catalysed hydrostannolysis using Bu_3SnH.[926,927]

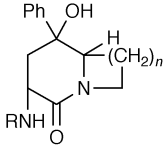
Electrochemical oxidation characteristics of cysteine differ from those of homocysteine because of differences in hydrophobicity and structures of their metal complexes.[928] This explanation may need to be modified in the light of the report that cysteine affects the much slower autoxidation of homocysteine, which is capable of reducing cystine to cysteine.[929] A means of preserving homocysteine-containing clinical samples from oxidative changes, by the addition of 3-deaza-adenosine, has been proposed.[930] Selenocysteine and selenomethionine undergo aerobic decomposition under protein hydrolysis conditions and during ion-exchange purification.[931]

Reaction of S,S′-dibenzyl-N,N′-1,3-propylenediyl bis-L-cysteine with ^{99m}Tc

at pH 12 and further elaboration leads to technetium[99^m]-L,L-propylene-dicysteine.[932]

Unexpected flexibility is shown by *Beauveria bassiana* in its conversion of N-phthaloyl D- or L-methionine and -ethionine into the S_S-sulfoxides.[933] Laboratory preparation of sulfoxides of methyl S-methyl N-Z-L-cysteinate and the corresponding methionine using tert-butyl hydroperoxide in supercritical CO_2 has been demonstrated to lead to the anti-isomer.[934] The sulfate anion radical, generated by KrF-laser photolysis (248 nm) of $K_2S_2O_8$, brings about oxidation of methionine and its methyl ester through a 3-electron radical cation intermediate.[935]

S-(Aminoiminomethyl)amides of cysteic and homocysteic acids have been prepared for their potential as mimics of arginine.[936] Ornithine lactams (119, R = TFA or Z; and its epimer as minor product) result from photoinduced ε-H abstraction followed by cyclization of the resulting 1,6-biradicals, from 2-amino-4-oxo-4-phenylbutanoylamines.[937] N^{ω}-Substituted arginine derivatives are effectively prepared from ornithine through mild condensation with $ArSO_2N{=}C(SMe)_2$.[938] A lysine–arginine crosslink develops through reaction of glucose with bovine serum albumin, hydrolysis and isolation giving 2-(5′-carboxypentyl)amino-4-(5′-carboxypentyl)-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-*b*]azepine (120).[939] This shows some instability under the



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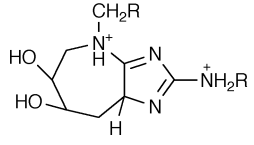
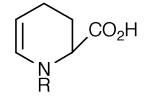


(120) R = $(CH_2)_3CH(NH_2)CO_2^-$



(121) R = Z or Boc

conditions of acid hydrolysis, perhaps explaining why it has not been isolated in real situations before, but otherwise it behaves similarly to the lysine crosslink pentosidine (see also ref. 258). tert-Butyl (9R,10S,11E,13S)-9,10-epoxy-13-hydroxyoctadec-11-enoate undergoes ring-opening with N-acetyl-lysine 4-methylcoumarin-7-ylamide, and the search is on for products of aminolysis of α,β-unsaturated epoxides by protein-bound lysine.[940] Hippuryl-arginine and -lysine react differently with glyoxal at 40 °C, in the obvious way with the former compound but sluggishly with the lysine side-chain, reaction only occurring significantly at 80 °C to give N^{ε}-(carboxymethyl)lysine in low yield.[941] This lysine derivative has been identified as a constituent of proteins,[942] and the N^{ε}-hexanoyl analogue is formed with hippuryl-lysine by reaction with the lipid hydroperoxide, hydroperoxyoctadeca-1,3-dienoic acid.[943] Similar studies of reactions of lysine-containing peptides with *trans*-2-hexenal have been described,[944] and the crosslink formed between lysine residues in adjacent polypeptide chains, through reaction with (E)-4-hydroxy-2-nonenal, has been confirmed to be a 2-alkyl-2-hydroxy-1,2-dihydropyrrolin-3-one imine.[945]

A new look at these processes involving lysine with oxidized species would

be desirable, in view of the easy hydroperoxydeamination of hydrazino side-chains [prepared from lysine derivatives with N-Boc-3-(4-cyanophenyl)-oxaziridine]. Air oxidation in the presence of bicarbonate ions gives the hydroperoxide, which is readily reduced (*e.g.* with a water-soluble phosphine) to 6-hydroxynorleucine.[946]

Simpler heterocyclic syntheses involving lysine include the N-substituted 5,6-dehydropipecolic acid (121; R = Z or Boc) formed from Z- or Boc-L-lysine with a cell suspension of *Rhodotorula graminis*.[947] (S)- or (R)-2,4-Diamino-butanoic acid gives (3,4,5,6-tetrahydropyrimidinyl)glycines through reaction with imino-ethers derived from glycine, serine or tyrosine.[948] Lysine protection strategies have been optimized over the years as far as the familiar protecting groups are concerned, and reliable recipes for preparation of N^{α}-Z-L-lysine[949] and $N^{\alpha}N^{\varepsilon}$-bis-Boc- and N^{α}-Z- N^{ε}-Boc-L-lysine[950] [*via* the copper(II) complex of N^{ε}-Boc-L-lysine]. Protected arginine cyclic aminals result from $LiAlH_4$ reduction of the arginine Weinreb amide to aldehyde, coupling through the aminal oxygen to a linker unit attached to a solid phase giving a scaffold on which acyl derivatives of the arginine α-amino group were prepared.[951]

Sulfur-containing modifications of the side-chain functional groups of ornithine, citrulline and arginine, *e.g.* (S)-$H_3N^+CH[(CH_2)_3N{=}C(SMe)NHOH]CO_2^-$, have shown promise as nitric oxide synthase inhibitors.[952]

Aromatic groups in amino acid side-chains provide the site for electrophilic substitution, exploited for assorted reasons: assisting analysis, improving cell receptor response, and isotopic labelling are only a few of these. Aqueous phenylalanine gives tyrosine and DOPA through 'heavy ion irradiation' (350 MeV neon ions),[953] and m-tyrosine formation from phenylalanine has been advocated as a sensitive means of detecting hydroxyl radical formation in aqueous media (though this should be followed by diode array or electrochemical devices since HPLC procedures are liable to introduce artifacts).[954] Conversion of DOPA into 6-hydroxyDOPA through use of standard chemical and electrochemical oxidation protocols proceeds *via* dopaquinone.[955] More conventional laboratory substitution protocols have provided 3′-bromo- or iodo-4′-hydroxyphenylglycines,[956] N^{α}-Fmoc-4′-phosphonomethyl-L- and D-phenylalanines,[957] 4′-(diethylphosphonophenylazo)-phenylalanine,[958] 4′-(tert-butylthio)phenylalanine [from 4′-iodo-phenylalanine with $Bu^tSH/Pd_2(dba)_3.CHCl_3$],[959] (S,S)-isodityrosine (coupling of protected L-phenylalanine 4′-boronic acid with 4′-O-benzylDOPA[960] and with aryl halides,[961] and 3-nitrotyrosine (UV absorption at λ_{max} 358 nm).[962] *In vivo* non-enzymic reduction of nitrotyrosine to aminotyrosine involves a haem with thiols.[963] Iodination of aqueous tyrosine in a liquid macrocycle-containing membrane by KI/I_2,[964] and formation of a thymine-tyrosine adduct, 3′-[(1,3-dihydro-2,4-dioxopyrimidin-5-yl)methyl]-L-tyrosine, from L-tyrosine and 5-(hydroxymethyl)uracil *via* radical intermediates,[965] illustrate applications of less familiar procedures. Pd/Cu-Mediated Stille coupling with Me_4Sn after iodination with Barluenga's reagent (Ipy_2BF_4) offers a useful methylation procedure targeted at the phenolic moiety of a tyrosine derivative.[966]

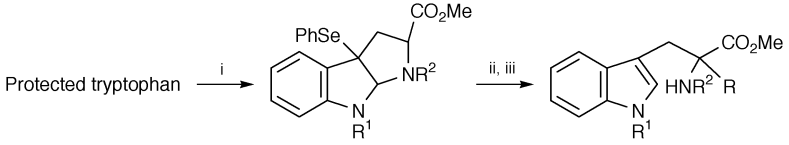
The hydroxy group of '3-hydroxytyrosine' (*i.e.*, 3′-hydroxyphenylalanine) is

the focus of attempts to create the biaryl ether bridge in syntheses of 14-membered macrocycle-containing antibiotics, a new solid-phase S_NAr approach employing an o-nitrofluorophenyl partner offering flexibility.[967]

[^{18}F]Labelling is being explored in several laboratories, providing potential tumour-imaging materials: 3- and 5-[^{18}F]fluoro-L-o-tyrosines (by use of MeO^{18}F)[968] and 5-[^{18}F]fluoroDOPA (by use of H^{18}F /BF_3)[969] from the amino acids themselves, and 6-[^{18}F]fluoro-L-DOPA by [^{18}F]fluorodestannylation with [^{18}F]acetyl hypofluorite in $CFCl_3$,[970] mixture of ring-[^{18}F] and [^{18}F]adducts by [^{18}F]fluorination of (R)- or (S)-(E)-β-fluoromethylene-m-tyrosine,[971] and O-(2-[^{18}F]fluoroethyl)-L-tyrosine.[972] Radio-iodinated α-methyl-L-tyrosine is easily prepared from the amino acid using Chloramine-T/I_2.[973]

Histidine reacts efficiently with the lipid oxidation product hexanal (see lysine above), to give side-chain aminols.[974] Side-chain N-tritylation of protected histidines leads inexorably to the N$^{\tau}$-trityl derivative; the conclusion has been reached[975] that prospects are poor for preparing the N$^{\pi}$- trityl isomer that would provide for racemization-free histidylation of a growing peptide chain. However, N$^{\pi}$-allyloxycarbonylmethyl protection has been established.[976] Side-chain attachment to a trityl-resin can be a useful prelude to further reactions at histidine functional groups.[977] L-Histidine anions contribute low EES as catalysts for the reduction of carbonyl compounds by a trialkoxysilane.[978]

Tryptophan chemistry that is above the routine level is shown in its reaction with N-phenylselenylphthalimide (Scheme 25), allowing α-alkylation of this



Reagents: i, *N*-phenylselenyl phthalimide; ii, LDA–THF–78 °C; iii, MeI or *p*-Br$C_6H_4CH_2$Br

Scheme 25

amino acid with inversion of stereochemistry,[979] and in 2′-(α-C-mannosyl)ation using a stannylacetylene as a novel coupling reagent with an aldose.[980] Routes to three types of tetrahydro-β-carbolines to which a 5- or 6-membered heterocycle is attached have been described.[981] Swern oxidation of methyl N-acetyl-L-tryptophanate proceeds *via* a tetrahydro-β-carboline (intramolecular attack of N$^{\alpha}$ on the indole C-2 site) with methylthiomethyl-substituted indoles featuring among the products.[982] The indole moiety of an L-tryptophan derivative undergoes substitution with 1,2-anhydro-3,4,6-tri-O-benzyl-β-D-mannopyranose to give C^2-α-D-[C-mannopyranosyl]-L-tryptophan, a member of a novel sub-class of 'glyco–amino acid'.[983]

6.4 Effects of Electromagnetic Radiation on Amino Acids. – Most of the studies under this heading concern tyrosine and tryptophan, but the usual almost total exclusion of other amino acids is not sustained this year.

Hydroxyl radicals formed by radiolysis of ^2H_2O solutions of amino acids under anaerobic conditions induce ^{1}H–^{2}H exchange at C–H bonds to the

extent of 3–8%.[984] N-Centred radicals have been detected in radiolysis or photoionization of aqueous N-phenyl- and N-chloroglycine,[985] while the superoxide radical anion and indole-centred radicals have been detected in corresponding studies of N-acetyltryptophan[986] and three radicals have been generated by γ-irradiation of a single crystal of N-acetyltyrosine.[987]

UV photolysis of N-arenesulfonylamino acids causes sulfonamide cleavage, a promising deprotection option but contradictory results on accompanying structural changes need to be brought into line; the mechanism of the process for N-toluene-p-sulfonylglycine involves intramolecular electron or proton transfer,[988] and the general reaction gives a low return of deprotected amino acids as a result of oxidative decarboxylation.[989] 300 nm Photolysis of N-acetoacetyl-α-amino acid esters gives complex mixtures through Norrish type I reactions (H atom abstraction with concomitant radical cleavage and radical recombination),[990] whereas β-2,2′-dinitrobenzhydryl N-methyl D-aspartate is cleanly cleaved into the free acid.[991] Photolysis of glycine, alanine, and proline, and other common amino acids with functional side-chains (hydroxyproline, arginine, lysine and histidine) that are found in collagen, has been investigated at higher energies (193 nm laser irradiation).[992] Cationic and neutral radicals arise in photo-oxidation of aqueous tryptophan sensitized by $PtCl_6^-$ salts, due to the intervention of Cl_2^- radicals.[993] Intense circularly-polarized irradiation (XeF 351 nm laser source) of threonine and methionine causes deamination and decarboxylation and the D-enantiomers appear to be degraded to a greater degree relative to their L-isomers.[994]

Fluorescence of N-dansyl-S-nitroso-homocysteine is enhanced during its denitrosation by thiols, and this effect can be exploited in a quantitative assay.[995] More conventional fluorescence studies relate to methyl L-tyrosinate {quenching by mesoporphyrin II, 2-[(2-hydroxyethyl)thio]-3-methyl-1,4-naphthoquinone},[996] N-acetyl-L-tyrosinamide (3-photon excitation with 780–855 nm femtosecond titanium sapphire laser),[997] and L-tryptophan [quenching by lanthanum(III) ions,[998] and sensitivity of phosphorescence features to local environment[999] including tryptophan trapped in silica glass[1000]]. Fluorescence features of branched tryptophan derivatives is modified by hydrogen-bonded dendritic microenvironment.[1001] Photoproducts of tryptophan could have roles in light-regulated biosynthesis, since cytochrome P gene expression is affected by their presence.[1002]

7 Analytical Methods

7.1 Introduction. – Reviews spanning several analytical techniques deal with amino acid analysis of proteins,[1003] and applications in amino acid analysis of currently emerging chromatographic and other instrumental methods.[1004] Methods for the analysis of particular amino acids that are diagnostic of metabolic disorders have been intensively studied, and, while pyridinoline and its deoxy-analogue are mentioned in later subsections, there are methods being advocated (immunoassay;[1005] and the rather easier automated chemilumines-

cence assay[1006]) that fall outside the main categories of technique into which this section is divided. 3-Nitrotyrosine has special current interest since it arises in proteins through a pathway starting with nitric oxide.[1007]

7.2 Gas–Liquid Chromatography. – All the conventional methods continue to be developed, with greater confidence in absolute configurational assignments with emphasis on the modification of commercial chiral stationary phases (CSPs). The amino acids of hydrolysed pyoverdins derivatized as N(O,S)-perfluoroacylated alkyl esters and separated over Permabond Chirasil-Val,[1008] and a closely similar study using Chirasil-γ-Dex[1009] and N-trifluoroacetyl selenomethionine isopropyl ester over L-valine butylamide-modified Chirasil-L-Val[1010] illustrate the general style of current work. A new CSP with chiral resorc[4]arene basket-type selector bonded through diamide groups to a dimethyl polysiloxane shows good enantiomer selectivity towards methyl esters of N(O,S)-trifluoroacetylamino acids.[1011]

GLC of N-trifluoroacetyl 2,6-diaminopimelic acid isopropyl esters over Chirasil-L-Val to provide D:L-ratios,[1012] and configurational analysis in the same way, of pipecolic acids in plasma,[1013] of N-aminoethylamino acids after cyclization to piperazin-2-ones with trifluoroacetic anhydride,[1014] employ the standard off-the-peg analytical protocol.

Sulfur-containing amino acids have an above-average share of the papers in this section, due to the clinical relevance of homocysteine monitoring. This amino acid can be analysed together with methionine and cysteine as the N,S-alkoxycarbonyl alkyl esters,[1015] or after S-pyridylethylation with vinylpyridine then tert-butyldimethylsilylation, with ^{2}H- and ^{13}C-labelled analyte as internal standard.[1016] An identical approach has been applied to the analysis of methyl N,S-di-ethoxycarbonylcysteinate,[1017] and to the identification of novel related amino acids in plants (ref. 16).[15]N-Labelled internal standard is appropriate for GLC-MS analysis of S-nitrosocysteine employing $HgCl_2$ cleavage into nitrite and ^{15}N-nitrite.[1018] Spiking with U-^{13}C-labelled amino acids, followed by TBDMS-derivatization, offers a sensitive assay of plasma amino acids.[1019]

Alternative derivatization protocols have been illustrated with GLC analysis of N-carboxymethylserine (as the N,O-diacetyl methyl ester derivative),[1020] 5-hydroxylysine and lysine content of collagen (as the N-trifluoroacetyl n-propyl ester derivative),[1021] GLC-MS analysis of 3-nitrotyrosine as its penta-fluorobenzyl derivative,[1022] tyrosine and substituted tyrosines derivatized using N-methyl-N-(tert-butyldimethylsilyl)trifluoracetamide.[1023]

7.3 Ion-exchange Chromatography. – Some novel variants of classical amino acid analysis protocols are coming to prominence, anion exchange separation followed by amperometric quantitation comparing well with ninhydrin colorimetry.[1024] Interpretation of bimodal integrated amperometric waveforms permit analysis of underivatized amino acids at less than 1 picomole levels,[1025] and carbohydrates do not have to be cleared from samples since they do not interfere.[1026]

Cation-exchange separation of amino acids using evaporative light-

scattering detection offers low sensitivity (more than 200 picomole sample is required).[1027]

7.4 Thin-layer Chromatography. – Enantiomeric analysis of aromatic amino acids is conveniently accomplished on commercially available chiral stationary phases.[1028] The quantitation of L-tyrosine and L-DOPA in samples is feasible when their TLC spots contain more than 0.7 μg and 1.5 μg respectively.[1029]

7.5 High-performance Liquid Chromatography. – Broad-ranging coverage of protein protocols includes reviews of amino acid analysis based on HPLC.[1030] Hydrophobic interaction chromatography has been investigated with amino acids and peptides; amino acids are not retained sufficiently so that useful application of the method is unlikely.[1031] Polymeric stationary phases whose properties are affected by pH and temperature changes, *viz*. irregular poly-(ether)s, have shown merit in separations of amino acids and peptides.[1032]

Underivatized amino acids carrying chromophoric or electrochemically-active groupings are easily detected after HPLC separation, though other detection methods, notably mass spectrometry but also laser-based polarimetry (0.5–50 microgram samples)[1033] and evaporative light-scattering after ion-pair reversed-phase HPLC separation,[1034] are also appropriate (the sensitivity of this detection technique[1035] is 0.5–1 mg mL^{-1}).

Analysis of phenylalanine and tyrosine that exploits their inherent fluorescence (λ_{ex} 215 nm, λ_{em} 283 nm; N-methylphenylalanine as internal standard),[1036] and similar procedures applied to tryptophan and its metabolites,[1037] N-acetyl-S-nitrosocysteine (λ_{max} 333 nm, exploited in an assay for nitrate and nitrite),[1038] S-adenosyl-L-methionine and -L-homocysteine,[1039] tyrosine O-sulfate have been reported.[1040] Electrochemical detection procedures have been applied to a crop of sulfur-containing amino acids: taurine,[1041] S-sulfocysteine,[1042] cysteine and N-acetylcysteine using a novel cobalt ferricyanide electrode,[1043] 5-(S-cysteinyl)DOPA.[1044] Papers covering homocysteine are collected later in this section, together with papers on HPLC analysis of other clinically important amino acids.

Electrochemical detection underpins an HPLC assay of 5-hydroxytryptophan.[1045] An unusual amperometric technique relies on the reaction of electrogenerated bromine species with underivatized amino acids.[1046]

Mass-spectrometric detection allied with HPLC is now a standardized operation, as with phenylalanine and tyrosine quantitation in blood spots based on stable isotope dilution.[1047] The high sensitivity of this method, and its further advantage in yielding spectra that can be interpreted to supply structural information, is underlined by detection for the first time of N′- and 2-(β-D-hexopyranosyl)-L-tryptophans and related conjugates in human urine.[1048]

Ligand exchange HPLC is represented in a use of the copper(II) complex of poly(divinylbenzene)-immobilized L-proline for estimation of D:L-ratios for samples of common amino acids.[1049]

Homocysteine has gained importance as a clinical marker for cardiovascular

disease, and several new studies have led to refined analytical procedures. Classical ion-exchange analysis is not sufficiently sensitive, and relative advantages of the other standard HPLC approaches have been considered,[1050] which has also been coupled with improved analysis of cysteine.[1051-1055] Emphasis has been given to electrochemical detection[1056,1057] and to colorimetry,[1058] with fluorophoric derivatization (use of 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonamide[1059] and closely-related reagents[1060,1061] to introduce the SBD fluorophore; use of other classical derivatization procedures, *e.g.* to prepare the OPA derivative[1062] or the 7-N,N-dimethylaminobenzenesulfonyl-4-(2,1,3-benzodiazolyl)thiocarbamoyl derivative, λ_{ex} 385 nm, λ_{em} 515 nm[1063]). OPA derivatization has been applied to cysteine after tagging the thiol group with N-(1-pyrenyl)maleimide.[1064]

The levels of pyridinoline and its deoxy-analogue in physiological samples continue to be considered as valuable markers for osteoporosis and bone degradation, and HPLC estimation using established protocols has been reported,[1065-1067] one of the current studies[1068] including another crosslinking amino acid, desmosine, in its assay.

3-Nitrotyrosine is another current target for which HPLC assays have been developed. This trace constituent of modified proteins gives an electrochemical signature permitting its detection with satisfactory sensitivity.[1069] Reviews of methods for analysis of this physiological marker for nitrogen oxides and oxyacids,[1070] together with assays for 3-chlorotyrosine,[1071,1072] N-nitrosoproline,[1073] and 2-oxohistidine,[1074] and glycine betaine,[1075] have been published.

Derivatization of amino acid mixtures and HPLC separation remains the most favoured approach to general amino acid analysis in the absence of special circumstances, and some of the methods chosen most often have been mentioned above for homocysteine. Further examples of analysis as o-phthaldialdehyde (OPA) derivatives, usually relying on fluorescence quantitation, have been published for N-isobutyroyl-D- or L-cysteine,[1076] γ-carboxyglutamic acid,[1077] 2,6-diaminopimelic acid,[1078] isotope-enriched amino acids (mass spectrometric detection),[1079] amino acids in a single human polymorphonuclear leukocyte.[1080] The last-mentioned example underlines the sensitivity of this approach, which can be enhanced by using o-naphthalenedialdehyde as reagent instead of OPA, illustrated for S-adenosylmethionine and -homocysteine.[1081] Careful sample preparation is particularly important with the OPA procedure, and cleansing using a strong cation exchange resin is recommended.[1082]

N-Phenylthiocarbamoylamino acids continue to give good service in this context, illustrated for glutamine analysis (a particularly difficult analytical problem for proteins) using the Pico-Tag protocol, release of the amino acid depending on successive treatment of bovine milk protein with pronase E, aminopeptidase M, and prolidase.[1083] Phosphatidylserine[1084] is another problem amino acid that has been successfully analysed as its PTC derivative, and more general amino acid mixture analyses[1085] have been described that employ this approach. Cyclized PTC-amino acids (*i.e.* PTHs) are supported with a voluminous HPLC literature; their improved analysis benefits from

careful control of gradient and column temperature.[1086] Fluorescent thiohydantoins formed with (R)-(−)-DBD-pyridyl isothiocyanate have proved suitable for the determination of D:L-ratios for amino acid samples.[1087]

Several other fluorescent amino acid derivatives are gaining approval for sensitive HPLC analysis: N-Fmoc (amino acids in Z-DE spots;[1088] detection supported by electrospray MS;[1089] analysis of lysine[1090]). The related procedure employing (+)-1-(9-fluorenyl)ethyl chloroformate as reagent has been used for analysis of the imino acid N-methyl-D-aspartic acid after clearing primary amines from samples by OPA derivatization followed by extraction.[1091] Another amino acid of interest as a cellular constituent and requiring sensitive reliable analysis, D-leucine in rat hippocampus, has been quantified by HPLC over a CSP, at the one femtomole level after derivatization with NBD fluoride.[1092]

Many of the foregoing examples illustrate well-known procedures, and dansyl- and dabsyl-amino acids are also no strangers in this context. The use of the former for analysis of O-(β-1-galactosyl)hydroxylysine in serum[1093] and for demonstrating separation of enantiomers for dansyl-D,L-phenylalanine by HPLC over α-acid glycoprotein,[1094] and of the latter in sensitive amino acid analysis,[1095] indicate current interests in novel chromatographic applications. Newer derivatization reagents that have been advocated, in a search for reliable trace amino acid analysis, are 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (giving DMS derivatives, λ_{ex} 318 nm, λ_{em} 406 nm, reaching below 5 femtomoles of analyte[1096]) and carbazole-N-(2-methyl)acetyl chloride (giving CMA derivatives, λ_{ex} 335 nm, λ_{em} 360 nm, 10–65 femtomoles of analyte[1097]). The former reagent has been applied to the estimation of as little as 1–5 femtomoles of proline and hydroxyproline in amino acid mixtures after removal of primary amines using OPA (as above).[1098] Results for the analogous use of acridone-N-acetyl chloride have been published.[1099]

Condensation products of amino acids with pyrroloquinoline quinone have been assessed by HPLC with mass spectrometric structure determination.[1100]

7.6 Capillary Zone Electrophoresis (CZE), and Related Analytical Methods. – The topic has settled into established categories of routine amino acid analysis which are closely related, from the point of view of sample preparation and detector response, to HPLC methodology. A review of 1997–8 literature has appeared.[1101]

Free amino acids are amenable to CZE assay [tryptophan, 40 pg sample with detection at 280 nm;[1102] O-phosphorylated serine, threonine and tyrosine;[1103] 3-methylhistidine;[1104] DNA–histidine complexes in isoelectric histidine buffers;[1105] cysteine and homocysteine;[1106,1107] aromatic amino acids[1108]]. Points of interest from these studies include favourable comparison with HPLC assays for homocysteine, and accurate estimation of D:L-ratios when buffers include a chiral additive (cyclodextrin), also seen for ligand exchange CZE [copper(II)–N-alkyl-4-hydroxy-L-proline derivatives], MEKC[1109] [hydroxy-L-proline–surfactant buffers],[1110] and capillary isotacho-

phoresis of N-(2,4-dinitrophenyl)-D,L-norleucine (D:L-ratio determination) with a β-cyclodextrin-containing buffer.[1111] The CZE separation of a mixture of 82 inorganic anions, organic acids including amino acids, and carbohydrates provides a dramatic illustration of the power of the method, though this example depends on the use of highly alkaline buffers so limiting the range of potential applications.[1112] Post-column o-phthaldialdehyde–2-mercaptoethanol treatment allows laser-induced fluorescence quantitation of common amino acids.[1113]

Amino acids have been subjected to standard CZE procedures after derivatization with OPA-2-mercaptoethanol (D:L-ratios for aspartic acid using β-cyclodextrin buffer),[1114] dansyl chloride (D:L-ratios using N-alkoxycarbonyl-L-amino acids as chiral buffer surfactant additive),[1115] fluorescein isothiocyanate (γ-carboxyglutamic acid,[1116] D:L-ratios using β- and γ-cyclodextrins in buffers,[1117] and amines formed by Hofmann rearrangement of N-acetylamino acid amides[1118]), illustrating the two predominant approaches. The last-mentioned derivatives of amino acids extracted from the Murchison meteorite permit sub-attomole quantitation including D:L-ratio determination (SDS-γ-cyclodextrin buffer), when the CZE-on-a-chip technique is applied, and give data closely similar to those already reported (Volume 30, p. 2) for HPLC analysis.[1119] Derivatization efficiency by aliphatic isothiocyanates has been investigated as a function of reaction time, temperature, and other parameters.[1120] PTHs have been detected after CZE separation, through thermo-optical absorbance data,[1121] and analysis of N^{α}-Fmoc derivatives of lysine and methylated lysines separated by two-dimensional electrophoresis has been supported by mass spectrometric detection.[1122]

7.7 Assays for Specific Amino Acids. – Modifications of well-known colorimetric assays have been described, for histidine (coupling with diazotized p-aminoacetophenone followed by electrochemical quantitation),[1123] and for the estimation of a large amount of cysteine in the presence of a small amount of cystine.[1124] For this, a subtractive analysis stage in which an excess of N-ethylmaleimide is added, and unreacted reagent quenched with D,L-homocysteine, is coupled with dithioreitol reduction of cystine and ninhydrin analysis as usual.

The exquisitely specific immunoassay approach to amino acid analysis is not covered routinely in this Specialist Periodical Report. One paper covers a technique showing some breadth of application, in which surface plasmon resonance detection has been applied to an Igs immunosensor mounted on a chiral disc using a competitive antibody assay; this allows differential response of amino acid enantiomers and is a highly sensitive technique.[1125]

Amperometric biosensors of traditional design based on enzymes immobilized on an electrode are dedicated to L-glutamic acid assay (thermophilic L-glutamate dehydrogenase with NADP;[1126] L-glutamate oxidase,[1127] peroxidase with L-glutamate oxidase,[1128] glutamate dehydrogenase and NADH oxidase,[1129] L-glutamate decarboxylase coupled with a CO_2 electrode[1130]), L-alanine/α-ketoglutarate/L-glutamic acid assay (L-alanine aminotransferase

with L-glutamate oxidase, with chemiluminescence exploited as a measure of the H_2O_2 produced[1131]), and L-lysine assay (peroxidase and lysine oxidase[1132]). A flow injection analysis protocol for glutamic acid and glutamine based on L-glutamate dehydrogenase and L-aspartate aminotransferase depends on spectrophotometric quantitation of NADH generated from the analytes.[1133] A review of the literature of 1997 gives thorough coverage of the different categories of amino acid assays using biosensors.[1134]

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2
Peptide Synthesis

BY DONALD T. ELMORE

1 Introduction

As in the previous Report,[1] many reviews have been written and are cognate to particular sections as follows; Section 2.1,[2] Section 2.4,[3] Section 2.5,[4–6] Section 2.6,[7–23] Section 2.7,[24] Section 3.1,[25–29] Section 3.3,[30] Section 3.4,[31–34] Section 3.5,[35–43] Section 3.6,[44] Section 3.7,[45] Section 3.8,[46–53] Section 3.9[54–62] and Section 3.10.[63–66] Lack of space does not allow all references to Section 2 to be reported in detail and some references are included in Section 3.9.

2 Methods

2.1 Amino-group Protection. – The design of a nonaqueous aprotic system for dissolving free amino acids[67] could be useful in preparing N-protected derivatives. The solvent system consists of *N,N*-dimethylformamide, a tertiary base and an inorganic salt from Groups 1 or 2. Acylation is effected using e.g. Boc_2O, Z-OSu or Fmoc-OSu. Some experimentation has been carried out with the solvent system in the coupling step of peptide synthesis. An improved synthesis of α-Z-Lys-OH has been described.[68] It involves making the ε-Boc derivative, introduction of the α-Z-group using benzyl *N*-succinimidyl carbonate followed by removal of the Boc group with tosic acid. The Z-group has also been introduced using BzlOCOCl at neutral pH in presence of activated Zn powder.[69] A new type of reagent (1) has been designed for the introduction of *N*-alkoxycarbonyl groups.[70] Removal of *N*-Boc groups can be effected using $AlCl_3$ as a Lewis acid.[71] This method requires more detailed examination in the peptide field. Since thioxopeptides (alternatively named thionopeptides) are sensitive to acids and bases, the report[72] that $Mg(ClO_4)_2$ in MeCN or $ZnCl_2$ in tetrahydrofuran can remove Bpoc or Dde groups without affecting the >C=S moiety solves a serious problem. A similar study reports that Fmoc groups can be removed during SPPS without affecting thioester groups.[73] The preferred reagent consisted of a mixture of 1-methylpyrrolidine, hexamethyleneimine and Bu^tOH. A new amino protecting group has been described.[74] The prop-2-ynyloxycarbonyl function (POC) can be introduced using prop-2-ynyl chloroformate under basic conditions and can be detached under

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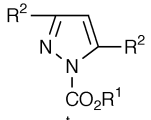
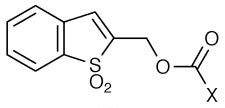
neutral conditions in presence of benzyltriethylammonium tetrathiomolybdate using sonication.

Carpino's group continues to add to the peptide chemist's synthetic repertoire. The 1,1-dioxobenzo[b]thiophene-2-yl-methyloxycarbonyl (Bsmoc) group (2) has been briefly reported before but has now been described in detail as have Bsmoc amino acid fluorides.[75] Use of the Bsmoc group has an advantage over the Fmoc group since a wash with water or saturated NaCl rather than with acidic phosphate removes all byproducts. In addition, removal of the Bsmoc group can be achieved under less basic conditions thereby decreasing the possibility that the Asp-Gly sequence, if present, will produce the amino succinimide moiety. The Bsmoc group can be used in SPPS and its light-absorption properties permit resin loading to be followed. The related 2-(t-butylsulfonyl)-2-propenyloxycarbonyl (Bspoc) group (3) is more labile to bases than is (2), presumably because there is less steric hindrance.[76] Bspoc amino



(1) R^1 = Me, Et, Bu^t or Bzl, R^2 = H or Me



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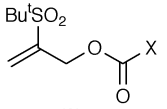
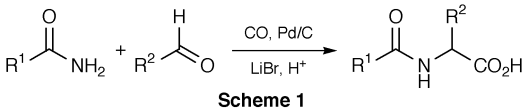


(3)

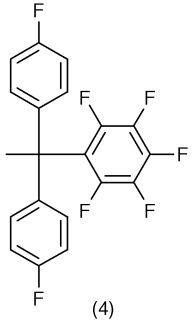
acids are easily obtained and converted into the acyl chlorides using $SOCl_2$ in CH_2Cl_2. Peptide coupling reactions can be conveniently carried out in a two-phase system followed by removal of the Bspoc group with a secondary amine immobilized on silica. 2-(4′-Nitrophenylsulfonyl)ethoxy-carbonyl (Nsc) derivatives of hydroxy acids have been prepared.[77] The general route involves *N*-acylation with 2-(4-nitrophenyl-thio)ethoxycarbonyl chloride of the methyl ester of the amino acid, followed by hydrolysis of the ester and then oxidation of the product with Na_2MoO_4 and H_2O_2. The behaviour of the Nsc and Fmoc groups have been compared as base-labile groups in SPPS.[78] The former has a slight edge since deprotection can be monitored at 380 nm and less rearrangement of Asp residues occurs during deprotection. The related 2-(2′,4′-dinitrophenylsulfonylethyl)-ethoxycarbonyl group has also been briefly examined for protecting amino groups but more information is required before giving a verdict on its potential value.[79] The same comment applies to the 2-(trimethylsilyl)ethylsulfonyl group.[80] Two developments in the use of Alloc group protection have been described. $Pd(PPh_3)_4$ is used for deprotection in the presence of an amine/BH_3 complex as scavenger for allyl groups in CH_2Cl_2 at room temperature.[81] The technique can be used in SPPS. Alloc derivatives of amino acids undergo tandem deprotection and coupling with various carboxy-activated partners when treated with $PhSiH_3$ in the presence of catalytic amounts of $Pd(PPh_3)_4$.[82]

A Pd-catalysed reaction (Scheme 1) offers a new route to *N*-acyl amino acids.[83] Although the choice of accessible acyl groups adumbrates problems with loss of chirality in coupling reactions, enzymic coupling techniques could provide a convenient route to some peptides that might be useful in the design of certain potential drugs.



Scheme 1

2.2 Carboxyl-group Protection. – An alternative route to methyl esters avoiding the use of diazomethane is available.[84] The carboxy acid is treated with $LiOH.H_2O$ in dry tetrahydrofuran (10–30 min), then reacted with Me_2SO_4 (0.5–1.0 equivalents) under reflux (0.5–3 h). The solvent is distilled off and the residue is diluted with $NaHCO_3$ solution and then extracted with Et_2O. Modified trityl groups (4,5) have been proposed for the protection of the γ-carboxyl group of Glu.[85] These are markedly more stable than simple trityl esters but are easily cleaved by $\geq 1\%$ CF_3CO_2H in CH_2Cl_2 in the presence of $Pr^i{}_3SiH$ as scavenger. Consequently, these protecting groups can be removed without affecting Bu^t groups if present. 9-Fluorenylmethyl esters of amino



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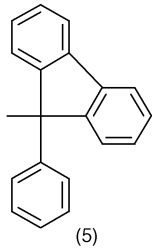


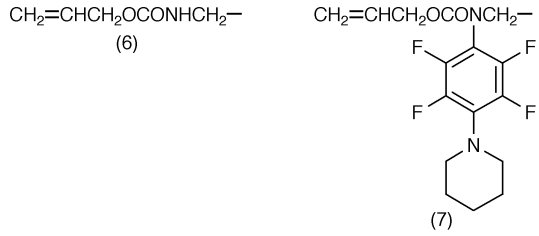
(5)

acids can be prepared by reacting 9-fluorenylmethyl chloroformate with *N*-protected acids at 0 °C in the presence of $Pr^i{}_2EtN$ as base and DMAP as catalyst.[86] The reaction probably proceeds through an unsymmetrical anhydride that loses CO_2. The formation of a succinimide ring from an Asp residue occurs quite frequently in the presence of acid or base. It has now been reported to occur during a coupling step if an allyl group is used to protect the β-carboxy group of Asp when the latter residue is followed by Gly.[87] It occurs in the absence of base if an excess of coupling agent is used. Choline esters can be used in the presence of other protecting groups that are sensitive to acids and bases because choline esters can be cleaved at pH 6.5 and room temperature in the presence of horse serum butyrylcholine esterase.[88] No other groups are affected. This promises to be a valuable technique provided that chemists are not deterred by a little enzymology; it is to be hoped that Waldmann is not a voice crying in the wilderness. Removal of the enzyme should be easily achieved by either ultrafiltration or affinity chromatography on an antibody to the enzyme. It could easily be incorporated into a solid-phase protocol. The phenylhydrazide group can also be used as a protecting group.[89] Removal of the group involves two steps. Initially, the hydrazide is

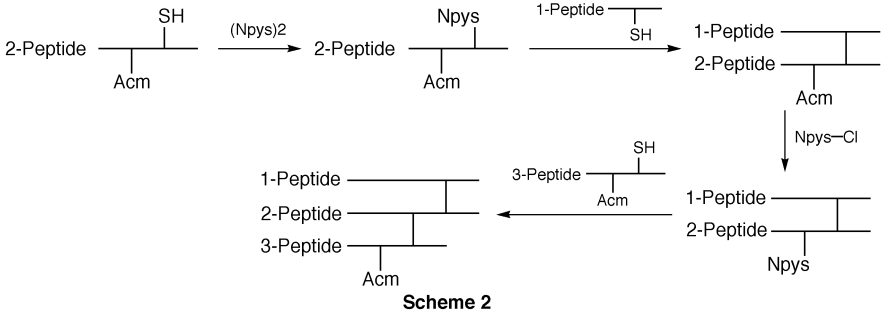
oxidized with mushroom tyrosinase to give an acyl diazene which then hydrolyses spontaneously at pH 7 in phosphate buffer.

2.3 Side-chain Protection. – There have been further applications of adamantyl-based protecting groups.[90] The 2-Adoc group has been used to protect both the ε-amino group of Lys and the hydroxy group of Tyr. Further, the β-carboxy group of Asp was protected with the 2-adamantyl ester group in the synthesis of fragments of *Sulfolobus solifaturicus* RNase.[91] Because the *O*-Bzl group is somewhat labile to acid when used to protect Ser, the *O*-cyclohexyl group has been investigated.[92,93] Direct alkylation of Boc-Ser-OH with 3-bromocyclohexene gave a moderate yield of the cyclohexenyl derivative. This could be hydrogenated in the presence of PtO_2 to give a good yield of *N*-Boc-*O*-Chx-Ser-OH which could be used in SPPS. A different approach uses the 2,4,5-tris(octadecyloxy)benzyl group in liquid-phase syntheses since the high molecular weight of the product permits purification by gel filtration.[94] Protection of the π-nitrogen atom in the imidazole ring of histidine has been further studied and shown to be feasible and satisfactory with the *im*-allyloxymethyl group.[95] Deprotection is effected with a Pd(0)-catalyst. In contrast, attempts to prepare Fmoc-His(π-Trt)-OH regio-specifically from several intermediates bearing removable τ-substituents were unsuccessful and the prospects for a more successful outcome in any future study were regarded as poor.[96] Peptides containing both Trp and cystine can be synthesized using the 2,4-dimethylpent-3-yloxycarbonyl (Doc) group to protect the indole *N*-atom.[97] The indolyl side-chains of Trp can crosslink in neat CF_3CO_2H. One possible solution involves assembling a peptide containing dihydrotryptophan (Dht).[98] Dht peptides do not crosslink in strong acid and the Dht residues can be converted into Trp residues subsequently by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. The problem associated with the possible oxidation of any cysteine residues by the latter reagent remains to be solved. Reaction of cysteine with an epoxide or alkyl halide in presence of NaOEt/EtOH followed by treatment with Boc_2O provides a simple one-pot synthesis of *N*-Boc-*S*-alkylated cysteine derivatives.[99] A new cleavage cocktail for the Fmoc-based SPPS of Met peptides has been reported that minimizes oxidation of Met residues.[100] Two allylic groups have been described for the protection of thiols and particularly Cys.[101,102] The allyloxycarbonylaminomethyl group (Allocam) (6) is introduced by reaction of the thiol with *N*-hydroxymethyl-carbamic acid allyl ester. Deprotection is effected by Pd(0)-catalysed hydrostannolysis with Bu_3SnH in presence of CH_3CO_2H. It is stable to bases when removing Fmoc groups. Alternatively, the *N*-[2,3,5,6-tetrafluoro-4-(*N*′-piperidino)-phenyl]-*N*-allyloxycarbonylaminomethyl group (Fnam) (7) can be used. It is readily deprotected by Pd(0)-catalysed allylic cleavage in the presence of various nucleophiles. Like the Allocam group, it is stable to bases.

2.4 Disulfide Bond Formation. – Me_2SO can not only oxidize peptides containing two cysteinyl residues at positions *i* and *i*+5, but the solvent acts as a 'chaperon' in assisting disulfide formation and thus minimizing other folding



routes such as β-strand formation.[103] In the synthesis of endothelin, a mixture of Me_2SO and CF_3CH_2OH favoured formation of the 1–4 disulfide bond. 2,2′-Bispyridyl disulfide [$(Pys)_2$] is reported[104] to effect intramolecular disulfide formation rapidly in peptides. Reactants and products are easily separated by reverse-phase chromatography. A different approach involves synthesis of a peptide containing two cysteine residues on MBHA resin and oxidation with CCl_4 containing $Bu_4N^+F^-$.[105] The oxidation can also be effected in solution after release of the precursor peptide from the resin. Conditions were found for the oxidation of the linear precursors of endothelins by H_2O_2 without affecting the side chain of Met.[106] In the synthesis of Kalata B1, a cyclic polypeptide with a cystine knot structure embedded in a cyclic polyamide structure, it was found that if disulfide bonds were formed before cyclization, the correct folded structure was formed only in a partly hydrophobic solvent. If cyclization preceded oxidation, however, some correct product was formed in aqueous solution and considerably improved yields were obtained in presence of hydrophobic solvents.[107] A new strategy has been developed for the regio-selective interstrand disulfide bridging of multiple cysteine peptides.[108] One problem concerns the tendency for thiol/disulfide exchange reactions to occur if $(Pys)_2$ is used to form disulfide links. This scrambling reaction can be suppressed by using 5,5′-dinitro-2,2′-bispyridyl disulfide [$(Npys)_2$] at pH 4.5–5.5 which is possible due to the better leaving-group character of 5-nitro-pyridyl-2-thione (Scheme 2). It is of interest that in the biosynthesis of insulin from the linear precursor, the Cys^6-Cys^{11} intrachain link forms first and this is followed rapidly by the formation of the two interchain disulfide links.[109]

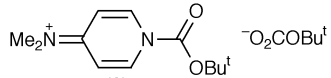


Scheme 2

2.5 Peptide Bond Formation. – Further evidence has been adduced that addition of Cu^{2+} ions in coupling reactions produced by carbodiimides

suppresses loss of chiral purity.[110,111] This is true even when the *N*-terminal residue of the amino component is Sar or when Boc-Phe-MeAla-OH was coupled to Phe-OBzl. The preferred system consists of *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) and HOBt. A mixture of $OCHNMe_2$, a tertiary base such as pyridine and an inorganic additive, such as halide of Groups 1 or 2, is not only a good solvent for amino acids,[67] but also for some peptides and therefore provides a suitable system for peptide coupling reactions[112] and for inverse peptide synthesis.[113] A mixture of $CHCl_3$ and PhOH is claimed to be a good solvent system for the segment coupling of sparingly soluble protected peptides.[114] No loss of chirality was detected when EDC with HOOBt was used.

N-Formyl Ala dipeptides can be synthesized by reaction of an amino acid ester with 2-tribromomethyl-3-formyl-4-methyl-5-oxazolidinone.[115] Protected dipeptides can be easily synthesized using Boc_2O, DMAP, C_5H_5N in tetrahydrofuran.[116] The reaction is sluggish in the absence of DMAP. Success is attributed to the intermediate formation of (8) which facilitates the nucleophilic addition of the *N*-protected amino acid at the Boc group of (8). There



(8)

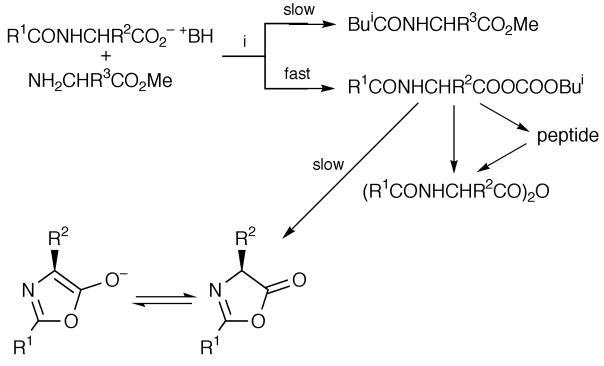
was no detectable loss of chiral purity. A third method of dipeptide synthesis starts with either trityl or 9-phenyl-9-fluorenyl amino acids.[117] These are converted into *N*-carboxy-anhydrides with either $COCl_2$ or triphosgene and coupled with another amino acid in tetrahydrofuran with no loss of chirality. There is a warning,[118] however, that the preparation of *N*-carboxy-anhydrides using $COCl_2$ can result in a product that is contaminated with HCl and the hydrochloride of the amino acid. These can be removed by washing with H_2O and $NaHCO_3$ at 0 °C. A fourth method for synthesizing dipeptides uses 5′-(aminoacyl)-adenylates, in the absence of a tRNA, which when complexed to a tRNA synthetase can generate dipeptides in the presence of excess amino acids or other appropriate nucleophiles.[119] Does all this attention to the synthesis of dipeptides adumbrate the development of methods of synthesis of large peptides starting from dipeptides using enzymic methods of coupling and perhaps using genetically engineered enzymes with closely defined substrate specificities?

Fmoc amino acid chlorides can be generated *in situ* from bis(trichloromethyl) carbonate and then used in SPPS for difficult couplings.[120] Fmoc chlorides of small peptides have been used to prepare slightly larger peptides.[121] The use of aryl esters has decreased, although a method of preparation from aryl sulfonates has been reported[122] which is virtually identical to that published by this Reporter over 30 years ago. The penta-fluorophenyl ester of an Fmoc amino acid has been described.[123]

The use of carbodiimides for peptide assembly has decreased, but Sakakibara has published a paper[124] strongly suggesting that these reagents

should not be abandoned too precipitately. Fragments of about 10 residues were coupled in solution using $EtN{=}C{=}N(CH_2)NMe_2$ (EDC) with HOOBt giving rise to angiogenin (123 residues), human midkine (121 residues), human pleiotrophin (136 residues) and *Aequoria* green fluorescent protein (238 residues). Moreover, Carpino has shown[125] that segment coupling using *N,N′*-diisopropylcarbodiimide and HOAt in CH_2Cl_2 gave very good yields of product with very little loss of chirality. Interestingly, the crystal structure of HOAt has been determined[126] and the compound dimerizes in solution as a result of hydrogen-bonding between hydroxy groups and aza-nitrogen atoms. Determination of the optimal amount of HOBt, HOOBt and HOAt to be added in peptide assembly using EDC in alcohol solution showed that less than an equimolar amount of additive suppresses the competitive formation of ester.[127] Coupling of phthaloyl amino acids and racemic amino acids using DCC/HOBt gives the LD-dipeptide preferentially.[128]

The nature of the side-chain of the *C*-terminal residue has little effect. Further study of the HOCt additive reported last year has revealed that most amino acids couple without loss of chiral purity.[129] An exception is Fmoc-His(Trt)-OH, although chiral purity is preserved at 0 °C. The transfer active ester condensation technique reported last year has been successfully applied[130] to the construction of a chimeric peptide consisting of a ubiquitin fragment (67–76) coupled to a fragment of histone 2A (114–128) via an isopeptide bond involving the *C*-terminal Gly residue of the ubiquitin and the ε-amino group of Lys[119] in the histone fragment. The formation of isomeric peptides involving coupling a Lys residue has been studied.[131] Coupling with Bu^iOCOCl gave high yields of product in which the ε-amino group is acylated irrespective of the acylating amino acid derivative. In contrast, when BOP-Cl was used, acylation of the α-amino group heavily predominates especially with bulky amino acids. The use of Bu^iOCOCl for peptide synthesis can be kinetically controlled (Scheme 3) thus affording an increased yield of desired product with retention of chiral integrity.[132] The carboxy group of the *N*-protected amino acid is activated in the presence of the *C*-protected amino acid. Formation of the unsymmetrical anhydride is much faster than the



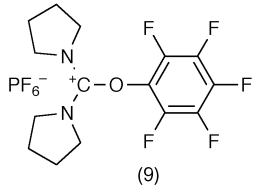
Reagents: i, Bu^iOCOCl, base (B)

Scheme 3

acylation of the amino ester with $Bu^{i}OCOCl$. Although the unsymmetrical anhydride can slowly form the oxazolone with consequent loss of chiral purity, the competing reaction of the anhydride with the amino ester is much faster thus affording high yields of optically pure product.

In the synthesis of the [Abu,[20.31] HOTic[22]]hEGF (20–31) fragment of EGF, difficulty was encountered in the attachment and deprotection of the two *N*-terminal residues.[133] Better results might have been obtained by using one of Carpino's techniques such as the use of Fmoc amino acid chlorides and arenesulfonyl protection. Another coupling difficulty was encountered[134] involving the formation of pyrrolidide derivatives from slow reactions of activated carboxylates with nucleophilic amines when using PyAOP, PyBOP or PyBrOP. This side reaction was attributed to the presence of small quantities of pyrrolidine in the coupling reagent. The side reaction was avoided by recrystallizing the reagent before use. Coupling reactions using a pentafluorophenyl-substituted reagent (HPyOPfp) (9) were often found to be accompanied by extensive loss of chiral purity;[135] this was attributed to formation of oxazolone. Addition of HOAt overcame this problem as a result of transesterification and the faster coupling relative to oxazolone formation.

Several new coupling reagents have been described. The order of presentation is not significant; a complete assessment of their value awaits independent reports from other workers. Benzotriazol-1-yloxy-*N,N*-dimethylmethaniminium hexachloroantimonate (BOMI)[136] (10) is much better than DCC for preserving chiral integrity in the coupling of Z-Gly-Phe-OH and Val-OMe and is comparable to BOP, HBTU, HBPyU and HBPipU. Chiral purity is well preserved in tetrahydrofuran or MeCN, but is extensively lost in $OCHNMe_2$. 3-(Diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (11) (DEPBT) is a crystalline reagent that effects peptide bond formation in nearly quantitative yields and with only a small loss of chiral purity.[137] 5-(1*H*-Benzotriazol-1-yloxy)-3,4-dihydro-1-methyl-2*H*-pyrrolium hexachloroantimonate (12) (BDMP) effects peptide bond formation in both solution syntheses and SPPS.[138] Good retention of chiral purity was observed in Young's test in comparison with four other reagents, but only small peptides have so far



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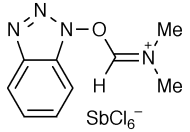

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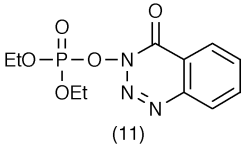

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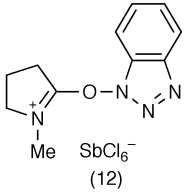

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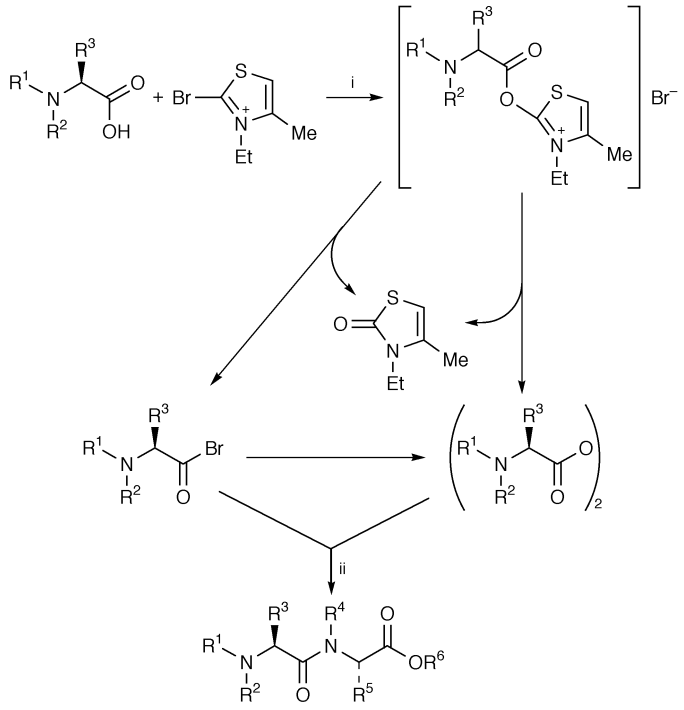
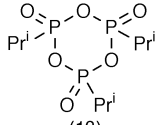


R^1 = Z, Fmoc or Boc; R^2,R^4 = H, Me or alkyl; R^3,R^5 = amino acid side-chain; R^6 = Me, Et, Bu^t or Bzl
Reagents: i, base; ii, $R^4NHCHR^5CO_2R^6$

Scheme 4

been synthesized with this method. 2-Bromo-3-ethyl-4-methylthiazolium tetrafluoroborate (BEMT) has been synthesized and tested as a peptide coupling reagent, especially for very hindered amino acids such as α-*C*-dialkylamino acids.[139] The proposed mechanism of peptide synthesis is outlined in Scheme 4. Carpino's group have produced a novel type of coupling reagent,[140] 2-propane-phosphonic anhydride (13) (T3P), that is suitable for

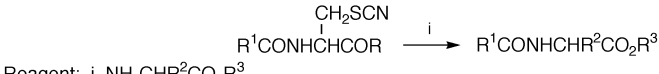


(13)

both segment coupling and head-to-tail cyclization of sterically hindered peptides. Uronium salts derived from 2-mercaptopyridine-1-oxide also gives good yields, including the so-called difficult couplings, with high retention of chiral purity.[141] The relationship between the structure and reactivity of aminium and uronium coupling reagents has been advanced.[142] The reagents considered all contain the >N-CR^+-N< moiety and it is argued that the reactivity of such reagents is governed by structural factors in the putative transition state. Clearly, delocalization of the π-electron density from the N

atoms towards the carbocation would reduce the positive charge on the latter and disfavour nucleophilic attack. Electron delocalization is minimal if the N atoms have a pyrimidal configuration. Theoretical structural studies showed that the best structure to promote nucleophilic attack on the carbocation occurs when the >N-CR-N< moiety occurs in a pyrrolidino ring.

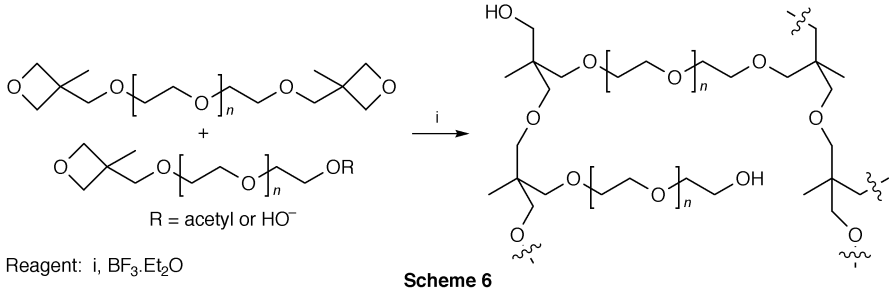
The native chemical ligation technique of protein synthesis has been further examined and applied. For example, the peptide bond between the *C*-terminal residue of one fragment and the *N*-teminal Cys residue of the other fragment (X-Cys) is possible where X is any of the 20 amino acids that occur in natural proteins.[143] Fully active human secretory phospholipase A_2 (124 residues) was assembled from four fragments (1–27, 28–58, 59–87 and 88–124). A novel method for synthesizing peptides uses unprotected peptides in solution.[144] The formation of a peptide bond involves the reaction between a peptide with a *C*-terminal Cys residue and peptide with a free α-amino group (Scheme 5). Note



Scheme 5

that the Cys residue is lost so coupling can theoretically be effected at any point in the peptide sequence. Small quantities of byproducts may arise (e.g. R_1CO_2H, and the dehydropeptide derived from elimination of HSCN from the *S*-cyanopeptide component). Other methods of native chemical ligation have used solid phase methods and are discussed in the next section.

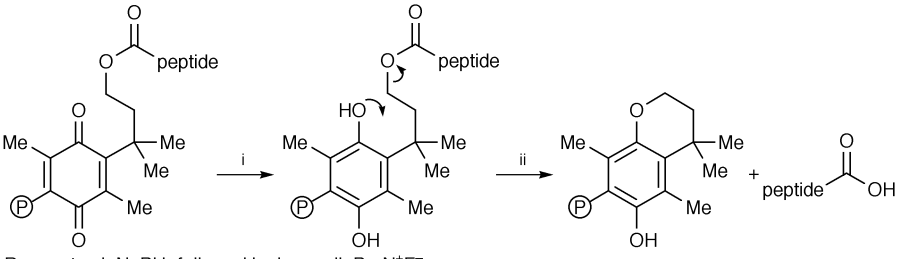
2.6 Peptide Synthesis on Macromolecular Supports. – Copolymers of styrene and butanediol dimethacrylate covering a range of cross-linking densities have been prepared.[145] The products had excellent swelling properties in all solvents normally used for SPPS. They were stable to exposure to CF_3CO_2H, 20% piperidine in $OCHNMe_2$, aqueous NaOH, NH_2OH and liquid NH_3. The resins could easily be functionalized with $-CH_2Cl$, $-CH_2NH_2$ or $-CH_2OH$ groups. Several small peptides were synthesized thereon in high purity and yield. A similar resin made from 1,6-hexanediol diacrylate and polystyrene led to easier peptide bond formation in shorter time and with higher yield than was found with polystyrene-divinyl copolymer.[146] Several supports based on polyethylene glycol (PEG) have been designed.[147,148] A peptide aldehyde can be generated on the support that can undergo a variety of characteristic reactions. Reaction of PEG with oxetane derivatives followed by polymerization catalysed by $BF_3.Et_2O$ afforded an open structure (Scheme 6) that was accessible to molecules as large as enzymes thus allowing enzyme-catalysed peptide extension or modification to be easily carried out. Low-loading copolymers of PEG and polystyrene have been described earlier and the concept has been extended to high-loading resins that helps to improve yields where difficult coupling steps are involved.[149] A simple method for generating chloromethyl polystyrene on a multiple pin support has been described.[150] Dendrimers for the assembly of libraries of small peptides have been synthe-



Scheme 6

sized in the solid phase.[151] A high loading paramagnetic support has been made by encasing beads of magnetite in high cross-linked polystyrene containing chloromethyl groups.[152] A detailed study has been made of the conditions necessary to remove the *N*-terminal Boc group without disturbing the linkage between the peptide and the resin.[153] At the same time, the conditions necessary to detach the completed peptide from the resin with HF were established. Thus, the decreasing order of acid stability for resins was as follows; (i) benzhydrylamine resin > (ii) 4-methylbenzhydrylamine resin ≅ (iii) 4-oxymethyl-phenylacetamidomethyl resin > (iv) chloromethyl resin and for *C*-terminal amino acids; Phe > Gly ≅ His ≅ Asp. Cleavage times with HF were ≈ 6 h for (i) and 2–3 h for (ii) and (iii) when Phe was *C*-terminal. Also, for peptide sequences longer than about 40 residues, (i) is preferable to (ii). Although this may be regarded as rather pedestrian research, it is attention to detail of this sort that can mean the difference between obtaining a trace yield and one which is commercially viable. More studies of this kind are desirable, e.g. with the PEG resins designed by Meldal and co-workers.[147]

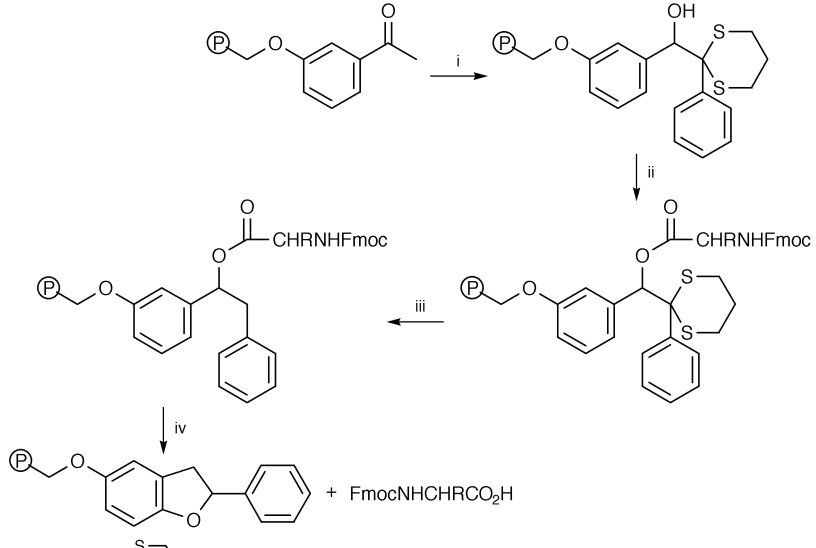
Also pertinent to the preceding discussion is the continuing plethora of described linkers offering methods for preserving the peptide during the assembly process or a choice of conditions for product detachment without chemical modification. 4-(3-Hydroxy-4-methylpentyl)phenylacetic acid is proposed for use with Boc chemistry because (a) the linker-peptide bond is slightly more stable than when the Pam resin is used and (b) benzyl groups are more stable to cleavage with acid.[154] A redox-sensitive resin linker for the SPPS of peptides modified at the *C*-terminus has been designed.[155] The linker contains a quinone moiety that is readily reduced by sodium hydrosulfite (Scheme 7).



Scheme 7

The generation of a hydroxy group and the presence of a 'trimethyl lock' facilitates the formation of a lactone and the simultaneous detachment of the peptide derivative. Although this method does not involve either the removal of a protecting group or the introduction of an activating group, it is very closely allied to the use of the safety catch procedure described in the following paragraph. Perhaps some readers will regard the separation as hair splitting. The second of the two papers describes a later variation that permits the preparation of the peptide with a free carboxy group. Although this methodology has been applied only to the synthesis of short peptides, there is no obvious reason why it should not work satisfactorily with bigger molecules. A silyl ether based linker for the synthesis of protected glycopeptides has been developed.[156] (α,α-Dimethyl-4-nitrobenzyl)dimethylsilyl chloride is reacted with the primary hydroxy group in the side-chain of a glycoamino acid derivative. The nitro group is reduced to allow coupling with the resin using succinic anhydride. Protected glycopeptides are detached by fluoridolysis. A photochemically labile carbamate-based linker permits easy detachment of peptide that can be identified by electrospray mass spectroscopy.[157]

Interest has been revived in safety-catch linkers as proposed by Kenner and Sheppard thirty years ago. For example, the linker may contain a benzoin moiety in which the carbonyl group is protected by a 1,3-dithian group (safety-catch). The hydroxy group on the linker is the site of attachment of the peptide under construction. The safety catch is removed by oxidation and the peptide is detached by photolysis at 350 nm[158] (Scheme 8). The sulfonamide type of linker and safety catch has been considerably improved most notably by using iodoacetonitrile rather than diazomethane to alkylate the sulfonamide nitrogen



Reagents: i, Li(S,S); ii, $FmocNHCHRCO_2H$, $Pr^iN{=}C{=}NPr^i$; iii, CF_3SO_3Me or HIO_4; iv, *hν* 350 nm

Scheme 8

atom.[159] The latter is readily attacked by a nucleophile with scarcely any loss of chiral purity. The power of this methodology was soon proved by the synthesis of an analogue of diptericin, an 82-residue antibacterial glycoprotein produced by insects in response to immunological challenge.[160] The structural changes in the diptericin analogue included the substitution of Gly^{25} by Cys required in the Kent native ligation method and the replacement of Asp^{29} and Asp^{45} by Glu in order to avoid aspart-imide formation. The Backes and Ellman method has been successfully applied to the synthesis of 'head-to tail' cyclic peptides. The cyclization was initiated by removal of *N*-terminal Trt by CF_3CO_2H and then addition of Pr^i_2EtN.[161] Kent[162] has described a further development, 'solid phase chemical ligation', of his approach to the synthesis of large polypeptides with minimal protection and mild coupling methods. Molecular assembly can be effected in either the conventional C→N direction or the more unusual N→C direction. The successful synthesis of a phospholipase A_2 molecule containing 118 amino acids and 6 disulfide bonds makes this paper compulsive reading.

The anchoring of an amino acid through its side-chain offers the possibilities of being able to extend the polypeptide chain in either direction and effecting 'head-to-tail' cyclization while still attached to the support. The phenolic hydroxy group of Tyr can undergo a Mitsunobu type reaction in order to establish a link to a support.[163] In a formally similar approach, the imidazole group of histidine and related compounds can be attached to a trityl resin in order to produce cyclic peptides.[164] The general philosophy of assembling a peptide on a solid support and forming a final peptide bond before release can lead to either a peptide with a *C*-terminal alkylamide group[165] or a cyclic peptide.[166] An aromatic aldehyde group on the resin is the site for attachment of an allyl ester of an amino acid by reductive amination followed by peptide assembly on the generated secondary amino group. The assembled peptide can either be detached giving a peptide alkylamide or can be cyclized after liberating an amino group in a peptide side-chain as a target for an activated carboxy group. This technique uses Boc chemistry, but a related method using Fmoc protection has been described for the synthesis of peptide 4-nitroanilides and thioesters.[167]

Kent's method of solid phase chemical ligation is not the only route to large proteins. Ramage has reported the synthesis of deglycosylated human erythropoietin[168] (166 residues) using ethyl 1-hydroxy-(1*H*)-1,2,3-triazole-4-carboxylate in conjunction with DIC for carboxyl activation. Synthetic enzymes and even multienzyme conjugates will probably be commercially available in the near future.

The use of Hmb backbone modification to avoid intra- and inter-molecular hydrogen bonding during peptide assembly has not received much attention this year. A comparative study[169] of the use of Hmb-protected amino acids and the incorporation of pseudoproline into appropriate analogues suggested that the latter approach was preferable because of the difficulty in obtaining good yields when Hmb amino acids were involved in coupling reactions. Indeed, it has been recommended that Hmb-Gly residues should be incorpo-

rated using N^{α}-Fmoc-Hmb-Gly-OH rather than *N,O*-bis-Fmoc-Hmb-Gly-OPfp in order to limit steric hindrance.[170] In the coupling of short peptides to resin-bound fragments, it has been customary to use a substantial excess of the peptide in the soluble phase in order to maximize yields. An approach that appears rather obvious in retrospect uses dry resin carrying the fragment with a free amino group and a small excess of the soluble fragment in a volume of solvent sufficient to cause swelling of the resin.[171] Each coupling step is followed by capping with 2,4-dinitrofluorobenzene; detection of deletion peptides is thus simplified. The technique was tested by synthesizing a fragment (78–90) of HIV-1 proteinase; the yield was vastly improved compared to the standard methodology. An accelerated method of SPPS has been described and tested.[172] Using Boc chemistry with the HATU coupling reagent in Me_2SO, 10–15 residues per hour could be coupled. The method works satisfactorily with so-called difficult sequences. Suitable methods for monitoring the various steps in SPPS improve the rate of sequence assembly. For example, Boc group removal can be followed spectrophotometrically since CO_2 bubbles produce a fringe of spikes accompanying the peak.[173] Again, near-infrared multispectral imaging can be used to monitor peptide coupling.[174] Fast scanning and high sensitivity at 1529 nm monitors the concentration of amino groups on beads or the increase in absorption at 1483 nm follows the production of amide groups during the coupling stage. Monitoring at 1529 nm also plots the removal of Fmoc groups. Finally, the use of gaseous HF allows the fast detachment of peptide from the support at the end of a synthesis.[175,176] Polypropylene is a suitable material for construction of a reaction vessel. Evacuation of the reaction vessel before admitting HF permits faster filling and reaction. If desired, acid-sensitive protecting groups on side-chains can be removed by a preliminary treatment with CF_3CO_2H before the step with HF. If it seems that all the improvements in technique leave little new for the chemist to do, fresh problems continue to emerge. In the SPPS of Trp peptides on a Wang resin, unexpected alkylation of the indole nucleus can occur and this is not controlled by addition of standard scavengers.[177] Strangely, the problem did not occur if Trp was the *C*-terminal residue.

2.7 Enzyme-mediated Synthesis and Semisynthesis. – Proteinases continue to be used occasionally to esterify acylamino acids.[178] Optimase M-440 from *B. licheniformis* has been used to synthesize amino acid esters of disaccharides.[179] Similarly, α-chymotrypsin catalyses the enantioselective amidation of chiral amines by Z-Phe-OCH_2CO_2H.[180] Improved yields of 'Aspartame' precursors are still being sought.[181] Small peptide derivatives have been made using subtilisin in MeCN or as a SDS complex in an alcoholic medium.[182,183] $[E]/[S]$ ratios were about 10^{-5}. Elastase from *Pseudomonas aeruginosa* has been used to synthesize *N*-protected dipeptide amides in aqueous methanol.[184] The kinetics have been studied of a solid-to-solid peptide synthesis using thermolysin with Z-Gln-OH and H-Leu-NH_2 as substrates.[185] Preheating of the substrates and ultra-sonication during reaction had little effect. The substrates formed a salt during the process. Enzymic peptide synthesis in frozen solution

has been further studied.[186] An apparatus was constructed to shock-freeze large volumes of solution. The enhancement of α-chymotrypsin activity after freeze-drying in the presence of 18-crown-6 has been further examined.[187] After a gap of a few years, peptide synthesis in reverse micelles using α-chymotrypsin[188–190] and the esterification of Z-Ala-OH by sorbitol catalysed by papain[191] have been studied.

