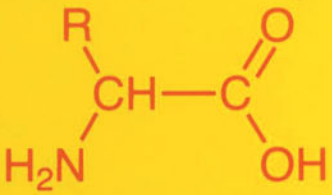

SPECIALIST PERIODICAL REPORTS

AMINO ACIDS, PEPTIDES AND PROTEINS

VOLUME 29

SENIOR REPORTER J.S. DAVIES

THE ROYAL SOCIETY OF CHEMISTRY

A Specialist Periodical Report

Amino Acids, Peptides and Proteins

Volume 29

A Review of the Literature Published during 1996

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THE ROYAL SOCIETY OF CHEMISTRY

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Preface

This 29th Volume in the series, represents the steady toil over many months of a core of Reporters whose names have appeared in previous volumes. Their dedication is greatly appreciated. However, we do welcome 'new blood' into this volume, in that Chapter 3 has this year been written by Dr Anand Dutta, who, from his pharmaceutical company background, has been able to augment authoritatively the pharmacophoric interest in the many peptide analogues now being produced. We also welcome for the first time new authors, Drs Farkas and Sóvágó from Hungary, for our biennial Chapter on Metal Complexes of Amino Acids and Peptides. We are truly grateful for everyone's input into this volume.

Peptide and protein science has diversified into a very broad swathe of science, creating a feeling amongst the core researchers that the discipline may have lost some of its past thrust and suffers from indifferent support from both governmental and pharmaceutical sources. Yet during the gestation of this book, there was sufficient confidence shown by Japanese workers to organise the First International Peptide Symposium at Kyoto in December 1997, and at that meeting any feelings of despondency were strongly disputed at a forum session by Dietrich Brandenburg and Tetsuo Shiba, who convinced the audience that an annual world output of 25,000 papers on peptides should convince everyone of the buoyancy of the subject. The references reviewed in this Volume continue to come from a very wide range of Journals. However the title of a core Journal in the field, the *International Journal of Peptide and Protein Research*, appears for the last time as it was incorporated with *Peptide Research* at the end of 1996 to become the *Journal of Peptide Research*.

The buoyancy in the field of solid phase peptide synthesis is reflected in a complete issue of *Methods in Enzymology*, Vol. 289 (Academic Press, 1997) being dedicated to the topic. Fundamentals of all aspects of synthesis and structural elucidation of peptides have been the subject of two books, *Peptide, Chemie und Biologie,* by H.-D. Jakubke (Spektrum Akademischer Verlag, 1996) and *Chemical Approaches to the Synthesis of Peptides and Proteins,* by P. Lloyd-Williams, F. Albericio and E. Giralt (CRC Press, 1997). So a new generation of practitioners can cut their weaning teeth on the contemporary scene in peptide science, using these and other text books, and our hope is that this volume will once again fulfil the role of reporting the significant literature at the frontiers of the subject.

Apart from the Reporters, a crew under the stewardship of Janet Freshwater at the RSC have also been of tremendous help in seeing this volume into port.

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Contents

Abbreviations

The abbreviations for amino acids and their use in the formulation of derivatives follow in general the 1983 Recommendations of the IUB–IUPAC Joint Commission, which were reprinted as an Appendix in Volume 16 of this series. These are also published in:

Eur. J. Biochem., 1984, **138**, 9-37; *Int. J. Pept. Protein Res.*, 1984, **24**, after p.84; and *J. Biol. Chem.*, 1985, **260**, 14-42.

A complete listing of the single-letter codes for amino acids appeared in the Abbreviations section of Volume 24 of these Reports, together with structures for the closely related BOP family of coupling reagents.

Chapter authors have been encouraged annually to include new abbreviations in their texts. With the ever increasing diversification in structures, lists of unusual abbreviations are periodically compiled. Some examples are listed below.

Abo	2-azabicyclo[2.2.2]octane-3-carboxylic acid
Abu	α-aminobutyric acid
A_2bu	2,4-diaminobutyric acid
ACCA	4-aminocyclohexanecarboxylic acid
εAhx	6-aminohexanoic acid
Aib	α-aminoisobutyric acid
Aic	2-aminoindan-2-carboxylic acid
A_2pr	2,3-diaminopropionic acid
Atc	2-aminotetralin-2-carboxylic acid
Ava	5-aminopentanoic acid
Aze	azetidine-2-carboxylic acid
Cha	3-cyclohexylalanine
Cpg	α-cyclopentylglycine
Cpp	1-mercaptocyclohexaneacetic acid, *or* β-mercapto-β,β-cyclopentamethylenepropionic acid, *or* Pmp *(below)*
*c*PzACAla	*cis*-3-(4-pyrazinylcarbonylaminocyclohexyl)alanine
Dab	2,4-diaminobutyric acid
Dap	2,3-diaminopropionic acid
Dbf	3-(2-dibenzofuranyl)alanine
Dip	3,3-diphenylalanine
Dph	α,α-diphenylglycine
Dpr	2,3-diaminopropionic acid
Gly(Ph)	phenylglycine
Har	homoarginine
Hib	α-hydroxyisobutyric acid
Hyp	*trans*-4-hydroxyproline

Iva	isovaline
Mpt	*trans*-4-mercaptoproline
1-Nal	3-(1-naphthyl)alanine
2-Nal	3-(2-naphthyl)alanine
Nap	β-(1′-naphthyl)alanine
Oic	octahydroindolecarboxylic acid
Opt	*O*-phenyltyrosine
3-Pal	3-(pyridyl)alanine
Pen	penicillamine
Phg	phenylglycine
Pip	pipecolic acid
Pmp	β,β-pentamethylene-β-mercaptopropionic acid, *or* Cpp *(above)*
Qal	3-(3-quinolyl)alanine
Qua	quinoline-2-carboxamide
Sar	sarcosine
Thi	β-thienylalanine
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid

1
Amino Acids

BY GRAHAM C. BARRETT

1 Introduction

The literature of 1996 is covered in this Chapter, which aims to report and appraise newly-published knowledge of the chemistry of amino acids. Biological aspects are given prominence only where the chemical interest is enhanced by explaining the life science context.

Literature citations forming the basis for this Chapter have been obtained from *Chemical Abstracts* (Volume 124, Issue no. 11 to Volume 126, Issue no. 9 inclusive), and from papers consulted in major Journals that have consistently been used by authors of relevant material.

The expanding volume of the relevant literature continues to demand ingenuity in somehow getting a litre of wholesome nourishment into the half-litre pot that this Chapter represents, and restrictions have been placed on citations of the patent literature and material of a more routine nature. Authors who repeat-publish and over-fragment their material are responsible to a significant extent for the ever-increasing number of references for this Chapter, and this Reviewer's conscience rests easily when grouping such papers together without detailed comment on each of them.

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 oxy-acid analogues and also in sulfonic acid analogues, is continuing to grow. Methods applicable for the synthesis of α-aminoalkaneboronic acids (Refs. 65, 146, 147), α-aminoalkanesulfonic acids (Refs. 154, 845), and α-aminoalkanephosphonic acids and other phosphorus oxyacids (Refs. 32, 62, 80, 82, 85, 87, 88, 152, 326, 374, 437, 843) are usually derived from extensions of standard methods in the amino acid field, and representative examples of syntheses of amino oxyacid analogues are described, side-by-side with corresponding methods for amino carboxylic acids, in appropriate locations in this Chapter.

2 Textbooks and Reviews

References collected in this Section do not represent the total coverage for this year, since many reviews are located with their primary literature in appropriate sections in this Chapter.

Amino Acids, Peptides and Proteins, Volume 29

Books on protein analysis and peptide topics inevitably relate to amino acids as well.[1] New books[2] and Conference Proceedings[3] are a continuing support for all those working in this flourishing field, and are especially useful to those entering it for the first time and depending on a general background in organic and biological chemistry. Material presented at Conferences is usually published elsewhere, or is already on its way to the primary literature by the time that the Proceedings Volumes appear, and studies covered in this Chapter are linked only to their journal sources even if accessible also through Conference Proceedings.

Reviews cover the twenty-first amino acid utilized in normal ribosome-mediated protein synthesis, selenocysteine[4] (see also the report[5] on incorporation into proteins by modified *E.coli* of numerous non-natural amino acids), uses of tert-leucine[6] and trans-4-hydroxy-L-proline[7] as chiral starting materials for organic synthesis, (S)-2,3-diaminopropanoic acid,[8] lipidic α-amino acids,[9] chemical modification of amino acid side-chains for studies of protein function,[10] the detection of amino acids in samples collected on Mars,[11] naturally-occurring proline analogues,[12] analysis methods for D-α-amino acids and discussion of their *in vivo* roles,[13] free-radical reactions in the synthesis of amino acids,[14] cation π-binding between aromatic amino acid side-chains in host-guest complexes,[15] synthesis and uses of α-trifluoromethyl-α-amino acids,[16] and L-carnitine biochemistry.[17] As with earlier issues of the 'Methods in Molecular Biology' series, useful material on amino acids science is contained in current Volumes,[18] and the same applies to American Chemical Society Symposia.[19]

3 Naturally Occurring Amino Acids

3.1 Isolation of Amino Acids from Natural Sources – New solutions to problems of winning of amino acids from mixtures, by conventional or novel methods are collected in this Section. Protein hydrolysis is important both in terms of reliability of analytical data, and as a source of certain amino acids on a preparative scale. The recovery of tryptophan (86–88%) from seaweed is considerably better by aqueous alkaline hydrolysis than by mercaptoethanesulfonic acid degradation[20] (though the latter method is claimed to give >90% recovery of tryptophan from proteins when dithioglycollic acid is added[21]). Degradation accompanying conventional acid hydrolysis of a protein is even greater for cysteic acid than for serine.[22] A new enzyme, actinase, that catalyses the hydrolysis at both peptide bonds that anchor a tyrosine residue within a protein, may be widely useful; for example, the tyrosine content (12%) is released from silk through its action.[23]

Further details (see Vol. 28, p. 3) of the application of an emulsion liquid membrane consisting of di-(2-ethylhexyl)phosphoric acid, Span 80, and kerosene, for concentrating alanine from aqueous solutions, have been published.[24] An organic membrane has been formulated for the separation by nanofiltration of arginine, glutamic acid, and serine from an aqueous solution containing 15 amino acids,[25] and N-benzyloxycarbonyl-L-aspartic acid and L-phenylalanine methyl ester hydrochloride can be concentrated and separated from each other in

solutions in organic solvents, by using reverse osmosis membranes.[26] N-Acylation of protein hydrolysates has been claimed[27] to provide easier separation, though the well-known further reactions undergone by N-acylamino acids and acylating agents may create more confusion. A novel approach, temperature-swing chromatography, has been applied to the preparative scale separation of a mixture of arginine, histidine, and lysine.[28] Conventional large-scale ion-exchange purification methods have concentrated on the removal of inorganic impurities from amino acid mixtures.[29]

An effective solvent system for amino acids and their derivatives is dimethylformamide containing a strong acid (TFA, HBF_4, TosOH *etc.*) together with an excess of tertiary amine with pK less than 6 (pyridine is recommended).[30] Derivatization of amino acids, *e.g.* by acylation, is claimed to be feasible in such systems, but it should be appreciated that troublesome side-reactions unique to amino acids would be expected for syntheses carried out in such media.

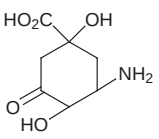
Crystallization techniques leading to pure products are routine final steps in isolations of amino acids from mixtures, and new data are contained in studies of aspartic acid and phenylalanine.[31]

3.2 Occurrence of Known Amino Acids – This section could be very extensive, but it does not include routine papers mentioning familiar amino acids in their predictable locations in the biosphere; therefore, only those papers describing familiar amino acids in unusual locations (extraterrestrial; Ref. 11), or describing more unusual amino acids in natural locations, are considered.

Glycine appears in hydrolysates of bacterial lipopolysaccharides, and is concluded to be an integral constituent.[32] L-Arginine appears in *Coccinellidae subcoccinella-24-punctata* as its N^{α}-quinaldyl derivative,[33] and in the marine ascidian *Leptoclinides dubius* as corresponding p-hydroxybenzoyl and 6-bromo-1H-indolyl-3-carbonyl derivatives;[34] also present are N^{α}-(1H-indolyl-3-carbonyl)-D-arginine, N^{α}-(6-bromo-1H-indolyl-3-carbonyl)-L-histidine, and the rare amino acid L-enduracidin. Assignments of structure to konbamide[35] (from the sea sponge *Theonella sp.*) and related cyclic peptides are shown to be incorrect by synthesis of tryptophan-containing analogues of the proposed peptides, and creation of D- and L-2-bromo-5-hydroxytryptophan residues by NBS bromination.[36] Hypusine [N^{ε}(4-amino-2-hydroxybutyl)-L-lysine] is formed by transfer of the side-chain substituent from spermidine to a lysine residue in a protein.[37]

The opines [α-(N-carboxyalkylamino)alkanoic acids; ten such compounds are uniquely located in 43 crown gall tumours] have been surveyed.[38] N^{ε}-Carboxymethyl-L-lysine is an advanced glycation end-product of proteins *in vivo*, and is thought to arise by reaction with compounds formed by lipid peroxidation since it can be generated *in vitro* by copper-catalysed oxidation of mixtures of proteins with polyunsaturated fatty acids.[39] Crosslinking amino acids released from proteins *in vivo*, that then find their way into clinical samples, are attracting increasing interest since they are thought to be markers of human bodily deterioration; deoxypyridinoline is one of these (a marker for osteoporosis; see also Refs.1048,1075-1078) and its identification in urine together with hydroxyproline has been studied.[40]

Biosynthesis by *Streptomycetes* of 3-amino-5-hydroxybenzoic acid involves a new variant of the shikimate pathway, since 3,4-dideoxy-4-aminoarabinoheptulosonic acid, 5-deoxy-5-amino-3-dehydroquinic acid (aminoDHQ, 1) and 5-deoxy-5-amino-3-dehydroshikimic acid (2) are present in cultures.[41] Pyridazomycin (3) is biosynthesized from ornithine, glycine, and a C-4 unit.[42] The antimetabolite YS-460 from a *Streptomyces* sp. turns out to be identical with furanomycin.[43] Nostocyclin, from newly-discovered *Nostoc* strains, extends the list of cyclodepsipeptides of cyanobacteria (blue-green algae) that contain the 3-amino-6-hydroxy-2-piperidone moiety.[44]



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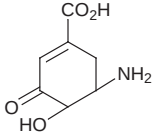

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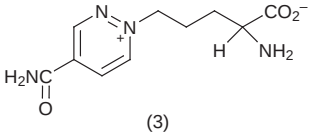

(3)

3.3 New Naturally Occurring Amino Acids – *Neocosmospora vasinfecta* contains (2S,3R,4R,6E)-2-acetylamino-3-hydroxy-4-methyloct-6-enoic acid (structure assigned on the basis of synthesis by aldolization of tert-butyl isocyanoacetate and routine elaboration; Section 4.2).[45]

3.4 New Amino Acids from Hydrolysates – This section covers new amino acids that occur as residues in larger structures that can in principle be broken down by hydrolysis.

The most prolific growth area under this heading is the group of cyclic dipeptides (*alias* dioxopiperazines), and new examples include diatretol (4; from *Clitocybe diatreta*)[46] and the four cyclotryprostatins (5) from *Aspergillus fumigatus* BM 939 that are responsible for inhibition of cell cycle progression.[47]

Studies of the fluorescent 'crossline', a lysine residue in proteins that has become post-translationally modified through condensation with D-glucose, have been reported.[48] A di-isotyrosine isolated from hydrolysates of cell walls of tomato cell cultures has been shown to be a biphenyl rather than a diaryl ether.[49]

Of course, the field covered by this section includes acylated amino acids as well as unusual peptides and crosslinked proteins. The first-mentioned of these

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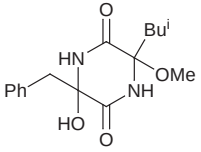
Three-dimensional features of molecules are depicted throughout this Chapter as follows: horizontally ranged atoms and bonds and ring atoms are to be understood as being in the plane of the paper; substituent atoms and groups attached to these are to be understood to be ABOVE the page if ranged LEFTWARDS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



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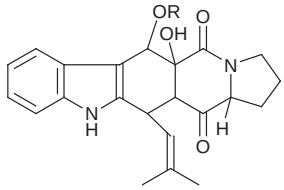
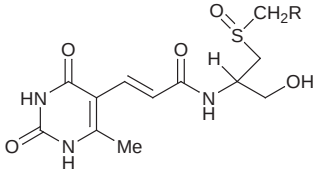


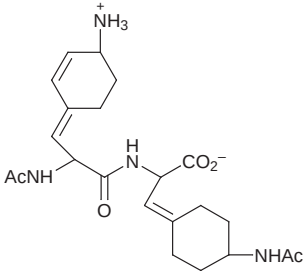
(5) a: R = H
b: R = Me
c: as a, epimeric at OH
d: as c, C=O in place of CH(OR)

classes is represented in the modulators of the proliferation of mammalian cells, the sparoxomycins A1 and A2 (6; these are epimers at the sulfoxide chiral centre).[50] The trypsin inhibitor radiosumin (7), from the blue-green alga *Plectonema radiosum*, contains unsaturated moieties that are novel in the amino acid context,[51] while clathramides A and B (8) are novel bromopyrroles from the



(6) A$_1$; R = 

A$_2$; R = 



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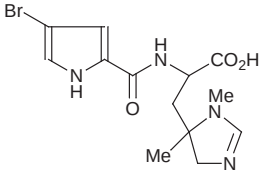

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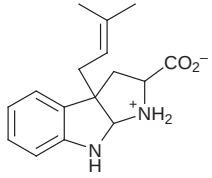

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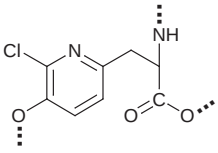

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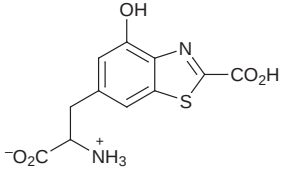


(11)

sponge *Agelas clathrodes*.[52] The chymotrypsin inhibitor oscillatorin (from the toxic freshwater cyanobacterium *Oscillatoria agardhii*) is a cyclic decapeptide that contains 3a-cis-1,2,3,3a,8,8a-hexahydro-3a-(3-methyl-2-butenyl)-pyrrolo[2,3-b]-indole 2-carboxylic acid (9).[53] Progress with the structure determination of kedarcidin has been reported; its incompletely defined chromophore contains partial structure (10).[54]

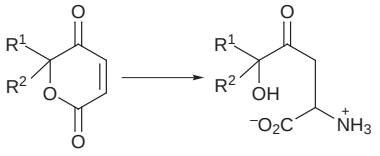
The novel amino acid (11) is released from pheomelanins through alkaline hydrogen peroxide treatment at room temperature.[55]

4 Chemical Synthesis and Resolution of Amino Acids

4.1 General Methods for the Synthesis of α-Amino Acids – Routine methods based on the amination of carbonyl compounds, carboxylation of amines, and alkylation of glycine derivatives continue to provide the main material under this heading.

Significant modifications providing improvements to standard methods are noticeable, and over the years there have been continuous developments, including improved deprotection conditions [alkaline hydrolysis of N-phthaloyl-glycine and acid hydrolysis of N-(o-carboxybenzoyl)glycine in aqueous organic solvents] for the Gabriel synthesis.[56] Attempted aminolysis of dimethyl 1-bromocyclopropane-1,2-dicarboxylate in methanol gave the methyl ether.[57] Amination through azidolysis of α-halogenonitriles followed by reduction of the azide grouping and hydrolysis of the nitrile is another standard approach, illustrated in this year's literature with 1-halogeno-D-tetra-O-acetylglycopyranosyl cyanides giving monosaccharides carrying amino and carboxy groups at the anomeric carbon.[58] Azidolysis of cyclic carbonates (using NaN_3)[59] or oxiranes (using 1,1,3,3-tetramethylguanidinium azide with a simple transition-metal salt as catalyst)[60] *en route* to trans-1,2-amino alcohols (see also Refs. 125, 128 *etc*.) is the first step in an easy introduction of a primary amine function. Reductive addition of hydrazoic acid to γ-keto-α,β-unsaturated dicarbonyl compounds, *e.g*. lactones (12), offers an alternative approach to this amination protocol.[61] Lithiated cycloalkanephosphonate esters[62] and N-acylmethylanilides[63] have undergone electrophilic azidation (diphenyl phosphorazidate) followed by reduction (H_2/Pd) and hydrolysis, to lead to α-aminoalkanephosphonic acids and α-amino acids, respectively.

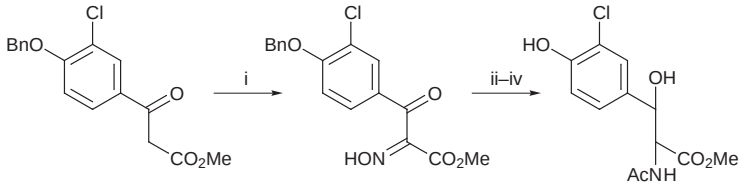
Vinylglycinol has been obtained by Pd(naphthalene)-catalysed amination of butadiene mono-epoxide by phthalimide.[64] α-N-Boc-Aminoalkaneboronate



(12)

esters have been obtained through amination (BocNHNa) of α-halogenoalkaneboronates.[65]

Nitrosation of β-ketoesters (Scheme 1) giving α-oximino-esters, and electrophilic amination of the corresponding α-hydroxy ester with di-tert-butyl azodicarboxylate to give the α-hydrazino β-hydroxy ester, are the initial steps for two standard amination protocols used for the syntheses of the vancomycin constituents, syn- and anti-β-hydroxy 3-chlorotyrosines, respectively.[66] β-Alkoxy-α-oximino-esters have been prepared from oxiranes and hydroxylamine,[67] and easily-prepared α-oximinoalkanephosphonates are readily reduced ($NaBH_4$) to α-aminoalkanephosphonates.[68]



Reagents: i, BuONO, HCl; ii, Zn, AcOH; iii, H_2, 140 bar, $RuBr_2$[(*R*)-MeOBiphep]; iv, H_2/Pd–C

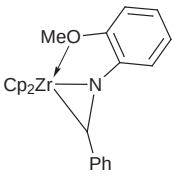
Scheme 1

Another 'named' method also based on amination, the Strecker synthesis, has been used with carbonyl compounds derived from protected D-glyceraldehydes to give (2S,3S)- and (2R,3S)-2-amino-3,4-dihydroxybutanoic acids;[69] the Strecker synthesis gives poor yields when attempting the synthesis of amino acids bearing electron-withdrawing groups such as 4-pyridyl.[70] A close relative of the method is involved in a synthesis of phenylglycine from benzaldehyde, $CHCl_3$, KOH and ammonia; an identical brew has been studied over many years and this time inclusion of β-cyclodextrin in the reaction mixture is shown to generate stereoselectivity (to an extent that is not clear from information in the abstract).[71]

Ugi solid-phase syntheses, one (employing 1-isocyanocyclohexene)[72] giving α-amino acid derivatives *via* a munchnone intermediate, and a related approach[73] giving hydantoin-4-imides in another way (the isocyanide is tethered to the solid phase[74]), have been studied. These are being studied in the context of the generation of combinatorial compound libraries, as is a route to 5-alkoxyhydantoins employing α-hydroxyalkanoylated Merrifield resin ($BnONH_2$ followed by ArNCO).[75] The rich reactivity profile of the munchnone intermediate involved in these studies can be exploited in other ways, *e.g.* 1,3-dipolar cycloadditions with alkynes leading to pyrroles (see also Ref. 72).[76]

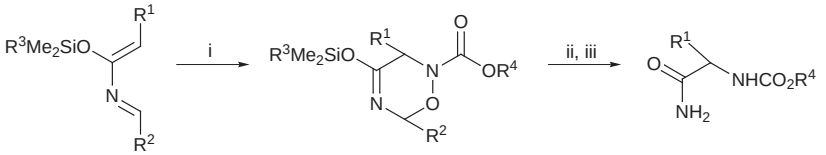
A '5-centre-4-component reaction' (aldehyde, L-amino acid, isocyanide, and alkanol) leads to homochiral 1,1′-iminodicarboxylic acid derivatives.[77] Reactions of chiral imino aziridines (the synthetic equivalent of the condensation of three of the four components of the classical Ugi reaction) with alkanoic acids give N-acylamino acid amides with very little racemization, as well as analogues resulting from Mumm rearrangement.[78] Optically-active zirconaziridines (13) can be

trapped with ethylene carbonate (a 'CO_2 synthon') as they are formed, and the resulting complex generates α-amino-α-methyl esters, or gives phenylglycinamides when isocyanates are added; optically-active substrates generate poor enantioselectivity.[79]



(13)

Amination of keto-acids leading to α-aminoalkylphosphonic acids has been demonstrated, employing benzhydrylamine and reduction of the resulting Schiff base with $K(OAc)_3BH$; a synthesis of phosphohomoserine lactone is included.[80] Amination of azadienes through cycloaddition to arylnitroso compounds (Scheme 2) gives N-arylamino acids through reductive cleavage.[81] Mitsunobu amination of α-hydroxyphosphonates formed from dibenzyl phosphite and aldehydes is an effective route to N-hydroxy-α-aminophosphonates.[82]