The versatility of enzyme-catalysed peptide synthesis can be enhanced by (i) controlled mutagenesis of the enzyme, (ii) chemical modification or (iii) a combination of (i) and (ii). Mutants (S166C and M222C) of subtilisin from *B. lentus* were chemically modified by reaction of the thiol group with $MeSO_3SR$.[192] The new enzymes were able to accept some D-amino acids as acyl donors. They were also able to accept α-branched amino acid amides as acyl acceptors in the S′ pocket whereas the wild-type enzyme will not. Again, in the synthesis of Ac-Phe-Lys-OH, the carbobenzyloxy derivative of α-chymotrypsin gave a better yield than when native enzyme was used.[193]

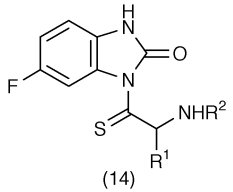
The choice of ester as acyl donor can influence the kinetics of peptide synthesis. Carbamoylmethyl esters are excellent donors in reactions catalysed by α-chymotrypsin.[194] The use of inverse substrates has been cited before and some further examples have been reported this year. Bovine or *S. griseus* trypsin will accept 3-guanidinophenyl or 3-(guanidino-methyl)phenyl ester as acyl donor substrates.[195] The advantage of this approach lies in the resistance of the product to enzymic hydrolysis after the loss of the guanidinated product. Similar experiments can be carried out using thrombin as the enzyme for catalysis.[196] Conditions can be optimized by the choice of solvent, pH and concentration of acyl acceptor. A comparative study has been made of positional isomers of guanidinonaphthyl esters as acyl donors.[197] Esters of 4-guanidino-1-naphthol were the best substrates for bovine trypsin. The method proved to be useful for the synthesis of peptides containing α,α-dialkyl amino acids. Enzyme specificity can be influenced by physical conditions. In the synthesis of peptides from Z-Ala-Ala-Phe-OH using porcine pepsin, derivatives of unnatural amino acids such as homophenylalanine, 4-nitrophenylalanine and *S*-methylcysteine were good second substrates.[198] Other unnatural substrates tested as potential substrates in enzyme-catalysed syntheses included di- and tri-peptide derivatives containing a trifluoromethyl group on the α-carbon atom.[199] Dipeptide derivatives have been produced from 3-trimethylsilylalanine using thermolysin.[200] In fact, the Z-silylamino acid is a better substrate than Z-Leu-OH. On the other hand, trimethylsilylalanine methyl ester is not accepted as the amino component by thermolysin.

This section concludes with brief accounts of syntheses or modifications of peptides involving enzyme-catalysed steps. Vasoactive intestinal peptide (VIP) contains one Gln and three Lys residues and so can act as both an amino acceptor and a donor substrate for tissue transglutaminase.[201] Gln^{16} reacts as an acceptor with $NH_2(CH_2)_3NH_2$, putrescine, cadaverine, spermidine, spermine and H-Gly-OEt. In addition, Lys^{21} can react with the side-chain of Gln^{16} both intra- and inter-molecularly. Transglutaminase has also been used to effect the formation of a covalent bond between the two chains of the sweet

protein, monellin.[202] The synthetic A chain was extended at the *N*-terminus with the sequence *K*GK. The B chain was elongated at the *C*-terminus with the LL*Q*G sequence. Exposure to transglutaminase catalysed the formation of an interchain amide link between the italicized residues. When the oxidized B chain of insulin was exposed to the action of trypsin,[203] a dimer of the fragment B^{23}–B^{29} was formed in up to 15% yield by transpeptidation. Peptide aldehydes can be prepared by using an acylpeptide ester to acylate an amino alcohol in the presence of subtilisin 72 supported on macroporous silica. The product is then oxidized with Me_2SO/Ac_2O.[204] Alternatively, the acylpeptide ester can be coupled to an aminoaldehyde semicarbazone in the presence of subtilisin. Good yields of products were obtained by both methods. The biosynthesis of bacterial cell wall or murein offers some potential targets in the search for new antibiotics. The first six cytoplasmic intermediates have been synthesized enzymically.[205] Four of the stages involve the formation of amide bonds involving in turn, L-alanine, D-glutamic acid, mesodiamino-pimelic acid and D-alanyl-D-alanine. A molecule of ATP is consumed at each of these steps.

Alcalase has been used to prepare di- and tri-peptide conjugates of 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid, a cytotoxic drug.[206] It must be confessed, however, that the design and synthesis of prodrugs has not produced the pharmacological successes that were hoped for. The use of glycosidases for the production of glycopeptides still attracts some attention but requires more effort to produce the potentially important outcomes that might be expected. Lactose-β-galactosidase from *E. coli* and *A. oryzae* forms galactosidases using lactose as substrate, but the enzyme is extensively denatured by high concentrations of organic solvent and lactose becomes insoluble under these conditions. At lower concentrations of solvent, presumably hydrolysis becomes important. Thus Aloc-Ser-OMe gave 28% of the *O*-galactoside, Aloc-(Galβ-)Ser-OMe, in water containing 8–15% of MeCN, EtCOEt, Me_2CO or EtOAc.[207] Perhaps substrate capture by continuous recycling through an insoluble affinity support would give improved yields. Using an oligosaccharide rich in mannose gave a glycopeptide derivative of calcitonin when incubated with an endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae*.[208]

2.8 Miscellaneous Reactions Related to Peptide Synthesis. – The guanidination of the ω-amino group of Orn or Lys derivatives has been used to prepare the corresponding Arg or Har compounds during the last 50 years. Derivatives of ω-*N*-substituted Arg can be made using $ArSO_2N{=}C(SMe)_2$,[209] $EtN{=}C(SO_3)$-NEt[210] or 1*H*-pyrazole-*N*-propyl-1-carboxyamidine.[211] The long known reaction of 1,3-diketones with the guanidino group of Arg residues in proteins has found a new application. 4,6-Dioxoheptanoic acid reacts with the Arg side-chain at pH 9.2 (e.g. in pyoverdin) and the resulting carboxy group has been used to crosslink with e.g. cephalexin.[212] Other possible applications come to mind such as the coupling of an epitopic peptide to a carrier protein, the immobilization of enzymes and the attachment of various reporter groups to proteins as an aid for conformational studies. Distantly related, is the report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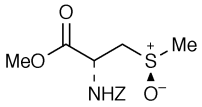
(15)

of a new biotinylating agent.[213] Bis(pyridine)iodonium tetrafluoroborate is a new reagent for introducing iodine adjacent to phenolic hydroxy groups and thus potentially for radioimmunoassays.[214] Protected hydroxy groups do not react. The pentachlorophenyl esters of *N*-Boc amino acids or peptides can be reduced to the corresponding alcohols using $NaBH_4/I_2$ in tetrahydrofuran.[215] Esters of *N*-tosylated or *N*-tri-fluoroacetylated amino acids and peptides can be *N*-allylated using allyl carbonate in the presence of Pd(0) at room temperature under neutral conditions.[216] A new type of thioacylating agent (14) has been described[217] and this should simplify the synthesis of endothiopeptide analogues of biologically active peptides. Peptides of β-nitro-α-amino acids can undergo a β-elimination reaction when refluxed with e.g. Pr^i_2NH in $CHCl_3$ for 48 h giving rise to α,β-dehydro-α-amino acid residues.[218] Oxidation of the sulfur atom in Met and Cys derivatives using Bu^tOOH shows no stereoselectivity in conventional solvents but in supercritical CO_2 the major product is (15).[219] Finally, peptides containing *N*-methylaminoisobutyric acid (NMeAib) are sensitive to acid hydrolysis. The bond linking NMeAib to the following amino acid is ruptured.[220] It is postulated that the carbonyl oxygen atom of the amino acid preceding NMeAib is proximate to the carbonyl carbon atom of NMeAib and acts as an internal nucleophile leading to cleavage via an oxazolinium ion intermediate. This could be an annoying complication in the synthesis of peptides of NMeAib and related *N*-alkylated bulky amino acids.

3 Appendix: A List of Syntheses Reported Mainly in 1999

Peptide/Protein	*Ref.*
3.1 Natural Peptides, Proteins and Partial Sequences. –	
Adaptor protein Grb2	
Potent inhibitors of the Grb2-SH2 domain	221, 222
Nonphosphorylated peptide that binds to Grb2-SH2	223
Aequorea	
Green fluorescent protein	124, 224
Amelogenin	
Analogue of *C*-terminal sequence	225
β-Amyloid	
Alzheimer's Aβ1-42 amyloid peptide and analogues	226, 227
Angiotensin	

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3

Analogue and Conformational Studies on Peptides, Hormones and Other Biologically Active Peptides

BY ANAND S. DUTTA

1 Introduction

The subject matter included this year is broadly similar to that included last year.[1] Although most of the publications covered in this chapter were published in 1999, a few of the 1998 publications not covered last year have been included. This is especially so in the case of peptides not discussed last year due to space restrictions. No work published in patents or in unrefereed form (such as conference proceedings) has been included. Non-peptide ligands acting on the peptide receptors (agonists and antagonists) and non-peptide inhibitors of various enzymes have again been included in individual sections. However, due to space limitations, the structure–activity studies on non-peptide series of compounds are not described in detail. Only the more potent compounds from each series are highlighted to give an idea about the structural types displaying the desired activity and pharmacokinetic profile. Throughout this chapter, amino acids are referred to by their three letter codes following standard nomenclature. For the naturally occurring L-amino acids, no stereochemistry is specified in the text.

2 Peptide Backbone Modifications and Di-, Tri-peptide Mimetics

Examples of peptide bond replacements and peptidomimetics incorporated in individual biologically active peptides and enzyme inhibitors (discussed below individually) are not included in this section. This section contains other publications which either describe synthetic details of pseudopeptide moieties or peptidomimetics or their incorporation in peptides not discussed in individual sections.

2.1 Aza, Hydrazinoaza and Aminoxy Peptides. – Synthetic routes to pseudopeptides such as Boc-AzTic-Leu-OMe, Ac-AzTic-Gly-OMe and AzTic-Leu-OMe (**1**), incorporating the conformationally constrained AzTic residue, have been reported and conformational properties of these peptides have been

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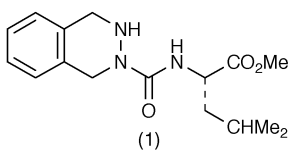
studied.[2] Peptoids containing hydrazino and hydrazinoaza moieties (*e.g.* **2**) and aminoxy amino acids have been synthesised.[3,4] Conformations of aminoxyamino acid-containing peptides like Boc-(NH-O-CH(CH_2CHMe_2)-CO)$_n$ -OBu^t (n = 1, 2, 3, 4 and 6) (*e.g.* **3**) have also been investigated.[4]



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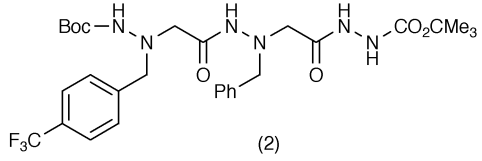

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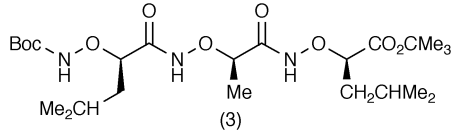
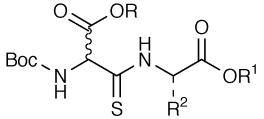


(3)

2.2 ψ[CSNH], ψ[CH_2NH], ψ[PO_2CH_2], ψ[hydroxyethylene], ψ[dihydroxyethylene], ψ[CH_2CH_2-N(isopropyl)-CO] and Retro-inverso Peptide Analogues. – Thioamides (**4**) {Boc-D/L-Ama(OEt)ψ[CSNH]Tyr(Bzl)-OMe, Boc-D/L-Ama(OMe)ψ[CSNH]Tyr(Bzl)-OTmse, Boc-D/L-Ama(OEt)ψ[CSNH]Tyr-OBu^t, Boc-D/L-Ama(OMe)ψ[CSNH]Tyr(Bzl)-OBzl, Boc-D/L-Ama(OMe)ψ[CSNH]Phe-OBu^t, Boc-D/L-Ama(OEt)ψ[CSNH]Phe-OBu^t, Boc-D/L-Ama(OEt)ψ[CSNH]Phe-OMe, Boc-D/L-Ama(OMe)ψ[CSNH]Leu-OBu^t, D/L-EthylMal(OMe)ψ[CSNH]Phe-OMe, D/L-AllylMal(OEt)ψ[CSNH]N(L-1-phenyl)ethylamide} were synthesised (by thionation of the corresponding peptides with Lawesson's reagent) and reduced (Raney nickel) to give the corresponding ψ[CH_2NH] analogues (**5**).[5] Incorporation of ψ[CH_2NH] in larger peptides was achieved by ligation (reductive amination) of a peptidyl aldehyde with resin bound amino peptide. No epimerization took place during the aldehyde preparation or the reductive amination step.[6] The preparation of Aspψ(PO_2CH_2)Ala phosphinic pseudopeptide (**6**) and the corresponding Glu analogue was reported using phenyl group as the carboxyl synthon.[7]



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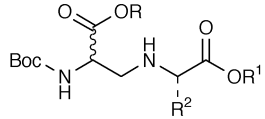

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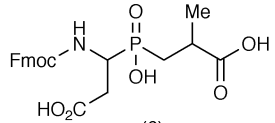
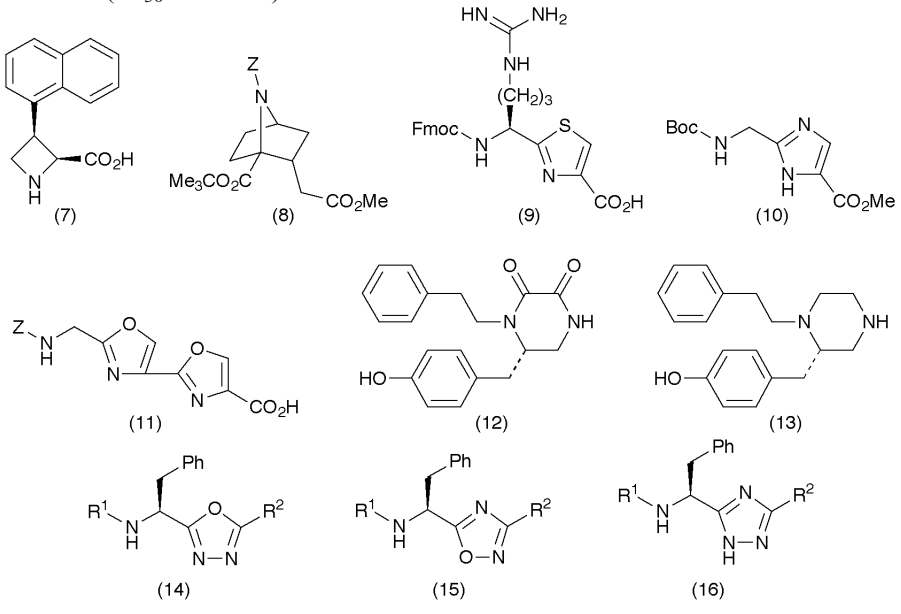


(6)

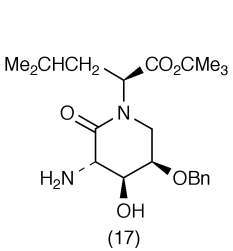
Synthetic routes for non-symmetric dihydroxyethylene dipeptide isosteres were reported starting from dihydroxynitriles.[8] Ab initio calculations of the representative α-hydroxy ketomethylene dipeptide isostere (2*S*,5*S*)-5-amino-2-hydroxy-4-oxohexanoic acid [(NH_2-CH(R)CO-CH_2CH(OH)COOH)] are described.[9] Synthesis of an octapeptide derivative Lys-Alaψ[CH_2CH_2-N(isopropyl)-CO]Tyr-Asn-Phe-Ala-Thr-Nle-NH_2 {Alaψ[CH_2CH_2-N(isopropyl)-CO]-Tyr = -NH-CH(Me)-CH_2CH_2-N(isopropyl)-CO-Tyr} has been reported.[10] The NMR structures of a 19-mer peptide corresponding to the major

antigenic region of foot-and-mouth disease virus (Gly-Ser-Gly-Val-Arg-Gly-Asp-Phe-Gly-Ser-Leu-Ala-Pro-Arg-Val-Ala-Arg-Gln-Leu) and its retro-enantiomeric analogue were determined in aqueous solution and both peptides were shown to exhibit similar folding features. However, the retro-inverso analogue appears to be more rigid than the parent peptide and contains five atypical β-turns.[11]

2.3 Rigid Amino Acid, Di-, Tri-peptide and Turn Mimetics. – Syntheses of several conformationally constrained amino acids containing Phe, Tyr, Trp and His, Nal (**7**), Glu (**8**) and Leu side chains and thiazole (**9**), imidazole (**10**) and oxazole (**11**) derivatives containing Arg and Gly type of structures were reported.[12–15] Other constrained structures incorporating amino acid side chains include 1,6-disubstituted 2,3-diketopiperazines (**12**), 1,2-disubstituted piperazines (**13**) and piperazine-2,3,5-triones.[16,17] The synthesis of 1,6-disubstituted 2,3-diketopiperazines and 1,2-disubstituted piperazines was achieved from resin-bound reduced *N*-acylated amino acids (Tyr, Phe, Ala) and four carboxylic acids (phenylacetic acid, acetic acid, isobutyric acid and cyclohexane carboxylic acid). Similar to structure **9** containing Arg side chain, 1,3,4-oxadiazole, 1,2,4-oxadiazole, and 1,2,4-triazole ring systems (**14**–**16**, R^2 = -COOH or -CH_2COOH) containing a Phe side chain have been reported. Some of these mimetics were incorporated as Phe-Gly replacements in dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, SP). Some of the dermorphin analogues displayed affinities for the μ-receptor (IC_{50} = 12–31 nM) in the same range as dermorphin itself (IC_{50} = 6.2 nM). The SP pseudopeptides showed considerably lower affinities (IC_{50} > 1 μM) for the NK_1 receptor than SP itself (IC_{50} = 1.5 nM).[18]



Synthetic details of a number of dipeptide mimetics,[19–22] various turn mimetics[23–29] and β-strand mimics[30,31] were reported. A review describing 5-azidomethyl tetrahydrofuran-2-carboxylates as carbohydrate-derived dipeptide isosteres was published.[19] Examples of dipeptide mimetics include a 3-amino-2-piperidone as a Leu-Ser mimetic (**17**)[20] and a tetrahydropyrimidinone derivative as a Pro mimetic (**18**).[22] Single-crystal X-ray analysis of Ac-D-Ala-(cyclo)Asn-NHMe (**18**) shows a high degree of structural similarity to a known proline-containing dipeptide. Examples of various turn mimetics include compounds **19** and bicyclic lactams like **20** which possess structural similarity to the two central residues of a β-turn. Some of the turn mimetics were incorporated into Pro-Leu-Gly-NH_2 and the resulting compounds like **21** (R = isobutyl, butyl and benzyl) were shown to be active in an *in vivo* model of apomorphine-induced rotational behaviour in the 6-hydroxydopamine-lesioned rats.[27–29] Based on the work reported earlier, two additional β-strand mimics (**22**, **23**) were synthesised.[30] β-Strand mimics **22** (composed of a 5-amino-2-methoxybenzoic acid unit linked by a diacylhydrazine group to a fumaramide unit) and **23** (composed of a 5-amino-2-methoxybenzoic acid unit linked by a diacylhydrazine group to a peptide) were coupled to Phe-Ile-Leu by means of 1,2-diaminoethane diurea turn units to form artificial β-sheets. NMR studies revealed that **22** and **23** derivatives adopt hydrogen-bonded antiparallel β-sheet conformations. *N*-Methylated 3,5-linked pyrrolin-4-ones like **24** are reported as privileged nonpeptide scaffold which are able to mimic not only the extended β-sheet/β-strand conformations, but also diverse conformations including those analogous to β-turns and helices.[31]



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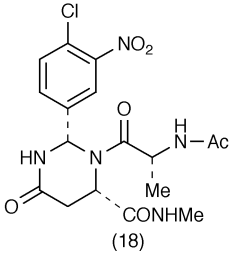

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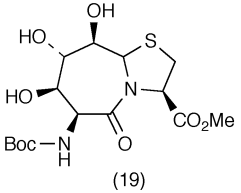

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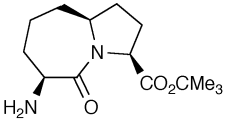

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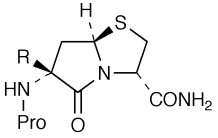

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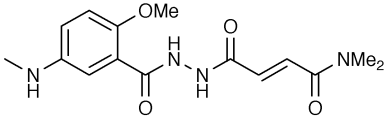

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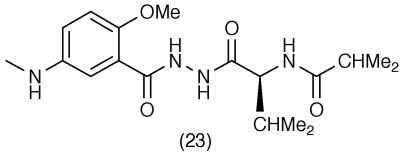

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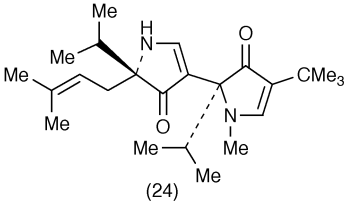

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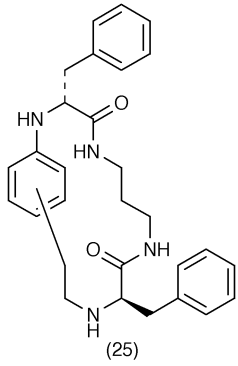
In addition to the mimetic structures mentioned above, influence of N- and C-terminal capping and unnatural amino acids like α,α-dialkyl amino acids and D-amino acids in maintaining a desired structure was also studied.[32–35] In a series of cyclic pentapeptides, derived from the C-terminal CCK-4 fragment enlarged with Asp^1 (Asp-Trp-Met-Asp-Phe), all D-amino-acid-substituted peptides showed beta II′-turn conformations with the D-amino acid in the $i+1$ position, excepting the D-Asp-containing peptides.[33] A 14-residue synthetic, amphiphilic α-helical peptide model system [Lys-Leu-X-Glu-Leu-Lys-Gln-Lys-Leu-X-Glu-Leu-Lys-Gln] was used to study the helix stabilising effects of a series of four bridges [-CH_2-NH-CO-Ph-NH-CO-CH_2-, -CH_2-NH-CO-Ph-CH_2-NH-CO-, -NH-CO-CH_2-Ph-NH-CO-CH_2- and -NH-CO-CH_2-Ph-CH_2-NH-CO-; all *p*-substituted].[35] These bridges were used to link positions 3 and 10 of the model peptides. In aqueous solution and in 50% (v/v) trifluoroethanol–water, the most effective bridge for helix stabilisation consisted of a 4-(aminomethyl)phenylacetic acid residue (AMPA) linked by amide bonds to the side chain functional groups of a (*S*)-2,3-diaminopropionic acid residue (Dap) in position 3 of the model peptide and an aspartic acid residue in position 10. This Dap^3(AMPA), Asp^{10} bridge was about as effective as two Lys(*i*), Asp(*i*+4) lactam bridges incorporated linking residues 3 and 7, and 10 and 14, in the same model peptide sequence.

3 Cyclic Peptides

Cyclic peptide analogues of biologically active peptides are included in the sections dealing with individual peptides (Section 4). Sequences, synthetic routes and biological activities of other cyclic peptides isolated from natural sources are presented here. Synthetic routes to cyclic peptides and depsipeptides on various solid supports either by attaching the first amino acid through the C-terminal carboxyl group, α-nitrogen atom or the side chain functional groups are reported and work on safety catch linkers and monitoring techniques has been published.[36–42] For example, synthesis of a cyclic peptide containing a *R*-3-hydroxy-13-methyltetradecanoic acid residue, cyclo-(Gln-Leu-D-Leu-Val-Asp-D-Leu-Ile-O-CH((CH_2)$_9$-$CHMe_2$)-CH_2CO), was achieved by using a cyclisation-cleavage method with oxime resin.[38] A 4-alkoxybenzyl-derived linker that anchors the C-terminal amino acid to the resin through the α-nitrogen atom was used to synthesise the cytotoxic heptapeptide, stylostatin.[39] Head-to-tail histidine containing cyclopeptides were synthesised by a three-dimensional orthogonal strategy (Fmoc/*t*-butyl/allyl) *via* anchoring the imidazole ring to trityl resin.[41] A β-turn directed cyclisation of simple peptidomimetics like **25** and analogues containing leucine residues in place of Phe and -NH(CH_2)$_2$NH- and -NH(CH_2)$_4$NH- groups in place of the -NH(CH_2)$_3$NH- group are reported.[43] Methods for the synthesis of valinomycin, dolastatin and didemnin analogues were reported.[44–47]

New cyclic peptides have been isolated from natural sources.[48–57] Cyclolinopeptides B-E were isolated from the seeds of *Linum usitatissimum*, and their



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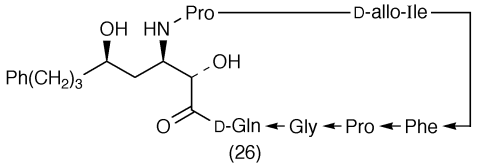
structures were elucidated as c(Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile), c(Pro-Pro-Phe-Phe-Val-Ile-Met(O)-Leu-Ile), c(Pro-Phe-Phe-Trp-Ile-Met(O)-Leu-Leu) and c(Pro-Leu-Phe-Ile-Met(O)-Leu-Val-Phe), respectively, by 2D NMR and chemical degradation methods.[48] Cyclolinopeptides B and E showed immunosuppressive activity. Structures of cycloquamosins A–G, isolated from the seeds of *Annona squamosa*, were found to be c(Gly-Ser-Phe-Gly-Pro-Val-Pro), c(Gly-Leu-Met-Gln-Pro-Pro-Ile-Thr), c(Gly-Leu-Met(O)-Gln-Pro-Pro-Ile-Thr), c(Ser-Tyr-Tyr-Pro-Gly-Gly-Val-Leu), c(Gly-Gly-Val-Leu-Ser-Tyr-Tyr-Tyr-Pro), c(Gly-Ala-Pro-Ala-Leu-Thr-Thr-Tyr) and c(Gly-Tyr-Pro-Met-Thr-Ala-Ile-Val).[49] Examples of cyclic peptide produced by the toxic cyanobacteria and *Theonella* sponge include compounds **26**–**29**. Nostophycin (**26**) displayed weak cytotoxic activity and the theonellapeptolide-related cyclic depsipeptides (**28**, R^1 = -SOMe, R^2 = H; R^1 = H, R^2 = Me) showed antimicrobial activity. [Hysp^2] and [Hap^2]Didemnin B (**30**, R = sec-butyl and H, respectively), isolated from the tunicate *Tridemnum cyanophorum*, had IC_{50}s of 21–184 nM in inhibiting the growth of human tumour cell lines and also inhibited the growth of multidrug-resistant cell lines in a dose dependent manner.[54]

Leualacin (**31**) (R = H, X = O), a cyclic depsipeptide isolated from the fungus *Hapsidospora irregularis*, and its backbone/amide-modified analogues (R = H or Me and X = O or NH) were synthesised. Amide analogue (R = H, X = NH) exhibited stronger vasodilatory (isolated rat aortic rings) effects. It also strongly inhibited collagen- and arachidonic acid-induced platelet aggregations with IC_{50}s of 0.6 μM and 2.0 μM, respectively. The corresponding *N*-methyl analogue (R = Me, X = NH) was inactive in both the tests.[55] Analogues of cryptophycin 1, a potent tumour-selective depsipeptide isolated from the terrestrial blue-green algae, containing various substituents at the two methylene carbons in the β-Ala residue (**32**) were synthesised. In some of the analogues, the β-Ala residue was replaced by α-amino acids (Ala, Val, Leu, Phe and Pro). In comparison to cryptophycin (IC_{50} 0.24 nM), a few of the analogues containing various substituted β-alanines [*e.g.* R^1 = H, R^3,R^4 = Me; R^1 = Me, R^3,R^4 = H] were about 2-5-fold more potent and many other analogues [*e.g.* R^1 = isobutyl, phenyl or benzyl, R^3,R^4 = H] were less potent. Analogues containing α-amino acids were also much less potent (IC_{50} values 13 to >1500 nM). In the murine pancreatic adenocarcinoma model, none of



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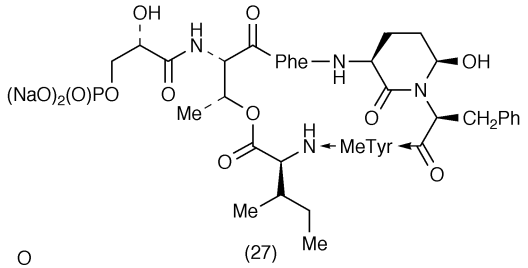

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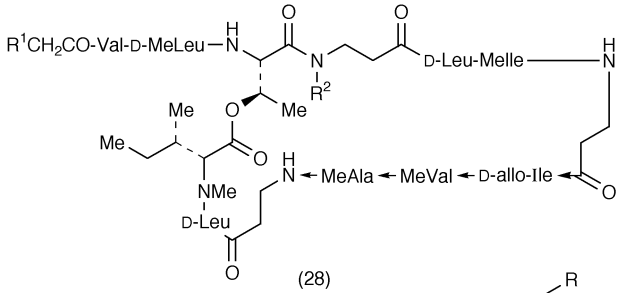

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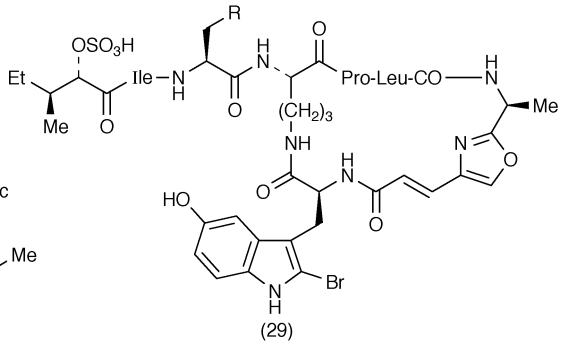

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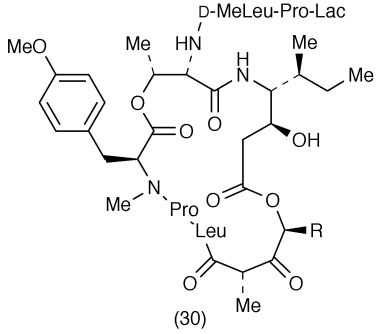

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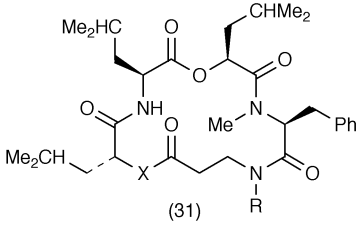

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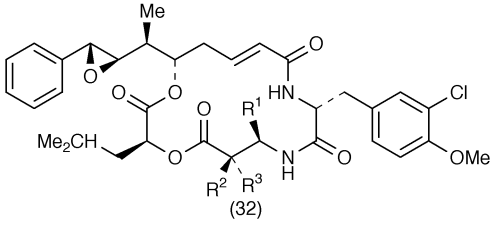


(32)

the compounds tested showed significant antitumour activity.[56] The cyclic depsipeptide, sansalvamide, c(Leuψ(CO-O)Leu-Val-Leu-Phe), isolated from the mycelium of a fungus exhibited selective *in vitro* cytotoxic effect toward COLO 205 colon and SK-MEL-2 melanoma cancer cell lines (IC_{50}s 3.5–5.9 μg ml^{-1}).[57]

4 Biologically Active Peptides

Reviews on the role of peptides in human brain diseases, hypertension, angiogenesis, cancer anorexia-cachexia syndrome, G-protein receptors and transferrin receptors have been published.[58–65]

4.1 Peptides Involved in Alzheimer's Disease. – Only the work related to the involvement of β-amyloid, β-amyloid precursor proteins and enzymes involved in the processing of β-amyloid precursor proteins is discussed in this section. Reviews on the role of peptides in Alzheimer's disease have been published.[66–70] Unlike other familial Alzheimer's disease-linked β-APP mutations, overexpression of a mutated β-amyloid precursor protein (Val^{715}Met-β-APP) in human HEK293 cells and murine neurones reduces total Aβ production and increases the recovery of the physiologically secreted product, APPα. Val^{715}Met-β-APP significantly reduces Aβ40 secretion without affecting Aβ42 production in HEK293 cells.[71] The transgenic mouse, which over-expresses mutant human amyloid precursor protein (Val^{717} replaced by Phe^{717}), progressively develops many of the neuropathological symptoms of Alzheimer's disease. Immunisation with $Aβ_{42}$ either before the onset of Alzheimer's disease-type neuropathologies or at an older age when Aβ deposition and several of the subsequent neuropathological changes were well established, prevented the development of β amyloid plaque formation and other Alzheimer's-like neuropathologies.[72] Plasma proteins including albumin, α1-antitrypsin and immunoglobulins A and G are potent inhibitors of Aβ polymerisation. These proteins are also present in cerebrospinal fluid, but at low concentrations having little or no effect on Aβ.[73] Acetylcholinesterase may play a role in the neurodegeneration observed in Alzheimer brain. Stable acetylcholinesterase-Aβ complexes were found to be more neurotoxic than those formed without the enzyme.[74] Polymerisation studies on selected Ab peptide fragments revealed that the shortest fibril-forming sequence was Aβ(14–23). Substitutions in this decapeptide impaired fibril formation and deletion of the decapeptide from Aβ(1–42) inhibited fibril formation completely.[75] Molecular modelling of Aβ(14–23) oligomers in an antiparallel β-sheet conformation displayed favourable hydrophobic interactions stabilised by salt bridges between all charged residues.

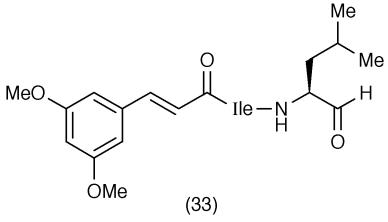
Solid phase synthesis of Alzheimer's Aβ1–42 and analogues, [Glu^7, Asn^7, Ala^7, Asp^{11} and $isoGlu^{11}$]-Aβ1–42, was reported using Fmoc-amino acid fluorides as coupling agents.[76] The aggregation and neurotoxic properties of Aβ1–42 and its isomers with an isoaspartyl residue at position 7 or 23,

[Aβ1–42(isoAsp^7) and Aβ1–42(isoAsp^{23})], were investigated.[77] Aβ1–42(isoAsp^{23}) aggregated more strongly than native Aβ1–42 and showed significant neurotoxicity, while the aggregation ability and neurotoxicity of Aβ1–42-(isoAsp^7) was weak. Aggregation properties of an Aβ fragment, Aβ(25–35) in pure form and in the presence of different phospholipid vesicles have also been reported.[78] Pure peptide aggregated with time, forming fibrils with β-structure. Phospholipid vesicles formed by negatively charged phospholipids accelerated the aggregation of the peptide. However, the presence of vesicles formed by a zwitterionic phospholipid slowed down the aggregation process. Aβ isolated from neuritic plaque and vascular walls of the brains of patients with Alzheimer's disease has been shown to contain significant quantities of Aβ peptides which begin at residue Glu^3 or Glu^{11} in the form of pyroglutamyl residues. To investigate the effects of these N-terminal modifications on the biophysical properties of Aβ, several pyroglutamyl peptides, Pyr-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val, Pyr-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val, Pyr-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys and Pyr-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys, were synthesised.[79] Using various techniques, the pyroglutamyl-containing peptides were shown to have greater aggregation propensities than the corresponding full-length peptides.

Proteolytic processing of the amyloid precursor protein by various enzymes (β- and γ-secretase, caspase and calpain) leading to the Aβ-peptide, the main component of the amyloid plaques found in Alzheimer's disease patients, has been studied.[80–89] New membrane-bound aspartyl protease(s) with β-secretase activity were identified.[80,81] Both Aβ40 and Aβ42 were shown to be generated by a single γ-secretase by using enzyme-linked immunosorbent assays selective for Aβ40 or Aβ42 and five structurally diverse γ-secretase inhibitors [L-menthyloxycarbonyl-Leu-Leu-H, Z-Leu-Cha-$CF_2CONHCH_2CH(Me)Et$, Z-Leu-Nle-H, Z-Trp-Leu-H, Z-β-Ala-Leu-H] and Boc-Cha-Ile-(2*S*,3*R*,4*S*)-2-amino-1-cyclohexyl-3,4-dihydroxy-6-methylheptane.[82] Substrate specificity of γ-secretase was examined by mutating various residues within or adjacent to the transmembrane domain of the amyloid precursor protein and then analysing Aβ production from cells transfected with these mutant proteins. Aβ production was also analysed from a subset of mutants that showed shifts in γ-secretase cleavage in the presence or absence of pepstatin, an inhibitor of γ-secretase activity. The results indicated that γ-secretase's cleavage specificity was primarily determined by location of the γ-secretase cleavage site of amyloid precursor protein with respect to the membrane, and that γ-secretase activity was due to the action of multiple proteases exhibiting both a pepstatin-sensitive and a pepstatin-insensitive activity.[84] Mutation studies (replacement of all residues outside the Aβdomain with Phe) in the C-terminal fragment (99 residues) of the amyloid precursor protein were carried out to determine the effect of these mutations on the cleavage specificity of γ-secretase ($Aβ_{42}/Aβ_{40}$

ratio).[86] Compared with the wild-type fragment, mutations at Val^{44}, Ile^{47}, and Val^{50} led to decreased $A\beta_{42}/A\beta_{40}$ ratios, whereas mutations at Thr^{43}, Ile^{45}, Val^{46}, Leu^{49}, and Met^{51} led to increased $A\beta_{42}/A\beta_{40}$ ratios [Ile^{45}Phe showing 34-fold increase]. Unlike the other mutations, Val^{44}Phe mutant was processed mainly to $A\beta_{38}$.

Modified-peptide and non-peptide inhibitors of amyloid β-peptide production and polymerisation were reported.[90–93] Aβ-derived peptides of fifteen residues [Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala, cholyl-His-Asp-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe, cholyl-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala, cholyl-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe] and some of the N-terminally modified smaller peptides containing all L- or all D-amino acid sequences [cholyl-Gln-Lys-Leu-Val-Phe-Phe, cholyl-Lys-Leu-Val-Phe-Phe, cholyl-Leu-Val-Phe-Phe-Ala, cholyl-D-Leu-D-Val-D-Phe-D-Phe-D-Ala, cholyl-D-Leu-D-Val-D-Phe-D-Phe-D-Ala-NH_2] were found to be inhibitory of Aβ polymerisation.[90] Many of the smaller peptides like cholyl-Leu-Val-Phe-Phe-Ala-OH, the corresponding all-D-amino acyl analogue peptide acid and amide retained inhibitory activity and were both stable in monkey cerebrospinal fluid for 24 h. In an another approach, based on linking a recognition element for Aβ to a disrupting element designed to interfere with Aβ aggregation, peptides of 4–8 residues composed of overlapping sequences within the 15–25 domain were synthesised, along with hybrid compounds containing those recognition sequences coupled to a lysine hexamer.[91] None of the recognition peptides altered Aβ aggregation kinetics and only two, Lys-Leu-Val-Phe-Phe and Lys-Leu-Val-Phe, had any protective effect against Aβ toxicity. The hybrid peptide Lys-Leu-Val-Phe-Phe-Lys-Lys-Lys-Lys-Lys-Lys altered Aβ aggregation kinetics and aggregate morphology and provided significantly improved protection against Aβ toxicity compared to the recognition peptide alone. In an effort to identify inhibitors of Aβ production and to probe the amino acid sequence specificity of the protease(s) responsible for the production of this peptide, a number of dipeptide aldehydes (starting from Z-Val-Phe-H) were combinatorially synthesised and evaluated for their inhibitory properties.[92] The most active dipeptide aldehydes [*e.g.* 3,5-dimethoxycinnamamide-isoleucinyl-leucinal (**33**), IC_{50} 9.6 μM] were those that possessed hydrophobic amino acids at both the P_1 and P_2 positions. Compound **33** was approximately 10-fold more potent than Z-Val-Phe-H. In immunoprecipitation experiments using antibodies directed toward either Aβ1–40 or Aβ1–42, compound **33**



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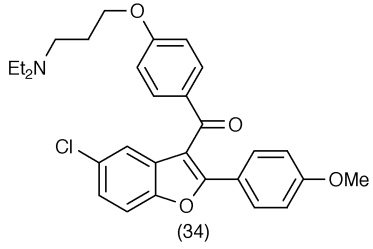


(34)

preferentially inhibited the shorter 1–40 form of Aβ. In a non-peptide series of compounds, benzofuran derivatives like **34** were identified as inhibitors of fibril formation in the β-amyloid peptide. The inhibition afforded by the compounds seems to be associated with their binding to β-amyloid, as identified by scintillation proximity binding assay.[93]

4.2 Antimicrobial Peptides. – Reviews on various antibiotic peptides and genomic approaches to antimicrobial drug discovery have appeared.[94–97]

4.2.1 Antibacterial Peptides. As in previous years, many new antibacterial peptides have been isolated from various natural sources.[98–110] Tylopeptins A [Ac-Trp-Val-Aib-D-Iva-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Leu-ol] and B [Ac-Trp-Val-Aib-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Leu-ol] possessing an acetylated N-terminal residue, fourteen amino acids, and leucinol as the C-terminal amino alcohol were isolated from the methanol extract of the fruiting body of the mushroom *Tylopilus neofelleus*. These peptides were active against some Gram-positive bacteria, but inactive against pathogenic fungi and Gram-negative bacteria.[98] Many citropin peptides were present in the secretion from the granular dorsal glands of the Blue Mountains tree-frog *Litoria citropa*. Two major peptides, Gly-Leu-Phe-Asp-Val-Ile-Lys-Lys-Val-Ala-Ser-Val-Ile-Gly-Gly-Leu-NH_2 and Gly-Leu-Phe-Asp-Ile-Ile-Lys-Lys-Val-Ala-Ser-Val-Val-Gly-Gly-Leu-NH_2 and a minor peptide, Gly-Leu-Phe-Asp-Ile-Ile-Lys-Lys-Val-Ala-Ser-Val-Ile-Gly-Gly-Leu-NH_2, are wide-spectrum antibacterial peptides.[99] Two cysteine-rich antimicrobial peptides [Myticin A = His-Ser-His-Ala-Cys-Thr-Ser-Tyr-Trp-Cys-Gly-Lys-Phe-Cys-Gly-Thr-Ala-Ser-Cys-Thr-His-Tyr-Leu-Cys-Arg-Val-Leu-His-Pro-Gly-Lys-Met-Cys-Ala-Cys-Val-His-Cys-Ser-Arg and Myticin B = His-Pro-His-Val-Cys-Thr-Ser-Tyr-Tyr-Cys-Ser-Lys-Phe-Cys-Gly-Thr-Ala-Gly-Cys-Thr-Arg-Tyr-Gly-Cys-Arg-Asn-Leu-His-Arg-Gly-Lys-Leu-Cys-Phe-Cys-Leu-His-Cys-Ser-Arg] were isolated from haemocytes and plasma of the mussel *Mytilus galloprovincialis*.[100] The two peptides display antibacterial activity against Gram-positive bacteria, whereas only myticin B is active against the fungus *Fusarium oxysporum* and the Gram-negative bacteria *Escherichia coli* D31.

Four N- to C-terminal cyclic cystine-knot peptides of 29–31 residues, katala [c(Cys^1-Thr-Cys^3-Ser-Trp-Pro-Val-Cys^8-Thr-Arg-Asn-Gly-Leu-Pro-Val-Cys^{16}-Gly-Glu-Thr-Cys^{20}-Val-Gly-Gly-Thr-Cys^{25}-Asn-Thr-Pro-Gly), Cys^1 to Cys^{16}, Cys^3 to Cys^{20} and Cys^8 to Cys^{25} disulfide bonds], circulin A [c(Cys^1-Ser-Cys^3-Lys-Asn-Lys-Val-Cys^8-Tyr-Arg-Asn-Gly-Ile-Pro-Cys^{15}-Gly-Glu-Ser-Cys^{19}-Val-Trp-Ile-Pro-Cys^{24}-Ile-Ser-Ala-Ala-Leu-Gly), Cys^1 to Cys^{15}, Cys^3 to Cys^{19} and Cys^8 to Cys^{24} disulfide bonds], circulin B [c(Cys^1-Ser-Cys^3-Lys-Asn-Lys-Val-Cys^8-Tyr-Arg-Asn-Gly-Val-Ile-Pro-Cys^{16}-Gly-Glu-Ser-Cys^{20}-Val-Phe-Ile-Pro-Cys^{25}-Ile-Ser-Thr-Leu-Leu-Gly), disulfide bonds as in katala] and cyclopsychotride [c(Cys^1-Ser-Cys^3-Lys-Ser-Lys-Val-Cys^8-Tyr-Lys-Asn-Ser-Ile-Pro-Cys^{15}-Gly-Glu-Ser-Cys^{19}-Val-Phe-Ile-Pro-Cys^{24}-Thr-Val-Thr-Ala-Leu-Leu-Gly), disulfide bonds as in circulin A] and their analogues were tested against various strains of microbes.[101] Katala and circulin A were specific for the Gram-

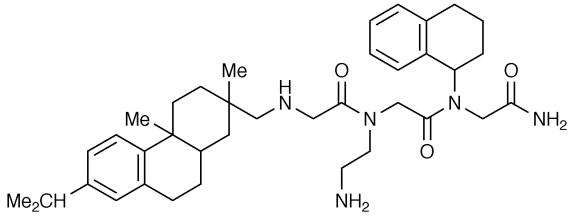
positive *Staphylococcus aureus* (MIC 0.2 μM). However, circulin B and cyclopsychotride were active against both Gram-positive and Gram-negative bacteria. All four cyclic peptides were moderately active against two strains of fungi, *Candida kefyr* and *Candida tropicalis*, but were inactive against *Candida albicans*. These macrocycles were cytotoxic and lysed human red blood cell (LD_{50} 400 μM).

Zelkovamycin, a cyclic peptide containing Gly, Ala and several unnatural amino acids like 2-aminobutanoyl, 2-amino-2-butenoyl, Sar, 1,3-thiazoyl, 7-methoxytryptophanyl and 2-methyldehydrothreonyl residues was isolated from *Streptomyces*.[108] Biochemical analysis of a secreted *agr*-encoded peptide isolated from culture supernatants of *Staphylococcus aureus* identified peptides with an unusual thiol ester-linked cyclic structure. The synthetic thiolactone/lactone peptides (*e.g.* Tyr-Ser-Thr-Cys-Asp-Phe-Ile-Met, Gly-Val-Asn-Ala-Cys-Ser-Ser-Leu-Phe and Gly-Val-Asn-Ala-Ser-Ser-Ser-Leu-Phe, C-terminal carboxyl linked to the Cys or Ser^5 side chain) exhibited biological activity *in vivo* in a mouse protection test.[109] Structure of a microcin group of peptide antibiotics (microcin J25) produced by *Enterobacteriaceae* was reported to be a cyclic peptide [c(-Val^1-Gly-Ile-Gly-Thr-Pro-Ile-Ser-Phe-Tyr-Gly-Gly-Gly-Ala-Gly-His-Val-Pro-Glu-Tyr-Phe^{21}-)].[110] The 21-residue peptide showed high resistance to most of endoproteases and exhibited antibiotic activity towards *Salmonella newport* and several *E. coli* strains (MIC ranging between 0.01 and 0.2 μg ml^{-1}). A peptide with antibacterial activity was purified from the cattle tick (*Boophilus microplus*) gut contents. The synthetic peptide was active against Gram-positive bacteria and fungi.[111] Based on the antimicrobial activity of bovine apolipoprotein A-II against *Escherichia coli* and the yeast *Saccharomyces cerevisiae* reported earlier, the active domain of apolipoprotein A-II was identified using synthetic peptides.[112] A peptide corresponding to C-terminal residues Leu^{49}-Thr^{76} [Leu-Thr-Pro-Phe-Phe-Lys-Lys-Ala-Gly-Thr-Asp-Leu-Leu-Asn-Phe-Leu-Ser-Ser-Phe-Ile-Asp-Pro-Lys-Lys-Gln-Pro-Ala-Thr] exhibited significant antimicrobial activity against *E. coli*, but not against *S. cerevisiae*. Experiments using amino-acid-substituted peptides indicated that the residues Phe^{52}-Phe^{53}-Lys^{54}-Lys^{55} were required for the activity.

Antimicrobial activities[113–118] and conformational properties[119–121] of several synthetic peptides were reported. Peptides D2A21 [Phe-Ala-Lys-Lys-Phe-Ala-Lys-Lys-Phe-Lys-Lys-Phe-Ala-Lys-Lys-Phe-Ala-Lys-Phe-Ala-Phe-Ala-Phe] and D4E1 [Phe-Lys-Leu-Arg-Ala-Lys-Ile-Lys-Val-Arg-Leu-Arg-Ala-Lys-Ile-Lys-Leu] were active against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[113] Analogues of the peptide GS14, c(Val-Lys-Leu-Lys-Val-D-Tyr-Pro-Leu-Lys-Val-Lys-Leu-D-Tyr-Pro), designed on the basis of gramicidin S, were synthesised to discover peptides with high antimicrobial activity coupled with low haemolytic activity. Each amino acid was replaced by the corresponding D-amino acid. In comparison to GS14, several of the peptides showed enhanced antibacterial activity.[115] Two of the best peptides of the series, c(Val-Lys-Leu-D-Lys-Val-D-Tyr-Pro-Leu-Lys-Val-Lys-Leu-D-Tyr-Pro) and c(Val-Lys-Leu-D-Lys-Val-D-Tyr-Pro-Leu-Lys-Val-D-Lys-Leu-D-Tyr-Pro) with LPS binding affinities in the range of 50–93 μM and the haemolytic

activities in the range of 150–200 μg ml^{-1}, showed much better therapeutic index (haemolytic activity/antibacterial activity).

The active site of a 43 amino acid residue beetle (*Allomyrina dichotoma*) defensin, effective against methicillin-resistant *Staphylococcus aureus*, was identified by measuring the anti-bacterial activity of 64 overlapping 12-mer peptides against *S. aureus*. A Leu-Cys-Ala-Ala-His-Cys-Leu-Ala-Ile-Gly-Arg-Arg-NH_2 (19L-30R-NH_2) fragment showed the greatest activity against both Gram-positive and Gram-negative bacteria. N-Terminally truncated fragments (8–10-mer peptides) still had strong anti-bacterial activity. However, C-terminally truncated fragment was much less potent. The Ala-His-Cys-Leu-Ala-Ile-Gly-Arg-Arg-NH_2 fragment and its analogues [Ala-Leu-Arg-Leu-Ala-Ile-Arg-Arg-Arg-NH_2, Ala-Leu-Leu-Leu-Ala-Ile-Arg-Arg-Arg-NH_2, Ala-Trp-Leu-Leu-Ala-Ile-Arg-Arg-Arg-NH_2, Ala-Leu-Tyr-Leu-Ala-Ile-Arg-Arg-Arg-NH_2 and Ala-Leu-Trp-Leu-Ala-Ile-Arg-Arg-Arg-NH_2] exhibited about 3-fold and 9–12-fold higher activity against *S. aureus* than did the 19L-30R-NH_2 fragment, and these analogues were effective against methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa* isolated from patients.[117] These oligopeptides showed no haemolytic activity and did not inhibit the growth of murine fibroblast cells. *N*-acylated or D enantiomer peptide derivatives based on the sequence Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys in lactoferricin B demonstrated antimicrobial activities greater than those of lactoferricin B against bacteria and fungi.[118] The most potent peptide, conjugated with an 11-carbon-chain acyl group [CH_3-$(CH_2)_9$-CO-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys], showed two to eight times lower MIC than lactoferricin B. Peptoids (*N*-substituted glycine oligomers) were discovered as antibacterial agents by screening combinatorial chemistry libraries for bacterial growth inhibition. *In vitro*, the peptoid **35** and some of its analogues were active in the range of 3 to 12 μg ml^{-1} against a panel of Gram-positive and Gram-negative bacteria which included isolates which were resistant to known antibiotics.[122,123]



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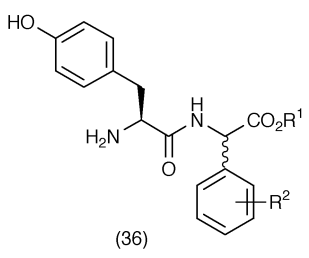
Work on bacterial proteinases and their inhibitors has been published.[124–132] Proteinases isolated from *Pseudomonas* sp., 101 and *Xanthomonas* sp. T-22 are examples of unique carboxyl proteinases which are insensitive to aspartic proteinase inhibitors, such as pepstatin, diazoacetyl-D/L-norleucine methyl ester, and 1,2-epoxy-3(p-nitrophenoxy)propane.[124,125] Subsite preferences for these enzymes were determined by using a series of synthetic chromogenic substrates, Lys-Pro-Ile(P_3)-Glu(P_2)-Phe*Phe(NO_2)-Arg(P_2')-Leu (P_3') [Phe*-

Phe(NO_2) is the cleavage site] with systematic substitutions at the P_3, P_2, P_2', and P_3' positions. The best substrate for *Pseudomonas* sp. 101 enzyme had Leu at the P_2 position, and that for *Xanthomonas* sp. T-22 enzyme had Ala at the P_3 position. Both enzymes preferred such charged amino acid residues as Glu, Asp, Arg, or Lys at the P_2' position. Tyrosyl aryl dipeptide inhibitors of *S. aureus* tyrosyl tRNA synthetase have been identified.[126] A crystal structure of the enzyme complexed to one of the inhibitors shows occupancy of the tyrosyl binding pocket coupled with interactions to key catalytic residues. With *S. Aureus* tyrosyl tRNA synthetase Tyr-Tyr was shown to inhibit the enzyme (IC_{50} 3.8 μM) while Tyr-Phe dipeptide was inactive. Various Tyr-phenylglycine derivatives (**36**, R^1 = H or Me, R^1 = 2-OH, 3-OH, 4-OH, 3,4-diOH, 2,3,4-triOH and 2-F) were synthesised and tested as inhibitors of the enzyme. The tri-hydroxy derivative **37** was the most potent inhibitor of the enzyme (IC_{50} 0.51 μM). Specific inhibition of urease activity has been proposed as a possible strategy to fight *Helicobacter pylori*. Peptides which specifically bind and inhibit *H. pylori* urease were identified by screening random libraries displayed on filamentous phage.[127] A 24 amino acid peptide, Thr-Phe-Leu-Pro-Gln-Pro-Arg-Cys-Ser-Ala-Leu-Leu-Arg-Tyr-Leu-Ser-Glu-Asp-Gly-Val-Ile-Val-Pro-Ser, and a hexapeptide (Tyr-Asp-Phe-Tyr-Trp-Trp) inhibited the enzyme with K_i values of 47 and 30 μM, respectively. Both peptides specifically inhibited the activity of *H. pylori* urease but not that of *Bacillus pasteurii* up to a concentration of 50 μM.



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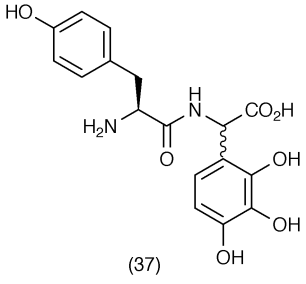
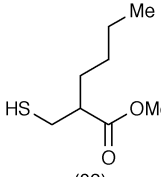


(37)

A spectrophotometric assay was developed for peptide deformylase (metalloenzymes cleaving the *N*-formyl groups from *N*-blocked methionine polypeptides).[128] This assay employs peptide mimetics as deformylase substrates which, upon enzymatic removal of the N-terminal formyl group, rapidly release free thiols (quantitated using Ellman's reagent). Using the assay, a variety of peptide analogues that contain β-thiaphenylalanine or β-thiamethionine as the N-terminal residue were found to be substrates of the peptide deformylase from *E. coli*. Sequence specificity of the *E. coli* peptide deformylase was examined by using a peptide library that contains all possible N-terminally formylated tetrapeptides (constructed on TentaGel resin).[129] Peptide sequence analysis revealed a consensus sequence, formyl-Met-X-Z-Tyr (X = any amino acid except Asp or Glu; Z = Lys, Arg, Tyr, or Phe), for the *E. coli* enzyme. Inhibitors of bacterial deformylase were prepared.[130–132]

Peptide aldehydes (Z-Leu-Nle-H, Ac-Leu-Leu-Nle-Hac-Leu-Leu-Met-H, Ac-Leu-Val-Phe-H, Ac-Leu-Val-Lys-H, Ac-Leu-Leu-Arg-H and Ac-Tyr-Val-Ala-Asp-H), especially the aldehydes containing a methional or norleucinal, inhibited recombinant peptide deformylase from Gram-negative *E. coli* and Gram-positive *B. subtilis*. N-Z-Leu-norleucinal was the most potent competitive inhibitor which inhibited the zinc-containing metalloenzymes, *E. coli* and *B. subtilis* deformylase (K_i 26.0 and 55.6 μM, respectively).[130] Based on the minimal substrates of the enzyme like For-Met-OCH_3, For-Nle-OCH_3 and For-Nle-Arg-NH_2, deformylase inhibitors were designed by incorporating features from other metalloproteinases.[131] Compounds **38** and **39** behaved as competitive inhibitors of peptide deformylase with K_i values of 52 and 2.5 μM, respectively. Evidence that **39** binds inside the active site cavity of peptide deformylase, while keeping intact the 3D fold of the protein, was provided by NMR. The structures of the protein-inhibitor complexes of both the cobalt and the zinc containing *E. coli* peptide deformylase bound to the transition-state analogue **40** were reported.[132] The proteins for both deformylase-inhibitor complexes show the same fold as for the native enzyme and the inhibitor **40** adopts an extended conformation and fits into a hydrophobic cavity located near the metal site.



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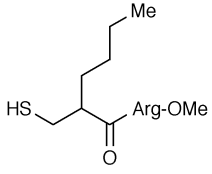

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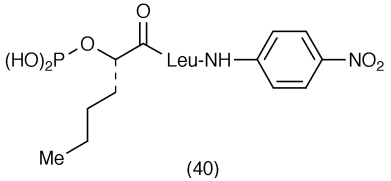

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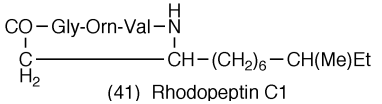
4.2.2 Antifungal Peptides. Rhodopeptins, novel cyclic tetrapeptides (composed of a β-amino acid and three α-amino acids) with antifungal activity were isolated from *Rhodococcus* sp.[133–135] The peptides showed high *in vitro* antifungal activity against *C. albicans* and *Crytococcus neoformans* and no activity against bacteria. The structures of rhodopeptins C1, C2, C3, C4 and B5 were c(-Gly-Orn-Val-3-amino-10-methyldodecanoyl-) (**41**), c(-Gly-Orn-Ile-3-amino-10-methyldodecanoyl-), c(-Gly-Orn-Val-3-amino-12-methyltridecanoyl-), c-(-Gly-Orn-Val-3-amino-12-methyltetradecanoyl-) and c(-Gly-Lys-Val-3-amino-13-methyltetradecanoyl-), respectively. Antifungal activity and cytotoxicity of a novel membrane-active peptide, Lys-Lys-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-Lys, obtained by combinatorial chemistry, was investigated.[136] The peptide inhibited the growth of various pathogenic fungi isolated from patients and of fluconazole-resistant fungi at concentrations of 2 to 32 μg ml^{-1}. Pseudopeptides [Lysψ(CH_2NH)Lys-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-D-Lys-NH_2, Lysψ(CH_2NH)Lysψ(CH_2NH)Val-Val-Phe-Lys-Val-Lys-



(41) Rhodopeptin C1

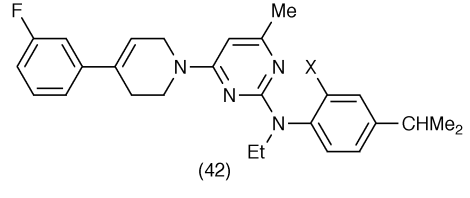
Phe-Lys-D-Lys-NH_2, R_1NHCH_2CO-Lys-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-D-Lys-NH_2, R^1NHCH_2-ψ($CONR^1$)CH_2CO-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-D-Lys-NH_2, R^2NHCH_2ψ-($CONR^2$)CH_2CO-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-D-Lys-NH_2, Lysψ(CH_2 OCONH)Lys-Val-Val-Phe-Lys-Val-Lys-Phe-Lys-D-Lys-NH_2, $R^1 = -(CH_2)_3$-NMe_2 and $R^2 = CH_2CH_2NH_2$] corresponding to a membrane-active peptide were synthesised and compared with the parent peptide.[137] The pseudopeptides showed greater resistance to serum proteinases (*e.g.* Lysψ(CH_2NH)-Lysψ(CH_2NH)Val-Val-Phe-Lys-Val-Lys-Phe-Lys-D-Lys-NH_2 had a half life of 240 min compared to 7 min for the parent peptide) and similar antimicrobial activities to that of the parent peptide without haemolytic activity.

4.3 ACTH/CRF Peptides. – The physiological actions of ACTH peptides are mediated by at least two receptor subtypes and a soluble binding protein. Although the earliest functions of these peptides may have been associated with osmoregulation and diuresis, a constellation of physiological effects associated with stress and anxiety, vasoregulation, thermoregulation, growth and metabolism, metamorphosis and reproduction have been identified in various vertebrate species.[138–140] Evolution, physiology and the role of corticotropin-releasing factor in depression and anxiety disorders have been reviewed.[141,142] The nature of the CRF binding protein in the synovial fluid of rheumatoid arthritis patients has been investigated.[143] The results showed that synovial fluid samples contained intact CRF binding protein and a 10 kDa C-terminal fragment. Only the N-terminal fragment of the recombinant protein bound human CRF.

Reduced amide bond replacements (ψ[CH_2NH]) between residues 6–9 in $oCRF_{(5-41)}$ resulted in less potent analogues (<1% that of oCRF). Similar replacements in the longer peptide, $hCRF_{(4-41)}$, led to more potent compounds.[144] Some of the analogues (7ψ8, 8ψ9, and 9ψ10) were 2–4 times more potent than hCRF and 3–7 times less potent than the parent [D-pro^4, Nle^{21,38}]-$hCRF_{(4-41)}$. In a series of cyclic peptides, O-alkylation of Ser^7 also gave less potent compounds. c(30–33)[Ser(OMe)^7, D-Phe^{12}, Nle^{21}, Glu^{30}, Lys^{33}, Nle^{38}]Ac-$hCRF_{(7-41)}$ was found to exhibit full efficacy and 40% of the potency of c(30–33)[D-Phe^{12}, Nle^{21}, Glu^{30}, Lys^{33}, Nle^{38}]Ac-$hCRF_{(7-41)}$. Other substitutions at position 7 including aminoglycine and alkylated and/or acylated D- or L-aminoglycines reduced potency. The most potent analogue in this series, c(30–33)[D/L-Agl(Me,Ac)^7, D-Phe^{12}, Nle^{21}, Glu^{30}, Lys^{33}, Nle^{38}]Ac-$hCRF_{(7-41)}$, was 60–80% as potent as the Ser^7 analogue.[144] Based on the earlier observation that substitution by an α-methyl amino acid and a few other changes in a CRF antagonist {c(30–33)[D-Phe^{12}, Nle^{21}, Glu^{30}, Lys^{33}, Nle^{38}]$hCRF_{(12-41)}$} resulted in a compound with a longer duration of action, c(30–33)[D-Phe^{12}, Nle^{21}, αMeLeu^{27}, Glu^{30}, Lys^{33}, Nle^{38}]Ac-hCRF(9–41), additional analogues containing two or more α-methyl amino acids were synthesised.[145] Whereas the introduction of αMe-Leu at positions 27 and either 18, 37, or 40 resulted in increases in duration of inhibitory action in the adrenalectomized rat, the same substitution at positions 27 and either 15, 17,

19, or 41 led to short acting analogues. Cyclo(30–33)[D-Phe^{12}, Nle^{21}, αMeLeu^{27}, Glu^{30}, Lys^{33}, Nle^{38}, αMeLeu^{40}]Ac-hCRF$_{(9-41)}$ was one of the most efficacious analogues of this series (> 4 h inhibition of ACTH secretion at 25 μg/adx rat, i.v., >24 h inhibition at 100 μg/adx rat, s.c.).

A number of publications on non-peptide antagonists of ACTH have appeared.[146–157] Several of these were based around a 2-anilinopyrimidine or -triazine structure (*e.g.* **42**–**45**). Both CRA1000 (**42**, X = SMe) and CRA1001 (**42**, X = Br) inhibited [I^{125}]ovine CRF binding to membranes of COS-7 cells expressing the rat CRF$_1$ receptor (IC$_{50}$s 30 and 38 nM, respectively) without affecting [I^{125}]sauvagine binding to membranes of COS-7 cells expressing the rat CRF$_{2\alpha}$ receptor. The compounds were also without affinity for the CRF$_{2\beta}$ receptor when examined using rat heart. In mice, orally administered CRA1000 and CRA1001 showed anxiolytic- and antidepressant-like properties in various animal models.[146,147] One of the triazine derivatives **43** (hCRH$_1$ K_i 32 nM) also demonstrated improved pharmacokinetic profile in the rat (19% oral bioavailability at 30 mg kg^{-1}) as well as in the dog (20% oral bioavailability at 5 mg kg^{-1}).[148] N-Aryltriazolo- (**45**) and -imidazopyrimidines and -pyridines were also investigated for oral bioavailability, high plasma levels, and duration of action and some of these showed good pharmacokinetic profile.[154] Other examples of non-peptide CRH antagonists include compounds **46**–**48**.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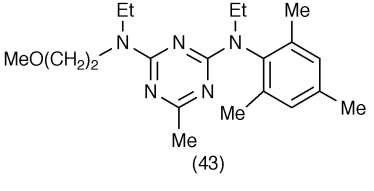
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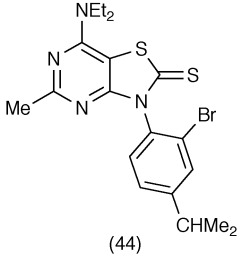
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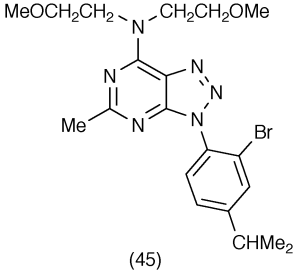
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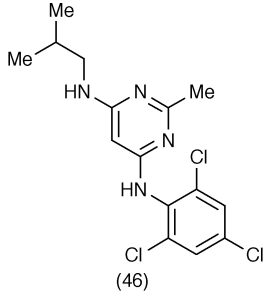
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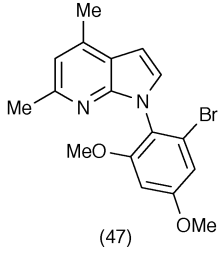
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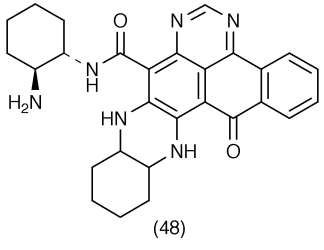
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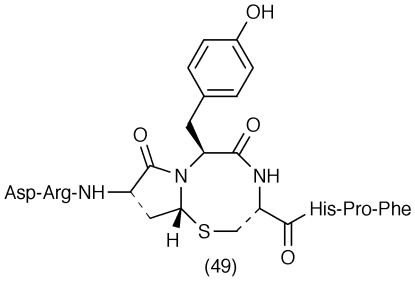
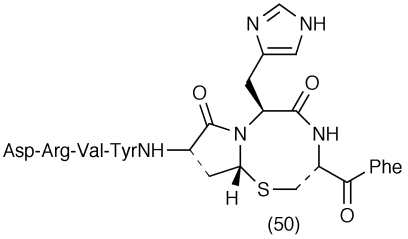
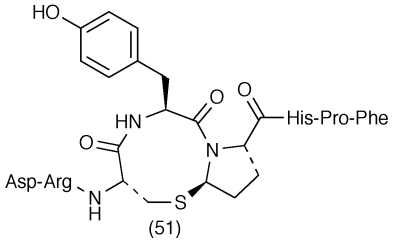
4.4 Angiotensin II Analogues and Non-peptide Angiotensin II Receptor Ligands. – Various aspects of angiotensin(1–7) and angiotensin II receptors and antagonists were reviewed.[158–162] AT_1 receptor-associated protein that interacts with the C-terminal cytoplasmic domain of the AT_{1a} receptor and affects signalling was isolated by employing a mouse kidney cDNA library.[163] Overexpression of AT_1 receptor-associated protein in COS-7 cells caused a marked inhibition of AT_{1a} receptor-mediated activation of phospholipase C. Selective angiotensin-receptor subtype antagonists revealed that AT_1 and AT_4 receptors mediated the response of angiotensin(1–7).[164] Structural properties of bovine AT_4 receptors from adrenals, kidney, heart, thymus, bladder, aorta, and hippocampus indicated that, apart from the hippocampal receptor, which was significantly smaller and did not appear to possess other disulfide-linked subunits, other receptors had similar properties.[165] Angiotensin II epitope recognition to AT_1 and AT_2 receptors was investigated by using amino acid substituted and radio-labelled photoreactive angiotensin analogues.[166–168] Although modifications of all angiotensin II side chains affected binding to the AT_2 receptor to nearly similar extent, binding to the AT_1 receptor was significantly affected by modifications at side chain positions 2, 4, 6 and 7. Interactions between Tyr^4 and Phe^8 of angiotensin II with Asn^{111} and His^{256} of the AT_1 receptor, respectively, are essential for agonism.

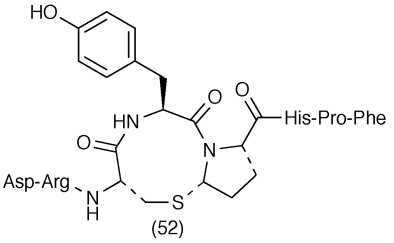
Angiotensin II analogues containing constrained tripeptide mimetics (**49**–**54**) were synthesised.[169,170] Only one of these conformationally constrained analogues (**54**) exhibited AT_1 receptor affinity (K_i 750 nM). Other analogues were not active up to a concentration of 10 μM. Non-peptide antagonists of angiotensin II like **55** were reported.[171] Compound **55** (K_i 0.26 nM for AT_1 receptor) was more potent than losartan in spontaneously hypertensive rats after oral administration.

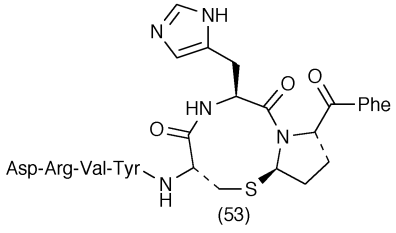
4.5 Bombesin/Neuromedin Analogues. – The role of bombesin and its mammalian homologue, gastrin-releasing peptide (GRP), as growth factors in various tumours (*e.g.* colon and prostate cancers) was examined.[172–174] High levels of bombesin receptors were shown to be expressed in human colon carcinoma Isreco1 cell line. Exposure to bombesin resulted in an increase of intracellular calcium concentrations. Bombesin (1 nM) induced cell spreading at 24 h, stimulated adhesion of Isreco1 cells to collagen I-coated culture dishes and increased [H^3]thymidine uptake in a dose-dependent manner.[172] Expression of GRP and its receptors was also examined in randomly selected colon cancers. Coexpression of both ligand and the receptor was seen with equal frequency in stage A and D cancers and was only detected in <3% metastases. No difference was seen in patient survival between those whose tumours did or did not express GRP and its receptors. It was suggested that GRP is a mitogen but not a clinically significant growth factor in human colon cancers.[173] Gastrin-releasing peptide receptors were detected (using I^{125}-Tyr^4-bombesin as radioligand), often in high density, in invasive prostatic carcinomas and also in prostatic intraepithelial proliferative lesions. Well-differentiated carcinomas had a higher receptor density than poorly differentiated ones.[174]

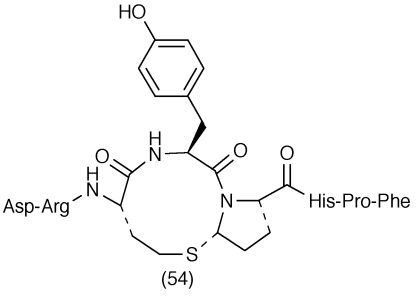


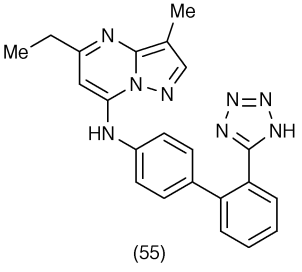












Affinities of various bombesin analogues were assessed against bombesin receptor subtype 4 (BB_4) expressed in CHO-K1 cells. [D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) (K_i 0.4 nM) and an iodinated derivative, I^{125}-[D-Tyr^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14), were found to have high affinity for the BB_4-receptor.[175] [D-Phe^6, βAla^{11}, Phe^{13}, Nle^{14}]Bn(6–14) also showed affinity for other receptor subtypes [K_i values at rat pancreatic acini (GRP receptor), recombinant neuromedin B receptor transfected BALB 3T3 cells and hBRS-3-transfected BALB 3T3 cells were 0.99, 0.36 and 8.9 nM, respectively]. [Phe^{13}]bombesin [K_i 0.96 nM for the BB_4-R receptor] was much less potent at

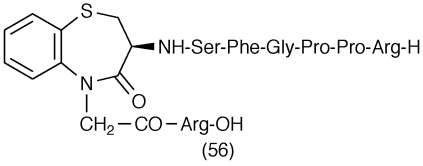
the hBRS receptor [K_i values at rat pancreatic acini, rNMB- and hBRS-3-receptors were 0.77, 6.0 and 6600 nM, respectively]. [Phe^6]bombesin(6–13)-hexylamide [K_i 18 nM for the BB_4-R receptor] was much less potent at the other receptors [K_i values at rat pancreatic acini, rNMB and hBRS-3 receptors were 100, >10000 and 3200 nM, respectively]. Many other analogues of bombesin like [D-Phe^{12}]-BN, [Leu$^{13}\psi$(CH_2NH)Leu^{14}]-BN, [D-Phe^{6,12}, Leu^{14}]-BN, [D-Phe^6, Leu$^{13}\psi$(CH_2NH)Cpa^{14}]-BN(6–14)-, [D-Phe^6]-BN(6–13)-NH_2, [D-Phe^6]-BN(1–13)-NH_2, [D-Phe^6]-BN(6–13)-$NHNH_2$, [D-Phe^6]-BN(6–13)-$NHNMe_2$ were much weaker ligands at the BB_4-R receptors (K_i values >1000 nM). Bombesin pseudo-peptide analogues containing a hydroxamide function at the C-terminal end [D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOBzl and D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-NHOH] were evaluated for their affinity at rat pancreatic acini and 3T3 cells and in some other *in vitro* assays.[176] Both peptides recognised bombesin receptors with high affinity ($K_i = 7$ and 5.8 nM on rat pancreatic acini, and 4.1 and 7.7 nM on 3T3 cells, respectively). The -NHOBzl analogue behaved as a potent agonist in stimulating amylase secretion from rat pancreatic acini (50-fold less potent than bombesin) and stimulated thymidine accumulation in 3T3 cells, while the -NHOH analogue antagonised bombesin-stimulated amylase secretion ($K_i = 22$ nM) in rat pancreatic acini and had no effect on 3T3 cells.

4.6 Bradykinin Analogues. – The role of serine endopeptidase, prolyl endopeptidase and metallopeptidases were determined in the degradation of bradykinin.[177,178] In the kidney the endopeptidase activities were found to be present throughout the nephron. Equimolar fragments of bradykinin were detected in the early proximal tubule (Arg^1-Pro^7, Phe^8-Arg^9, Arg^1-Gly^4, Phe^5-Arg^9), late proximal tubule (Arg^1-Phe^6, Arg^1-Pro^7, Gly^4-Pro^7, Gly^4-Arg^9), late distal tubule (Arg^1-Gly^4, Phe^5-Arg^9, Arg^1-Phe^5, Ser^6-Arg^9, Gly^4-Arg^9 and [des-Arg^9]bradykinin) and urine (Phe^8-Arg^9, Phe^5-Arg^9, Arg^1-Phe^5, Ser^6-Arg^9, Arg^1-Pro^7, Glu^4-Pro^7, Gly^4-Arg^9 and [des-Arg^9]bradykinin).

Alanine scanning studies on the B_1 receptor selective antagonist, desArg^{10} Hoe140 (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-OH) (pA_2 rat ileum 6.9) indicated that, with the exception of Ser in position 7 {[Ala^7, des-Arg^{10}]-Hoe140 pA_2 rat ileum 6.9} and, to a lesser extent, D-Arg in position 1 {[Ala^1, des-Arg^{10}]-Hoe140 pA_2 rat ileum 6.5} and Hyp in position 4 {[Ala^4, des-Arg^{10}]-Hoe140 pA_2 rat ileum 6.4}, Ala substituted analogues were less potent. The most critical residues appeared to be Pro in position 3 and the C-terminal dipeptide D-Tic-Oic; Ala replacement at these positions resulted in a total loss of activity.[179] Moreover, replacement of Gly^5 by Ala reverts the activity of desArg^{10}-Hoe140 to that of an agonist {[Ala^5, des-Arg^{10}]-Hoe140 pD_2 rat ileum 5.7}. The central tetrapeptide Pro-Hyp-Gly-Xaa in des-Arg^{10}-Hoe140 and B-9858 (Lys-Lys-Arg-Pro-Hyp-Gly-Igl-Ser-D-Igl-Oic-OH) were replaced with linear alkyl spacers of variable length.[180] The analogue of des-Arg^{10}-Hoe140 containing the 11-aminoundecanoic acid as spacer, MEN 11575 [D-Arg-Arg-NH-$(CH_2)_{10}$-CO-Ser-D-Tic-Oic-OH], was found to be slightly more potent than the unmodified peptide ($pA_2 = 7.1$) as a kinin B_1 receptor

antagonist in the rat ileum longitudinal smooth muscle assay. Moreover, in contrast to desArg^{10}-Hoe140, MEN 11575 was devoid of residual agonist activity at the kinin B_1 receptor (rat ileum) and antagonist activity at the kinin B_2 receptor. Replacement of the linear spacer in MEN 11575 by other residues led to less potent analogues [D-Arg-Arg-Ac$_7$c-Ser-D-Tic-Oic, D-Arg-Arg-Gly-Ac$_7$c-Gly-Ser-D-Tic-Oic, D-Arg-Arg-β-Ala-Ac$_7$c-β-Ala-Ser-D-Tic-Oic, D-Arg-Arg-γ-Abu-Ac$_7$c-γ-Abu-Ser-D-Tic-Oic, D-Arg-Arg-Ac$_6$c-Ser-D-Tic-Oic, D-*Arg-Arg-Ac$_8$c-Ser*-D-Tic-Oic and D-Arg-Arg-Ac$_9$c-Ser-D-Tic-Oic] (pA_2 values 5.0–5.9).

MEN 11270 [D-Arg-Arg-Pro-Hyp-Gly-Thi-c(Dab-D-Tic-Oic-Arg)], a conformationally constrained derivative of the B_2 kinin receptor antagonist Hoe140, bound with high affinity to the B_2 kinin receptor (W138 human fibroblasts), inhibiting ^{3}H-bradykinin with a pK_i value of 10.3 (Hoe140 pK_i 10.6). In the human umbilical vein contraction assay, MEN 11270, shifted the concentration–response curve to bradykinin (pA_2 8.14) but did not affect the concentration–response curve to the B_1 agonist Lys[des-Arg^9]-bradykinin.[181] Incorporation of non-peptidic conformationally restricting residues in bradykinin and Hoe140 led to compounds like **56**–**59**. Compounds **56** and **57** competed with [H^3]bradykinin binding to the human cloned B_2 receptor (K_i 13 and 0.7 nM, respectively). Both compounds were full bradykinin B_2 receptor agonists on the human umbilical vein (pD_2 6.6 for **56** and 6.8 for **57**) and rat uterus (pD_2 7.2 for **56** and 7.5 for **57**) preparations with the same efficacy as bradykinin.[182] In an attempt to increase the potency of **57**, both its N-terminal part and the benzothiazepinone moiety were modified.[183] Substitution of the D-Arg residue by a Lys led to a 10-fold more potent bradykinin B_2 ligand [**58**, K_i 0.07 nM, pD_2 7.1)], retaining full agonist activity on human umbilical vein. Modification of the benzothiazepinone moiety led to compound **59** which exhibited a higher agonist activity ($pD_2 = 7.4$) than **57** (pD_2 6.8).



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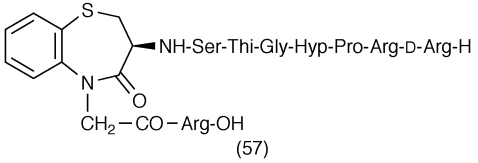

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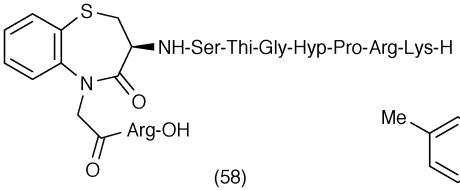

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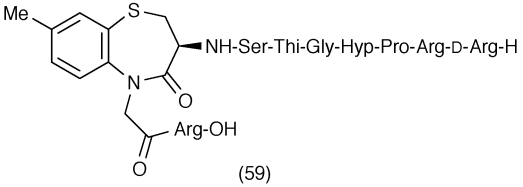

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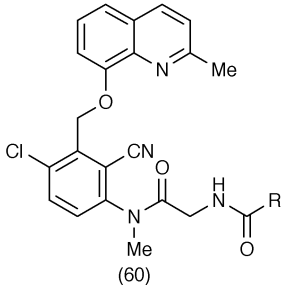
α-MePhe containing analogues of Ac-Lys-[D-β Nal^7, Ile^8]-desArg^9-bradykinin were prepared and the peptides {Ac-Lys-[($^{\alpha}$Me)Phe^5, D-β Nal^7, Ile^8]des Arg^9-, Lys-Lys-[($^{\alpha}$Me)Phe^5, D-β Nal^7, Ile^8]desArg^9-, and Ac-Lys-Lys-[($^{\alpha}$Me)Phe^5, D-β Nal^7, Ile^8]desArg^9-bradykinin} were evaluated in two B_1 receptor bioassays, the human umbilical vein, and the rabbit aorta.[184] Their antagonistic activities were compared with those of the (Lys-[Leu^8]desArg^9- and [Leu^8]desArg^9-bradykinin). The three α-MePhe analogues showed antagonistic potencies (pA_2) at both the human (8.8, 7.7, and 8.7, respectively) and rabbit (8.6, 7.8, and 8.6, respectively) B_1 receptors. No antagonistic effects (pA_2<5) were observed on the B_2 receptors. The B_1 antagonists were resistant to *in vitro* degradation by purified angiotensin-converting enzyme from rabbit lung. The N^{α}-acetylated peptides were resistant to aminopeptidases from human plasma.

A number of non-peptide antagonists of bradykinin were reported.[185–187] In the 8-quinoline series of compounds, **60** (R = -NH-$(CH_2)_4$-NH_2, -CH=CH-C_6H_5-(*m*-OMe) or -CH=CH-C_6H_5-(*p*-CF_3)] (B_2 receptor K_i values 0.1–0.3 nM), replacements for the R group and the chloro and cyano groups gave less potent compounds.[185] Compound **60** [R = -NH-$(CH_2)_4$-NH_2) inhibited bradykinin-induced guinea pig ileum contraction (EC_{50} 4.1 nM). In the rabbit jugular vein preparation, 78% inhibition of bradykinin-induced contraction was achieved at a concentration of 10 μM. The more potent compounds in the 4-benzothiazoles series included **61** [R = -CH=CH-C_6H_5-(*p*-Me) or -CH=CH-C_6H_5-(*m*-OMe)] (B_2 receptor K_i values 1.3–1.8 nM). Examples of other non-peptide bradykinin antagonists include benzodiazepine (**62**) (K_i 9.2 μM) and piperazine (**63**) (28–36% inhibition of histamine and bradykinin at 0.1 μM) derivatives.



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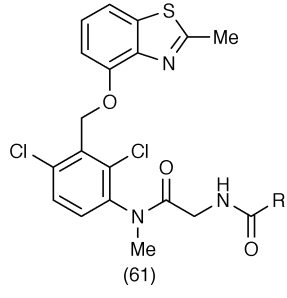

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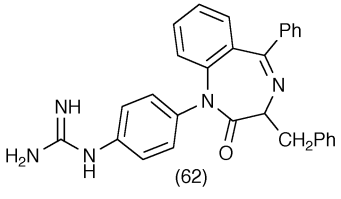

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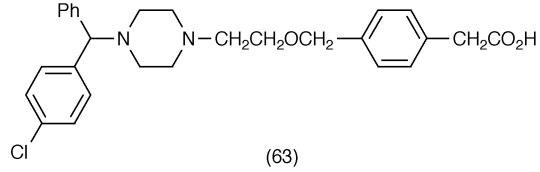

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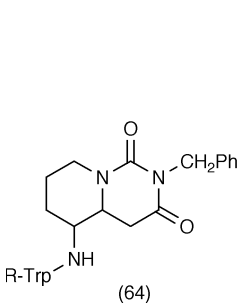
4.7 Cholecystokinin Analogues. – The role for CCK and leptin in the regulation of body weight was investigated.[188] A single intraperitoneal injection of CCK (1–2 μg kg^{-1}) given 2–3 h after leptin (2–5 μg, icv.) reduced

body weight and food intake over the ensuing 48 h more than did leptin alone. Gastrin-17-NH_2 and its analogues extended at the C-terminus by a glycine residue or by the remaining progastrin sequence and C-terminal progastrin fragments (Ser-Ala-Glu-Asp-Glu-Asn and Gly-Arg-Arg-Ser-Ala-Glu-Asp-Glu-Asn) were tested for histamine release from the vascularly perfused rat stomach.[189] C-Terminally extended gastrins induced histamine release which was inhibited by the gastrin/CCK_B antagonist L-740,093, but had to be given in concentrations 100-fold higher than amidated gastrin-17 to produce comparable effects. The two carboxy-terminal flanking peptides (Ser-Ala-Glu-Asp-Glu-Asn and Gly-Arg-Arg-Ser-Ala-Glu-Asp-Glu-Asn) were tested at 52 nM and did not induce histamine release.

Analogues of the previously reported tryptophan derivatives as potent and selective CCK_1 receptor antagonists were prepared to explore the structural requirements at the Boc-tryptophan domain.[190] Results of the CCK binding and *in vitro* functional activity evaluation showed that replacement of the acid-labile Boc group with 3,3-dimethylbutyryl or *tert*-butylaminocarbonyl conferred acid stability to the analogues. Compounds **64** [R = Bu^t-CH_2CO- (CCK_1 IC_{50} 4.4 nM, CCK_2 >10000 nM) or Bu^t-NHCO- (CCK_1 IC_{50} 0.91 nM, CCK_2 >10000 nM)] retained a high potency and selectivity in binding to CCK_1 receptors, as well as an *in vivo* antagonist activity against the acute pancreatitis induced by caerulein in rats. Oral administration of compounds also produced a lasting antagonism to the hypomotility induced by CCK-8 in mice. Other amino acid based CCK antagonists included β-phenylalanine derivatives like **65** (R^1 = Boc or Z) (free acids or trimethylsilylethyl esters) which may be considered as conformationally constrained dipeptoids containing Trp and β-Phe.[191] The compounds were more potent in inhibiting [^{3}H]propionyl-CCK-8 binding to rat pancreas (CCK_A) than to rat cortex membranes (CCK_B). Examples of non-peptide ligands acting at CCK receptors include compounds **66**–**68**.[192–195] The 9-membered structure **66** exhibited antagonistic activity at the CCK_A receptor with a 54-fold selectivity over the CCK_B/gastrin receptor (IC_{50}s 0.36 and 19.5 μM against CCK_A and CCK_B receptors, respectively).[192] SR146131 (**67**) inhibited the binding of [I^{125}]-Bolton Hunter-sulfated CCK octapeptide to the human recombinant CCK_1 receptor (IC_{50} 0.56 nM) with high selectivity (300-fold less potent at the CCK_2 receptor).[193] It behaved as a full agonist with an efficacy comparable with that of sulfated CCK octapeptide (EC_{50} 1.38 nM, intracellular calcium release in NIH-3T3 cell line expressing human recombinant CCK_1 receptor). SR146131 inhibited gastric acid and gallbladder emptying in mice (ED_{50} 66 and 2.7 μg kg^{-1} p.o., respectively) and reduced food intake in fasted rats (from 0.1 mg kg^{-1}, p.o.), non-fasted rats in which food intake had been highly stimulated by the administration of NPY (from 0.3 mg kg^{-1}, p.o.), in fasted gerbils (from 0.1 mg kg^{-1}, p.o.) and in marmosets maintained on a restricted diet (from 3 mg kg^{-1}, p.o.).[194] A similar compound **68** was an antagonist of CCK.

4.8 Complement-related Peptides. – Excessive levels of proinflammatory peptides such as the anaphylatoxin C5a are associated with immunoinflamma-



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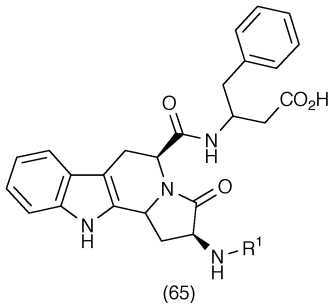

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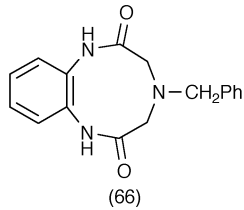

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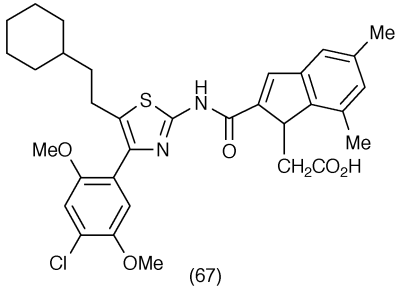

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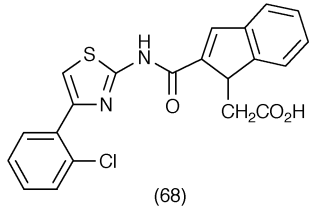

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tory diseases. A series of small peptides derived from the C-terminus of C5a were shown to be C5a receptor antagonists. Further SAR studies on the complement receptor antagonist MePhe-Lys-Pro-D-Cha-Trp-D-Arg are described.[196] Replacement of the C-terminal D-Arg by Arg led to a 20-fold loss in antagonist potency and the replacement of the N-terminal MePhe by Phe resulted in 60–70-fold loss in antagonist potency. A few of the N-terminally extended analogues, Lys-Phe-Lys-Pro-D-Cha-Trp-D-Arg, Phe-Lys-Phe-Lys-Pro-D-Cha-Trp-D-Arg, $C5a_{12-20}$-Ahx-Phe-Lys-Pro-D-Cha-Trp-D-Arg and $C5a_{12\text{-}20}$-Ahx-Gly-Gly-Gly-Gly-Phe-Lys-Pro-D-Cha-Trp-D-Arg, were similar in potency to the parent peptide (IC_{50}s 0.07–0.5 μM). Replacement of the Lys residue by ornithine or diaminobutyric acid residues also resulted in compounds [Ac-Phe-Orn-Pro-D-Cha-Trp-D-Arg and Ac-Phe-Dap-Pro-D-Cha-Trp-D-Arg] comparable in potency to MePhe-Lys-Pro-D-Cha-Trp-D-Arg.

The N- to C-terminal and side chain to C-terminal cyclisations also led to antagonists. Although c(Phe-Lys-Pro-D-Cha-Trp-D-Arg) was much less potent, c(MePhe-Lys-Pro-D-Cha-Trp-D-Arg) was only about 20-fold less potent. The side chain to C-terminal cyclic peptides, Phe-c(Lys-Pro-D-Cha-Trp-D-Arg), Phe-c(Lys-Pro-D-Cha-Trp-Arg), Phe-c(Orn-Pro-D-Cha-Trp-D-Arg), Phe-c(Orn-Pro-D-Cha-Trp-Arg), Phe-c(Dab-Pro-D-Cha-Trp-D-Arg), Phe-c(Dab-Pro-D-Cha-Trp-Arg), Phe-c(Dap-Pro-D-Cha-Trp-Arg), Ac-Phe-c(Orn-Pro-D-Cha-Trp-Arg), Ac-Phe-c(Orn-Pro-D-Cha-Trp-D-Arg), Ac-Phe-c(Lys-Pro-D-Cha-Trp-D-Arg) and Ac-Phe-c(Dab-Pro-D-Cha-Trp-D-Arg) were either similar or somewhat more potent (IC_{50}s 0.02–2.4 μM) than the linear hexapeptide. Ac-Phe-c(Orn-Pro-D-Cha-Trp-Arg) was the most potent cyclic peptide (IC_{50} 0.02 μM). Conformation of this peptide was studied using NMR.[196] One of the cyclic peptides, Phe-c(Orn-Pro-D-Cha-Trp-Arg), was

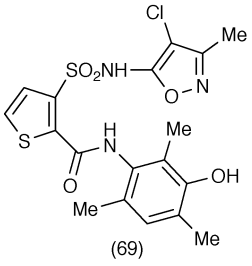
tested for its ability to antagonise the neutropenic effects of both C5a and endotoxin in rats.[197] Administration of the cyclic peptide (0.3–3 mg kg^{-1}, i.v.) did not affect the levels of circulating polymorphonuclear leukocytes but, when given 10 min prior to C5a, it inhibited the C5a-induced neutropenia by up to 70%. At a slightly higher dose (0.3–10 mg kg^{-1}), the peptide also inhibited *E. coli* lipopolysaccharide-induced neutropenia in rats when the rats were pre-treated with the peptide.

4.9 Endothelin Analogues. – Chemical and biological aspects of endothelin research were reviewed.[198–201] Incubation of big ET-1 with recombinant human matrix metalloproteinase-2 (MMP-2, gelatinase A) resulted in the specific cleavage of the Gly^{32}-Leu^{33} bond of big ET-1.[202] Moreover, the resultant peptide ET-1(1–32) exerted greater vasoconstrictor effects than big ET-1 [ET-1(1–32) ED_{50} 4 pmol< ET-1(1–21) ED_{50} 27.7 pmol< bigET-1 ED_{50} 45.7 pmol]. Thus vascular MMP-2 may be involved in the regulation of vascular reactivity by cleaving big ET-1 to yield the vasoconstrictor peptide, ET-1(1–32).

A number of publications on the non-peptide antagonists of endothelin acting at ET_A, ET_B or both the receptor subtypes were reported.[203–213] Examples of some of the structurally different antagonists include compounds **69**–**79**. Further modifications in substituted anilinothiophenesulfonamide series of ET_A-selective antagonists (reported earlier) showed that an additional substituent at the 6-position of the anilino ring further increases the potency.[203] In addition, a wide range of functionalities at the 3-position of the 2,4,6-trisubstituted ring increased ET_A selectivity by ~10-fold while maintaining *in vitro* potency. Compound **69** (TBC2576) was one of the more potent and ET_A-selective compounds with improved stability (serum half-life of 7.3 h in rats). Another series of ET_A-selective compounds includes pyrrolidine carboxylic acid derivatives like A-127722 (**70**, R = OMe). Modifications of the 2-substituent on the pyrrolidine ring led to compounds with alkyl groups at the 2-position which possessed improved ET_A selectivity, with the best of these compounds (**70**, R = pentyl) (ET_A IC_{50} 2.5 nM and ET_B IC_{50} 47.3 μM) showing nearly 19,000-fold selectivity.[204] A similar compound (**70**, R = n-hexyl) at a dose of 10 mg kg^{-1} in rats showed about 40% oral bioavailability. A more selective (> 25,000-fold) ET_A antagonist **71** (A-216546) inhibited [I^{125}]endothelin-1 binding to cloned human ET_A and ET_B receptors (K_i 0.45 and 13,000 nM, respectively) and blocked endothelin-1-induced arachidonic acid release and phosphatidylinositol hydrolysis (IC_{50} of 0.59 and 3 nM, respectively).[205] In isolated vessels, **71** inhibited endothelin ET_A receptor-mediated endothelin-l-induced vasoconstriction, and endothelin ET_B receptor-mediated sarafotoxin 6c-induced vasoconstriction (pA_2 8.29 and 4.57, respectively). A-216546 was orally available in rat, dog and monkey and blocked endothelin-1-induced presser response in conscious rats.

Replacement of the dialkylacetamide side chain in the above series of the pyrrolidine carboxylic acid containing ET_A-selective antagonists resulted in a complete reversal of receptor selectivity, preferring ET_B over ET_A. Administra-

tion of a single oral dose of the ET_B-selective (4000-fold) antagonist A-192621 (**72**, 30 mg kg^{-1}) blocked the ET-1-induced depressor effect.[206] When administered for three days (30 mg kg day^{-1}), the antagonist increased the mean arterial blood pressure. Administration of an ET_A-selective antagonist returned the elevated blood pressure to normal values. A similar compound (**73**, A-308165) demonstrated over 27,000-fold selectivity favouring the ET_B receptor (IC_{50} values 77360 and 3.2 nM, respectively, for human ET_A and ET_B receptors).[207] Examples of other non-peptide antagonists include compounds



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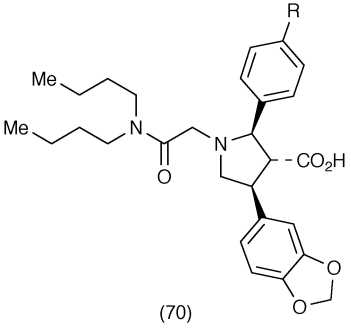

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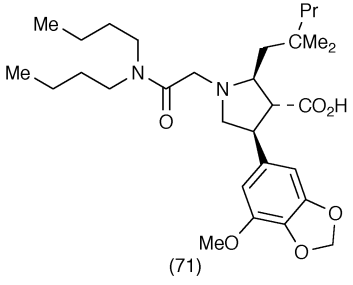

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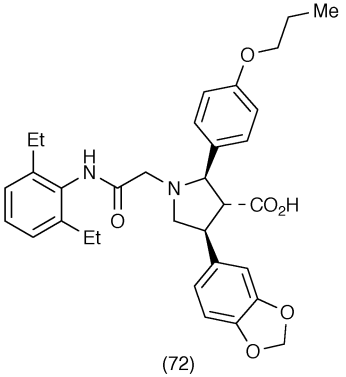

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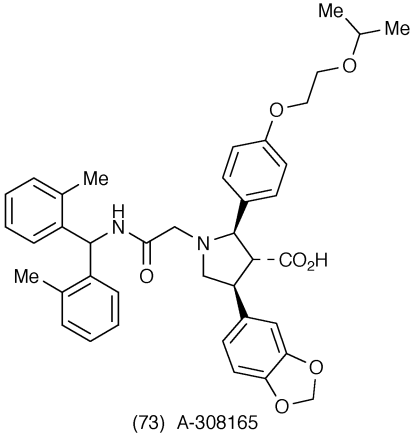

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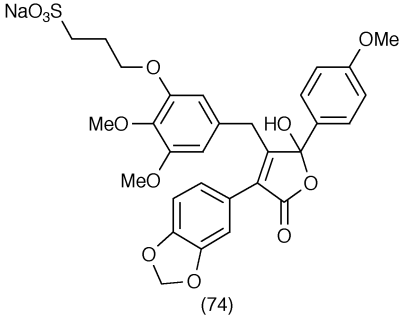

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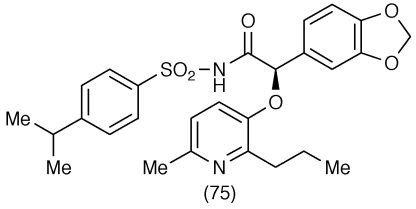

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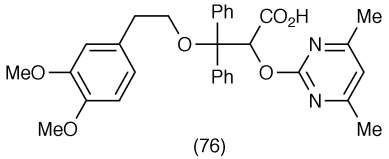

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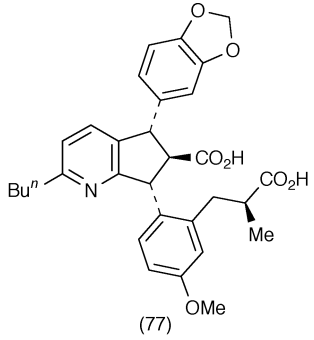

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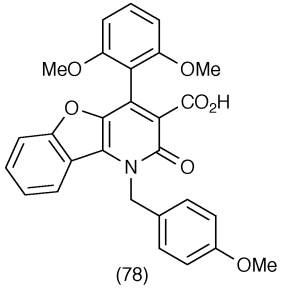

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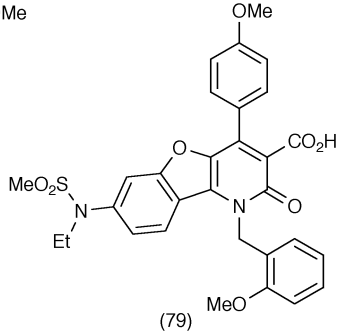

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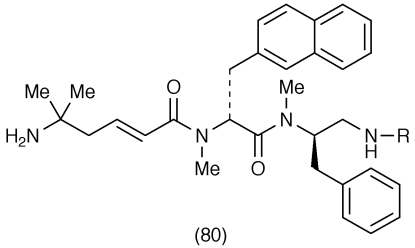
74–**76**.[208–210] Oral administration of **75** (K_i 0.11 and 25 nM, respectively, at ET_A and ET_B receptors) (1–10 mg kg^{-1}) caused dose-dependent inhibition of the presser response to exogenous ET-1 in conscious normotensive rats.[209] Compound **75** (10–100 mg kg^{-1}, p.o.) also reduced blood pressure in deoxycorticosterone acetate-salt hypertensive rats, spontaneously hypertensive rats and stroke-prone spontaneously hypertensive rats. Compound **78** is a functional antagonist for the ET_A receptor with a pA_2 value of 6.3 and compound **79** is a functional antagonist for the ET_B receptor with a pA_2 value of 6.9.[212]

4.10 Growth Hormone-releasing Peptide and Non-peptide Analogues. – Work on the antagonistic analogues of growth hormone-releasing hormone, orphan receptor involvement in pulsatile growth hormone release and physiological role and clinical utility of growth hormone secretagogues was reviewed.[214–216] Purification and identification of a G-protein-coupled receptor through which synthetic small molecule growth hormone secretagogues release growth hormone was reported previously. Endogenous ligand for this receptor has now been identified in rat stomach. The 28 amino acid peptide [Gly-Ser-Ser(O-

$CO(CH_2)_6$-OMe)-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-Ala-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg] called 'ghrelin' contains a serine residue in position 3 which is *n*-octanoylated.[217] The acylated peptide specifically releases growth hormone both *in vitro* and *in vivo*, and O-*n*-octanoylation at Ser^3 is essential for the activity. The human sequence of ghrelin was identified [Gly-Ser-Ser(O-$CO(CH_2)_6$-OMe)-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg] by screening a human stomach cDNA library.

In an attempt to prepare hGH-RH antagonists with a high and protracted activity, analogues of [PhAc-Tyr^1, D-Arg^2, Phe(*p*-Cl)^6, Abu^{15}, Nle^{27}]hGH-RH(1-29)-NH_2 containing Arg, D-Arg, Nle, homoarginine (Har), and other substitutions were prepared.[218] The more potent antagonists with extended duration of action include [PhAc-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Arg^9, Abu^{15}, Nle^{27}, D-Arg^{29}]hGH-RH(1-29)-NH_2, [PhAc-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}]hGH-RH(1-29)-NH_2, [PhAc-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Arg^9, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}]hGH-RH(1-29)-NH_2 (JV-1-36), and [PhAc-Tyr^1, D-Arg^2, Phe(4-Cl)^6, Har^9, Tyr(Me)^{10}, Abu^{15}, Nle^{27}, D-Arg^{28}, Har^{29}]hGH-RH(1-29)-NH_2 (JV-1-38). Analogue JV-1-36 showed the highest GH-RH antagonistic activity *in vitro* and also induced a strong and prolonged inhibition of GH release *in vivo* for at least 30 min. The antagonist JV-1-38 was slightly less potent than JV-1-36 both *in vitro* and *in vivo* but proved to be very long-acting *in vivo*, suppressing the GH-RH-induced GH release even after 60 min. One of the GH-RH antagonists, phenylacetyl-[D-Arg^2, Phe(*p*-Cl)^6, Abu^{15}, Nle^{27}]hGH-RH(1-28)-agmatine, was used to study the mechanism by which the antagonists inhibit the growth of various tumours.[219] In nude mice bearing xenografts of U-87MG human glioblastomas, the peptide reduced levels of telomerase activity as compared with controls. When U-87 glioblastomas, H-69 small cell lung carcinomas, H-23 non-small cell lung carcinomas, and MDA-MB-468 breast carcinoma cells were cultured *in vitro*, addition of the antagonist (3 μM) also inhibited telomerase activity.

New GH secretagogues like **80**–**85** were reported.[220–226] NN703 (**80**, R = Me) stimulated GH release from rat pituitary cells with a potency and efficacy similar to that of GHRP-6. The oral bioavailability of NN703 was about 30% and plasma half-life was about 4 hours.[220] An analogue of NN703 containing a C-terminal sulfonamide functionality (**80**, R = SO_2Me) showed high activity in a *in vitro* rat pituitary model (EC_{50} 2.7 nM).[221] Compound **81** (EC_{50} 1 nM) was obtained by screening acylated dipeptide libraries synthesised on solid support using a PAL resin.[222] Examples of other dipeptide based GH secretagogues include compounds **82**–**85**. The 4-thiazolyl analogue **82** (*S* isomer) showed increased potency in the rat pituitary cell GH release assay (EC_{50} 0.5 nM) and in beagles (44% bioavailability).[223] The corresponding *R* derivative was about 3-fold less potent in the *in vitro* GH release assay and about 4-fold less potent *in vivo* when administered orally. One of the spiroheterocyclic GH secretagogues (**83**, X = SO_2) had an EC_{50} of 1.4 nM in the rat pituitary cell assay.[224] A number of other analogues containing an -O-, -CH_2-, -S- or an -SO- group in place of X and Trp or hPhe residues in place of



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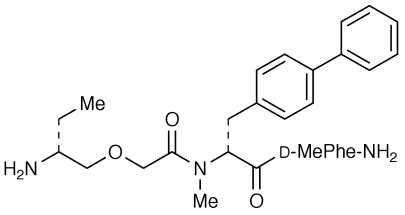

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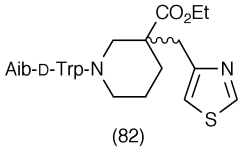

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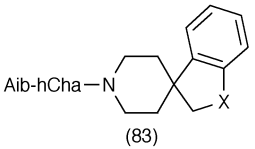

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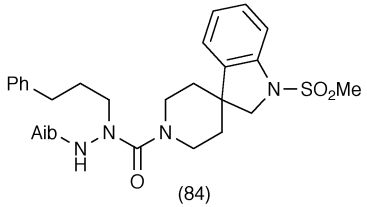

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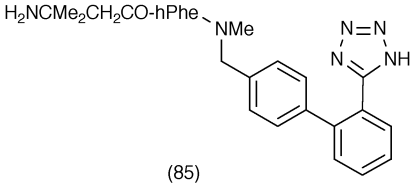

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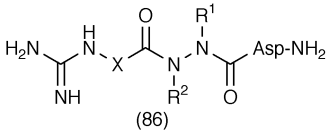
hCha or -$(CH_2)_3$-Ph, -CH_2-O-CH_2Ph groups in place of the hCha side chain were also potent agonists (EC_{50} 1.1–21 nM).

4.11 Integrin-related Peptide and Non-peptide Analogues. – Various aspects of cell adhesion molecules including integrin antagonists, extracellular matrix and integrin signalling, role of cellular adhesion molecules on vascular smooth muscle cells and the role of α_V integrins during angiogenesis were reviewed.[227–230]

4.11.1 IIb/IIIa Antagonists. As in previous years, most of the work in the integrin area has been concentrated in the field of peptide/non-peptide IIb/IIIa antagonists as platelet aggregation inhibitors. Two platelet aggregation inhibitors, ussuristatin 1 and 2, were isolated from the venom of Chinese viper (*Agkistrodon ussuriensis*). The sequences of both peptides (71 amino acids) showed high similarities to those of other disintegrins.[231] Ussuristatin 1 had a typical Arg-Gly-Asp sequence while in ussuristatin 2, the corresponding sequence was Lys-Gly-Asp. Ussuristatin 1 suppressed platelet aggregation induced by ADP, collagen, thrombin, and epinephrine with IC_{50} 17–33 nM. Ussuristatin 2 also inhibited the platelet aggregation, but the IC_{50}s were about ten times higher.

Linear Arg-Gly-Asp-mimetics containing an aza-amino acid instead of glycine (synthesised by solid phase methodology) displayed differential activities against αIIbβ3 and αvβ3 integrins.[232] For example, in comparison to c(Arg-Gly-Asp-D-Phe-Val) (IC_{50}s 0.0015 and 3.7 μM against αvβ3 and αIIbβ3, respectively), compound **86** [$R^1 = R^2 = H$, NH-X-CO = -$NH(CH_2)_4CO$-] (IC_{50}s

6.8 and >100 μM against αvβ3 and αIIbβ3, respectively) was >4000-fold less potent at the αvβ3-integrin ligand binding assays. Peptide **86** [R^1 = H, R^2 = Me, NH-X-CO = δ-aminovaleric] (IC_{50}s >100 and 2.7 μM against αvβ3 and αIIbβ3, respectively) was equipotent to the cyclic peptide in the αIIbβ3-integrin ligand binding assay and much less potent in the αvβ3-integrin ligand binding assays. Compound **86** [R^1 = H, R^2 = Me, X = Cyh (4-aminocyclohexylcarbonyl)] showed similar selectivity profile (IC_{50}s >100 and 22 μM against αvβ3 and αIIbβ3, respectively). Aza-peptide **86** [R^1 = Me, R^2 = H, NH-X-CO = δ-aminovaleric] and [R^1 = Me, R^2 = H, X = Cyh] were inactive (IC_{50}s >100 μM against αvβ3 and αIIbβ3) and compound **86** [R^1 = R^2 = H, NH-X-CO = Cyh] showed similar potency against both integrins (IC_{50}s 4–5 μM). Replacing the Arg in the Arg-Gly-Asp tripeptide by a variety of cationic structures led to inhibitors of platelet aggregation and fibrinogen-receptor binding.[233] Compound **87**, which contained an amidinophenyl structure as the cationic moiety, showed high inhibitory potency.



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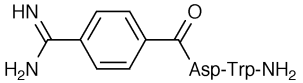

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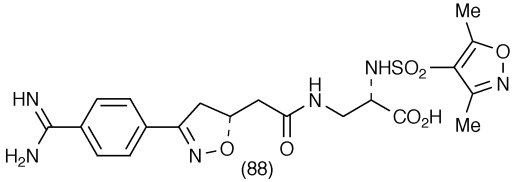
Diaminopropionic acid derivatives containing various conformationally restricting residues between the two important charged groups were reported as Arg-Gly-Asp mimetics.[234–239] Many of these were potent inhibitors of platelet aggregation. Isoxazolylsulfonamide derivative **88** (DMP 802) demonstrated a prolonged duration of action after i.v. and p.o. dosing and high-affinity for resting and activated platelets.[234] Similar compounds bearing a phosphoramidate group α- to the carboxylate moiety (**89**, R = H, Me, Et, *i*-Pr, *n*-Bu or -CH_2-CH=CH_2) were found to bind GPIIb/IIIa with high affinity (IC_{50}s 0.55–2.6 nM) and were potent antagonists of ADP mediated platelet aggregation.[235] Isoxazoline containing mimetic **90** was an antagonist of both $\alpha_v\beta_3$ (IC_{50} 0.7 nM) and IIb/IIIa (IC_{50} 0.34 nM).[236] A thienothiophene containing analogue **91** inhibited *ex vivo* platelet aggregation in dogs at a dose of 5 mg kg^{-1}, i.v. An oral dose of 50–90 mg kg^{-1} followed by low daily doses of 10 mg kg^{-1} was sufficient to maintain 80% inhibition of *ex vivo* platelet aggregation over several days.[238] Replacement of the N-terminal 3-pyridyl group in **91** by -$(CH_2)_2$-O-Et, phenyl, 4-chlorophenyl and 2-thienyl groups resulted compounds with similar potency in the platelet aggregation assays. The conformationally restricted analogue **92** inhibited platelet aggregation (IC_{50} 17 nM) and showed good oral activity in dog (40% inhibition of *ex vivo* platelet aggregation at a dose of 2 mg kg^{-1} for 4 h).[239] A tyrosine based Arg-Gly-Asp mimetic (**93**) inhibited ADP-induced platelet aggregation with an IC_{50} value of 0.25 μM.[240]

A number of non-peptide analogues based on the Arg-Gly-Asp sequence have been reported.[241–244] Selected examples, all inhibiting platelet aggregation after oral administration, include compounds **94**–**97**. The nipecotamide deriva-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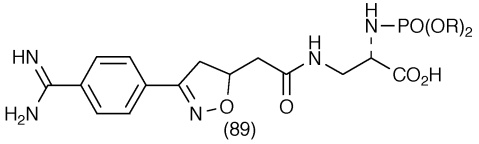
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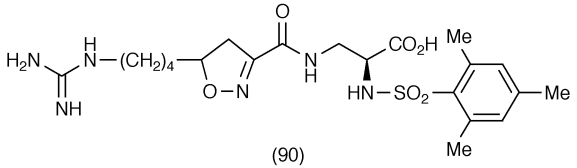
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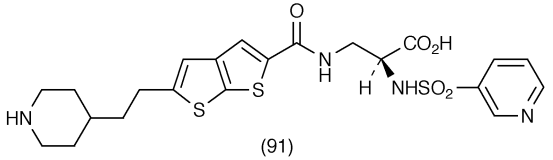
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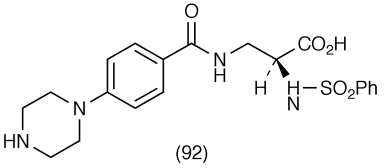
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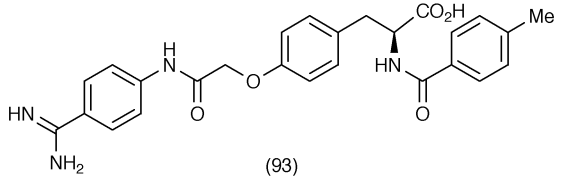
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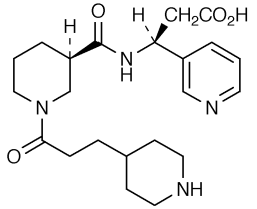
tive **94** displayed significant *ex vivo* antiplatelet activity on oral administration (1.0 mg kg^{-1}, 16% bioavailability) and was found to be efficacious in several *in vivo* thrombosis models.[241] Isoquinolone derivative **95** and its analogues in which the isoquinolone ring was replaced by tetralin, isoquinoline, tetralone or benzopyran inhibited platelet aggregation in platelet rich plasma (IC_{50} 0.06–0.19 μM).[243]

4.11.2 $\alpha_v\beta_3$ Antagonists. Role of the integrin $\alpha_v\beta_3$ in bone disorders and tumour growth has been discussed previously and several additional papers have appeared this year. A method for coating implants using integrin-specific peptide ligands has been reported. Cyclic pentapeptide [c(Arg-Gly-Asp-D-Phe-



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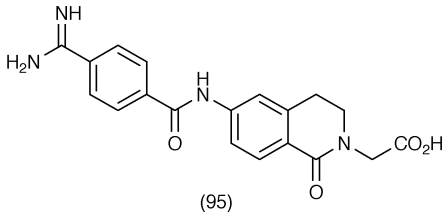

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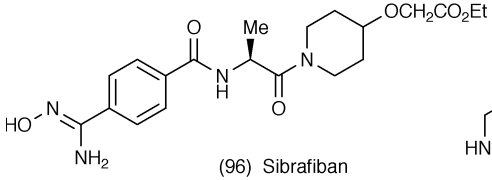

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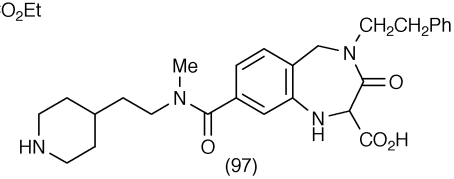

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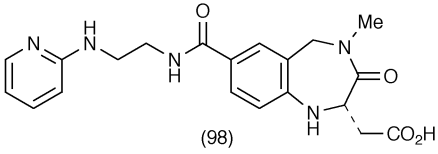
Lys), selective for $\alpha_V\beta_3$ and $\alpha_V\beta_5$] was coupled to a suitable acrylic acid spacer (*e.g.* CH_2=CH-CONH$(CH_2)_5$-CONH-CH_2CH_2O-CH_2CH_2O-CH_2COOH or CH_2=CH-CONH$(CH_2)_5$-CONH-CH_2CH_2O-CH_2CH_2O-CH_2CONH-CH_2CH_2O-CH_2CH_2O-CH_2COOH) through the Lys side chain and the peptide was then radically polymerised onto the poly(methyl methacrylate) graft.[245] The graft material surfaces bound osteoblasts, stimulated their proliferation and triggered biological tissue regeneration.

The influence of N-methylation on the binding affinity and selectivity of an $\alpha_v\beta_3$ antagonist c(Arg-Gly-Asp-D-Phe-Val) was investigated.[246] The ability of peptides to inhibit the binding of vitronectin and fibrinogen to the isolated, immobilised $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ receptors was compared with that of the linear peptide Gly-Arg-Gly-Asp-Ser-Pro-Lys [IC_{50}s against $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ 210 nM and 1.7 μM, respectively]. Corresponding values for the cyclic peptides against $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ were: c(Arg-Gly-Asp-D-Phe-Val) (2.5 nM and 1.7 μM), c(MeArg-Gly-Asp-D-Phe-Val) (55 nM and 5.2 μM), c(Arg-Sar-Asp-D-Phe-Val) (45 nM and >10 μM), c(Arg-Gly-MeAsp-D-Phe-Val) (560 nM and >10 μM), c(Arg-Gly-Asp-D-MePhe-Val) (14000 nM and >10 μM), c(Arg-Gly-Asp-D-Phe-MeVal) (0.58 nM and 0.86 μM). c(Arg-Gly-Asp-D-Phe-MeVal) was one of the most active and selective compounds in inhibiting vitronectin binding to the $\alpha_v\beta_3$ integrin. Detailed conformational studies (in solution by ^{1}H NMR in H_2O and DMSO-d_6 and molecular modelling simulations) on cyclic peptides containing the Arg-Gly-Asp-Asp-Val and the Arg-Gly-Asp-Tyr(Me)-Arg pharmacophore were reported.[247]

Incorporation of a 2-aminopyridine arginine mimetic into the 3-oxo-1,4-benzodiazepine-2-acetic acid integrin antagonist series led to nonpeptide vitronectin receptor antagonists with oral activity.[248] For example compound **98** (K_i 3.5 nM for $\alpha_V\beta_3$ and 28000 nM for $\alpha_{IIb}\beta_3$) showed between 4–14% oral bioavailability in the rat and dog. A similar analogue containing a phenyl group in place of the pyridyl group was less potent in the *in vitro* assays (K_i 22 μM for $\alpha_V\beta_3$ and >50 μM for $\alpha_{IIb}\beta_3$). A Gly-Asp mimetic analogue SB 265123 (**99**) (K_i 4.1 nM for $\alpha_V\beta_3$, 1.3 nM for $\alpha_V\beta_5$, 18000 nM for $\alpha_5\beta_1$, and 9000 nM



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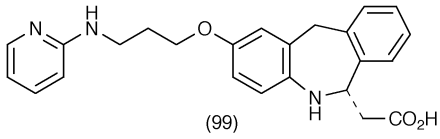

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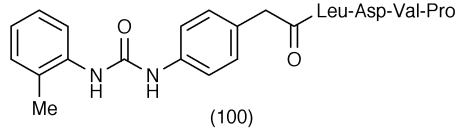
for $\alpha_{IIb}\beta_3$) displayed 100% oral bioavailability in rats, and was orally active *in vivo* in the ovariectomized rat model of osteoporosis.[249]

4.11.3 $\alpha_4\beta_1$ and $\alpha_5\beta_1$ Antagonists. Roles of these integrins in cellular trafficking and various diseases have been discussed.[250–253] Cyclic peptide inhibitors of VLA-4 and fibronectin/VCAM-1 interaction, *e.g.* c(Ile-Leu-Asp-Val-NH-$(CH_2)_5$CO) were reported.[254] Several of these inhibitors like c(Ile-Leu-Asp-Val-NH$(CH_2)_5$CO) and c(Ile-Leu-Asp-Val-NH$(CH_2)_4$CO) blocked VLA-4/VCAM-1 and VLA-4/fibronectin interaction in *in vitro* assays and inhibited oxazolone and ovalbumin-induced contact hypersensitivity responses in mice. The compounds did not affect cell adhesion mediated by two other integrins [VLA-5 ($\alpha_5\beta_1$) and LFA-1 ($\alpha_L\beta_2$]. Cyclic peptides with a much smaller ring structure like c(Ile-Leu-Asp-Val-NH$(CH_2)_2$CO) were inactive in the *in vitro* and *in vivo* assays. *p*-Aminophenylacetyl-Leu-Asp-Val derivatives containing various non-peptide residues at the N-terminal end are reported as inhibitors of integrin $\alpha_4\beta_1$. In comparison to Ile-Leu-Asp-Val (IC_{50} 66 μM in a Jurkat cell/VCAM-immunoglobulin fusion protein binding assay) and Tyr-Leu-Asp-Val (IC_{50} 12.6 μM), compounds like Ph-CO-NH$C_6H_4CH_2$CO-Leu-Asp-Val, Z-NH$C_6H_4CH_2$CO-Leu-Asp-Val and Ph-NH-CO-NH$C_6H_4CH_2$CO-Leu-Asp-Val were more potent. Compound **100** (BIO-1211) showed activity in an antigen-induced bronchoconstriction and airway hyper-responsiveness model in sheep. In various integrin adhesion assay, **100** showed activity against $\alpha_4\beta_7$, $\alpha_1\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_L\beta_2$ and $\alpha_{IIb}\beta_3$ integrins at much higher concentrations.[255,256] Other VCAM-1/integrin interaction inhibitors include compounds like **101** which contain a naphthalene-based template.[257]



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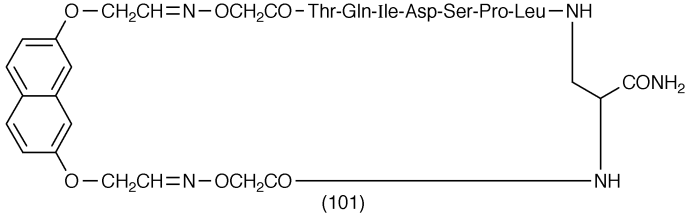

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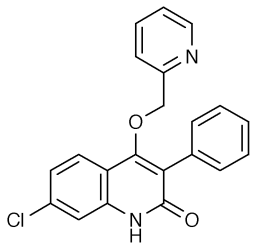
4.12 LHRH Analogues. – A review on cancer chemotherapy based on targeting cytotoxic peptide conjugates to their receptors on tumours has

appeared.[258] Targeted cytotoxic peptide conjugates are hybrid molecules composed of a peptide (like LHRH, bombesin or somatostatin) carrier which binds to receptors on tumours and a cytotoxic moiety (*e.g.* doxorubicin).[259] Conjugation of LHRH analogues [GnRH-III, Pyr-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2], Ac-D-Trp-D-Phe(p-Cl)-D-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-Ala-NH_2 and Ac-D-Trp-D-Phe(p-Cl)-D-Trp-Ser-Tyr-D-Lys-β-Asp(α-NEt_2)-Leu-Gln-Pro-D-Ala-NH_2] through lysyl side chains and a tetrapeptide spacer, Gly-Phe-Leu-Gly (X), to a copolymer, poly(*N*-vinylpyrrolidone-co-maleic acid) increased antiproliferative activity toward MCF-7 and MDA-MB-231 breast, PC3 and LNCaP prostate, and Ishikawa endometrial cancer cell lines in culture and against tumour development by xenografts of the breast cancer cells in immunodeficient mice.[260] Mechanisms of GnRH-induced desensitization of LH secretion have been investigated.[261,262]

To obtain transdermally deliverable analogues of LHRH, hydrophobic derivatives of [D-Lys^6]GnRH were synthesised by attaching various aliphatic acids to the N^{ε}-amino side chain of Lys. Analogues with 12-carbon or shorter aliphatic acids retained agonist activity comparable to that of [D-Lys^6]GnRH. [D-Lys-lauryl^6]GnRH was shown to have a longer duration of action *in vivo*, as compared to [D-Lys^6]GnRH. The transdermal penetration efficiency of hydrophobic peptides was gradually lowered in increasingly hydrophobic analogues.[263]

Non-peptide antagonists of LHRH were discovered by screening the company (Merck) collection for binding affinity to the rat GnRH receptor.[264,265] The substituted quinolone derivative (**102**, IC_{50} 10 μM) was further modified by addition of an alkyl amine at the 4-position, a 3,5-dimethylphenyl group at the 3-position and 6-nitro-7-chloro-substitution of the 1*H*-quinolone core. The most potent compound (**103**) which possesses the 4-(2-piperidinylethyl) group and 3-(3,5-dimethylphenyl) group on the optimised quinolone core had an IC_{50} of 32 nM in the same binding assay.



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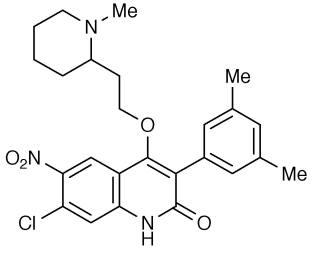

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4.13 α-MSH Analogues. – Aspects of melanocortin receptors and biology were reviewed.[266,267] A number of publications on agouti-related protein, an endogenous antagonist of melanocortin action, and its N- and C-terminal fragments have appeared.[268–271] Conformations of α-MSH analogues leading to ligand-receptor interaction and selectivity were analysed by measuring receptor-binding and cAMP-generating activity in CHO cell lines stably

transfected with rMC_3R and hMC_4R, as well as the NMR structures of chemically synthesised α-MSH analogues.[272] Compared with [Ahx^4]α-MSH, the linear peptide Ahx^4-Asp-His^6-D-Phe-Arg-Trp-Lys^{10} revealed a preference for the MC_4R. Truncation of Ahx^4 and Asp^5 of the linear heptapeptide decreased the receptor-binding and cAMP-generating activity. Whereas the solution conformation of Ahx-Asp-His-D-Phe-Arg-Trp-Lys revealed a stable type I β-turn structure, [Gln^6]-analogue revealed a tight γ-turn composed of Gln^6-D-Phe^7-Arg^8. Replacement of the His^6 residue by Gln, Asn, Arg or Lys decreased not only the receptor binding, but also the cAMP-generating activity in both the MC_3R and the MC_4R.

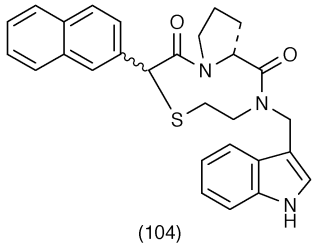
Activities of MSH ligands were also examined in *in vitro* binding assays (rat MC_3 and MC_4 receptors) as well as in melanocortin-induced behaviour in the rat grooming behaviour.[273] [D-Tyr^4]melanotan-II {Ac-c[D-Tyr^4, Asp^5, D-Phe^7, Lys^{10}]α-MSH(4-10)-NH_2} and RMI-2001 (Ac-cyclo-[Cys^4, Gly^5, D-Phe^7, Cys^{10}]α-MSH-NH_2) showed significantly higher affinity and potency on the MC_4 receptor as compared to the MC_3 receptor. Nle-γ-MSH (Ac-Tyr-Val-Nle-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2) was the only ligand with higher affinity and potency on the rat melanocortin MC_3 receptor. SHU9119 (Ac-cyclo-[Nle^4, Asp^5, D-Nal(2)^7, Lys^{10}]α-MSH-(4-10)-NH_2) and RMI-2005 (Ac-cyclo-[Cys^4, Gly^5, D-Nal(2)^7, Nal(2)^9, Cys^{10}]α-MSH-(4-10)-NH_2) inhibited α-MSH-induced melanocortin receptor activity *in vitro*, as well as α-MSH-induced grooming behaviour.

Receptor affinities of α-MSH, γ-MSH and analogues were compared on MC_3 and MC_4 receptor subtypes.[274] Although the MC_3R and MC_4R both recognised α-MSH [Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2], the affinity of γ-MSH was 50-fold higher for MC_3R. In comparison to their affinities at MC_4 receptor subtypes, α-MSH, [Nle^4]α-MSH, γ_2-MSH, γ_1-MSH, Lys-γ_2-MSH, [Nle^3]-γ_2-MSH, and [Nle^4]Lys-γ_2-MSH were 10–30 -fold more potent at MC_3R. In general, analogues of α-MSH, [Lys^1, Nle^4]-, [Val^3, Nle^4]-, [Gly^5, Nle^4]-, [Asp^{10}, Nle^4]-, [Nle^4, Gly^5, Asp^{10}]-, [Nle^4, Arg^{11}]-, [Nle^4, Phe^{12}]- and [Nle^4, Gly^{13}]α-MSH, bound to both MSH receptor subtypes but their affinities were greater at the MC_3 receptor subtypes. Similar pattern was also observed in the case of γ_2-MSH analogues. Ac-Lys-γ_2-MSH-NH_2, Ac-[Ser^1, Nle^4]Lys-γ_2-MSH-NH_2, Ac-[Ser^3, Nle^4]Lys-γ_2-MSH-NH_2, Ac-[Nle^4, Gly^{10}]Lys-γ_2-MSH-NH_2, Ac-[Nle^4, Lys^{11}]Lys-γ_2-MSH-NH_2, Ac-[Nle^4, Pro^{12}] Lys-γ_2-MSH-NH_2, Ac-[Nle^4, Val^{13}]Lys-γ_2-MSH-NH_2 and Ac-[Nle^4, Gly^{10}, Pro^{12}]Lys-γ_2-MSH-NH_2 were all more potent at the MC_3 receptor subtypes.[274]

Several disulfide bridge containing MSH cyclic peptide analogues [c(Cys-Glu-Pro-D-Nal-Arg-Trp-Gly-Cys) (HS015), c(Cys-Glu-Glu-D-Nal-Arg-Trp-Gly-Cys) (HS017), c(Cys-Glu-Gly-D-Nal-Arg-Trp-Gly-Cys) (HS023), c(Cys-Arg-His-D-Nal-Arg-Trp-Gly-Cys-Asp-Arg-Phe) (HS016), c(Cys-Glu-His-Gly-D-Nal-Arg-Trp-Cys) (HS018), c(Pro-Tyr-Arg-Cys-Glu-His-D-Nal-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp) (HS019), c(Cys-Glu-His-D-Phe(di-Cl)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp) (HS028), c(Cys-Glu-His-D-Phe(*p*-F)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp) (HS029) and c(Cys-Glu-His-D-Phe(*p*-NO_2)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp) (HS030)] were tested for their affinities on cells

expressing the MC_1, MC_3, MC_4 and MC_5 receptors.[275] Several of the more potent compounds like HS019, HS028, HS029 and HS030 were more potent against MC_4 receptors (0.95–21 nM) and about 20–200-fold less potent at the remaining three receptors. Some of the less potent compounds like HS017, HS023 and HS018 also showed higher affinity at the MC_4 receptors (K_i values 3640, 138 and 604 nM, respectively) in comparison to the other three receptors (K_i values 1700–>300,000 nM). Cyclic peptides like HS015 and HS016 did not show much selectivity. One of the analogues, c([Ac-Cys^{11}, D-Phe(dichloro)^{14}, Cys^{18}, Asp-NH_2^{22}]-β-MSH_{11-22}) (HS028) showed high affinity (K_i values against MC1, MC3, MC_4 and MC_5 receptors 60.1, 73.7, 0.95 and 211 nM, respectively) and high MC_4 receptor selectivity (80-fold) over the MC_3 receptor. HS028 antagonised α-MSH induced increase in cAMP production in transfected cells expressing the MC_3 and MC_4 receptors but it seemed to be a partial agonist for the MC_1 and MC_5 receptors. Chronic icv. administration of HS028 by osmotic minipumps significantly increased both food intake and body weight in a dose-dependent manner without tachyphylaxis for a period of seven days.[275]

Melanocortin MC_1 receptor ligands were identified by using a β-turn motif containing library of compounds.[276] Compounds **104** [D-Pro in the *i*+2 position and side chains of Nal(2′) and Trp in the *i*+1 and *i*+3 positions, respectively] and **105** [D-Lys in the *i*+2 position and Trp and Phe side chains in the *i*+1 and *i*+3 positions, respectively] displayed EC_{50} values of 42.5 and 63.4 μM, respectively.



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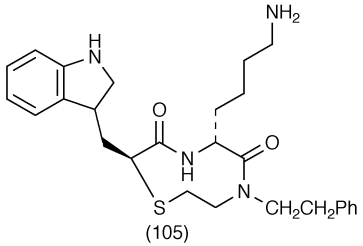

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4.14 MHC Class I and II Analogues. – Different aspects of peptide presentation by the major histocompatibility complex (MHC) class I and class II are reviewed.[277–281] Complete sequence and gene map of an MHC, a region on chromosome 6 essential to the immune system, was reported.[282] It was estimated that about 40% of the expressed genes may have immune system function. Sequence of the region that determines rapid allograft rejection in chickens, the chicken MHC, has also been reported.[283] This 92-kilobase region of the B locus contains only 19 genes, making the chicken MHC roughly 20-fold smaller than the human MHC. Crystal structures of MHC bound to glycopeptides were reported.[284,285] Conformational change accompanying peptide binding to class II MHC proteins were investigated by using gel filtration, dynamic light scattering, analytical ultracentrifugation and circular dichroism techniques.[286] Conformational variants of class II MHC/peptide

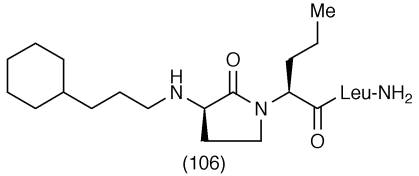
complexes induced by N- and C-terminal extensions of minimal peptide epitopes are also described.[287] The role of MHC class II molecules in susceptibility to diabetes has been discussed.[288,289]

An approach to design structurally modified, peptidase-resistant and biologically active analogues of human tumour antigen MAGE-1.A1 was reported.[290] This led to the design of a minimally modified analogue of MAGE-1.A1, [Aib^2, MeSer^8]MAGE-1.A1 [Glu-Aib-Asp-Pro-Thr-Gly-His-MeSer-Tyr], which was highly peptidase-resistant and bound to MHC and activated MAGE-1.A1-specific anti-melanoma CTLs. Other active compounds included MeGlu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr, Glu-Ala-Asp-Pro-Thr-Gly-D-His-Ser-Tyr and Glu-Ala-Asp-Pro-Thr-Gly-His-MeSer-Tyr. The interaction of Ac-1-11 (N-terminal peptide of myelin basic protein which induces experimental autoimmune encephalomyelitis) to MHC class II molecules was studied as a model system for therapeutic intervention in the autoimmune response seen in experimental autoimmune encephalomyelitis.[291] The synthetic random amino acid copolymer (Copolymer-1, Copaxone) which binds to various class II MHC molecules and inhibits the T cell responses to several myelin basic protein antigens has been shown to suppress experimental autoimmune encephalomyelitis, slow the progression of disability, and reduce relapse rate in multiple sclerosis. Further studies have shown that the polymer is also a T-cell receptor antagonist of the 82–100 epitope of the myelin basic protein.[292]

A tetrapeptide derivative EtOCO-Phe-Arg-Nva-Leu-NH_2 was reported previously to inhibit the binding of haemaglutinin HA307-319 peptide to purified DRB1*0101. Role of the peptide backbone was evaluated by studying *N*-methyl and $\psi(CH_2NH)$ analogues.[293] In comparison to EtOCO-Phe-Ala-Ala-Leu-NH_2 (IC_{20} 0.27 μM), only one of the *N*-methyl analogues, EtOCO-Phe-Ala-MeAla-Leu-NH_2, retained comparable potency (IC_{20} 0.32 μM). The other three analogues, EtOCO-MePhe-Ala-Ala-Leu-NH_2, EtOCO-Phe-MeAla-Ala-Leu-NH_2 and EtOCO-Phe-Ala-Ala-MeLeu-NH_2, were less potent (IC_{20} values 1.9–88 μM). Similarly, the reduced amide bond containing peptides EtOCO-Phe$\psi(CH_2NH)$Arg-Nva-Leu-NH_2, EtOCO-Phe-Arg-Nva$\psi(CH_2NH)$ Leu-NH_2 and EtOCO-Phe-Val$\psi(CH_2NH)$Nva-Leu-NH_2 were less potent (IC_{20}s 1.2–45 μM) than the parent tetrapeptides (IC_{20}s 0.001 μM). Except the lactam analogues (**106** and **107**, IC_{20}s 0.76 and 0.34 μM, respectively), the remaining *R* or *S*-series of lactam-containing analogues were also less potent (IC_{20}s 3.1–>50 μM). However, some similar analogues **108** (IC_{50} 25 nM) and **109** (IC_{50} 1.26 μM) were moderately potent. Incorporation of α-aza-amino acids in compound **106** was attempted to improve stability to enzymatic cleavages. Compound containing an azaleucineamide in place of Leu-NH_2 was much less potent (IC_{50} >50 μM). In comparison, replacement of the Leu-NH_2 by AzLeu-OEt or AzNle-OEt resulted in moderately potent compounds.[294] The AzNle compound **110** was equipotent to compound **106** (IC_{50}s 0.76 and 0.78 μM, respectively). Some other AzNle analogues (**111**, R = H, NHC(NH)-NH_2 or OH) were similar in potency (IC_{50}s 0.24, 0.21 and 0.16 μM, respectively).

The design and synthesis of pyrrolinone-peptide hybrid ligands (*e.g.* **112**) for

the rheumatoid arthritis-associated class II MHC HLA-DR1 protein are described.[295] The hybrids incorporate bispyrrolinones as tetrapeptide mimics for amino acids Val-Lys-Gln-Asn (residues 309–312) of the virus hemagglutinin peptide HA 306–318 (Pro-Lys-Tyr-Val-Lys-Gln-Asn-Thr-Leu-Lys-Leu-Ala-Thr). Bioaffinity studies revealed that hybrid ligand **112** bound the HLA-DR1 protein with affinity ($IC_{50} = 137$ nM) comparable to that of the native HA 306–318 peptide ($IC_{50} = 89$ nM). Non-peptidic high-affinity ligands for class I MHC proteins were obtained by substituting oligomers of (*R*)-3-hydroxybutanoate and (or) β-homoalanine for the central part (Phe-Val-Thr-Ile-Gly) of a HLA-B27-restricted T-cell epitope of viral origin (Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys) (**113**, X = NH or O).[296] Some of the analogues [Gly-Arg-Ala-(*R*-β-HAla)$_4$-Lys, Gly-Arg-Ala-(*S*-β-HAla-*R*-β-HAla)$_2$-Lys, Gly-



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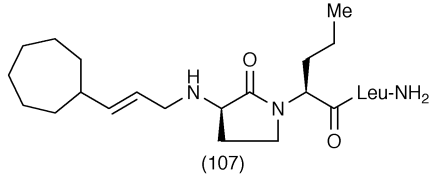

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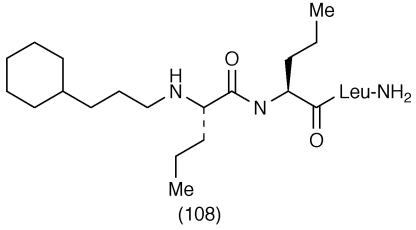

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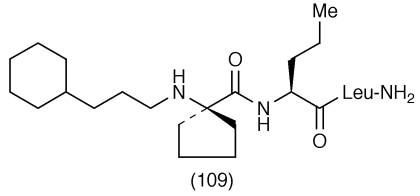

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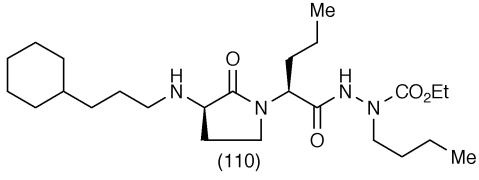

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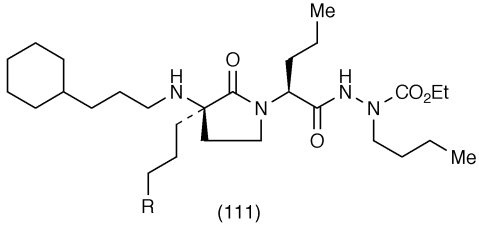

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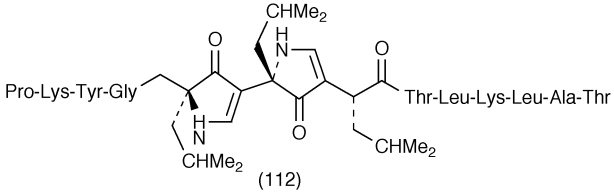

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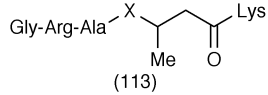

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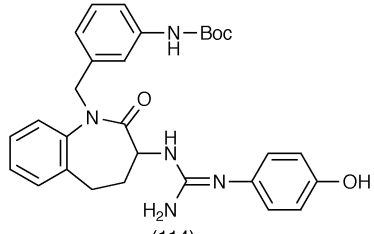
Arg-Ala-(*S*-β-HAla)$_4$-Lys] presented an affinity similar to that of the parent peptide.