Reagents: i, $R^4OCON{=}O$, CH_2Cl_2, r.t.; ii, MeOH; iii, Na–Hg in MeOH

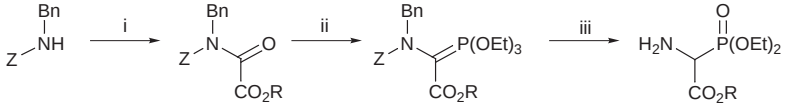
Scheme 2

[3,3]-Trichloroacetimidate rearrangement of mono-protected *syn*-allylic diols leading to allylic amines is followed by successive ozonolysis and Jones oxidation in a standard route to α-amino acids.[83] Full details have been reported for the preparation of trichloroacetimidates.[84]

Carboxylation of amines is rarely used for α-amino acid synthesis, though addition of diphenyl phosphite to an imine to give a diphenyl N-alkyl-α-aminophosphonate illustrates the principle [$DMTO(CH_2)_2N{=}CH_2 \rightarrow DMTO(CH_2)_2NHCH_2P(O)(OPh)_2$; see the coverage of PNAs in the later Section 4.10].[85] Hydrophosphonylation of a cyclic imine is enantioselective when a chiral titanium-diol complex (20 mol%) is used as catalyst.[86] Equivalent phosphonylation of imines by polymer-bound H-phosphonates gives α-aminophosphonates,[87] and polymer-bound imines give α-aminophosphonic acids by reaction with $HP(OTMS)_2$.[88] Amidocarbonylation (acetamide, CO, H_2, with an aldehyde and $PhCCo_3(CO)_9$[89] or with benzyl chloride, to give DL-phenylalanine[90]) and a

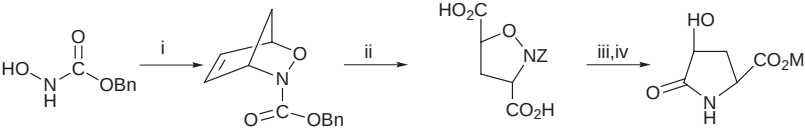
closely-related route to N-α-acylaminophosphonates [$HP(O)(OR^1)_2$ + R^2CHO + R^3X-$CONH_2$][91] have been developed further. Carboxylation and amination are combined in a three-component synthesis of aldehydes, primary amines, and silanes [R^1CHO + R^2NH_2 + Me_3Si-Nu → $R^1CH(NHR^2)Nu$] that leads to β-aminoketones, β-amino esters, or α-aminonitriles,[92] while trifluoromethylated enamino- and imino-esters are formed from an iminophosphorane, TFAA, and an organozinc reagent.[93]

Isocyanoacetates undergo transition metal-catalysed aldolization by ketones to give β-disubstituted β-hydroxy-α-amino acids.[94] Close relatives of keto acids, the oxalylcarbamates $R^1R^2NCOCO_2Et$, give α-diethylphosphonyl-α-amino acids by reaction with triethylphosphite and steps shown in Scheme 3[95] (the later Section 4.7 deals with α-hetero-atom-substituted α-amino acids). α-MethylDOPA has been obtained through an 8-step route from diethyl methylmalonate, Hofmann rearrangement being the key step that introduces the eventual amino group in a synthesis of 2-carbamoyl-2-methyl-3-[3,4-(methylenedioxy)phenyl]propanoic acid.[96] The 1,2-oxazoline formed by hetero-Diels-Alder addition of cyclopentadiene to benzyl N-hydroxycarbamate and oxidation of the adduct *in situ* with tetrabutylammonium periodate, has been used for the synthesis of γ-substituted glutamic acids by hydrogenation and ring opening (Scheme 4), and in principle could be more widely applicable.[97]



Reagents: i, $NaN(TMS)_2$ then $ClCOCO_2R$; ii, $P(OEt)_3$,toluene, reflux 7 h; iii, TMSBr then H_2/Pd–C

Scheme 3

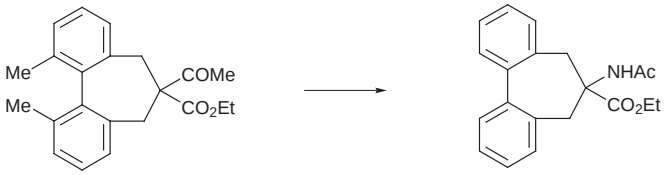


Reagents: i, Bu_4NIO_4 + cyclopentadiene; ii, $KMnO_4/Bu_4NHSO_4$; iii, $Me_3O^+BF_4^-$, Pr^i_2NEt; iv, H_2/Pd–C

Scheme 4

Bucherer-Bergs synthesis (see also Ref. 941) of N^{α}-Fmoc-N^{γ}-Boc-4-aminopiperidine-4-carboxylic acid from the corresponding piperidin-4-one,[98] preparation of αα-dialkylglycine esters through Beckmann rearrangement of correspondingly substituted β-keto-esters,[99] and Schmidt rearrangement of the ethyl αα-dibenzylacetoacetate (14),[100] illustrate further standard methods.

A curious observation, that aldoses react with propylamine, N^{α}-acetyl-L-



(14)

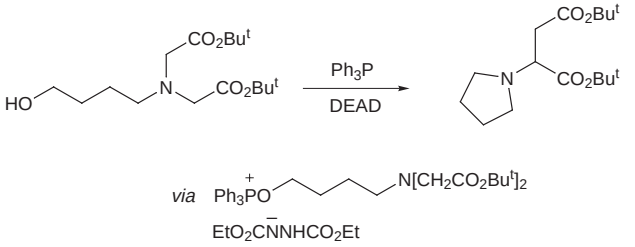
lysine, glycine, or alanine, to give N-propyl alanines and (4S,5R)-5,6-dihydroxy-2-propylamino-γ-hexanolactone, is accounted for by the intervention of methylglyoxal formed through the Maillard reaction (see Section 6.2). This is unlikely to develop into a general amino acid synthesis.[101]

Diethyl acetamidomalonate, the glycine synthon surviving from classical times of amino acid synthesis, is still being given regular employment, leading to protected allylglycines suitable for osmylation by OsO_3, for a synthesis of 5-hydroxy-4-oxonorvaline,[102] and leading to tyrosine analogues,[103] (2S)-amino-3-cyclopropylpropanoic acid,[104] and 2-amino-5,5-dimethylhexanoic acid using 4,4-dimethylpentyl bromide as alkylating agent.[105] A new variant of this route involves alkylation of organo-iron complexes of $EtO_2CCH(NH_2)CO_2(CH_2)_2SiMe_3$, which leads to diastereoisomerically-pure samples.[106] Mono-ethyl acetamidomalonate serves in a corresponding way for the synthesis of α-acylamino-β-ketoesters.[107] Oxazolones are also long-serving synthons, useful for the synthesis of α-hydroxymethyl-phenylglycine and phenylalanine[108] and other αα-dialkylglycines (see Vol. 28, p.23; and Ref. 179).[109]

Glycine Schiff bases, *e.g.* $Ph_2C{=}NCHR^1CO_2R^2$ (R^1 = H; see also Refs. 336, 397) and homologues (R^1 = alkyl), give 4-(phosphonomethyl)phenylalanine and homologues through alkylation,[110] and $Ph_2C{=}NCH_2CN$ (see also Refs.100, 370) has been used similarly. Double alkylation of ethyl cyanoacetate by 1,2-dibromoethane constitutes the crucial step in a synthesis of 1-aminocyclopropane-1-carboxylic acid, successive treatment with aqueous sodium hydroxide, H_2O_2, and Br_2-NaOH generating the required functional groups.[111]

N-Phenylsulfonyl DL-α-bromoglycinate esters undergo nucleophilic substitution with alkylaluminium complexes, to give modest asymmetric induction when homochiral reagents are used.[112] Weakly-activated glycine derivatives participate in aldol reactions: Boc-sarcosine benzyl ester and acetone give N-methyl hydroxy-DL-valine;[113] a protected glycine TMS ketene acetal adds to an aldehyde to give (2- and 6-fluoro-threo-dihydroxyphenyl)serines.[114] Boc-Glycine and excess LDA and alkyl bromides $BrCH_2XCN$ give α-cyanoalkylglycines from which N^{ω}-hydroxyindospicine and p-hydroxyamidinophenylalanine have been prepared [X = $(CH_2)_3$ and p-C_6H_4, respectively].[115] N-(δ-Hydroxybutyl)-N-tert-butyloxcarbonylmethyl-glycine esters undergo unusual intramolecular alkylation (Stevens rearrangement; see also Vol. 27, p. 9, and Ref. 275) under Mitsunobu conditions, so far limited to generating aspartic acid derivatives (Scheme 5).[116]

A wide range of standard approaches to aspartic acid and glutamic acid derivatives, starting from ethyl N-(diphenylmethylidene)glycinate,

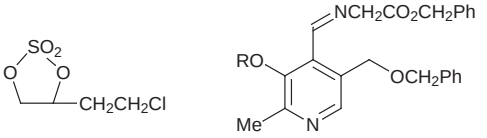


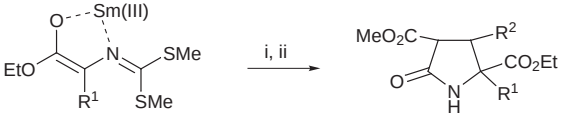
Scheme 5

$Ph_2C{=}NCH_2CO_2Et$, is covered in a paper describing α-alkylation, αα-dialkylation, and phase-transfer-catalysed Michael addition reactions,[117] and alkylation of this Schiff base by methyl 4-bromocrotonate has been optimized for syntheses of 2,3,4-trisubstituted prolines and α-cyclopropylglycines,[118] and 'methanoprolines' through cyclization following alkylation with the cyclic sulfate (15) formed from (S)-butane-1,2,4-triol,[119] and a corresponding route to (1S,2R)-allonorcoronamic acid.[120] α-Alkylation of glycine aldimines formed from novel pyridoxamine models (16) mimics an *in vivo* mechanism and leads to αα-dialkylglycine esters after hydrolysis.[121] α-Vinylation of N-(benzylidene)glycinonitrile using non-activated alkynes under basic conditions has been described.[122] tert-Butyl N-(p-chlorobenzylidene)glycinate gives novel 1,1′-binaphthyl-substituted analogues of aminoisobutyric acid, a new chiral atropisomeric amino acid (a close relative has been prepared from 14, Ref. 100).[123]

Cycloaddition of samarium(III) azomethine ylides to αβ-unsaturated esters (Scheme 6) gives αα-disubstituted-γ-carboxypyroglutamates.[124]

The growth of interest in routes to β-amino alcohols[125] and 1-amino-2,3-diols,[126] also the long-standing awareness of the usefulness of α-aminonitriles,





Reagents: i, $R^2CH{=}CHCO_2Me$; ii, 1M HCl

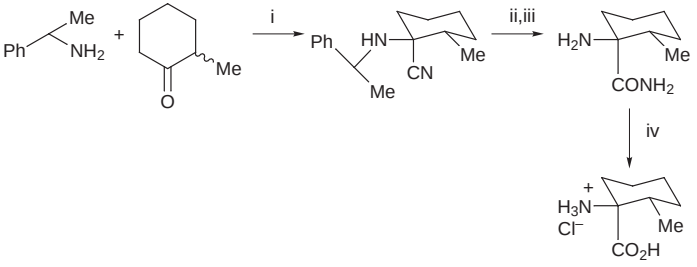
Scheme 6

has generated careful studies of the final stages of their use as precursors of α-amino acids. Kinetics of the selective hydration of the last-mentioned family (alkaline H_2O_2)[127] and oxidative cleavage of aminodiols as a route to lipidic amino acids (Ref.126), have been established. Rearrangement of homochiral dialkylaminomethyloxiranes using TMSOTf gives trans-β-amino alcohols.[128]

4.2 Asymmetric Synthesis of α-Amino Acids – A survey of the general field of asymmetric synthesis[129] includes several methods that are relevant to the amino acid context. Coverage of routes to fluorinated amino acids (Ref. 327) includes reviews of synthesis methods that are also applicable to amino acids more generally. A complete issue of the journal *Amino Acids* has been taken over by reviews of asymmetric synthesis of α-amino acids (*e.g.*, Refs. 9, 131, 225, 327, 369, 432). Uses of chiral auxiliaries or kinetic enzymic resolution to provide homochiral αα-dialkylglycines have been reviewed.[130]

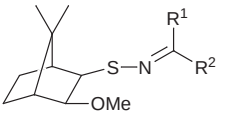
The preceding section of this Chapter mentions classical routes that have been extended to provide asymmetric syntheses, and further examples are provided here. Strecker synthesis catalysed by homochiral dioxopiperazines (cyclo-L-histidyl-L-norarginine and the L-Phe-L-Arg analogue) gives L-amino acids (see also Ref. 998),[131] and alternative asymmetric Strecker syntheses of a vancomycin component from a benzyl-protected isovanillin with trimethylsilyl cyanide and (S)-phenylglycinol,[132] and of all four stereoisomers of 1-amino-2-methylcyclohexanecarboxylic acid [using (S)- or (R)-2-methylbenzylamine; Scheme 7],[133] are notable. A simpler system is represented by hydroxylamination of isobornyl α-cyanoalkanoates using O-(diphenylphosphinyl)hydroxylamine, to give (R)-α-(2-aminomethyl) α-amino acids.[134] Enantiopure sulfinamides ArS(O)N=CHR give best results with diethylaluminium cyanide in Strecker syntheses of L-phenylglycine and L-tert-leucine when 1 equivalent of propan-2-ol is present.[135] (+)-Camphor-derived sulfenimines (17) undergo enantioselective cyanation (TMSCN) *en route* to L-α-amino acids.[136] Chiral α-hydrazinonitriles are formed highly diastereoselectively from chiral α-hydrazones [aliphatic aldehyde condensed with (S)-1-amino-2-methoxymethyl-indoline] through reaction with TMSCN in the presence of diethylaluminium chloride.[137]

Asymmetric electrophilic α-amination using di-tert-butyl azodicarboxylate



Reagents: i, $ZnCl_2$, various solvents; ii, conc. H_2SO_4; iii, H_2/Pd–C; iv, conc. HCl

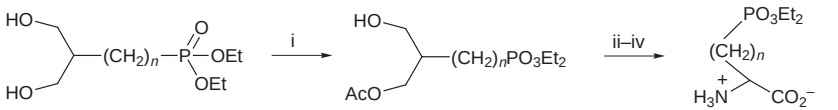
Scheme 7



(17)

with Ph_3P has been illustrated through the conversion of a chiral β-hydroxyester, $Me_2C{=}CHCH_2CH_2CH(OH)CH_2CO_2Me$, into both enantiomers of trans-3-hydroxypipecolic acid.[138] The diol formed by Sharpless asymmetric dihydroxylation of a substituted styrene has been elaborated into the corresponding (R)-phenylglycine component of vancomycin through the same amination procedure.[139]

Uses for proteins in biomimetic amination continue to be demonstrated, this year for a novel system in which an adipocyte lipid-binding protein enclosing a pyridoxamine cofactor has been used for the reductive amination of α-keto-acids.[140] Conventional uses for enzymes in fermentative production of common amino acids is covered in the next Section of this Chapter; unusual asymmetric desymmetrization processes are attracting more interest, not least because such routes can give both enantiomers of the target amino acid. Thus, *cis*-6-hydroxymethylpipecolic acid enantiomers have been obtained through lipase-catalysed partial hydrolysis of *cis*-2,6-bis(acetoxymethyl)-N-benzyloxycarbonyl piperidine,[141] and the reverse process, lipase-catalyzed mono-acylation of 2-(ω-phosphonoalkyl) propane-1,3-diols followed by routine elaboration (Scheme 8), has led to L-α-amino acids carrying aliphatic phosphonate side-chains.[142] Methods established earlier (*e.g.* Vol. 22, p. 30) involving β-methylaspartase bioconversions of alkylfumarates have been used in routes to (2S,3S)-3-methyl- and isopropyl-aspartic acids.[143] A target amino acid for mycestericin D synthesis has been obtained through condensation of benzyloxybutanal with glycine catalysed by L-threonine aldolase, achieved under kinetic control leading to high erythro/threo selectivity.[144]

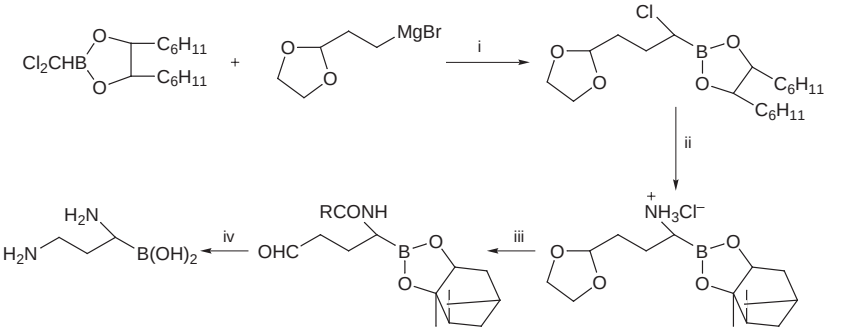


Reagents: i, lipase PS/vinyl acetate/organic solvent; ii, $RuCl_3$–$NaIO_4$; iii, DPPA, Et_3N, BnOH; iv, routine development of functional groups

Scheme 8

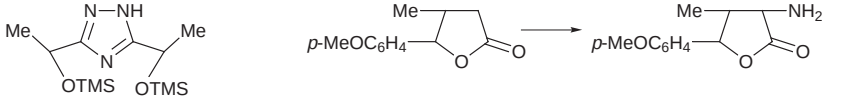
Amination of triflates of α-hydroxyacids by N-benzyl-ω-(alkoxycarbonylamino)alkylamines or N-benzyl-ω-(N-Boc-amino)alkylamines has been studied as a route to N^{α}-(ω-aminoalkyl)amino acids.[145] Amination is the anti-climax of a route to α-amino boronic acids starting with a spectacular elaboration sequence of a chiral boron synthon (Scheme 9);[146] this route uses a (+)-pinanediol-derived

boronic ester, also used in a synthesis of (R)-1,4-diaminobutane-1-boronic acid hydrochloride (an L-ornithine analogue) involving the B-(3-azidopropyl) synthon, and the same ensuing stages.[147] Use of the chiral triazole (18) for enantioselective amination of α-bromo acid esters gives the expected product that can act as a protected synthon, alkylation leading to αα-dialkylglycines.[148] Azidolysis after electrophilic bromination of the TMS enol ether of the butenolide (19) leads to the N-terminal residue of nikkomycins B and B_x.[149]



Reagents: i, $ZnCl_2$; ii, transesterification with (+)-pinanediol; iii, LHMDS, then HCl (3 eq); iv, H_2NOH, then H_2–cat., H_3O^+

Scheme 9



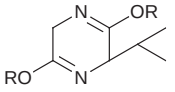
(18) (19)

Addition to imines amounting to the introduction of a carboxylic acid function can be easily biased towards a particular enantiomer, for example using imines of (R)-(+)-camphor to which addition of the 5-methylthiazole carbanion has been demonstrated.[150] Stereocontrolled introduction of a carboxy group into an alkylamine is usually a multistage process, as in the preceding example and in Pummerer rearrangement of homochiral α-(tolylsulfinylmethyl)alkylamines [*e.g.*$TolS(O)CH_2CR^1R^2NHZ \rightarrow HOCH_2CR^1R^2NHZ$][151] (see also Ref. 174). All four stereoisomers of phosphothreonine $MeCH(OH)CH(NH_2)PO_3H_2$ have been prepared from the N-trimethylsilyl lactimine and O-trimethylsilyl diethyl phosphite, and exploiting the inversion accompanying Mitsunobu amination of α-hydroxy-β-silyloxyphosphonate.[152] Addition of an organometallic reagent to the protected benzylimine $BnOCH_2CH(OBn)CH{=}NBn$ prepared from D-glyceraldehyde, and elaboration of the deprotected diol, gives either a D-α-amino acid or a β-amino acid depending on choice of route.[153]

2-Aminoalkanesulfonic acids are best prepared through substitution by sulfite of methanesulfonates of protected 2-aminoalkanols.[154]

(S)-$Ph_2C{=}NCH(CO_2Bu^t)CR(CO_2Me)_2$ (R = H) formed from diethyl malonate and the corresponding Schiff base of acetoxyglycine with $Pd(OAc)_2$/(2S,4S)-BPPM, can be alkylated (R = Me, allyl, benzyl) through a standard phase-transfer catalysis protocol.[155]

The routes that are based on chiral glycine synthons have continued to dominate the current literature covering the asymmetric synthesis of α-amino acids. The Schollkopf bis-lactim ether route provides the following (further examples are covered elsewhere in this Chapter, Refs. 399, 406): all four isomers of 3-(4-chlorophenyl)glutamic acid [alkylation of the (S)-bis-lactim ether (20) through Michael addition to methyl *cis*-4-chlorocinnamate gives a 56:40-mixture of (2R,3S)- and (2*R*,3*R*)-diastereoisomers],[156] the novel bis(amino acid) (21) through $Pd(OAc)_2PPh_3$-catalysed alkylation by 2,3-dibromopropene,[157] 2-alkanethio-3,5-dimethoxy-L-phenylalanine,[158] (2R)-6-Z-amino-2-Boc-amino-hex-4-ynoic acid (a constrained analogue of D-lysine),[159] and 2,3-*anti*-2-amino-3-substituted 4-phosphonobutanoic acids and 2-amino-6-phosphono-4-hexenoic acid through addition to *E*-alkenyl- and 1,3-butadienylphosphonates.[160] A synthesis of (S)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propanoic acid [(S)-AMPA] involves a novel KF-promoted 1,3-dipolar cycloaddition to give 3-bromo 4-hydroxymethyl-5-methylisoxazole, used in a standard alkylation protocol employing the D-valylglycine-derived bis-lactim ether, (20).[161] L-(p-Boronо)phenylalanine has been prepared through this protocol, relying on chymotrypsin resolution for attainment of full homochirality.[162] The D-penicillamine-derived bis-lactim ether offers easier handling and better stereodiscrimination than the standard valine analogue in a synthesis of L-propargylglycine.[163] A brief review of Schollkopf methodology has appeared.[164]



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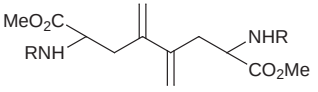
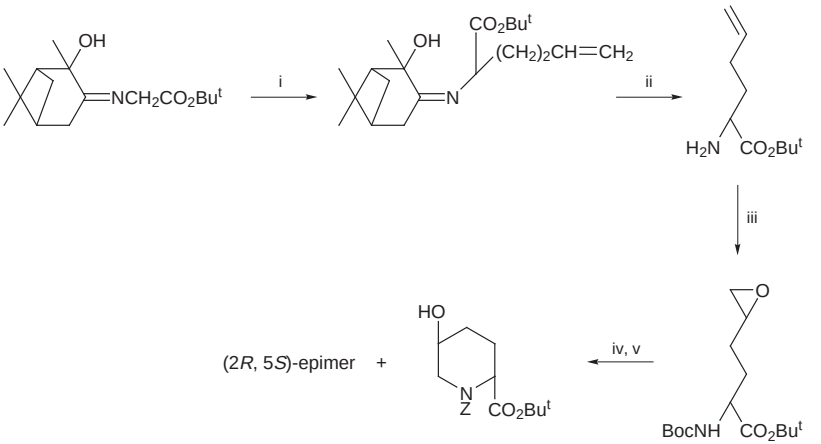


(21)

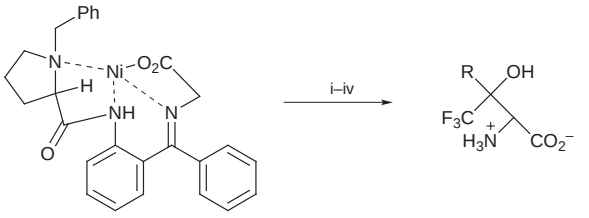
Oppolzer's camphorsultam has served well for the preparation of enantiomers of p-carboranylalanine and 2-methyl o-carboranylalanine,[165] and N-benzyloxy-allylglycines[166] (see also Refs. 422, 483), and chiral glycine synthons based similarly on Schiff bases have been used for the synthesis of cis- and trans-5-hydroxy-D-pipecolic acid (Scheme 10).[167] An unusual variation uses *N*-acryloyl (2S)-bornane-10,2-sultam in a 1,3-dipolar cycloaddition to the nitrone (4-$MeOC_6H_4)_2CHN(O){=}CHCO_2Et$, leading to the usual isoxazolidine from which N-Boc-(4S)-4-hydroxy-L-glutamic acid is obtained through routine stages.[168]