4.15 Neuropeptide Y (NPY) Analogues. – Some biological aspects of NPY were reviewed.[297] Five NPY receptors belonging to the rhodopsin-like G-protein-coupled, 7-transmembrane helix-spanning receptors (Y_1-, Y_2-, Y_4-, Y_5- and Y_6-subtypes) were cloned previously. The Y_2-receptor subtype expressed in a human neuroblastoma cell line and in transfected CHO cells was characterised by using photoaffinity labelling and antireceptor antibodies.[298] Human NPY and the Gln^{34} to Pro^{34} mutant were characterised by CD spectroscopy.[299] The role of NPY in food intake has been discussed.[300,301] Central administration of a Y_1 receptor antagonist, [(Ile-Glu-Pro-Daba-Tyr-Arg-Leu-Arg-Tyr-NH_2)$_2$ cyclic (2,4′),(2′,4)-diamide] blocked NPY-induced feeding in food-deprived monkeys but had no effect on food intake in satiated monkeys.

Various non-peptide antagonists of NPY were reported.[302–306] Screening of the company (Shionogi) compound library led to a benzazepin-2-one derivative as a weak Y_1 receptor antagonist lead (IC_{50} 1.6 μM). Chemical modifications gave a potent NPY Y_1 antagonist (**114**; IC_{50} 43 nM), which had no affinity for NPY Y_2 and Y_5 receptors.[303] Additional SAR studies resulted in compound **115** (R = 6-benzothiazolyl) which competitively inhibited [I^{125}]peptide YY binding to Y_1 receptors in human neuroblastoma SK-N-MC cells (K_i = 5.1 nM). Many other analogues [R = 5-indolyl, 6-benzofuryl, 5-benzothienyl, 6-benzothienyl, 5-benzothiazolyl, 5-indazolyl or 5-benzoxazolyl] were about 2–5-fold less potent. Compound **115** inhibited the Y_1 receptor-mediated increase in cytosolic free Ca^{2+} concentration (SK-N-MC cells) and antagonised the Y_1 receptor-mediated inhibitory effect of peptide YY on



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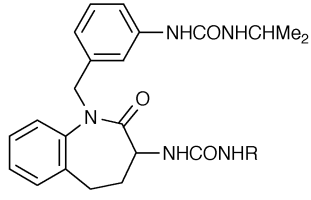

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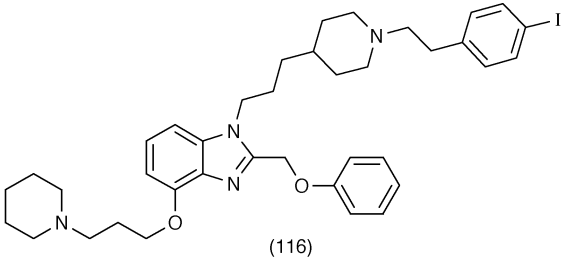

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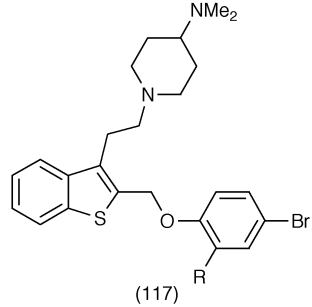

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gastrin-induced histamine release (rat enterochromaffin-like cells). The antagonist showed no significant affinity in many other receptor binding assays including Y_2, Y_4, and Y_5 receptors.[304] Other non-peptide antagonists of NPY include examples from benzimidazole and benzo[*b*]thiophene series of compounds.[305,306] The most potent and Y_1-selective compounds from these series were **116** and **117** (R = -CH_2OH, -CH_2OMe or -CN, K_i values 11–15 nM).

4.16 Opioid (Neuropeptide FF, Enkephalin, Nociceptin, Deltorphin and Dynorphin) Peptides. – Neuropeptides FF [Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2], AF [Ala-Gly-Glu-Gly-Leu-Ser-Ser-Pro-Phe-Trp-Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH_2], and SF [Ser-Leu-Ala-Ala-Pro-Gln-Arg-Phe-NH_2], originally identified on the basis of similarity to the molluscan neuropeptide Phe-Met-Arg-Phe-amide, have been claimed to have wide-ranging functions in the mammalian central nervous system, including pain modulation, opiate function, cardiovascular regulation, and neuroendocrine function. Neuropeptides FF gene was cloned from human, bovine, rat, and mouse, and the mRNA was shown to encode for all three of the identified peptides.[307] Biological results on a neuropeptide FF analogue [D-Tyr-Leu-MePhe-Gln-Pro-Gln-Arg-Phe-NH_2] have been reported.[308]

A number of new enkephalin analogues have been reported.[309–311] 2,6-Dimethyl-Tyr (D or L Dmt^1) analogues of Leu-enkephalin and Tyr-D-Arg-Phe-β-Ala-NH_2 were synthesised and their enzymatic stabilities (against aminopeptidase M and rat brain homogenate), *in vitro* activities and receptor binding affinities were compared with those of parent peptides. [L-Dmt^1]enkephalin exhibited 4-fold higher stability against aminopeptidase-M and possessed increased activities in guinea pig ileum (187-fold) and mouse vas deferens (131-fold) assays, and in rat brain receptor binding assays (356-fold at μ- and 46-fold at δ-receptors) as compared to enkephalin.[309] L-Dmt^1-D-Arg-Phe-β-Ala-NH_2 also exhibited increased activities in the ileum (46-fold) and vas deferens (177-fold) assays, and in the binding assays (69-fold at μ- and 341-fold at δ-receptors) as compared to the parent peptide. [D-Dmt^1]enkephalin and D-Dmt^1-D-Arg-Phe-β-Ala-NH_2 exhibited activities with diminished or lesser potency than the parent [L-Dmt] peptide in all assays.

Biological activities of [D-Ala^2, Leu^5]-enkephalyl-Arg (Tyr-D-Ala-Gly-Phe-Leu-Arg, dalargin) analogues were investigated in several *in vitro* tissue preparations.[310] [Ala^2]-dalargin was 19 times less potent than dalargin, and its pharmacological activity was peptidase sensitive. The ratio of δ/μ-activity for [Ala^2]-dalargin was 6.78 and K_B was 7.9 nM. [Met^5]-dalargin was equipotent to delargin in the myentric plexus, but was more potent in the vasa deferentia of hamster and mouse (K_B 5.5 nM). Dalarginamide was more potent and selective for μ opioid receptors than dalargin, whilst dalarginethylamide, though equipotent to dalarginamide in the myentric plexus, was more potent at δ opioid receptors (K_B 5.0 nM). [D-Phe^4]-dalarginamide and [D-$MePhe^4$]-dalarginamide were inactive. [$MePhe^4$]-dalarginamide possessed the highest potency and selectivity for μ-opioid receptors (δ/μ-activity ratio 0.00053; K_B = 2.6 nM).

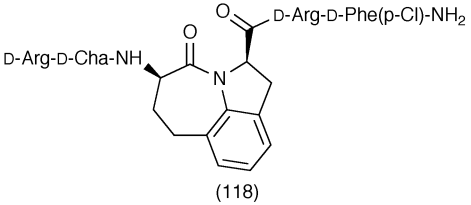
The synthesis and biological activity of two fragments of the opioid peptide biphalin (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr) (binding IC_{50} 2.6 and 1.4 nM at the δ and μ receptors, respectively), showed that Tyr-D-Ala-Gly-Phe-NH-NH<-Phe is the minimal fragment necessary to express equal affinities and the same biological activity profile (IC_{50} 15 and 0.74 nM at the δ and μ receptors, respectively) as the parent biphalin.[311] The replacement of N′-Phe with other L or D-lipophilic amino acids (D-Phe, Nle, D-Nle, Tyr and Trp) resulted in analogues still more potent at the μ receptor subtypes (IC_{50} 16–70 nM) than at the D receptor subtypes (IC_{50} 0.88–5.9 nM).

Opiate analogues containing β-methyl-2′,6′-dimethyltyrosine-L-tetrahydroisoquine-3-carboxylic acid (TMT-Tic), Dmt-Tic and N,2′,6′-trimethyltyrosine (Tmt) were reported.[312–314] (2*S*,3*R*)TMT-L-Tic-OH inhibited G protein activation (58% of basal) in membranes prepared from CHO cells transfected with cDNA of the human δ-opioid receptor (EC_{50} 0.72 nM), suggesting that the peptide was an inverse agonist at the human D-opioid receptor.[312] *N*-Methylation of Dmt-Tic analogues enhanced antagonism while *N*-piperidine-1-yl, *N*-pyrrolidine-1-yl, and *N*-pyrrole-1-yl were detrimental. Dmt-Tic-X (X = -$NHNH_2$, -$NHCH_3$, -NH-1-adamantyl, -NH-tBu, -NH-5-tetrazolyl) analogues had high affinities (K_i 0.16-1 nM) with variable affinities at different receptor subtypes to yield non-selective or weakly-selective analogues.[313] *N,N*-$(Me)_2$Dmt-Tic-NH-1-adamantane exhibited dual receptor affinities and potent antagonism (pA_2 9.06) with agonism (IC_{50} 16 nM). H-Dmt-HTic-OH (methylene bridge between C of Tic and carboxylate function) yielded a peptide with high affinity (K_i 0.85 nM) and antagonism (pA_2 8.85) without bioactivity. Dmt-Tic-Ala-X (X = -$NHCH_3$, -OCH_3, -NH-1-adamantyl, -NHtBu) exhibited high affinities (K_i = 0.06 to 0.2 nM), but only H-Dmt-Tic-Ala-NH-1-adamantane and H-Dmt-Tic-Ala-$NHBu^t$ yielded receptor antagonism (pA_2 9.29 and 9.16, respectively). Tyr-Tic-Phe-Phe-NH_2 analogues containing Tmt in place of Tyr^1 were synthesised.[314] Dmt-Tic-Phe-Phe-NH_2 and Dmt-Ticψ[CH_2NH]-Phe-Phe-NH_2 retained a mixed μ agonist/δ antagonist profile whereas Tmt-Tic-Phe-Phe-NH_2 was a partial μ agonist/δ antagonist and Tmt-Ticψ[CH_2NH]-Phe-Phe-NH_2 was a μ and δ antagonist. In the rat tail flick test, Dmt-Ticψ[CH_2NH]Phe-Phe-NH_2 given icv produced a potent analgesic effect (ED_{50} 0.04 μg; morphine ED_{50} 0.11 μg). It produced less acute tolerance than morphine but still a certain level of chronic tolerance. Unlike morphine, Dmt-Ticψ[CH_2NH]Phe-Phe-NH_2 produced no physical dependence upon chronic administration at high doses (up to 4.5 μg h^{-1}) over a 7-day period.[314]

The neuropeptide nociceptin [Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln] (an endogenous ligand for the orphan opioid-like receptor ORL-1, also known as orphanin FQ) and nocistatin (Thr-Glu-Pro-Gly-Leu-Glu-Glu-Val-Gly-Glu-Ile-Glu-Gln-Lys-Gln-Leu-Gln), processed from the same 176 amino acids precursor prepronociceptin, have been reported previously. Many other biological studies on nociceptin and analogues like [Phe^1ψ(CH_2-NH)Gly^2]nociceptin(1-13)-NH_2 have been reported.[315–320] For example, given intrathecally (0.1 to 10 nmol), nociceptin produced dose-dependent inhibition of the tail-flick response in both non-diabetic and diabetic

mice. The antinociceptive effect of nociceptin was not antagonised by pre-treatment with selective μ-, δ- or κ-opioid receptor selective antagonists.[316] The effects of a range of nociceptin C-terminal truncated fragments and [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin (1-13)-NH_2 were investigated on nociceptin receptor binding, glutamate release from rat cerebrocortical slices, inhibition of cAMP accumulation in CHO cells expressing nociceptin receptor and electrically-evoked contractions of the rat vas deferens.[317] [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)-NH_2 was as potent as nociceptin(1-13)-NH_2. The order of potency for nociceptin fragments to inhibit cAMP accumulation was nociceptin-NH_2 $\geqslant$ nociceptin = nociceptin(1-13)-NH_2 > nociceptin(1-12)-NH_2 >> nociceptin(1-11)-NH_2. [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)-NH_2 was a full agonist with a pEC_{50} value of 8.65. Nociceptin-NH_2 and [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)-NH_2 both inhibited K^+ evoked glutamate release. In rat vas deferens nociceptin inhibited electrically evoked contractions with a pEC_{50} of 6.63. Although [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)-NH_2 displayed a small but consistent agonist activity, it acted as a competitive antagonist (pA_2 6.76) in the rat vas deferens. In the Freund's adjuvant-induced rat model of arthritis, both nociceptin and [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)-NH_2 act as anti-opioid peptides.[319]

Ligands for ORL1 and κ-opioid receptors were identified by screening a synthetic peptide combinatorial library (2×10^7 β-turn-constrained peptides) in binding assays on four structurally related receptors, the opioid receptor-like ORL1 and the human opioid receptors μ, δ, and κ. One peptide (**118**) displayed comparable affinity and partial agonist activity toward all four receptors.[321] Another peptide (**119**) showed selectivity for the ORL1 receptor and displayed



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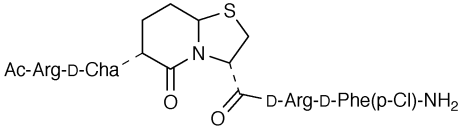

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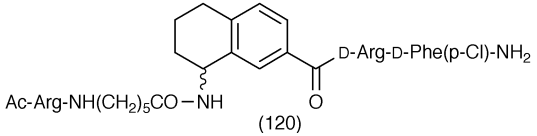

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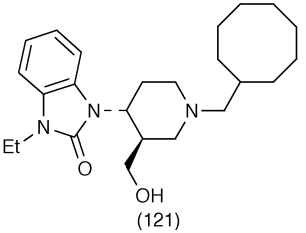

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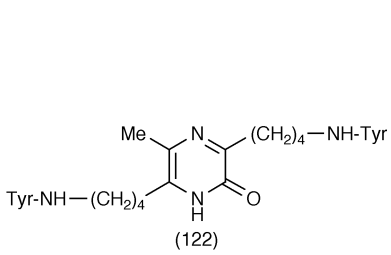
antagonist activity at ORL-1 and agonist activity at opioid receptors. Other peptides like **120** were more potent at the κ receptors. Non-peptide ligands for the ORL-1 receptor were identified starting from a chemical library lead which showed high affinity for ORL-1 (IC_{50} 200 nM) but poor selectivity over μ- and κ-receptors.[322] The benzimidazol-2-one derivative **121** (J-113397) binds to ORL-1 (IC_{50} 2.3 nM) with a selectivity greater than 600-fold over μ- (IC_{50} 2200 nM), κ- (IC_{50} 1400 nM), and δ-receptors (IC_{50} >10000 nM)and inhibits ORL-1.

Piperazine, piperazinone, piperidine and pyrazinone-based opiate ligands were reported.[323–326] Pyrazinone derivatives like **122** displayed higher μ-opioid receptor binding affinity (K_i (61 nM) and selectivity (K_i μ/δ 31).[323] 4-Benzyl-piperazine derivatives (**123**, R = -CH_2-O-CH_2-Ph, -Me or -CH_2OH) were nearly equipotent at the δ receptor (IC_{50} 11–38 nM) whereas **123** (R = -CH_2Ph) was much less potent. At the μ receptor, **123** (R = -Me or -CH_2OH) displayed an IC_{50} of 3–8 μM, but **123** (R = -CH_2-O-CH_2-Ph or -CH_2Ph) were less potent (IC_{50}s 26–46 μM).[324] Extensive modification of the piperazine nucleus in SNC80 (a non-peptidic receptor agonist reported earlier) led to several compounds like **124**, **125** (R = H or benzyl) and **126** (R = H or benzyl) which strongly bound to the δ receptor (K_i 1–12 nM) but the binding affinities of these compounds for the μ and κ receptors were negligible, indicating δ opioid receptor subtype selectivity.[325] Racemic piperidine derivative **127** showed good affinity and selectivity for the δ-receptor (K_i values for μ, δ and κ receptors 1212, 11.9 and 3284 nM, respectively).[326] The corresponding *trans* isomer showed K_i values for μ, δ and κ receptors 1589, 126 and 8695 nM, respectively. Functionally, **127** behaved as an agonist at the δ-receptor with no measurable stimulation of either the μ or κ receptor subtypes and was devoid of any measurable amount of antagonist activity for any opioid receptor.

Intracellular recording was used to study the effects of eight opioid tetrapeptides with similar amino acid sequences, namely endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2), endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), morphiceptin (Tyr-Pro-Phe-Pro-NH_2), hemorphin-4 (Tyr-Pro-Trp-Thr), Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2), Tyr-Pro-Trp-Gly-NH_2, Tyr-D-Arg-Phe-Sar and Tyr-D-Arg-Phe-Lys-NH_2, on neurones of the rat locus coeruleus, using a submerged brain slice preparation.[327] All the tetrapeptides inhibited the spontaneous firing of all neurones of the locus coeruleus tested. Higher concentrations also caused hyperpolarization of the neurones and a reduction in input resistance. These inhibitory effects were rapidly and completely reversed by D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2, a selective μ-opioid receptor antagonist. The antinociceptive effects of endomorphins are thought to be mediated through distinct μ_1 and μ_2 subtypes of μ-opioid receptor.[328]

Opioid receptor affinities, selectivity and analgesic activity in mice (s.c. injection) of dermorphin and deltorphin analogues β-O- and α-C-glycosylated on the C-terminal amino acid are reported.[329] The glycosylated analogues [Tyr-D-Ala-Phe-Asp-Val-Val-[βGlc(Ac)$_4$]Ser-NH_2, Tyr-D-Ala-Phe-Asp-Val-Val-(βGlc)Ser-NH_2, Tyr-D-Ala-Phe-Gly-Tyr-Pro-[βGlc(Ac)$_4$]Ser-NH_2, Tyr-D-Ala-Phe-Gly-Tyr-Pro-(βGlc)Ser-NH_2, Tyr-D-Ala-Phe-Gly-Tyr-Pro-[αGal(Ac)$_4$]Ser-NH_2,



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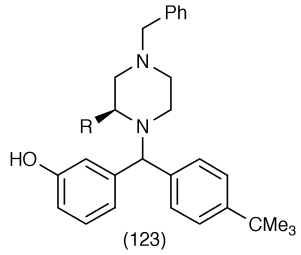

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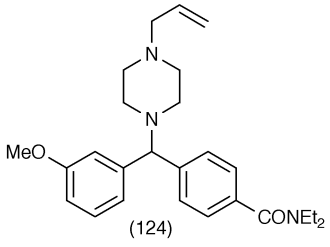

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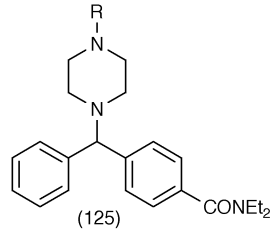

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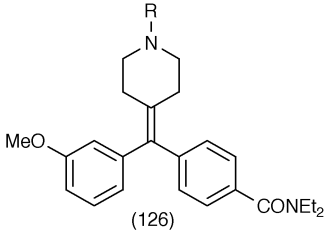

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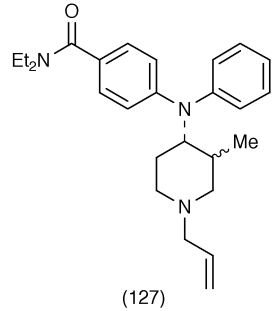

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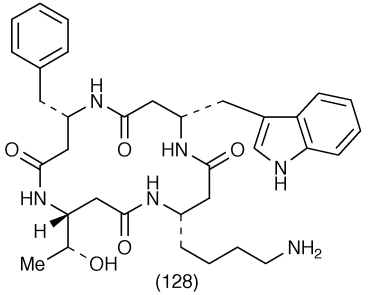
Tyr-D-Ala-Phe-Gly-Tyr-Pro-(αGal)]Ser-NH_2, Tyr-D-Ala-Phe-Asp-Val-Val-[αGal $(Ac)_4$]Ser-NH_2 and Tyr-D-Ala-Phe-Asp-Val-Val-(αGal)Ser-NH_2] were more potent as analgesic agents than the parent peptides. The nature of the dynorphin A processing enzyme in the brain was investigated.[330] The enzyme, a thiol-sensitive metalloprotease, had a neutral pH optimum. Specific inhibitors of other metallopeptidases such as enkephalinase (enkephalin generating neutral endopeptidase) did not inhibit the dynorphin A processing activity. In contrast, specific inhibitors of angiotensin converting enzyme inhibited the activity. A chimeric peptide, JVA-901 (Ac-Tyr-Lys-Trp-Trp-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2) (K_i values for κ, μ and δ receptors 19.8, 251 and 5320 nM, respectively) was obtained by combining the N-terminal acetylated derivative of the tetrapeptide Ac-Tyr-Lys-Trp-Trp-NH_2 (reported earlier) (K_i values for κ, μ and δ receptors 1367, 3590 and 3478 nM, respectively) with residues 5-11 of [D-Ala^8]dynorphin A(1-11)-NH_2 (Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2). In comparison to [D-Ala^8]dynorphin A(1-11)-NH_2 (K_i values for κ, μ and δ receptors 0.19, 1.97 and 12.2 nM, respectively), JVA-901 was less potent at all the receptor subtypes.[331] However, in comparison to the tetrapeptide, it retained significant activity at the κ receptor subtypes.

4.17 Somatostatin Analogues. – Work on the role of somatostatin and its receptors in various forms of cancer, gastrointestinal tract and pathophysiology of rheumatoid arthritis was published.[332–346] Biological results on a cytotoxic analogue of somatostatin consisting of 2-pyrrolinodoxorubicin linked covalently to the octapeptide RC-121 (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Cys-Thr-NH_2) were reported. In nude mice bearing xenografts of PC-3 human androgen-independent prostate cancer, administration of the analogue inhibited tumour growth (62–74% decrease in tumour volume and 61–71% reduction in tumour weight after 4–7 weeks).[347] Results on clinical studies using long-acting, depot injection form of somatostatin analogues like octreotide (sandostatin) and vapreotide were reported.[348–351] Work on different receptor subtypes of somatostatin including genomic structure, transcriptional regulation and localisation in various cell types was published.[352–356] Ligands for the orphan somatostatin-like receptor 1 were obtained from rat brain extracts.[357] Partial peptide sequencing of the two peptides with high agonist activity [Met-Leu-Arg-X-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-X-X-Gln-X and Asp-Phe-Asp-Met-Leu-Arg-X-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-X-X-X-X] revealed that one peptide was identical with the neuropeptide melanin concentrating hormone (Human MCH = Asp-Phe-Asp-Met-Leu-Arg-Cys-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Gln-Val) and the other represented a truncated version of MCH. *Xenopus oocytes* expressing the MCH receptor responded to nM concentrations of synthetic MCH.

SAR studies (K_i values at hsst-1, -2, -3, -4 and -5 receptors, respectively) on cyclic hexapeptides related to c(Pro^6-Phe-D-Trp-Lys-Thr-Phe^{11}) [K_i >1000, 5.1, 129, >1000 and 20 nM] were reported.[358] The Pro^6 was replaced with N-substituted glycine residues like Nphe (*N*-benzylglycine) [K_i >1000, 6.9, 253, >1000 and 100 nM], (*S*) βMeNphe [(*S*)-N-[(α-methyl)benzyl]glycine]-[K_i >1000, 29, 797, 987 and 87 nM], (*R*) βMeNphe [(*R*)-*N*-[(α-methyl)benzyl]-glycine] [K_i >1000, 2.3, 425, >1000 and 33 nM] or Nnal [*N*-naphthylmethyl]-glycine) [K_i >1000, 32, >1000, >1000 and 830 nM]. The incorporation of Nphe in place of Pro^6 and Nal in place of Phe^7, [Nphe^6, Nal^7 analogue], led to a compound which binds potently to the hsst2 and has increased selectivity towards this receptor (weaker binding to hsst3 and hsst5 receptors) [K_i >1000, 3.5, 204, >1000 and 54 nM ($hsst_5/hsst_2 = 15.2$ and $hsst_3/hsst_2 = 57.1$)] compared with the parent compound. The K_i values for the corresponding [Nphe^6, Nal^{11}] analogue were >1000, 3.7, 88, >1000 and 25 nM ($hsst_5/hsst_2 = 6.7$ and $hsst_3/hsst_2 = 23.8$). The analogues with β-methyl chiral substitutions in the aromatic peptoid side chain and Nal in position 7 or 11 bind effectively to the hsst2 and hsst5 receptors {K_i values at hsst-1 to -5 receptors, respectively: [(*R*)βMeNphe^6, Nal^7] >1000, 7.1, 380, >1000 and 10.3 nM; [(*S*)βMeNphe^6, Nal^7] >1000, 3.1, 198, >1000 and 20.3 nM; [(*R*)βMeNphe^6, Nal^{11}] >1000, 2.7, 125, >1000 and 10.7 nM; [(*S*)βMeNphe^6, Nal^{11}] >1000, 7.25, 267, >1000 and 27.6 nM}. Conformations of many of these analogues were studied using ^{1}H-NMR in DMSO and computer simulations involving distance geometry and molecular dynamics simulations.[359] The results indicate that the [Nphe^6, Nal^7] and [Nphe^6, Nal^{11}] compounds adopt a preferred

backbone conformation which can be described as folded about residues 7 and 10.

A cyclic tetrapeptide (**128**) containing β-amino acids was synthesised as a somatostatin ligand.[360] Although the peptide showed affinity for all the five receptors of somatostatin, it was much less potent than somatostatin and octreotide. Screening of heterocyclic β-turn mimetic libraries (based upon the Trp-Lys motif found in the turn region of somatostatin) against a panel of the five cloned human somatostatin receptors ($hSST_1$-$hSST_5$) led to somatostatin receptor ligands like **129**–**131** which bound to the five receptor subtypes.[361] Compound **129** was relatively more selective for the $hSST_2$ receptor subtype (IC_{50} 158 nM) and **130** showed higher affinity against $hSST_3$ and $hSST_5$ subtypes (IC_{50} 135 and 120 nM, respectively). The turn mimetic **131** was more potent at the $hSST_5$ receptor subtype (IC_{50}s 501, 1585, 3090, 1047 and 87 nM, respectively, against hSST1, -2, -3, -4 and -5 receptor subtypes). Many proline (**132**, R = Ph or 3-indolyl; R = Ph and MeLys or D-Lys in place of Lys) (**133**) or



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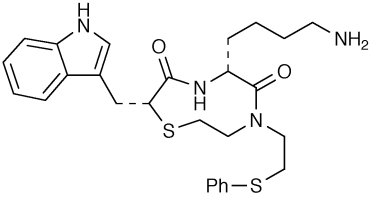

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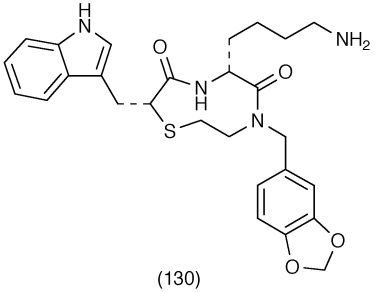

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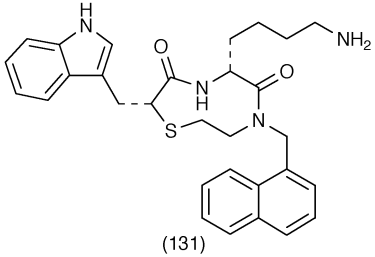

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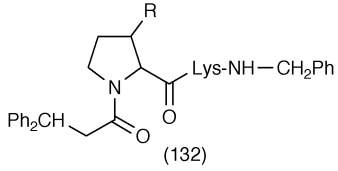

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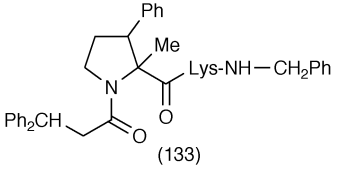

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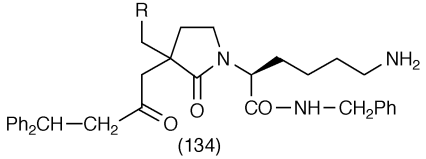

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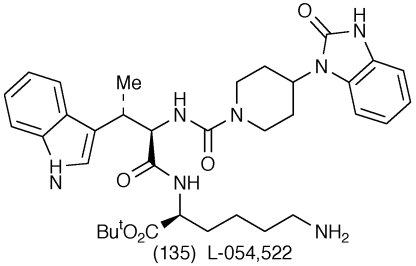
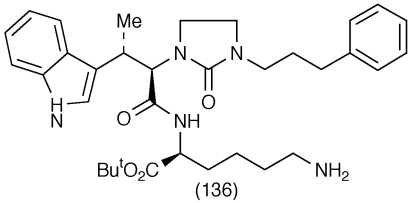
γ-lactam derivatives (**134**, R = Ph or 3-indolyl) bearing either an aryl group such as a phenyl or 3-indolyl in position 3 of the proline moiety or on the 3-methyl chain of the γ-lactam skeleton were prepared as non-peptide mimics of somatostatin/sandostatin.[362] Binding assays showed that these proline and γ-lactam derivatives had weak affinity (IC_{50} 7–64 μM) for somatostatin receptors on membranes of rat cerebral cortex.

Non-peptide, receptor selective agonist analogues of somatostatin were reported.[363] Modification of the urea group in **135** (reported earlier) like cyclisation between both urea nitrogen atom *via* a 2 carbon linker resulted in compounds with better oral bioavailability. For example, compound **136** (K_i $hSSTR_2$ 3.6 nM) showed about 5% oral bioavailability and compound **137** displayed about 15% oral bioavailability. Compound **138** (K_i $hSSTR_2$ 8.5 nM, K_i $hSSTR_3$ 8.4 μM and K_i $hSSTR_5$ 1.1 μM) was the most orally bioavailable compound of the series (64%).



(135) L-054,522



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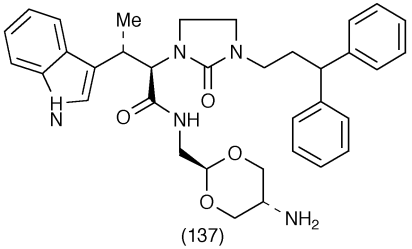

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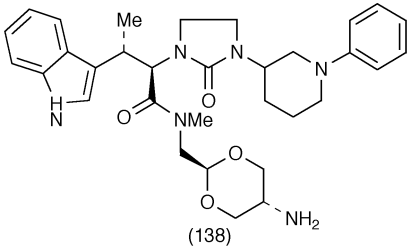

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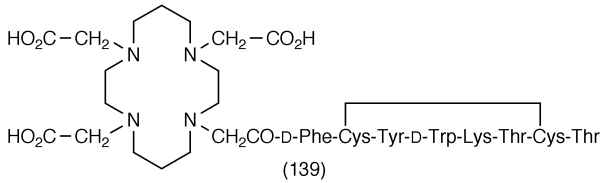
Several antagonists of somatostatin were reported.[364] Further SAR studies on one of the antagonists, Nal-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Nal-NH_2 (K_i values 348, 81, 171, >1000 and 524 nM against $hSST_1$, $hSST_2$, $hSST_3$, $hSST_4$ and $hSST_5$, respectively), are reported. Several of the analogues, like Trp-c[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2, Phe(*p*-F)-c[D-Cys-Pal-D-Trp-Lys-Tle-Cys]-Nal-NH_2, Cpa-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-Nal-NH_2, retained significant antagonist activity at $hSST_2$, $hSST_3$ and $hSST_4$ receptors but were much less potent at $hSST_1$ and $hSST_5$ receptors. Compounds like Nal-c[D-Cys-Pal-D-Trp-Lys-Ile-Cys]-Nal-NH_2, Phe(*p*-F)-c[D-Cys-His-D-Trp-Lys-Val-Cys]-Phe(*p*-F)-NH_2 and Phe(*p*-F)-c[D-Cys-Pal(2)-D-Trp-Lys-Val-Cys]-Phe(*p*-F)-NH_2 retained significant activity at $hSST_1$ and $hSST_3$ receptors and were less potent at $hSST_2$, $hSST_4$ and $hSST_5$ receptors. Compounds like Cpa-c[D-Cys-Phe-D-Trp-Lys-Thr-Cys]-Nal-NH_2, Cpa-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-Nal-

NH_2, Bpa-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Bpa-NH_2 and Iph-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Iph-NH_2 were more potent at $hSST_2$ and $hSST_5$ or $hSST_2$, $hSST_3$ and $hSST_5$ and less potent at the remaining receptor subtypes. One of the $hsst_2$ receptor antagonists, PRL-2903 [Phe(*p*-F)-c(D-Cys-Pal-D-Trp-Lys-Tle-Cys)-Nal-NH_2 (injected i.v. at maximal effective doses) increased gastric acid secretion by 2–10-fold from basal values within 30 min in urethane-anaesthetised rats.[365]

Labelled (^{64}Cu- or ^{111}In-) somatostatin analogues have been synthesised for use in tumour diagnosis and therapy.[366–368] These analogues (*e.g.* **139** and **140**) retain the biological activities associated with the parent peptide.



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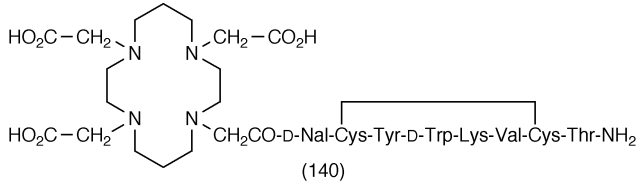

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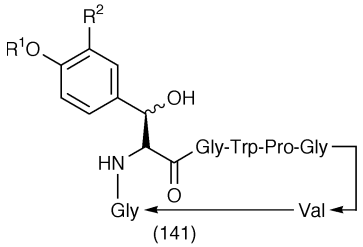
4.18 Tachykinin (Substance P and Neurokinins) Analogues. – Total synthesis of the cyclic heptapeptide **141** (R^1 = Me, R^2 = 2-methyl-butyl), a substance P antagonist 70 times more potent than the naturally occurring cyclic peptide WIN66306 (R^1 = H, R^2 = prenyl), established the stereochemistry of the β-OH group in the isoprenyltyrosine moiety in **141** as *R*.[369] Chemical modifications were carried out on the dual NK_1/NK_2 ligand Z-Gly-Leu-Trp-OBzl$(CF_3)_2$ with a view to optimising affinities for both NK_1 and NK_2 receptors.[370] Replacement of the Gly residue by other amino acids increased affinities for NK_1/NK_2 receptors or induced selectivity for the NK_1 receptor. Several analogues (**142**, n = 2 or 5 or Aib in place of -$NH(CH_2)n$CO-) were the most potent at the NK_1 receptors (K_i 2–8 nM at NK_1 and 160–800 nM at the NK_2 receptors). An analogue of **142** (phenylglycine in place of -$NH(CH_2)n$CO-) was the most selective peptide for the NK_1 receptors (K_i 25 nM at NK_1 and >5000 nM at the NK_2 receptors). Chemical modifications on a conformationally constrained tryptophan based NK_1 competitive antagonist led to hNK_1 selective ligands like **143**, **144** and **145**. Free indolylmethyl and Z carbamate groups were shown to be essential for NK_2 receptor affinity.[371]

To study the steric and electrostatic requirements for molecular recognition at position 6 of a NK_1 receptor agonist, [Nle^{10}]neurokinin A(4-10), two series of peptide analogues, (a) *p*-substituted analogues, [*p*-X-Phe^6, Nle^{10}]NKA-(4-10), where X = F, Cl, Br, I, NH_2, NO_2, and (b) [X^6, Nle^{10}]NKA(4-10)



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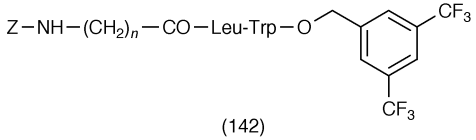

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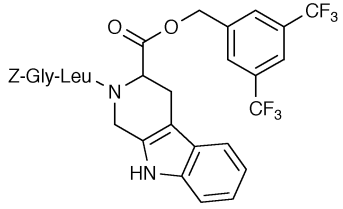

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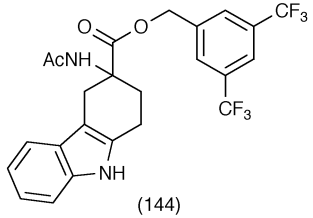

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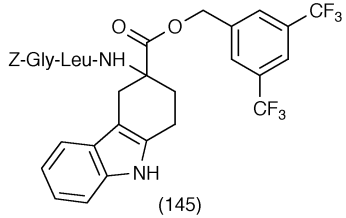
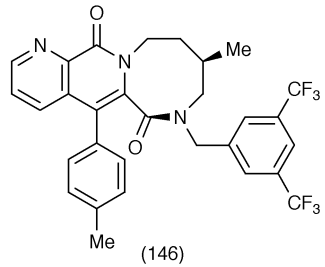


(145)

(X = D-Phe, Trp or Cha), were synthesised.[372] Competition binding experiments with [H^3]NKA were performed using cloned human NK_2 receptors expressed in CHO cells. Antagonistic and agonistic properties of the analogues were studied using an *in vitro* functional assay with hamster tracheal rings. The rank order of potency of agonists was [Nle^{10}]NKA(4-10) (K_i 12.7 nM) ([*p*-F-Phe^6, Nle^{10}]NKA(4-10) (K_i 44 nM) > [*p*-NH_2-Phe^6, Nle^{10}]NKA(4-10) (K_i 156 nM) > [*p*-Cl-Phe^6, Nle^{10}]NKA(4-10) (K_i 1422 nM) ≈ [*p*-NO_2-Phe^6, Nle^{10}]NKA(4-10) (K_i >2000 nM) > [Trp^6, Nle^{10}]NKA(4-10) (K_i >2000 nM). Size and planarity of the aromatic side chain were important for the biological activity, whereas electron-donating and electron-withdrawing properties of the *p*-substituent were less important.

A number of non-peptide antagonists acting at the NK_1, NK_2 or NK_3 receptors were reported.[373–375] Examples include compounds **146** (NK_1-selective), **147** (NK_1/NK_2-selective) and **148** (NK_3-selective).

4.19 Thyrotropin-releasing Hormone Analogues. – An analogue of TRH (**149**, JTP-2942) competed with [H^3]-Me-TRH for the binding sites in rat brain *in vitro*, and its inhibitory effect was approximately 17 times less than TRH (K_i values 673 and 39.7 nM, respectively).[376] Intravenous injections of **149** (0.3–3 mg kg^{-1}) and TRH (3 and 10 mg kg^{-1}) produced a significant reduction of [^{3}H]-Me-TRH binding sites in rat brain. Although the decrease by TRH was maximal 10 minutes after the injection and declined rapidly with time, the



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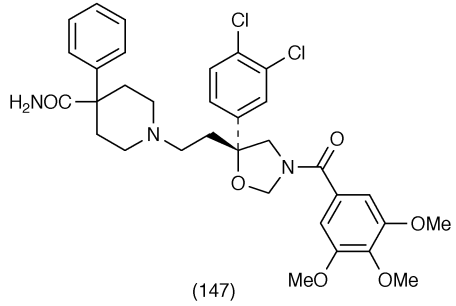

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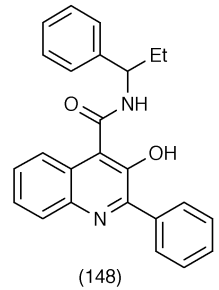
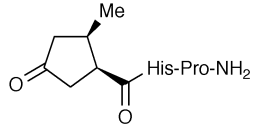
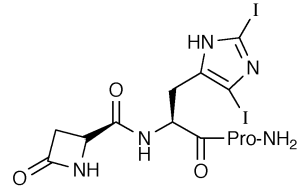


(148)

decrease by **149** (1 and 3 mg kg^{-1}) was maximal after 30 minutes and it lasted for 120 minutes ($t_{1/2}$ of 19.3–29.9 min). Thus **149** appears to exert potent and sustained occupation of brain TRH receptors under *in vivo* conditions. Another analogue of TRH (**150**) with substitutions at the NH_2-terminus and imidazole ring was shown to retain the neuroprotective action of TRH-like compounds while decreasing their autonomic, analeptic, and endocrine effects.[377] Rats administered with **150** (1.0 mg kg^{-1}, iv) 30 min after lateral fluid percussion brain injury showed marked improvement in motor recovery compared with vehicle-treated controls. Treatment of mice subjected to moderate controlled cortical impact brain injury improved both motor recovery and cognitive performance in a water maze place learning task. In injured rats, no autonomic or analeptic effects were observed with **150** and endocrine effects were significantly reduced, in contrast to those found with some other N-terminal-substituted TRH analogues.



(149) JTP-2942



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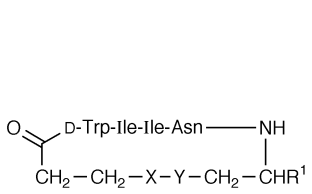
4.20 Vasopressin and Oxytocin Analogues. – *4.20.1 Oxytocin Peptide and Non-peptide Analogues.* Analogues of oxytocin containing D-Trp, 2-amino-1,2,3,4-tetrahydronaphthalene-1-carboxylic acid (Atc) or 1,2,3,4-tetrahydro-β-carboline-1-carboxylic acid (Car) with *R* or *S* configurations in position 2 were

synthesised, and their receptor bindings were tested on isolated guinea-pig uterus, rat liver and rat kidney inner medulla plasma membranes.[378] The binding to the oxytocin receptor was somewhat decreased for the Atc isomers and dramatically decreased for both *R*- and *S*-Car, while the D-Trp-containing analogue displayed a relatively high receptor affinity. However, the V_1 receptor affinities were almost the same as those of the parent peptide for the Car-containing analogues and dramatically decreased for the S-Atc substituted analogue, which has a relatively high oxytocin/V_1 receptor selectivity of 44.5. Based on the earlier reported antagonist of oxytocin, [D-Tyr(Et)^2, Thr^4, Orn^8]oxytocin (Atosiban), analogues were synthesised in which the C-terminal Pro-Orn-Gly-NH_2 and some of the amino acids in the ring were replaced.[379] Several analogues [**151**, R^1 = -CO-MeOrn-NH_2, -$CONH_2$, -$(CH_2)_4$-NH_2 and -$(CH_2)_3$-NH_2] [X and Y = -S-CH_2- or -CH_2S-] were synthesised and shown to be antagonists of oxytocin. Parallel and antiparallel heterodimers (*e.g.* **152** and **153**) were synthesised that combine into a single molecule oxytocin and the vasopressin V_2-antagonist d$(CH_2)_5$[D-Ile^2, Ile^4]-Arg vasopressin. Biological studies revealed that both the parallel and antiparallel chimeras lack pressor activity, have low uterotonic activity, and have diuretic activities comparable to that of the monomeric V_2-antagonist.[380] Sodium excretion was dependent on experimental conditions. With a 4% water load, both chimeras display effects similar to that of an equimolar mixture of oxytocin and V_2-antagonist, *i.e.* lower sodium excretion than that resulting from administration of oxytocin alone but higher than that when V_2-antagonist was administered alone. However, when no water load was used, the parallel chimera proved to be more effective in promoting sodium excretion than either oxytocin alone or an equimolar mixture of oxytocin and V_2-antagonist.



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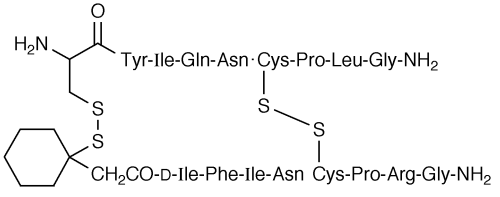

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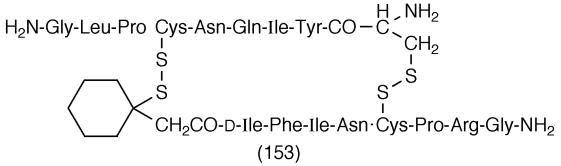

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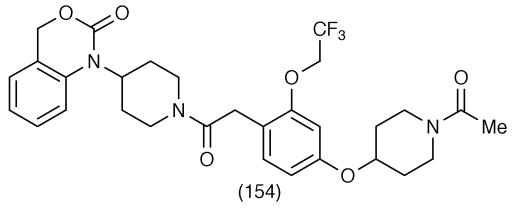
SAR studies on a non-peptide oxytocin antagonist reported earlier (L-371,257; K_i = 9.3 nM) have led to the identification of a related series of compounds containing an *o*-trifluoroethoxyphenylacetyl core (*e.g.* **154**, L-374,943; K_i 1.4 nM) which are orally bioavailable and have significantly improved potency *in vitro* and *in vivo*.[381] In a functional assay using isolated



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rat uterine tissue, **154** was shown to be a competitive antagonist of oxytocin (pA_2 9.2). *In vivo*, the compound had about 20% oral bioavailability.

4.20.2 Vasopressin Peptide and Non-peptide Analogues. Solid phase synthesis of [Arg^8]-vasopressin methylenedithioether, an analogue containing an extra methylene group between the two sulfur atoms of Cys^1 and Cys^6 is described.[382] The uterotonic *in vitro* (1.4 IU mg^{-1}), pressor (55.3 IU mg^{-1}), and antidiuretic (46.5 IU mg^{-1}) activities of the compound were reduced in comparison to [Arg^8]-vasopressin by one order of magnitude. Analogues of Arg-vasopressin in which the Phe^3 residue was replaced by thienylalanine, Cha, Nle, Leu, Nva, Val, α-aminobutyric acid, Ala, Gly, hPhe, Tyr, Trp, Nal(2), Pro, 2-aminotetraline-2-carboxylic acid, Ser, Thr, Gln, Asp, Glu, Arg, Lys and Orn were evaluated for agonistic and antagonistic activities in *in vivo* antidiuretic (V_2-receptor) and vasopressor (V_{1a}-receptor) assays and in *in vitro* oxytocic assays.[383] The results indicated that the aliphatic amino acids Cha, Nle, Leu, Nva and Val were well-tolerated at position 3 with retention of high levels of antidiuretic activity and significant gains in both antidiuretic/vasopressor and antidiuretic/oxytocic selectivities relative to Arg-vasopressin. [Thi^3]Arg-vasopressin was a more potent antidiuretic and oxytocic agonist than Arg-vasopressin and was equipotent with Arg-vasopressin as a vasopressor agonist. The antidiuretic, vasopressor and oxytocic potencies of the remaining peptides were significantly reduced.

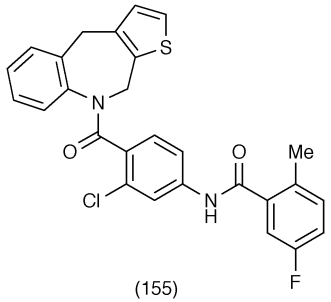
Analogues of Arg-vasopressin, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4]Arg-, d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4]Arg-, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Tyr-$NH_2{}^9$]Arg- and d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4, Tyr-$NH_2{}^9$]Arg-vasopressin, were evaluated for agonistic and antagonistic activities in *in vivo* antidiuretic, vasopressor and in *in vitro* oxytocic assays.[384] Six of the hypotensive peptides, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Eda^9]Arg-, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg-$NH_2{}^9$]Arg-, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Eda^9]Arg-, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Eda^9←Tyr^{10}]Arg-, d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, desGly^9]Arg- and d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Lys^8]Arg-vasopressin (Eda = ethylenediamine) were more potent than d$(CH_2)_5$[D-Tyr $(Et)^2$, Arg^3, Val^4]Arg-vasopressin. d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Arg^7, Eda^9←Tyr^{10}]Arg-vasopressin, a radioiodinatable ligand containing a retro-Tyr at the C-terminal end, was ten times more potent than d$(CH_2)_5$[D-$Tyr(Et)^2$, Arg^3, Val^4, Tyr-$NH_2{}^9$]Arg- and d$(CH_2)_5$[D-$Tyr(Et)^2$, Lys^3, Val^4, Tyr-$NH_2{}^9$] Arg-vasopressin.

Photoactivatable and fluorescent ligands were developed for labelling cells

expressing the human V_{1a} receptor subtype and to identify binding domains.[385,386] Fluoresceinyl and rhodamyl groups were coupled by an amide link to side-chain amino groups at positions 1, 6, and 8 of vasopressin peptide antagonists through different positions on the fluorophore, to give tetraethylrhodamyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2, 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Lys(5-carboxyfluoresceinyl)-Pro-Arg-NH_2, 4-HOPh-$(CH_2)_2$-CO-D-Tyr(Me)-Phe-Gln-Asn-Lys(5- or 6-carboxytetramethylrhodamyl)-Pro-Arg-NH_2, 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5- or 6-carboxyfluoresceinyl)-NH_2, and 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5- or 6-carboxytetramethylrhodamyl)-NH_2. The closer to the C-terminus the fluorophore, the higher the affinities of the fluorescent derivatives for the human vasopressin V_{1a} receptor transfected in CHO cells. 4-HOPh$(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5-carboxytetramethylrhodamyl)-NH_2 had a K_i value of 70 pM as determined by competition experiments with [I^{125}]-4-ROPhCH_2CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 and showed a good selectivity for human V_{1a} receptor versus human oxytocin (K_i 1.2 nM), human vasopressin V_{1b} (K_i (27 nM), and human vasopressin V_2 (K_i > 5000 nM) receptor subtypes.

Non-peptide antagonists of vasopressin (including V_{1a}-receptor specific antagonists, OPC 21268 and SR 49059, V_2-receptor specific antagonists, SR 121463 A and VPA 985, and combined V_{1a}/V_2-receptor antagonists, OPC 31260 and YM 087) were reviewed.[387] The antagonists are primarily based around benzazepine and benzodiazepine structures and many of these are more potent at the V_2 receptor subtypes.[388–395] Examples of some of the antagonists include compounds **155**–**160**. Compound **157** (IC_{50}s 200 and 2.9 nM, respectively, against V_1 and V_2 receptor subtypes) when administered orally to rat (1–10 mg kg^{-1}) showed an ~18-fold increased urine volume at the highest dose in comparison with control rat.[390] The IC_{50} values of **158** and **159** against V_2 receptor were 0.772 and 0.216 nM, respectively.[392] The ED_{300} values (dose required to increase three times the urine volume of the control rats; oral administration) of **158** and **159** were 0.22 and 0.78 mg kg^{-1}, respectively. The V_2 selective antagonist OPC-41061 (**160**) significantly increased urine volume 2 hour after oral administration (3-fold increase achieved at a dose of 0.54 mg kg^{-1}).[393]

4.21 Miscellaneous (Insulin, Scavenger Receptor Ligands, Chemokine Receptor Antagonists, N-type Calcium Channel Blockers, Urotensin and Cytotoxic Peptides). – Structure, function and design aspects of insulin analogues were reviewed.[396] Work on the class A and B type scavenger receptors has been published.[397,398] Screening against human embryonic kidney (HEK-293) cells transfected with scavenger receptor subtypes led to small molecule antagonists like **161**. Ligands for the CCR_1 receptor (MIP-1α and RANTES; implicated in a number of chronic inflammatory diseases like multiple sclerosis and rheumatoid arthritis) were identified by high throughput screening. A series of 4-hydroxypiperidines inhibited the binding of MIP-1α and RANTES to the recombinant human CCR_1 chemokine receptor.[399] Further SAR studies of this



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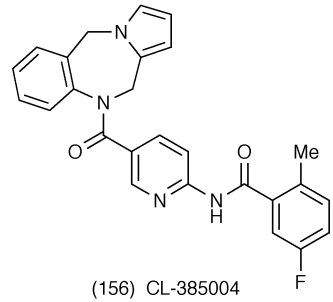

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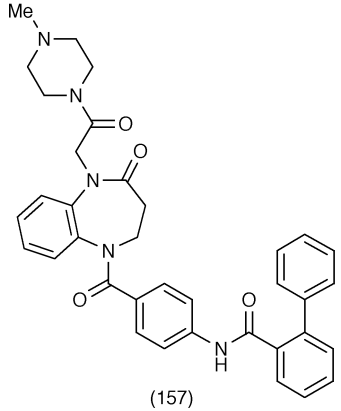

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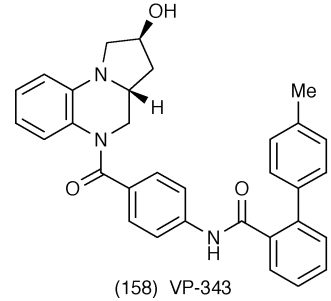
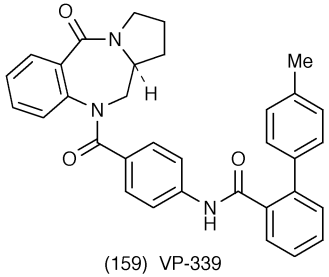
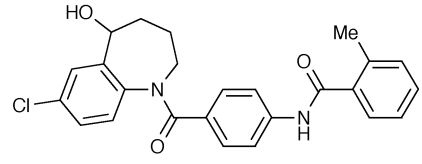


(158) VP-343

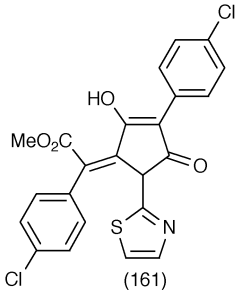


(159) VP-339



(160) OPC-41061

template structure resulted in receptor antagonists like **162** (K_i 52 nM) which showed at least 200-fold selectivity for inhibition of CCR1 over other human 7-TM receptors, including other chemokine receptors. A number of publications on N-type calcium channel blockers based on *N*,*N*-dialkyl-dipeptidyl-amine structures have appeared.[400–402] The MeLeu-Tyr derivative **163** (PD 173212) was active in the *in vitro* IMR-32 (human neuroblastoma cells) assay as well as in the in *vivo* audiogenic seizure model.[401] The Leu derivative **164** (IC_{50} 180 nM in the IMR-32 cell assay) blocked neuronal N-type calcium channels in superior cervical ganglion neurones (71% at 3 μM, IC_{50} 1.8 μM) and showed significant activity in preventing tonic seizures (80% protection at 30 mg kg^{-1}). Compound **164** also blocked voltage-gated sodium channels (41% at 3 μM).[402]



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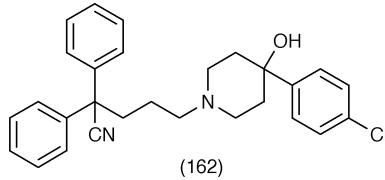

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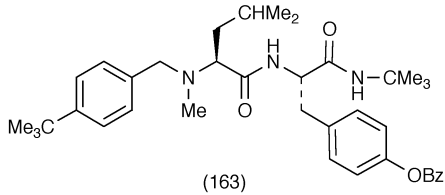

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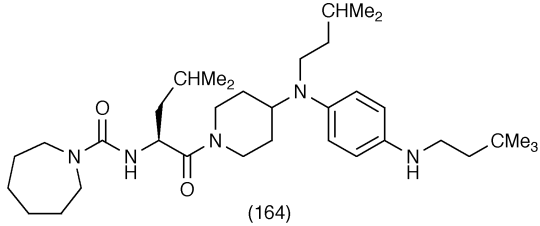

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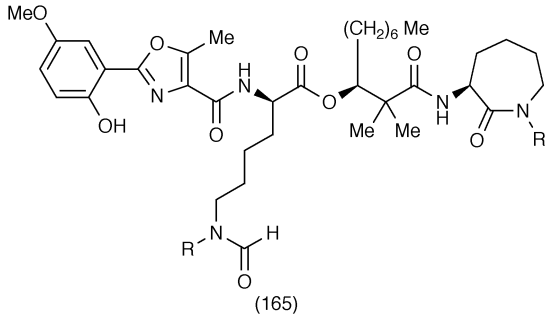
An orphan human G-protein-coupled receptor homologous to rat GPR14 and expressed predominantly in cardiovascular tissue has been shown to function as a urotensin-II {a vasoactive 'somatostatin-like' cyclic peptide [Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val] originally isolated from fish spinal cords and recently cloned from man} receptor.[403] Human urotensin-II caused contraction in all non-human primate arterial vessels studied, including both elastic and muscular arteries (6-28-fold more potent than endothelin-1). Koshikamide A_1, a new cytotoxic linear peptide [$MeOCH_2CO$-Phe-MeVal-MeAsn-Me*a*Ile-MeVal-MeLeu-Asn-Phe-Pro-Pro] was isolated from a marine sponge, *Theonella* sp. The peptide showed moderate cytotoxicity against P388 leukemia cells (IC_{50} 2.2 μg ml^{-1}).[404] Two new lipopeptides, amamistatins A (**165**, R = OH) and B (dimethoxy derivative of **165**), were isolated from an actinomycete. Amamistatins A and B showed growth inhibition for human tumour cell lines.[405] The IC_{50} values of amamistatin A were 0.48, 0.56 and 0.24 μM against MCF-7 breast, A549 lung and MKN45 stomach cancer cell lines.

5 Enzyme Inhibitors

Like last year, most of the work this year has been on converting enzymes, HIV protease, farnesyltransferase, various matrix metalloproteases and



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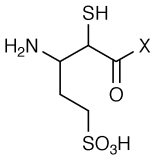
thrombin inhibitors. Involvement of proteases in apoptosis and cellular regulation has been reviewed.[406,407] Work on conformational aspects of inhibitor design based enzyme–substrate interactions in the transition state,[408] protease and protease inhibitor assays using biotinylated casein coated on a solid phase[409] and a rapid method to identify exo-protease inhibitors[410] has been published.

5.1 Aminopeptidase Inhibitors. – To study the physiological roles of the membrane-bound zinc-aminopeptidase A (glutamyl aminopeptidase, EC 3.4.11.7), an exploration of aminopeptidase A active site was performed by a combinatorial approach using (3-amino-2-mercapto-acyl)dipeptides able to fit its S_1, S_1', and S_2' subsites.[411] This analysis confirmed that the S_1 subsite is optimally blocked by a glutamate or isosteric residues and demonstrated that the S_1' subsite is hydrophobic whereas the S_2' subsite recognises preferentially negatively charged residues derived from aspartic acid. The optimisation of these structural parameters led to inhibitors like **166** (X = Ile-Pro(3-COOH) or Ile-Asp) which inhibited aminopeptidase A (K_i 3–4 nM). Some of these also inhibited NEP (K_i 5–200 nM), ACE (K_i >200 nM) and aminopeptidase N (K_i >5000 nM).

α-Aminophosphinic acids bearing a hydrophobic side chain interacting with the S_1 subsite inhibited aminopeptidase N (a monomeric or heterodimeric type II membrane-bound zinc exopeptidase), an enzyme widely distributed in mammalian tissues including the CNS, the kidney, the intestine and the lung. Coupling of these α-aminophosphinic acids with an analogue of Phe-Phe, which has been shown to recognise efficiently the S_1' and S_2' subsites of aminopeptidase N, provided the most potent compounds (**167**, R = Me or benzyl) (K_i 0.6–1.5 nM).[412] Inhibitors of membrane-bound aminopeptidase P (involved in the degradation of bradykinin in several vascular beds) were developed from an inhibitor of aminopeptidase P called apstatin (**168**) (IC_{50} 2.9 μM, human enzyme). The most potent inhibitor was obtained by replacing the N-terminal residue (**169**, IC_{50} 0.23 μM). Apstatin analogues lacking the alanine or having hydroxyproline in place of the proline in the second position had reduced affinity.[413] Certain thiol-, carboxyalkyl-, and hydroxamate-containing compounds like **170** (IC_{50} 48 μM) were inhibitory in the low μM range.



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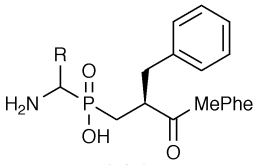

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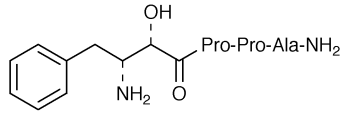

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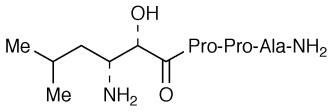

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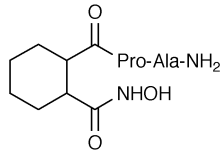

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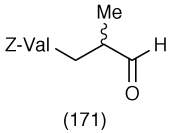
5.2 Calpain Inhibitors. – Role of calpain in adipocyte differentiation was highlighted[414] and inhibitors of calpain like **171**–**174** were reported.[415–417] The ketomethylene phenylalanal and alanal analogues of Z-Val-Ala-H and Z-Val-Phe-H were significantly less potent than the corresponding dipeptide aldehydes. K_i values for compounds **171** and **172** against chicken gizzard smooth muscle calpain were 45 μM and 2.5 μM, respectively.[415] Even the more potent analogue **172** was about 250-fold less potent than Z-Val-Phe-H. In a series of P′-extended α-ketoamide inhibitors of calpain I, compound **174** was the most potent compound of the series (K_i 8 nM).[417] Various other substituents at either end of the -Phe-$CONHCH_2CH_2NHSO_2$- moiety gave somewhat less potent compounds (K_i 14–1100 nM).



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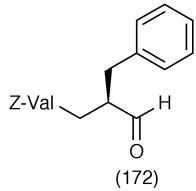

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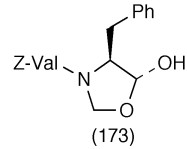

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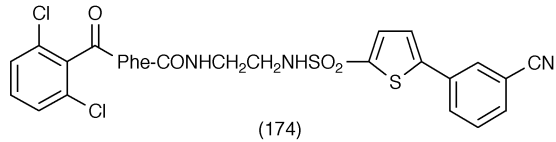

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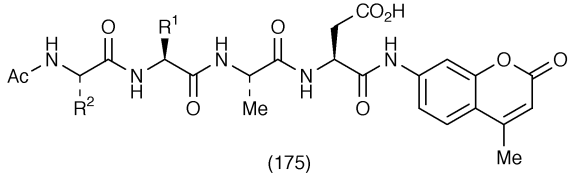
5.3 Caspase Inhibitors. – Structure, activation, substrates and functions of mammalian caspases (a family of cysteine-dependent aspartate-directed proteases) during apoptosis were reviewed.[418] It was suggested that both receptor-induced (CD95 and tumour necrosis factor) and chemical-induced apoptosis result in a similar time-dependent activation of caspases-3, -7, -8, and -9 in Jurkat T cells and human leukemic U937 cells.[419] In receptor-mediated apoptosis, the caspase inhibitor, Z-Val-Ala-Asp fluoromethyl ketone, inhibited apoptosis prior to commitment to cell death. However, Z-Val-Ala-Asp fluoro-

methyl ketone inhibited chemical-induced apoptosis at a stage after commitment to cell death. A proteolytic mechanism was proposed for caspase-1 involving polarisation of the scissile carbonyl by the His^{237} imidazolium group.[420] By evaluating many aspartic ketone based inhibitors with different types of prime-side groups (*e.g.* acyloxymethyl, aryloxymethyl, arylthiomethyl, alkylthiomethyl, acylamino-oxymethyl and sulfonylaminomethyl), the inhibitory behaviours were classified as reversible, inactivating, or bimodal (*i.e.* reversible inhibition followed by slow inactivation).

A strategy for the synthesis of a tetrapeptidyl substrate combinatorial array (**175**) directed toward the caspases was reported. In the fluorogenic tetrapeptide substrates, P_1 was kept as Asp (required by caspases) and P_2 (caspase 1 can accommodate various residues in this position) was kept as alanine.[421] Diversity was introduced in the P_3 and P_4 positions. Testing of this set of substrates with caspases 1 and 4 gave substrate hydrolytic profiles characteristic of each caspase, and permitted the identification of efficiently processed substrates. $(Z\text{-Asp-Glu-Val-Asp})_2$-Rhodamine (**176**) was characterised as a sensitive fluorogenic substrate for the determination of caspase-3 activity.[422] The inhibitory effect of serpin (a family of serine proteinase inhibitors) analogue proteinase inhibitor 9 (cloned from a placental cDNA library on the basis of its similarity to the cytoplasmic antiproteinase PI6) which contains an acidic residue in the putative specificity-determining position of the reactive-site loop was investigated against caspases.[423] The hydrolysis of peptide substrates by caspase-1 (interleukin-1β-converting enzyme), caspase-4 and caspase-8 was inhibited by the inhibitor in a time-dependent manner. The hydrolysis of a tetrapeptide substrate by caspase-3 was not inhibited by the serpin analogue proteinase inhibitor 9.



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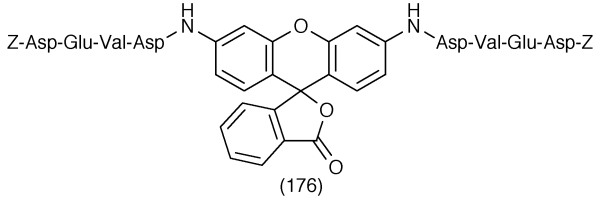

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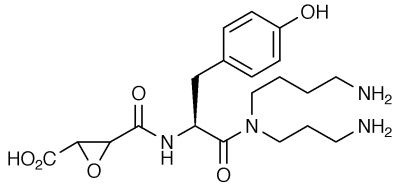
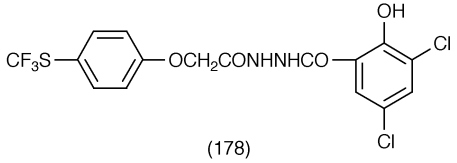
5.4 Cathepsin Inhibitors. – Publications on cathepsins B, D, F, G, J, K, L, P, S, V and X have appeared. The peptides derived from the pro-region of cathepsin B (lysosomal cysteine protease) inhibited the enzyme in a pH-dependent manner. This pH dependency was eliminated either by the removal of a portion of the enzyme's occluding loop through deletion mutagenesis or

by the mutation of either residue Asp^{22} or His^{110} to alanine; *e.g.*, the mutant enzyme His^{110}Ala was inhibited by its propeptide (K_is 2.0 nM at pH 4.0 and 1.1 nM at pH 6.0).[424] An inhibitor of cathepsins B and L (**177**, WF14861) consisting of *trans*-epoxysuccinic acid, L-tyrosine and spermidine was obtained from the culture mycelium of a fungus strain *Colletotrichum* sp. No. 14861. WF14861 also showed inhibitory activities against bone derived crude protease and other cysteine proteases *in vitro*.[425,426]



(177) WF14861

Fluorogenic substrates for cathepsin D (a lysosomal aspartyl protease) [A-Tyr-Phe(NO_2)-Leu-Leu (A = Ala-Arg-Pro-Lys-Pro-Leu-Leu-, Arg-Pro-Lys-Pro-Leu-Leu-, Pro-Lys-Pro-Leu-Leu-, Lys-Pro-Leu-Leu- or Pro-Leu-Leu-) and B-Phe(NO_2)-Tyr-Leu-Leu (B = Arg-Pro-Lys-Pro-Leu-Leu-, Pro-Lys-Pro-Leu-Leu-, Lys-Pro-Leu-Leu- or Pro-Leu-Leu-) Phe(NO_2)] were synthesised and digested by cathepsin D and pepsin.[427] The hydrolysis rate constants (k_{cat} K_m) of B-Phe(NO_2)-Tyr-Leu-Leu for cathepsin D were same or 2–3 times greater than A-Tyr-Phe(NO_2)-Leu-Leu. On the other hand, those of B-Phe(NO_2)-Tyr-Leu-Leu for pepsin were the same or 4–20 times greater than A-Tyr-Phe(NO_2)-Leu-Leu. The hydrolysis rates of the substrates by both enzymes increased with the increase in the peptide chain length. The best substrate for cathepsin D was Arg-Pro-Lys-Pro-Leu-Leu-Phe(NO_2)-Tyr-Leu-Leu. Using a solution phase parallel synthesis approach, small molecule cathepsin D inhibitors were obtained by the coupling of acyl chlorides, sulfonyl chlorides and carboxylic acids with nitrogen nucleophiles.[428] Compound **178**



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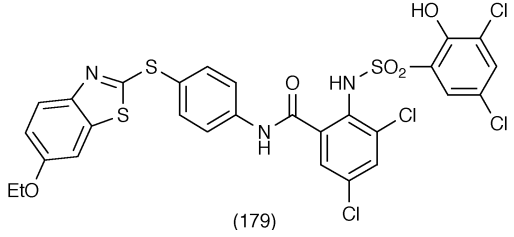


(179)

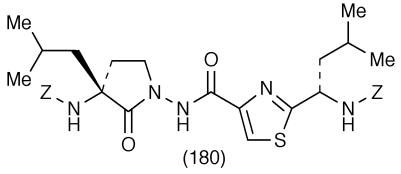
was one of the more potent cathepsin D inhibitor of the series (IC_{50} 320 nM). Chemical optimisation of a weakly active cathepsin D inhibitor (identified by high throughput screening) led to the discovery of inhibitors like **179** (IC_{50} 250 nM).[429]

A cDNA encoding cathepsin F (cysteine proteinase belonging to the papain family) was cloned from a human prostate cDNA library. This cDNA encodes a polypeptide of 484 amino acids, with the same domain organisation as other cysteine proteinases, including a hydrophobic signal sequence, a pro-domain, and a catalytic region. However, the propeptide domain was unusually long and distinguished cathepsin F from other proteinases of the papain family.[430] A series of intramolecularly quenched fluorogenic peptides based on the sequences of various serpin loops were synthesised as fluorogenic substrates for human cathepsin G and assayed as substrates for cathepsin G and other chymotrypsin-like enzymes including chymotrypsin and chymase.[431] Replacement of Leu-Leu in *o*-aminobenzoyl-Thr-Leu-Leu-Ser-Ala-Leu-Gln-N-(2,4-dinitrophenyl)ethylenediamine (EDDnp) by Pro-Phe produced a highly sensitive substrate of cathepsin G. Molecular modelling studies of a peptide substrate bound into the cathepsin G active site revealed that, in addition to the protease S_1 subsite, subsites S_1' and S_2' significantly contribute to the definition of the substrate specificity of cathepsin G.