A novel approach using a cobalt(III)-chelated glycinate involves the addition of carbanions illustrating "asymmetric transformations of the second kind",[169] and a new chiral Schiff base (Scheme 11; closely shadowing voluminous earlier work by the same research group, using an L-proline-based glycine Schiff base) has been used for the preparation of (2S,3S)-β-hydroxy-β-trifluoromethyl-α-amino acids,[170] and for asymmetric aldol reactions with trifluoromethyl ketones



Reagents: i, LHDMS, $CH_2{=}CH(CH_2)_2I$; ii, H_3O^+; iii, Boc_2O then MCBA; iv, LiBr, then TBDMSCl, then NaH/DMF; v, Bu_4NF, THF

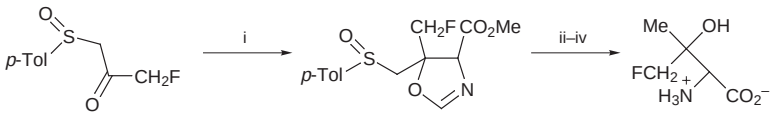
Scheme 10



Reagents: i, CF_3COR/DBU, MeCN; ii, aq. AcOH; iii, HCl/MeOH, iv, Dowex-H^+/NH_4OH

Scheme 11

to give (2S,3S)-3-trifluoromethyl-3-alkylserines in 90–98% diastereoisomeric excess.[171] The earlier-established chiral glycinate Schiff base nickel complex just referred to has been used for a synthesis of o-, m-, and p-fluoro-L-phenylalanines through standard methodology.[172] Further novelty is exhibited in the use of the ester of N-benzylideneglycine formed with an (S)-binaphthol, giving predominantly D-amino acids through routine alkylation and work-up.[173] Aldol reactions are not limited to Schiff bases, as in examples in the preceding text, with copper(I)-catalysed addition of (S)-3-fluoro-1-(p-tolylsulfinyl)acetone to methyl isocyanoacetate leading to stereoisomers of 3-fluoromethyl-threonines (Scheme 12;[174] see also Refs. 45, 385). The absolute stereochemistry of one of these products (22 in Scheme 12) was determined by X-ray crystal structure analysis. The stereochemistry of gold(I)-catalysed asymmetric aldolization of isocyanoacetic acids with fluorobenzaldehydes is rationalized as a consequence of *si*-face to *si*-face interaction, as in (23).[175] Palladium(0)-catalysed alkylation of the



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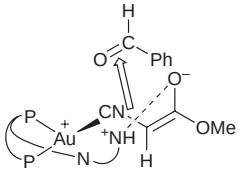
Reagents: i, $CNCH_2CO_2Me$; ii, H_2O, $CHCl_3$; iii, Raney nickel, methanol buffer pH 5.2; iv, 1 M HCl

Scheme 12



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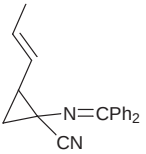
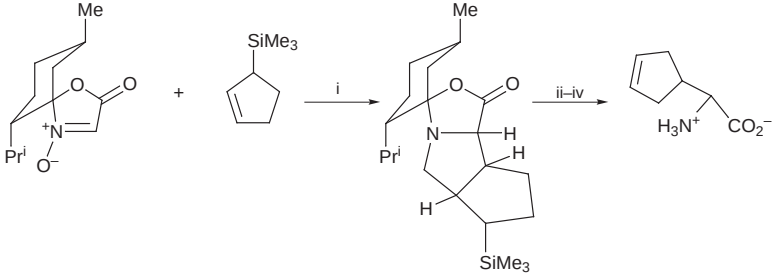


(24)

carbanion of the achiral Schiff base $Ph_2C{=}NCH_2CN$ with (4S)-1-chloropent-2-en-4-ol, followed by Mitsunobu $S_{N'}$ cyclization, gives the cyclopropane (24).[176]

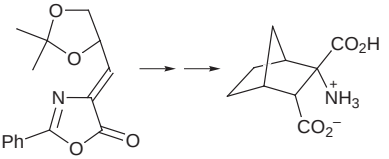
An oxazol-5(4H)-one is essentially a Schiff base of an α-amino acid, and although asymmetric syntheses based on 4-alkylation have been standard practice for many years, a novel approach based on the cycloaddition reactivity of the nitrone of an oxazol-3(2H)-one has been described (Scheme 13), and illustrated for (2S,1′S)-cyclopent-2-enylglycine.[177] An N-mannofuranosyl nitrone added to methylenecyclopropane gives a 4:1-mixture of regioisomeric isoxazolidines, the major product being isomerized in xylene at 137° to give the virginiamycin component (2S)-4-oxopipecolic acid, from which the novel cis-4-hydroxy-analogue was obtained by L-Selectride reduction.[178] Further results from the project directed at asymmetric synthesis of oxazol-5(4H)-ones of (S)-αα-dialkylglycines employing L-phenylalanine cyclohexylimide as recoverable chiral auxiliary (see Vol.28, p. 23, and also Ref. 109) describe the preparation of α-bromomethyl-S-



Reagents: i, $BF_3.Et_2O$; ii, OH^-; iii, H_2/Pd; iv, BF_3

Scheme 13

alanine.[179] The (R)-glyceraldehyde-derived oxazol-5(4H)-one (25) has appeared in several papers describing amino acid syntheses, *e.g.* the conformationally-constrained L-aspartic acid analogue (26) obtained from the Diels-Alder adduct with cyclopentadiene,[180] and the corresponding cyclohexenone formed with the Danishefsky diene.[181] Lithium enolates of glucose-derived bicyclic oxazinones (27) are alkylated easily, with high diastereoselectivity but giving only modest reaction yields.[182] A more routine addition pathway is demonstrated in Michael addition of the carbanion of ethyl benzylideneglycinate to trans-$CF_3CH{=}CHCO_2Et$, ensuing cyclization leading to a cis-trans mixture of ethyl 3-trifluoromethylpyroglutamate;[183] further results from this study include the corresponding use of the homochiral Schiff base from tert-butyl glycinate and 2-hydroxypinan-3-one to give the separate enantiomers of the same product. (+)-8-Phenylmenthyl N-benzylideneglycinate has been used for synthesis of eudistomidin B (28) through alkylation with the appropriate chiral iminium ion and showing the expected double diastereodifferentiation.[184] The titanium enolate of the Schiff base formed between ethyl glycinate and (1R,2R,5R)-hydroxypinanone [shown to adopt the (E)-configuration through X-ray crystal analysis] undergoes enantioselective aldol reactions as illustrated by preparations of pure (1R,2S)-chloramphenicol base and of D-allo-threonine.[185] Conventional diastereoselective



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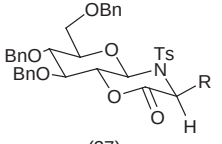

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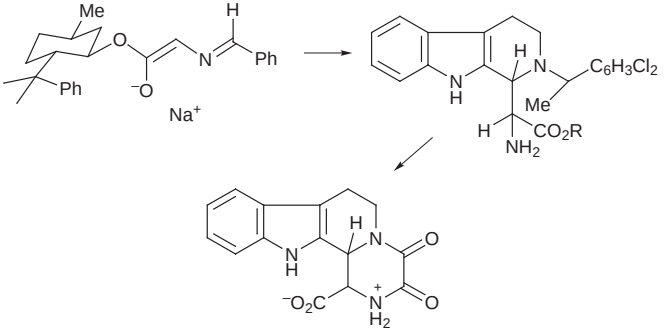
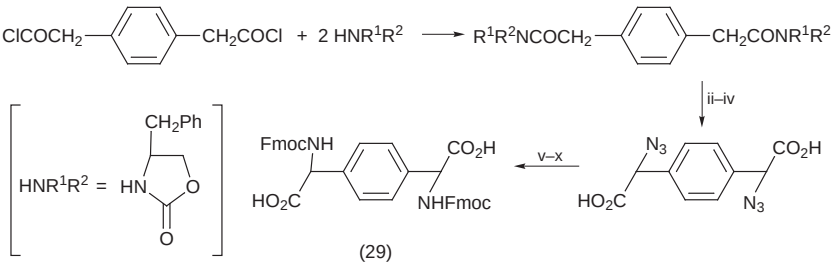


(28)

alkylation (LDA/alkyl halide) of this substrate or its equivalent (thiazol-2-yl in place of the ester function) is described in a project aimed at a mechanistic understanding of the process (two hypotheses are presented),[186] including molecular orbital calculations for three-dimensional details of the alkylation transition state.[187]

A brief review of Seebach methodology (in other words, the use of chiral oxazolidinones and imidazolidinones derived from glycine for the asymmetric synthesis of amino acids) has appeared.[188]

The (R)- and (S)-oxazolidinones introduced by Evans continue to be useful, and new examples prepared from cis-2-amino-3,3-dimethylindan-1-ol through a standard sequence[189] have been described. A general high-yielding route to 4-alkoxycarbonylimidazolidin-2-ones has been established.[190] '(S,S)-Phenylbis(glycine)' (29 and its m-isomer; Scheme 14) have been prepared from (S)-4-benzylox-

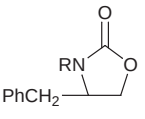


Reagents: i, Bu^{n}Li, THF,–78 °C; ii, KHDMS, THF; iii, $ArSO_2N_3$; iv, AcOH; v, $SnCl_2$; vi, Boc_2O; vii, LiOH; viii, TFA; ix, HMDS, TMSCl; x, FmocCl

Scheme 14

azolidin-2-one through N-acylation with benzenediacetyl dichlorides and routine steps thereafter,[191] exemplifying this approach. D- and L-Carboranylalanines have been prepared through addition of the same chiral auxiliary to allylcarborane.[192] The synthon was used as the boron enolate of its N-bromoacetyl derivative with 4-fluoro-3-nitrobenzaldehyde to give an amino acid moiety of orienticin C.[193] Azidolysis was used to introduce the amine function and other well-established steps completed these preparations. 3-(4-Hydroxyphenyl)-prolines (conformationally-constrained tyrosine analogues) have been prepared through this standard protocol, with cyclization accomplished at the α-azidoalkanoate stage of the route.[194] Alkylation of the lithium enolate of $Ph_2C{=}NCH_2CO_2Et$ with the N-acylated oxazolidinone (30; R = $BrCF_2CH{=}CHCO$-) and further synthesis stages, gives trans-3,4-difluoromethano-glutamic acid.[195] All four stereoisomers of the highly conformationally constrained amino acid β-isopropylphenylalanine have been prepared from commercially available (4R)- or (4S)-4-phenyloxazolidin-2-one,[196] and an unjustly criticized synthesis of (3R)- and (3S)-piperazic acids from a 5-bromopenta-

noyloxazolidinone and di-tert-butyl azodicarboxylate has been rigorously authenticated.[197] Related oxazolidinones (31) and (32; R = H) have been used for asymmetric synthesis of β-benzoylaminophenylalanine (an analogue of the taxol side-chain) from (31),[198] and to reveal an unexpected self-addition by-product (32; R = PhCONHCHPh) accompanying alkylation of (32) by diphenylmethyl bromoacetate.[199] α-Methyl-L-phenylalanines carrying halogen substituents in the benzene ring are accessible from the imidazolidinone (33 in Scheme 15).[200] tert-Butyl 1-methyl-2-oxoimidazolidine-4-carboxylate continues to prove interesting in the context of the dynamic kinetic resolution of its 3-(2-bromopropionyl) derivative (see Vol.28, p. 13, and Ref. 532), used for Gabriel synthesis of L- or D-alanine, the outcome depending on the nitrogen nucleophile.[201]



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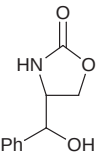

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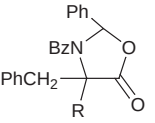

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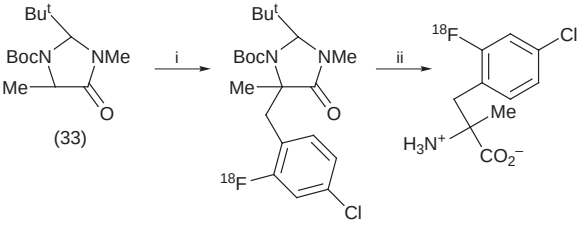
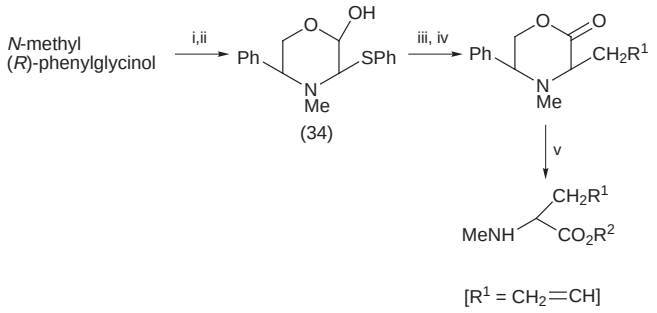


Reagents: i, $ArCH_2I$; ii, H_3O^+

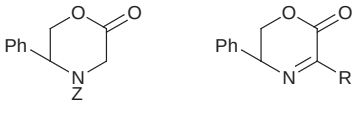
Scheme 15

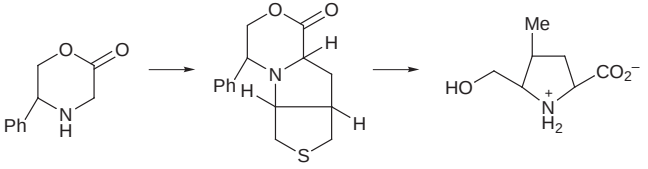
(R)-Phenylglycinol-derived morpholines, *e.g.* (34) produced by condensation with glyoxal (Scheme 16; R^1 = $CH{=}CH_2$ or $CMe{=}CH_2$),[202] and the more familiar morpholinone (35) formed from ethyl bromoacetate followed by N-protection in a 'one pot' synthesis,[203] are further 'chiral glycine' synthons that have been put to use, the former for a synthesis of N-methyl-D-allylglycine and its homologues. α-Substituted alanines are obtainable through methylation of the morpholinone (36) prepared from (R)-phenylglycinol and an α-keto-ester,[204] a variation on the established use of azomethine ylides generated from this synthon for stereospecific proline synthesis, illustrated most recently for 5-hydroxymethyl-4-methylproline (Scheme 17),[205] and 3,4-dicarboxy-,[206] 2-phenyl-3,4-di(methoxycarbonyl)-[207] and 2-methyl-prolines.[208] Alkylation of the ephedrine-based morpholinone gives (2S,3S,6S)-2,3-methano-2,6-diaminopimelic acids and (2S,3S,6R)- and (2R,3R,6S)-isomers *via* the lithium enolate (37),[209] and N-Fmoc-4-(phosphonodi-



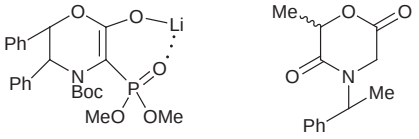
Reagents: i, glyoxal; ii, PhSH; iii, TMSCl, then $CH_2{=}CHCH_2SiMe_3 + ZnBr_2$; iv, oxalyl chloride + DMSO; v, routine hydrolysis, hydrogenolysis

Scheme 16





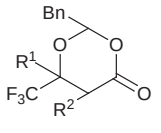
Scheme 17



fluoromethyl)-L-phenylalanine has been prepared from the same synthon.[210] Further syntheses of D- or L-amino acids by enantiospecific alkylation of N-[(S)-1-phenylethyl]morpholin-2,5-diones (38) have been published.[211]

The dioxanone (39) has been introduced for α-amino acid synthesis as substrate for amination by BocN=NBoc, and demonstrated for asymmetric synthesis of (2R,3S)- and (2S,3R)-2-amino-3-trifluoromethyl-3-hydroxyalkanoates.[212] A novel synthon (40) shows considerable promise, from the point of view that the L-prolinol moiety can be recycled; further development is needed (moderate optical purity in preparations of N-methyl-L-allylglycine and related L-amino acids).[213]

The 'chiral glycinamide' $NH_2CH_2CONMeCHMeCH(OH)Ph$ derived from ψ-ephedrine (see Vol. 28, p.12) continues to show promise, *e.g.* for the synthesis of L-2′-azatyrosine[214] and an amino acid carrying the pyridoxamine moiety (41), noting that hydrolytic removal of the chiral auxiliary needs harsh conditions that cause partial loss of Boc groups.[215] Syntheses of representative D- and L-N-methylamino acids have also been achieved[216] with this approach, with the claim that the chiral auxiliary is easily removed by hydrolysis in hot water or in aqueous dioxane.



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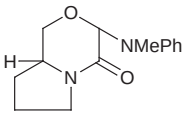

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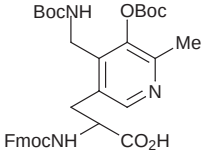

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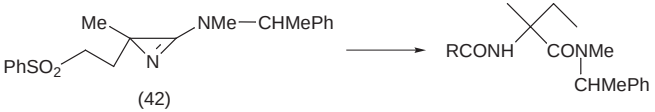
Claisen rearrangement of chiral allyl esters of N-Boc-glycine leading to β-methyl-D-aspartic acid after oxidation ($RuCl_3/NaIO_4$) of the resulting D-alkenylglycine has extended established methodology (see also Section 4.11).[217]

Other routes employing starting materials with amino and carboxy groups in place, but in a latent form (see also Section 6.3), include chiral azirines [*e.g.* 42, used in a synthesis of L-isovaline, *alias* (S)-α-methyl-α-ethylglycine],[218] and chiral aziridines. The lactone (43; R = Ac) from D-ribose, when treated with BF_3/MeOH, generates the novel 2,3-iminoglutamate (44; the first example of the synthesis of an L-glutamic acid derivative from a carbohydrate) but this treatment gives (4S)-hydroxy-(3S)-methoxy-L-glutamic acid with the analogue (43; R = Z).[219] Examples of the use of the latter class of synthon include a synthesis of αα-disubstituted glycines by nucleophilic ring-opening of 2,2-dialkyl derivatives;[220] analogous syntheses of both α- and β-amino acids using magnesium bromide, sodium iodide or sodium bromide, respectively,[221] and using copper-'catalysed' Grignard reagents.[222] A new enantioselective synthesis of N-arylaziridine-2-carboxylic acids involves addition of N-acyl-N-arylhydroxylamines to tert-butyl acrylate using a quaternary salt of a *Cinchona* alkaloid as phase-transfer catalyst.[223]



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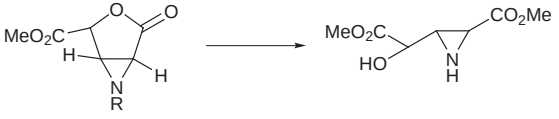
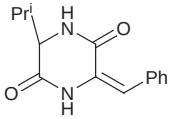


(43) (44)

Chiral α-hydroxy-β-lactams can be made to undergo oxidative rearrangement to give N-carboxyanhydrides of αα-dialkylglycines (see also Vol. 28, p. 19 and Ref. 885).[224]

The usual crop of papers dealing with asymmetric hydrogenation of achiral precursors of α-amino acids provides small improvements here and there, to well-established methodology. A broad review of the hydrogenation of 'dehydro-amino acids' catalysed by homochiral rhodium(I) complexes has appeared,[225] and better than 90% e.e. can be secured with the PROPRAPHOS ligand,[226]and with related catalysts.[227] Another typical example of this approach concentrates on the assessment of other chiral diphosphines, *e.g.* 2,3-bis(silyloxy)-1,4-bis(di-phenylphosphino)butanes, for use as ligands for rhodium(I) hydrogenation catalysts acting on methyl α-acetamidoacrylate; these turn out to be rather unselective in giving 19-23 % enantiomeric excess (e.e.).[228] D-Mannitol-derived chiral diphosphine catalysts generate e.e. of 24 - 80% in this process.[229] The use of Rh^+ complexed to solid-phase supported homochiral peptides carrying phosphine side-chains offers a promising new variant.[230]

A related approach with a long history (and even worse discrimination) is illustrated by the hydrogenation of the oxime of pyruvic acid catalysed by palladium-alumina in the presence of a homochiral alkaloid (best result: 26% e.e. using ephedrine).[231] Where the hydrogenation substrate is chiral, as with the dioxopiperazine (45), good results can be obtained with simple hydrogenation catalysts (*e.g.* 45 → L-threo-product in 91% e.e.) but with extraordinary depen-dence on structure; thus, hydrogenation of the bis(N-Boc) derivative of (45) gives the L-erythro-derivative in 95% e.e.[232] Reduction of 2-(N-arylimino)-3,3,3-tri-fluoropropionates by a chiral borane gives (R)-trifluoroalanine in only 62% e.e.[233]



(45)

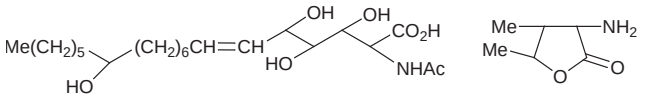
Asymmetric synthesis of *vic*-amino-alcohols, illustrated by oxa-Michael addi-tion of (-)-N-formyl norephedrine to nitroalkenes and $NaBH_4$-Pd/C reduction including removal of the chiral auxiliary,[234] provides close relatives of the amino acids that are useful in synthesis (see also Refs. 125, 126).

4.3 Synthesis of Protein Amino Acids and Other Naturally Occurring α-Amino Acids – A number of papers describing the synthesis of amino acids conforming to this title appear elsewhere in this Chapter, because they are used primarily to demonstrate novel applications of synthetic methodology. This section carries papers that describe applications of established methodology for achieving specific targets.

The use of enzymes and whole-cell techniques for the synthesis of familiar amino acids is an inescapable feature of this section, year after year, but only

representative citations for this commercially-important topic can be accommodated in the space available. Reviews cover the production of threonine and lysine from a *Corynebacterium* sp. and a *Brevibacterium* sp.,[235] of L-lysine and other L-amino acids using mutants of *Bacillus methanolicus*,[236] of L-tryptophan from glucose using *E. coli* engineered with the *Enterobacter aerogenes* tryptophanase gene,[237]and microbiological production of L-tyrosine.[238] A general review has appeared,[239] and the use of methanol-utilizing bacteria in this context has been surveyed.[240] New results in this category include L-serine formation from methanol and glycine acted upon by *Methylobacterium* sp. MN43,[241] and a study of parameters determining optimum L-phenylalanine formation by *E. coli* AT2471.[242] The *E. coli* mutant W1485lip2 that generates pyruvic acid has been transformed for use for L-tryptophan production when presented with ammonia and indole,[243] and reductive amination of pyruvic acid employing a NADH regeneration system and alanine dehydrogenase (or the leucine equivalent) has been accomplished.[244] Methyl L-phenylalaninate is an unusual outcome from phenol-ammonia lyase-containing cells of *Rhodotorula glutinis*.[245]

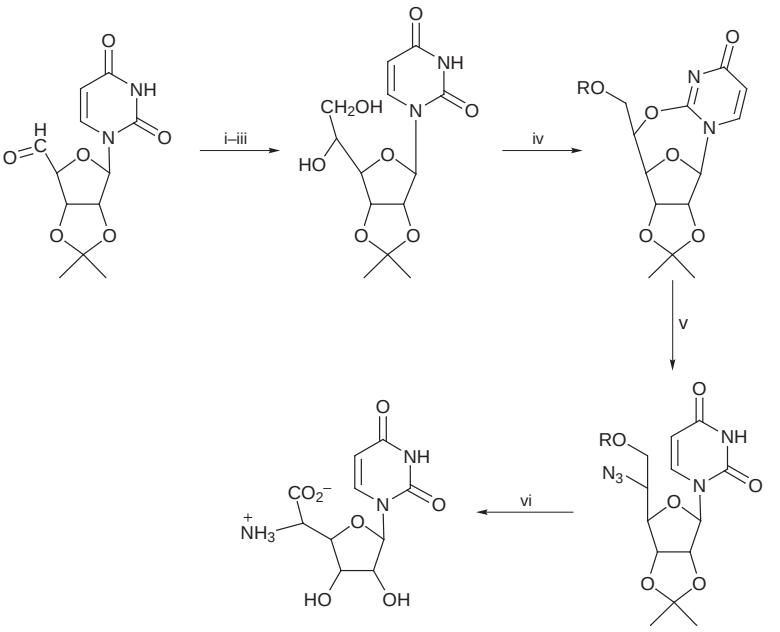
Sphingofungin D (46; the N-acetyl derivative of asperfungin) has been obtained from myo-inositol and (R)-epoxyoctane, thus establishing the (R)-configuration at C-14 based on secure understanding of the stereochemical basis of this nucleophilic ring-opening process.[246] (+)-Polyoxamic acid also offers a challenge for exploring synthesis methodology, and the modest selectivity that often accompanies deracemization of acyclic allyl esters *via* π-allylpalladium intermediates does not cause complications in a route to (E)-4,5-epoxypent-2-en-1-ol, ring-opening with phthalimide being followed by dihydroxylation and established steps.[247] Two new stereoselective syntheses of characteristic metabolites of *Quararibea funebris* have been based on the lactone of (2S,3S,4R)-γ-hydroxyisoleucine (47), including amination of β-angelicalactone.[248]



(46) (47)

An ingenious strategy for placing an amino acid moiety with correct stereochemistry appears in a synthesis of uracil polyoxin C (Scheme 18).[249] Other natural amino acids with heterocyclic side-chains, (S)-β-pyrazolylalanine and (S)-quisqualic acid, are both conveniently accessible from (S)-tert-butyloxycarbonylaziridine-2-carboxylate through nucleophilic ring opening.[250]

Natural cyclic α-imino acids regularly targetted for synthesis include L-proline, for which a new route from N-protected L-2-amino-5-bromopentanoic acid esters prepared from L-glutamic acid, and a similar approach to 1-aminocyclopropanecarboxylic acid, starting from 2-aminobutanoic acid, has been described.[251] Both enantiomers of cis-4-hydroxyproline have been obtained by double iodocyclization of the diene (48) prepared from the (6R)- and (6S)-forms of N-[(S)-1-



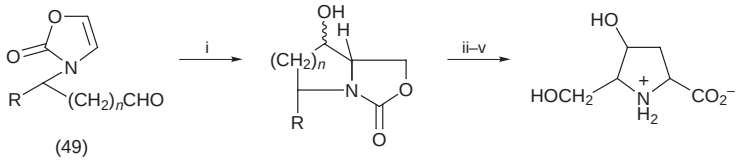
Reagents; i, $AcCN_2P(O)(OMe)_2$; ii, H_2/Pd–C; iii, AD-mix-d; iv, O-protection, then DBU/DMF/70 °C; v, NaN_3; vi, routine steps

Scheme 18

(48)

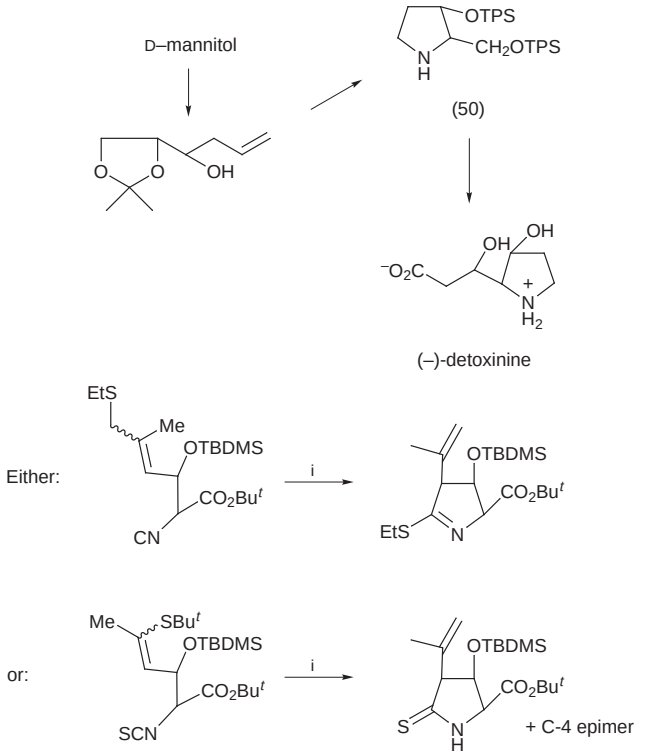
phenylethyl]-6-methylmorpholin-2,5-dione (see also Ref. 211),[252] and a corresponding approach to (+)- and (-)-bulgecinines has been described.[253] An alternative synthesis of (+)-bulgecinine starts with the O-stannyl ketal of the N-substituted oxazolin-2-one (49 in Scheme 19).[254] cis- and trans-3-Hydroxy-D-prolines and (+)-detoxinine (the non-natural isomer) have been synthesized from D-mannitol (see also Ref. 307) *via* (50).[255]

A broadly-applicable route to prolines based on thiol-mediated free radical isomerization has been worked out (Scheme 20)[256] that generates all the structural features of α-kainic acid, except the 3-carboxymethyl group. Extension of this route introduces this feature by intramolecular alkylation *via* a cyclic sulfone involving the isopropenyl group.[257] Trimethylstannyl radical carbocyclization of a diene (51) derived from L-serine, leading to a 2,3-trans-/3,4-cis- and 2,3-trans-/3,4-



Reagents; i, Bu_3SnH/AIBN, reflux 5h; ii, TBSCl [for n = 1, R = $PhCH_2OCH_2$]; iii, H_2/Pd–C; iv, $RuCl_3$–$NaIO_4$; v, NaOH in 10% aq. EtOH

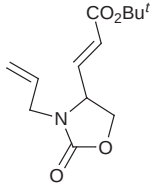
Scheme 19



Reagents; i, Bu_3SnH/AIBN

Scheme 20

trans- mixture of isomers (2.8:1) in high yield and favouring the natural stereochemistry, has been used for syntheses of (-)-α-kainic acid and (+)-α-allokainic acid.[258] Hydroxyl group-directed heterogeneous catalytic hydrogenation of an enamide (52) establishes the cis-C-3/C-4 stereochemistry in a route to acromelic acid analogues.[259] Stereocontrol is a well-established feature of carbocyclizations, and this step is a feature of a high-yield route to kainoids (Scheme 21) using N,N-



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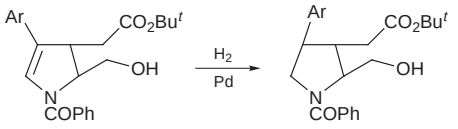

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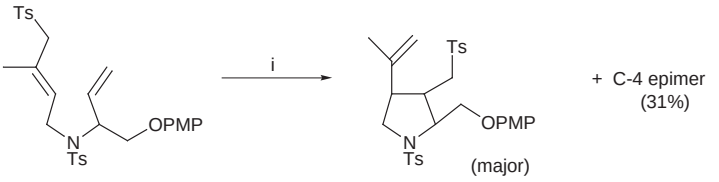


Reagents: i, TsSePh (0.15 equiv.), *hν*, C_6H_6

Scheme 21

di-allyl-N-toluene-p-sulfonylamines.[260] The synthesis of kainoids has been reviewed.[261]

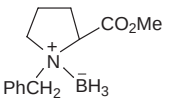
4.4 Synthesis of α-Alkyl Analogues of Protein Amino Acids and of Other Natural α-Amino Acids – This section expands year by year, seeming to suggest a growing interest in these particular homologues of the protein amino acids. The derivatives have their own importance, particularly in enzyme inhibition studies, and synthetic methods undergoing establishment for access to α-amino acids in general are often easily extended to their α-alkyl analogues, as illustrated in preceding sections of this Chapter as well as with papers collected here. Results from Ohfune's group on routes to αα-dialkylglycines have been reviewed.[262]

Several projects described in the preceding sections covering general synthesis methods, include αα-dialkylglycine syntheses. α-Methyl serine, needed for the synthesis of the novel immunomodulator (+)-conagenin, has been obtained through different routes: Katsuki-Sharpless oxidation of methallyl alcohol to give (S)-2-methylglycidol, and rearrangement of the derived trichloroacetimidate,[263] and by an application of the Schollkopf procedure.[264] α-Methylphenylalanine and α-methyl-β-phenylserine have been prepared through ring-opening of N-(toluene-p-sulfinyl)aziridine-2-carboxylic acid.[265]

Direct α-alkylation of an α-amino acid derivative is a common approach. The most familiar commercially-available derivatives are often the least satisfactory for this purpose, as seen in carbanion generation from N-Boc hydroxy-L-proline by LDA followed by reaction with an alkyl halide, resulting in only 20% diastereoisomer excesses and modest yields.[266] An extraordinary set of results has been collected for α-benzylation of methyl N-benzyl-L-prolinate in the presence of BH_3-Me_2S [the (S)-product is favoured (54% yield) by the simplest benzylation protocols but with LDA/18-crown-6/HMPA, the (R)-enantiomer is formed], including an X-ray crystal analysis of the intermediate (53).[267]

Electrochemical α′-methoxylation (see also Refs. 882, 883), substitution to give an α′-phenylthio group, α-methylation and reductive removal of the phenylthio group has been used to prepare non-racemic α-methyl-proline and α-methyl-pipecolic acid.[268]

An effective substrate is an imidazolinone from (R)-4-hydroxyphenylglycine (C-2-epimer of 33; N-benzoyl, R = 4-$PhCH_2O$-C_6H_4-), providing the basis for the synthesis of (S)-(+)-α-methyl-(4-carboxyphenyl)glycine;[269] the same target, studied independently from the point of view of establishment of its absolute configuration through X-ray crystal analysis,[270] was obtained by resolution of the racemate through N-(L-leucin)ylation and anion exchange separation of the resulting diastereoisomers followed by hydrolysis. Asymmetric alkylation of α-amino acid esters by use of a novel pyridoxal model (54) carrying a chiral ionophore (the Na^+ ion plays an important role) has been reported.[271]



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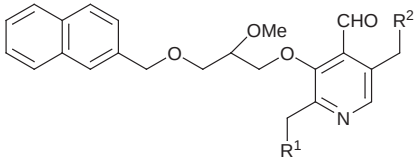


(54) R^1–R^2 = (*S*)-$S(CH_2)_5S$–

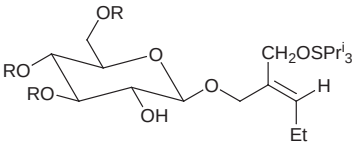
α-Halogenomethylated protein amino acids are a subject of interest in several laboratories, as potential inhibitors of certain enzymes. Their synthesis is therefore bringing out some ingeneous approaches; thus, regioselective alkylation of imines $XCF_2CH(=NCO_2R)CO_2Me$ provides α-chlorodifluoromethyl- and α-bromodifluoromethyl-α-amino acid esters,[272] while addition of HCN to homochiral enamines $TolS(O)CH=C(CHF_2)NH_2$ has been used to start syntheses of (R)-α-difluoromethylalanine and (S)-α-difluoromethylserine.[273] Both enantiomers of α-trifluoromethylbutyrine have been prepared in the same way from homochiral alkyl p-tolylsulfoxides.[274] Methyl 3,3,3-trifluoro-2-diazopropionate, in the presence of catalytic amounts of copper and di-rhodium tetra-acetate, forms ammonium ylides with amines and amides that undergo [1,2]-Stevens rearrangement (see also Ref. 116) to give α-trifluoromethyl-N,N-dialkylamino acid esters.[275]

4.5 Synthesis of α-Amino Acids Carrying Alkyl Side-Chains, and Cyclic Analogues – This section is devoted for the most part, to the synthesis of near analogues of familiar natural α-amino acids. A variety of differing motives underlies the choice of targets.

L-tert-Leucine is gaining in importance as a chiral auxiliary (Ref. 6) and its preparation by reductive amination of trimethylpyruvate esters with leucine dehydrogenase can become a continuous process when NADH that is required is regenerated by formate dehydrogenase.[276]

'Methano' analogues of the protein amino acids have served well as conformationally-restricted models for structure-activity studies, and a general approach employing stereoselective cyclopropanation (Et_2Zn/CH_2I_2) of a D-glucose-derived chiral auxiliary (55) has been described (amination is achieved by a Curtius rearrangement at a late stage).[277] A new route to '(-)-(Z)-2,3-methano-L-glutamic acid', *i.e.* a stereoisomer of 1-amino-2-carboxymethyl cyclopropanecarboxylic acid, has been described, proceeding *via* cyclopropanation of a D-glyceraldehyde-derived aminopentenoate to a protected (-)-(Z)-cycloaspartic acid derivative, and final Arndt-Eistert chain extension.[278] The same approach provides (-)-isomers of allocoronamic acid, allonorcoronamic acid, (Z)-2,3-methanohomoserine, and (Z)-2,3-methanomethionine.[279] Three stereoisomers of N-protected 2,3-methanomethionine have been prepared through conventional methods suitable for large-scale production, two from (S)-1-O-benzylglycerol, the other through ammonolysis of the malonate (56).[280] 2,3-Methanophenylalanine, prepared by cyclopropanation of the protected dehydro-amino acid, adds a further example to the list.[281] A mixture of cis/trans 2,3-methanotryptophans has been obtained by cyclopropanation of the corresponding azlactone,[282] and 2,3-methanopipecolic acid has been prepared through (3-chloropropylcyclopropan)ation of methyl N-benzylideneglycinate followed by cyclization.[283] An alternative route to methano-pipecolates[284] and methano-kainoids[285] exploits the recently acquired understanding of relevant radical cyclization methodology (*cf* Ref. 258), and is based on cyclization of trimethylstannyl radicals attached through a methylene group to suitably structured acrylates and acrylamides.

Carboxycyclopropyl-L-glycines, *alias* 3,4-methano-L-glutamic acids, have been generated from the pyroglutamate derivative (57).[286] The general synthesis of 1-aminocyclopropane-1-carboxylic acids, a subject that has been reviewed,[287] has also been developed strongly, in fulfilment of the interest in conformationally-restricted amino acids that has attracted several groups to the 'methano-amino acids'. Cyclopropanation of the oxazinone derivative (58) using the Corey ylide $CH_2{}^-S^+(O)Me_2$ gives access to 1-amino-2-alkylcyclopropanecarboxylic acids.[288] Further examples include a synthesis of (2S,1′S,2′S,3′R)-2-(2′-phenyl-3′-carboxycycloprop-1′-yl)glycine, which is a potent glutamate receptor antagonist,[289] and a synthesis of all sixteen stereoisomers of 2-(2′-carboxy-3′-phenylcyclopropyl)glycine starting from the racemic aldehyde.[290] The first example of a [1,1,1]-propellane analogue (59), which incorporates the structural feature of linearity of the side-chain carboxy group with the chiral centre, has been reported.[291]



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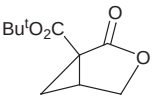

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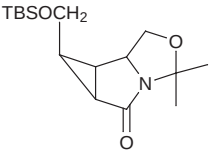

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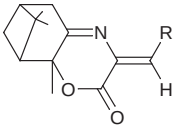

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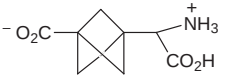
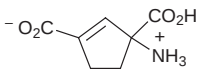


(59)

Tri-anions created from cyclopent-1-ene-1,3-dicarboxylic acids have been aminated with chloramine, to give the corresponding βγ-unsaturated-α-amino acids (60).[292] Four 1-amino-2-phenyl-1-cyclohexanecarboxylic acids have been prepared through asymmetric Diels-Alder addition of 1,3-butadiene to chiral (E)-2-cyanocinnamates, and their N-acryloyl derivatives used in Diels-Alder reactions with cyclopentadiene; poor diastereoselectivity was shown.[293] Diels-Alder reactions of the 'dehydroamino acid' derivatives [61; or the equivalent oxazolin-5(4H)-one][294] and a homochiral 2-substituted 4-methylideneoxazolidinone[295] lead to the 1-aminocyclohexene-1-carboxylic acid (62) and a bicyclo[2.2.2]octane analogue. An analogous bicyclo[2.2.1]heptane (63) has been prepared.[296]



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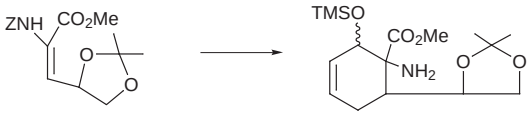
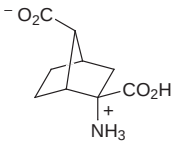


(61) (62)

This Section in all preceding Volumes has reflected the prominent role given in current research to aziridinecarboxylic acids, azetidinecarboxylic acids, and other analogues of proline and pipecolic acid, and their importance continues to be reflected in novel synthetic approaches. Aziridination of αβ-unsaturated esters by ethoxycarbonylnitrene, generated by α-elimination from 4-NO_2-$C_6H_4SO_2ONHCO_2Et$ using CaO, gives good yields;[297] and even higher yields of the same products have been claimed from the interaction of hexahydro-1,3,5-triazines and alkyldiazoacetates in the presence of tin(IV) chloride, though rather poor diastereoselectivity is achieved using N-[(S)-1-phenylethyl]triazines.[298]

General synthesis protocols have been illustrated leading to trans-2-carboxy-



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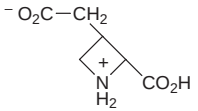
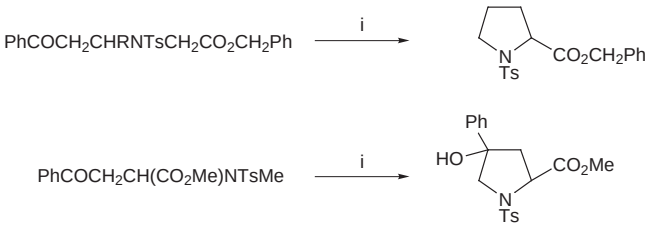


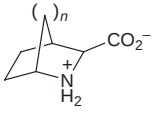
(64)

azetidine-3-acetic acid (64), its diastereoisomers, and homologues,[299] to 4-hydroxy-4-phenyl-L-proline and other hydroxyprolines (Scheme 22),[300] and to highly functionalized 2-methylthio-Δ^1-pyrroline-5-carboxylic acid esters, the latter being formed through diastereoselective 1,3-dipolar cycloaddition of samarium(III)-azomethine ylides $(MeS)_2NCR{=}CH(OSm)OEt$ to αβ-unsaturated esters (as in Scheme 6, Ref. 124).[301] Resin-bound αβ-unsaturated carbonyl compounds have been converted into substituted prolines through cycloaddition to methyl N-benzylideneglycinate, and released from the solid phase by trifluoroacetic acid after N-acylation.[302] The same outcome has been accomplished with a resin-bound azomethine ylide adding to maleimide, a process cryptically described as a 'three-component 1,3-dipolar cycloaddition'.[303] Trifluoromethyl azomethine ylides formed from N-trifluorothioacetyl pyrrolidine have been added to αβ-unsaturated esters and analogues to give bicyclic proline analogues with nitrogen at the ring junction;[304] isomeric bicyclic proline analogues may be prepared by Birch reduction of O-methyl-L-tyrosines, followed by acid-catalysed aminocyclization, giving cis-6-hydroxyoctahydroindole-2-carboxylic acid.[305] Yet another type of bicyclic proline analogue (65) is accessible through cycloaddition of cyclopentadiene or cyclohexadiene to the chiral iminium ion derived from (+)-1-phenylethylamine with freshly prepared benzyl glyoxylate $PhCH_2O_2CCHO$, followed by hydrogenation (H_2/Pd-C).[306]



Reagents: i, *hν*

Scheme 22



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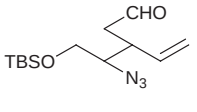


(66)

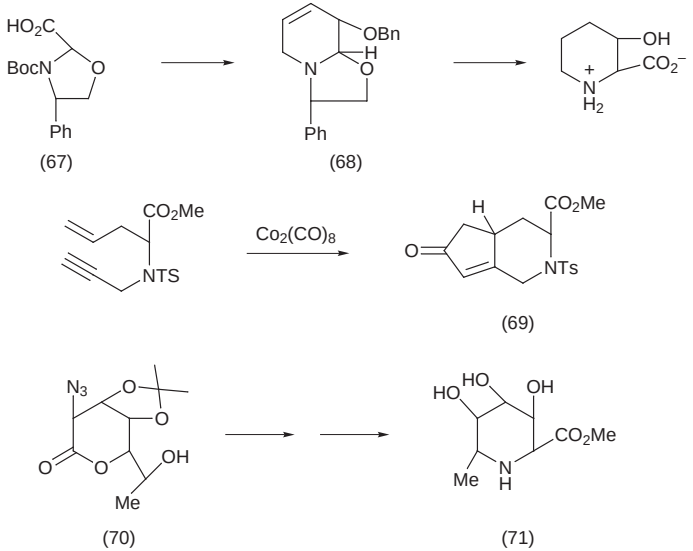
D-Mannitol has been developed into γ-azido-aldehydes (66; see also Ref. 255) and iminophosphoranes derived from them undergo Staudinger - aza-Wittig

cyclization under mild conditions to give 3-, 3,5- and 3,4,5-substituted D-prolines.[307]

7-Azabicycloheptane-based amino acids have been prepared by transannular alkylation of 5-bromoethylproline prepared from L-glutamic acid.[308]

3-Carboxypipecolic acids (*i.e.* piperidine-2,3-dicarboxylic acids) can be accessed by aza-annulation of enamino esters of a morpholinone of (S)-phenylglycinol (*cf* 34), using acryloyl chloride or a homologous alk-2-enoyl halide.[309] (2S,3S)-3-Hydroxy analogues have been obtained from the (R)-phenylglycinol-derived oxazolidine-2-carboxylate (67) *via* (68),[310] and racemic 3-methyl- and ethyl-pipecolate esters have been obtained from the triflate of commercially-available 3-hydroxypyridine-2-carboxylic acid through Pd-catalysed cross-coupling.[311] Bicyclic pipecolates, *e.g.* (69) have been prepared through an unusual intramolecular Pauson-Khand reaction (either solution or solid-phase modes) applied to intermediates prepared from either (S)-allylglycine or RS-propargylglycine.[312] The readily available[313] L-rhamnose-derived azidolactone (70) leads to trihydroxypipecolic acids, *e.g.* (71),[314] and has also been used to prepare spirohydantoins and spirodioxopiperazines joined at the anomeric position, through HOBr oxidation (Ref. 313). (2S,4R)- and (2R,4S)-4-Hydroxypipecolic acid lactones are formed (60:40) through iminium ion cyclization of the N-(3-butenyl)-(S)-α-methylbenzylamine adduct.[315] A short synthesis of β-amino-alkanols based on the pipecolic acid structure exploits the spontaneous cyclization, after deprotection, of the products of Sharpless asymmetric epoxidation of 7-N-phthalimidohept-2-enols.[316]

Cyclization of hydrazones of α-keto acid esters, or δ-hydrazinopentanoates, provides 6-substituted perhydropyridazine-3-carboxylic acid esters.[317]



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4.6 Models for Prebiotic Synthesis of Amino Acids – Some 40 years after the classic Miller-Urey experiment, which remains the inspiration for most of the ensuing research, this topic continues to generate new information, illustrated with the formation of amino acids in a $N_2/H_2O/CH_4$ mixture bombarded with high energy protons (a major constituent of cosmic radiation).[318] A review has appeared.[319]

A study using UV radiation (254 nm) acting on allyl alcohol in aqueous ammonium hydroxide illustrates the mild energy requirements established in recent years that lead to the generation of amino acids in organic - inorganic media. No fewer than ten aliphatic protein amino acids have been formed in this particular reaction mixture, together with 2-amino-2-methylpropanoic acid and β-alanine.[320] Formaldehyde and HCN are more plausible prebiotic building blocks and lead to amino acids and other organic compounds now known to be essential to life. Aspartic acid, alanine and valine, together with adenine and other heterocyclic compounds, are formed *via* diaminomaleonitrile in this system. A role for montmorillonite as a template for bringing these reactants together has been strongly advocated.[321]

The current interest in deep sea hydrothermal vents as the prebiotic, and presumably continuing, source of amino acids, has been reviewed.[322]

4.7 Synthesis of α-Alkoxy-α-Amino Acids, and Analogous α-Hetero-atom Substituted α-Amino Acids – Protected α-hydroxy- and α-alkoxy-α-amino acids are intermediates in syntheses of hetero-atom analogues, for example the preparation of N-Fmoc-α-(tert-butyloxycarbonylamino)-glycine from Fmoc carbamate, glyoxylic acid, and tert-butyl carbamate.[323] N-Alkyl analogues may be prepared through amination of the protected α-hydroxyglycine in the same way, or through amination (using BocNHR + NBS) of the α-alkylthioglycine $FmocNRCH(SPr^i)CO_2H$ prepared from the corresponding α-hydroxyglycine derivative.[324] H_2SO_4-Catalyzed O-alkylation of methyl N-Z-α-hydroxyglycinate has been achieved (see Refs. 882, 883), and remarkably, this aminal can be saponified to give an N-protected intermediate suitable for use in peptide synthesis.[325]

The condensation of oxalyl carbamate with triethyl phosphite gives the phosphorane $BzlO_2CN(Bzl)C(CO_2R)=P(OEt)_3$ from which α-diethylphosphonylglycine esters $H_2NCH[P(O)(OEt)_2]CO_2R$ are readily obtained through phosphorus functional group conversion (Me_3SiBr or HBr/AcOH) and hydrogenolysis (H_2-Pd/C).[326] α-Diethylphosphonylglycine derivatives are useful in synthesis (Refs. 95, 355, 370).

Section 4.4 carries some further examples of the preparation and use of α-alkoxy-α-amino acid derivatives.

4.8 Synthesis of α-(ω-Halogeno-alkyl)-α-Amino Acids – The year has been a prolific one in this field, producing a crop of reviews of the general field of fluorinated amino acids,[327] of fluorinated amino acid precursors of amine neurotransmitters,[328] and of γ-fluoro-α-amino acids.[329] (S,S)-δ-(Fluoromethyl)-ornithine has received attention in a review[330] that acknowledges the importance

of this compound as an ornithine aminotransferase inhibitor. Numerous papers in this Symposium Volume are of broader interest in amino acid synthesis, for example a detailed coverage of the use of asymmetric aldol reactions leading to enantiopure amino acids,[331] and routes to fluorinated cyclopropanes for use in the preparation of 'fluoromethano'-amino acids[332] (see also Section 4.5).