The role of cathepsin K (a cysteine protease present in human osteoclasts) in osteoporosis in cathepsin K-deficient mice was discussed.[432] The X-ray structure of human pro-cathepsin K at 2.8 Å resolution was reported.[433] The structure of the mature enzyme domain within pro-cathepsin K was similar to that of mature cathepsin K. A portion of the propeptide occupies the active site cleft of cathepsin K. The fold of the propeptide of pro-cathepsin K was similar to that observed in pro-cathepsins B and L. Since type I collagen is the most abundant component of extracellular matrix of bone and regarded as an endogenous substrate for the cysteine proteinases in osteoclastic bone resorption, fragments of this protein (157–192, Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser) were investigated as substrates for the cathepsins.[434] Cathepsins K and L cleaved the fragment at different specific sites. The major cleavage sites for cathepsin K were Met^{159}-Gly^{160}, Ser^{162}-Gly^{163} and Arg^{165}-Gly^{166}, while those for cathepsin L were Gly^{166}-Leu^{167} and Gln^{180}-Gly^{181}.

Starting from the aldehyde-based inhibitors like Z-Leu-Leu-Leu-H, a series of α-heteroatom substituted ketones were synthesised as inhibitors of cathepsin K. The phenoxymethyl ketone Z-Leu-Leu-CH_2OPh inhibited cathepsin K (IC_{50} 3.7 nM).[435] The corresponding 4-phenyl benzyl ether derivative Z-Leu-Leu-CH_2OPh-4-Ph, also a potent inhibitor of cathepsin K, was about 3-fold less potent inhibitor of cathepsin L and about 60-fold less potent inhibitor of cathepsin B. In the Z-Leu-Leu-CH_2OMe series, the C-terminal Leu replacement by D-Leu, Glu, Lys, Phe, Ile, Ser, Gly and Ala gave much less potent inhibitors. Non-peptide inhibitors of cathepsin K like **180**–**183** were reported.[436,437] Diaminopyrrolidinone **180**, a moderately potent inhibitor of

human osteoclast cathepsin K (K_i 33 nM), was about 3-fold less potent than the corresponding linear analogue **181**. However, **180** was more stable to cathepsin K than **181**. Diacylhydrazine **181** loses all ability to inhibit the processing of a fluorescent substrate (Z-Phe-Arg-AMC) by cathepsin K after a one hour pre-incubation time with the protease. In contrast, **180** retains almost complete inhibitory potency after the same pre-incubation period. In compounds like **182**, α-isobutyl-*para*-biphenylacetyl was used as a mimic of Z-Leu. Compound **183** was equipotent to Z-Leu analogue **182**.



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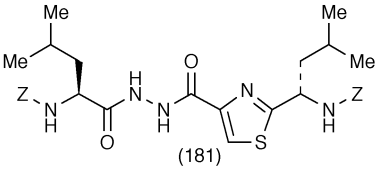

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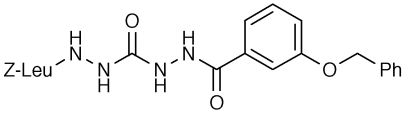

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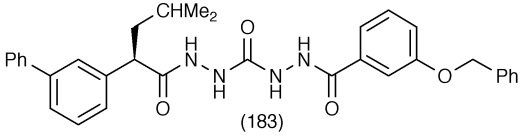
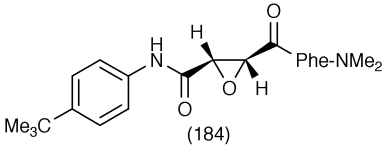


(183)

Inhibitors for cathepsin L and cathepsin S were developed using computer modelling techniques. Several of these compounds like **184** and **185** specifically inhibited cathepsin L at a concentration of 10^{-7} M *in vitro*, while almost no inhibition of cathepsins B, C, S and K was observed.[438] Some of the compounds were stable to enzymes present in mouse liver and small intestine homogenates (1 h), and showed selective inhibition for hepatic cathepsin L *in vivo*.



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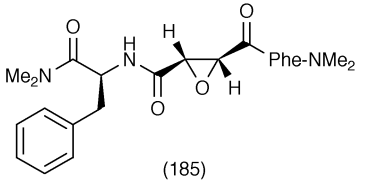
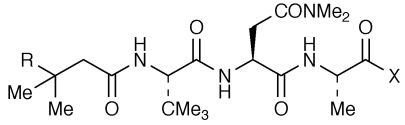


(185)

5.5 Cytomegalovirus and Rhinovirus 3C Protease Inhibitors. – The conformational properties of **186** (R = X = Me) and the N-*tert*-butylacetyl- analogue (R = Me, X = H) were investigated.[439] Whereas these compounds were weak inhibitors of the human cytomegalovirus protease, their activated carbonyl analogues (R = Me, X = CF_3, CF_2-CF_3 or $CONHCH_2Ph$) were 1000-fold more potent (IC_{50} 0.1–1.1 μM). NMR studies demonstrated that N-*tert*-butylacetyl

analogue exists in solution as a relatively rigid and extended peptide structure and that the bulky side chains, notably the P_3 *tert*-butyl group, greatly contribute to maintaining this solution conformation.

A number of non-peptide inhibitors (*e.g.* **187**–**192**) of the human cytomegalovirus protease were reported.[440–446] In the case of hydroxylamine derivatives like **187** and **188** (IC_{50} 14–60 nM), covalent modification of the enzyme occurs through adduct formation at Ser^{132} (**187**) or Cys^{138} (**188**).[441] The IC_{50} value (anti HSCV activity in the Hs68 cell line) of compound **189**, obtained by optimising a screening lead, was 0.0004 μg ml^{-1}.[443] In a series of monobactams possessing a heterocyclic residue at C-4 (*R*), inhibitors containing a heterocycle such as 2-furyl, 2-thiophenyl, 4-methyl-2-tetrazole and 2-benzothiazole were found to be active in a plaque reduction assay. The IC_{50} values for the benzothiazole derivative **191** were 2.7, 43 and 11 μM, respectively, against human cytomegalovirus protease, bovine pancreatic α-chymotrypsin and plaque reduction assay.[445] A fluorogenic β-lactam derivative **192** was used for rapid determination of the active enzyme.[446]



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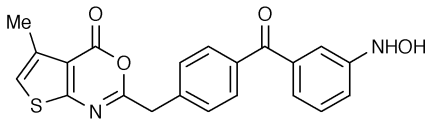

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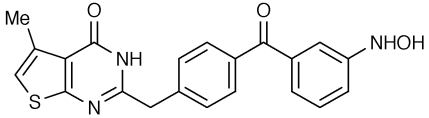

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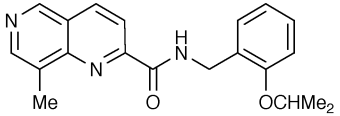

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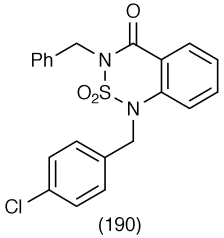

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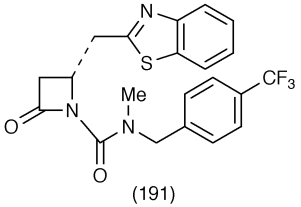

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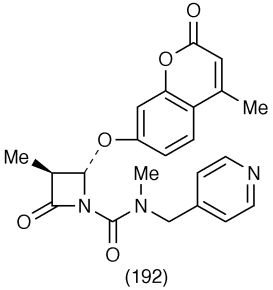
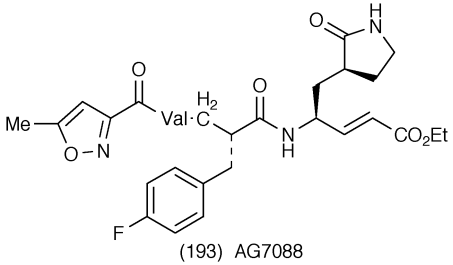


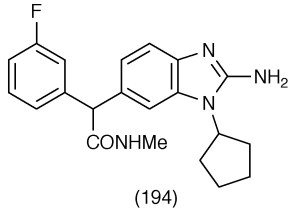
(192)

Aza-peptide-based substrates and inhibitors of human rhinovirus 3C protease were reported.[447] Boc-Val-Leu-Phe-AzGln-OPh was a slow-turnover substrate that gave transient (1–2 h) inhibition as it underwent hydrolysis. Boc-Val-Leu-Phe-AzGly-OPh gave very slow but essentially irreversible inhibition. Boc-Val-Leu-Phe-AzGln-Gly-Pro-NHiBu did not show any substrate or inhibitor like properties against the enzyme. Tripeptide-derived inhibitors incorporating N-terminal modifications and C-terminal Michael acceptor moieties were evaluated as irreversible inhibitors of the cysteine-containing human rhinovirus 3C protease.[448–451] One of the most potent inhibitors (**193**,

AG7088, antirhinoviral $EC_{90} \approx 0.10$ μM), did not inhibit thrombin, chymotrypsin, trypsin, elastase, cathepsin B and calpain at a concentration of 10 μM for a period of 10–15 min. The peptide was stable in dog and human plasma (half-life >60 min) but degraded rapidly in rat plasma (half-life <2 min). Orally active inhibitors of rhinovirus replication like **194** have been reported.[452]



(193) AG7088



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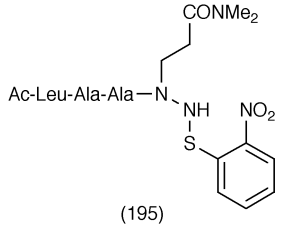
Hepatitis A virus 3C proteinase (a cysteine proteinase) is essential for cleavage of the initially synthesised viral polyprotein precursor to mature fragments and is therefore required for viral replication *in vivo*. Since the enzyme generally recognises peptide substrates with Gln at the P_1 site, several analogues having an AzGln residue were synthesised.[453] Sulfenamide **195** (IC_{50} ~100 μM) gave time-dependent inactivation of the enzyme due to disulfide bond formation with the active site cysteine thiol, as demonstrated by electrospray mass spectrometry. Sulfonamide **196** was a weak competitive inhibitor (IC_{50} ~75 μM). The haloacetyl AzGln analogues Ac-Leu-Ala-Ala-AzGln-CH_2Cl and Ac-Leu-Ala-Ala-AzGln-CH_2Br were time-dependent irreversible inactivators of hepatitis A virus 3C proteinase and were shown to alkylate the active site thiol.



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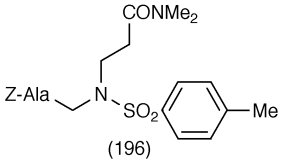

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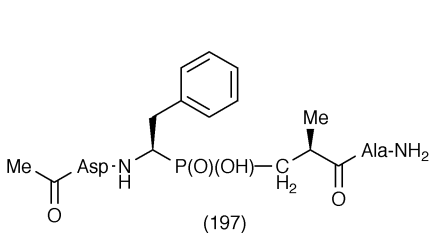
5.6 Converting Enzyme [Angiotensin (ACE), Neutral Endopeptidase (NEP), Endothelin, TNF-α Convertase and Interleukin-1β (ICE)] Inhibitors. – Some aspects of angiotensin converting enzyme and a novel family of proteases with a disintegrin and metalloproteinase domains (ADAMTS) were reviewed.[454–456] Primary structures of putative zinc metalloproteases ADAM-TS5, ADAM-TS6, and ADAM-TS7 were reported.[457] The mechanism of substrate binding and catalysis of endopeptidase EC 5.4.24.15, a zinc metalloendopeptidase broadly distributed within the brain, pituitary, and gonads and able to cleave neuropeptides such as neurotensin, bradykinin, and LHRH, was investigated by *in vitro* mutagenesis and subsequent protein expression studies.[458] The

active site of the enzyme exhibited an His-Glu-X-X-His motif. Mutation studies confirmed the importance, for binding and catalysis, of the residues His^{473}, Glu^{474}, and His^{477} within this motif along with Glu^{502}. Alterations to these residues reduced enzymatic activity against both a putative physiological substrate and a synthetic quenched fluorescent substrate as well as binding of the specific active site-directed inhibitor, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Ala-Tyr-p-aminobenzoate. Crystal structures of α-mercaptoacyldipeptides [HS-CH(CH_2Ph)CO-Gly-(5-Ph)Pro, HS-CH(CH_2Ph)CO-Phe-Tyr and HS-CH((CH_2)$_4CH_3$)CO-Phe-Ala] bound to thermolysin active site have been reported.[459]

5.6.1 Angiotensin Converting Enzyme and Neutral Endopeptidase Inhibitors. A number of peptides composed of 2–7 amino acid residues [Thr-Phe, Leu-Tyr, Tyr-Leu, Ala-Phe, Ile-Tyr, Val-Phe, Ile-Val-Tyr, Val-Phe-Pro-Ser, Thr-Ala-Pro-Tyr, Thr-Val-Pro-Tyr, Thr-Val-Val-Pro-Gly, Asp-Ile-Gly-Tyr-Tyr, Asp-Tyr-Val-Gly-Asn, Thr-Tyr-Leu-Gly-Ser, Gly-Gly-Val-Ile-Pro-Asn and Ala-Pro-Gly-Ala-Gly-Val-Tyr] (IC_{50}s < 20 μM against ACE) were identified from the wheat germ hydrolysate.[460] By screening phosphinic peptide libraries, a phosphinic peptide [Ac-Asp-Pheψ(PO_2CH_2)Ala-Ala-NH_2, RXP 407 (**197**)] capable of differentiating the two ACE active sites was identified.[461] The Ac-Asp-$_{(L)}$Pheψ(PO_2CH_2)$_{(L)}$Ala-Ala-NH_2 peptide bound to the N-terminal site with a much higher affinity than to the C-terminal site (K_i 12 nM and 25 μM, respectively). The C-terminal amide, N-terminal acetyl groups and aspartic acid side chain in the P_2 position appear to be important for potency and selectivity. Ac-Asp-Pheψ(PO_2CH_2)Ala-Ala-OH, Asp-Pheψ(PO_2CH_2)Ala-Ala-NH_2 and Ac-Ala-Pheψ(PO_2CH_2)Ala-Ala-NH_2 were less selective (K_i values 2–15 and 7–800 nM, respectively, at N- and C-terminal binding sites). Synthesis of a conformationally constrained analogue **198** of the ACE inhibitor idrapril was reported. The proline derivative **198** did not inhibit ACE.[462] Potential role of mixed ACE and neutral endopeptidase inhibitor in the treatment of heart failure was discussed[463] and compounds like **199** and **200** inhibiting both the enzymes were reported.[464,465] The thiol derivative **200**



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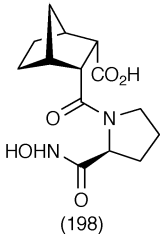

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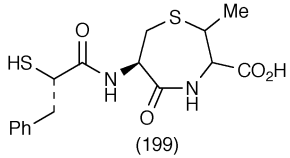

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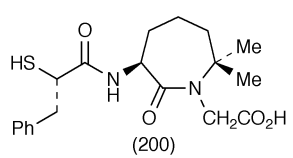

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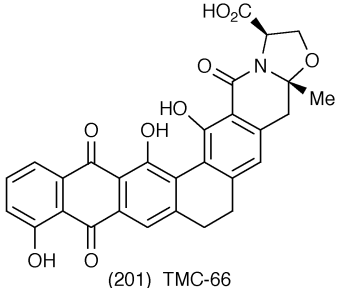
(K_i against rabbit lung ACE 5.3 nM and against rat kidney NEP 16 nM), obtained by further work around BMS-186716 (omapatrilat, reported earlier) was effective in lowering blood pressure in several animal models including spontaneously hypertensive and DOCA salt hypertensive rats.

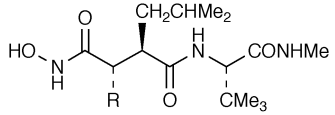
5.6.2 Endothelin Converting Enzyme Inhibitors. The existence of endothelin converting enzyme in smooth muscle cells and cultured human vascular endothelial cells was demonstrated.[466,467] The substrate specificity of ECE-1 (a zinc metalloendopeptidase) was compared to neprilysin, an enzyme related in amino acid sequence to ECE-1. Unlike neprilysin (a mammalian cell-surface peptidase involved in the metabolism of numerous biologically active peptides), ECE-1 was found to have minimal activity against substrates smaller than hexapeptides, such as Leu-enkephalin.[468] Larger peptides such as neurotensin, substance P, bradykinin, and the oxidised insulin B chain were hydrolysed by ECE-1 as efficiently as big endothelin-1, a known *in vivo* substrate. Identification of the products of hydrolysis of six peptides indicates that ECE-1 has a substrate specificity similar to that of neprilysin, preferring to cleave substrates at the amino side of hydrophobic residues. Since ECE-1 cleaves big ET-3 to a significantly lesser extent than Big ET-1, the conformational properties of the two peptides were studied by CD spectroscopy and homology modelling.[469] The results indicated that both peptides can adopt the same overall fold except in the C-terminal residues, 34–38 in big ET-1 and 34–41 in big ET-3. The differences in affinity between big ET-1 and big ET-3 for ECE-1 were assigned to the sequence variations in the local region of the cleavage site. A new endothelin converting enzyme inhibitor, TMC-66 (**201**) (IC_{50} 2.9 μM) was isolated from the fermentation broth of *Streptomyces* sp. A5008 and its structure elucidated by spectroscopic analyses.[470]



(201) TMC-66

5.6.3 TNF-α Convertase Inhibitors. Tumour necrosis factor convertase (a metalloproteinase closely related to matrix metalloproteinases) inhibitors were designed on succinate-based hydroxamic acids.[471,472] The introduction of bulky substituents into these succinate-based hydroxamic acids (*e.g.* thioethers, sulfonamides, and ethers) showed improved potency against the enzyme. Most of the analogues with a sulfur [**202**, R = PhS-, 3,4-$(MeO)_2$ C_6H_3S-, 4-$(CN)C_6H_4S$-, 4-$(MeO_2S)C_6H_4S$-, 3,5-$Cl_2C_6H_3S$-, 2-Cl-4-FC_6H_3S-, (8-quinoline)CH_2S-, MeS- and EtS-] or a nitrogen [**202**, R = -Ph-SO_2NH-, 4-(Ac-

NH)C_6H_4-SO_2NH-, 3,5-$Cl_2C_6H_3$-, 2-(CN)C_6H_4-SO_2NH-, 2-Cl-4-FC_6H_3-SO_2NH-, 2,4,6-(^{i}Pr)$_3C_6H_2$-SO_2NH-, thiophene-2-SO_2NH-, pyridine-3-SO_2NH-, 4-(HOOC)C_6H_4-SO_2NH-, 4-BrC_6H_4-SO_2NH-, naphthalene-, 1-SO_2NH-, naphthalene-2-SO_2NH-, quinoline-8-SO_2NH-, quinoline-6-SO_2NH-, isoquinoline-5-SO_2NH-, 4-(oxo)-3,4-dihydroquinazoline-8-SO_2NH-, 4-(oxo)-3,4 dihydroquinazoline-6-SO_2NH-, 4-(pyridine)$CH_2CH_2SO_2$NH-] link in the side chain were potent inhibitors of the enzyme (IC_{50}s 0.4–4 nM). One of the sulfonamide derivatives, **202** [R = 4-(oxo)-3,4-dihydroquinazoline-6-SO_2NH-] was one of the more potent and stable compound of the series (TNF-α convertase IC_{50} 0.57 nM; blood IC_{50} 0.28 μM).



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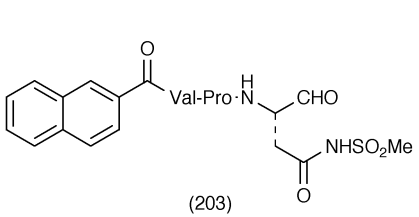
5.6.4 Interleukin 1β Converting Enzyme (IL-1β) Inhibitors. A three-dimensional quantitative SAR study using the comparative molecular field analysis method was performed on a series of IL-1β inhibitors.[473] Further work on the IL-1β inhibitors like 2-NapCO-Val-Pro-Asp-CH_2OPh was reported.[474] Compound **203** showed high potency in both the enzyme and cell based assays (IC_{50}s 38 nM and 0.23 μM, respectively) and inhibited LPS-primed ATP-induced IL-1β release in mice. The crystal structure of the complex of **203** and ICE revealed further interactions of **203** with ICE in the P_4 (naphthoyl group) and P_1 (methyl group of the methanesulfonamidecarbonyl group) positions. Replacement of the naphthyl group in 2-NapCO-Val-Pro-Asp-CH_2OPh by other heterocyclic groups did not improve inhibitory activity. P_2 modifications achieved by incorporating N-substituted glycine residues indicated that compounds like **204** were similar in potency (IC_{50} 13 nM) to **203**. In **203**, replacement of -$NHSO_2$Me group by -NHOH and -NHOPh groups led to a significant reduction in potency (IC_{50}s 1300 and 600 nM, respectively). Compounds **203** and **204** were also effective in inhibiting IL-1β release from LPS-stimulated human monocytic cells (IC_{50}s 770 and 230 nM, respectively). The corresponding IC_{50} value for 2-NapCO-Val-Pro-Asp-CH_2OPh was 900 nM. Chiral synthesis of an ICE inhibitor **205** was reported.[475]

5.7 Elastase Inhibitors. – Peptide and non-peptide (**206**–**209**) inhibitors of elastase were reported.[476–482] Total syntheses of depsipeptide elastase inhibitors YM-47141 (**206**, R = Ph-CH_2CO-) and YM-47142 (**206**, R = Me_2CH-CH_2CO-) were reported.[476] Compound **207** was active both *in vitro* (human sputum elastase) and *in vivo* assays (31–55% inhibition of human leukocyte elastase-induced lung haemorrhage in mice at 10–30 mg kg^{-1}, p.o.).[478] Some compounds related to **207** and **208** also inhibited α-chymotrypsin and thrombin. A series of metallopeptides (*e.g.* **209**), synthesised by solution and solid-phase methods, exhibited specificity in inhibiting human neutrophil



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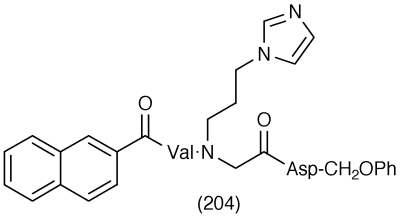

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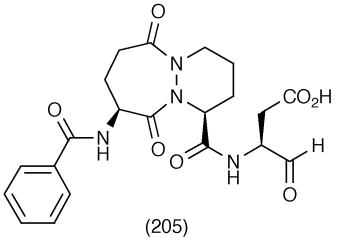

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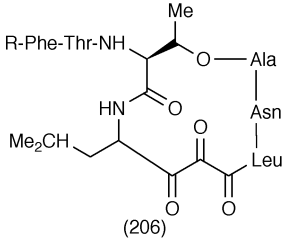

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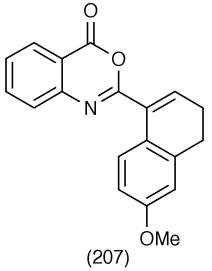

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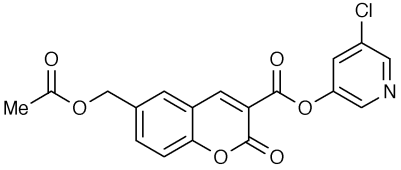

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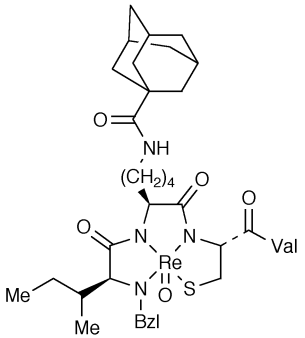
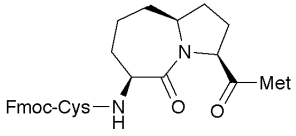


(209)

elastase.[482] The rigid backbone conformation of this rhenium metal peptide was similar to a reversed-turn structure. Compound **209** [Ile-Lys-Cys-Val derivative] was about 400-fold more potent in inhibiting human leukocyte elastase (K_i 9.7 μM) than porcine pancreatic elastase. The corresponding linear peptide without Re was much less potent (K_i 270 μM). The C-terminal aldehyde derivative of compound **209** was about 2-fold more potent.

5.8 Farnesyltransferase Inhibitors. – Work on farnesyl protein transferase inhibitors based on the C-terminal tetrapeptide of the Ras protein and non-

peptide inhibitors has continued with the aim of improving potency and oral bioavailability. The reverse-turn mimetic tetrapeptide based inhibitors like **210** and **211** were weak inhibitors (IC_{50} 5–7 μM). The free peptides (without the Fmoc group) did not show any effect on farnesylation.[483] The inhibitors like **212** (IC_{50} 0.61 nM) were potent inhibitors of the enzyme.[484] The corresponding methyl ester analogue (FTI-277) was less potent (IC_{50} 63 nM) as an enzyme inhibitor but retained significant activity in the cell-based assays and inhibited H-Ras processing (IC_{50} 100 nM). Synthesis of a library of secondary benzylic amines based on **212** followed by further chemistry led to **213** (IC_{50} of 0.20 nM and an EC_{50} of 4.4 nM). *In vivo* tests in a nude mouse xenograft model of human pancreatic cancer (MiaPaCa cells) showed that oral dosing of **213** gave rise to attenuation of the growth of this tumour cell line.[485] Many other analogues of **212** containing cysteine replacements were prepared.[486–492] The *p*-chlorophenylfuran ether **214** showed 32% oral bioavailability in the mouse, 30% in rats, and 21% in dogs. Replacement of the *p*-chlorophenyl substituent



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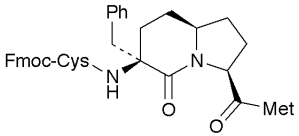

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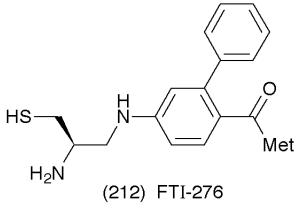

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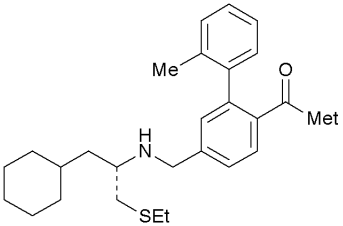

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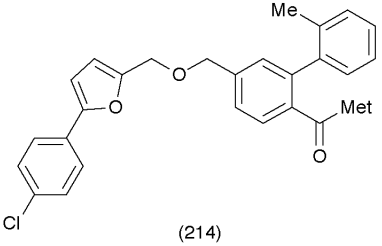

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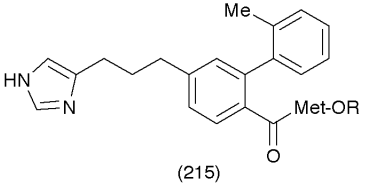

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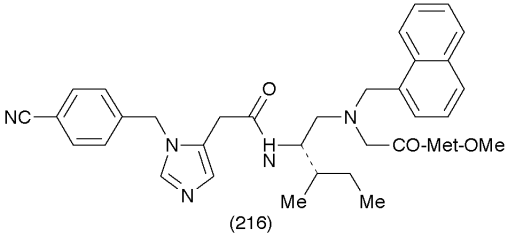

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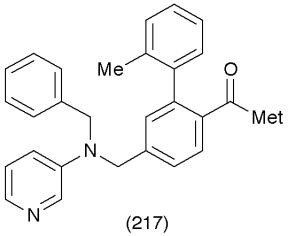
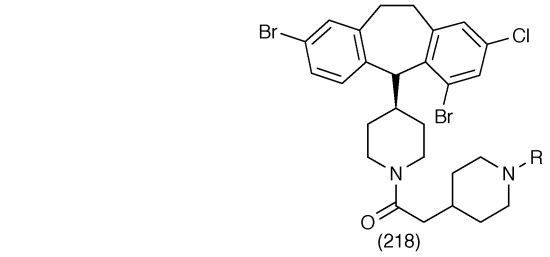


(217)

on the furan ring by other substituted phenyl groups or pyridyl groups resulted in at least 10-fold reduction in the *in vitro* potency.[487] Another non-thiol-containing inhibitor (**215**, FTI-2148) was selective for FTase (IC_{50} 1.4 nM) over GGTase I (IC_{50} 1700 nM), whereas the corresponding analogue containing Leu in place of Met was selective for GGTase I (IC_{50} 21 nM) over FTase (IC_{50} 5600 nM). The methyl ester prodrug of **215** was effective at suppressing oncogenic H-Ras constitutive activation of mitogen-activated protein kinase and human tumour growth in soft agar. The prodrug suppressed the growth of the human lung adenocarcinoma A-549 cells in nude mice by 33–91% in a dose-dependent manner. Combination therapy of the prodrug with either cisplatin, gemcitabine, or taxol resulted in a greater antitumour efficacy than monotherapy.[488] Another methyl ester prodrug **216** also showed activity in several *in vivo* tumour models.[489] The Met derivative **217** (IC_{50} 0.1 nM) and an analogue (pyridine ring replaced by a benzene ring) (IC_{50} 1.4 nM) demonstrated significant *in vivo* efficacy in nude mice inoculated with MiaPaCa-2, a human pancreatic tumour-derived cell line.[491]

A number of publications describing additional SAR studies on the previously reported tricyclic and piperazine-derived farnesyltransferase inhibitors have appeared.[493–498] Analogues of **218** (R = -$CONH_2$, -CONHMe, -$CONHCH_2COOH$, -$CONHCH_2CONH_2$, -$CONHCH_2COOEt$, -SO_2Me, -SO_2Ph, -SO_2NH_2, -SO_2NMe_2 and many other heterocyclic ring containing amides) were potent inhibitors of the enzyme (IC_{50}s 5–100 nM).[497] Oral bioavailability varied between 5–76%. In the piperazine-derived series of inhibitors, compounds like **219** (IC_{50} <0.18 nM) at a dose of 14 mg kg day^{-1} blocked tumour growth in mice implanted with H-*ras*-transformed cells.[498] About 60–70% inhibition of K-*ras* tumour was achieved with **219** at a dose of 1.4 mg kg^{-1} day^{-1}. In addition to the above mentioned inhibitors, several other non-peptidic inhibitors of farnesyltransferase were reported.[499–503] Examples of these are illustrated by structures **220**–**223**. The benzodiazepine derivative **220** (IC_{50} 24 nM) produced 85% phenotypic reversion of Ras transformed NIH 3T3 cells at 1.25 μM and had an EC_{50} of 160 nM for inhibition of anchorage-independent growth in soft agar of H-Ras transformed Rat-1 cells.[499] Compound **221** completely inhibited Ras farnesylation in intact cells (120 μM concentration), inhibited the growth of LIM1899 colon carcinoma cells, NIH3T3 and v-H-Ras transformed NIH3T3 cells (IC_{50}s 70–180 μM) and colony formation in soft agar of v-H-Ras transformed NIH3T3 cells (75% inhibition at 100 μM).[500] Non-peptide inhibitors like **222** were much more potent against protein geranylgeranyltransferase (IC_{50}s against geranylgeranyltransferase-I and farnesyltransferase 44 and 10000 nM, respectively). This selectivity was retained in whole cells where **222** blocked the geranylgeranylation of Rap-1A without affecting the farnesylation of small GTP-binding proteins such as Ras.[503]

5.9 HIV Protease Inhibitors. – In addition to the discovery of peptidic and non-peptidic inhibitors of the enzyme, efforts have been made to control human HIV-1 gene expression by unnatural peptides and to prevent dimerisa-



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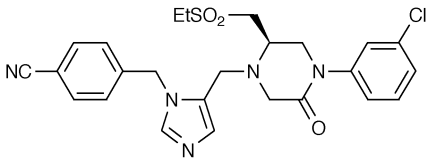

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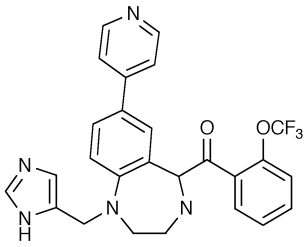

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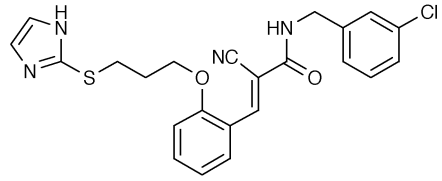

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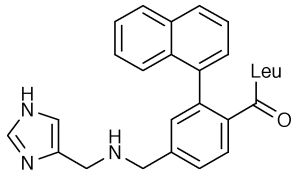

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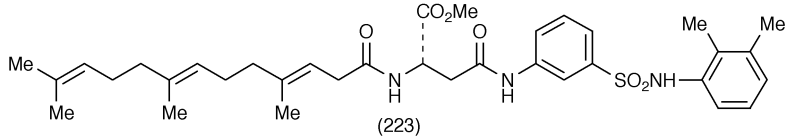
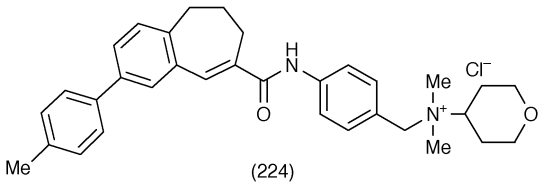


(223)

tion of the enzyme which plays an important role in its activity. The role of cytokines in the virus life cycle has been further explored because several chemokine receptors (*e.g.* CCR5 and CXCR4) have emerged as the predominant cofactors, along with CD4, for cellular entry of HIV-1 *in vivo*.[504,505] Using a molecular modelling approach, attempts have been made to delineate common molecular determinants that might be related to coreceptor activity for M-tropic HIV-1 entry.[506] Antagonists for the CCR5 and CXCR4 have been identified. TAK-779 (**224**) antagonised the binding of RANTES to CCR5-expressing CHO cells and blocked CCR5-mediated Ca^{2+} signalling.[507] It antagonised CCR2b to a lesser extent but did not affect CCR1, CCR3 or CCR4. Compound **224** also inhibited the replication of R5 HIV-1 clinical isolates as well as a laboratory strain at a concentration of 1.6–3.7 nM in peripheral blood mononuclear cells, though it was totally inactive against T-cell line-tropic (CXCR4-using or X4) HIV-1. Bis-tetraazamacrocycles like AMD3329 (**225**) inhibited the binding of a specific CXCR4 mAb and the Ca^{2+} flux induced by SDF-1, the natural ligand for CXCR4. The *m*-phenylenebis-

(methylene)-linked dimer **225** displayed the highest antiviral activity in this series of compounds, exhibiting EC_{50}s against the cytopathic effects of HIV-1 and HIV-2 replication of 0.8 and 1.6 nM, respectively.[508] Furthermore, **225** also interfered with virus-induced syncytium formation (EC_{50} 12 nM). A series of peptides corresponding to the N-terminal sequence of RANTES was also investigated as a coreceptor-directed anti-HIV-1 agent.[509] The N-terminal derivative, Ac-[Ala^{10}]-RANTES(1-10)-NH_2 (Ac-Ser-Pro-Tyr-Ser-Ser-Asp-Thr-Thr-Pro-Ala-NH_2) was slightly less potent (51–69% inhibition at 10–100 nM) than the recombinant RANTES (53–95% inhibition at 10–100 nM). Ac-[Ala^{10}]-RANTES(6-10)-NH_2, was the smallest anti-HIV-1 peptide (43–46% inhibition at 10–100 nM).



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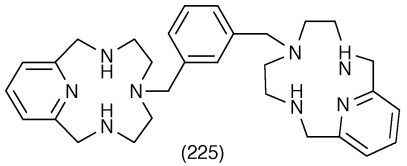

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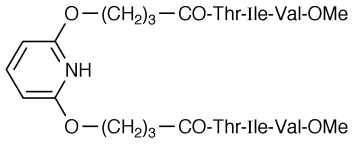
A synthetic all D-amino acid peptide derived from the Tat sequence (37-72, Cys-Phe-Thr-Thr-Lys-Ala-Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln-Gly-Ser-Gln-Thr-His-Gln-Val-Ser-Leu-Ser-Lys-Gln) inhibited Tat trans-activation *in vitro* and *in vivo*. A mutated D-Tat peptide (Gly^{44}-Gln^{72}) where all Arg residues in the RNA-binding region were substituted with Ala was inactive.[510] The importance of each side chain of a cross-linked interfacial peptide inhibitor of HIV-1 protease dimerisation [HO-Trp-Leu-Thr-Ile-Gln-Pro-CO-$(CH_2)_{14}$-CO-Ser-Thr-Leu-Asn-Phe-OH] was evaluated using an alanine scanning approach.[511] Whereas the parent inhibitor had an IC_{50} value of 350 nM, values for the mutants ranged from 280–9200 nM. Replacement of the Trp residue by Ala led to the least potent inhibitors (26-fold less potent) and the replacement of the Gln and Ser by Ala resulted in inhibitors with similar potency to the parent peptide. The replacement of Leu, Thr, Ile, Leu, Asn and Phe residues by Ala had a small effect (<4-fold reduction in potency) on the HIV protease inhibition. To inhibit the dimerisation process, conformationally constrained peptides were evaluated.[512] Inhibitions (submicromolar range) were obtained with compounds containing tripeptidic or tetrapeptidic arms attached to a pyridinediol- (**226**) or naphthalenediol-based (**227**) scaffold.

Cyclic depsipeptides papuamides A, B, C and D were isolated from Papua New Guinea collections of the sponges *Theonella mirabilis* and *Theonella swinhoei*. In addition to glycine, alanine, and threonine, these peptides contain



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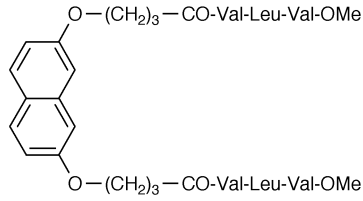

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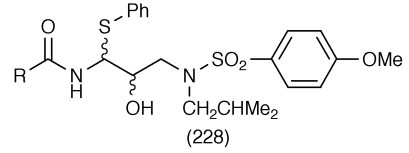
a number of unusual amino acids including 3,4-dimethylglutamine, β-methoxytyrosine, 3-methoxyalanine, and 2,3-diaminobutanoic acid or 2-amino-2-butenoic acid, 3-hydroxyleucine, homoproline and 2,3-dihydroxy-2,6,8-trimethyldeca-(4*Z*,6*E*)-dienoic acid residues.[513] Papuamides A and B inhibited the infection of human T-lymphoblastoid cells by HIV-1_{RF} *in vitro* ($EC_{50} \sim 4$ ng ml^{-1}). Papuamide A was also cytotoxic against a panel of human cancer cell lines with a mean IC_{50} of 75 ng ml^{-1}. A number of publications on statin-based inhibitors of HIV-1 protease have appeared.[514–522] Examples from this class of inhibitors include compounds **228**–**231**. Analogues of **228** (*e.g.* R = *t*-Bu-O-, Ph-CH_2-O- *etc.*) showed *in vitro* anti-HIV activities ranging from EC_{50} 0.1–1 μM (infected MT4 cells), and IC_{50} 10 nM–1 μM (enzyme inhibition).[515] In a series of C_2-symmetric inhibitors (**230**, R = bromo, 3-nitrophenyl, thienyl, pyridyl, phenethyl) (P_1/P_1' positions), all the compounds inhibited the enzyme (K_i values 0.09 to 3.8 nM).[518] SAR studies in the α-hydroxy-β-amino acid series of inhibitors led to compounds like **231** which displayed wide spectrum of antiviral activity.[519–522] For example, the allophenylnorstatine-containing dipeptide (**231**, JE-2147) was a potent inhibitor active against a wide spectrum of HIV-1, HIV-2, SIV, and various clinical HIV-1 strains *in vitro*. JE-2147 showed an elimination half-life of 94 min after i.v. administration, and the oral bioavailability was estimated to be 33–37% in the non-fasting and fasting conditions. A single oral dose of 25 mg kg^{-1} exhibited plasma levels exceeding the *in vitro* antiviral IC_{95} (52 nM) for more than 12 h in dogs.[521,522] Non-peptide inhibitors of HIV-1 protease have been discovered in the dihydropyrone (**232** R = OH or NH_2) and symmetric and unsymmetric cyclic urea (**233**) and sulfonyl urea series of compounds.[523–532]

5.10 Matrix Metalloproteinase Inhibitors. – Some aspects of metalloproteinase inhibitors were reviewed.[533,534] Publications highlighting the role of this class of enzymes in various forms of cancers, connective tissue remodelling, high density lipoprotein-induced cholesterol efflux from human macrophage foam cells and inflammation have appeared.[535–541] A cysteine residue, conserved in the propeptide domain of all MMPs was shown to be essential for maintaining the MMPs in an inactive state. It was suggested that the sulfhydryl group of this cysteine residue was coordinated to the catalytic Zn^{2+} ion and that interruption of this interaction caused activation (cysteine-switch mechanism). The structure of proMMP-2 (X-ray crystal structure) reveals how the propeptide shields the catalytic cleft and that the cysteine switch may operate



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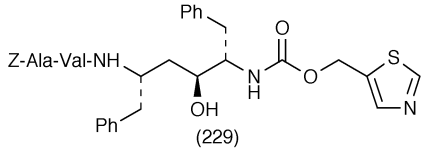

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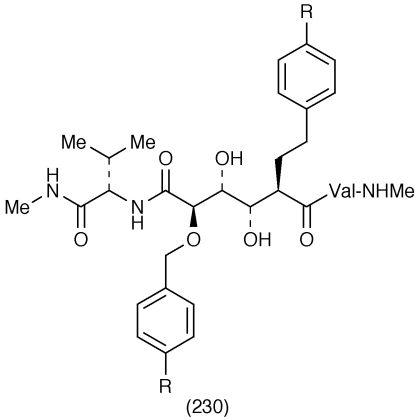

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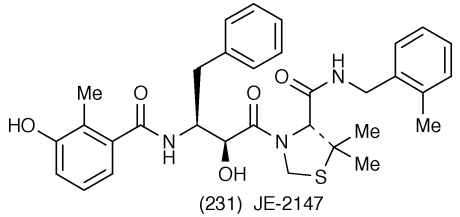
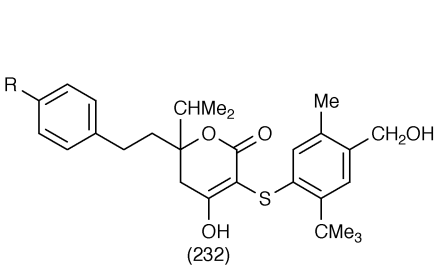


(231) JE-2147



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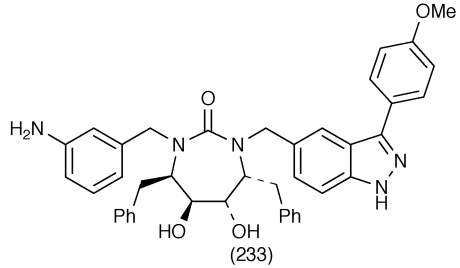

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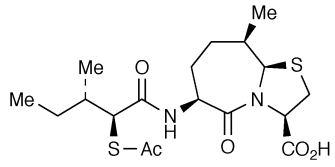
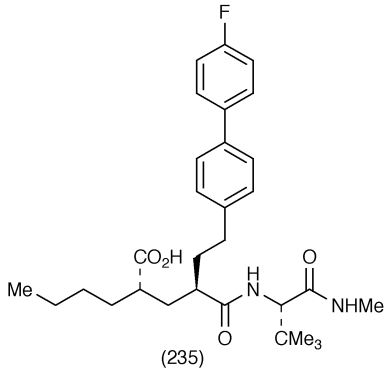
through cleavage of loops essential for propeptide stability.[542] Synthetic routes to metalloprotease inhibitors like **234** and **235** were reported.[543–545] Polymer supported reagents were used to generate an array of variously substituted hydroxamic acid derivatives (**236**) as potential inhibitors of matrix metalloproteinases.[546]

Many matrix metalloproteinase inhibitors containing a hydroxamate, thiol, carboxylate or phosphinic acid group were reported along with many other non-peptide inhibitors.[547–569] Although many of the hydroxamate-based compounds were potent inhibitors of metalloproteinases, none of the compounds demonstrated high selectivity.[547–558] In the Tic series (**237**), the *p*-methoxybenzenesulfonyl group could be replaced by a large number of substituents without any significant effect on the MMP-8 and MMP-3 inhibitory activity (IC_{50}s 2–100 nM). Removal of the Boc group in **237** resulted in slight reduction in potency (IC_{50} 30 and 5 nM, respectively, against MMP-3 and MMP-8) but replacement of the hydroxamic acid by a carboxyl group led to about a 1000-fold reduction in potency.[548] In a series of proline-based inhibitors, compounds like **238** inhibited several of the metalloproteinases



(234) ER-40133



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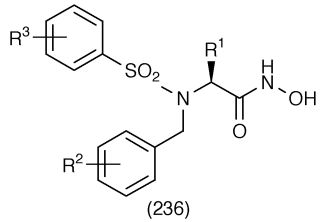

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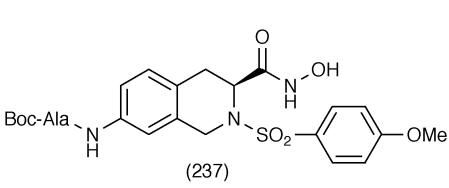
[IC_{50}s 212, 5, 19, 3393 and 3 nM, respectively, against MMP-1, -2, -3, -7 and -13].[549] A series of thiazine- and thiazepine-based inhibitors like **239** (IC_{50}s 0.7–41 nM against MMP-1, -2, -3, -7, -8, -9 and -13) and sulfonamide-based inhibitors like **240** [IC_{50}s 6, 300, 400, 10 and 40 nM, respectively, against MMP-1, -2, -3, -8 and -13] and **241** [R = H, -S-CH_2Ph, -S-$CH_2C_6H_4$*p*-Ph, -S-$CH_2C_6H_4$*p*-OCH_2Ph] [IC_{50}s 0.7–150 nM against MMP-1, -2, -3, -9 and -13] were potent, broad-spectrum inhibitors.[550–552] Conformationally restricted inhibitors like **242** (R = H, Ac, Boc or $PhSO_2$) inhibited MMP-1, -3, -8 and -9.[554,555] SAR studies around hydroxamic acid based inhibitors generated dual inhibitors of phosphodiesterase 4 and metalloproteinases. For example, compounds **243** [K_i values 0.03, 2, 0.01 and 0.5 μM against PDE4, MMP-1, -2 and -3, respectively] and **244** [K_i values 0.001 μM against PDE4 and >10 μM against MMP-1, -2 and -3] inhibited both series of enzymes.[558]

Examples of thiol and phosphinic acid based inhibitors include compounds **245**–**247**.[559–565] The IC_{50}s for **245** against MMP-13, -1, -3 and -8 were 2, >10,000, 150 and 36 nM, respectively. For the corresponding analogue containing OPh in place of -SPh, the IC_{50}s against MMP-13, -1, -3 and -8 were 0.5, 1500, 500 and 4 nM, respectively.[559,560] Analogues of **246** containing Tyr-NHMe, Ala-NHMe, $NHCH(CH_2OH)CMe_3$ or -$NHCH_2CMe_3$ group in place of the P_2' *tert*-leucine residue were less potent inhibitors of MMP-1 (IC_{50}s 2.3–20 μM).[563] Replacement of the N-terminal 4-$PhCH_2$ group in the Tyr-NHMe series with H, 2-Ph, 3-Ph, 4-Ph, 3-$PhCH_2CH_2$-, 4-$(CH_3)_2CHCH_2$- or 4-cyclohexylCH_2- group resulted in less potent analogues (MMP-1 IC_{50}s 1.9–15 μM). Side chain replacements in the P1′ position (R^1 = -CH_2CHMe_2, -CH_2CH_2Me, -$CH_2CH_2CF_3$, -CH_2cyclopropyl, -CH_2cyclobutyl, -$CH_2CH_2CHMe_2$, -CH_2CH_2Ph, -CH_2cyclohexyl, -CH_2CH_2cyclohexyl or -$(CH_2)_4OPh$ groups gave compounds with varying levels of inhibitory activities against



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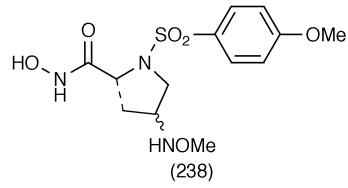

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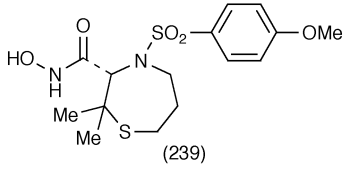

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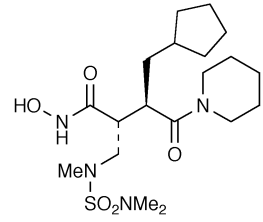

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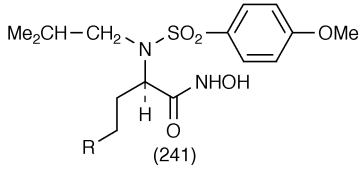

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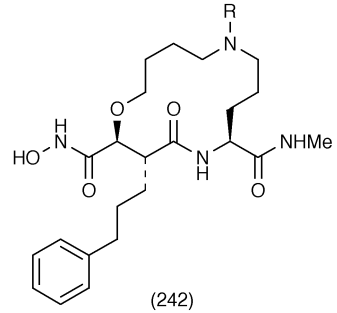

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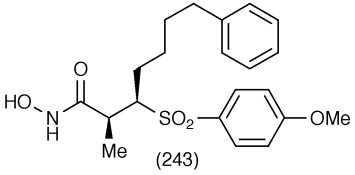

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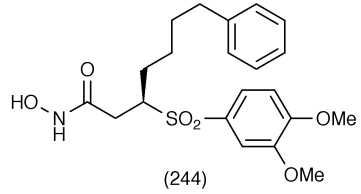

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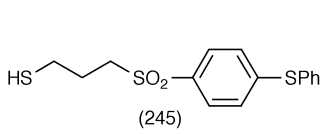
MMP-1 and MMP-13. Phosphinic pseudo-tripeptides like **247** inhibited many of the metalloproteinases (K_i values 36, 24, 117, 5, 7, 0.9 and 32 nM, respectively, against MMP-1, 2, 7, 8, 9, 11 and 14).[565] Examples of other non-peptidic inhibitors include compounds **248**–**250**.[566–569] The D-Trp analogues **249** (R = -nBu, -O-nBu or -OPh; IC_{50}s 3.9–10 nM against MMP-2 and 2–2.7 μM against MMP-9) displayed 230–614-fold selectivity. The corresponding D-Val analogues retained similar potency against MMP-2 but lost selectivity due to improved potency against MMP-9 (IC_{50}s 210–160 nM).[567] Diketopiperazine derivatives (**250**, R = Ph or *p*-substituted phenyl groups like -Ph-OMe, -Ph-OPh, -Ph-OBu, -Ph-Et, -Ph-Ph) were relatively more potent against collagenase 1 (IC_{50}s 21–108 nM) than against gelatinase B (IC_{50}s 570–4400 nM).[569]



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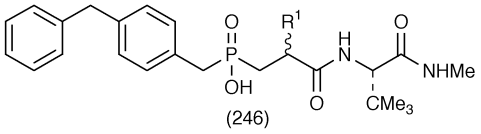

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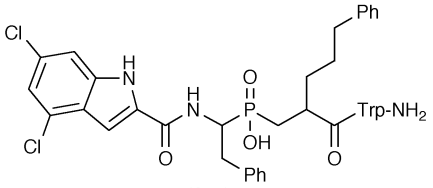

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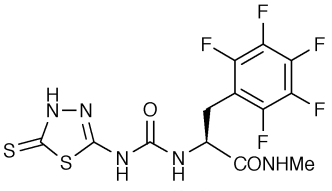

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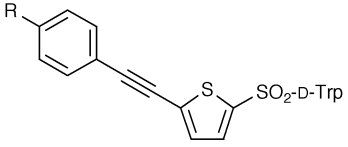

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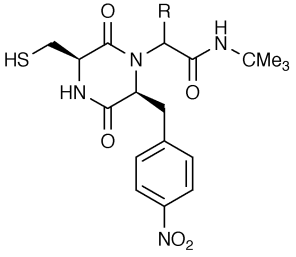

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5.11 Protein Phosphatase Inhibitors (Ser/Thr or Tyr). – Role of protein phosphatases in processes underlying learning and memory formation was highlighted.[570] Protein tyrosine phosphatases were implicated in the negative regulation of insulin signalling. Gene disruption studies indicated that phosphatases like PTP_{1B} may have a role in modulating insulin sensitivity and fuel metabolism and may represent a potential therapeutic target in the treatment of type 2 diabetes and obesity.[571] The insulin resistance caused by overexpression of PTP_{1B} in rat adipose cells (but not PTPα) was reversed by treating the transfected cells with protein tyrosine phosphatase inhibitor (Ac-Asp-Ala-Asp-Glu-F_2Pmp-Leu-NH_2) containing the phosphotyrosyl mimetic difluorophosphonomethyl Phe.[572]

Mca-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-NH_2 (Mca = 7-methoxycoumarin-4-yl)acetyl and DNP = 2,4-dinitrophenyl group) was reported as a fluorogenic substrate for protein tyrosine phosphatases.[573] Inhibitors of protein tyrosine phosphatase were reported.[574–577] Based on the earlier observation that PTP_{1B} contains two proximal aromatic phosphate binding sites, bis(aryldifluorophosphonates) were synthesised. Several of the compounds [$(HO)_2P(O)CF_2$-*p*-$C_6H_4CH_2CH_2CO$-$N(CH_2CH_2NH$-$COCH_2CH_2C_6H_4$-*p*-CF_2-$P(O)(OH)_2)_2$ and $N(CH_2CH_2NH$-$COCH_2CH_2C_6H_4$-*p*-CF_2-$P(O)(OH)_2)_3$] exhibited selectivities for PTP_{1B} *versus* PTPα, LAR, and VHR.[574] Peptides containing two adjacent Phe(CF_2P) residues like Glu-Phe(CF_2P)-Phe(CF_2P) were also potent and selective inhibitor of PTP_{1B}. The tripeptide inhibited PTP_{1B} with an IC_{50} of 40 nM, which was at least 100-fold lower than

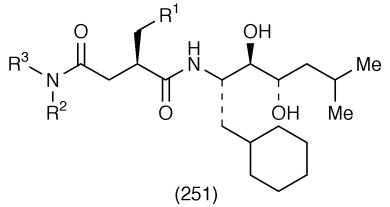
with other PTPs (CD45, PTPβ, LAR and SHP-1). A second tripeptide, Pro-Phe(CF_2P)-Phe(CF_2P), was most potent against PTPβ, with an IC_{50} of 200 nM, and inhibited PTP_{1B} with an IC_{50} of 300 nM.[575] Based on the results from previously reported molecular modelling analyses of the interactions between the inhibitor microcystin and the serine-threonine protein phosphatases 1 and 2A, additional analogues of microcystin LA were synthesised and some were found to be more selective.[578]

5.12 Renin and Other Aspartyl Proteinase Inhibitors. – Various aspects of the renin–angiotensin system were reviewed.[579–581] Scanning mutagenesis was used to identify the amino acids which determine the site selectivity of prorenin cleavage by human cathepsin B *in vitro*. Co-expression assays in AtT-20 cells were used to test for the ability of cathepsin B to cleave human prorenin within cells.[582] N-terminally substituted analogues of pepstatin were synthesised with the aim of forming a covalent bond to various bioadhesive polymers.[583] These analogues [R-CO-Val-Val-NH-CH(CH_2CHMe_2)-CHOH-$(CH_2)_5$-Me (R = Me_3-C-O-, $PhCH_2O$-, $PhCH_2CH_2$- and Me_2-CH-CH_2-)] displayed 10–30-fold reduced-inhibitory activity when compared to pepstatin A. Compounds substituted at the N-terminus by a shorter N-acyl group like propionyl or cyclopropylcarbonyl showed further reduced activity. The presence of an amide or a urethane moiety at the N-terminus had no effect on enzyme inhibition.

A series of non-peptidic renin inhibitors having a 2-substituted butanediamide moiety at the P_2 and P_3 positions were identified.[584,585] Some of the compounds [**251**; R^1 = cyclopropyl, R^2 = H, R^3 = $PhCH^2$-; R^1 = cyclopropyl, R^2 = $PhCH_2$-, R^3 = Me; R^1 = cyclopropyl, R^2 = $PhCH_2$-, R^3 = $Me_2NCH_2CH_2$-] were poor inhibitors of renin (IC_{50}s 450-650 nM). Many other analogues [R^1 = cyclopropyl, R^2 = cyclohexylCH_2-, R^3 = Me_2NCOCH_2-; R^1 = cyclopropyl, R^2 = (*S*)-cyclohexylCHMe, R^3 = Me_2NCOCH_2-; R^1 = 2-thienyl, R^2 = (*S*)PhCHMe, R^3 = Me_2NCOCH_2-] were potent inhibitors of renin (IC_{50}s 10–15 nM). Compound **252** (IC_{50} 1.4 nM) displayed oral activity in a sodium depleted normotensive cynomolgus monkey at a dose of 3 mg kg^{-1}. An analogue of **252** containing a methyl group in place of the 2-PyrCH_2CH_2-group (IC_{50} 0.8 nM) only showed activity at a dose of 10 mg kg^{-1} when given orally. Substituted piperidine derivatives, based on the leads obtained by random screening, were reported as non-peptide inhibitors of renin.[586,587] Tetrahydroquinoline derivative (**253**) (IC_{50} 0.67 nM against recombinant human renin and 37 nM against human plasma renin) displayed potent and long lasting (20 h) blood pressure lowering effects after oral administration (1 and 3 mg kg^{-1}) to sodium-depleted conscious marmosets. The piperidine derivatives also inhibit plasmepsin I and II from *Plasmodium falciparum*.[588]

Inhibitors of the aspartic proteinases plasmepsins I and II from the human malaria parasite *Plasmodium falciparum* are being sought as novel antimalarial agents.[589,590] A number of plasmepsin II aspartyl protease inhibitors were identified using combinatorial chemistry and structure-based design. The best inhibitors (**254**, X = O or NH; K_i 1.3–1.9 nM) showed between 3- and

15-fold selectivity toward plasmepsin II over cathepsin D, the most closely related human protease.[591] A series of 1,3-diamino-2-propanol derivatives [R^4-CONH-CH(R^1)-CH(OH)-CH_2-N(R^2)-CO-R^3] [$R^1 = R^2 = H$; $R^1 = H$, R^2 = isobutyl; R^1 = isobutyl, $R^2 = H$; $R^1 = R^2$ = isobutyl] (*e.g.* **255**) were synthesised on solid phase as potential aspartic acid protease inhibitors.[592]



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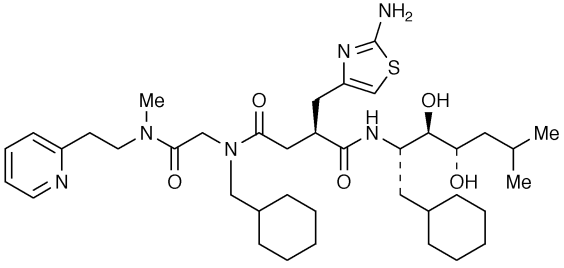

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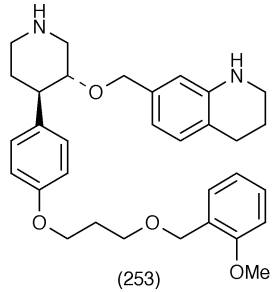

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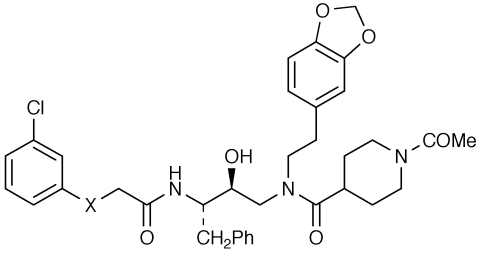

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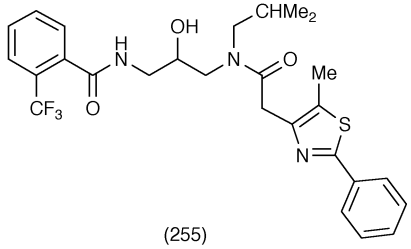

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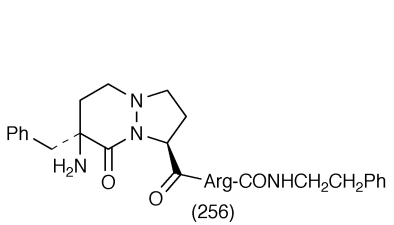
5.13 Thrombin Inhibitors (Serine Protease) and Thrombin Receptor Ligands. – *5.13.1 Thrombin Inhibitors.* A review on synthetic inhibitors of thrombin and factor Xa has appeared.[593] An anti-thrombin peptide (anophelin), isolated from the salivary glands of the mosquito *Anopheles albimanus*, inhibited thrombin-induced platelet aggregation, thrombin esterolytic activity on a synthetic substrate, and thrombin cleavage of fibrinogen.[594] A large number of publications on various structural types of thrombin inhibitors have appeared.[595–623] Examples of some of these structural types are illustrated by compounds **256**–**263**. Using constrained dipeptide mimetics (D-Phe-Pro templates), inhibitors like **256**–**258** containing α-ketoamide or α-ketoheterocycle at the C-terminus were prepared.[595–598] One of the more potent and selective compounds (**257**, IC_{50}s <1 and 590 nM, respectively, against thrombin and trypsin) was active in a rat arterial thrombosis model but did not have much

oral bioavailability. Replacement of the 3-amidinopiperidinealanine **257** by 3-amidinophenylalanine reduced both potency and selectivity (IC_{50}s 57 and 970 nM, respectively, against thrombin and trypsin).[598] Compound **258** based on a piperazinedione template (K_i 1.2 nM; >500-fold less potent against trypsin) showed poor oral bioavailability.[599,600] Amongst the arylsulphonyl derivatives compounds like **259** and **260** were potent inhibitors of thrombin.[604–610] In an *in vivo* rat model of venous thrombosis, **259** inhibited thrombus formation (nearly complete inhibition a dose of 30 mg kg^{-1}) when administered orally (bioavailability 55%, 4 h duration) one hour before induction of stasis.[605] The arylsulfonylpropargylglycinamide derivative **260** (K_i values 5, 19,000, >30,000 nM, >200,000 and >200,000 respectively, against thrombin, factor Xa, trypsin, plasmin and t-PA) also demonstrated oral activity at a dose of 30 mg kg^{-1} in rats.[608]



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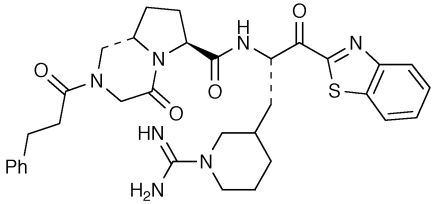

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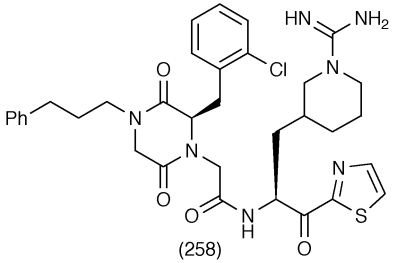

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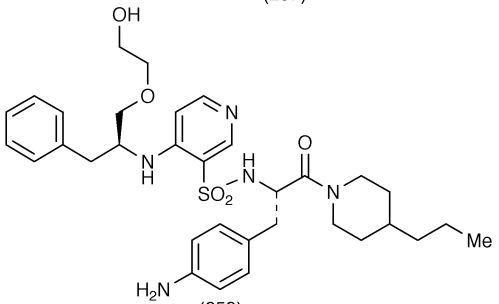

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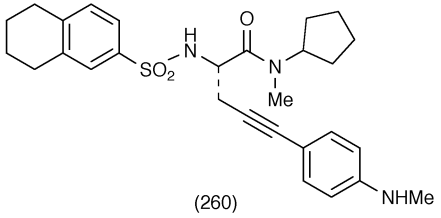

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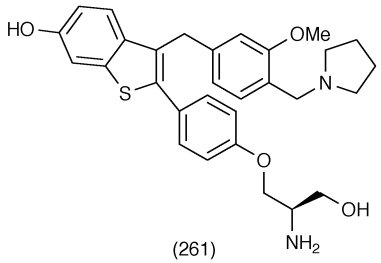
Non-peptide inhibitors of thrombin include compounds based around benzothiophene and other ring systems and cyclic and linear oligocarbamate derivatives.[614–621] Examples of benzothiophene derivatives include compound **261** (K_i 0.4 nM) which demonstrated antithrombotic efficacy in a rat model of thrombosis after infusion at a rate of 0.3–5 mg kg^{-1} h^{-1} (ED_{50} 2.3 mg kg^{-1} h^{-1}).[617] Oligocarbamate thrombin inhibitors were identified through the screening of diverse cyclic trimer, cyclic tetramer, and linear tetramer libraries.

Whereas the cyclic trimer oligocarbamate ligands bound thrombin with modest affinity, a cyclic tetramer (**262**) inhibited thrombin with an apparent K_i of 31 nM. Linear oligocarbamate tetramers bound thrombin with inhibition constants in the 100 nM range.[321]



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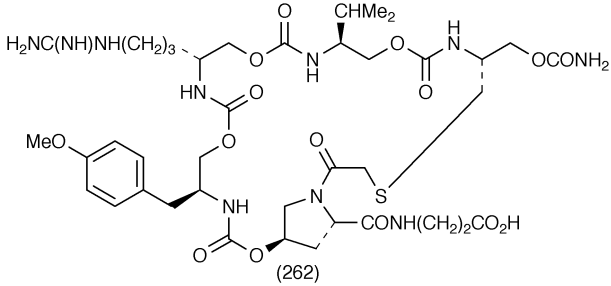

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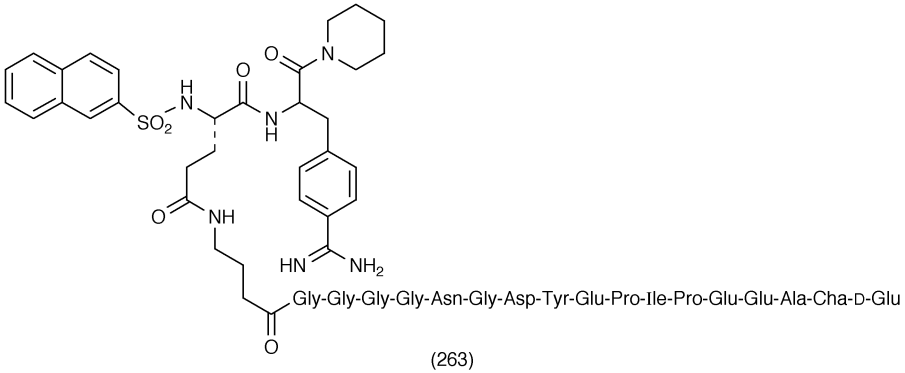
Synthetic bivalent thrombin inhibitors were reported consisting of an active site blocking segment, a fibrinogen recognition exosite blocking segment, and a linker connecting these segments. The bivalent inhibitors bound to the active site and the fibrinogen recognition exosite simultaneously. Various arginyl ketomethylene isosteres Argψ-[CO-CH_2-X]P_1' were incorporated into the bivalent inhibitors as P_1–P_1' segment to eliminate the scissile bond. One of the inhibitors, D-Cha-Pro-Argψ[CO-CH_2-S]Gly-$(Gly)_4$-Asp-Tyr-Glu-Pro-Ile-Pro-Glu-Glu-Tyr-Cha-D-Glu-OH showed the lowest K_i value of 0.35 pM, which was comparable (K_i 0.23 pM) to that of recombinant hirudin.[622] Another bivalent inhibitor consisting of a benzamidine-based active-site-blocking segment, a fibrinogen recognition exosite inhibitor and a peptidic linker connecting these fragments (**263**) was characterised as a slow, tight binding inhibitor of thrombin (K_i 0.29 pM).[623] For **263** a significantly reduced plasma clearance was observed after intravenous injection in rats compared with hirulog-1.