Fluorination of ethyl N-acetyl-DL-leucinate using CF_3OF causes γ-fluorination of the side-chain, as well as the expected formation of the N-fluoramide.[333] Indirect routes leading to (2S,3S)- and (2S,3R)-3-fluoroaspartic acids start with esters of D-tartaric acid, aminolysis of the derived epoxide followed by substitution with retention of the hydroxy group using Et_2NSF_3, to give the former target, and fluoride ring-opening of the cyclic sulfate of tartaric acid, followed by substitution with inversion of the triflated hydroxy group by azide providing the diastereoisomer.[334]

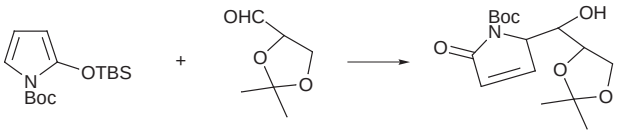
A synthesis of methyl (S)-hexafluorovalinate has been established, in which (R)-(+)-1-phenylethylamine undergoes anti-Michael addition to $(F_3C)_2C{=}CHCO_2Me$. This gives a 52:48-mixture of diastereoisomers from which the (S,R)-hydrochloride fortunately happens to crystallize.[335] Michael addition of the ethyl benzylideneglycinate anion to 3-chloro-4,4,4-trifluorocrotonate, and routine ensuing steps, has led to diethyl 3-(trifluoromethyl)-DL-glutamate.[336]

4,4-Difluoro-DL-glutamic acid has been prepared through aldol addition of ethyl nitroacetate to the ethyl hemiacetal of a difluorinated aldehyde,[337] and the procedure has also been used to give the 3,3-difluoro-analogue.[338] The activation of the side-chain function by the neighbouring fluorine atoms in the 4,4-difluoro-compound allows selective conversions to be implemented to give the glutamine and ornithine analogues.[339]

4.9 Synthesis of α-(ω-Hydroxyalkyl)-α-Amino Acids – Approaches to the synthesis of the archetypal members of this family, serine and threonine, and analogues, are mentioned elsewhere in this Chapter (Sections 4.2, 6.3).

A review has appeared of asymmetric syntheses of β-hydroxy-α-amino acids through aminolysis of chiral epoxy-acids, including the synthesis of the cyclosporin constituent MeBmt using methylamine in this reaction.[340]

The broad principles of aldolization of glycine and its analogues, to give β-hydroxy-α-amino acids, have become well entrenched in current methodology. These principles have been illustrated further in an exploration of routes to anti-α-alkyl-7-hydroxy-α-amino acids from chelated metal enolates formed from N-protected alanine, ethylglycine, valine, or phenylalanine by treatment with LDA and a metal salt.[341] The pyrrole (72) is a hidden form of glycine (though it is better viewed as a four-carbon synthon of wider applicability in organic synthesis), and its aldolization with the increasingly useful glyceraldehyde synthon (73; *cf* also 61) forms the basis for a synthesis of both enantiomers of trans-2,3-cis-3,4-dihydroxyproline.[342] A different route to these imino acids has already been described (Vol. 21, p. 29). Corresponding aldolization of Grignard reagents with the Garner aldehyde (a close relative of 73; see 121, and Section 6.3) leads to β-hydroxy-α-amino acids with only moderate asymmetric induction.[343]



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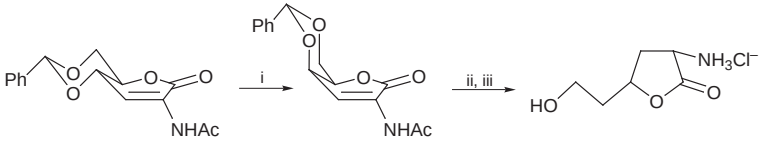
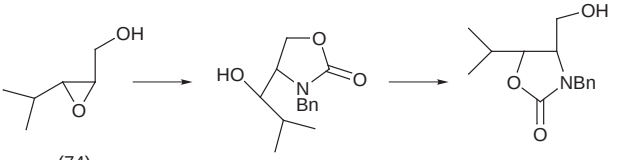
γ-Hydroxy-α-amino acids are formed through 1,3-dipolar cycloaddition of alkenes to nitrones $BnN^{+}(O^{-})=CHCO_2H$; this gives cis-isoxazolidines when the reactants are mixed at room temperature, but gives trans-isomers when triethylamine is also present.[344]

Routes to specific targets are often endowed with particularly interesting synthetic features, or may arise serendipitously from a quite different objective. The syntheses of (2S,4R,5R)-4,5,6-trihydroxynorleucine and of 5-hydroxynorvaline that have been described starting from D-glucosamine, involve precursors derived from an unusual rearrangement of a 5,6-dihydro-2-pyrone (Scheme 23).[345] The key steps in a valuable enantioselective route to βω-dihydroxyalkyl amino acids are Sharpless asymmetric dihydroxylation of αβ-unsaturated esters, 4-nitrobenzenesulfonylation of the more acidic α-hydroxy group, and azidolysis.[346] The same approach to allo-threonine and other β-hydroxy-α-amino acids, *via* azidolysis of the cyclic sulfate of the dihydroxylation product, has been shown to be viable on a large scale.[347] A new asymmetric route to all four isomers of 3-hydroxyleucine (needed for a synthesis of lactacystin) involves conversion of the appropriate homochiral epoxide (*e.g.* 74) into an oxazolidinone with BnNCO, and isomerization.[348]



Reagents: i, $SnCl_4$; ii, H_2/Pd–C; iii, 2M HCl, 100 °C, 12 h

Scheme 23



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4.10 Synthesis of N-Substituted α-Amino Acids – This has become more than a routine topic, with only the opines (Ref. 38) previously offering a high point of interest. N-Aminoalkylglycines carrying one or other of the four nucleobases

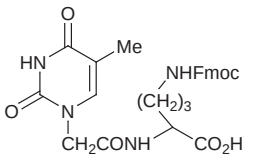
linked to the nitrogen atom have generated interest as precursors of peptide analogues of nucleic acids [known as 'peptide nucleic acids' (PNAs); the topic has been reviewed[349]].

Unremarkable chemistry is involved in the preparation of PNAs suitable for solid-phase synthesis [$H_2N(CH_2)_2NHCH_2CO_2H \rightarrow$4-methoxytrityl-$NH(CH_2)_2N(COCH_2X)CH_2CO_2H$; X = nucleobase].[350,351] The synthesis of homologues (carrying the nucleobase at the β-position of the N-ethyl group) has been reported,[352] and thymine-based PNAs prepared from L-lysine, serine, glutamic acid, aspartic acid, and isoleucine, $H_2N(CH_2)_2N(COCH_2T)CHRCO_2H$ (T = thymin-1-yl), have been described.[353]

N-Protected L- and D-ornithine carrying a thymine-bearing side-chain to provide PNAs of alternative structures (75) have been prepared and found to be suitable for routine peptide synthesis.[354]

4.11 Synthesis of α-Amino Acids Carrying Unsaturated Aliphatic Side-Chains – Citations of papers describing syntheses of amino acids in this category can also be found in the later Section 6.2 if another amino acid is used as the starting material. However, uses of glycine synthons are dealt with here, since they are fundamental to many classical amino acid syntheses.

Amino acids with olefinic side-chains continue to be prepared in all their familiar forms, though with new substitution patterns; αβ-unsaturated amino acids ('dehydro-amino acids') are represented by dienes (76), formed for use in an azinomycin synthesis from a dialkoxyphosphonylglycine through Horner-Emmons condensation with an aldehyde,[355] and 2-amino-3,3-difluoropropenoates, $CF_2=C(NRAr)CO_2Et$ (R = alkyl), prepared from the imino ester $CF_3C(=NAr)CO_2Et$ (Ar = p-MeO-C_6H_4) by treatment with organometallic reagents.[356] (E)-N-Acetyl-dehydrotryptophan ethyl ester and its Z-isomer have been prepared through condensation of indole with ethyl α-nitro-β-ethoxyacrylate $EtOCH=C(NO_2)CO_2Et$ and ensuing steps, as a 1:1-mixture that was separated by flash chromatography.[357] Configurational assignment rested on interpretation of ^{1}H-NMR spectra; the Z-isomer was established by this study to be formed by the incubation of N-acetyl-L-tryptophan ethyl ester with L-tryptophan 2′,3′-oxidase.



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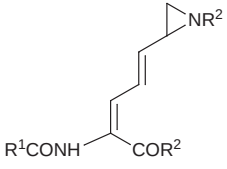

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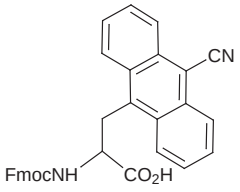
α-Chlorovinyl- and α-bromovinyl-α-amino acids have been prepared from N-trifluoroacetyl α-vinyl analogues through additions of benzeneselenyl chloride or bromide, respectively, and pyrolytic elimination of the derived sulfoxides.[358]

Further work on the ester enolate Claisen rearrangement route[359] has been published (see also Ref. 217, and Vol. 28, p. 16) extending its use to the preparation of polyhydroxylated γδ-unsaturated amino acids,[360] and to βγ- and γδ-unsaturated amino acids when starting with allyl esters of dehydroamino acids.[361] The route leads satisfactorily to α-alkyl-γδ-unsaturated amino acids starting from a variety of α-amino acids,[362] and to sterically-demanding targets (including demonstration of diastereo- and enantioselectivity when the reactant was used in the form of a metal chelate including quinine).[363] Allenic side-chains can be introduced through the equivalent route, involving the rearrangement of propargylic esters.[364] Alternative routes have been explored, including highly enantioselective allylation of oximes of α-ketoesters $BnON{=}CR^1CO_2R^2$, for the preparation of allylglycine and its homologues, including the α-methyl-α-amino acid.[365]

The synthesis of ethynylglycine, and of other βγ-alkynyl-α-amino acids, has been reviewed.[366] 'δ-Acetylenic amino acids' have been obtained through Pd-mediated Heck-type arylation and by Simmons-Smith homologation of terminal alkynes for use in alkylation of N-benzylideneglycine esters.[367]

4.12 Synthesis of α-Amino Acids with Aromatic or Heteroaromatic Groupings in Side-Chains – Common amino acids carrying functional groups in side-chains continue to serve as starting materials for the preparation of aryl and heteroaryl analogues, and current examples are covered later in Section 6.2. More fundamental approaches include enzymatic synthesis (tyrosine phenol-lyase) of fluorinated tyrosines from fluorophenols and ammonium pyruvate,[368] and laboratory syntheses of tyrosine analogues using diethyl formamidomalonate.[369] A new synthesis of 3,4-dimethoxyphenylalanine (a fluorescence quenching amino acid) and a synthesis of the fluorescent cyanophenanthrene analogue ('Flu'; 77) have been described, through alkylation of $Ph_2C{=}NCH_2CO_2Bu^t$.[370] Cobalt-mediated alkylation of (4R)- and (4S)-3-acetoacetyl-4-benzyloxazolidin-2-ones [30 → MeCOCHRCOX → AcNHCHRCOX *via* Schmidt rearrangement of the azidoamide], using alkyl halides that are known to react through radical intermediates, such as benzylic halides, has been used to prepare enantiopure diphenylmethyl, fluoren-9-yl, and adamant-1-yl glycines.[371] Corresponding use of the imidazolidinone chiral auxiliary is illustrated in a route to the biphenyl side-chain (78).[372]



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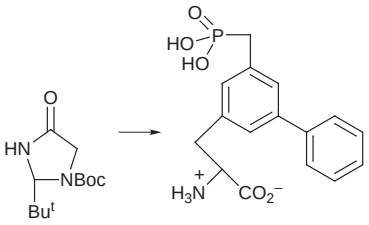
Increasing effort is being put in to the discovery of amino acids that affect NMDA receptors, and modification of the aromatic side-chains of common



(78)

amino acids has led to useful lead compounds. A modified di-iodotyrosine (79) provides a new example;[373] the phosphonate analogue of L-phenylalanine, $PhCH_2CH(NHCOPh)P(O)(OMe)_2$[374] and (2S,4S)-2-amino-4-(4,4-diphenylbutyl)-pentane-1,5-dioic acid[375] are among several other amino acids cited in this section that were prepared for their pharmacological potential. Many more analogues prepared from phenylalanine, tyrosine and tryptophan are listed in Section 6.3.

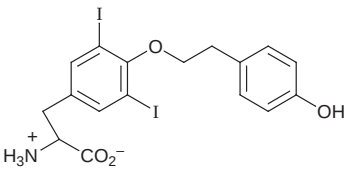
The synthesis of novel excitatory amino acids carrying isoxazolyl side-chains for use in related receptor studies has continued to develop using established methodology (see Vol. 28, p. 33), with 2-amino-4-(3-hydroxy-5-methylisoxazol-4-yl)butanoic acid emerging as a highly selective metabotropic compound at the $mGlu_6$ receptor.[376] The thymine derivative (80) is an 'alanine PNA' (see Section 4.10) prepared from the L-serine-derived β-lactone.[377]

Syntheses of other heteroaromatic relatives include (81) and (82) as powerful bidentate ligands for metal ions, *e.g.* for zinc(II),[378] and the thiatryptophan (83), prepared by Pd-mediated Heck cyclization of the benzoyloxime ether of ethyl (E)-2-oxo-5-bromo-3-pentenoate.[379] (S,S)-Pyridin-2,6-diyl bis(alanines) (84) have been prepared from 2,6-pyridine dicarbaldehyde through Horner-Emmons condensation with a protected dialkoxyphosphonylglycine followed by asymmetric hydrogenation $[H_2/Rh\{(COD)(R,R\text{-}DIPAMP)\}BF_4]$, after finding that double Heck coupling with Boc-amino acrylate esters was unsuccessful.[380] A synthesis of N^{in}-methyl-7-azatryptophan starts with the readily available amino acid.[381] cis- and trans-2,3-Methanotryptophans feature in an application of oxazolones in amino acid synthesis (Ref. 282).

Indoline 2,2-biscarboxylates (85) have been prepared from diethyl bromomalonate and o-bromomethyl-N-trifluoroacetylanilines.[382]

4.13 Synthesis of α-(N-Hydroxyamino) Acids – A number of papers discussed in other Sections deal with these compounds which are usually prepared from α-oximino esters (Refs. 379, 424).

4.14 Synthesis of α-Amino Acids Carrying Aminoalkyl Groups, and Related Nitrogen Functional Groups, in Side-chains – β-(Phenylamino)-phenylalanine has been prepared as a mixture of diastereoisomers through condensation of trans-2-oxo-1,5-diphenyl-4-imidazolidineamide (*cf* 30; NH in place of ring O, CO_2H at C-4) with N-benzylideneaniline, followed by hydrolysis.[383] In a similar way to a



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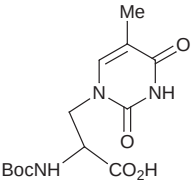

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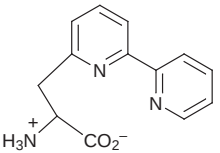

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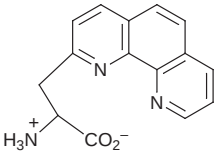

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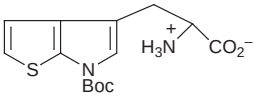

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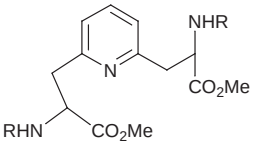

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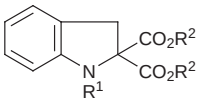

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standard synthesis of β-hydroxy-α-amino acids, amino analogues have been prepared from methyl isocyanoacetate by AuCl(cyclohexylisocyanide) catalysed addition to imines.[384]

α-Amino-β-lactams are formed as a mixture of isomers from triethylamine-induced condensation of tetrachlorophthalimidoacetyl chloride with an imine, but the trans-isomer predominates under microwave irradiation.[385] Another use for these synthons, for peptide synthesis, has been explored.[386]

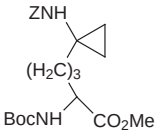
The L-lysine analogue (86) has been prepared from N-Boc-2,2-dimethyl-5-(3-hydroxypropyl)oxazolidine through a lengthy sequence of functional group manipulations from the hydroxy group, notable for a phosphonate-promoted cyclopropanation of the $Et_2O_3PCH(CO_2Et)CH_2CH_2CH_2$ side-chain.[387] An arginine analogue $R^1N{=}C(NH_2)NH(CH_2)_3CH(NH_2)P(O)(OH)R^2$ has been prepared in a search for novel nitric oxide synthase inhibitors.[388]

Further details have been published of a nitrogen analogue of S-adenosylmethionine (Vol. 28, p. 34) that has been prepared from L-glutamic acid and D-adenosine.[389]

4.15 Synthesis of α-Amino Acids Carrying Sulfur-, Selenium- or Tellurium-containing Side-chains – (2R,3R)-3-Mercaptoaspartic acid is readily obtained through sulfenylation of a protected β-aspartyl enolate dianion using a novel reagent, 4-methylphenyl 2,4-dimethoxybenzylthiosulfonate.[390]

A classical route to seleno- and telluro-methionines, based on ring-opening of DL-2-(acetylamino)butyrolactone with MeSeLi or MeTeLi, employs aminoacylase resolution to obtain enantiomerically-pure samples.[391]

4.16 Synthesis of α-Amino Acids Carrying Phosphorus Functional Groups in Side-chains – A synthesis that exemplifies recent interests in the evaluation of potential competitive NMDA antagonists has led to α-amino acids carrying alkanephosphonate side-chains modified with arene and heteroarene spacers (87, X, Y, Z = N or CH; and the benzofuran analogue).[392] The kynurenine analogues (88) carrying phosphinic acid or methyl phosphinate functional groups in place of the keto-group have been described.[393] A typical route to N-Boc- and N-Fmoc 4-[diethyl phosphono(difluoromethyl)]-L-phenylalanine using the β-iodoalanine organozinc synthon (see also Section 6.3, especially Refs. 923-925) employs Pd-mediated coupling to the appropriate iodoarene.[394]



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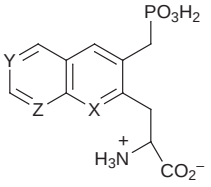

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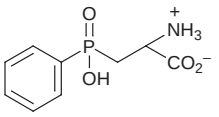


(88)

Silylation of the phenolic function of tyrosine and its analogues is recommended prior to diethyl phosphite - CBr_4 treatment leading to O-phosphotyrosines.[395]

The 4-(phosphonomethyl)pipecolic acid (89), formed through standard olefin - iminium ion cyclization protocol, is a potent and specific NMDA antagonist.[396]

4.17 Synthesis of α-Amino Acids Carrying Boron Functional Groups in Side-chains – Standard routes to DL-o-carboranylalanine and its tyrosine and phenylalanine analogues have been illustrated and developed.[397] The aliphatic compound was obtained by building the carboranyl function on to the N-benzylidene propargylglycine side-chain and the others were prepared by alkylation of the Schiff base with the appropriate benzyl bromide. Curtius rearrangement of 1,12-bis(hydroxycarbonyl)-p-carborane in tert-butanol gives the p-(N-Boc-amino) polyhedral-p-carborane,[398] and other standard routes are Schollkopf synthesis of (S)-5-[2-methyl-1,2-dicarba-closo-dodecaboran(12)-1-yl]-2-aminopentanoic acid[399] and Pd-mediated coupling of iodophenylboronic acid with the L-serine-derived β-lactone to give 4-borono-L-phenylalanine.[400]

An alternative aldolization route using ethyl isocyanoacetate with 4-[1,2-dicarbadodecaboran(12)-1-ylmethoxy]benzaldehyde has led to the corresponding 3-hydroxytyrosine analogue,[401] and p-boronophenylserine (90) has been prepared in the same way.[402]

4.18 Synthesis of Isotopically Labelled α-Amino Acids – The importance of these compounds is, as ever, to satisfy the need for materials either for metabolic and mechanistic studies in the laboratory and *in vivo*, or for clinical investigations. Their synthesis has been reviewed.[403]

Derivatives are listed in order of increasing relative atomic mass of the label. The well-established unpredictability of direct [^{1}H]–[^{2}H]-exchange resulting from drastic treatment of solutions of amino acids in ^2H_2O is further revealed in results for mixtures kept at 400°C and 390 bar pressure.[404] Rapid exchange of the α-proton occurs, but decarboxylation and failure to achieve exchange elsewhere in the molecule limits the value of this approach; a similar outcome, but with racemization and destruction of side-chain functional groups, accompanies attempted exchange in alkaline media under the same conditions.[405] More appropriate access to non-racemic α-[^{2}H]-labelled α-amino acids uses a chiral dioxopiperazine, and, as with deuteriation of 3,4-dehydro-L-proline and routine elaboration to give (2S,3S,4R)-[3,4-$^2H_2$]glutamic acid, also places the label with known stereochemistry.[406]

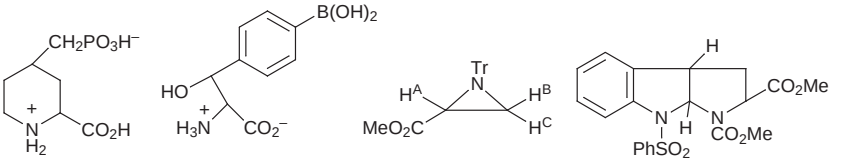
Enzyme-catalysed processes are also appropriate in some cases, and bacterial tryptophan synthase seems to offer a breakthrough for the preparation of α-[^{2}H]-labelled L-enantiomers of alanine, serine, methionine, phenylalanine, asparagine and glutamine, as well as tryptophan.[407] An alternative classical route involving exchange of the α-proton by acetylation and ^2H_2O quenching followed by acylase resolution has been used for preparations of 2S-[2-^{2}H]-tryptophan and -kynurenine.[408] Routes to 2S-O-phosphohomoserine and its [2-^{2}H]- and [3-^{2}H]-isotopomers announced earlier (see Vol. 28, p. 37) and starting from 2S-[2-^{2}H]aspartic acid, have been fully described.[409]

Detailed information is given for the preparation of stereospecifically-[^{2}H]-labelled aziridines (91) starting from corresponding (2S)-isoserines.[410] These products are useful in the synthesis of a variety of β-[^{2}H]-labelled α-amino acids with securely-known stereochemistry. Earlier reports (see Vol. 28, p. 37) are now backed up with full details of routes to (2S,4S)-5,5′-dihydroxy-[5,5-$^2H_2$]leucine and its (2S,4R)-epimer starting with either L-pyroglutamic acid or with 4-hydroxy-L-proline,[411] and the route to (2S,4R)-[5,5,5-$^2H_3$]leucine from L-pyroglutamic acid.[412]

An alternative preparation of chirally β-[^{2}H]-labelled α-amino acids using diacetone-D-glucos-3-ulose has led to (3R)- and (3S)-[3-^{2}H]-(2S)-serine (Scheme 24).[413] More routine syntheses include preparations of [$^2H_9$]- and [$^2H_6$]lysines from [$^2H_{10}$]cyclohexanone through polyphosphoric acid-induced rearrangement of the oxime.[414]

^{11}C-Labelled amino acids are of value in clinical tomography, and synthetic methodology needed to provide them may seem routine, because it is chosen on the basis of reliability, and, above all, a need for speed imposed by the short half-life of the label, and depends on the particular ^{11}C-labelled intermediates available. Nevertheless, these syntheses offer a challenge, to provide a fascinating exercise in economy of time and motion in the laboratory; synthesis of ^{11}C- and ^{18}F-labelled amino acids has been reviewed.[415] Simple acetylation to give α-N-(1-[^{11}C]acetyl)aminoisobutyric acid,[416] and esterification by [^{11}C]methyl iodide to

give N^{ω}-nitro-L-arginine [^{11}C]methyl ester (to be used for *in vivo* studies of this nitric oxide synthase inhibitor),[417] is in contrast to a more central role for the same reactant in classical amino acid synthesis in routes to [^{11}C]aminoisobutyric acid[418]and to α-[^{11}C]methyl-L-tryptophan.[419] The last-mentioned preparation from the well-established tryptophan synthon (92), involving the successive generation of its anion with LDA, deprotection (TFA and alkaline hydrolysis), and HPLC purification, occupied 30-35 minutes.



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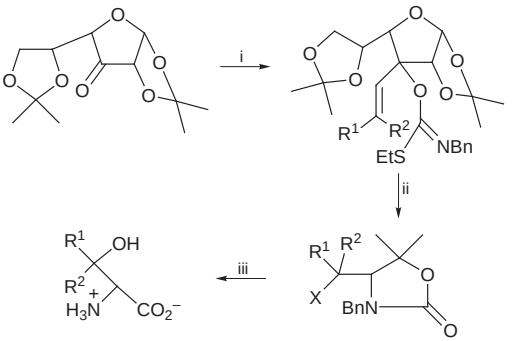


R^1 = H, R^2 = ^{2}H
and R^1 = ^{2}H, R^2 = H

Reagents: i, $R^1R^2C{=}CHMgBr$, then $EtSCCl{=}NBn$; ii, Br^+, H_3O^+, then X = Br → X = OH; iii, [O], H_3O^+

Scheme 24

Standard oxazolidinone methodology has been used for syntheses of 3-[^{14}C]-(2S,3R)-2-amino-3-(3,4-dihydroxyphenyl)-3-hydroxypropanoic acid ('L-DOPS'),[420] and of (3R)-[4-$^2H_3$]valine, L-[^{15}N]isoleucine and L-[^{15}N]alloisoleucine[421] (involving enzymic asymmetric amination of intermediate keto acids in the latter study). Oppolzer's camphorsultam auxiliary has been used for the preparation of L-[1,2-$^{13}C_2$]amino acids,[422] and when acylated by the $(MeS)_2C{=}^{15}N^{13}CHR^{13}CO$-grouping and subjected to familiar manipulations, has (for the first time, it is claimed) provided L-amino acids labelled with ^{2}H, ^{13}C, and ^{15}N.[423] Hydroxyamino acids have been prepared through Zn/H^+ reduction of the [^{15}N]oxime of the N-acylcamphorsultam.[424] Standard protocol for the

conversion of glutamic acid into lysine employing $Na^{13}CN$ has given the [6-^{13}C]isotopomer.[425]

The CNS imaging agent (S)-(2-[^{18}F]fluoro-4,5-dihydroxyphenyl)-2-methyl-L-alanine has been obtained from the corresponding acetophenone,[426] and 6-[^{18}F]fluoro-O-pivaloyl-L-DOPA has been prepared, using [^{18}F]acetyl hypofluorite, for similar metabolic studies;[427] in this preparation, 2,6-[^{18}F]difluoro-L-DOPA is a significant side-product.[428] The preparation of α-methyl-p-chloro-o-[^{18}F]fluorophenylalanine has been described in an earlier Section (Ref. 200).

Nucleophilic aromatic substitution of N-trifluoroacetyl 6-trifluoroacetoxymercurio-L-DOPA ethyl ester forms the basis of a preparation of 6-[^{125}I]iodo-L-DOPA.[429]

A synthesis of [^{75}Se]selenomethionine[430] has been claimed as a 'first', but the route was established many years ago (Vol. 25, p. 44).

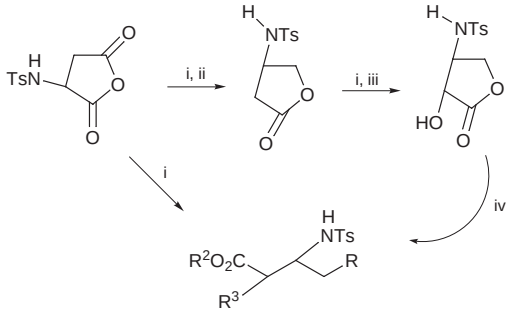
4.19 Synthesis of β-Amino Acids and Higher Homologous Amino Acids – Reviews of routes to β-amino acids and their α-substituted homologues,[431] and of stereoselective syntheses of β-amino acids,[432,433] have appeared.

Routes from α-amino acids have continued to be attractive, especially for the synthesis of β-amino acids carrying an α-chiral centre. A lengthy and rather routine approach from N-Fmoc- or N-Boc-L-α-amino acids *via* the α-amino aldehyde and the propargyl alcohol to give $XNHCHRCH(OH)C{\equiv}CH$ (further protected as the tetrahydropyranyl ether) followed by generation of the carboxy group has been explored.[434] Homologation of α-Z-amino aldehydes into α-benzeneselenyl-β-amino aldehydes provides opportunities for further homologation to give amino oxiranes and δ-amino acids,[435] and aldolization of α-Boc-amino aldehydes with 4-isopropyloxazol-5(4H)-one (a nucleophilic aldehyde equivalent) gives β-amino-α-hydroxyalkanoic acids.[436] Diethyl 2-amino-1-hydroxy-3-phenylpropylphosphonate has been obtained by the addition of diethyl phosphite to an N-protected L-phenylalaninal.[437]

Diazomethyl esters of α-amino acids have long been used (Wolff rearrangement) for homologation, and have been newly studied for conversion into β-amino acid esters through rearrangement using silver benzoate in the presence of triethylamine and a nucleophile (*e.g.* $HOCH_2CH_2CMe_2OH$).[438]

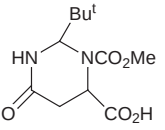
L-Aspartic acid is a special case in this context, being both an α- and β-amino acid; elaboration of its α-carboxy group (see also Section 6.3) into a particular α-substituent is illustrated for a range of syntheses of (R)-3-aminobutanoic acids (Scheme 25), including the preparation of 2-hydroxy-analogues through electrophilic hydroxylation.[439] A route to (R)-3-amino-4-phenylbutyric acid involves a more straightforward use of aspartic acid, as the carbonyl component in Friedel-Crafts acylation of benzene.[440] D-Asparagine, converted into the nitrile $Bn_2NCH(CH_2OH)CHRCN$, leads to αβ-disubstituted β-amino acids.[441] The preparation from L-asparagine and pivaldehyde of the general purpose β-amino acid synthon (93) has been carefully worked out.[442] Alkylation of (93; H in place of CO_2H, PhCO in place of CO_2Me) gives β-substituted β-alanines.[443]

Among acyclic natural α-amino acids, the pre-eminent synthetic challenge remains ADDA, and a new synthesis combines (S,S,E)-$PhCH_2CH(OMe)CH$-



Reagents: i, $NaBH_4$; ii, LDA, TMSCl; iii, NaHDMS, $PhSO_2N$(O)$CHPh$; iv, TMSI, then R_2CuLi

Scheme 25



(93)

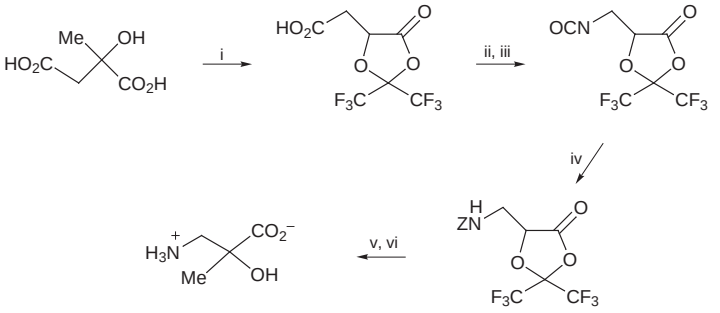
MeCH=CMeI, prepared from (S)-lactic acid, and (S,S,E)-$Me_3SnCH{=}CHCH(NHBoc)CHMeCO_2Me$ prepared from D-aspartic acid, in a $[Cl_2PdMeCN)_2]$-catalysed cross-coupling reaction.[444] An equivalent route from $PhCH_2CH(OMe)CHMeCH{=}CMeCH_2P^+Ph_3\ Br^-$ and $OHCCH(NHBoc)CHMeCO_2Me$ has been reported.[445] A quite different approach[446] starts from D-glucose and places the β-amino acid moiety into the molecule at the last stage through development of the carboxy group from the acetal of a 4-aminopyran.

Routes to β-amino acids from other α-amino acids are covered in the later Section 6.3.

A variety of other β-alanine synthons is being developed, *e.g.* the isocyanate prepared from (S)-(+)-citramalic acid, as used in the preparation of (S)-α-methylisoserine derivatives (Scheme 26).[447] More distant relatives of β-alanine such as the protected syn-2R-amino-1,3,4-triol (94) obtained from D-iso-ascorbic acid,[448] and the anti-α-aminoalkyl epoxides (95)[449] are being evaluated; the latter synthons are particularly useful as cationic β-aminoalkanol equivalents, and have been prepared independently[450] from 5-chloromethyl-4-methoxyoxazolidin-2-one. β-Aminonitriles, *e.g.* that formed from the β-azidonitrile derived from glucosamine,[451] are also latent β-amino acids.

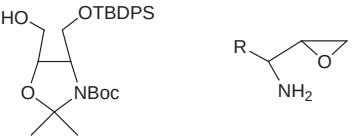
The construction of more complex structures, achieved by starting with a β-amino acid, is covered mostly in the later Section 6.3.

The other synthesis approaches to β-amino acids can be grouped together on the basis of (i) β-amination of a carbonyl compound -C-C-COR ('N + C_3



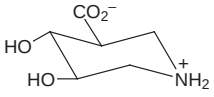
Reagents: i, $(CF_3)_2CO$, DMSO; ii, $SOCl_2$; iii, $TMSN_3$/toluene/80 °C; iv,BnOH, $CHCl_3$; v, H_2O, Pr^iOH; vi, H_2/Pd–C

Scheme 26



(94) (95)

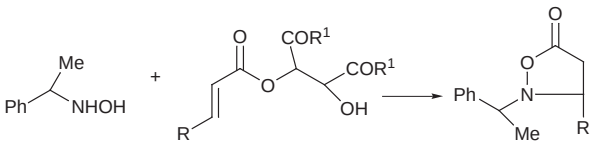
condensation'; see also Refs.80, 92), (ii) β-carboxylation of an ethylamine (-N-C-C- + 'CO_2'), or (iii) condensation of N-C + C-C units. The simplest approach to amination is represented by azidolysis of anhydrides of β-chloro-acids, and routine development to give the amino group.[452] Greater complexity is seen in the development from the primary azide formed from D-arabinose, through numerous stages to the new glucuronic acid-based iminosugar (96), a pipecolic acid isomer that is a potent β-glucuronidase inhibitor.[453] Conversion of an acid chloride of thiomalic acid into an isocyanate and routine conversion into carbamates yields DL-isocysteine.[454] (S)-2-(Aminomethyl)butanedioic acid has been prepared through stereoselective alkylation of the sodium enolate of an Evans acyloxazolidinone (30; R = vinyl or 3,4-dimethoxyphenyl group) with methyl bromoacetate, followed by oxidation of R into carboxyl, then Curtius rearrangement.[455]



(96)

Diastereoselective conjugate addition of lithium (α-methylbenzyl)benzylamide to αβ-unsaturated esters has been illustrated further, for syntheses of the δ-hydroxy-β-lysine constituent of negamycin[456] and of (2S,3S)- and (2R,3S)-2,3-

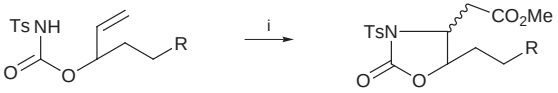
diaminobutanoic acids.[457] Other amination processes are represented in hydroxyamination of an αβ-unsaturated ester (Scheme 27),[458] and asymmetric aminohydroxylation of alkenes, *e.g.* ethyl trans-crotonate with chloramine-T/$K_2OsO_2(OH)_4$/dihydroquinine/phthalazine gives ethyl (2R,3S)-N-(toluene-p-sulfonyl)-3-amino-2-hydroxybutanoate in 74% e.e.[459] The viability of this route has been realized through recently-disclosed mild de-sulfonylation conditions. Biomimetic Miller cyclization of a β-hydroxy acid O-methyl hydroxamate into an N-methoxy-β-lactam,[460] biomimetic base-catalysed transamination of fluorinated β-keto-esters with benzylamine to give β-fluoroalkyl-β-amino acids,[461] and a related approach to give syn-β-amino-α-hydroxyacids starting from βγ-unsaturated α-ketoesters[462] have been reported.



Scheme 27

β-Enamino esters can be reduced to α-amino esters using sodium triacetoxyboron hydride in acetic acid, with good diastereoselectivity and enantioselectivity if the N-substituent is a homochiral grouping.[463] Reduction of homochiral α-hydroxy-βγ-unsaturated nitriles (formed through R-oxynitrilase-catalysed addition of HCN to αβ-unsaturated aldehydes) gives α-hydroxy-β-amino acids, as illustrated in a synthesis of isoserines.[464] Direct hydroxylation of enolates of N-substituted 3-amino-3-phenylpropionate, to give the taxol component (2R,3S)-3-phenylisoserine, can be accomplished using the oxodiperoxymolybdenum-pyridine-HMPA complex,[465] and the same target has been attained both through the equivalent aminolysis of epoxides formed from cinnamates using the cobalt(III)-DMG - O_2 reagent,[466] and through the longer route from homochiral trans-epoxy ester to anti-bromohydrin (using $MgBr_2$ etherate) followed by azidolysis.[467] L-Isoserinal has been prepared from the N-benzylimine of D-glyceraldehyde acetonide (73).[468] 1-Arylthio-1-nitroalkenes formed from α-Boc-amino aldehydes and (4-tolylthio)nitromethane are converted into 5-carboxy-oxazolidin-2-ones (instead of the expected epoxides), using lithium tert-butyl peroxide.[469] These are protected forms of anti-α-hydroxy-β-amino acids; the diastereoisomeric pair derived from 3-amino-2-hydroxyvaleric acid has been separated into threo and erythro forms,[470] and their relative stereochemistry assigned through interpretation of ^{1}H-NMR spectra. The threo-isomer was used for the determination of absolute configuration of the 3-amino-2-oxovaleric acid constituent of poststatin [established to be (S)], through resolution and assignment to it of (2R,3S)-configuration, followed by oxidation and comparison with the natural material.

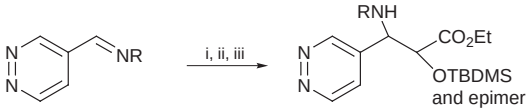
Wacker aminocarbonylation (synchronous carboxylation and intramolecular amination of an alkene) has been applied for a synthesis of cyclic β-amino acids (Scheme 28).[471]



Reagents: i, $PdCl_2$, $CuCl_2$, CO, methyl orthoacetate

Scheme 28

Imines can be condensed with silyl enolates, a process illustrated in a route to isoserines that also emphasizes the need for a separation step when particular stereoisomers are required (Scheme 29),[472] and this well-established general route has been reduced to its simplest form, the condensation of an aldehyde, a chiral amine, and silyl enolate using $Yb(OTf)_3$ as catalyst.[473] Good diastereoselectivity accompanies the use of methyl L-valinate in this route, which is equivalent to the $Yb(OTf)_3$-catalysed addition of N-(α-aminoalkyl)benzotriazoles to silyl enolates.[474] Simple enol ethers $F_2C{=}CROMe$ react with N-acyliminium ions $R^1CH{=}N^+HCO_2Et$ [formed from $R^1CH(NHCO_2Et)_2$ with Tf_2O] to give β-amino-α,α-difluoroketones,[475] and a similar use of an imine is described in Ref. 153. Bromoacetates of chiral alcohols condense easily with imines under the influence of activated zinc, with high diastereoselectivity in certain cases.[476] Achiral trimethylsilylimines ArCH=NTMS add chiral boron enolates derived from the boron reagent Men_2BBr [Men = menth-2-yl from (+)-menthone] and an α-halothioacetate XCH_2COSBu^t to give α-halo-β-amino thioesters and used for a synthesis of hydroxymethylaziridines.[477] β-Amino thioesters are obtainable also from polymer-supported thioketene silyl acetals and imines through scandium triflate catalysis.[478] Nucleophilic ring-opening of N-tosyl aziridines gives a mixture of β- and γ-amino acids.[479]



Reagents; i, TBDMSO—CH═C(OEt)OTBDMS; ii, $ZnCl_2$; iii, crystallization

Scheme 29

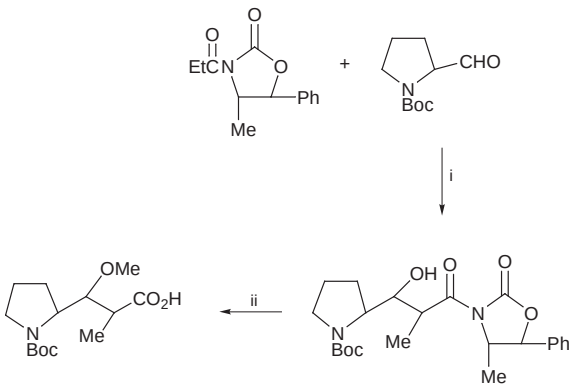
Standard β-lactam synthesis using $(4\text{-}MeOC_6H_4)N{=}CHCF_3$ and the ketene $PhCH_2OCH{=}C{=}O$ leads on to a preparation of methyl syn-3-trifluoromethylisoserinate.[480]

3-Aminoalkanols are prime synthesis targets for uses outside the amino acid field, but routes to them can also serve for β-amino acid synthesis. An example is the synthesis of 1-TBDMS ethers of protected syn- and anti-3-aminoalkane-1,2-diols, selective deprotection and oxidation of the anti-isomer giving (2R,3S)-3-phenylisoserine.[481] Pig liver esterase de-symmetrization of $ZNHCH(CO_2Me)_2$

and tert-butyl esterification of the resulting carboxy group gives (R)-β-lysine after elaboration of the CO_2Me group ($CO_2Me \rightarrow CH_2OH \rightarrow CH_2CN \rightarrow CH_2CH_2NH_2$).[482] A similar chain extension has been exemplified for the standard Oppolzer camphorsultam protocol [$R^*O_2CCH(OAc)CN \rightarrow R^*O_2CC$-$Me(OH)CH_2NH_2$; R* = (1S,2R,4R)-1-dicyclohexylsulfamoylisobornyl residue] for a synthesis of (2R)-α-methylisoserine.[483] A route to 3-aminoalkanols through Michael addition of nitrones $R^1CH{=}NR^2{=}O$ to allyl sulfones $CF_3CH{=}CHCH_2SO_2Ph$ proceeds *via* 5-trifluoromethyl isoxazolidines.[484]

A synthesis of (R)-carnitine from $ClCH_2CO_2Et$ (Grignard addition, reduction, amination) employs dibenzoyl (+)-tartaric acid for resolution,[485] and amine analogues have been prepared through azidolysis of (R)-carnitine β-lactam.[486]

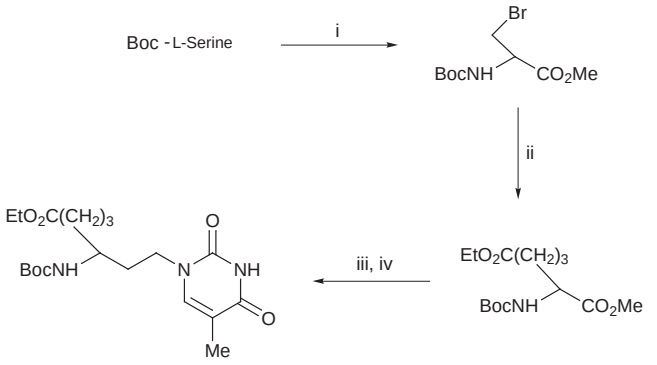
As with other homologues, routes to homochiral γ-amino acids that start from D- or L-α-amino acids should be among the first to be considered. An effective route to dolaproline starting from (S)-prolinal (Scheme 30) illustrates a standard approach, involving Bu_2BOTf-catalysed condensation with a homochiral oxazo-



Reagents: i, Bu_2BOTf, NEt_3; ii, routine steps

Scheme 30

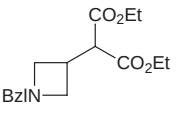
lidinone (*cf* 30)[487] and a protected L-isoleucinal has been condensed similarly with N-[(methylthio)acetyl]oxazolidinone to start a route to dolaisoleucine (3R,4S,5S)-$EtCHMeCH(NHR)CH(OMe)CH_2CO_2H$.[488] Chiral β-amino alcohols condense with lactams to give 2-(ω-aminoalkyl)oxazolines that may be α-alkylated to give enantiomerically-pure ω-amino acids,[489] although another approach[490] employs (R)-phenylglycinol to generate 2-(β-cyanoalkyl)oxazolines *via* 2-alkenyl analogues, and then $LiAlH_4$ reduction and hydrolysis (the oxazoline C-2 becomes the carboxy group) leading to (S)-4-amino-3-methylbutanoic acid (β-methyl-GABA).[491] Other examples of similar uses of α-amino acid-derived synthons are dealt with in Section 6.3. Arndt-Eistert homologation of β-amino acids prepared from Boc-L-serine (Scheme 31) has been used to prepare new PNAs (see Section 4.10).[492]



Reagents: i, CBr_4, PPh_3; ii, Vitamin B_{12a}/ethyl acrylate; iii, $CO_2Me \longrightarrow COCHN_2 \longrightarrow (CH_2)_2Br$; iv, thymine

Scheme 31

γ-Amino acids have been synthesized through other straightforward approaches, such as a route from epichlorhydrin, to azetidin-3-ylacetic acid *via* the malonate (97),[493] and to the NMDA receptor antagonist milnacipran *via* the lactone (98) formed with phenylacetonitrile using $LiNEt_2$ in THF followed by azidolysis and hydrogenation ($CH_2OH \rightarrow CH_2NH_2$; similarly in Ref. 452).[494] Electrochemical reduction of oximes of 2-hydroxy-4-oxoalkanoic acids gives the corresponding γ-amino acids.[495] A new synthesis of the CNS-active drug vigabatrin (γ-vinylGABA) employs phthalimide for asymmetric amination of butadiene mono-epoxide based on differentiation within a π-allylpalladium complex, and homologation by malonate of the resulting 2-phthalimidobut-3-en-1-ol.[496] An efficient GABOB synthesis from D-mannitol arises from subjecting the 3-aminopropan-1,2-diol synthon (99) to ring-opening by cyanide.[497] Another chiral auxiliary approach involves the Evans N-acyloxazolidinone (30; see also Ref. 192),



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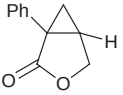

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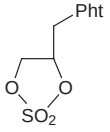
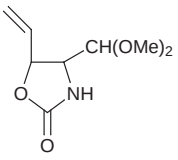


(99)

cyanomethylation giving 2-substituted 4-aminobutanoic acids through the conversion $R \rightarrow NH_2$.[498] An approach to γ-amino-β-ketophosphonic acids is described in Ref. 843. The D-glucosamine-derived starting material (100) has provided a lengthy entry to calyculin constituents, *viz*. (2S,3S)-dihydroxy-(4S)-amino acids,[499] and to other threo-β-hydroxy-γ-amino acids such as (3S,4S)-statine and (3S,4S)-AHPPA.[500] This approach bears comparison with the route from anti-3-aminoalkane-1,2-diols to syn- or anti-β-hydroxy-γ-amino acids, with asymmetric

epoxidation of allylic alcohols as a key step, and Ti-promoted oxirane-opening, as illustrated with a cyclohexylstatine synthesis,[501] and with further statine syntheses by the same research group using essentially the same approach (nucleophilic opening of a chiral oxirane). Thus, cyclohexylnorstatine was obtained from either (E)-4-cyclohexylbut-2-en-1-ol,[502] or (S)-2-methylbutan-1-ol *via* the oxazolidine (101).[503] An equivalent use of the homochiral N-Boc-aziridine (102) has been illustrated for the synthesis of statines and analogues,[504] also formed by alkylation of imines formed from (R) or (S)-1-phenylethylamine with (S)-$HOCH_2$-CH(OBn)CHO.[505] Further results from azaenolate α-alkylation or nucleophilic addition to a homochiral hydrazone (Vol. 28, p. 44) include a statine synthesis.[506] Hydrogenation of chiral tetramic acids formed by dicyclohexylcarbodi-imide-induced condensation of N-protected α-amino acids with Meldrum's acid gives homochiral cyclic statines.[507] The products formed from the rhodium carbenoid formed from the decomposition of the glycine-derived diazo-acetoacetamide $MeO_2CCH_2N(Ac)COC(N_2)CO_2Me$ include N-acetyl isopyroglutamates.[508]



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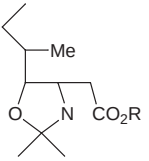

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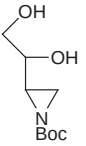
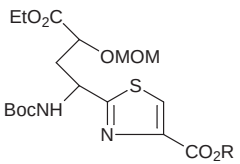


(102)

Cycloaddition of chiral enol ethers to dichloroketene, then Beckmann ring expansion to give a γ-lactam, offers an alternative approach to β-hydroxy-γ-amino acids, and has been developed into a (-)-statine synthesis.[509] A synthesis of the α-hydroxy-γ-amino acid fragment (103) of nosiheptide starts from L-pyroglutaminol.[510] Chiral αβ-acetylenic γ-amino acids and GABA analogues have been prepared either through pyrolysis of chiral aminoacyl phosphorus ylides $R^1O_2CNR^3CHR^2COC(=PPh_3)CO_2Et$,[511] or from (S)-N-Boc-amino aldehydes through the Corey-Fuchs protocol.[512] Palladium(0)-catalysed rearrangement of allylic sulfoximines leads to αβ-alkenyl γ-amino acids (Scheme 32), though the route has been established only for racemic substrates;[513] a Ru-catalysed metathesis process (Scheme 33) is a spectacular route to these compounds.[514] The starting point (104) for the synthesis of carbanucleoside analogues has been prepared by asymmetric Pd-mediated desymmetrization of meso-cyclopent-2-ene-1,4-diols and functional group incorporation into the cis-isomer.[515]



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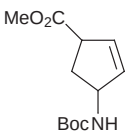