5.13.2 Thrombin Receptor Ligands. The G-protein-coupled receptors stimulated by thrombin (proteinase-activated receptor-1: PAR_1) or by trypsin (PAR_2) are activated by the proteolytic unmasking of anchored N-terminal receptor-activating sequences (Ser-Leu-Ile-Gly-Lys-Val and Ser-Leu-Ile-Gly-Arg-Leu for human and rodent PAR_2 and Ser-Phe-Leu-Leu-Arg and Ser-Phe-Phe-Leu-Arg for human and rodent PAR_1). Short synthetic peptides based on



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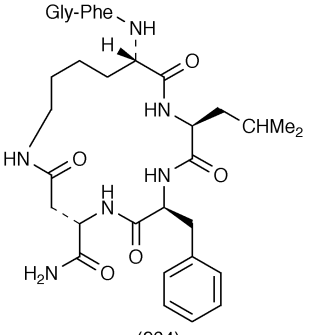
these N-terminal activating sequences can, in isolation, activate either PAR_1 or PAR_2. The PAR_2-activating peptides can mimic the action of trypsin in activating PAR_2, but they are unable to activate the PAR_1 thrombin receptor. In contrast, thrombin receptor-activating peptides derived from the human PAR_1 receptor sequence (*e.g.* Ser-Phe-Leu-Leu-Arg-NH_2) have been observed to activate both PAR_1 and PAR_2. Work on additional thrombin receptor agonist and antagonist ligands has been published.[624–632] The PAR_1/PAR_2 selectivity of various compounds was investigated.[625] Thr-Phe-Leu-Leu-Arg-NH_2, Ala-Phe(*p*-F)-Arg-Cha-hArg-Tyr-NH_2, *trans*-cinnamoyl-Phe(*p*-F)-Arg-Leu-Arg-Orn-NH_2 and Ala-Phe(*p*-F)-Arg-Cha-Cit-Tyr-NH_2 were most selective for the PPR_1 receptor (IC_{50}s 0.14–2.5 and 17–>100 μM at PAR_1 and PAR_2 receptors, respectively). Some other peptides, *e.g.* Ser-Phe-Leu-Leu-Arg-NH_2, Ser-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-Phe-NH_2 and Mpr-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2 (IC_{50}s 2.2–17 and 8.7–33 μM at PAR_1 and PAR_2 receptors, respectively), were nearly equipotent at both the receptors. Platelet aggregation activities of the above compounds were compared with the PAR_1 selective activities of the above compounds. All peptides except Mpr-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2 (up to 20 μM) and Met-Ser-Arg-Pro-Ala-Cys-Pro-Asn-Asp-Lys-Tyr-Glu were platelet agonists, with EC_{50}s in the range of 0.1–10 μM. Mpr-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2 was an inhibitor of both thrombin-mediated and Ser-Phe-Leu-Leu-Arg-NH_2-mediated platelet aggregation. Both Mpr-Phe-Cha-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH_2 and Met-Ser-Arg-Pro-Ala-Cys-Pro-Asn-Asp-Lys-Tyr-Glu proved to be poor antagonists of Ser-Phe-Leu-Leu-Arg-NH_2 in the platelet aggregation assay (IC_{50} > 200 μM).[625]

Macrocyclic peptide analogues of Ser-Phe-Leu-Leu-Arg-Asn were synthesised and evaluated *in vitro*. In general, the compounds were much less potent in inducing platelet aggregation relative to Ser-Phe-Leu-Leu-Arg-Asn-NH_2 and did not act as antagonists of α-thrombin.[626] Derivative **264** was the most potent macrocycle in activating PAR_1 (EC_{50} 24 μM). Replacement of the Gly-Phe residues by Gly-Phe(3-F), β-Ala-hPhe and 4-Abu-hPhe had a small effect on the agonist activity (EC_{50}s 37–63 μM). Other dipeptide containing analogues (β-Ala-Phe, 4-Abu-Phe, Ser-Phe, β-Ala-Phe(3-F), 4-Abu-Phe(3-F) and

Ser-Phe(3-F) were inactive at a concentration of 50 μM. Analogues of **264** containing various dipeptides along with an Arg residue in place of the Phe residue in the ring structure were inactive (5–16% platelet aggregation at 50 μM). Only the analogues containing an Ala-Phe or Gly-Phe(4-F) dipeptide along with the ring arginine showed agonist activity (EC_{50}s 60–66 μM). A series of heterocycle-peptide hybrids composed of a tripeptide segment (*e.g.* Cha-Arg-Phe) and an N-terminal heterocyclic group also behaved as full PAR_1 agonists.[627] Aminotriazole derivatives like **265** and the corresponding analogue containing Phe in place of Cha were nearly as potent as Ser-Phe-Leu-Leu-Arg-Asn-NH_2 in inducing platelet aggregation (EC_{50} 0.7–1 μM). The aminotriazole moiety could be replaced with other substituted heterocycles while maintaining agonist potency. Some such compounds exhibited mixed PAR_1 agonist-antagonist activity. Photoactivatable analogues of the thrombin receptor antagonist, *N-trans*-cinnamoyl-Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2, were prepared with benzophenone substitutions at the N-terminal, Leu, or Arg positions.[628] The analogues [**266**; R^1 = *trans*-cinnamoyl, benzoyldihydrocinnamoyl; R^2 = Leu-Arg, Bpa-Arg (Bpa = 4-benzoylphenylalanine), Leu-



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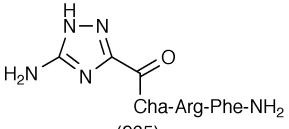

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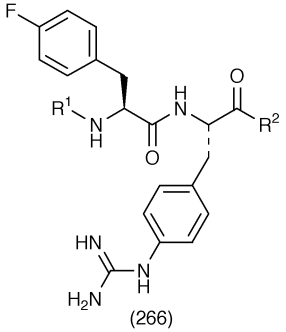

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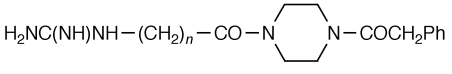

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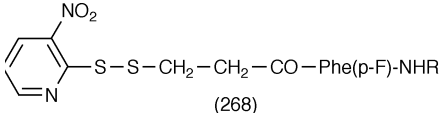

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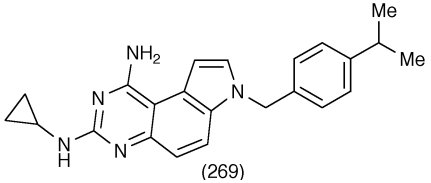

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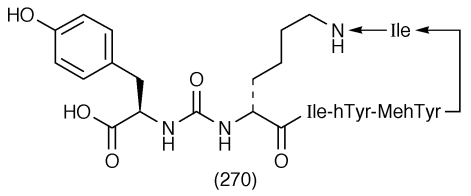
Bpa, Bpa-Arg-Orn, Bpa-Arg-Orn(propionyl), Bpa-Arg-Orn(biotinyl), Leu-Bpa-Orn, Leu-Bpa-Orn(propionyl), Leu-Bpa-Arg-Orn(biotinyl)] retained antagonist activity (IC_{50}s 0.3–13 μM). C-Terminal extension of the analogues with ornithine(biotin) did not alter antagonist potency. The photoaffinity analogues inhibited Ser-Phe-Leu-Leu-Arg-Asn-NH_2-induced platelet aggregation. Examples of other antagonists include Ser-Phe(p-F)-Aad-Leu-Arg-Asn-Pro-NH_2 (IC_{50} 115 μM), and compounds like **267**, **268** [R = -$CH(C_6H_5)_2$ and -CH_2-CH-$(C_6H_5)_2$] and **269**.[629–632] Amongst the 1,4-disubstituted piperazines (**267**, $n = 1$, 2, 3 and 5) carrying features of Phe and Arg residues present in the pentapeptide Ser-Phe-Leu-Leu-Arg, compound **267** ($n = 5$) was the most potent compound (EC_{50} 150 μM) in a rat aorta relaxation assay. The nonpeptide did not show agonist activity and inhibited thrombin and Ser-Phe-Leu-Leu-Arg-NH_2-induced but not the collagen-induced platelet aggregation.[630]

5.14 Miscellaneous [Aggrecanase, Carboxypeptidase, Dipeptidyl-peptidase, Prolyl Endopeptidase, Protein Tyrosine $K_i$nase, Serine Proteases Including Chymase and Tryptase] Inhibitors. – Proteolytic cleavage of the aggrecan core protein (a key event in arthritic diseases) is believed to be mediated by a putative proteinase, aggrecanase. Various attempts have been made to purify, characterise and clone the enzyme.[633–637] Anabaenopeptins G (**270**, Ile carboxyl linked to D-Lys side chain amino group) and H (**270**, Tyr residue outside the ring replaced by Arg) were isolated from the cultured cyanobacterium *Oscillatoria agardhii* (NIES-595) as potent carboxypeptidase A inhibitors.[638] 2-Benzyl-2-methylsuccinic acid and several hydroxamate derivatives were identified as inhibitors for carboxypeptidase A.[639,640] In the hydroxamate series of compounds, N-formyl-N-hydroxy-β-Phe derivatives [HCO-N(OH)-CH_2-CH(CH_2Ph)-COOH, $PhCH_2CH_2CO$-N(OH)-CH_2-CH(CH_2Ph)-COOH and $PhCH_2CH_2CH_2CO$-N(OH)-CH_2-CH(CH_2Ph)-COOH] were moderately potent inhibitors of carboxypeptidase (K_i 0.98–1.2 μM). The corresponding phenylacetyl analogue, $PhCH_2CO$-N(OH)-CH_2-CH(CH_2Ph)-COOH, was somewhat more potent (K_i 0.32 μM). All the other analogues containing an acetyl, propionyl and benzoyl groups were less potent (K_i 4.9–6.5 μM). Thiocarbamate inhibitors for carboxypeptidase G_2 [*e.g.* *N*-(p-methoxybenzenethiocarbonyl)amino-L-Glu were used to investigate *in vitro* antibody-directed enzyme prodrug therapy approaches.[641]

A general solid-phase method for the preparation of mechanism-based cysteine protease inhibitors was reported.[642] Some pseudo-peptide analogues of thiol proteinase inhibitors were reported.[643] Among them, Suc-Ala-Val-Val-Alaψ(CH_2-NH)Ala-*p*NA and Suc-Ala-Val-Val-ψ(CH_2NH)-Ala-Ala-*p*NA showed a stronger inhibitory activity compared with parent peptide such as Suc-Ala-Val-Val-Ala-Ala-*p*NA. Cysteine proteases are being considered as targets for the development of new antiparasitic chemotherapy.[644,645] A series of vinyl sulfones like **271** inhibited cysteine protease falcipain and parasite biological activities *in vitro*. The *N*-methyl piperazine urea derivative **271** showed activity in an *in vivo* model when administered orally twice-a-day for



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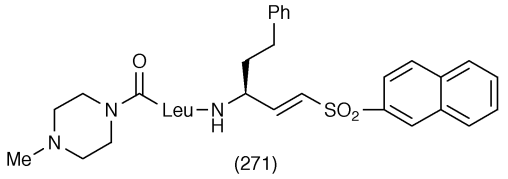

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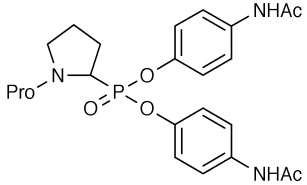
four days. A number of other inhibitors of cysteine proteases have been reported.[646–649]

Inhibitors of dipeptidyl peptidase IV (*e.g.* **272** and **273**) were reported.[650,651] Compound **273** was one of the more selective inhibitors of a series [IC_{50}s 57.8 and >309.2 μM respectively, against DPP-IV and aminopeptidase N], while the others also showed inhibitory activity toward aminopeptidase N. Solution-phase automated parallel synthesis of a Tic-based library (2560 members) was used to identify inhibitors of a parasitic prolyl endopeptidase secreted by *Trypanosoma cruzi*. Pyrrolidine derivatives like **274** (IC_{50} 9 nM) proved the most potent inhibitor.[652] Several other analogues with different N-terminal substituents were less potent (IC_{50}s 21–55 nM).



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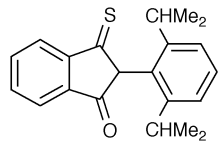

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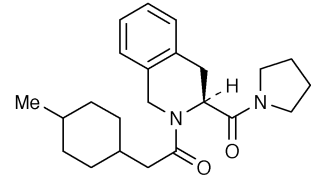

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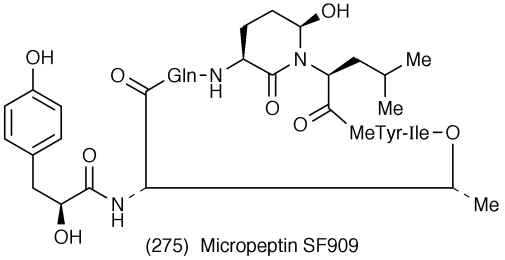
Reviews on various aspects of protein kinases have appeared.[653–658] Peptide and non-peptide inhibitors of protein kinase (tyrosine, serine and threonine) inhibitors have been reported.[659–667] Myristoylation of peptides was used as an approach to enhances their ability to cross intact plasma membranes and thus inhibit intracellular protein kinases. Using insulin-secreting β-cells, it was demonstrated that myristoylation alters the specificity of pseudosubstrate peptides such that all myristoylated peptides tested [Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile and Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile] acted as protein kinase C inhibitors.[659] Four lactam bridge constrained analogues [Glu-Asp-c(Glu-Glu-Tyr-Thr-Lys), Glu-Asp-c(Asp-Glu-Tyr-Thr-Orn), Glu-Asp-c(Glu-Glu-Tyr-Lys)-Ala and Glu-Asp-c(Asp-Glu-Tyr-Orn)-Ala] were screened for their suitability as c-Fgr and Syk tyrosine kinase substrates. In general cyclisation decreased the peptide phosphoryl-ability; however, the sequence containing the greatest lactam ring, Glu-Asp-

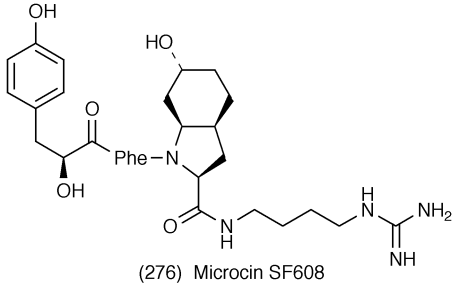
c(Glu-Glu-Tyr-Thr-Lys), resulted in a selective substrate for Syk tyrosine kinase.[660] A nonapeptide (Arg-Lys-Lys-Tyr-Lys-Tyr-Arg-Arg-Lys-NH_2), obtained by combinatorial approaches, was shown to be a selective inhibitor of myosin light chain kinase (IC_{50} 50 nM, inhibited calmodulin-regulated kinase II only at 4000-fold higher concentrations and did not inhibit cyclic AMP-dependent protein kinase).[662] Analogues of the peptide containing conformationally constrained *cis*-4-aminocyclohexanecarboxylic acid (Ach) at positions 4, 5 and 6 and multiple combinations of these positions were also evaluated as myosin light chain kinase and calmodulin-regulated kinase II inhibitors. Peptide Arg-Lys-Lys-Tyr-Ach-Tyr-Arg-Arg-Lys-NH_2 was as active and selective as the parent peptide. A peptide with two Ach residues at positions 5 and 6 (Arg-Lys-Lys-Tyr-Ach-Ach-Arg-Arg-Lys-NH_2) was about 3-fold less potent. Other peptides containing Ach residues at position 4 [Arg-Lys-Lys-Ach-Lys-Tyr-Arg-Arg-Lys-NH_2, Arg-Lys-Lys-Ach-Ach-Tyr-Arg-Arg-Lys-NH_2, Arg-Lys-Lys-Ach-Lys-Ach-Arg-Arg-Lys-NH_2 and Arg-Lys-Lys-Ach-Ach-Ach-Arg-Arg-Lys-NH_2)] were much less potent. Inhibitors of cyclin-dependent kinase were reported.[663–665] In a series of $p21^{Waf1/Cip1}$ peptide fragments, the most potent peptide [Gly-Arg-Lys-Arg-Arg-Gln-Thr-Ser-Met-Thr-Asp-Phe-Tyr-His-Ser-Lys-Arg-Arg-Leu-Ile-Phe-Ser-Lys-Arg-Lys-Pro] bound to proliferating cell nuclear antigen and inhibited cyclin-dependent kinase activity. Some of the analogues containing a single Ala substitution were similar in potency to the parent peptide, but analogues with multiple Ala replacements were less potent. The peptide chemically linked to an antennapedia peptide (to improve cell permeability) exhibited growth inhibition that resulted from necrosis in human lymphoma CA46 cells. In another series of compounds, peptides linked to either HIV Tat or the Antennapedia homeodomain protein (penetratin) [Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Pro-Val-Lys-Arg-Arg-Leu-Asp-Leu and Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Pro-Val-Lys-Arg-Arg-Leu-Phe-Gly] killed U2OS osteosarcoma cells in a dose-dependent fashion.[665]

Trypsin and chymotrypsin inhibitors, micropeptins SF909 (**275**), SF995 and microsin SF608 (**276**), were isolated from the hydrophilic extract of a *Microcystis* sp. waterbloom.[668] Micropeptin SF909 inhibited chymotrypsin (IC_{50} of 4.0 μg ml^{-1}) while micropeptin SF995 and microsin SF608 inhibited trypsin (IC_{50}s 0.2–0.5 μg ml^{-1}). Phenylalanine derivative **277** inhibited α-chymotrypsin, trypsin, human cathepsin G and porcine elastase [IC_{50}s 2.6, 540, 8.0 and 7.0 nM, respectively].[669] The D-Leu derivatives like **278** gave time-dependent irreversible inhibition of α-chymotrypsin and other serine proteases.[670] Analogues with the (*R*)-configuration at C3 (**278**) were the most potent against α-chymotrypsin. The corresponding (*S*)-analogue was less potent against α-chymotrypsin, but was more potent against elastase. Replacement of amide bonds in peptides by sulfonamide moieties resulted in peptidosulfonamides with an increased stability towards pepsin, trypsin and Carlsberg C subtilisin catalysed degradation.[671] Half-lives of compounds Tyr-Gly-Gly-Phe-Leuψ(CH_2SO_2)-NH_2, Tyr-Gly-Gly-Pheψ(CH_2SO_2NH)Leu-NH_2, Tyr-Gly-Glyψ(CH_2SO_2NH)Phe-Leu-NH_2, Tyr-Glyψ(CH_2SO_2NH)Gly-Phe-Leu-NH_2, Tyr-

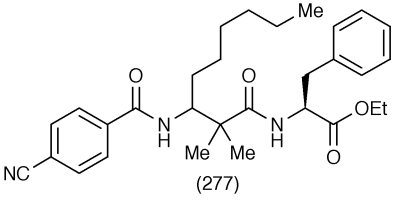
Gly-Gly-Pheψ(CH_2SO_2NH)Leu-OH, Tyr-Gly-Glyψ(CH_2SO_2NH)Phe-Leu-OH and Tyr-Glyψ(CH_2SO_2NH)Gly-Phe-Leu-OH, were between 1.5 to >7 hours when treated with pepsin. In comparison to the unsubstituted analogue Asp-Phe-Gly-Asn-Lys-Thr-Phe-Gly-Tyr, the sulfonamide analogues Asp-Phe-Gly-Asn-Lys-Thr-Pheψ(CH_2SO_2NH)Gly-Tyr and Asp-Pheψ(CH_2SO_2NH)Gly-Asn-Lys-Thr-Pheψ(CH_2SO_2NH)Gly-Tyr were much more stable against trypsin and Carlsberg subtilisin.



(275) Micropeptin SF909



(276) Microcin SF608



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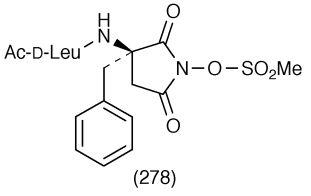

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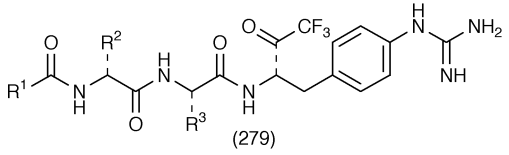
Inhibitors of human plasma and tissue kallikrein were reported.[672–675] Trifluoromethylketone inhibitors like **279** (R^1 = adamantyloxy, t-butyloxy or morpholine; R^2 = t-butyl, isopropyl, benzyl, phenethyl; R^3 = benzyl, 2-naphthyl or H) inhibited plasma kallikrein, plasmin and tissue kallikrein. The IC_{50} values for **279** (R^1 = adamantyloxy, R^2 = t-butyl and R^3 = benzyl) against kallikrein, plasmin and tissue kallikrein were 0.002, 0.12 and 47.3 μM, respectively. Compound **279** (R^1 = morpholine, R^2 = t-butyl and R^3 = benzyl) was one of the more selective inhibitors (IC_{50}s 0.028, 0.56 and >2500 μM, respectively, against plasma kallikrein, plasmin and tissue kallikrein).[672] Pseudo-peptide analogues of a plasma kallikrein selective inhibitor PKSI-527 (**280**) containing CH_2-NH amide bond replacements did not exhibit any detectable inhibitory activity against plasma kallikrein, plasmin, urokinase,

thrombin or trypsin.[674] A number of chymase and tryptase inhibitors were reported.[676–680] Chymase inhibitors like **281** (R^1 = 4-CN-Ph and R^2 = 2-phenylethyl) inhibited human chymase, α-chymotrypsin, human cathepsin G, porcine pancreatic elastase and porcine pancreatic trypsin (IC_{50}s 270, 2.1, 1.4, 36 and 300 nM, respectively). In comparison, **281** (R^1 = Ph and R^2 = 2-phenylethyl) was more potent against chymase and lost activity against all the other enzymes (IC_{50}s 20, 18, 64, 64 and 2700 nM, respectively, against human chymase, α-chymotrypsin, human cathepsin G, porcine pancreatic elastase and porcine pancreatic trypsin). Symmetrical bisbenzamidines able to bridge two adjacent active sites were explored as human lung tryptase inhibitors. The most potent compounds [**282**, n = 5 or 6] (K_i values <0.01 nM) showed >100,000-fold selectivity when tested against trypsin and plasmin. The corresponding sulfonamide derivatives (both amide bonds replaced by -SO_2NH-) were much less potent (K_i values 250–3150 nM). In comparison to the *p*-substituted benzamidines, the compounds containing *m*-substituted benzamidines were also much less potent.[679]



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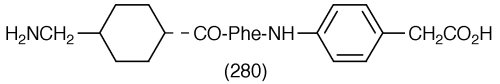

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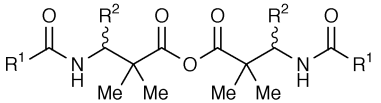

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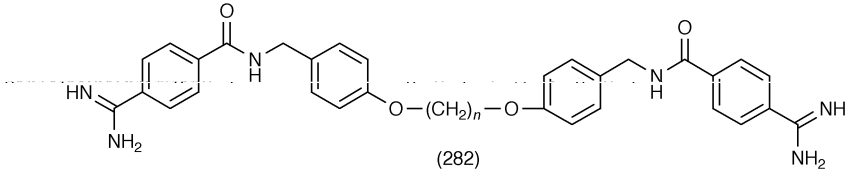

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6 Phage Library Leads

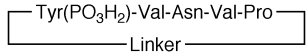
The use of phage display technology was reported in the identification of peptide binding sites.[681] Using this methodology, the receptor for the Cys-Gly-Phe-Glu-Cys-Val-Arg-Gln-Cys-Pro-Glu-Arg-Cys (potentially containing two disulfide bonds) peptide which binds to mouse lung vasculature after an iv injection was shown to be a membrane dipeptidase, a cell-surface zinc metalloprotease involved in the metabolism of glutathione, leukotriene D_4, and certain β-lactam antibiotics. By screening phage display libraries, a set of peptides which bind 14-3-3 proteins (involved in various signal transduction pathways controlling cell proliferation, transformation, and apoptosis) was identified.[682] One of the peptides, Pro-His-Cys-Val-Pro-Arg-Asp-Leu-Ser-Trp-

Leu-Asp-Leu-Glu-Ala-Asn-Met-Cys-Leu-Phe, exhibited a high affinity for different isoforms of 14-3-3 with estimated K_D values of $7–9 \times 10^{-8}$ M. Ganglioside binding peptides were obtained from a phage-displayed pentadecapeptide library.[683] Three synthetic pentadecapeptides [Asp-Phe-Arg-Arg-Leu-Pro-Gly-Ala-Phe-Trp-Gln-Leu-Arg-Gln-Pro, Gly-Trp-Trp-Tyr-Lys-Gly-Arg-Ala-Arg-Pro-Val-Ser-Ala-Val-Ala and Val-Trp-Arg-Leu-Leu-Ala-Pro-Pro-Phe-Ser-Asn-Arg-Leu-Leu-Pro] inhibited the binding of cholera toxin B subunit to the GM1 monolayer with an IC_{50}s 24, 13 and 1.0 μM, respectively. Publications on the use of phage libraries in the production of antibodies have appeared.[684,685]

7 Protein–Protein Interaction Inhibitors

7.1 SH2 and SH3 Domain Ligands. – Using an NMR-based screen, a series of novel phosphotyrosine mimetics [aromatic compounds containing one or two carboxyl groups (K_D values 1–12 mM compared to 0.3–0.4 mM for pTyr and Ac-pTyr-OEt)] were discovered that bind to the SH2 domain of Lck.[686] Based on a phage library based non-phosphorylated disulfide linked 11-mer peptide lead, thioether cyclised and backbone cyclised peptides [Cys-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys-NH_2 (disulfide bridge), Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys(CH_2CO-)-NH_2 [Cys(CH_2CO-) linked to N-terminal Glu amino group], Gly-Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys (CH_2CO-)-NH_2 [Cys(CH_2CO-) linked to N-terminal Glu amino group] and c(Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr)] were synthesised.[687] The thioether peptide Glu-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys(CH_2CO-)-NH_2 showed equipotent binding affinity for the Grb2-SH2 domain (IC_{50} 10–15 mM) when compared to the parent peptide. The N-terminal glycine extended thioether and backbone cyclised analogues were inactive. Replacement of the Glu residue in the above non-phosphorylated cyclic peptide, c(CH_2CO-X-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-NH_2, by N^{α}-aminoadipic acid or γ-carboxyglutamic acid resulted in 5–30-fold improvement in potency.[688] The most potent peptide of the series, c(CH_2CO-Gla-Leu-Tyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-NH_2 (IC_{50} 640 nM), was 25–30-fold less potent than the phosphorylated tyrosine-containing cyclic peptide c(CH_2CO-Ala-Leu-pTyr-Glu-Asn-Val-Gly-Met-Tyr-Cys)-NH_2 (IC_{50} 23 nM). Cyclic peptides with high affinity and specificity toward the SH2 domain of the growth factor receptor-binding protein Grb2 were also obtained in a series exemplified by **283**. Replacement of the cystine in the cyclic heptapeptide cyclo(Cys-pTyr-Val-Asn-Val-Pro-Cys) [**283**, linker group = -Cys-SS-Cys-] (IC_{50}s 0.06 μM against Grb2-SH2 and 38 μM against Src-SH2) by D-α-acetylthialysine or D-α-lysine gave c(pTyr-Val-Asn-Val-Pro-(D-α-acetyl-thiaLys)) and c(pTyr-Val-Asn-Val-Pro-(D-α-acetyl-Lys)), which showed 10-fold improved binding relative to that of the control peptide Lys-Pro-Phe-pTyr-Val-Asn-Val-Glu-Phe.[689] The D- and the corresponding L-thialysine analogues were similar in potency (IC_{50}s 0.06–0.11 μM against Grb2-SH2 and 55–88 μM against Src-SH2). In comparison to Ac-

(thiaLys-pTyr-Val-Asn-Val-Pro) (Lys side chain linked to Pro carboxyl), the cyclic peptide containing a Lys residue in place of the thialysine was much less potent against Src-SH2 domain binding (IC_{50}s 0.07 μM against Grb2-SH2 and >150 μM against Src-SH2).



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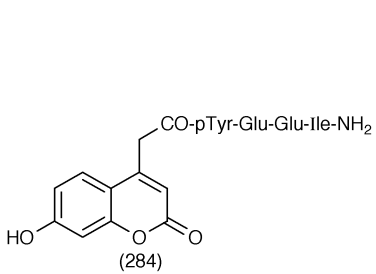
Using a combinatorial library approach, compounds like **284** were designed to target the SH2 domain of Lck[1] and Fyn[1], Src tyrosine kinase family members known to participate in T cell activation. The affinity of compound **284** (K_Ds 35 and 150 nM, respectively, for Lck and Fyn SH2 domain) was the highest.[690] It displayed significantly weaker affinity for the SH2 domains of PLCγ1 (K_D 4.9 μM), the p85α subunit of PI3 kinase (K_D 9.3 μM) and Grb2[1] (K_D 11.3 μM). SAR studies in a series of phosphopeptides containing α,α-disubstituted cyclic α-amino acids (Ac_nc, 3–7 carbons in the ring) at the X_{+1} position of the minimal recognition motif of Grb-SH2 [Ac-Tyr(PO_3H_2)-X_{+1}-Asn-NH_2] showed Ac-Tyr(PO_3H_2)-Ac_nc-Asn-NH_2 ($n = 6$ or 7) to be the most potent.[691] Other compounds of similar structure [mAZ-pTyr-Ac_6c-Asn-NH_2 (IC_{50} 120 nM), mAZ-pTyr-pTyr-Asn-NH_2 (IC_{50} 235 nM), mAZ-pTyr-(α-Me)pTyr-Asn-NH_2 (IC_{50} 11 nM), mAZ-pTyr-Tyr-Asn-NH_2 (IC_{50} 497 nM), mAZ-pTyr-(α-Me)Tyr-Asn-NH_2 (IC_{50} 1098 nM), mAZ-pTyr-(α-Me)Phe(4-COOH)-Asn-NH_2 (IC_{50} 153 nM), mAZ-pTyr-(α-Me)Phe(4-CH_2-COOH)-Asn-NH_2 (IC_{50} 198 nM)] were also reported.[692] Additional changes at the N-terminal and Asn positions led to compounds like **285**–**287**.[693–701] In comparison to the indol-1-yl-propylamine derivative (**286**, R = H, IC_{50} 1.2 nM),



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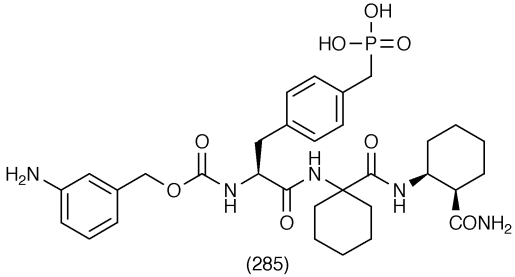

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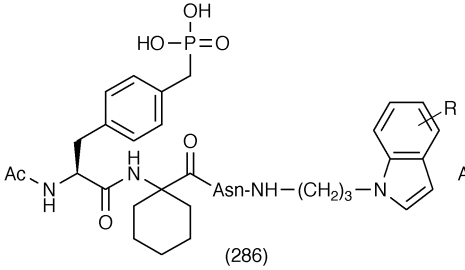

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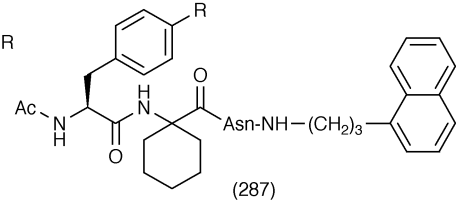
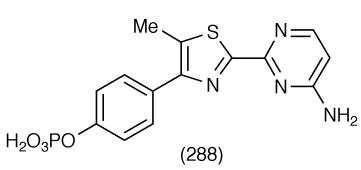


(287)

substituted analogues (R = 5-Me, 5-OH and 5-OMe) were marginally more potent (IC_{50}s 0.3–0.9 nM). Other analogues (R = 2-Me, 3-Me, 5-Cl and 5-NMe_2) were less potent (IC_{50}s 3.4–81 nM) in inhibiting the binding of the phosphorylated carboxy-terminal intracellular domain of EGF receptor to the Grb2-SH2. Replacement for the phosphotyrosyl residue in **287** [R = -$CH_2P(O)(OH)_2$] by 4-(O-carboxymethyl)-Tyr residue (R = -OCH_2COOH) led to a 1000-fold loss in potency (IC_{50} 65 μM). However, the carboxymethyl-Phe analogue (R = -CH_2COOH) (IC_{50} 2.5 μM) was only about 50-fold less potent. The 4-carboxydifluoromethyl-Phe analogue (R = -CF_2COOH) was also less potent (IC_{50} 28 μM). Replacement of the N^{α}-acetyl group by N^{α}-oxalyl group in these compounds enhanced binding affinity 4–10-fold.

A number of publications on non-peptidic inhibitors of SH2 domain interaction have appeared.[702–709] Examples of the non-peptidic compounds include structures **288**–**292**. The IC_{50} value for compound **288** was 25.9 μM. In comparison, the IC_{50} values for Ac-pTyr-Gly-Asn-NH_2 and Ac-pTyr-Val-Asn-NH_2 were 67 and 4.32 μM, respectively.[702] Analogues of **289** (IC_{50} 7 μM) with different substituents in place of the $(CH_2)_6Me$ group and Gln and Trp side chains in place of the Glu side chain were less potent (IC_{50} 20–300 μM).[704] A series of 1,2,4-oxadiazole analogues were potent and selective SH2 inhibitors of the tyrosine kinase ZAP-70, a potential therapeutic target for immune suppression.[705,706] Compound **290** was the most potent of the series (IC_{50} 1 μM). Analogues containing other groups in place of -CH_2-(2-naphthalene) and Ala and Gln side chains in place of the Ser (CH_2OH) side chain were less potent (IC_{50} 3–100 μM). This series of compounds showed selectivity (>50-fold) over the closely related tyrosine kinase Syk, as well as other SH2-containing proteins such as Src and Grb2. The Tyr moiety in the series could be replaced with non-peptidic functional groups (*e.g.* **291**) without a substantial loss of binding affinity [IC_{50}s 4–7 μM against ZAP-70, >500 μM against Syk and 54–210 μM against Src].[707] The 1,4-benzodiazepine derivative **292**, obtained by screening a diverse combinatorial library against protein tyrosine kinases Src, Yes, Abl, Lck, Csk, and fibroblast growth factor receptor, had an IC_{50} of 73 μM against Src, 2- to 6-fold lower than against other protein tyrosine kinases.[708] The inhibitor was found to be non-toxic to the AFB-13-human fibroblasts cells and inhibited the colony formation of HT-29 colon adenocarcinoma cells that are dependent on Src activity.

With the aim of interrupting the growth factor-stimulated Ras signalling pathway at the level of the Grb2-Sos interaction, a peptidimer, made of two identical proline-rich sequences from Sos linked by a lysine spacer, was designed using structural data from Grb2 and a proline-rich peptide complexed with its SH3 domains.[709] The peptidimer (**293**) showed high affinity for Grb2 (K_D 40 nM) whereas the monomer (Val-Pro-Pro-Pro-Val-Pro-Pro-Arg-Arg-Arg-Lys) was much less potent (K_D 18 μM). The control peptide in which one of the peptides was replaced by a scrambled proline rich sequence was also much less potent (K_D 16 μM). For *in vivo* studies, **293** was coupled at the C-terminus of the lysine residue to a 16 amino acid-long peptide (Ahx-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys) corresponding



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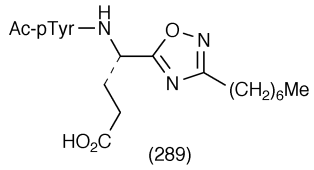

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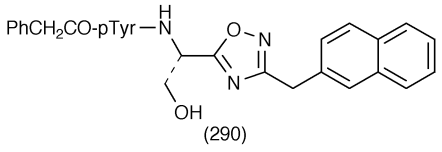

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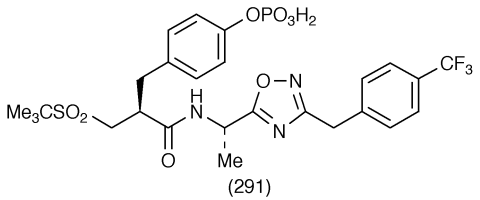

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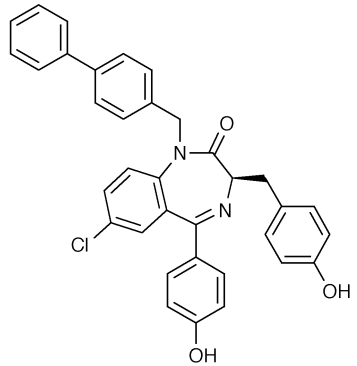

(292)

Val-Pro-Pro-Pro-Val-Pro-Pro-Arg-Arg-Arg—Lys-OH
Val-Pro-Pro-Pro-Val-Pro-Pro-Arg-Arg-Arg—

(293)

to the third helix of the homeodomain of Antennapedia which has been shown to deliver biologically active peptides inside living cells by a non-receptor-derived process. At 10 μM, the conjugate inhibited the Grb2-Sos interaction (100%) and MAP kinase (ERK1 and ERK2) phosphorylation (60%) without modifying cellular growth of ER 22 cells. At the same concentration, the conjugate also inhibited both MAP kinase activation induced by NGF or EGF in PC12 cells, and differentiation triggered by NGF. When tested for its antiproliferative activity, the conjugate was an efficient inhibitor of the colony formation of transformed NIH3T3/HER2 cells grown in soft agar, with an IC_{50} of around 1 μM.

8 Advances in Formulation/Delivery Technology

The stability of peptides and proteins in the solid-state is an important aspect of formulation. Reactions can occur in the solid-state, leading to degradation and inactivation of these agents. A review has been published summarising the major chemical reactions affecting proteins and peptides in the solid-state.[710]

An asparagine-containing hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) was shown to be deamidated in lyophilised poly(vinyl alcohol) and poly(vinyl pyrrolidone) polymers at 50 °C. The rate of Asn-hexapeptide deamidation increased with increasing water content.[711,712] The major degradation products were isoAsp, Asp and cyclic imide (Asu) hexapeptides. The dominance of isoAsp and Asu indicated the formation of the cyclic imide as the major route of deamidation. The cyclic imide derivative was rapidly hydrolysed to generate isoAsp and Asp hexapeptides. Lipid-based delivery systems for the transdermal and dermal delivery of protein pharmaceuticals were reviewed.[713] Lipoamino acid and liposaccharide conjugates of a somatostatin analogue (TT-232) were synthesised to modify the physicochemical properties of the parent peptide. Experiments *in vitro* showed that many compounds modified at the N- and/or C-terminus with lipid or sugar moieties retained the biological activity of the parent compound. However, only in one case, improved absorption of the peptide conjugate across Caco-2 cell monolayers was observed.[714] Work on biodegradable poly(lactic/glycollic) and poly(4-hydroxy-l-proline ester) polymers has been published.[715–717] A copoly (D/L-lactic/glycollic acid) polymer was shown to consist of random sequences of lactic and glycollic acid by using fast atom bombardment (FAB)-tandem mass spectrometry.[715] N-terminal 4-imidazolidinone prodrugs of Leu-enkephalin and coumarin-based esterase-sensitive cyclic prodrugs of Gly-Arg-Asp peptidomimetic analogues were reported.[718,719] Orally bioavailable formulations of insulin have been reported.[720–722]

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4
Cyclic, Modified and Conjugated Peptides

BY JOHN S. DAVIES

1 Introduction

The major source of listed papers covering the calendar year 1999 has again been CA Selects[1] on Amino Acids, Peptides and Proteins (up to Issue 12, June 2000). Although this source lists abstracts of relevant conference reports, such as those of the 15th American Peptide Symposium,[2] the compilation in this review has adhered strictly to refereed papers and articles. Comprehensive coverage has again been enhanced by scanning the index pages of Journals, either manually or *via* the Web of Science databases[3] on the Internet.

Glycopeptides and cyclodepsipeptides continue to generate the longest subdivisions of this chapter. In last year's Report,[4] two major reports on the total synthesis of vancomycin were included. Further reports of total synthesis have appeared this year again and are reviewed under the glycopeptide antibiotics section. A general review covering significant areas of this Report has also been published.[5]

2 Cyclic Peptides

2.1 General Considerations. – Cyclisation yields often depend on the size of ring being formed and the amino acids in the linear precursor. However, a new ring closure/ring contraction process[6] has even allowed the synthesis of cyclo(Ala-Phe-Leu-Pro-Ala) which has not been possible by conventional protocols. The sequence in Scheme 1 summarises the intramolecular ring contraction process which is key to the use of the photolabile auxiliary group. In a model cyclopentapeptide cyclisation utilising pentafluorophenyl esters for activation, it has been revealed[7] that addition of HOAt increases the reaction rate and decreases oxazolone formation, which is a source of the epimerisation of the C-terminal residue. The efficiency of HOAt-based coupling agents, however, seems to have been exceeded[8] in examples involving sterically hindered amino acids by 2-propanephosphonic anhydride (T3P) (1). Head to tail cyclisation on solid phase has utilised[9] Kenner's safety catch sulfonamide linker, which obviates the need for anchoring at the side-chains. Key features of the method are summarised in Scheme 2. In the synthesis of MK-678 cyclo(MeAla-Tyr-D-Trp-Lys-Val-Phe), the best yield (52%) involved cyclisa-

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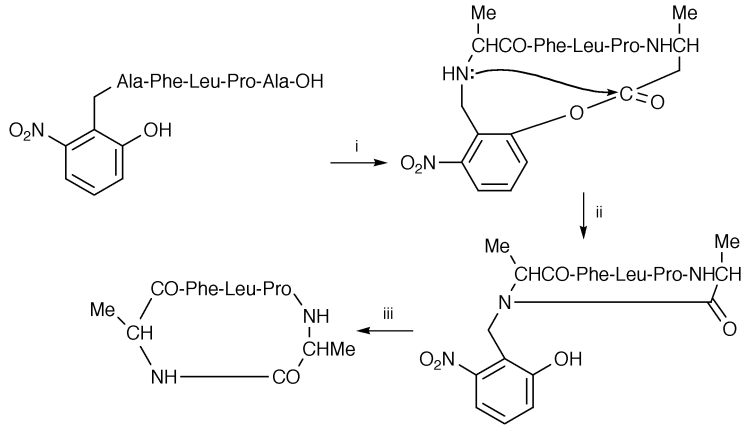
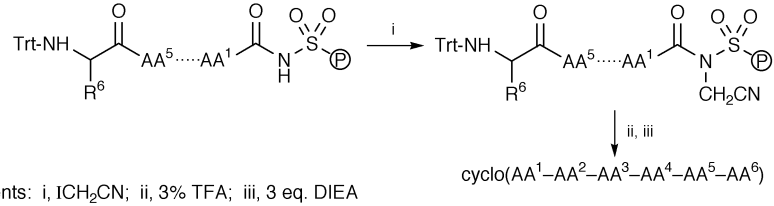


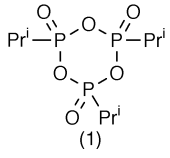
Reagents: i, BOP/DIEA 65°C/1 h; ii, O⟶N acyl transfer; iii, *hν* (366 nm)

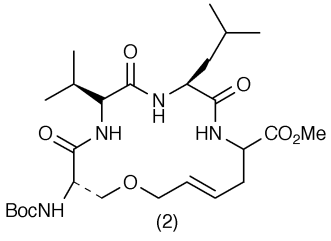
Scheme 1



Reagents: i, ICH_2CN; ii, 3% TFA; iii, 3 eq. DIEA

Scheme 2

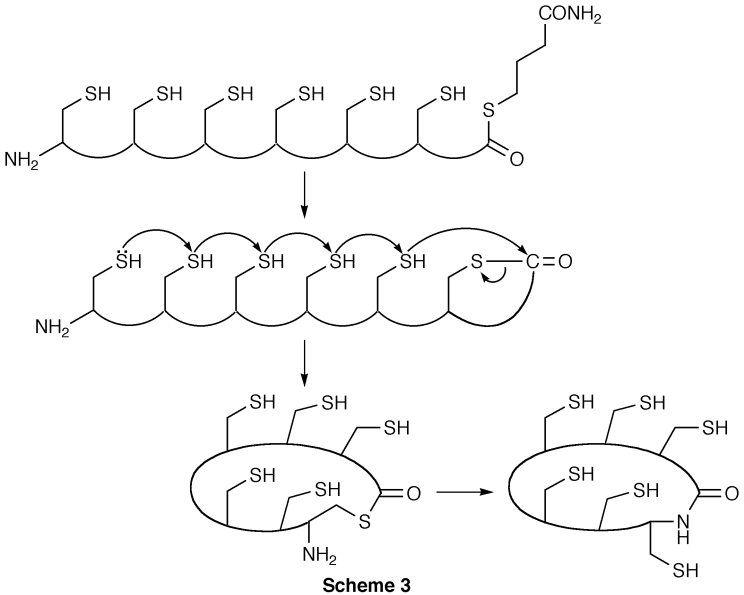




tion between Tyr (C-terminal) and D-Trp. Peptidomimetics possessing heterodetic rings such as in (2) can be accessed[10] *via* the Claisen rearrangement of suitable allylic ester precursors.

As the combinatorial interest has expanded to all kinds of pharmacophoric compounds, its roots in producing libraries of peptides, peptoids and cyclic peptides by solid phase synthesis has been reviewed.[11] An orthogonal cyclisation strategy[12] utilising unprotected peptides to form libraries of lactones and

lactams has been developed. Ag^+ ion coordination of the N-terminal end with C-terminal thioesters permit long range acyl migration, so that unprotected linear peptides ranging from 5 to 16 amino acid residues have been cyclised to give mixtures or pure forms. A thioester at the C-terminus, an N^{α}-cysteine and at least one internal free thiol is the fundamental requirement for the thia-zip reaction[13] for the synthesis of large cyclic peptides. An internal thiolactone formation initiates the reaction, followed by successive thiol–thiolactone exchanges leading to a large *N*-aminothiolactone which undergoes irreversible *S* to *N*-acyl isomerisations as depicted in Scheme 3. A 31-residue cyclic peptide, cyclopsychotride CT13, has been produced in this manner. Head to tail backbone cyclisation of proteins has not in the past been explored, but an intramolecular chemical ligation, using a biosynthetic process,[14] has been used to construct a cyclic version of a Src homol.3 domain.



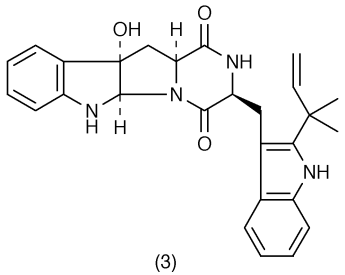
Scheme 3

A facile preparation[15] of cyclic disulfides from cysteine residues has utilised tetrabutylammonium fluoride (TBAF) in CCl_4. This is also applicable to on-resin work. The kinetics of cyclisation to form disulfide links *via* air oxidation at different temperatures has been studied using a MHC class II cyclic peptide vaccine.[16] Air oxidation at pH 10 at 37–55 °C over 2 hours proved to be an efficient approach to the desired product.

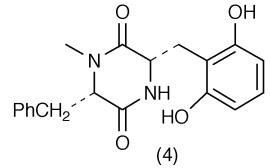
While the mass spectral fragmentation patterns for linear peptides have benefited from the nomenclature system of Roepstorff, Fohlmann and Biemann, it is now the turn of cyclic peptides and cyclodepsipeptides to be provided[17] with fragment descriptors. Efficient Monte Carlo schemes have been worked out for the simulation of complex cyclic peptides,[18] and the *cis*/*trans* isomerisation in proline containing cyclic peptides.[19] The assessment of

the efficiency of three minimisation algorithms has been carried out[20] using cyclo(D-Pro-Ala-Ala-Ala-Ala) and cyclo(Asn-Pro-Phe-Val-Leu-Pro-Val).

2.2 Cyclic Dipeptides (Dioxopiperazines). – Structure (3) represents the main structure (okaramine J) of a series of congeners isolated[21] from *Penicillium simplicissimum* ATCC90288, while the marine *Aspergillus* sp has yielded[22] the fungistatic dioxopiperazine (4) which contains the unusual 2,6-dihydroxyphenylalanine residue. The streptomyces strain which produces albonoursin (5) has also been found[23] to catalyse its formation from cyclo(Phe-Leu). Two species of penicillium, *P. dipodomyis* and *P. nalgiovense*, have been found[24] to be sources of dipodazine (6).



(3)



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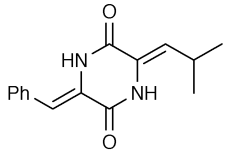

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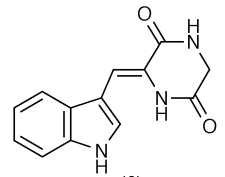
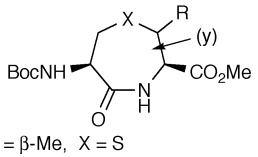
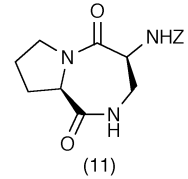


(6)

Some of the structures synthesised under the name cyclic dipeptides are not of the dioxopiperazine type. Thus in work on antihypertensive agents[25] the thiazepinones (7–9) have been synthesised as conformationally restricted dipeptide mimetics, while the Pummerer rearrangement applied to sulfoxide (10) yields a number of heterocyclic products.[26] Constrained *trans*-Pro amides in either D or L-Pro form (11) have also been synthesised.[27]



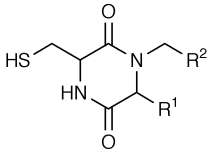
(7) R = β-Me, X = S
(8) R = α-Me, X = S
(9) = at position (y), R = Me, X = S
(10) = at position (y), R = Me, X = S^{+}—O^{-}

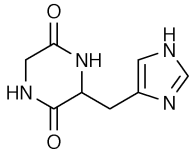


(11)

A previously published combinatorial library procedure (*Tetrahedron* 1997, **53**, 6573) for dioxopiperazine formation *via* the Ugi reaction has been

developed further[28] for the formation of inhibitors of collagenase-1. A library of compounds based on (12) was produced. Head to tail His cyclopeptides can be synthesised[29] by attaching the imidazole residue to a trityl resin *via* the starting support Fmoc-His(Trt-Resin)-OAllyl. Libraries of dioxopiperazines such as (13) have also emanated from this procedure. 3-Alkylidene-2,5-piperazinediones (14, $RR^1 = CH_2$, $CHCHMe_2$, CH_2Ph) in the presence of HBr can produce *N*-heteroaromatic derivatives as precursors of quaternary amino acids.[30] An HPLC-ESMS study[31] on C-terminal prolyl peptides has been tuned to look for deletion sequences due to dioxopiperazine formation in solid phase synthesis. Dioxopiperazine formation from H-Ala-Pro-NH_2 has been studied[32] in a large number of different solvents. Reaction rates are retarded by solvents able to stabilise charged solutes, or solutes that are H-donors or acceptors. Epidithiopiperazine-2,5-diones of the general structure (15), including gliotoxin, when studied[33] electrochemically undergo a one-electron reduction, rather than a two-electron cleavage as seen in acyclic disulfides.


(12)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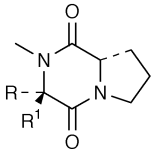
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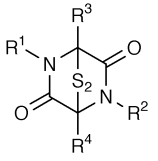
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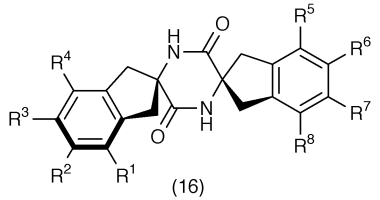
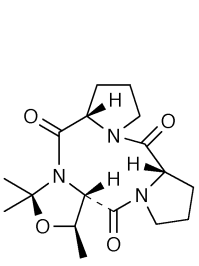

(16)

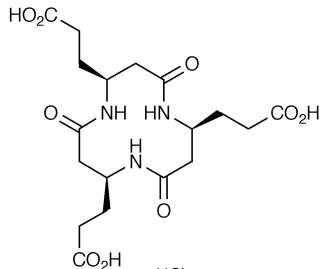
Crystallographic and spectroscopic methods have been applied[34] to cyclo(Trp-Pro) and cyclo(Trp-Trp) DMSO solvate crystals. The dioxopiperazine ring appears to be a more planar boat form than comparable examples. Bis-lactim ethers from cyclo(Gly-Val) have also been studied[35] by X-ray crystallography. Aromatic residues introduced into the side chain at the Gly position had been thought of as residing over the heterocyclic rings. No evidence for this conformation was found. X-ray crystallography has been used[36] to characterise enantiomerically pure, racemic and meso forms of spiro dioxopiperazines exemplified by (16). Ladder-like intermolecular amide to amide H-bonding interactions were observed in all cases. Cyclo(His-Phe) in the presence of chiral auxiliaries has been shown[37] to catalyse the alcoholysis of 2-phenyl-4-benzyl-5(4*H*)-oxazolone by methanol. Using l-diisopropyl tartrate as auxiliary, and a series of alcohols, *N*-benzoyl-L-phenylalaninates with 20–39% e.e. were obtained.

2.3 Cyclotripeptides. – Homodetic cyclic peptides under this category are hard to find and to synthesise, so features have to be built into the backbone to

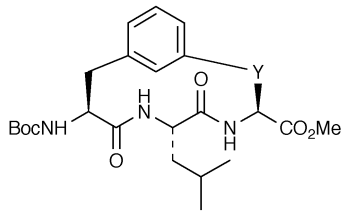
alleviate strain. Increasing the propensity of *cis*-amide bonds is one solution,[38] as seen in the synthesis of cyclo[Pro-Thr($\Psi^{Me,Me}$pro)-Pro] (17) from a linear precursor in 85% yield using PyBOP. Another solution is including β-amino acids in the backbone, but cyclo β-tripeptides have only received scant structural investigations because of their extreme insolubility. However, by exploiting the solubilising effect of LiCl in THF, a water-soluble cyclo[β^3-HGlu]$_3$ derivative (18) could be investigated.[39] NMR studies (D_2O) showed the predominance of a three-fold symmetrical conformation, with the rings stacking to form tube-like H-bond aggregates. Heterodetic cyclic tripeptide systems such as (19) and (20) have been produced[40] from chlorophenylalanine residues η^6-complexed to ruthenium which can undergo S_NAr cyclisations. Similar chemistry *via* an S_NAr cyclisation has yielded[41] two novel biphenyl ether analogues (21) and (22) of an inhibitor of HCV NS3 protease.



(17)

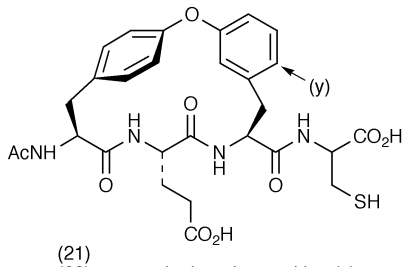


(18)



(19) Y = 

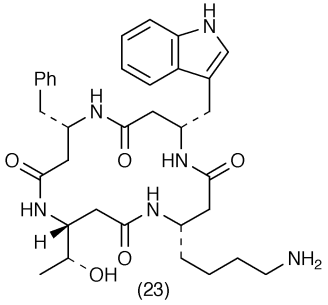
(20) Y = S



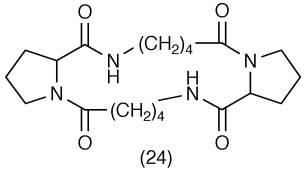
(21)
(22) *para*-substituted at position (y)

2.4 Cyclotetrapeptides. – As in the previous sub-section, the effect of using β-amino acids has been investigated[42] to assess whether such structures can mimic α-residues in their recognition by human receptors. Compound (23) is a mimic of octreotide but only had micromolar activity when compared to the nanomolar activity of the somatostatin analogue. β-Amino acid residues have also been found in naturally-occurring cyclotetrapeptides. Thus from *Rhodococcus spII* the antifungal rhodopeptins C1, cyclo(Gly-Orn-Val-3-amino-10-methyldodecanoyl), C2, cyclo(Gly-Orn-Ile-3-amino-10-methyldodecanoyl), C3, cyclo(Gly-Orn-Val-3-amino-12-methyldodecanoyl), C4, cyclo(Gly-Orn-Val-3-amino-12-methyldodecanoyl) and C5, cyclo(Gly-Lys-Val-3-amino-13-methyltetradecanoyl), have been characterised.[43] Synthesis was achieved by

cyclisation of protected linear precursors at the carboxyl group of the lipophilic β-amino acid residue using DPPA. According to the polarity of solvents cyclo (*S*-Ava-L-Pro)$_2$ (24) interconverts[44] between three conformers induced by the difference in the rotational states of the Pro $^{\alpha}$C–CO single bond. NMR data confirmed a C_2-symmetric conformation with an all-*trans* peptide backbone in all solvents.

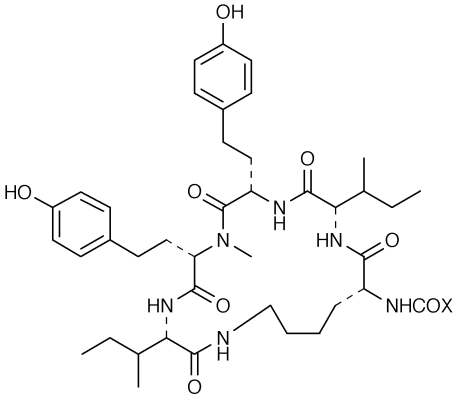


(23)

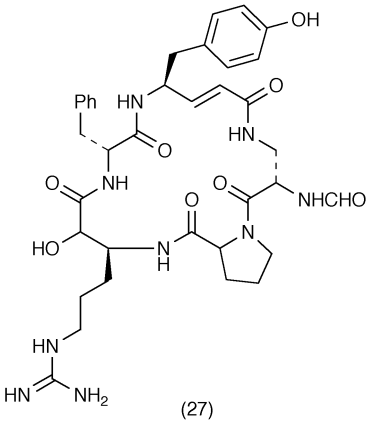


(24)

2.5 Cyclopentapeptides. – Nature continues to reveal its wealth of structures in this category. Cyanobacterium *Oscillatoria agardhii* (NIES-595) produces, amongst other anabaenopeptins, the potent carboxypeptidase A inhibitors, anabaenopeptins G (25) and H (26). Their structures were established[45] from deductions made from 2D NMR and chemical methods. Only one of the six new serine protease inhibitors isolated[46] contains a cyclic structure. This cyclic congener is the reduced form (27) (CHOH instead of CO) of cyclotheonamide A. Astin G (28), from the roots of *Aster tartaricus*, has been totally synthesised for the first time.[47] Attempts to cyclise linear precursors at the β-Phe-COOH using pentafluorophenyl esters or DPPA were unsuccessful but TBTU gave a 16% cyclisation yield. Two successful syntheses of (−)-motuporin (29) have been reported. Both reports used threonine derivatives as precursors of the *N*-methyldehydrobutyrine residue, as the dehydro unit seems to increase

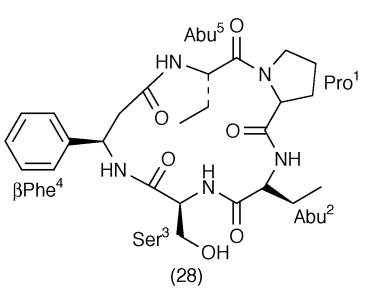


(25) X = Tyr
(26) X = Arg



(27)

epimerisation at the α-position adjacent to the activated carboxyl group in linear precursors. One synthesis[48] used the strategy of having valyl residue as C-terminal, thus incorporating the Adda residue as the last residue at the N-terminal position. Cyclisation at position (a) in (29) occurred in 79% yield using HATU/*N*-ethylmorpholine. The other synthesis[49] utilised cyclisation at point (b) in (29), when pentafluorophenyl diphenylphosphinate yielded 79% of (29), DPPA, 26%, and pentafluorophenyl ester 55%.



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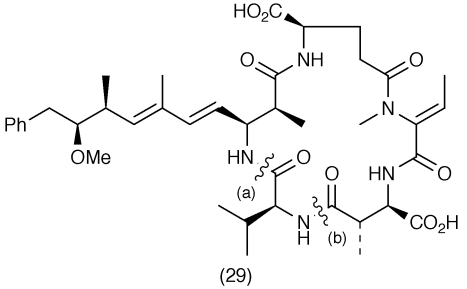
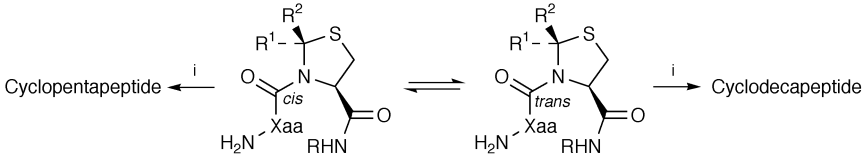


(29)

The influence of *N*-methylation of amide bonds in the selective $\alpha_v\beta_3$ antagonist cyclo(Arg-Gly-Asp-D-Phe-Val) on biological activity has been assessed.[50] The analogue cyclo(Arg-Gly-Asp-D-Phe-MeVal) came out as the best lead compound. Fmoc-Protocols on *o*-chlorotrityl resin were used in the synthesis, but only HOAt/HATU gave satisfactory couplings involving the *N*-methylated amino acids. Cyclisations were carried out either with DPPA or TBTU/HOBt at high dilution. Radio-labelled $\alpha_v\beta_3$ integrin antagonists have also been developed[51] for tumour targetting. Compounds synthesised were [^{125}I]-3-iodo-D-Tyr^{4}cyclo(Arg-Gly-Asp-D-Tyr-Val), [^{125}I]-3-iodo-Tyr^{5}cyclo (Arg-Gly-Asp-D-Phe-Tyr). NMR studies[52] have revealed that cyclisation to the monomer cyclopentapeptides, in comparison to cyclodecapeptides (dimers) in a series of Gly and Pro peptides, is strongly dependent on the *cis–trans* isomerisation. Scheme 4 shows the use of 2,2-dimethyl-1,3-thiazolidine-4-carboxylic acid as proline substitute. The *cis* rotamer yields monomers, while dimers are more prevalent for *trans* rotamers.



Scheme 4

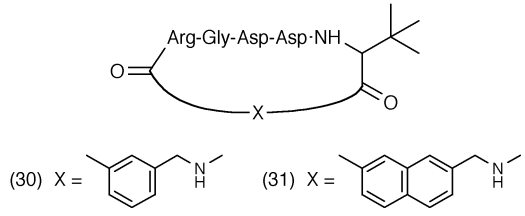
Cyclo(D-Trp-Pro-D-Lys-D-Trp-Phe) shows selectivity[53] for type 1 neurokinin receptor (NK1), and conforms to the DLDDL scaffold format which confers semi-rigidity for antagonism at the receptors. NMR studies confirmed a type IIβ turn over Trp^4-Trp^1 and a γ-turn at Phe. The cyclic peptide was

synthesised from Boc-Phe-D-Trp-Pro-D-Lys(TFA)-D-Trp-OBu^t, assembled in the solution phase and cyclised in 99% yield using DPPA/HOBt/DMAP. The cyclic tuftsin analogue cyclo(Thr-Lys-Pro-Arg-Gly) is 50 times more biologically active than tuftsin itself. Its NMR-derived conformation[54] in DMSO/water shows a type Vla turn centred over Lys-Pro, and a *cis*-rotamer at the Lys-Pro bond.

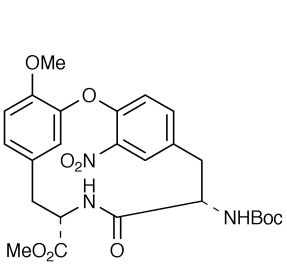
2.6 Cyclohexapeptides. – Monolayers of cyclo[(Cys-Lys)$_2$-Cys-Trp] and cyclo(Cys-Phe-Cys-Lys-Cys-Trp) on a gold surface have been tested[55] for their molecular recognition characteristics. These monolayers were able to discriminate between D and L-Arg and appeared to have a specific interaction with Lys. In the synthesis of these cyclopeptides cyclisation was carried out by TBTU/HOBt. In order to study[56] the effect of membranes on the binding of peptides to receptors, eight amphiphilic cyclopeptides based on Ada (L-α-aminodecanoic acid), Ahd (L-α-aminohexadecanoic acid) and Nhdg (hexadecyl glycine) have been synthesised and analysed by NMR in membrane-mimicking solvents. Four cyclohexapeptides seemed too rigid to be conformationally changed between isotropic and anisotropic environments. Three cyclopeptides, cyclo(Gly-Asp-Ahd-Ahd-Asp-Gly), cyclo(Asp-Ala-Nhdg-Ala-D-Asp) and cyclo (D-Asp-Ala-Nhdg-Ala-Asp), were highly flexible and unstructured in both environments while for cyclo(Asp-Asp-Gly-Ahd-Ahd-Gly) structure-inducing effects were seen in the membrane-like solvent. The somatostatin analogue L-363,301, cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe), has undergone[57–59] a thorough structure–activity study. The Pro residue has been replaced with *N*-2- aminoethylglycine, *N*-2-carboxyethylglycine and *N*-4-aminobutylglycine to assess the effects of positive/negative charges in the bridging region. Introduction of the carboxyethyl group was unfavourable to binding to recombinant human somatostatin receptors. Basic residues in the bridging region seem advantageous. NMR and distance geometry/molecular dynamics calculations[58] on the three analogues showed similar *cis–trans* orientations of the substituted peptide bonds, but while *cis* forms showed similar characteristics the *trans* forms showed larger variations. The longer chain base substituent seemed to have the necessary characteristics to bind to a negatively-charged domain on the receptors. DPPA/K_2HPO_4 activation for cyclisation has given[59] a series of L-363,301 analogues with Pro^6 replaced by *N*-benzylglycine, and *S* or *R*-*N*-[(α-methyl)benzyl]glycine, with L-1-naphthylalanine as a replacement for either Phe^7 or Phe^{11}. Binding to the hsst2 receptor was effective in all cases, but binding to hsst3 and hsst5 was variable.

Cyclo(Pro-Leu-Gly)$_2$ and cyclo(Pro-Leu-Gly)$_4$ have been synthesised[60] as representative of the C-terminal sequence of oxytocin. *N*-Hydroxysuccinimide esters of the precursor linear hexapeptide were used in cyclisation under high dilution conditions. The cyclohexapeptide in DMSO seemed to be fixed in the usual β-turn conformation but in $CDCl_3$ several conformations in rapid interconversion could be identified. The cyclododecapeptide showed more complicated NMR spectra in both solvents indicating a more random structure. Cyclo(Gln-Trp-Phe-β-Ala-Leu-Met), a new NK-antagonist, showed[61] in

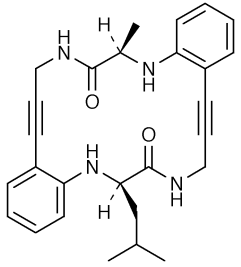
DMSO a main conformation having three intramolecular H-bonds, between MetNH and β-AlaCO, β-AlaNH and MetCO, and PheNH and MetCO. A type I β-turn with Gln and Trp and a γ-turn with Leu suggests that the extra methylene group of the β-Ala may relax some unfavourable restraints in the usual cyclohexapeptide backbone. Previously published data on cyclo(D-Pro-Phe-Ala-Ser-Phe-Phe) had indicated that at least two conformations had to be evoked to explain the NOE data. In a new study[62] the validity of the application of solvation parameters has been assessed *via* free energy calculations and a good agreement obtained between calculated and experimental NOE's and ^3J coupling constants. Detailed conformational characterisation,[63] using NMR data and molecular modelling simulations, has been carried out on pentapeptide RGD sequences attached to a sixth linking residue as in (30) and (31). The compounds were low to sub-nanomolar inhibitors of integrin $\alpha_v\beta_3$, and their conformation indicated that their $ArgC^{\beta}$–Asp^3C^{β} distance was within the tolerance needed for binding.



Previous syntheses of anti-tumour agent RAVII have reported poor yields in the synthesis of the top half of the molecule. A short route[64] to the top fragment (32) has involved cycloetherification *via* an intramolecular S_NAr reaction using fluoride displacement *ortho* to the nitro group. Macrocyclic heterodetic hexapeptide model (33) has been designed[65] to be an antiparallel β-sheet model. The carboxyl group of leucine, activated by DPPA/$NaHCO_3$ was the point of cyclisation in 28% yield. Macrocyclic systems represented by (34) and (35) have been synthesised[66] in the solution phase to represent cyclic versions of the tethered-ligand sequence SFLLRN believed to be the activation motif of thrombin receptor (PAR-1). The best activation of PAR-1 came from (35) but still with quite a moderate response of $EC_{50} = 24$ μM. Compounds such as (36) have been prepared[67] from $(Gly\text{-}Cys(R))_3OH$ precursors, where R

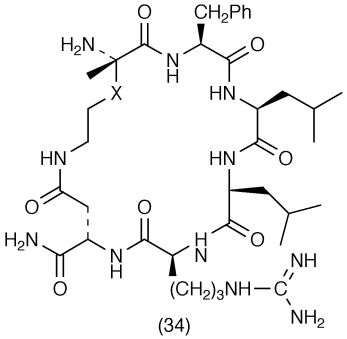


(32)

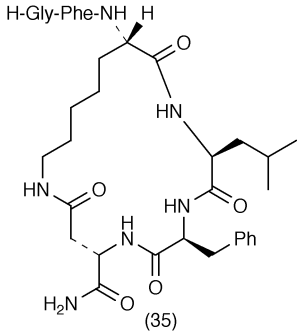


(33)

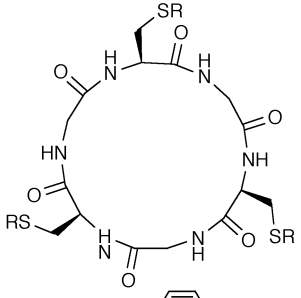
is a terpyPt^{II} complex. The cyclisation of the linear precursors was carried out by EDC/HOBt to give (36) and together with its cyclo-octa analogue they are being used as potential biosensors for protein folding studies. The hypothetical peptidostarand (37) has been investigated[68] as a valence tautomer of a cyclic hexapeptide. It has been calculated that its cyclohexaglycine analogue is 76 kcal mol^{-1} more stable than (37, R = H) but if R = CFO the peptidostarand becomes the more stable by 19 kcal mol^{-1}.

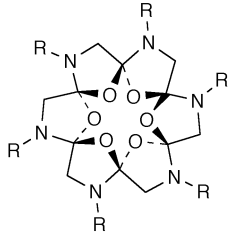


(34)

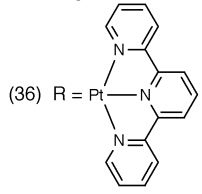


(35)

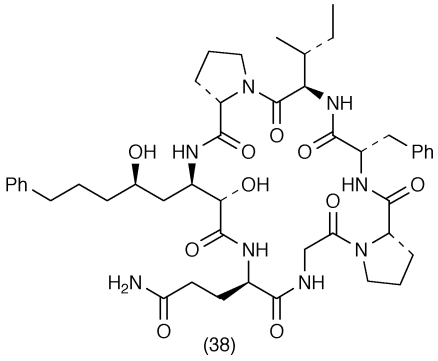




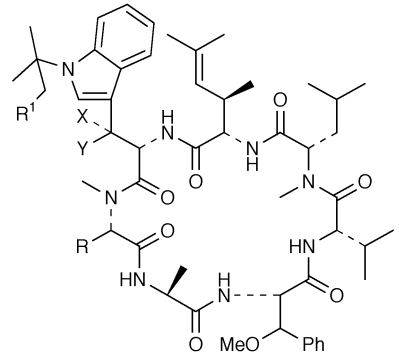
(37)

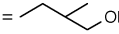


2.7 Cycloheptapeptides. – Two new cyclopeptides, glabrin D, cyclo(Pro-Pro-Val-Tyr-Gly-Pro-Glu), and a cyclo-octapeptide, glabrin C, cyclo(Pro-Gly-Tyr-Val-Leu-Ala-Leu-Val) have been isolated[69] from the seeds of *Annona glabra*. Nostophycin (38), related to the microcystins, has been characterised[70] as a constituent of the cyanobacterium *Nostoc* sp152, while a marine *Streptomyces* bacterium has generated[71] the anti-inflammatory cyclomarins A (39), B (40) and C (41). The latex of *Jatropha pohliana* is rich in cyclopeptides with phohlianin A, cyclo(Tyr-Pro-Leu-Gly-Val-Leu-Leu), B, cyclo(Tyr-Pro-Leu-Gly-Val-Leu-Leu-Leu), and the cyclo-octapeptide C, cyclo(Gly-Gly-Thr-Ile-Ile-Phe-Gly-Phe), being identified.[72] A type I β-turn around positions 5/6 with



(38)

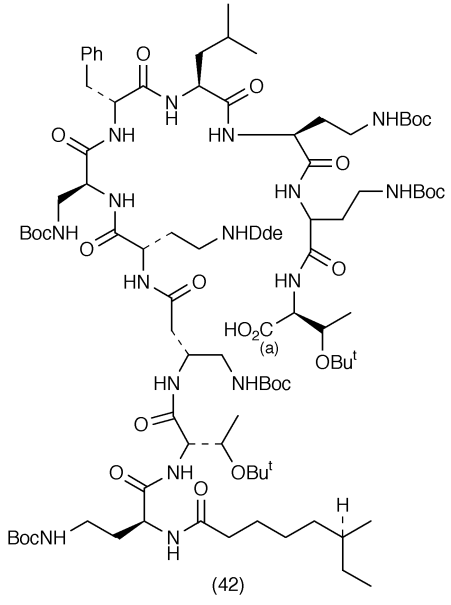


(39) R =  , R^1 =  , X = OH, Y = H

(40) R = Me_2CHCH_2, R^1 =  , X = OH, Y = H

(41) R = $HOCH_2CH(Me)CH_2$, R^1 = CH_2=CH, X = H, Y = OH

a β-bulge and a βVIa turn around Tyr-Pro gives the cyclohexapeptides a conventional conformation while pohlianin C has a type I β-turn at Gly-Thr and a type II β-turn at Phe-Gly. Linear precursor (42) of a Sasrin resin has worked well[73] as a precursor of polymyxin B1 (42, cyclised at position (a) and without Boc/Bu^t protection). Selective removal of Dde by hydrazine gave the necessary free amino group to attach to a DPPA activated COOH at link point (a). A 4 + 3 segment condensation has given[74] the linear precursor of phakellistatin 2, cyclo(Tyr^1-Pro^2-Phe^3-Pro^4-Ile^5-Ile^6-Pro^7). The cyclisation at the Pro^7 position proceeded in 50–65% yield with either TBTU, BOP-Cl, PyBroP or HOAt. TBTU gave consistently the highest yield.