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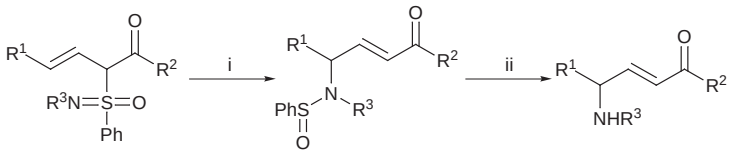


Reagents: i, $Pd(PPh_3)_4$, THF; ii, MeOH with trace of NEt_3

Scheme 32

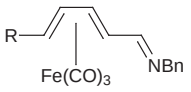
($FmCH_2$ = ferrocenylmethyl)

Reagents: i, $Ph_2C{=}CHCH{=}RuCl_2(PCy)_3$, benzene; ii, TFA, CH_2Cl_2; iii, $Et_3O^+BF_4^-$; iv, 0.4M HCl

Scheme 33

γ-Lactams are featured in a study of penicillin and cephalosporin analogues,[516] and have been built in to analogues of conformationally-constrained peptides.[517]

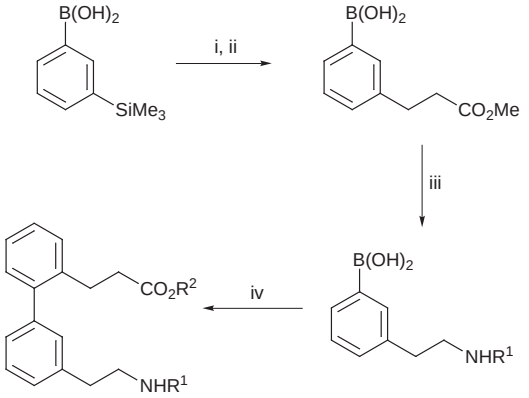
In the δ-amino acids context too, α-amino acids are valuable starting materials for the synthesis of their higher homologues, as illustrated in the cycloaddition to methyl acrylate of nitrile oxides formed *in situ* from N-protected α-amino aldehyde oximes, followed by acidolysis of the resulting isoxazoline to give δ-amino-αβ-unsaturated-γ-oxoesters $BocNHCHRCOCH{=}CHCO_2Me$.[518] (S)-5-Benzyl-5-amino-Z-pent-3-enoic acid is available from L-phenylalaninal by routine Horner-Emmons condensation[519] and by other homologation approaches (Ref. 435), including allylmagnesium bromide homologation of the Weinreb amide of L-phenylalanine, and then routine steps.[520] 1-Iminobutadiene-$Fe(CO)_3$ complexes (105) are Schiff bases, and may be alkylated at the imine carbon atom in the way that is so useful in α-amino acid synthesis; the resulting N-(butadienyl)amines have been elaborated into δ-amino acids.[521]



(105)

Chain extension of propargylamine with ethylene oxide, followed by oxidation, gives 5-aminopent-3-ynoic acid,[522] and hetero Diels-Alder reaction between 1,3-bis(tert-butyldimethylsilyloxy)-2-azabuta-1,3-diene and [60]fullerene gives the expected δ-valerolactam [*i.e.* a [60]fulleropiperidone].[523]

Miscellaneous examples of syntheses of ω-amino acids include amination by acylnitrene insertion leading to 1-amino-2-hydroxybicyclo[2.2.1]heptane-7-carboxylic acids.[524] Synthesis of biphenyl-based amino acids (Scheme 34) is a notable example of current practice in manipulations of benzene ring functional groups.[525]



Reagents: i, —$SiMe_3$ ⟶ —I ⟶ —CH=$CHCO_2Me$ by standard protocols; ii, H_2/Pd; iii, Curtius rearrangement (phosphoroazidate method); iv; Et *o*-bromocinnamate

Scheme 34

4.20 Resolution of DL-Amino Acids – Classical resolution procedures applied to DL-amino acids fall into the categories (i) preferential crystallization from racemates, and crystallization from a mixture of diastereoisomeric salts formed either with a homochiral acid or with a homochiral base; (ii) separation of a diastereoisomer mixture formed through derivatization with a homochiral reagent; (iii) chromatographic resolution and related processes; (iv) enantioselective hydrolysis, or a similar process, catalysed by enzymes or by other homochiral species. Categories (iii) and (iv) continue to be emphasized in the non-routine literature.

The solid form of (RS)-2-amino-3-chloropropanoic acid is a conglomerate, whereas its hydrochloride crystallizes as a racemic compound and is therefore resolvable through the preferential crystallization technique.[526] Separation by fractional crystallization, of salts formed with L-tyrosine hydrazide, has proved to be satisfactory for the resolution of DL-o- and p-fluorophenylalanines as their N-benzoyl derivatives,[527] and DL-pipecolic acid has been resolved through fractional crystallization of chiral Pd complexes.[528] (-)-Cinchonidine and (-)-quinine have been used for the resolution of DL-α-(hydroxymethyl)-phenylglycine and DL-α-(hydroxymethyl)-phenylalanine, respectively (Ref. 108). Diastereoisomeric derivatives formed between DL-α-amino acids and a salicylaldehyde carrying an optically-active 3-substituent are easily separated by conventional chromatography of their copper(II), zinc(II), or nickel(II) chelates.[529] Total

conversion of DL-phenylglycine into the (R)-enantiomer can be accomplished through conversion into a ketene and diastereoselective addition to (R)-pantolactone followed by transesterification and hydrazinolysis, though side-reactions will undermine the process with many common α-amino acids carrying functional groups in side-chains, and N-phthaloyl protection is necessary.[530] Chiral trans-dioxoruthenium(VI) porphyrins effect oxidation of DL-amino acid esters; the resulting achiral imines enter the complex and are accompanied by one enantiomer of the initial substrate.[531]

Dynamic resolution continues to attract interest (Vol. 28, p. 13; see also Ref. 201) with further examples based on N-(2-bromopropionyl)-(4R,5S)-1,5-dimethyl-4-phenylimidazolidin-2-one (total equilibration within the N-substituent by Bu_4NBr in favour of the R-configuration),[532] and epimerization of (S)-2-(2-Z-aminoisovaleroyl)-4-benzyloxazol-5(4H)-one.[533]

Proteolytic enzyme-catalysed hydrolysis continues to be widely used, discrimination between enantiomers being shown by subtilisin with phenylglycine ethyl ester;[534] α-chymotrypsin with β-(pyrid-2-yl)alanine[535] and (p-borono)phenylalanine (Ref. 162), with esters of azatyrosine [*i.e.* β-(3-hydroxypyrid-5-yl)alanine],[536] and with ethyl trans-3-(trifluoromethyl)pyroglutamate.[537] Anhydrous organic solvents can supply a suitable medium for protease-catalysed transesterification of N-trifluoroacetyl-DL-phenylalanine 2,2,2-trifluoroethyl ester with propan-1-ol,[538] and for papain-catalysed enantioselective hydrolysis of protected DL-4-carboxyglutamic acid esters[539] or conversion of the free acid into dipeptides.[540] Corresponding media used for CLEC-subtilisin with a range of amino acids and racemic alkylamines give protected L-aminoacyl-(S)-alkylamides.[541]

Aminoacylases can achieve the same outcome through enantioselective deacylation, illustrated for stereoisomer mixtures of 4-fluoroglutamic acid after separation of the erythro- and threo-DL-diastereoisomers,[542] and for amides of N-phenylacetyl anti-α-alkyl-β-amino acids using immobilized penicillin G acylase[543] (see also Ref. 408). Enantioselective pent-4-enoylation of racemic amino-alkanols catalysed by a serine acylase also accomplishes the same outcome.[544] A review has appeared covering results relevant to this area, covering also the use of a new N-acylamino acid racemase.[545] Another review covers exploitation for amino acid resolution of the amidase and aminopeptidase activity of *Pseudomonas patida*, *Mycobacterium neoaurum*, and *Ochrobactum anthropi*.[546] An aminoacylase together with a serine protease is advocated for the formation of L-ω-esters from α-N-acetylaminoalkanedioic acid diesters.[547] The promising results established for the exploitation of hydantoinases in this context are further illustrated with immobilized recombinant *E.coli*,[548] *Pseudomonas desmolyticum*,[549] and *Bacillus stearothermophilus* SD-1,[550] as used for the enantioselective hydrolysis of DL-hydantoins to give N-carbamyl-D-(p-hydroxyphenyl)glycine.

Lipases are also emerging as strong contenders for the resolution of amino acids in the form of their alkyl esters,[551] and are effective in organic solvents with alicyclic β-amino acids.[552] Transesterification of (1RS,2SR)-(2-TBDMS-oxymethyl)cyclopentanol with vinyl acetate benefits from lipase catalysis is followed by amination for a synthesis of both enantiomers of cis-pentacin [(1R,2S)- and (1S,2R)-2-aminocyclopentane-1-carboxylic acids]; the enzyme-catalysed kinetic

resolution approach is featured in this route.[553] Porcine pancreatic lipase has been found to be the most satisfactory lipase for the resolution of ethyl DL-2-amino-4-phenylbutyrate.[554]

An attractive practical proposition is offered by lipase- or α-chymotrypsin-catalysed hydrolysis of Schiff bases of DL-amino acids in aqueous MeCN (5:95).[555] The L-amino acid precipitates out, and more than half the initial amount of L-enantiomer can be obtained, as expected on the basis of asymmetric transformation.

β-Cyclodextrin-catalysed hydrolysis of DL-tryptophan isopropyl ester involves very restricted enantioselectivity, in leading to a predominance of the L-enantiomer.[556]

A widening variety of resolution techniques is being developed, based on physical interactions resulting in discrimination between enantiomers when they encounter chiral heterogeneous media. The classical test compound for such techniques applied to underivatized amino acids is DL-tryptophan; the L-enantiomer shows preferential transport through a (+)-poly[1-{dimethyl(10-pinanyl)silyl}prop-1-yne] membrane[557] and through an analogous homochiral supramolecular polymer membrane based on a poly(N-isopropylacrylamide) backbone.[558] Membranes formed from a cellulose acetate membrane carrying monoterpenes,[559] and membranes made from an acrylonitrile - styrene copolymer carrying a covalently-bonded all-L-tetrapeptide, have been studied; in the former case, dialysis and ultrafiltration rates are enhanced for the L-enantiomer for DL-tryptophan solutions, and in the latter case, the electrodialysis rate of N-acetyl-DL-tryptophan is enhanced for the L-enantiomer.[560] Porous poly(ethene) membranes have been rendered homochiral through grafting an epoxyalkylvinyl monomer, followed by reaction with an L-amino acid.[561] Following a long tradition exploiting natural homochiral materials as chromatographic media, the glycopeptide antibiotic teicoplanin has been used for the resolution of non-derivatized amino acids,[562] and polysaccharides and other CSPs are effective for the resolution of tryptophan derivatives.[563]

Molecule-imprinted polymers have continued to show promise for enantio-selection (and therefore, resolution of racemates), when the imprinting species is a homochiral amino acid derivative; the topic has been reviewed.[564] However, their use for the construction of membranes and chromatographic media still lacks a clear rationale that links the structure of the imprint with the structure undergoing chiral recognition. Thus, polymers prepared with monomers carrying the tetrapeptide H-Asp(O-cyclohexyl)-Ile-Asp(O-cyclohexyl)-Glu(O-Bzl)-OCH_2-grouping with Boc-L-tryptophan present from the start of the polymerization show preferential retention of several L-amino acids of diverse structural types (not only tryptophan, but also phenylalanine, alanine, arginine, and glutamic acid).[565] Polymers imprinted with this peptide permit D-tryptophan to pass more rapidly than its L-enantiomer.[566] Polymer-imprinting with antibody mimics has given CSPs showing enantiomer discrimination for amino acids.[567]

A report that ultrafiltration enhanced by chiral micelles effects the separation of amino acid enantiomers has required a correction to be published.[568]

More conventional chiral stationary phases (CSPs) applied to amino acid

resolution have been prepared; those carrying covalently-bonded L-proline-3,5-dimethylanilide and analogues,[569] and used for the chromatographic resolution of N-(3,5-dinitrobenzoyl)-DL-amino acid esters, appear to owe their high efficacy to 1:1-complex formation between the chiral moiety of the CSP and the L-enantiomer of the solute.[570] This arrangement in reverse, *i.e.* the CSP carries N-(3,5-dinitrobenzoyl)-(R)-phenylglycine linked to a polymer through the $-O(CH_2)_3SiMe_2O-$ linkage, is an effective medium for resolving DL-amino acid esters and amides[571] [a correction has been published[572] concerning preparative resolution using a hollow fibre membrane carrying N-(1-naphthyl)-L-leucine as chiral selector]. CSPs have been prepared either by bonding aminopropylsilica to Marfey's reagent followed by capping unreacted amino groups by N-trifluoro-acetylation, or by reaction with the closely-related reagent, N-5-(1-fluoro-2,4-dinitrophenyl)-L-phenylalanine tert-butyl ester and capping with butanoyl chloride after ester cleavage;[573] these show good discrimination between enantiomers of N-(2,4-dinitrophenyl)- and N-(3,5-dinitrobenzoyl)amino acid esters.

Lipids bonded to L-glutamic acid form highly oriented gels in benzene, and elution with water when N-dansyl-DL-phenylalanine is trapped in the gel causes the preferential release of the L-enantiomer.[574] Cyanuric chloride has already been established (Vol. 28, p.76) to be a useful, cheap compound to develop into a homochiral reagent, and has now shown its potential as a medium on which to build a CSP, through substitution of a chlorine atom by aminolysis with L-valinamide, for use in the resolution of N-dansyl-DL-amino acids.[575] Porous cross-linked poly(vinyl alcohol) carrying covalently-bonded L-proline-based groupings is an effective medium after complexation with copper(II) ions, for ligand-exchange chromatographic resolution of DL-amino acids.[576]

Common chromatographic media to which chiral species are adsorbed offer simple alternative CSPs for amino acid resolution, though the empirical nature of knowledge of their mode of action makes them attractive only to those with a predilection for gambling. (1→6)-2,5-Anhydro-3,4-di-O-methyl-D-glucitol adsorbed on silica gel is a good prospect as a CSP for the resolution of DL-amino acid salts,[577] and N-carboxymethyl-N-dodecyl-L-leucinol sodium salt[578] or copper(II) - N-octyl-(S)-phenylalanine N′-octylamide,[579] adsorbed on reversed phase C-18 silica gel, are effective in the resolution of free DL-amino acids and their esters and amides, using the ligand exchange chromatography principle.

The remaining topic under this heading, the mechanisms by which prebiotic enantioselection can be explained, continues to enjoy a loyal following, with well-worn themes that are rarely put to rest as a result of having waited in vain for experimental support. A review of mechanisms proposed under this heading suggests[580] that the order of events could not have been the generation of chiral polymers followed by their influence on racemic amino acids. Current themes and their practitioners have appeared in print.[581] A broad review of current theories has been published,[582] and a hybrid theory combining the parity violation mechanism with the long-respected Frank model has been considered.[583] Mirror symmetry-breaking ideas (see Vol. 28, p. 50) that invoke putative, unimaginably small, energy differences between enantiomers due to parity violation, as an explanation, may perhaps never be substantiated in the laboratory,[584] and energy

differences for β-decay electrons of opposite symmetry resulting from parity violation have been ruled out for frequently-observed spontaneous resolution by crystallization under racemization conditions; this study describes a bromo-fluoro-1,4-benzodiazepino-oxazole as a new example of this phenomenon that has led to high e.e. values, and stereoselective autocatalysis offers the best explanation for the result. Conventional concepts of the kinetics of enantioselective processes are an alternative explanation that has been considered within the mirror symmetry-breaking approach.[585] The enantioselection mechanism depending on a low temperature phase transition proposed for amino acids has been reviewed.[586]

5 Physico-chemical Studies of Amino Acids

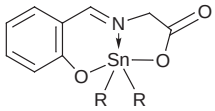
5.1 X-Ray Crystal Analysis of Amino Acids and Their Derivatives – Crystal structure details for many familiar L-(α-amino acids have been reinvestigated, and this adds to the impression that there has been a deluge of papers in this topic area. Structures have been determined at 120 K for L-isoleucine,[587] L-leucine,[588] L-cysteine,[589] DL-valine,[590] DL-norleucine (β-form),[591] and L-methionine and L-valine.[592] A new study of DL-glutamine has been reported.[593] An inelastic neutron scattering study of L-leucine[594] continues a series of similar applications of the technique, which has also been used in an investigation of samples in aqueous media to give direct evidence for a modified solvent stucture within the hydration shell of a hydrophobic amino acid.[595]

Salts of common amino acids have been studied: sarcosine sulfate,[596] L-argininium hydrogen squarate,[597] (R)-(-)-1-phenylglycinium hydrogen squarate,[598] picrates of DL-arginine, L-arginine, L-lysine, and L-ornithine,[599] arenesulfonates of glycine, L-alanine, and L-serine,[600] and amino acids with sulfonated azo dyes.[601]

Amino acid complexes have been studied: L-histidine - glyoxylic acid,[602] L-glutamic acid - 2-methylimidazole (1:1),[603] and sarcosine - sucrose (1:1).[604]

Amino acid derivatives have been studied: O-phospho-L- and -DL-threonine (structure comparisons with corresponding serine derivatives),[605] N-Boc-L-phenylglycine,[606] N-Boc-L-alanine,[607] N-benzyloxycarbonyl-L-serine tert-butyl ester,[608] N-acetyl (S)-isovaline methylamide,[609] ethyl N-(isopropylcarbamoyl-methoxyphosphonyl)-L-phenylalanine,[610] and L-histidine methyl ester dihydrochloride.[611]

Derivatives with structures more remote from the amino acids themselves have been subjected to X-ray crystal analysis so as to verify structures involved in reaction pathways, including amino acid synthesis routes. These are represented by (1S,6R,9S)-6-benzamido-9-hydroxymethyl-8-oxabicyclo[4.3.0]non-3-en-7-one,[612] (3RS,6SR,1′RS)-6-tert-butyl 3,6-dihydro-5-methoxy-3-methyl-3-(3-oxocyclohexyl)-2H-1,4-oxazin-2-one (verified as the structure of an intermediate in an α-methyl α-amino acid synthesis),[613] and novel five-coordinate tin(II)-amino acid derivatives, bicycloazastannoxides (106).[614] Recent controversy (*cf* Ref. 870) concerning the correct structure for the product formed from L-tryptophan and



(106)

pyrroloquinolinequinone (PQQ) has been settled by an X-ray structure determination showing the product to be an imidazolopyrroloquinoline (116).[615] X-ray data for proteins has been analyzed leading to comparisons of local structures with predicted conformations for individual amino acids.[616]

5.2 Nuclear Magnetic Resonance Spectrometry – Papers reviewed here cover either studies that employ more sophisticated instrumental techniques with amino acids, or studies yielding information that is of a non-routine nature.

Solid-state NMR data for crystalline amino acids providing rotation rates for the amino group show the dominant role of hydrogen bonding.[617] The same technique allows solid state molecular motion around carbon atoms within Boc-L-alanine to be quantified, and establishes heteronuclear dipolar coupling constants; the particular help that magic-angle spinning data can offer is demonstrated in this study.[618] ^{2}H-Spin-lattice relaxation times permit motion within the $N^{2}H_{3}$ group of labelled L-alanine to be assessed.[619] Deuteriated glycine samples have been assessed in a ^{2}H -^{1}H-cross-polarization kinetics study,[620] and determination of torsion angles Ψ for solid amino acids through a 2D-double quantum NMR study, has called for doubly-^{13}C-labelled amino acids.[621] High sensitivity INADEQUATE data for ethyl [N,N,O-$^{2}H_{3}$]-DL-tyrosinate has been converted into more conventional ^{13}C-^{13}C format.[622]

Triple resonance measurements can be exploited for direct NMR identification of certain amino acid types - those that lack $^{13}C^{\beta}$ - $^{13}C^{\gamma}$ coupling (glycine, alanine, cysteine, serine) and those for which C^{γ}-resonances are well-separated from other spectral features (aspartic acid, asparagine, phenylalanine, etc.).[623]

The relative stereochemistry of kainoids (3,4-disubstituted prolines) can be determined through ^{1}H-NMR in $^{2}H_{2}O$, concentrating on the resonances of the protons at C-2 and C-4; the 2,3-trans configuration is indicated when the resonance for H-2 appears at greater than 4.2 ppm in the p^{2}H-range 3 - 8.[624] ^{1}H-NMR chemical shift data has been interpreted to give information on intramolecular hydrogen bonding for amino acids.[625] Conformational information for glutamic acid and a series of its homologues carrying methyl groups at C-3 and C-4, and the 4-methylene derivative, has been obtained through interpretation of ^{1}H- and ^{13}C-NMR data and compared with molecular orbital calculations,[626] also applied to stereoisomers of aminocyclopentane-1,3-dicarboxylic acid.[627] A useful compilation has been published of ^{13}C-NMR data determined for the 20 common coded amino acids in a uniform sample environment (25°C, phosphate buffer, pH 7.3).[628]

NMR study of derivatives is an effective source of information on the solution behaviour of amino acids, *e.g.* the stabilization of the syn-rotamer of carbamate

derivatives of an α-amino acid by intramolecular hydrogen bonding with the carboxy group.[629] Suitably chosen derivatives give useful insights into peptide and protein behaviour, and determinations of cis-trans isomer ratios for the amide grouping of N-acetyl-L-proline N′-methylamide, and its cis and trans-5-tert-butyl homologues,[630] and of intramolecular hydrogen bonding within Me_2N-$CO(CH_2)_2CO$-Gly-NHMe and $Me_2NCO(CH_2)_3CO$-Gly-NHMe,[631] also benefit from FTIR data. Bis(amino acid) derivatives formed by acylation of representative amino acid esters (valine, phenylalanine, and proline) by 1,1′-ferrocenedicarboxylic acid adopt an intramolecularly hydrogen-bonded structure, as revealed by NMR and IR data.[632]

Determination of enantiomer ratios for amino acids by NMR methods already has a lengthy list of methods that exploit traditional physical principles, and new variants of these are the ^{1}H-NMR data for (R)-O-aryl lactic acid amides,[633] and ^{1}H- and ^{13}C-NMR data for C2-chiral palladium complexes [(2S,3S)-2,3-diaminobutanepalladium(aminoacidato)] dinitrates[634] of the target DL-amino acid sample. Another axially-chiral derivatization agent, 1,1′-binaphthalene-8,8′-diol, has been proposed for the determination, based on ^{1}H- and ^{13}C NMR, of absolute configuration of α-chiral carboxylic acids (see also Ref. 706).[635]

5.3 Optical Rotatory Dispersion and Circular Dichroism – As in other areas where new instrumental developments show their potential through dramatic extensions to existing knowledge, polarimetry is a newly-emerging and highly sensitive technique. The optical activity of crystalline L-glutamic acid has been determined[636] to establish solid state rotatory powers, including the temperature dependence of gyration tensor components, through the use of a high accuracy polarimeter. Instrumental details of a high sensitivity polarimeter, based on the magneto-optical principle and using a He-Ne laser monochromatic light source, have been published.[637]

Standard CD spectrometers have continued to give data that may be interpreted in terms of the absolute configuration of amino acids after derivatization so as to introduce a grouping that absorbs light in the accessible UV wavelength region, such as Pd(dmba)(acac), giving Cotton effects centred at 265-280 and 305-320 nm, with signs characteristic of absolute configuration, after complexation with α-amino acids.[638] A helical chirality characteristic of absolute configuration is induced into zinc biliverdin derivatives through complexation with an enantiomer of an α-amino acid ester, as revealed by CD and NMR data (M-helicity corresponds with the L-configuration).[639]

Information concerning solution conformations has been deduced from Raman optical activity data for N-acetyl-L-alanine N-methylamide.[640]

5.4 Mass Spectrometry – Only in the last few years has the non-routine literature on this topic shifted from an almost exclusive coverage of amino acid derivatives, to concentrate largely on the amino acids themselves. This is mainly due to the emergence of new ionization methods, but the delay is partly due to a lingering respect for the received wisdom that amino acids are involatile and therefore not suitable for mass spectrometry.