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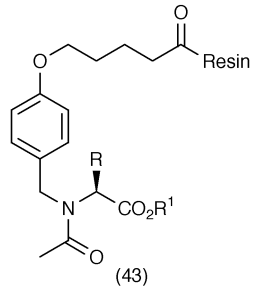
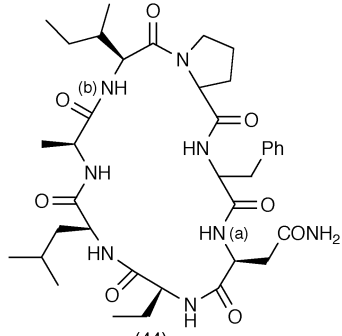
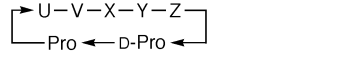


(43)

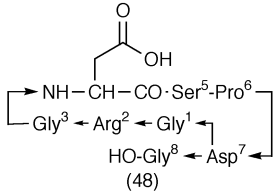


(44)

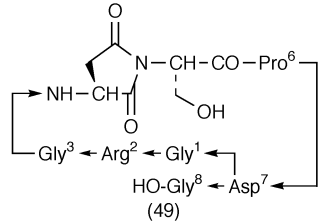
The resin linker represented in (43) allows anchoring *via* the α-nitrogen atom of a C-terminal residue and a test run of its capabilities has been carried out[75] in the synthesis of stylostatin (44). Sites (a) and (b) in (44) were selected for anchoring to the resin, to give two linear precursors and, using standard Boc strategies, on-resin cyclisation to (44) was achieved. The product profile of monomer and dimer formation was similar on solid phase as it was in the solution phase. It was the latter phase that was chosen[76] for synthesis of pseudostellarin D, cyclo-(Gly-Gly-Tyr-Pro-Leu-Ile-Leu), with the *p*-nitrophenylester at the Leu residue taking 10 days to achieve success. Structural mimetics of L2, L3 and H2 canonical forms in antibody hypervariable loops have included[77] the D-Pro-L-Pro templates as exemplified by (45)–(47). Only one major conformation could be detected by NMR of each analogue, and structures such as (46) and (47) bore good resemblance to L3 and H2 loops. In order to explain the increased propensity for vitronectin selective inhibitor (48) to convert into its cyclic imide counterpart (49), conformational analysis[78] of the two forms has been carried out using NMR techniques and molecular dynamics simulations. It was revealed that both (48) and (49) have a stable conformation in solution, and that the rearrangement is more influenced by the neighbouring group catalysis of the Ser^5CH_2OH than by the backbone conformation.



(45) U = Tyr, V = Arg, X = Asp, Y = Ala, Z = Met
(46) U = Phe, V = Tyr, X = Thr, Y = Gly, Z = Thr
(47) U = Asn, V = Thr, X = Tyr, Y = Ser, Z = Gly-Val



(48)

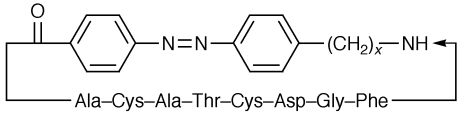


(49)

Table 1

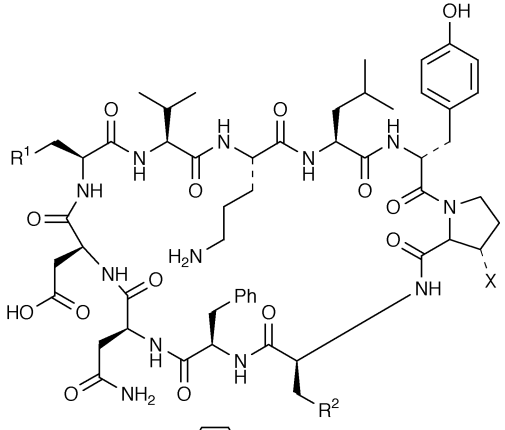
Name	*Source*	*Structure*	*Refs.*
Cyclosquamosin A	*Annona squanosa*	c(Gly-Ser-Phe-Gly-Pro-Val-Pro)	79
Cyclosquamosin B	*Annona squanosa*	c(Gly-Leu-Met-Gln-Pro-Pro-Ile-Thr)	79
Cyclosquamosin C	*Annona squanosa*	c(Gly-Leu-Met-Gln-Pro-Pro-Ile-Thr)	79
Cyclosquamosin D	*Annona squanosa*	c(Gly-Gly-Val-Leu-Ser-Tyr-Tyr-Pro)	79
Cyclosquamosin E	*Annona squanosa*	c(Gly-Gly-Val-Leu-Ser-$(Tyr)_3$-Pro)	79
Cyclosquamosin F	*Annona squanosa*	c(Gly-Ala-Pro-Ala-Leu$(Thr)_2$-Tyr)	79
Cyclosquamosin G	*Annona squanosa*	c(Gly-Tyr-Pro-Met-Thr-Ala-Ile-Val)	79
Cyclolinopeptide B	*Linum usitatissimum*	c(Pro-Pro-Phe-Phe-Val-Ile-Met-Leu-Ile)	80
Cyclolinopeptide C	*Linum usitatissimum*	c(Pro-Pro-Phe-Phe-Val-Ile-Mso-Leu-Ile)	80
Cyclolinopeptide D	*Linum usitatissimum*	c(Pro-Phe-Phe-Trp-Ile-Mso-Leu-Leu)	80
Cyclolinopeptide E	*Linum usitatissimum*	c(Pro-Leu-Phe-Ile-Mso-Leu-Val-Phe)	80
Psammosilenin A	*Psammosilene tunicoides*	c(Pro-Phe-Pro-Phe-Phe-Ala-Pro-Leu)	81
Psammosilenin B	*Psammosilene tunicoides*	c(Pro-Gly-Phe-Val-Pro-Phe-Thr-Ile)	81
Squarrin A	*Annona squamosa*	c(Pro-Mso-Tyr-Gly-Thr-Val-Ala-Ile)	82

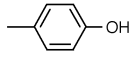
2.8 Cyclooctapeptides/Cyclononapeptides. – Table 1 summarises the structures that have been elucidated from natural sources. Cyclopeptides in the range cyclo-octa to cyclo-deca and including an Arg-Gly-Asp motif have been synthesised.[83] Their structures include cyclo(Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser), cyclo(Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser), cyclo(Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro), cyclo-(Arg-Gly-Asp-Phe-Pro-Ala-Ser-Ser), and cyclo(Arg-Gly-Asp-Cha-Pro-Ala-Ser-Ser). The linear precursors were made in the solution phase and cyclised using DPPA. The latter two examples in the list were the best for human platelet inhibition. Studies[84] of cyclic peptides at air–water interfaces indicate that nanotubes and aggregates are formed. Cyclo(Phe-D-MeAla)$_4$, as detected by X-ray diffraction, exhibits crystallinity in which the place of the peptide ring is parallel to the water interface, while cyclo[(Trp-D-Leu)$_3$-Ser-D-Leu] forms planar aggregates composed of several tubes lying parallel to the air–water interface. In contrast cyclo(Trp-D-Leu)$_4$ shows no great tendency to form ordered 2D arrays. PyBOP proved[85] to be the best agent for cyclisation at the Gly residue of (50) and (51). Preparation of a further disulfide ring between the two cysteines in (51) was more successful than in (50) due probably to the extra CH_2 unit. Both monocyclic and bicyclic forms are photoresponsive molecules which undergo *cis*/*trans* isomerism reversibly.

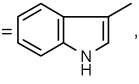


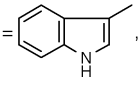
(50) $x = 0$
(51) $x = 1$

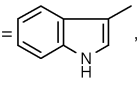
2.9 Cyclodecapeptides and Higher Cyclic Peptides. – A tropical marine bacterium produces[86] cyclodecapeptide antibiotics loloatins A(52), B(53),



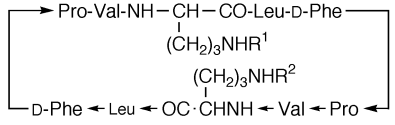
(52) R^1 =  , R^2 = Ph, X = H

(53) R^1 =  , R^2 = Ph, X = H

(54) $R^1 = R^2$ =  , X = H

(55) R^1 =  , R^2 = Ph, X = OH

C(54) and D(55). *In vitro* they exhibit antimicrobial activity against methicillin-resistant *Staph. aureus*, vancomycin-resistant enterococci, and drug resistant *Streptococcus pneumoniae*. Structural and conformational similarities with tyrocidine A were emphasised in this report. An unsymmetrically protected gramicidin S derivative (56) has opened up[87] an opportunity to produce other mono- and di-protected derivatives such as (57)–(59). The diprotected derivative (60) provided[88] the starting point for linking the two ornithine residues *via* methylene bridges ranging in size from monomethylene to pentamethylene. Most of the bridged analogues had similar antimicrobial activities to the parent molecule, the most potent being the trimethylene bridge. Two porphyrin groups have been attached[89] to gramicidin S *via* the ornithinyl side



(56) R^1 = H, R^2 = Tfa
(57) R^1 = Boc, R^2 = Tfa
(58) R^1 = Boc, R^2 = H
(59) R^1 = Boc, R^2 = NBS (*p*-nitrobenzenesulfonyl)
(60) $R^1 = R^2$ = NBS
(61) $R^1 = R^2$ = CO-*p*-C_6H_4-tritolylporphyrin

chains as defined in (61). The porphyrin residues on gramicidin S showed different circular dichroism properties. In order to understand better the mechanism of cell growth inhibition by the ion-complexing cyclopeptide antamanide, a series of linear and cyclic analogues have been synthesised.[90] Rudinger's version of the azide coupling method brought success in fragment condensations and in macrocyclisation. The Tyr^6-antamanide analogue cyclo(Phe-Tyr^6-Pro^7-Pro^8-Phe^9-Val-Pro-Pro-Ala) gave CD spectra similar to the Phe^6 parent, but Tyr^9-antamanide showed substantial differences.

Cyclic decapeptide template models such as (62) have been synthesised[91] *via* a 2-chlorotrityl resin support for linear assembly followed by HBTU/ cyclisation to make the homodetic ring. Air oxidation zipped up the disulfide ring as summarised in Scheme 5. Peptide (62) adopts a fold composed of two

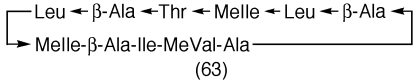
Reagents: i, 95% TFA; ii, Air oxidation

(62)

Scheme 5

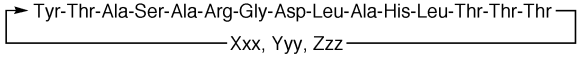
β-strands and two type II β-turns around Pro^4-Gly^5 and Pro^9-Gly^{10} from NMR data. Analogues of this template model have also been synthesised[92] and analysed in the same way. The loop III region of the platelet-derived growth factor (PDGF) β-chain has been mimicked[93] *via* cyclo(Arg^{73}-Lys-Ile-Glu-Ile-Val-Arg-Lys-Lys^{81}-Cys) with the C-terminal Cys being available for conjugation to a carrier protein. On-resin cyclisation, using allyl-protected glutamic acid anchored *via* its side-chain, was carried out using HATU/HOAt. The cyclic analogue produced an immunogen able to antigenically mimic the loop III, and could form a template to design future immunogens and agonists/antagonists of PDGF. In another development[94] of side-chain anchoring, this time linking Fmoc-Tyr-OMe to benzyl type resins by the Mitsunobu reaction, three cyclic analogues of neuropeptide Y have been synthesised. The three analogues, cyclo(β-Ala-Tyr-Pro-Ser-Lys-β-Ala-Arg-Gln-Arg-Tyr), cyclo(Ahx-Tyr-Pro-Ser-Lys-Ahx-Arg-Gln-Arg-Tyr) and cyclo-(Ala-Aib-Tyr-Pro-Ser-Lys-Ala-Aib-Arg-Gln-Arg-Tyr), each contains the N- and C-terminal tetrapeptide segments of neuropeptide Y, joined by different spacers, 6-aminohexanoic acid (Ahx), β-Ala, or Ala-Aib. Cyclisation on the resin was carried out using DIC/HOBt or HOBt/TBTU. In 30% trifluoroethanol the three analogues showed type I/III β-turn-structures.

The marine sponge *Theonella swinhoe* has yielded[95] the cyclic peptide barangamide A (63) which is the cyclopeptide analogue of the cyclodepsipeptide theonellapeptolide. In the latter structure the OH of the Thr residue in (63) is part of the ring structure. Crystal structures have been reported[96] for [O-Ac-4*R*-4-(*E*-2-butyl)-4,*N*-diMeThr]- and [*O*-Ac-4*R*-4-*E*-2-(4-bromobutyl)]-

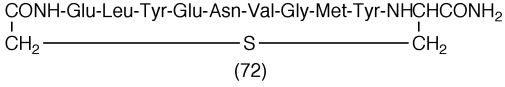


(63)

4,*N*-diMeThr]-cyclosporin A. Contrary to expectation, neither acetylation or bromination affects the conformation and packing of the parent molecule. Models of the antigenic side A of foot and mouth disease virus have been used[97] to study head to tail cyclisation. Structural variations are recorded in formulae (64)–(71), which were synthesised using three different cyclisation strategies. Cyclisation in the solution phase with minimal protection gave only 10% yield, but the other two strategies were based on side-chain anchoring through the Asp residue. The method of choice has become attachment of the Asp residue *via* its Cs salt, then a Boc/Bzl/OFm strategy. Amongst the cyclic analogues produced[98] as a result of a 11-mer lead compound showing inhibition of the interaction between growth factor receptors and the protein Grb-2, is a thio-ether bridged analogue (72) and a cyclic nonapeptide cyclo(Met-Tyr-Glu-Leu-Tyr-Glu-Asn-Val-Gly). This latter cyclic peptide was built up on a resin from the Gly terminal and cyclised in the solution phase in 45% using HATU/HOAt.



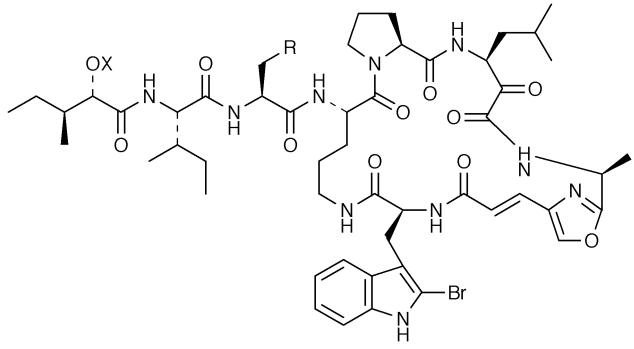
(64) no residues in Xxx, Yyy or Zzz
(65) Xxx = Gly
(66) Xxx = Cys
(67) Xxx = β-Ala
(68) Xxx = Ahx
(69) Xxx = Cys, Yyy = Gly
(70) Xxx = Gly, Yyy = Cys, Zzz = Gly
(71) Xxx = Gly, Yyy = Gly, Zzz = Cys



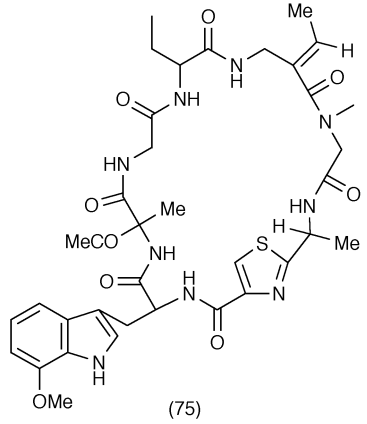
(72)

2.10 Peptides Containing Thiazole/Oxazole Rings. – This section this year reflects increased research activity in the total synthesis of naturally occurring compounds, but not without Nature producing further synthetic challenges for the future. Thus the *Theonella* sponge continues to supply novel structures[99] such as the keramamides M (73) and N (74) which are keramamide congeners possessing the unusual sulfate esters. Although peptides with sulfonate groups are well known, sulfate esters are rare. Zelkovamycin (75) from *Streptomyces* sp. K96-0670 has been shown[100] by NMR studies to be very similar to cyclic peptide antibiotics 21459A and B and nosiheptide.

In the synthesis of dolastatin I (76) present in *Dolabella auricularia*, the construction of the reactive oxazoline ring is kept until the last stages of the synthesis.[101] Serine is used in the sequence as its linear precursor, and after cyclisation (DPPA/Et_3N in 41% yield) the oxazoline ring is generated from the serine side-chain *via* the Mitsunobu reaction. In the total synthesis[102] of mallamide (77), a thioamide link built into the backbone became the penultimate precursor of the thiazoline ring using the Burgess reagent. DPPA activation of the prolyl carboxyl group was used for the initial cyclisation to



(73) R = Me, X = SO_3H
(74) R = Et, X = SO_3H



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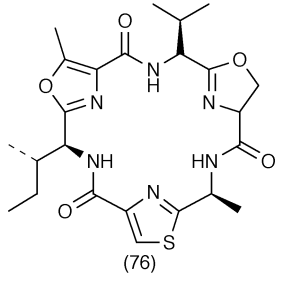
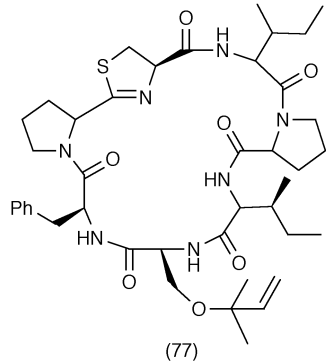


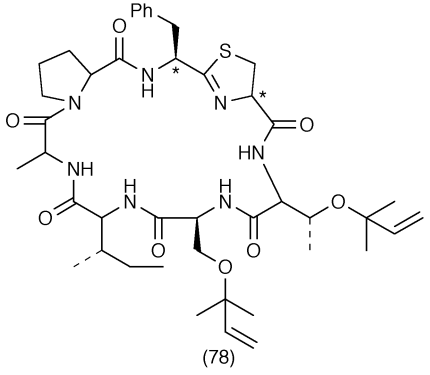
(76)



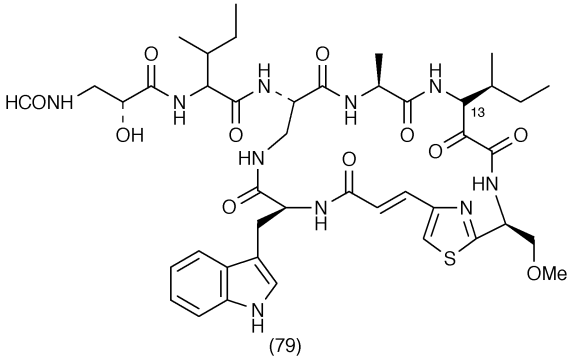
(77)

the cyclic peptide. The structure (78) previously assigned to trunkamide A from *Lissoclinum* sp. needs to be re-assessed in the light of the total synthesis of this structure.[103] The synthetic product differed from the natural product in its optical rotation, and in some aspects of its NMR spectra. Macrolactamisation was effected by HATU, and the authors suggest that the lack of agreement in data is due to assignments of stereochemistry at the * positions. A similar

situation has arisen after the total synthesis[104] of keranamide J (79). Differences in spectra between synthetic and natural forms suggest that a reassignment of the configuration at position 13 in the ring is necessary. Again cyclisation of a linear precursor was effected in 57% using DPPA activation of a C-terminal alanyl carboxyl.



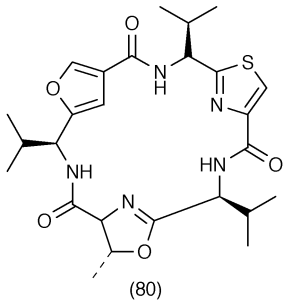
(78)



(79)

Enantiomerically pure oxazole, thiazole and oxazoline segments were inserted[105] into a linear precursor in the total synthesis of bistratamide D (80). Cyclisation at a C-terminal oxazoline generated the macrocyclic ring using HATU. Micrococcin P (81) has been totally synthesised[106] from protected fragments A–D as shown in the structure. The final macrocyclisation occurred at position (a) using BOP giving total coincidence between physical and spectral properties of the synthetic and natural products. The same strategy has been applied[107] to micrococcin P_1 (82) with similar success. Another synthesis[108] of (82) using the previously published Bycroft-Gowland structure for (82) gave spectra which were not identical with those of the natural product. The present authors[108] suggest that the stereochemistry of threonine derived thiazole might be in doubt in the original structure.

Thiangazole (83), a selective inhibitor of HIV-1, has been synthesised[109] from a linear precursor, with *S*-benzyl-2-methylcysteine residues functioning as precursors of the tris-thiazole rings and *O*-benzylthreonine amide for the



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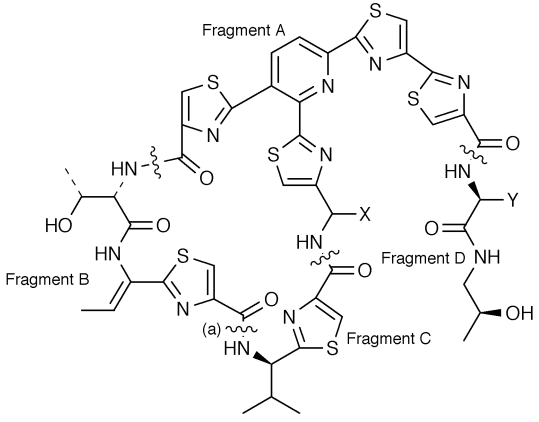
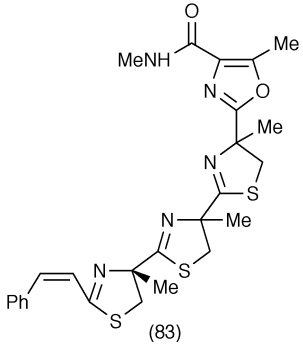


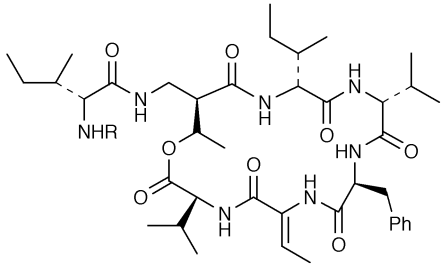
(81) X = CH_2=CH, Y = MeCH(OH)
(82) X = MeCH(OH), Y = CH_2=CH

oxazole ring. Dehydration to the ring structures took place using $TiCl_4$, followed by an acid-catalysed Robinson-Gabriel reaction. A thiazole-containing tetrapeptide has undergone[110] a one-pot cyclooligomerisation to produce cyclo-[Ile-Ser-D-Val-(Thz)-]$_n$, where $n = 2–19$. At low peptide concentration the cyclodimer with $n = 2$ predominated.

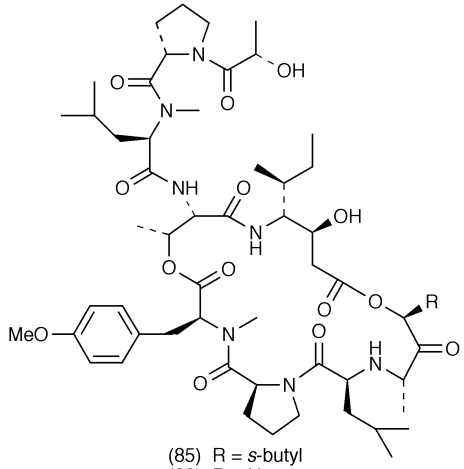
2.11 Cyclodepsipeptides. – This continues to be a popular backbone structure in products extracted from organisms in the marine environment. Thus kahalalide F (84) from the marine mollusk *Elysia rufescens*, already in preclinical trials against lung and colon cancers, has had its absolute stereochemistry scrutinised[111] [Hysp^2]- and [Hap^2]-didemnin B have been discovered[112] as new components (85) and (86) from the tunicate *Tridemnum cyanophorum*, while the sponges *Theonella mirabilis* and *Th. swinhoe* are a source[113] of the papuamides A–E (87–91). The absolute stereochemistry of the anti-inflammatory depsipeptides salinamides A (92) and B (93) have had to be revised,[114] and structures (94)–(96) have been worked out for the minor components, salinamides C, D and E respectively isolated from the marine



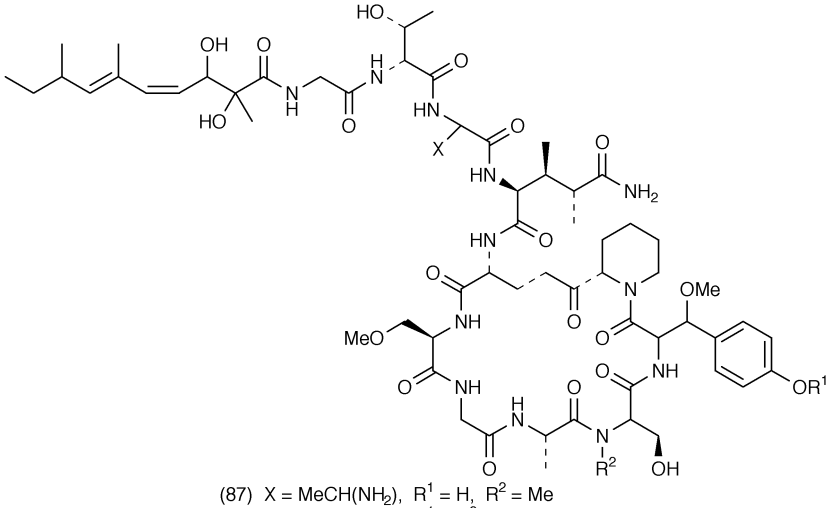
(83)



(84) R = MeHex-D-Val-Thr-D-Val-Val-D-Pro-Orn—

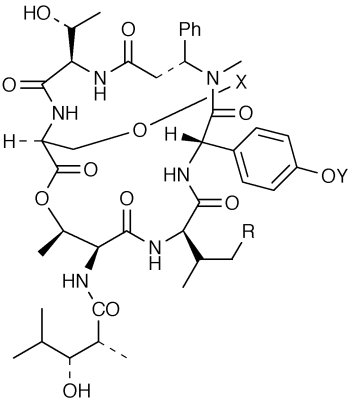


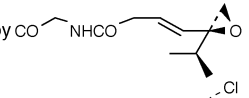
(85) R = *s*-butyl
(86) R = H

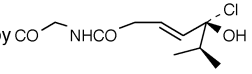


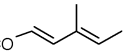
(87) X = MeCH(NH_2), R^1 = H, R^2 = Me
(88) X = MeCH(NH_2), R^1 = R^2 = H
(89) X = MeCH=, R^1 = H, R^2 = Me
(90) X = MeCH=, R^1 = R^2 = H
(91) X = MeCH(NHAc), R^1 = MeCO, R^2 = Me

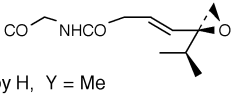
Streptomyces sp. CNB-091. The *Fusarium* marine fungus has yielded[115] sansalvamide (97) which is cytotoxic towards a large range of cancer cell lines, while the sponge *Cymbastela* sp. has had its family of geodiamolide structures augmented[116] with further structures for geodiamolides J–P and R. Figure 1 summarises the family of structures so far identified. Symplostatin from marine cyanobacterium *Symplora hydroides* has been assigned[117] the sulfoxide structure (98) of dolastatin 13 which gives rise to multiple signals in the NMR spectra due to the *R* and *S* forms at the sulfoxide. New jaspamide congeners B (99) and C (100) have been found[118] as minor constituents of the sponge *Jaspis*



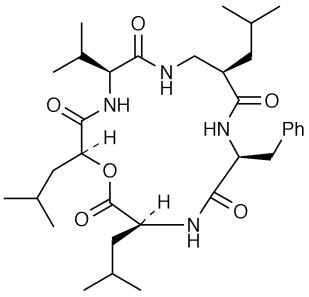
(92) R = Me, X–Y bridged by 

(93) R = Me, X–Y bridged by 

(94) R = Me, X =  , Y = Me

(95) R = H, X–Y bridged by 

(96) R = Me, X–Y bridged by H, Y = Me



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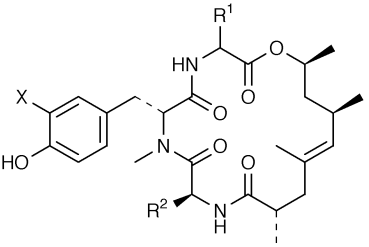
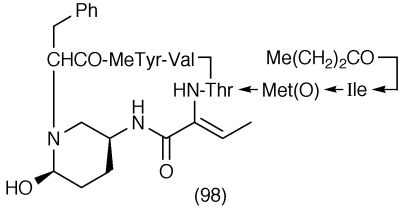
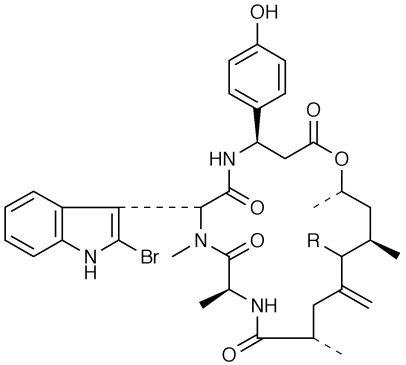


Geodiamolides	R^1	R^2	X
A	Me	Me	I
B	Me	Me	Br
C	Me	Me	Cl
D	Me	H	I
E	Me	H	Br
F	Me	H	Cl
L	CH_2OH	Me	I
M	CH_2OH	Me	Br
N	CH_2OH	Me	Cl
O	Me	CH_2OH	I
P	Me	CH_2OH	Br
Q	Me	CH_2OH	Cl
R	CH_2OH	H	I

Figure 1



(98)

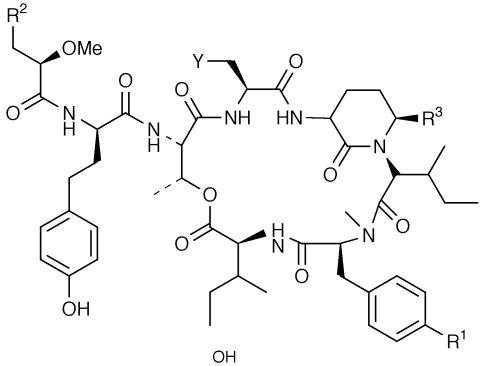


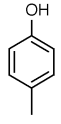
(99) R = O
(100) R = OH

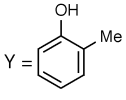
RCONH-Val-D-MeLeu-Thr-β-Ala-D-Leu-MeIle-β-Ala-D-Ile

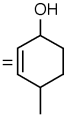
D-MeLeu-D-Leu-β-Ala- MeAla MeVal

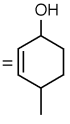
(101) R = MeSO
(102) R = MeCO

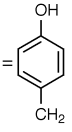


(103) R^1 = OMe, R^2 = OSO_3H, R^3 = OH, Y = 

(104) R^1 = OMe, R^2 = OSO_3H, R^3 = OH, Y = 

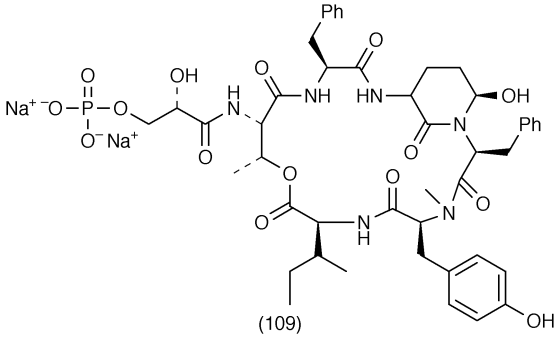
(105) R^1 = H, R^2 = OH, R^3 = OMe, Y = 

(106) R^1 = H, R^2 = OSO_3H, R^3 = OH, Y = 

(107) R^1 = H, R^2 = OSO_3H, R^3 = OH, Y = 

(108) R^1 = H, R^2 = OSO_3H, R^3 = OH, Y = $(CH_2)_3NH_2$

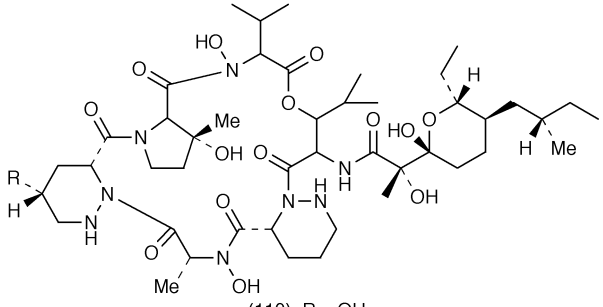
splendans, while *Theonella* sponge has yielded[119] theonellapeptolide congeners (101) and (102). Further congeners of oscillapeptin A (103), a serine protease inhibitor have been identified[120] as oscillapeptins B–F (104–108) isolated from three strains of cultured cyanobacterium *Oscillatoria agardhii*. Another cyano-



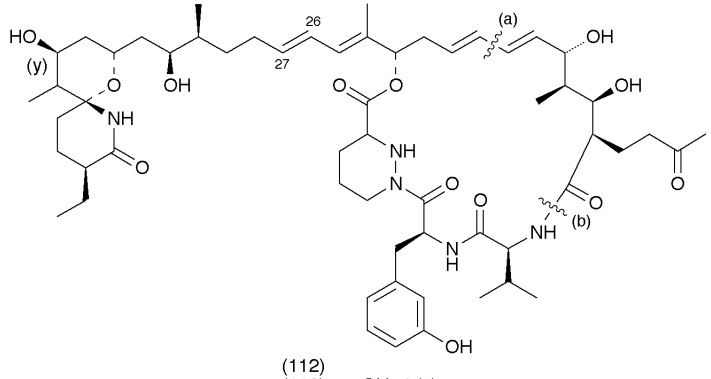
(109)

bacterium, *Microcystis aeruginosa*, produces[121] a very similar cyclodepsipeptide, micropeptin T 20 (109), which bears a novel phosphate group in the side-chain.

An actinomycete from soil, *Streptomyces anulatus*, produces[122] as well as valinomycin the cyclodepsipeptide montanastatin, cyclo(D-Val-L-Lac-L-Val-D-Hiv). Another *Streptomyces* sp. yields[123] polyoxypeptins A (110) and B (111), potent apoptosis-inducing cyclic depsipeptides. The presence of the new 3-hydroxy-3-methylprolyl residue was deduced from X-ray data. The struc-

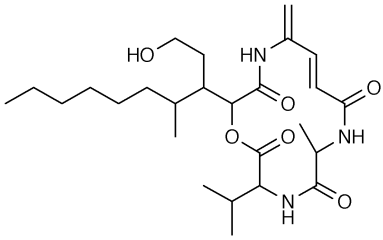


(110) R = OH
(111) R = H

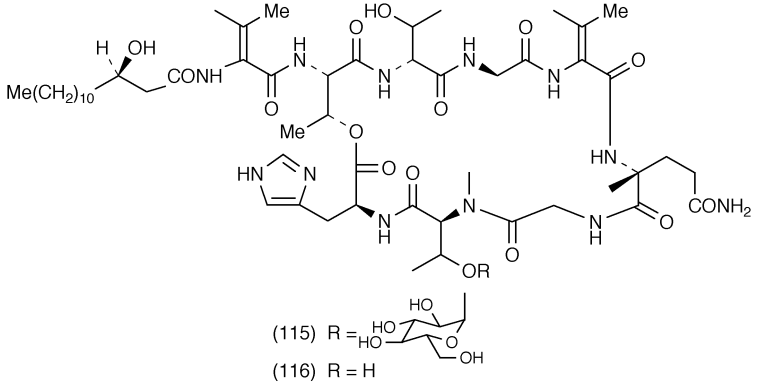


(112)
(113) no OH at (y)

tures add to the increasing preponderance of piperazic acid residues being discovered. In the sanglifehrins A (112) and B (113) the piperazic acid residue has the unusual feature of its β-nitrogen being involved in peptide bond formation.[124] Both of these compounds complex with cyclophilin A. Vinylamycin (114) has been isolated[125] from a *Streptomyces*, and shows antimicrobial activities against Gram-positive bacteria including MRSA. The major products found[126] in antibiotic W-10 fermentation complex have turned out to be two dehydropeptide lactones Sch 20562 (115) and 20561 (116), one being the aglycone of the other. The structures are closely related to the known herbicolins A and B.



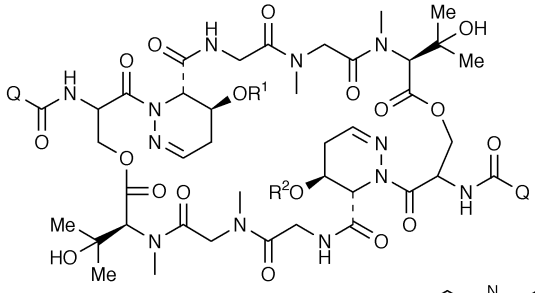
(114)



(115) R =

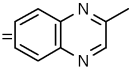
(116) R = H

Total syntheses have been published for many of the complex structures associated with this sub-section. In the synthesis of the core structure of sanglifehrin A (112) the key steps[127] involved combining two fragments *via* the amide bond at (b) and then completing the macrocyclic ring using an intramolecular Stille coupling at position (a). Another approach,[128] eventually aimed at total synthesis, has been to degrade the molecule regioselectively at double bond 26–27 in (112), and re-assemble fragments. Macrolide analogues of the sanglifehrin ring system have been prepared[129] *via* a cyclisation reaction which closes the ring at the diene unit. Preliminary[130] and full details[131] have been published on the total synthesis of quinoxapeptins A–C (117)–(119), potent inhibitors of HIV-1 and HIV-2RT. As the structures overlap with those of the luzopeptins A–C (120)–(122),[132] the common core of the two series was first synthesised and the different chromophore groups were added at the later

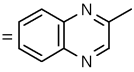


(117) $R^1 = R^2 =$ (1*S*,2*S*)-2-methylcyclopropylcarbonyl, Q =

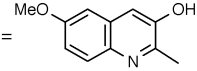
(118) R^1 = MeCO, R^2 = (1*S*,2*S*)-2-methylcyclopropylcarbonyl, Q =



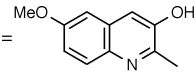
(119) $R^1 = R^2$ = H, Q =



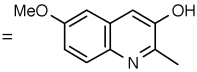
(120) $R^1 = R^2$ = Ac, Q =



(121) R^1 = H, R^2 = Ac, Q =

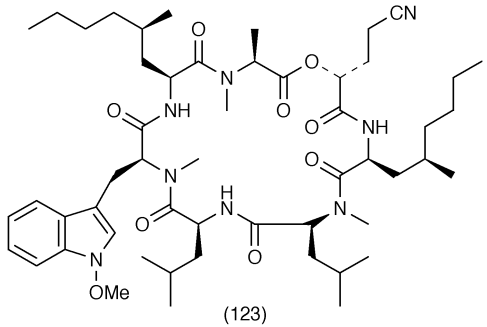


(122) $R^1 = R^2$ = H, Q =

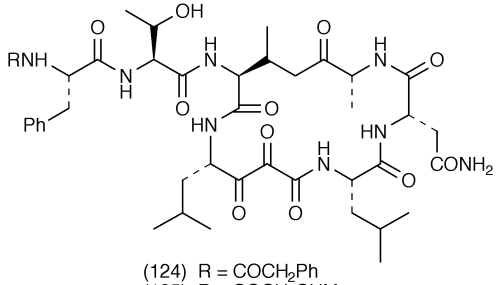


stages. In the synthesis of the common core, macrocyclisation was carried out in 66% of yield at the only secondary amide bond in the ring using EDCI/HOAt. It is interesting to note that the compound which gave the best inhibition of HIV-1 reverse transcriptase was (119), which was a synthetic intermediate on the path to total synthesis. The potent cell-adhesion inhibitor HUN-7293 (123) has been synthesised.[133] Efficient macrocyclisation at the MeLeu^3-Leu^4 amide bond, using BOP/DMAP in 80% yield, benefited from intramolecular H-bonding in the acyclic precursor, resulting in the pre-organisation of conformation prior to cyclisation. The depside link was incorporated into the acyclic precursor *via* a Mitsunobu esterification. The novel elastase inhibitors YM-47141 (124) and YM-47142 (125) possess the unusual tricarbonyl system within their macrocyclic ring. In their total synthesis,[134] phosphoranylidene ylides were used to 'mask' the tricarbonyl unit, and macrocyclisation was carried out in 59% yield at the Leu-Asn bond using DPPA/$NaHCO_3$.

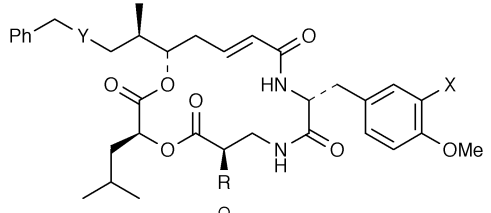
Cryptophycins-1 (126), -3 (127), -4 (128) and -24 (arenastatin A) (129) have been re-synthesised[135] using a convergent route from two fragments, one being the top half which involved synthesis of (5*S*,6*R*)-5-hydroxy-6-methyl-8-phenyl-octa-2(*E*),7(*E*)-dienoic acid. The bottom segment was joined to the top *via* the ester link before macrocylisation at the substituted Phe amino group using DPPA. Questions have been raised[136] about the stereochemical assignments in structure (130) assigned to epiantillatoxin, since the totally synthesised product



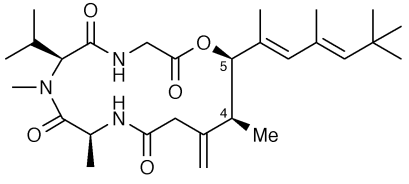
(123)



(124) R = $COCH_2Ph$
(125) R = $COCH_2CHMe_2$

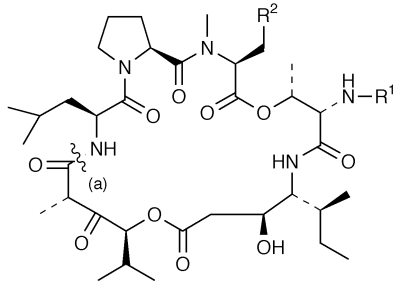


(126) Y =  , R = Me, X = Cl
(127) Y = =, R = Me, X = Cl
(128) Y = =, R = Me, X = H
(129) Y =  , R = H, X = H



(130)

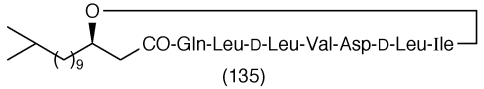
showed NMR data different from the natural product. Positions 4 and 5 would be likely centres for re-assignment of stereochemistry. In fact another total synthesis[137] of (130) has strongly supported a revised structure with a 4*R*,5*R* configuration rather than the 4*S*,5*R* in structure (130). DPPA was again instrumental in the macrocyclisation. The role of the diMeTyr[5] unit in



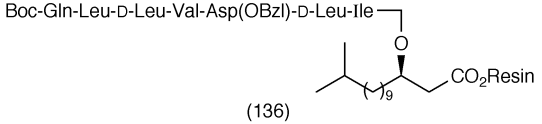
(131) R^1 = L-Lac-Pro-D-MeLeu, R^2 = (4MeO)PhCH_2–
(132) R^1 = L-Lac-Pro-D-MeLeu, R^2 = CH_2CHMe_2
(133) R^1 = L-Lac-Pro-D-MeLeu, R^2 = CH_2Ph
(134) R^1 = D-MeLeu, R^2 = CH_2Ph

didemnin B (131) has been explored[138] through to total synthesis of MeLeu and MePhe analogues (132) and (133). Both retained the activity of the original molecule, and it is interesting to note that although the changes at residue 5 seemed well away from the point of cyclisation '(a)', different activating conditions had to be used. A pentafluorophenyl ester proved successful (80% yield) in (132) while TBTU/DIEA brought success (55% yield) in (133). The influence of the side-chain R^1 on activity has been monitored[139] through the synthesis of analogues such as (134). The two ester bonds in the main ring were also successively replaced by amide bonds, which had an effect on conformation but did not affect the biological activity. Tamandarin A, closely related to didemnin B, has also been synthesised[140] for the first time. An efficient stereoselective synthesis of the isostatine unit and macrocyclisation with HATU/DIEA (63% yield) was a feature of the synthesis.

While most of the past syntheses of cyclodepsipeptides have been in the solution phase, an era is emerging where protocols are being adapted for their synthesis on solid phase. Thus cyclo-octadepsipeptide N-4909 (135), already synthesised in the solution phase has been re-synthesised[141] using an oxime resin, from a precursor (136) assembled from Boc-Leu-tetradeconic acid initially linked to the resin. A protocol has been devised[142] for incorporating depside links on solid phase. Tetrahydropyran derivatives of the hydroxy acids proved optimal for transient protection and DIC/DMAP was the condition of choice to make the depside links. A valinomycin analogue cyclo[Val-D-Man-D-Val-L-Lac]$_3$ where Man = mandelic acid and Lac = lactic acid was synthe-



(135)

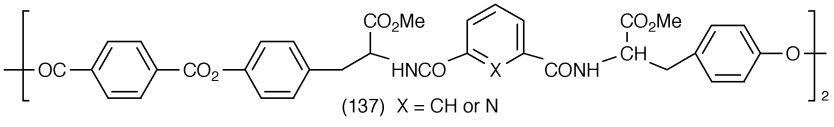


(136)

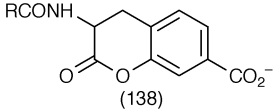
sised using the solid phase for assembly of the linear precursor with macrocyclisation being carried out in solution using the acid chloride (14% yield) or HATU (21%). The same authors[143] have also devised a colour test for monitoring the presence of free OH groups on solid phase. It involves formation of a tosylate, which is displaced by *p*-nitrobenzylpyridine, finally resulting in a strongly coloured internal salt in the presence of base. The HUN-7293 molecule (123) has also featured[144] in a site selective epimerisation reaction. On thionation of the most accessible MeLeu-Leu amide bond, the thioamide analogue can be *S*-alkylated and converted into a bridged 5-amino-oxazole which opens up to give the Leu residue with an inverted configuration. Of relevance to many cyclodepsipeptide syntheses is the availability of an efficient synthesis[145] of *N*-methyl-γ-amino-β-hydroxy acid.

In an assessment[146] of X-Ray and molecular modelling results it is concluded that in aureobasidin A, the four N-methylated amides are required for activity and conformational stability, as the non-methylated analogues did not secure a preponderance of the 'arrowhead-like' conformation usually seen in the natural form. The X-ray structure[147] of cyclo(Gly-Lac-Lac)$_2$ confirms two β-turns each of which involves a lactyl residue. Mass spectral fragmentation ions, useful in sequence determination, can be generated[148] *via* a highly specific sodium ion interaction that opens up the ring at the depside link. This approach has been applied to beauvericin, didemnin B and enniatin B1. The interactions of cations (Li^+, Na^+, Be^{2+} and Mg^{2+}) with cyclohexadepsipeptides composed of Gly and glycolic acids have been assessed[149] using *ab initio* calculations. Preferential co-ordination of the ions to the amide carbonyl, rather than ester oxygen atoms has been proven.

A new class of tyrosine based bridged cyclodepsipeptides has been synthesised[150] and given the family name tyrosinophanes, as represented by the general structure (137). Ring size can be varied but the potential for these structures serving as simple aromatic host molecules is exemplified by a K_{assoc} value of $8.95 \times 10^3 M^{-1}$ using *N*-methylacridinium hexafluorophosphate as the pyridinium guest. Several penicillin-like[151] and cephalosporin-like[152] β-lactamase inhibitors with typical structures represented by (138) have been synthesised. They represent the first δ-lactones that show substrate activity with class A and C β-lactamases. The antibiotic viomycin has been selected[153] for a study on the co-evolution of RNA and of proteins. It was concluded that 'smaller' molecules such as viomycin could play a role as selector molecules.



(137) X = CH or N

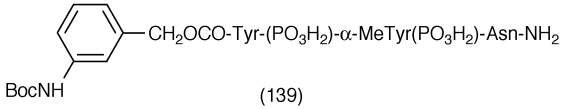


(138)

3 Modified and Conjugated Peptides

This section concentrates on peptides bearing non-peptidic conjugates attached to their side-chains. These are very often post-translational modifications to peptides, but are fundamental to their activity. Every year recently has seen an increase of activity in this field.

3.1 Phosphopeptides. – Although the full paper was not available to the Reporter, a one-step *O*-phosphorylation has been recorded[154] using bisalkyloxy-*N*,*N*-dialkylphosphoramidite followed by oxidation. The Association of Biomolecular Resource Facilities' peptide synthesis research group have assessed[155] the ability of member laboratories to synthesise phosphotyrosine peptides in a model sequence, H-Glu-Asp-Tyr-Glu-Tyr(PO_3H_2)-Thr-Ala-Arg-Phe-NH_2. All but four of the 33 samples submitted contained the correct product. Twenty of the 33 samples contained greater than 75% correct product, five contained less than 50%. All the laboratories used Fmoc chemistry, and no evidence of migration of phosphate to Tyr^3 was observed. Fmoc phosphotyrosine without side-chain protection had the edge over the protected derivatives. A new and high yielding method[156] for the preparation of the Fmoc-Tyr(PO_3H_2)-OPfp building block has been reported. When this was used for making fragments of murine adipocyte lipid binding protein, no pyrophosphates or other side-products were observed. Fmoc-L-(α-Me)Tyr (PO_3Bzl_2)-OH *via* its fluoride has been incorporated[157] into inhibitors of Grb2 SH2 domain. A peptide (139) showed the best inhibitory ability to date ($IC_{50} = 11 \pm 1$ nM). Evaluation of the merits of Fmoc-Tyr(PO_3Bzl,H)-OH, Fmoc-Ser(PO_3Bzl,H)-OH and Fmoc-Thr(PO_3Bzl,H)-OH has been done[158] using the multipin method and H-Ala-Ser-Gln-Gly-Xxx(PO_3H_2)-Leu-Glu-Asp-Pro-Ala-NH_2 (Xxx = Tyr, Ser or Thr) as a test peptide. After surveying 10 different coupling protocols, it became evident that all four DIC-based couplings resulted in incomplete incorporation. TBTU/HOBt/DIEA and HATU/HOAt/DIEA seemed to provide the most efficient incorporation of Fmoc derivatives. A novel polypeptide, poly[Ser(PO_3H_2)] has been synthesised[159] *via* the *O*-diphenylphospho-L-seryl-*N*-carboxyanhydride, followed by removal of protecting groups.

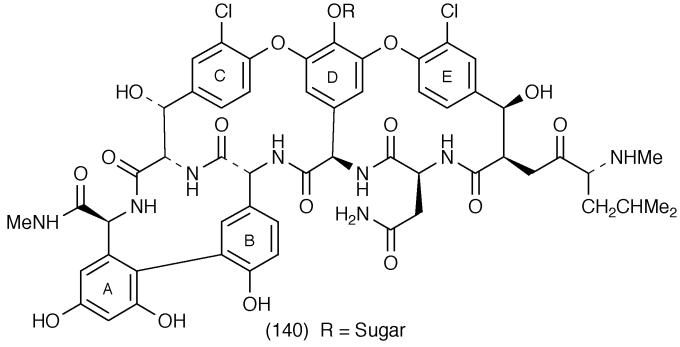


(139)

As phosphotyrosine itself is hydrolytically labile it is unsuitable in inhibitor design, as phosphatases and poor membrane penetration add to its disadvantages. Thus, there is an increasing interest in mimicking phosphotyrosine through the use of substituted phenylalanines. 4-Carboxymethyl-Phe and 4-carboxydifluoromethyl-Phe are known mimetics and have been produced[160] in protected guise with the synthesis of *N*-Fmoc-4-(OBu^{t}carboxymethyl)-Phe-OH and Fmoc-4-(OBu^{t}carboxydifluoromethyl)-Phe-OH for the preparation of

inhibitors. Their potential value as design units for Grb2SH2 domain inhibition has been evaluated.[161] Another mimetic 4-phosphonomethyl-Phe has proven to be a valuable tool, although having a chirally pure derivative for incorporation into a peptide had proved elusive. This has now been overcome by an enantioselective synthesis,[162] which does not require chiral induction, but derives its specificity from the racemisation-free nucleophilic substitution of lithium di-t-butyl-phosphite on to 4-bromomethylphenylalanine. Phosphopeptides can be purified[163] using affinity chromatography which is based on iron(III) immobilised on iminodiacetate-agarose gel. The method was successfully used in purification of seven enkephalin-related phosphorylated peptides, from crude solid-phase preparations.

3.2 Glycopeptide Antibiotics. – During 1998, as reported in this section last year, two very impressive total syntheses of vancomycin (140) were reported by the groups of Nicolaou and Evans. Full experimental details of the Nicolaou protocols have now been published in a four part series of papers.[164–167]

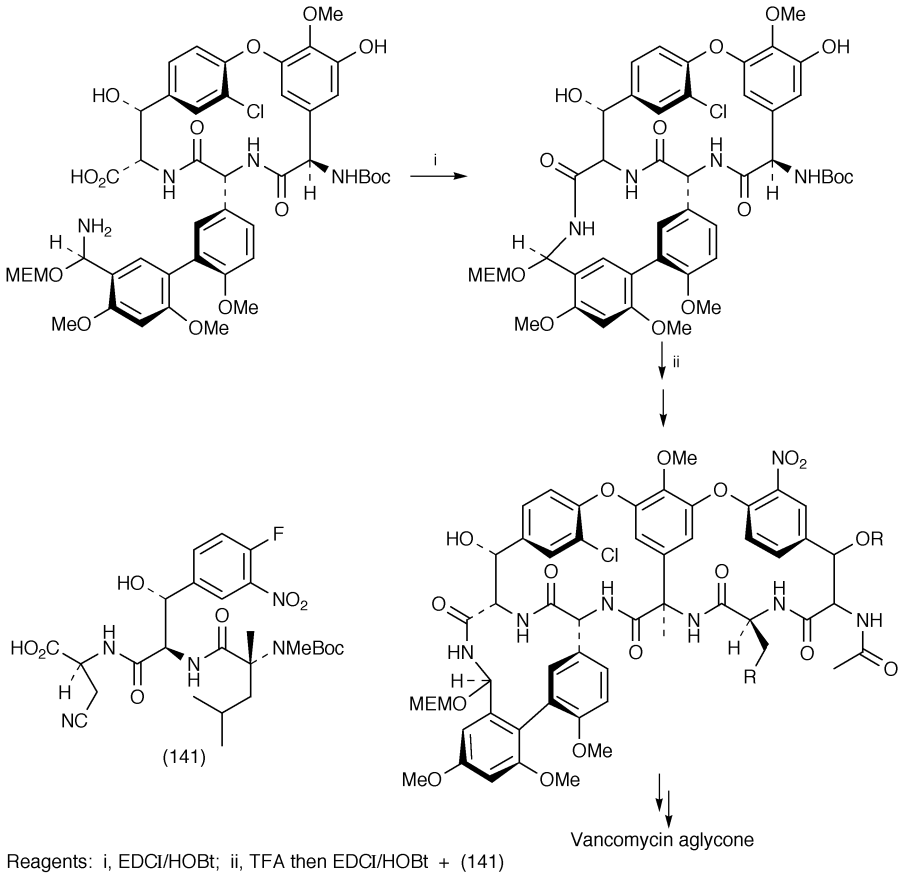


(140) R = Sugar

Complementary to the efforts of Nicolaou and Evans has been the work of Boger *et al.*, who now have published their own synthesis of the vancomycin aglycone.[168,169] Key to their strategy was the defined order of CD AB and DE ring closures which permitted selective thermal atropisomerism of the newly formed ring systems. This order also permitted recycling of the undesired atropisomers. Space does not permit inclusion of all details but the flavour can be deduced from Scheme 6.

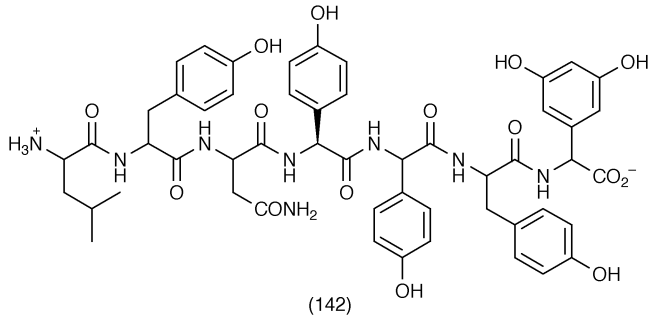
The current importance of the glycopeptide group of antibiotics is reflected in the authoritative reviews by expert practitioners in the field. Structure and mode of action of the vancomycins are covered in a 100-reference review,[170] while chemistry, biology and medicinal aspects are covered by a 376-reference review.[171] A wider perspective of the glycopeptides can be derived from reviews on antibacterial glycopeptide antibiotics[172] and on the synthetic and mechanistic studies on bleomycin A_2.[173]

Although total syntheses of the vancomycins have been reported as discussed above, a number of details of individual steps continue to enrich the literature for the vancomycins and related antibiotics. The impact of protecting

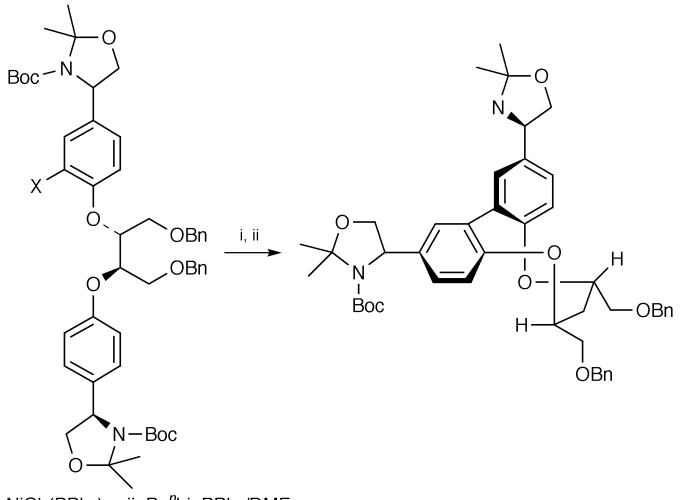


Reagents: i, EDCI/HOBt; ii, TFA then EDCI/HOBt + (141)

Scheme 6

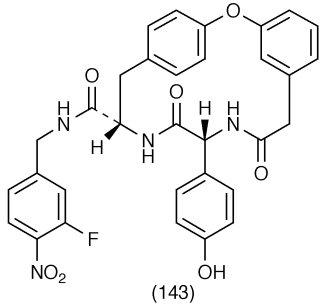


groups and the establishment of the stereochemical integrity of peripheral substituents on vancomycin CD and DE macrocyclisation have been reported.[174] Solid phase synthesis[175] of the putative heptapeptide intermediate (142) in vancomycin biosynthesis has been achieved using 2-chlorotritylresin, benzyl side-chain protection and allyloxycarbonyl groups for chain elongation.



Reagents: i, $NiCl_2(PPh_3)_2$; ii, Bu^nLi, PPh_3/DMF

Scheme 7



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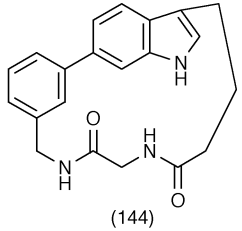
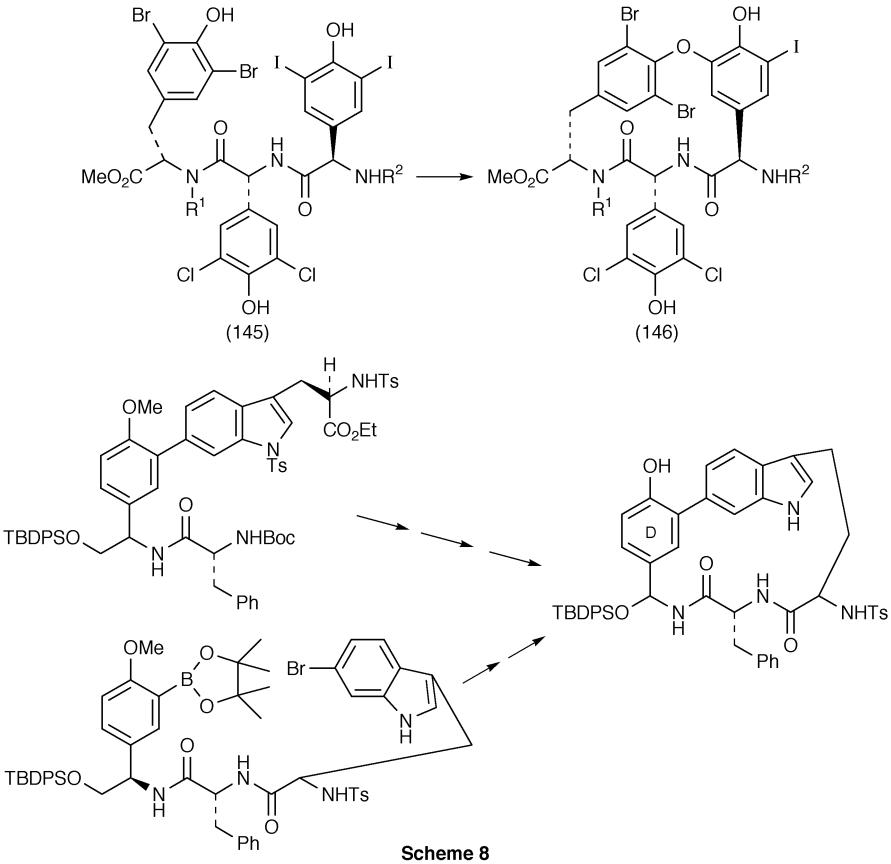


(144)

An intramolecular, Ni(0)-mediated approach has enabled[176] a biaryl coupling of a vancomycin synthon to take place with complete control of axial chirality. Scheme 7 summarises the details. Western (143) and eastern (144) sub-units of kistamycin have been synthesised[177] and, for (144) used Ni(0) mediated intramolecular cross-coupling. Thallium trinitrate has been shown[178] to be a successful reagent for phenolic oxidation of (145) to (146), which is the left hand segment of chloropeptin. The 16- and 17-membered DEF rings of chloropeptin and complestatin have been constructed by two approaches,[179] either by peptide backbone cyclisation under high dilution or by forming a C–C bond as a final step as summarised in Scheme 8. The C–C bond making appears to be faster.

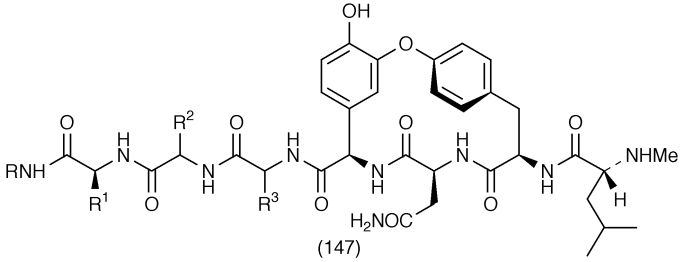
Efforts to modify the vancomycins, without total synthesis, have also been explored. To assess the contribution of the carbohydrate moiety, a protected aglycone of vancomycin has been converted[180] back into the natural form using sulfoxide glycosylation techniques. The N-terminus of vancomycin has been modified[181] by removing the leucyl residue by the Edman reaction and then replacing it with an extra amide NH to increase H-bonding. However, the



Scheme 8

modification does not enhance binding to *N*,*N*-diAc-Lys-D-Ala-D-Ala. Alteration[182] of the N-terminus of vancomycin to give a sp^2 centre at the α-carbon of residue-1 does not permit the leucyl hydrophobic chain to approach the ligand interface. Hence weaker binding results.

Covalent dimers of vancomycin, linked through the vancosamine sugar moieties have been synthesised[183] in one step in 69% yield. The dimers, qualitatively, seemed to give approximately the same response as natural vancomycin. A multivalent polymer[184] of vancomycin utilising linkages with the amino group of vancosamine has been reported to give 8–60 fold enhancement of potency against vancomycin-resistant enterocci. Combinatorial approaches have been used[185] to identify synthetic receptors which bind to Lys-D-Ala-D-Lac. The core macrocycle (147) was used as a fixed building block and amino acid members varied to give a 39,000 theoretical member library. A couple of library members were bound quite strongly to NAc$_2$-Lys-D-Ala-D-Lac and NAc$_2$-Lys-D-Ala-D-Ala. Two analogues of mucopeptide precursors found in vancomycin-resistant bacteria have been synthesised[186] by solid phase techniques. They were, *N*-Ac and *N*-docosanoyl-Gly-Ala-D-γ-Glu-



(147)

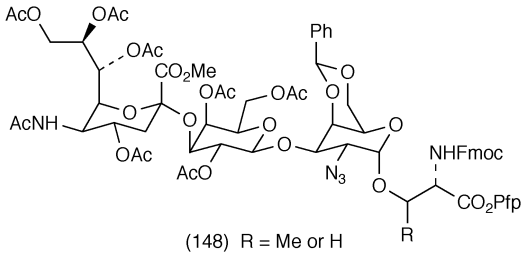
Lys(N-Ac)-D-Ala-D-Lac-OH, and their binding with chloroeremomycin assessed, when bound to an anchored ligand at the surface of a vesicle. Significant enhancement of binding was recorded.

A synthesis[187] starting from (*R*) and (*S*) Garner's aldehydes using stereocontrolled Grignard arylations has proved an efficient source of both diastereoisomers of the β-hydroxy-α-amino-acids in vancomycin. A chiral stationary phase incorporating teicoplanin has proved[188] suitable for enantioresolution of Boc-amino acids and even better for free amino acids.

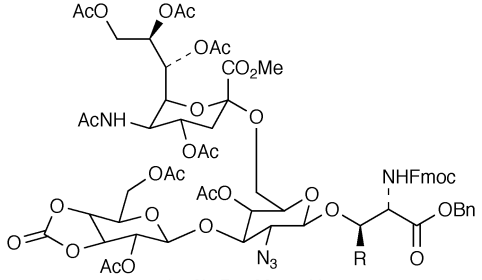
3.3 Glycopeptides. – Although publications under this section appear to be less numerous than usual, it is possible to sub-divide the output into O-linked and N-linked and other categories. However, some publications, especially reviews, transcend these boundaries and offer great insight into this important field. Thus recent developments in glyconjugates have been reviewed[189] and offer sections on glycopeptide assembly. Two reviews[190] explore the developing field of glycopeptide dendrimers, while a short review[191] has concentrated on the synthesis of glycopeptide mimetics, including C- and S-glycopeptides and glycopeptoids. The types and functions of glycopeptides found in nature and approaches to their synthesis have also been reviewed.[192]

Using deprotected C-glycopyranosyl ketones, and coupling in aqueous media, a convergent approach to O- and N-linked glycopeptide analogues has been devised.[193] In the move towards mimetics of natural links, C-linked isosteres have become popular, so publications in this area have been chronicled in a later sub-section.

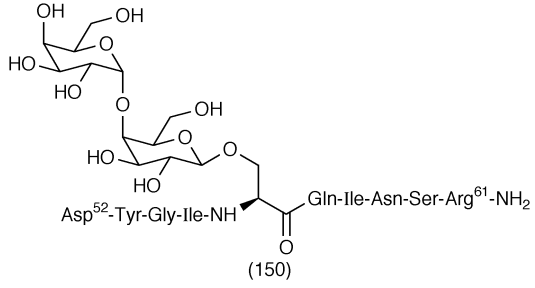
3.3.1 O-Glycopeptides. Synthesis remains the predominant theme in this selection of publications. A new block condensation approach[194] has been employed for the synthesis of sialyl (2→3)T-antigen trisaccharide, which after a series of manoeuvres was coupled with Fmoc-Thr/Ser-OPfp to give (148) as a derivatised building block. In a similar manner the *O*-linked derivatives (149) have been instrumental[195] in the synthesis of complex sialylated cell-surface antigens. The building block approach has also been applied[196] to the synthesis of sets of glycopeptides based on (150), which is [Galα→1(4)Galβ] O-linked to Ser^{56} in hen egg lysozyme (52-61). These analogues were used to probe the specificity of helper T cells. Two novel glycosyl building blocks (151) and (152), containing the T-antigen, have been synthesised[197] *via* glycosylation of



(148) R = Me or H



(149) R = Me or H



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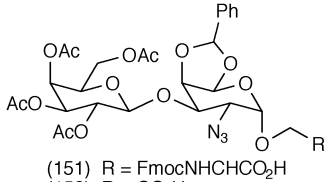
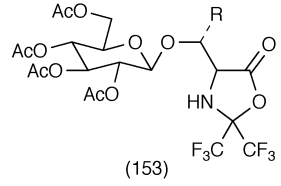


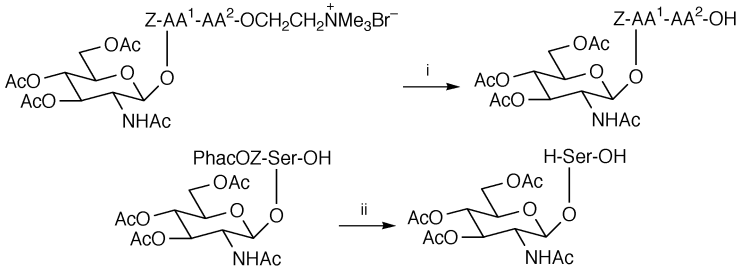
(151) R = FmocNHCHCO2H
(152) R = CO2H



(153)

Fmoc-homoserine and glycolic acid. A new protection/activation concept[198] for glycopeptide formation has been developed utilising the intermediate (153). This strategy offers the advantage of using ^{19}F NMR spectroscopy for monitoring the progress of the reaction.

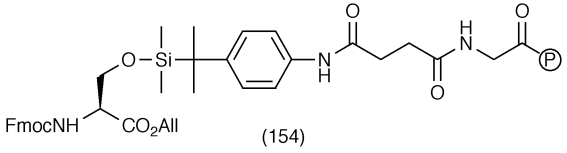
In a more general chemoenzymic approach,[199] glycosylated and phosphorylated peptides can be produced. The principle utilises enzymes to remove enzymatically labile protecting groups as represented by the two examples in Scheme 9, without affecting the glycosyl protecting groups and links. Short chromogenic and fluorogenic peptidyl-Arg-*p*-nitroanilides containing either



Reagents: i, butyryl choline esterase; ii, penicillin G acylase

Scheme 9

(β-Gal)Ser or (α-Glc)Tyr at P-2 and P-3 sites have been synthesised.[200] The hydrophilic sugar moieties increased the peptides' susceptibility to hydrolysis by trypsin, tissue kallikerin and rat tonin, but the effect with papain depended on the position of the sugar. A protected antigen building block, Boc-Ser(3,4,6-tri-OAc-D-GalNAc-1→α)-OH, has been used[201] in the solid phase assembly of multiple antigenic peptides such as, $[Ac\text{-}(Tn)_2\text{-}\gamma\text{-}Abu]_4\text{-}(Lys\text{-}\gamma\text{-}Abu)_2\text{-}Lys\text{-}\beta\text{-}Ala$, where Tn = D-GalNAc-1-α, immobilised on TentaGelS-NH_2. These compounds show promise for preparation of anti-tumour vaccines. The novel silyl linker (154) has been designed[202] for solid phase work with glycopeptide blocks, and has the advantage of the mild fluoridolysis step for release of glycopeptide from the resin. A successful protecting group strategy,[203] which overcomes the presence of Met, Tyr and Cys in glycopeptides, has evolved using (155) to make the corresponding diglycosylated portion of 256–270 type II collagen, Gly^{256}-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-HOLys(sugar)-Gly-Gln-Glu-Gly-Pro-Lys^{270}. Fmoc-Ser(2-acetamido-3,4,6-tri-OAc-2-deoxy-α-D-galactopyranosyl)-OH and its threonine analogue have been synthesised[204] as building blocks in *O*-glycopeptide synthesis.



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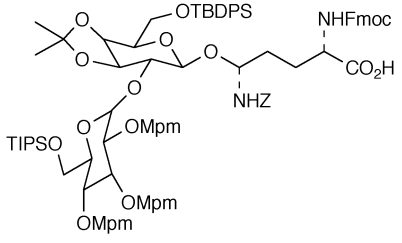
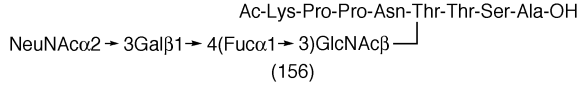


(155) Mpm = 4-methoxybenzyl

The effect of glycosylation on the conformation of peptides has been studied[205] by NMR, CD and molecular modelling using two series of

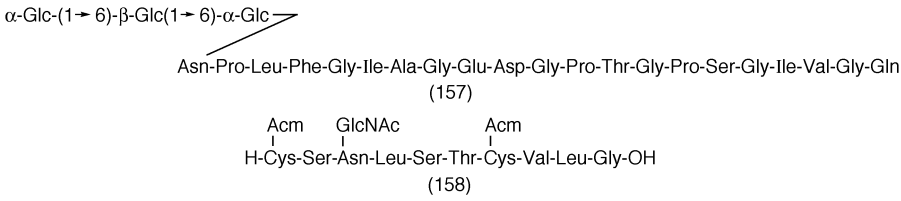
glycopeptides. GalNAc and Galβ→1(3)GalNAc derivatives of Ser and Thr were incorporated into two series of peptides, (I) Pro-Ala-Pro-Pro-Ser-Ser-Ser-Ala-Pro-Pro-Glu and (II) Ala-Pro-Pro-Glu-Thr-Thr-Ala-Ala-Pro-Pro-Thr, derived from tandem repeat sequences of human salivary mucin (MUC 7). Results show that the carbohydrate moiety on the Thr of series II is in close proximity to the peptide backbone. For Ser-linked examples (series I) the carbohydrates are more flexible, with more rotational freedom around the *O*-glycosidic bond. NMR studies[206] and molecular modelling work have been carried out on the *O*-Sialyl-Lewis-X model peptide (156) of the mucin domain of MAdCAM-1. There appears to be a delicate balance between hydrophobic (carbohydrate–carbohydrate and carbohydrate–water) interactions which may influence conformational changes in glycopeptides. Synthetic glycopeptides have assisted[207] in investigating the range of activity of drosocin and pyrrhocoricin derived from insects. Antibacterial activity of drosocin was generally increased by addition of Gal-GalNAc to the mid-chain positions, but pyrrhocoricin was more active without sugar units attached.



(156)

The crystal structure[208] of *N*-Z-*O*-(2,3,4,6-tetraOAc-β-D-Gal)-L-Thr-Aib-Aib-OBu^t has been obtained. The peptide backbone is fully extended at Thr, left-handed helical at Aib^2 and right-handed helical at Aib^3. Electron capture dissociation (ECD) in a Fourier Transform mass spectrometer gives favourable fragment ions to enable *O*-glycosylation sites to be localised in glycopeptides.[209] The mild character of the ECD technique provides far less loss of glycan in the fragment ions.

3.3.2 N-Glycopeptides. Nephritogenoside (157), isolated from the glomenural basement membrane of rats, has been synthesised[210] on solid phase using chlorotrityl-resin and a 3D orthogonal protection scheme based on Nps/Fmoc and benzyl groups. The strategy involved making three fragments, then attaching them at the Pro-Leu and Asp-Gly bonds. Prevention of aspartimide formation was covered by using Asp(OBzl) as the C-terminal residue of one fragment, attached directly to the trityl resin. An Asp residue just preceding an *N*-glycosylated Asn isomerised[211] readily to give the β-version as well, in the synthesis of an octadecapeptide spanning the fourth repeating unit of the human τ protein. This did not seem to occur when the Asn residue was not glycosylated. Glycopeptide analogues of eel calcitonin containing oligosaccharides such as (NeuAc-Gal-GlcNAc-Man)$_2$-Man-GlcNAc$_2$, (Gal-GlcNAc-Man)$_2$-Man-Glc-NAc$_2$ and (Man$_6$-GlcNAc$_2$) have been synthesised[212] through first of all preparing the GlcNAc analogue (158). The natural oligosaccharides were added by transglycosylation using *endo*-β-*N*-acetylglucosaminidase from *Mucor hiernalis*. Anomeric butyl glycosides of muramyl dipeptide have been synthesised[213] and tested for their adjuvant activity. Evaluation[214] of the effect



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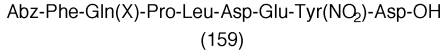
(158)



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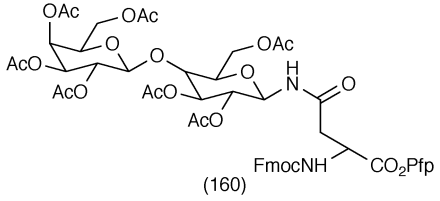
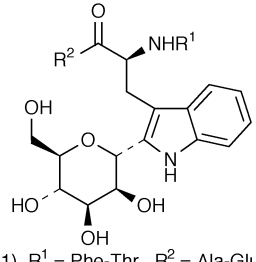


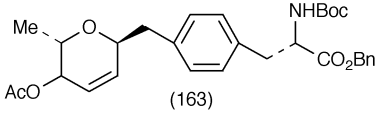
(160)

of glycosylation on enzymic stability of peptides has been obtained *via* the synthesis of N-linked Gln glycopeptides such as (159) with or without a carbohydrate at (X). The building block used in construction of the N-linked Glu was derivative (160). It was observed that glycopeptides are less susceptible to proteolytic enzyme degradation than the corresponding free peptides.

3.3.3 C-Linked and Other Linked Glycopeptides. As in last year's Report, there is once again justification in reviewing together under this heading an increasing trend towards mimetics of the natural glycopeptide links. However, it is not only mimetics that need to be highlighted, as the C-glycoside linkage shown in (161) was discovered naturally in 1994 in human ribonuclease. The stereospecific synthesis[215] of (162) has enabled its incorporation (as its tetrabenzyl derivative) into the pentapeptide (161). A first synthesis[216] of C-glycosyl tyrosine analogue (163) and Fmoc derivatives has provided the building blocks for use in the solid phase synthesis of C-glycopeptides.

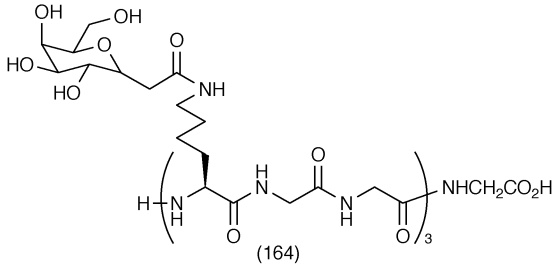


(161) R^1 = Phe-Thr, R^2 = Ala-Glu-Trp
(162) R^1 = H, R^2 = OH



(163)

Oxazolidine silyl enol ethers have been used[217] in the preparation of multigram quantities of α and β-Gal-CH_2-Ser isosteres. If the oxazolidine silyl enol ether was condensed with formyl$(OBzl)_4$-β-D-*C*-galactopyranoside in the presence of $BF_3{\cdot}Et_2O$ the β-linked C-glycoside was formed without anomerisation. On deoxygenation and cleavage this derivative afforded β-Gal-$(CH_2)_2$-Asn.



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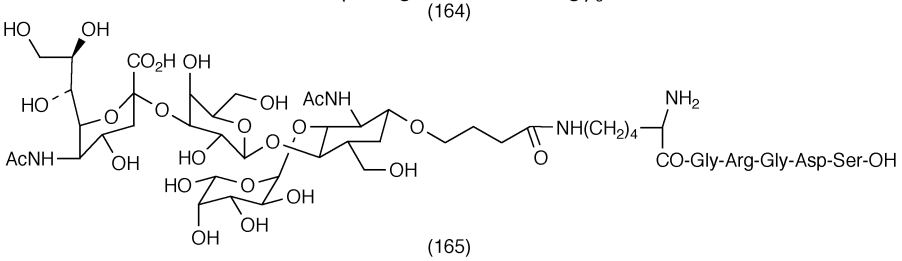
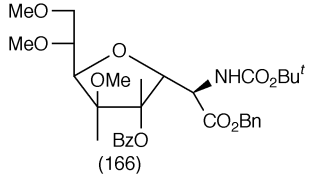


(165)

DuPHOS-Rh^+ catalysed asymmetric hydrogenation of enamide esters has been extended[218] to the preparation of the C-glycosyl serine analogue of the P^K trisaccharide [α-Gal(1→4)β-Gal(1→4)β-Glc-CH_2-serine]. Both α- and β-C-glycosides of *R*- and *S*-serine were prepared for investigation of the binding sub-unit of the shiga-like toxin. With the aim of understanding how native antifreeze glycoprotein (AFGP) inhibits ice crystal growth in organisms at subzero temperature, the C-linked structural mimic (164) has been synthesised.[219] A flexible lysyl based linker unit has been used[220] to cross-link an RGDS cell adhesion motif to a sialyl $Lewis^x$ ligand as in (165). A combination of chemical synthesis and chemoenzymatic strategies was used in the assembly.

C-Glycosyl amino acid derivatives such as (166), derived from free-radical cyclisations,[221] can be used as building blocks in combinatorial synthesis, while elaboration of an exo-glycal with subsequent rearrangement of a sulfone intermediate has yielded[222] *C*-glycosylserine. A hydroboration-Suzuki coupling of the same exo-glycal gave the *C*-glycosylasparagine analogue. Enantiomerically pure lactams have played a key role[223] in the synthesis of compounds such as (167) where R = H, Et, R^1 = Et, CH_2Ph and n = 1–3. The neoglycopeptide (168) or its acetate derivative has been synthesised[224] for testing the



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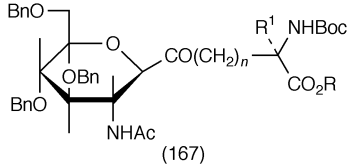
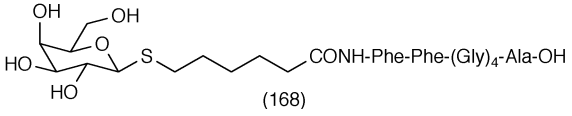


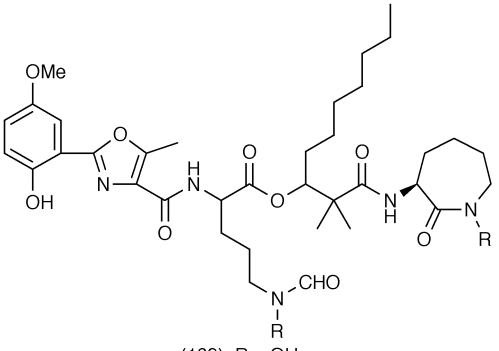
(167)



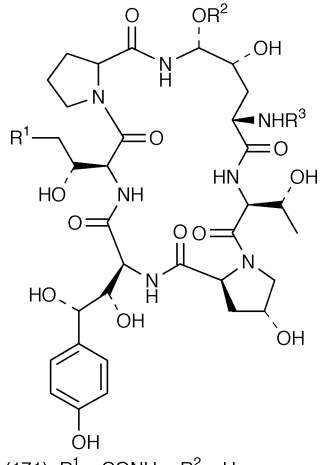
(168)

ability to bind to the lactose permease of *E. coli*, and to inhibit the transport of lactose. Very positive results were obtained for (168), derived from the resin bound heptapeptide H-Phe-Phe-$(Gly)_4$-Ala-OH and 2,3,4,6-tetraacetyl-5′-carboxypentyl-1-thio-β-D-galactopyranoside by different activation methods.

3.4 Lipopeptides. – Methods for the synthesis of lipidic amino acids and lipopeptides have been summarised in a review.[225] Two new lipopeptides, amamistatins A (169) and B (170), have been isolated from an actinomycete. Their structures are closely related to formobactin and nocobactin NA and show growth inhibition for human tumour cell lines.[226] The dimethyl myristoyl side-chain of pneumocandin B_o (171), a member of the echinocandin family, has been replaced[227] by the naphthoyl moiety as in (172), which is effective in treating systemic fungal infections.

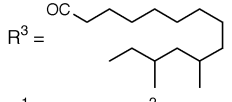


(169) R = OH
(170) R = H



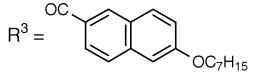
(171) $R^1 = CONH_2$, R^2 = H,

R^3 =

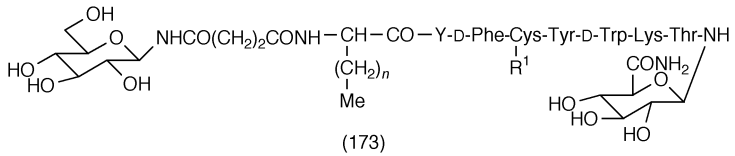


(172) $R^1 = CH_2NH_2$, $R^2 = (CH_2)_2NH_2$,

R^3 =



A method for *O*- and *S*-palmitoylation of non-protected peptides has been developed.[228] Excess palmitoyl chloride in 100% TFA for 10 min provides the necessary acidic conditions to prevent acylation of amino groups. So on prolonged treatment, peptides such as H-Gly-Cys-Phe-OH and H-Gly-Ser-Phe-OH are converted into *S*- and *O*-palmitoylated compounds respectively. *S*,*S*-Dipalmitoylated pulmonary surfactant protein-C model peptides show a substantial increase in α-helix content in dodecylphosphocholine micelles. Liposaccharide conjugates such as (173) of somatostatin analogue TT-232 have been synthesised[229] to study the effect on the physicochemical aspects of the molecules. *In vitro* experiments showed that lipid or sugar modifications at



(173)

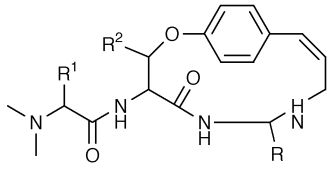
the N- and/or C-terminii did not affect the biological activity of the parent compound. Fluorescent-labelled lipopeptides have been prepared[230] to help in understanding the mechanism of their entry into the cell and their intracellular pathway. Solid phase chemistry was chosen to make the peptides which are based on a CTL-epitope. Pal-Lys(TMR)-$(Lys)_3$-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Lys(FL)-Leu-OH and Pal-Lys(FL)-$(Lys)_3$-Arg-Arg-Tyr-Pro-Asp-Ala-Val-Lys-(TMR)-Leu-OH were synthesised, where TMR = carboxytetramethylrhodamine and FL = carboxyfluorescein. These derivatives were able to induce antigen-specific cytotoxicity.

Derivatives of the lipopeptide tripalmitoyl-*S*-glycerylcysteine (Pam_3Cys) constitute highly potent non-toxic immunoadjuvants, and lipopeptide–antigen conjugates have found application as novel fully synthetic low MW vaccines. Their self-assembly and monolayer properties have now been studied[231] using transmission electron microscopy (TEM) and Brewster angle microscopy. Chirality of the glyceryl moiety and the addition of a Ser unit in the C-terminal position affected the aggregation and monolayer properties.

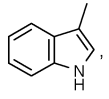
4 Miscellaneous Structures

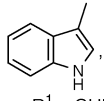
Many diverse structures have again been reported, which do not comfortably fit into the other main divisions of this chapter.

Cyclopeptide alkaloids have traditionally found themselves under this heading, and this year again new structures for waltherine A (174), and B (175)[232] and C (176)[233] have been given to the products from the bark of



(174) R = $PhCH_2$, R^1 = $-CH_2CH(Me)_2$, R^2 = $CHMe_2$

(175) R = $-CH(Me)CH_2Me$, R^1 =  , R^2 = $CHMe_2$

(176) R = Me, R^1 =  , R^2 = $CHMe_2$

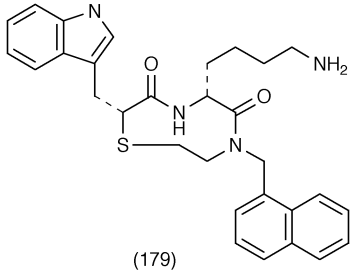
(177) R = CH_2CHMe_2, R^1 = $CHMe_2$, R^2 = Ph

(178) R = $-(CH_2)_3-$(Pro), R^1 = CH_2CHMe_2, R^2 = $CHMe_2$, *N*-oxide at Me_2N

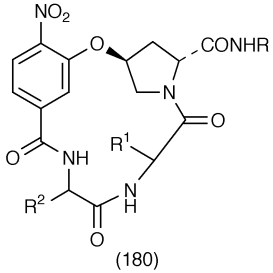
Waltheria douradinha. Integerrine (177) and an *N*-oxide analogue (178) have been isolated[234] from the Ecuadorian medicinal plant *Heisteria nitida.*

Cyclic lactam derivatives formed by bridging NH_2 and CO_2H groups of the side-chains of amino acids remain a popular means of restricting the conformations of peptides. For example four lactam bridges of analogues of the heptapeptide from the autophosphorylation site of Src have been synthesised,[235] by BOP catalysed cyclisation of the side-chains, to give H-Glu-Asp-c(Glu-Glu-Tyr-Thr-Lys)-OH, H-Glu-Asp-c(Asp-Glu-Tyr-Thr-Orn)-OH, H-Glu-Asp-c(Glu-Glu-Tyr-Lys)-OH and H-Glu-Asp-c(Asp-Glu-Tyr-Orn)-OH. In general, 'lactamisation' decreases the peptides' phorphorylability as PTK substrates although the first example in the list was a selective substrate of Syk tyrosine kinase. Fmoc and OFm orthogonal protection and on-resin cyclisation was the protocol adopted[236] to form lactam bridges such as H-Ser-Ala-Leu-Leu-c(Glu-Asp-Pro-Val-Gly-Lys)-NH_2 and H-Cys-Ser-Ala-Leu-Leu-c(Glu-Asp-Pro-Val-Gly-Lys)-NH_2, which bear the Asp^{281}-Pro-Val-Gly^{284} sequence of the glycoprotein gD-1 of the herpes simplex virus. N-Terminal to side-chain group cyclisation was also performed. The cyclic analogue Gln-c(Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu)-Asn-Pro-Val-NH_2 has been synthesised[237] to mimic the presumed 'cyclic' structure of the linear analogue based on the sequence 72–85 of Myelin Basic Protein Epitope (MBP). Both cyclic and linear analogues induced experimental allergic encephalomyelitis in animals.

Parallel syntheses and screening of β-turn mimetics such as (179) have been described[238] as a means of identifying heterocyclic ligands to somatostatin receptor type 5. Quite a number of macrocycles have been produced by S_NAr methodology. Thus 4-hydroxy-Pro analogue (180) was assembled[239] on solid

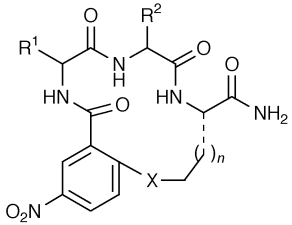


(179)

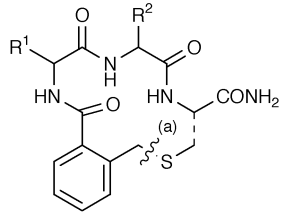


(180)

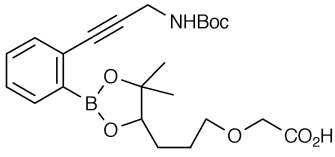
phase, the cyclic ether link being made from nucleophilic displacement of fluoride. The HO group of tyrosine has been coupled in the same way[240] to make further analogues. Extended turn peptidomimetic libraries of type (181) have also been formed[241] by nucleophilic displacement of fluoride on the NO_2-bearing aromatic ring. Compounds in this series, *e.g.* (181, $R^1 = CH_2COOH$, $R^2 = (CH_2)_4NH_2$, X = NH and $n = 0$, 1, 2 or 3) have been examined[242] by CD and NMR. All compounds adopt type I β-turns, but the most precise conformational mimetic existed in the analogue with $n = 1$. CD analysis has also been carried out[243] on O-analogue (181, $R^1 = CH_2COOH$, $R^2 = (CH_2)_4NH_2$, X = O and $n = 1$), and it revealed that stereochemical variation at the lysyl residue has the maximal effect on conformational diversity. An S_N2



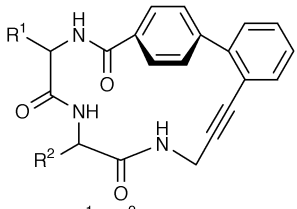
(181) X can be S, O or NH
R^1,R^2 and *n* can be varied



(182)



(183)

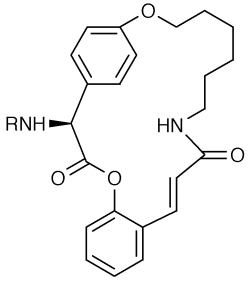


(184) $R^1 = R^2 = H$
(185) $R^1 = H$, $R^2 = CH_2CONH_2$

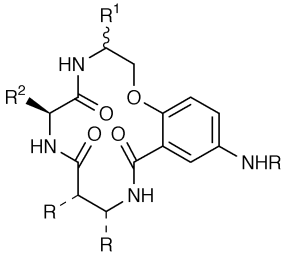
displacement between a benzyl bromide and the SH group of Cys [(a) in (182)] on solid phase has produced[244] a mimetic (182) with type I and type II β-turn conformations in solution. A Suzuki coupling[245] on solid phase, using a novel linker (183) has resulted in the preparation of (184) and (185).

In order to overcome their low intestinal mucosal permeability, RGD analogues incorporating coumarin-based esterase-sensitive groups have been developed[246] as cyclic prodrugs. Compounds such as (186) show higher membrane interaction potentials. The enhancement of stability of cyclic over linear peptides has been assessed[247] using molecular dynamics simulations, energy minimisations, and mass spectrometry of degradation fragments. Comparisons were made between H-Arg-Gly-Asp-Phe-OH and cyclo(1-6)Ac-Cys-Arg-Gly-Asp-Phe-Pen-NH_2, the latter cyclised *via* a disulfide bridge. It was found that as well as increased backbone rigidity the cyclic analogue had a salt bridge across Arg and Asp side-chains. Degradation of both linear and cyclic analogues seems to be influenced by the Asp-residue but above pH 8 the disulfide bond degraded. Ac-Pen-Arg-Gly-Asp-Cys-NH_2 has also been subjected to a similar simulation study[248] and compared with its disulfide bridged analogue. The cyclic analogue appears to be locked into a family of conformations, with a well-defined pharmacophoric conformation. Although this conformation can exist in the linear form, there are also many other conformations for the linear molecule to adopt. A combinatorial library based on (187) has been formed[249] on solid phase *via* an S_NAr fluoride displacement, and benefitted from the presence of a β-amino acid residue in the macrocycle.

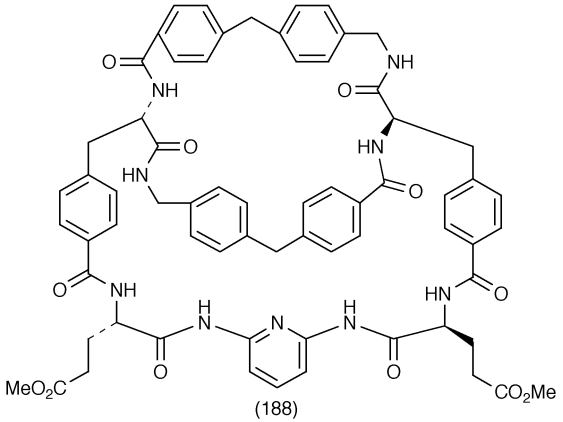
Synthetic receptor molecules have been popular developments in this section over recent years. In a project aimed at mimetics of vancomycin's attraction for bacterial cell wall's -Lys-D-Ala-D-Ala-OH triad, bowl-shaped macrobicyclic receptors such as (188) have been studied.[250,251] Compound (188) is a strong and selective receptor of Z-Ala-Ala-OH ($-\Delta G_{ass} = 25$ kJ mol^{-1}).



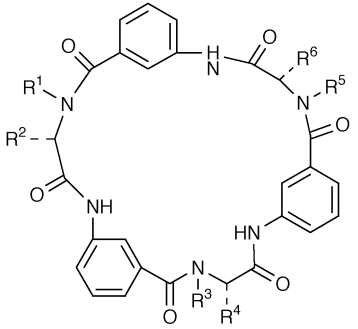
(186) R = SO_2Bu^n or $CO(CH_2)_2Ph$



(187)



(188)



(189) $R^1 = R^3 = R^5 = H$, $R^2 = R^4 = R^6 = (CH_2)_2CO_2H$

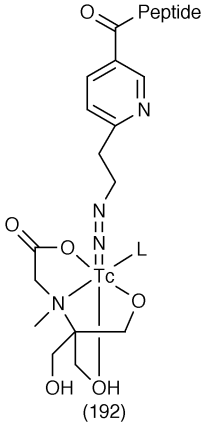
(190) $R^1 = R^2$, $R^3 = R^4$, $R^5 = R^6$ all equal to $-(CH_2)_3-$ (*i.e.* Pro)

H-Pen-Gly-Val-Asp-Val-Asp-Gln-Asp-Gly-Glu-Thr-Cys-OH

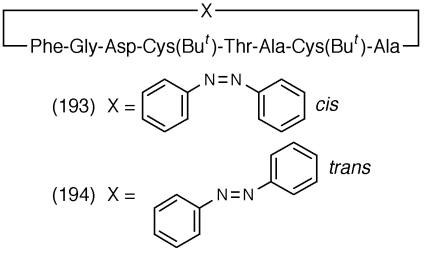
(191)

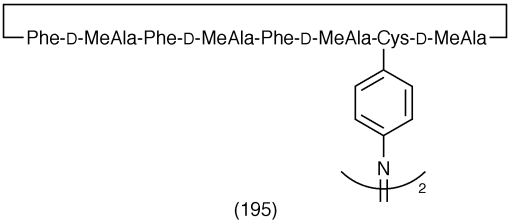
Receptor properties of (189) have been studied[252] with a number of physical techniques such as NMR. It binds to cations such as butyltrimethylammonium iodide, and to anions such as sulfonates and phosphonates, through H-bonding to peptide NH bonds. X-ray studies together with NMR techniques[253] on (190) have concluded that the shape of the molecule is similar to the cone

shape of the calixarenes. Again the aromatic subunits are involved in the cation-π interactions whilst anions interact with peptidyl NHs. Complexation of (191) to Ca^{2+} ions has been studied[254] by CD, NMR and molecular simulations with evidence accumulated for a 1:1 peptide calcium complex at low concentrations but multiple complexes occur at higher cation concentrations. The conformation is not drastically altered by complexation.



(192)





(195)

The success of ^{99m}technetium as a specific imaging agent has been developed further with its chelation to $GP_{IIb/IIIa}$ receptor antagonists for the early diagnosis of thrombus formation. The hydrazinonicotinamide complex (192) has been attached[255] to cyclo(D-Val-MeArg-Gly-Asp-Mamb) where Mamb is 3-amino-5-aminomethylbenzoic acid. Such complexes are now undergoing clinical evaluation as thrombus imaging agents. The peptide backbone[256] is much more conformationally relaxed in the *cis* form (193) than it is in the *trans* form (194). The *trans* form relaxes to the *cis* form on radiation with light. The

azopeptide (195) provides[257] a new photochromic supramolecular system that permits reversible switching between inter- and intra-molecular H-bonds, both in solution and in thin films.

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5
Current Trends in Protein Research

BY JENNIFER A. LITTLECHILD

1 Introduction

This article highlights some of the important areas developed in the field of protein research in 1999. This is a large research area, which is increasingly growing. Topics covered are protein folding, structural genomics, enzyme pathways, enzyme engineering, calcium containing proteins, proteins of the immune system and protein nucleic acid interactions especially RNA/protein interactions.

2 Protein Folding

An understanding of protein folding presents a major issue, especially now, due to the vast amount of data on primary sequence obtained from the genome sequencing projects. Also the increasing evidence of protein misfolding in disease has major implications. Much of the recent understanding of how proteins fold has come from new and improved experimental methodology. Collaborations between experimentalists and theoreticians have resulted in some new insights into the overall mechanisms involved. This approach has recently been reviewed by two leaders in the field, Professor C. Dobson and Professor M. Karplus.[1] One of the main experimental techniques that has been used to study the conformation of the denatured state of model protein systems is NMR spectroscopy. A recent study has been carried out using ^{15}N and ^{13}C labelling with the protein lysozyme as a model, in order to calculate the side chain conformations of the urea denatured state.[2] Individual residues appear to have preferred side-chain conformations in the denatured state – especially aromatic residues which were found to exist in hydrophobic clusters. Another new NMR approach is able to examine the hydration of denatured and molten globule proteins by magnetic relaxation dispersion.[3] The hydration of the native and molten globule state was found to be the same. It is possible to look at a 'folding energy landscape' as shown in Figure 1 to try and understand the route to the native state of the protein. Nuclear Overhauser Enhancement (NOE) data have been used to examine the conformation of SH3 protein domains from *Drosophilia* DrKN protein under native and denatured conditions.[4] Here the guanidium hydrochloride denatured species

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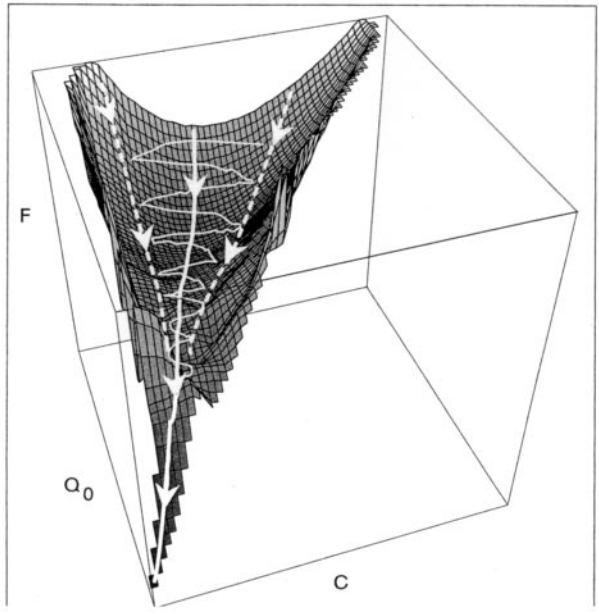

Figure 1 *Folding energy landscape showing the free energy (F) surface of a protein at a temperature T(T<Tm) of a fast-folding 27mer as a function of the fraction of native contacts (Qo) and the total number of the (native and non-native) contacts (C)*
(Reproduced with permission from Dobson and Karplus[1])

has a distinct compact structure that is thought to limit the search to find the native protein state.

The solvent trifluoroethanol (TFE) has been found to increase the secondary structure in denatured proteins. The addition of small amounts of TFE has been found to accelerate folding of acylphosphatase whereas high concentrations of TFE inhibit folding since incorrectly folded elements of secondary structure are thought to be trapped in the wrong conformation.[5] Secondary structure of proteins in different states can easily be examined by circular dichroism. The effect of TFE on folding pathways has indicated that studies with co-solvents should be interpreted with caution.[6] Site-directed mutagensis of proteins has been a popular technique to examine the effect of changes in specific amino acids to the overall folding pathway of proteins. The effect of H helix destabilisation mutations has been studied on the kinetic and equilibrium folding of apomyoglobin by Professor P. Wright and his group.[7] The effect of core mutations on the folding of a β sheet protein, CD2, has been studied;[8] this showed that intermediates early in folding have a native topology. Loop length variants of four-helix bundle protein ROP have suggested that loop closure is required for formation of rapidly formed intermediates.[9] In 'nature' protein folding is carried out in association with chaperonin proteins such as GroEL. It is important to establish that studies carried out *in vitro* mimic that which occus

in vivo. It has been demonstrated that GroEL accelerates the refolding of hen lysozyme without changing its folding mechanism.[10] The mechanism of chaperonin function has been discussed by Shtilerman *et al.*[11]

Other techniques developed to measure protein folding have been laser-flash photolysis, combined with triplet energy transfer. This has allowed measurement of contact times between donor and acceptor groups in various lengths of peptides to be measured from 20 ns to 30 ms time scale.[12] Photoinduced electron transfer has been used to follow the formation of the four-helix bundle protein cytochrome b_{562}.[13] A combination of stopped-flow and continuous-flow fluorescence measurements has allowed the full characterisation of the folding of the B1 domain of protein G.[14] Small angle X-ray scattering has been used to study the compactness of the denatured state in a submillisecond time scale.[15]

Diffusion collision theory has been applied to previously measured folding kinetics of the GCN4-pl peptide and this has been used to predict the effect of glycine mutations.[16] Theoretical interpretations of the folding of helical proteins have been published by Professor M. Karplus and colleagues,[17] and a lattice model analysis of multiple pathways with intermediates for the thermodynamics and kinetics of protein folding in general has been described.[18]

The group of Professor A. Fersht has continued his studies on folding of the small protein barnase.[19,20] This has involved generating peptide fragments starting at the N or C terminus and monitoring the importance of secondary and tertiary interactions in the folding pathway.

3 Structural Genomics and Protein Folding

With the high increase in genome sequencing currently occurring the need to determine how proteins fold based on their primary sequence has led to the emergence of the area of 'structural genomics'. Burley *et al.*[21] were early to recognise the importance of this area in their article where they discuss 'structural genomics beyond the human genome project'. Several databases contain information on protein structure such as PDB Protein Data Base, Prosite to look at three-dimensional patterns,[22] CATH to look at protein domain structure and SCOP, for example. The existing databases have been summarised in a review by Orengo *et al.*[23]

The number of protein structures determined by X-ray methods, NMR or homology modelling is increasing exponentially. As of the end of 1999 over 3000 structures were deposited. However, the number of new folds found is decreasing. It has been known for many years that a specific protein fold can recognise a particular cofactor such as NAD[24] or phosphate as described by Kinoshita *et al.*[25] The structural motif important for phosphate binding is common to various protein superfamilies. This is also true for metal binding motifs, for example the calcium binding motifs (discussed later in this review), other cofactor binding motifs and motifs that have to recognise other macromolecules such as DNA. This area of research will expand over the next few years with the ever increasing genomic and protein structural information.

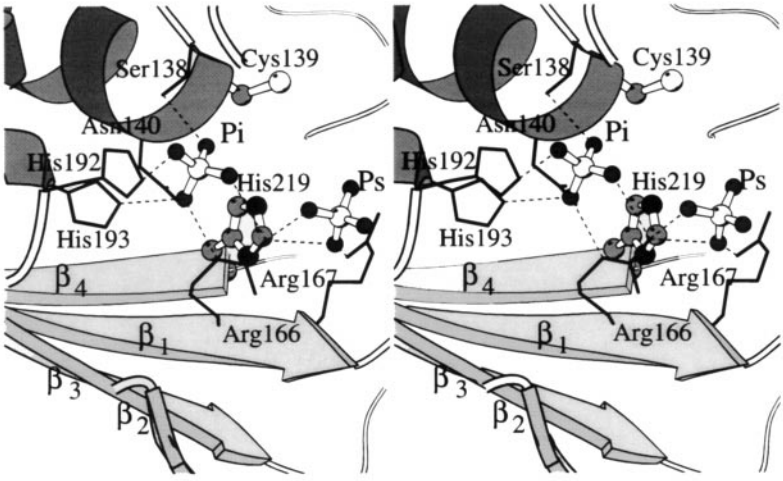

Figure 2 *A stereo view of the active site pocket from the outside towards the β-sheet of the catalytic domain. The sulfate ions located at the Pi and Ps sites, and the Cys139 and His219 implicated in the catalytic mechanism are shown as ball and stick models. Residues involved in the phosphate binding sites are shown as filled lines. Hydrogen bonds are shown by broken lines*
(Reproduced with permission from Isupov *et al.*[26])

4 Enzymes and Evolution

Many important enzymes are found throughout all species and it is interesting to compare their similarity and percentage identity. Living organisms are thought to be split into three kingdoms, the eukaryotes, the eubacteria and the archaea. The enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the archaea shows low sequence identity (16–20%) to its eubacterial and eukaryotic counterparts. The crystal structure of the apo GAPDH from *Sulfolobus solfataricus* has been determined by multiple isomorphous replacement at 2.05 Å resolution.[26] Significant differences from equivalent GAPDH enzymes are apparent at the secondary structure level. There is a relocation of the active site residues within the catalytic domain of the enzyme. Cys139 is located at the C-terminus of an α-helix in the same topological position as active site Cys149 in the *Bacillus stearothermophilus* eubacterial enzyme. In eubacterial and eukaryotic GAPDHs a conserved His176 which is located on strand β1 of the catalytic domain is thought to act as a base extracting the proton from the Cys149 during catalysis. However, there is no histidine residue at this position in the archaeal GAPDH structure. Instead, another residue conserved in archaea, His219 from the strand β4 of the catalytic domain, positions its imidazole group in about the same location (Figure 2). Two of the active site residues in the *S. solfataricus* enzyme, Arg166 and His219, seem to be laterally shifted across the β-sheet of the large domain in relation to the functionally equivalent pair of residues, Arg231 and His176, in

the *B. stearothermophilus* enzyme. Although archaeal and eubacterial/eukaryotic GAPDH enzymes have a related protein scaffold the residues implicated in the catalytic mechanism and the phosphate binding sites seem to be relocated between different structural elements. Only the active site cysteine and the preceding serine residue are conserved and remain in a similar position. One might speculate that this is due to the fact that an ancestral GAPDH enzyme had a similar fold and a related quaternary structure but had a low turnover and broad specificity using only a cysteine residue for catalysis. It seems that the active sites of archaeal and eubacterial/eukaryotic GAPDH enzymes eventually converged to a similar three-dimensional arrangement.

5 Enzyme Engineering

This can be carried out using mutagenesis in a rational or non-rational way. Rational redesign requires a knowledge of the three-dimensional structure of the enzyme obtained from crystallographic, NMR or modelling approaches. A novel approach to this problem has been described by the group of Jones[27] where a combination of chemical modification and mutagenesis has been used to tailor the specificity of the S1 pocket of subtilisin. A cysteine residue was introduced into the subtilisin which was then modified by a series of chemical groups. The normal preference of this enzyme for large hydrophobic residues in the P1 pocket has been switched to a preference for small and charged amino acids.

Standard mutagenesis has been used to convert linoleate 1,3-lipoxygenase into a 9-lipooxygenating species by the mutation of a single histidine residue to a valine residue.[28] Rather than a single residue the exchange of three loops has been used to convert 3-α-hydroxysteroid dehydrogenase into 20-α-hydroxysteroid dehydrogenase which changed specificity of the mammalian enzyme from androgens to progestins.[29] Catalytic function can be redesigned and this has recently been illustrated with the interchange of catalytic activity with the 2-enoyl-coenzyme A hydrogenase/isomerase superfamily.[30] This redesign involved changing eight residues and resulted in 4-chlorobenzoyl-CoA dehydrogenase being changed into crotonase. Asparate amino transferase has been changed into a L-aspartate β-decarboxylase by a triple active-site mutation.[31] O'Brien and Gerlt have reviewed the understanding of how mutations can change the balance between the original and the side reaction of an enzyme.[32]

Another completely different approach to rational design is to use natural multienzyme complexes such as polyketide synthases and to 'mix and match' individual modules. This approach has been exploited during 1999. The enzymes can be redesigned to change their catalytic properties or binding functions.[33–35]

Restriction enzymes have been engineered to change their substrate specificity by making them chimeric.[36] An engineered Cys_2H_5 zinc-finger protein has been redesigned to carry out highly efficient endonucleolytic cleavage of

RNA.[37] Other approaches to graft catalytic properties onto a protein template to create a scytalone dehydrotase enzyme have met with some success but lower than expectation.[38]

Artificial metalloenzymes have been made based on protein cavities.[39] Directed evolution is the opposite approach to rational design. This area has also received considerable interest recently since it has been shown that multiple properties of proteins can be optimised simultaneously and rapidly. In this way it is possible to produce enzymes that would not have evolved naturally since they would not have been subjected to the same conditions. This area of research has been reviewed by Arnold and Volkov.[40] Sometimes a combination of directed evolution and rational design is used, for example to produce a fungal peroxidase.[41] A combination of site-directed mutagenesis has been used with random mutagenesis by error prone PCR and *in vivo* DNA shuffling. An important contribution is the development of a peroxide-mediated cytochrome P450 hydroxylation system[42] which has been achieved by evolution of P450 monooxygenases that hydroxylate naphthalene in the absence of cofactors by using hydrogen peroxide as the source of oxygen.

Enzymes can be changed to increase their enantioselectivity[43] substrate specificity and catalytic efficiency[44] or to alter thermostability.[45] Hoseki *et al.* found that increased thermostability of up to 20 °C was conferred by an accumulation of 19 mutations on the protein surface. The references are only an example of the many papers published in this area in the last few years. Techniques involved are improving and can result in extremely high mutation rates such as the approach of saturation mutagenesis used with subtilisin by the group of Arnold.[46]

6 Enzyme Pathways

With so many crystal structures available it is now possible to examine the structures of every enzyme in a particular pathway. Probably one of the best known pathways is that of glycolysis where all of the structures can be compared to get some idea of their evolution. This is not always obvious. The only similarity in this case is that they all display a α/β hydrolase fold. In the case of other pathways such as the Rhamnose pathway there is an interest for therapeutic intervention. This pathway is found in bacteria and plants but not in humans. L-Rhamnose is a common component of the cell wall and the capsule of many pathogenic bacteria. Several enzymes in the pathway have been studied structurally and mechanistically in 1999.

The first enzyme of the pathway, Rm1A, catalyses the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate. The structure of a related nucleotide transfer enzyme *N*-acetylglucosamine-1-phosphate uridyltransferase has been reported in 1999 by Brown *et al.*[47] The structure is shown in Figure 3. The second enzyme of the pathway, Rm1B dTDP-D-glucose-4,5-dehydratase, has been reported by the group of Naismith.[48] The dTDP-4-dehydrorhamnose-3,5-epimerase and dTDP-4-dehydrorhamnose reduc-

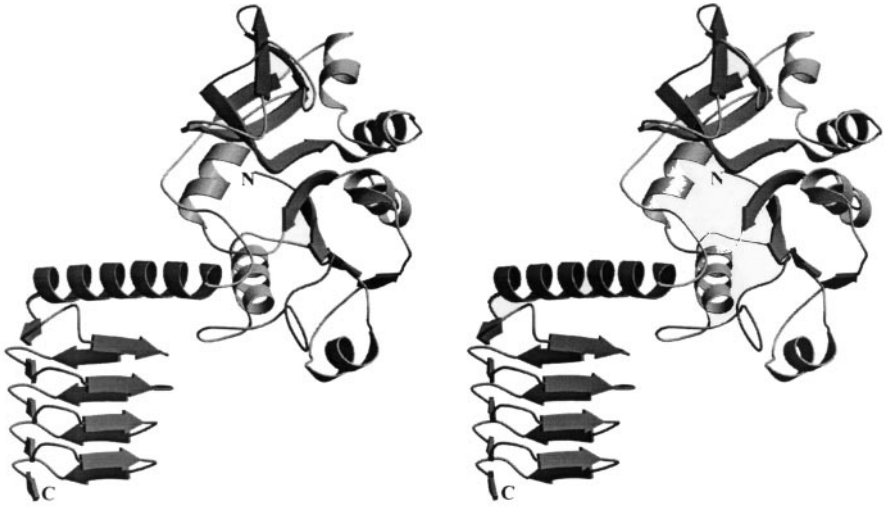

Figure 3 *Ribbon diagram of the overall view of the enzyme N-acetylglucosamine-1-phosphate uridyltransferase*
(Reproduced with permission from Brown *et al.*[47])

tase,[49,50] both from *Salmonella typhimurium*, have recently been characterised and represent Rm1C and Rm1D, the third and fourth enzymes in the pathway.

The crystal structure of Rm1C has also been recently reported by two groups.[51,52] This third enzyme catalyses an unusual double epimerisation reaction. The final and fourth enzyme of the pathway Rm1D has been studied structurally also by the group of Naismith.[53] This enzyme reduces the C4-keto function to generate the final product of dTDP-L-rhamnose as shown in Figure 4.

7 Calcium Containing Proteins

Calcium is an important metal which is recognised to be essential to human health. There have been many protein structures described where calcium binds to a specific protein motif. This binding site has been called the 'EF-hand' where one α helix winds around what would be the index finger and the other helix winds up the thumb. When the calcium binds between these two helices the 'thumb helix' moves. In some cases as described for the protein calmodulin,[54] this motif can also bind magnesium. The changes in protein conformational states as described above observed within the superfamily of proteins binding calcium have been summarised by Yap *et al.*[55]

7.1 Paravalbumin. – The crystal structure of EF-hand parvalbumin has been described in 1999 to atomic resolution (0.91 Å) by Declercq *et al.*,[56] and this is therefore the most accurate determination of a calcium binding protein

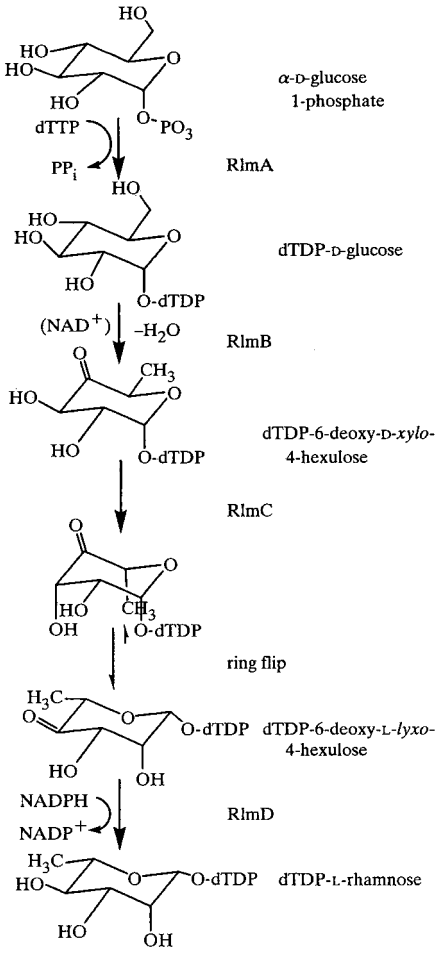

Figure 4 *The dTDP-L-rhamnose biosynthetic pathway*
(Reproduced with permission from Giraud *et al.*[53])

described to date. The metal ion affinity, co-ordination geometry and domain plasticity have been studied in paravalbumin by both experimental[57] and theoretical methods.[58] This latter paper uses energy calculations to understand the change in affinity of the EF-hand for Ca^{2+} and Mg^{2+} using information obtained from the high resolution structures.

7.2 S100 Proteins. – S100 proteins are regulated by calcium and have been found to undergo a conformational change when the metal binds.[59] Several papers have tried to understand the nature of this conformational change and what directly maintains the calcium binding by the protein.[60,61] The paper described by Rety *et al.* studies a mutant S100 enzyme and shows how the calcium loaded or 'open' conformation is maintained by a network of

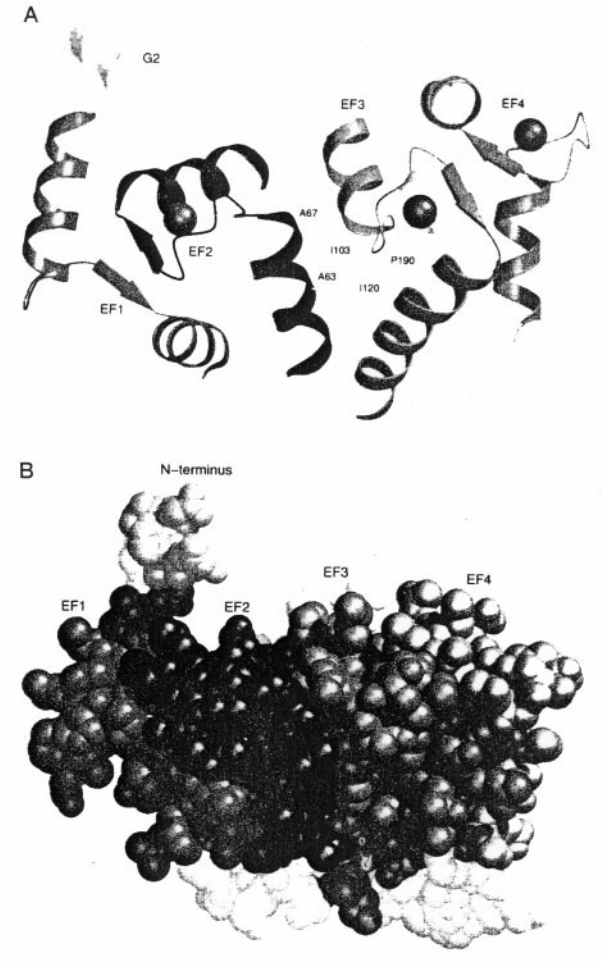

Figure 5a,b *Guanyl cyclase activating protein-2.* (a) *A schematic ribbon representation showing the four EF-1to EF-4 hands and the three bound calcium atoms in space filling mode;* (b) *A space-filled model of the protein structure showing the four EF-hands on one side of the molecule*
(Reproduced with permission from Ames *et al.*[64])

hydrogen bonds. The EF-hand domains occur in pairs in most proteins and can also be part of a large protein. The Eps15 homology domain, an important factor in receptor mediated endocytosis of growth factor receptors, is also related to S100 proteins.[62] In this case the pairs of EF-hands are separated by a flexible region.

7.3 Neurocalcin and Guanyl Cyclase Activating Protein-2. – Two new structures reported in 1999 are of neurocalcin[63] and guanyl cyclase activating protein-2.[64] The latter NMR structure contains four EF-hand motifs arranged

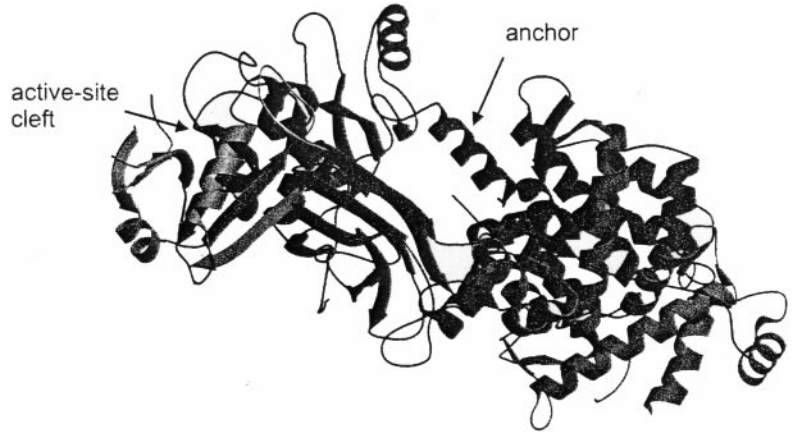

Figure 6 *A ribbon diagram of the structure of calpain showing the active site cleft and the helical anchor*
(Reproduced with permission from C.M. Hosfield, J.S. Elce, P.L. Davies and Z. Jia, *EMBO J.*, 1999, **18**, 6880–6889, by permission of Oxford University Press)

in a compact tandem array. This protein is a Ca^{2+} sensitive regulator of phototransduction in retinal photoreceptor cells. It contains a myristoylated amino terminus which is not present on this NMR structure. The EF-1 hand of this protein does not bind calcium (shown in Figure 5a) since it contains a Cys-Pro disabling sequence which is also found in the protein recoverin. In both of these structures the EF-hands are more compact and are located on one side of the molecule as shown for guanyl cyclase in Figure 5b.

7.4 Calpain. – Calpain is a thiol protease that is regulated by calcium. This molecule was not thought to have paired EF-hand domains as discussed above since the crystal structures reported earlier and a recent structure described in 1999[65] show that is contains five EF-hands in the C-terminal part of the large domain. However, the unpaired EF-hand pairs with the related EF-hand from another small subunit to form a homodimer. Alternatively the C-terminal domain of the large subunit and the small subunit in the heterodimer together form a complex that is superimposable onto the structure of a homodimer of the C-terminal domain. Hosfield *et al.* discuss the crystal structure of calpain and the basis for its Ca^{2+} dependent protease activity. They find a regulatory mechanism that is unusual for proteases. In calpain the catalytic cysteine residue is 10.5 Å away from the histidine and too remote to form a competent catalytic triad. The Ca^{2+} binding is proposed to induce a conformational change that reduces this distance to ~3.7 Å in order to form a catalytic triad for protease activity. A ribbon diagram of the structure of calpain is shown in Figure 6 showing the active site cleft and the anchor that inhibits active site assembly by associating with the regulatory subunit, thus restricting flexibility of the protease.

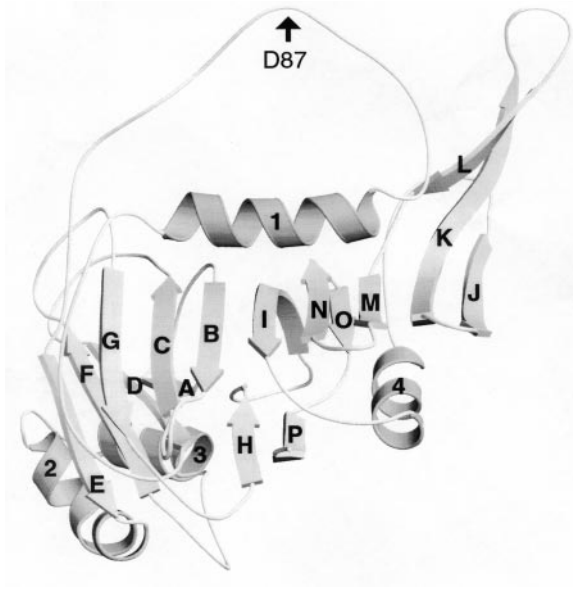

Figure 7 *The structure of the protein caspase. Ribbon drawing of the P35 monomer B. The scissile bond is marked with an arrow*
(Reproduced with permission from Fisher *et al.*[74]).

7.5 Calmodulin. – Calmodulin is a well studied Ca^{2+} binding protein but recent papers have tried to address the functional significance of the cooperative binding of the metal. This has been explored by NMR spectroscopy.[66,67] Also a recent NMR solution structure[68] has been reported for a complex of calmodulin with a binding peptide of the Ca^{2+} pump. In addition Osawa *et al.*[69] have studied a novel target recognition revealed by calmodulin in complex with Ca^{2+}-calmodulin kinase providing a further insight into the mechanism of calmodulin.

8 Other Interesting Proteins

8.1 Caspase-8. – The cysteine protease caspase-8 is one of the initiator enzymes in apoptosis or cell death.[70] Two papers in 1999 have described its structure, one at atomic resolution.[71,72] There are 14 caspase enzymes reported to date as reviewed by Wolf and Green.[70] They all show specificity in where they cleave proteins, which is always after an aspartic acid and a recognition sequence of at least four amino acids towards the N-terminal of the cleavage site. Caspase-8 falls into group 3 of this class of enzyme with a preference to cleave at the amino acid sequence (I/V/L)EXD (X = any amino acid). The caspases have to be activated by proteolysis of a zymogen and this process has been reviewed by Stennicke and Salvesen.[73] All caspase enzymes have a large and small domain. The heterodimer is composed of six β strands and two α

helices. The twisted β sheet has two of the α helices on one side and three on the other. Two of these heterodimers are associated. The structure of caspase-8 is shown in Figure 7. Since these enzymes have an important role in cell death they are important therapeutic targets. The structure of a macromolecular caspase inhibitor P35 from baculovirus has recently been described.[74] The structure shows a solvent exposed loop with the Asp-X cleavage site similar to that seen with serpin serine protease inhibitors.

8.2 Interactions of Proteins of the Immune System. – CD2/CD58 interaction. The human CD2 is a transmembrane glycoprotein found on T cells and other cells of the immune system. It is involved in cell–cell interactions and promotes the physical interaction with antigen-presenting cells that express its ligand CD-58. The structure of the complex has been described by Wang *et al.*[75] The nature of the interaction between the two adhesion domains utilises mainly charge interactions (10 salt bridges) and a small number of hydrophobic interactions. Although the structure of CD2 had been described several years ago, the structure of CD58 proves different. A novel approach was used to create a chimeric CD58–CD2 molecule which enabled a structure to be solved to a 1.8 Å resolution.[76] An NMR structure has also been reported for CD58 where mutations have been made to allow the unglycosylated form of the protein to be expressed in *Escherichia coli*.[77] The structures of both CD2 and CD58 are similar to the antibody Ig-like domains with a short stalk of a few residues at the cell surface.

8.3 TCR–pMHCII Complex. – The cell receptor (TCRV) is complex with a peptide and a major histocompatibility complex (MHCII) has been reported by Reinherz *et al.*[78] This complex shows a different orientation of docking to known complexes due to a protruding small β sheet characteristic of all class II MHC molecules.

9 Protein–Nucleic Acid Interactions

Two papers in 1999 have been published that summarise how proteins interact with DNA based on a structural analysis[79] and RNA.[80] The area of RNA–protein complexes has also been reviewed by two prominent researchers, Cusack[81] and Steitz.[82] It is known that the same stretch of polypeptide can change its conformation when binding to different regions of another protein. This has been described when a peptide has been engineered into different regions of protein G by Cregut *et al.*, where it can adopt an α helix or a β sheet conformation.[83]

It is now becoming increasingly accepted that RNA came before proteins in evolutionary terms. Several reviews have therefore addressed the interaction of RNA with peptides and the importance of induced fit.[84,85] The latter review addresses specific interactions with the acidic RNA and the basic arginine rich peptides.

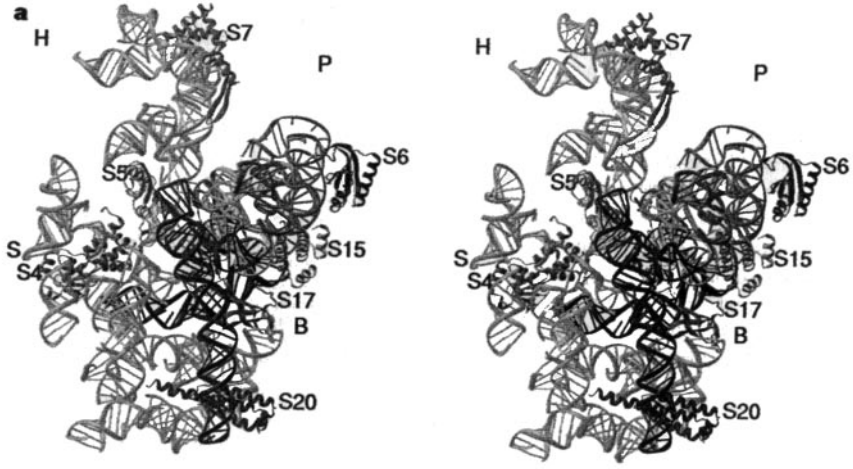

Figure 8 *A stereo view of the structure of the small ribosome subunit from* Thermus thermophilus*. The RNA is shown and the positions of the proteins of known structure S4, S5, S6, S7, S15, S17 and S20. H, head, P, platform, S, shoulder, B, body*
(Reproduced with permission from Clemons *et al.*[93])

HIV TAR RNA forms a complex with a peptide called Tat. This has been studied by NMR and it has been found that when Tat binds it 'locks' TAR into a single conformation in which an imino proton resonance is attributed to U23 of a base triplet. The kinetic stability of the so formed complex prevents rapid exchange of the imino proton with solvent and depends on an arginine rich peptide.[86]

Other approaches to study this interaction have been described. Wang *et al.* have used photo-cross-linking, and Huq *et al.* have studied interactions by tethered iron chelate analysis.[87,88] The same group have identified a tripeptide from a combinational library composed of D and L amino acids that binds the HIV-1 TAR and locks it into a different conformation to that described above.[89] This shows that the flexibility of RNA can be exploited to bind unnatural peptides as potential inhibitors to these RNA–peptide complexes. In turn the RNA architecture can dictate the conformation of a bound peptide. A study by Ye *et al.* describes how a single peptide can adopt different conformations when bound to different RNA molecules.[90]

The flexibility of these interactions provide a wealth of opportunities for the inhibition and moderation of a multitude of cellular functions. Based on structural information RNA binding zinc fingers have been made.[91,92]

9.1 Ribosome and Ribosomal Proteins. – The ribosome is the site where protein is built up in the cell dictated by the messenger RNA. In the last few years structural information on these large protein–RNA assemblies has rapidly advanced. The structure of both small and the large ribosome subunit was reported in 1999 to 5.5 Å and 5 Å resolution respectively.[93,94] This has

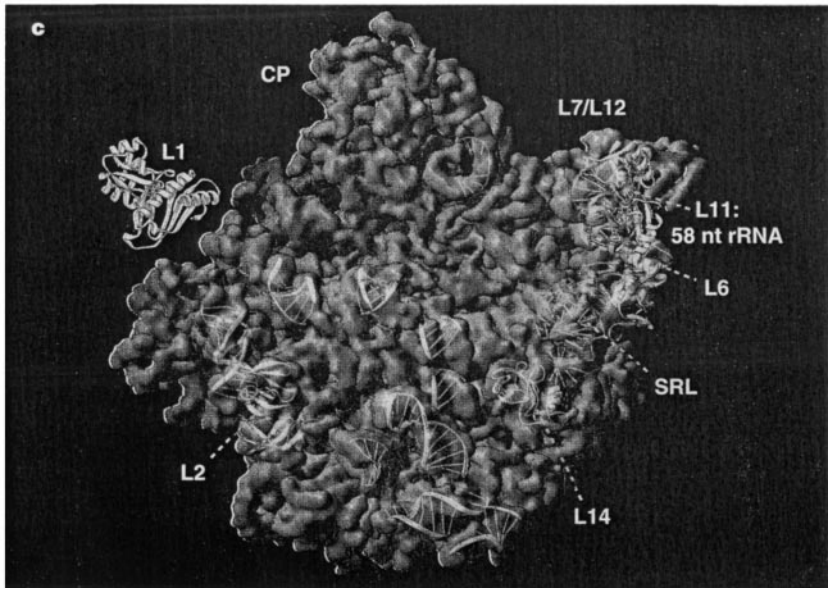

Figure 9 *Structure of the halophilic 50S ribosomal subunit. Some RNA helices and teraloops have been fitted into the density. The positions of proteins of known structure are shown as ribbon structures. CP, central protruberance*
(Reproduced with permission from Ban *et al.*[94])

allowed the rRNA and most of the known ribosomal protein structures to be located in the electron density map. The 30S ribosomal subunit from *Thermus thermophilus* can be visualised at this resolution such that the backbone of the ribosomal RNA and all seven of the small subunit ribosomal proteins whose structures were previously known can be positioned in the electron density map. The structure of the small subunit showing the rRNA and the proteins S4, S5, S6, S7, S15, S17 and S20 as described by Clemons *et al.*[93] is shown in Figure 8. The structure of the large 50S ribosomal subunit is from the halophilic archaeon *Haloarcula marismortui*. This shows the structure of the translation-factor-binding centre with the known crystal structures of proteins L6, L11 and L14, the sarcin-ricin loop RNA and the RNA sequence that binds L11 into the electron density map. A view of the 50S subunit showing the overall structure and the position of the proteins described above is shown in Figure 9 as described by Ban *et al.*[94] Also in 1999 the group of Noller have reported a crystal structure of the whole 70S *T. thermophilus* ribosome to 7.8 Å.[95] This provides important information between the spatial relationship between the different ribosomal components. The electron density of the 70S ribosome showing the interaction of the small and large ribosome subunit is shown in Figure 10a. This structure also allows the positioning of the transfer RNA molecules bound to the acceptor, peptidyl and exit sites as previously predicted from biochemical data, Figure 10b.

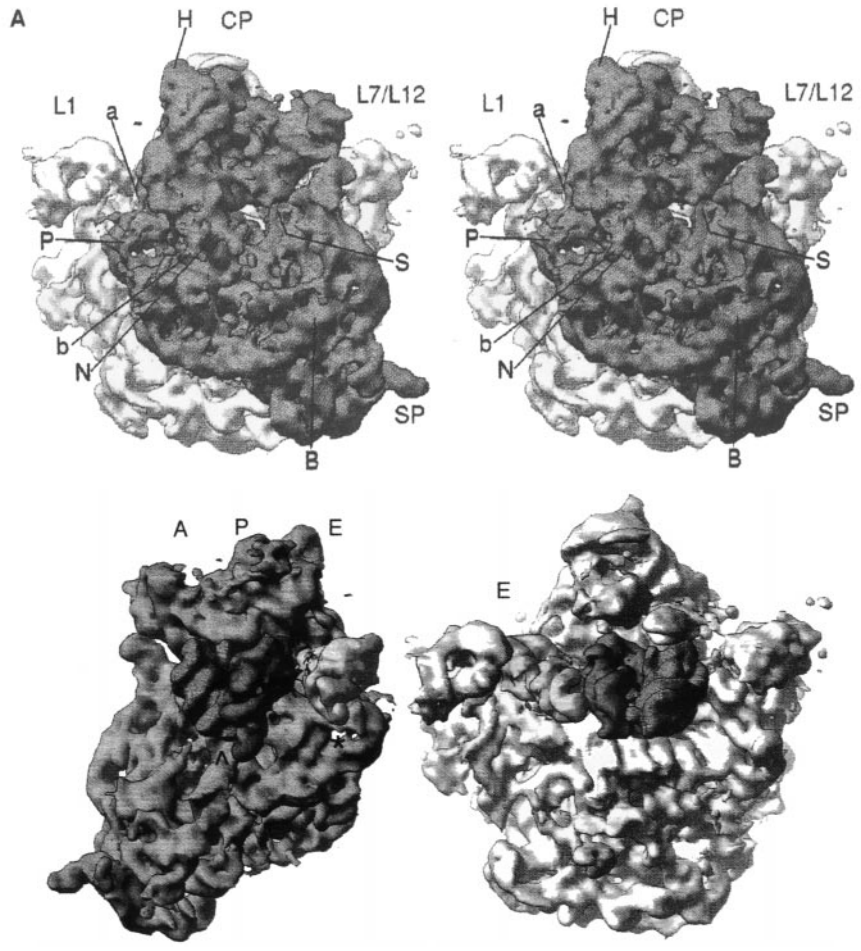

Figure 10 *Structure of the 70S* T. thermophilus *ribosome.* (a) *Stereo view of both subunits of the ribosome. The 30S subunit consists of the head (H), connected to the platform (P) and the body, (B), the neck (N), spur (SP), shoulder, (S) and contacts between the head and platform (a and b). The 50S subunit has the L1 stalk the central protruberance (CP) and the protein L7/L12 region.* (b) *The position of the three transfer RNA molecules on the ribosome, A, aminoacyl, P, peptidyl, E, exit, as viewed from the 30S interface (left) and towards the 50S interface (right)*
(Reproduced with permission from Cate *et al.*[95])

To obtain diffraction quality crystals of these large macromolecular assemblies it has been necessary to use ribosomes from unusual sources. The 30S small subunit and the 70S whole ribosome were from the thermophilic eubacteria *T. thermophilus* and the 50S large subunit was from a halophilic archaeon, which grows in conditions of high salt. The resolution of these

structures is ever increasing and further information on the detailed mechanism of protein synthesis will soon become available.

10 Summary

This article only covers a small amount of the literature appearing in 1999 on protein research. It addresses some of the important issues relating to the gene sequencing projects such as protein folding and structural genomics. Areas not covered in the review appearing in Volume 31 are specifically included.

An increasingly accepted idea that life evolved initially from an 'RNA world' is reinforced by the fact that RNA can act as a catalyst and is essential for the basic process of protein synthesis on the ribosome. We are starting to obtain details of the molecular structure of this large protein–RNA complex.

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