Evidence for aggregation of amino acid molecules ejected into the gas phase continues to accumulate; thus, tryptophan clusters of up to 5 molecules form during seeded supersonic expansion and ionization through laser desorption.[641] Although, at first sight, it is not surprising that no differences were found through this technique between data for L-, D-, and DL-tryptophan, it was a valid study in view of the different solid-state structures adopted by certain racemates in comparison with their enantiomers.[642] Individual molecules within heterogeneous clusters formed by mixed amino acid samples in the gas phase have been shown to participate in proton transfer reactions.[643] Kinetic energy release associated with the separation of a protonated dimer of an amino acid into protonated and neutral monomers has been evaluated.[644] The facility offered by mass spectrometry to study molecular complexes in the gas phase has been applied to determine the stoichiometry of αβ-cyclodextrin - protonated tryptophan complexes,[645] and a demonstration[646] (supporting a conclusion derived from ^{1}H-NMR data) that permethylated cyclodextrins complex preferentially (5:1) with the D-enantiomer of the methyl esters of phenylalanine or tryptophan. Complex formation between aluminium(III) salts, glycerol, and amino acids has been established by FAB-MS,[647] and gas-phase reactions of copper(I) and iron(I) ions with protein amino acids have been shown to be most prevalent with those that carry non-polar side-chains, suggesting that the ionized carboxy group is the prime reaction site.[648] Protonated nitric oxide reacts with glycine, alanine, and valine, and their N-methyl homologues, to produce the $[M\text{-}H]^+$ ion through hydride abstraction, which leads to iminium ions by loss of HNO_2 and CO.[649] Alkylation of glycine by ethylenehalonium ions $(CH_2)_2X^+$ (X = Cl, Br) has been observed in a similar study.[650]

Protonation of amino acids has been a long-running study and has promoted joint mass spectrometry - molecular orbital calculation studies, seen in a study of proton transfer from protonated glycinamide,[651] and to histidine, lysine, and their di- and tripeptides.[652] Protonated amino acids formed in the gas phase generate metastable ions that release H_2O and CO fragments.[653]

Although applied to less-common amino acids, mass spectrometric studies of L-cysteinylDOPA,[654] fluorinated β-hydroxy-β-phenylserines,[655] and the cross-linking amino acids pyridinoline and deoxypyridinoline[656] employ standard instruments. The last-mentioned study involves the continuous liquid flow/liquid secondary ion-ionization technique, and illustrates the high sensitivity of mass spectrometry to permit analysis at the 100 pmol level of these clinically-important amino acid markers (see also Refs. 663, 1049).

Standard derivatization protocols have been applied in the numerous papers in this year's literature, describing structure assignments using basic instrumentation. This topic is not covered thoroughly here because of its generally routine nature, but mechanistically-interesting results are illustrated by a structure assignment to the very intense *m/z* 171 ion generated by a modified McLafferty rearrangement of N-dansylamino acids and their methyl esters,[657] and a new fragmentation mechanism for leucine (as its N-heptafluorobutyryl isobutyl ester), assisted by the parallel study of the [1-^{13}C]isotopomer.[658] $[M\text{-}H]^-$ ions generated for N-phenylthiohydantoins are a reliable basis for characterization purposes.[659]

5.5 Other Spectroscopic Studies of Amino Acids – Infrared and Raman spectroscopy papers can only justify citation here if they are of exceptional interest from the point of view of either developing instrumentation, or new interpretations. While a vibrational frequency assessment of L-proline through FTIR - Raman study would be, by now, considered to be routine,[660] the use of photoacoustic FTIR for monitoring solid-phase reactions [four reaction steps from resin-bound S-benzyl-N-Boc-L-cysteine to resin-bound N-(p-cyanobenzoyl)-dehydroalanine] is a new application.[661] For other FTIR studies, see Refs. 630, 631. Three Raman tensors have been deduced from polarized Raman spectra of single crystals of N-acetyl-L-tryptophan.[662] Assistance in amino acid structural assignments given by IR studies is referred to in Refs. 607 and 632.

Other absorption spectroscopy studies that have yielded significant results, range from simple established techniques to sophisticated new methods; careful accumulation of accurate data for the standardization of analytical assays of pyridinoline and deoxpyridinoline (see also Refs. 656, 1049) based on UV absorption at 295 and 325 nm (ε values 5490 and 5785 L mol^{-1} cm^{-1} respectively for the former crosslinking amino acid, and 5160 and 5290 for the latter);[663] and absorption millimetre spectroscopy data at 31.42 GHz for solid samples of 19 amino acids, to reveal details of their hydration behaviour.[664] Solvatochromic parameters determined for N,N-dimethylvaline have been used to evaluate populations of zwitterionic tautomers in different solvents.[665]

ESR Spectra of glycine, DL-serine, DL-asparagine, and L-glutamic acid γ-irradiated at room temperature have filled gaps in the substantial literature on radical formation in solid amino acids.[666] Spectra of γ-irradiated hydrochlorides of methyl L-valinate and L-leucinate are best interpreted in terms of the location of the unpaired electron on the carbon atom adjacent to the isopropyl group.[667]

5.6 Physico-chemical Studies of Amino Acids – The simplest physico-chemical measurements for amino acids are usually made for their own sake, but also with the knowledge that improved understanding of their behaviour (in aqueous solutions in particular) will inevitably have some significance in the context of the living cell. Representative data in this category are partial molar volumes and isentropic compressibilities of aqueous solutions of N-acetyl amino acid amides,[668] and activity coefficients of amino acids in aqueous electrolytes[669] (determined by electrochemical methods with superior accuracy in comparison with standard techniques;[670] see also Ref. 725). Protonation constants have been reported for alanine,[671] and the effects of sodium dodecyl sulfonate on protonation equilibria of L-glutamic acid and L-ornithine have been determined.[672] Thermodynamic parameters have been collected, giving standard enthalpies of formation for ethyl NN-diethylalaninate and methyl and n-propyl N,N-dimethyl-aminoisobutyrate.[673] Enthalpies of sublimation and heat capacities have been determined for N-acetylamino acid amides.[674]

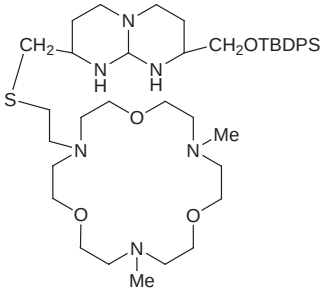
Intermolecular interactions involving amino acids continue to provide a major emphasis in this Section; calorimetric data giving a measure of hydrophobic interactions for N-acetylamino acids as a function of structure have been determined for binary aqueous solutions,[675] and further information has been

published[676] in this context for interactions between enantiomers of amino acids. Complexation constants have been determined for supramolecular aromatic amino acids with cyclodextrin - copper,[677] and for L-alanine β-naphthylamide hydrobromide with methyl β-cyclodextrin (by ultrafiltration).[678] Favourable interactions account for the 12,000 times stronger binding of L-arginine to RNA aptamers compared with the D-enantiomer,[679] for selective binding of amino acids to yeast tRNA in solutions at high ionic strength,[680] for enhancement by amino acids of pyrene - β-cyclodextrin binding,[681] and for binding to 1-(N-benzylamino)cyclopentyl O,O-diamylphosphonate.[682] In the latter case, the interaction accounts for the transport of amino acids across a Teflon matrix that has been impregnated with a solution of the aminophosphonate in o-nitrophenyl octyl ether, and similar explanations surround the transport of protonated amino acids by macrocyclic ligands[683] and transport of lipophilic lanthanide tris(β-diketonate) - valine and leucine complexes across CH_2Cl_2 membranes under neutral conditions,[684] enantioselective transport of amino acids across polymer membranes impregnated with a homochiral crown ether,[685] partition of amino acids in an aqueous two-phase system containing poly(ethylene glycol), with sodium sulfate, and mild surfactants[686]or in the same medium with dextran,[687] or on an ethylene oxide - propylene oxide copolymer,[688] and ordered aggregation of N-acylamino acid amphiphiles (*e.g.* N-stearoyl-L-valine) at liquid air interfaces.[689] The widely-used cationic surfactant, cetyltrimethylammonium bromide, showed only weak binding to 11 amino acids from an extensive series tested by TLC.[690] Free energy of adsorption data for amino acids at a hydrophobic and a hydrophilic surface (surprisingly, leucine is more strongly adsorbed than serine),[691] and for amino acids to silica[692] and to porous polymeric adsorbents[693] have been determined.

Classical separation procedures, such as the presentation of amino acids in aqueous ethanol to a weak acid cation exchanger, involve sorption as a major contributory mechanism.[694] The kinetics parameters for the DL-lysine - Amberlite IRA420 interaction have been evaluated,[695] and similar studies have involved Amberlite IR120[696] and a cellulose-based ion exchanger.[697] Partitioning of amino acids in water - butan-1-ol[698]and in other water - partially miscible alkanol mixtures[699] and assessing the distribution of the solute, gives considerable insight into structural factors that determine the magnitude of the ternary interactions involved. Enthalpic interaction coefficient data for amino acid - β-cyclodextrin complexes reveal the involvement of three different types of stabilizing interactions,[700] and similar detail is provided by interfacial tension measurements at the benzene - water interface for solutions of alanine, valine, leucine, and phenylalanine in the presence and absence of β-cyclodextrin.[701] The inclusion of the alkyl side-chain within the host, β-cyclodextrin, is claimed to contribute significantly towards the constructive interaction.

Host - guest interaction studies are well-represented again this year, with enantiospecific recognition of N-benzyloxycarbonylglutamic acid shown by chiral cage-like C_3-symmetric receptors,[702] and of aromatic amino acids in water or N-acetylamino acids in organic solvents, shown by cyclobis(paraquat - p-phenylene), a π-electron-deficient tetracationic cyclophane.[703] Discrimination to the

extent of 74% e.e. is found for a homo-oxacalix[3]arene - amino acid ester system[704] whereas a lower value (40% e.e. with phenylalanine) is shown for amino acid zwitterions enclosed by the chiral crown ether (107).[705] An axially-chiral derivatization agent, 1,1′-binaphthalene-8,8′-diol, has been found to bind a variety of amines and α-amino acids with significant chiral recognition (see also Ref. 635).[706] In a particularly thorough study of supramolecular bio-organometallic hosts, [(η^5-pentamethylcyclopentadienyl-rhodium(I)]-nucleobase, -nucleoside, and -nucleotide cyclic trimer complexes, it has been found that π - π interactions and hydrophobic interactions, as well as hydrogen bonding, contribute to the favoured complexation of L-enantiomers from aqueous solutions of DL-amino acids.[707]



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A clear indication of the complete shift from one structural type of aggregate to another is contained in the finding that long-chain bis(N-acyl)amides Z-Phe-NH$(CH_2)_{12}$NH-Phe-Z form gels, while analogues Z-Phe-NH$(CH_2)_3$Me form fibrils.[708] Information of this type obtained in the laboratory will contribute new insight into the nature of molecular interactions within the living cell, *e.g.* the conformational change induced into BkdR (the activator of the inducible bkd operon of *Pseudomonas putida*) by branched-chain L-amino acids.[709] Helix-forming propensities of basic αω-diamino acids, as constituents of peptides, increase with increasing length of the side-chain.[710]

Data from several routine optical measurements have been reported for single crystals of L-alanine.[711]

5.7 Molecular Orbital Calculations and Theoretical Studies for Amino Acids – Studies dwelling on continuing interests, concerned with conformational aspects in particular, have provided most of the papers in this Section over the years. A widening of the range of applications for molecular orbital calculations, and a significant increase in the number of studies published, is discernable.

Conformational calculations for N-formyl-L-serinamide[712] concentrate on backbone torsion angles, while a more extensive coverage under the same heading is provided for N-acetyl-N′-methyl-α-methyl-β-L-aspartamide[713] and other N-acetyl-N′-methyl-α-amino acid amides[714] including the 1-aminocyclo-

hexa-2,5-diene-1-carboxylic acid derivative[715] and the alanine derivative.[716] Models for solvation around the amino acid derivative are the particular concern of the last-mentioned study, also a feature of other studies [polarizing effect of the medium on conformations of amino acids;[717] structure of the (1:1)-glycine - water complex[718]].

The increasing proportion of papers dealing with underivatized α-amino acids is clearly a trend, covering electrostatic potential maps,[719] alanine in the gas phase,[720] proton - proton interactions for L-leucine and L-isoleucine,[721] and further development of the connectivity index proposed for the common protein amino acids.[722] Twenty stable conformations are predicted for β-alanine on the basis of calculations using standard protocols.[723]

Physical characteristics of amino acids and their common derivatives have been assessed through molecular orbital calculations: crystal lattice energies of amino acid hydrohalides,[724] activity coefficients of amino acids in water (see also Refs. 669, 670),[725] electronic spectral features of glycine and N-acetylglycine,[726] vibrational frequencies for L-proline and hydroxy-L-proline[727] and for L-asparagine,[728] and FTIR/Raman characteristics of alanine.[729] The structure of a glycine radical and its calculated ESR characteristics have been considered in relation to experimental values.[730] Certain conformers predicted for glycine and alanine have not been found in ultrasonic jet spectroscopy studies, and this is thought to be due to selective conformational relaxation of these conformers into lower energy species during spectroscopic study.[731]

Examples of applications of calculations for the solution of mechanistic problems include unimolecular decomposition of N-chloroglycine[732] and of anions of other N-chloro-amino acids,[733] and the C-H acidity of succinimides derived from N-acyl-L-aspartic acids.[734] The impetus for the last-mentioned study lies in the well-known enigma concerning the enhanced propensity for racemization shown by aspartic acid and asparagine residues in peptides.

There are several methods of determining helicity leagues for the twenty coded amino acids, reflecting their tendency, as constituents of peptides and proteins, to promote helix formation, and three of these methods have been critically appraised.[735] One helicity-ranking method has been considered in detail.[736] A review of the predominant conformations available to individual amino acids, together with a controversial approach in which structural and functional distinction of amino acids, one from another, in terms of atomic number and nucleon number, has appeared.[737]

6 Chemical Studies of Amino Acids

6.1 Racemization – Racemization of free amino acids is a topic with clearly-defined strands of continuing interest, mostly connected with kinetics and mechanism, with racemization rates for constituents in protein hydrolysates featuring strongly. A salutary lesson relevant to the preparation of analytical samples, that solid D- or L-leucine suffers racemization within a few minutes when powdered in a planetary gear mill,[738] extends a few earlier examples of

the same sort, though some of these reports have been shown to be non-reproducible.

Loyalty is shown by several research groups to particular problems, one stemming from stereoinversion at aspartic acid residues in fossil proteins, and in proteins with no turnover in living organisms. Thus, αA-and αB-crystallins of human eye lens are known to undergo residue-specific racemization at aspartyl residues, and the ensuing conformational changes result in insolubilization and this is connected with the development of cataracts.[739] A kinetic study of this process in a series of synthetic peptides, chosen as models for the crystallins, has shown that racemization rates are strongly dependent on the sequence neighbouring the aspartyl residue.[740] Human teeth also offer a reproducible protein sample, dentin, usually of reliable age, and careful attention to analytical details has featured in recent papers describing aspartyl racemization studies.[741] Retrievable DNA in ancient tissue samples showing a D/L-ratio >0.08 for their aspartic acid content cannot be endogenous, a conclusion based on the degraded DNA content of unadulterated samples.[742]

An increased racemization rate can be expected for aspartyl residues in fossil proteins, undermining the reliability of amino acid racemization data, if microbial degradation is one of the diagenetic processes at work.[743] Standard analytical procedures have been set out for assessing high levels of racemization of C-terminal aspartic acid[744] and of Fmoc-S-trityl-L-cysteine[745] during solid-phase peptide synthesis.

Deliberate laboratory racemization of L-amino acids has been practised for many years for the provision of D-isomers, exploiting the easy stereoinversion at C-4 of certain five-membered heterocyclic compounds enclosing the -NH-CHR-CO- grouping. Hydantoins belong to this family, and deuteriation rates vary widely within a series, showing the control exerted by the 5-substituent on the rate of the underlying S_E2 push-pull mechanism.[746] Homochiral thiazol-5(4H)-ones produced either through cyclization of N-thioacyl and N-alkylthiocarbamoyl-L-α-amino acids, or from peptides through sequencing chemistry (Edman and related methods), are racemized by trifluoroacetic acid but not by BF_3.[747]

6.2 General Reactions of Amino Acids – Papers are grouped under three main headings: (a) reactions at the amino group, (b) reactions at the carboxy group, (c) reactions involving both amino and carboxy groups. Papers covering (d) reactions at the α-carbon atom of α-amino acids are collected next, followed by (e) reactions of β- and higher homologous amino acids.

6.2.1 Reactions at the Amino Group – Mechanistic interest in the N-halogenation of amino acids and the synthetic implications of some ensuing processes have continued to feature in the literature. The repetitious nature of these papers, from more than one research group, illustrates the advertiser's technique - since so much is arriving in the literature on a narrow topic, it must be an important topic! - and others are attracted to it. Kinetic parameters of the N-bromination of amino acids using N-bromosuccinimide have been determined.[748] Rates of base-

induced elimination from N-halogeno-α-amino acids in aqueous media are determined significantly by the structure of the side-chains (see also Ref. 733).[749] The reactions lead on to give hydrolysis products, aldehydes and/or ketones and ammonia and/or primary amines in near-neutral conditions, or α-keto acids in basic media.[750] In support of earlier conclusions, decarboxylative dechlorination of N-chloroalanine is a concerted fragmentation process in aqueous media.[751] N-Bromoamino acid anions show a clear absorption maximum centred at 290 nm and this has facilitated the determination of kinetic aspects of the E_2 elimination that is characteristic of these species.[752]

Another routine area that continues to generate a large number of papers on the oxidation of amino acids can only be hinted at here, *e.g.* a significant rate-enhancing role exerted by chloride and sulfate ions on chloramine-T oxidations in aqueous solutions.[753]

Displacement of the amino group of D-serine by bromine (HBr, $NaNO_2$) initiates a route to (S,S)-3-prolylazetidin-2-one.[754]

Acylation reactions of amino acids of unusual interest are represented in photoinduced N-phenylacetylation through UV irradiation of 2′-disubstituted diazoacetophenones $ArCHN_2$ in the presence of amino acid esters,[755] highly selective acetylation (of primary amines in the presence of secondary amines) using N-acetyl-N-acyl-3′-aminoquinazolinones,[756] and enzymic acylation/deacylation [a classical 'resolution' technique for amino acids (Section 4.21; most of the year's papers are covered there)]. But enzymic N-alkylation is unusual, and is illustrated for opine synthesis from an L-amino acid and pyruvic acid using an opine dehydrogenase from *Arthrobacter*.[757] N-Formylation of an amino acid ester hydrochloride can be brought about using cyanomethyl formate.[758] A thorough assessment of conditions for N-phenylacetylation of L-tyrosine ethyl ester using ethyl phenylacetate and penicillin amidase in a benzene - aqueous salt medium has been published,[759] and the N-tetrachlorophthaloyl protecting group (removed with ethylenediamine) has been advocated.[760]

Mild conditions have been established for N-sulfonylation, effective for γ-substituted glutamates without risk of pyroglutamate formation.[761]

Naturally-occurring 3-oxohexanoyl-L-homoserine lactone can be prepared in moderate yield from the two components through the dicyclohexylcarbodiimide - hydroxybenzotriazole procedure,[762] and a solid-supported carbodiimide has been used to couple Mosher's acid to homochiral amines and amino acids.[763]

N-Alkoxycarbonylation would be thought of as an optimized procedure, in view of the wide use made of the resulting N-protected amino acids, but improved results for preparations of N-Fmoc derivatives using Fmoc-Cl can be secured, using a borate buffer (pH 11.4), with a reaction time of 40 minutes at room temperature.[764] Even histidine and tyrosine, normally needing special care, are derivatized efficiently through this procedure. N-Benzyloxycarbonylation using ZCl with DMAP in $ClCH_2CH_2Cl$ illustrates beneficial inverse phase-transfer catalysis[765] in simple cases, but the severe conditions that are needed (ZCl or Boc_2O/LiHDMS/THF/$-78^\circ C$) for N-protection of pyroglutamate esters[766] might be expected to generate side-products. Introduction of the Boc-group into α-alkylprolines and sterically-hindered αα-disubstituted glycines using Boc_2O is

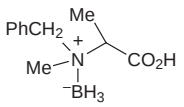
best accomplished in MeCN, but requires tetramethylammonium hydroxide to achieve solution, and four days for good yields![767]

New acid-labile N-alkoxycarbonyl groups [*e.g.* H_2C=CMeCONHCH$_2$C-Me$_2$(OCO)-] have been proposed.[768]

Removal of the Boc-group is the subject of even more papers this year: for side-chain tryptophan selective deprotection, silica gel under reduced pressure;[769] for removal in the presence of silyl ethers, saturated HCl in ethyl acetate;[770] for universal Boc removal, cerium(VI) ammonium nitrate catalysis in MeCN,[771] silicon tetrachloride with excess phenol in CH_2Cl_2,[772] or tin(IV) chloride in organic solvents (this avoids disruption of any thioamide groupings in the same molecule).[773] The removal of the increasingly widely-used N-Alloc-grouping using water-soluble palladium(0) catalysts has been reviewed.[774]

Pd/Cu-Catalysed N-arylation reactions, using aryl halides, give enantiomerically-pure products when L- or D-amino acids are used;[775] nucleophilic addition of amino acid butyl esters through nitrogen to quinones represents an alternative arylation procedure.[776] The preferred N-mono-methylation procedure for primary and secondary amines involves condensation with formaldehyde, followed by reduction, to ensure freedom from over-alkylation; use of microwave irradiation using formic acid as reaction medium is beneficial.[777] Fmoc-Amino acids are conveniently N-methylated by reaction with formaldehyde followed by reduction with triethylsilane,[778] while TFA-sensitive N-(2-hydroxy-4-methoxy)-benzyl Fmoc-amino acids are best prepared through attaching the amino acid to a chlorotrityl resin, conversion into the Schiff base, and $NaBH_3(CN)$ reduction.[779] The general alkylation process [RCHO/$NaBH_3(CN)$] leading to higher homologous N-alkylamino acids has been applied to amino acid amides.[780] Using $NaB(OAc)_3H$ for the reduction stage is claimed to lead to fewer problems.[781] In the presence of heteroaromatic acid amides, formaldehyde reacts with amino acid esters to give N-amidomethyl derivatives,[782] and an amino acid, an aldehyde, an isocyanide, and an alkanol react to give 1,1′-iminodicarboxylic acid derivatives.[783] Reductive alkylation of 5-aminopentanoic acid with N-Boc-piperidin-4-one is an essential stage of a semi-synthesis of the anti-tumour agent irinotecan.[784]

The borane adduct (108) of methyl L-alaninate has been separated from its epimer, and shown to undergo stereoselective α-alkylation in up to 82% e.e.[785]



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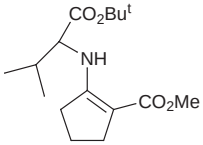
Enamines (109) formed from β-ketoesters with tert-butyl L-alaninate are representative of valuable intermediates in synthesis (*e.g.* of non-racemic acids[786]), and methyl N-(benzylidene)-L-valinate serves for the synthesis of R,S- or S,S-secondary homoallylic amines (the stereochemistry depending on the



(109)

nature of the allylmetal reagent used for addition to the imine moiety).[787] Near relatives, the homochiral azomethine ylides $XCH^{-}RN^{+}=CHY$, *e.g.* from (R)- or (S)-phenylglycine,[788] are even more versatile through their 1,3-dipolar cycloaddition reactivity, giving optically active pyrroloimidazoles.

The Maillard reaction, and associated reactions, continue to provide new products from the interactions of amino acids with carbohydrates (even though the reaction dates from 1912). These reactions (see also Refs. 101, 994) are increasingly recognized as having important roles *in vivo*, including higher organisms, as well as in food science and related areas, and the Maillard reaction has been reviewed[789] specifically from the human physiology perspective. The formation of Amadori rearrangement products, 1-(N-alkylamino)-1-deoxy-2-ketoses, through reductive amination of aldehydes by amino acids, has been demonstrated,[790] and the fructose - benzyl L-tyrosinate product has been studied.[791] The formation of Amadori compounds, an early stage in Maillard processes, has been shown to be reversible, thermolysis of N-(1-deoxy-D-fructo-pyranos-1-yl)-L-proline giving aldoses (identified after conversion of the reaction mixture into per-O-trimethylsilyl ethers).[792] α-N-Acetyl-L-lysinamide reacts with glucose in the usual Maillard fashion, but a product derived from two amino acid molecules and one glucose molecule is also formed, indicating that a search for a new protein crosslink could be worthwhile.[793] A 1,4,5-trideoxy-1-(N-alkylamino)-2,3-hexulos-4-ene formed from an Amadori product by double dehydration has been targeted in synthetic work, since its cyclization under physiological conditions may be relevant to protein crosslinking.[794]

The de-sulfonylation of protected amides has presented formidable problems, but a Bu_3SnH-AIBN induced radical reaction that requires neutral conditions (boiling toluene) is likely to be widely used. Since those working in the amino acid field could be main users, the method needs to be illustrated with more than just the one example used in this study.[795]

N-Phosphorothio-L-leucine derivatives $ROP(O)(S)NHCH(Bu^{s})CONH_2$ that carry a chiral phosphorus centre have been prepared as individual stereoisomers,[796] and reactions with uridine of N-(O,O-di-isopropyl)phosphoryl-L-threonine have permitted the construction of a nucleotide at the N-substituent.[797]

Z-Protected guanidino acids have been prepared from amino acids, trimethylsilyl chloride, NEt_3, ZNHC(SMe)=NZ in CH_2Cl_2,[798] and from the more useful mono-Mtr- or Pmc-protected analogues using essentially the same method.[799] Amidines $R^1R^2NCSN=CPhNHCHRCO_2Me$ have been prepared from amino acids using the chloroimidate as reagent.[800]

Incorporation of the amino group into heterocyclic structures is the result of

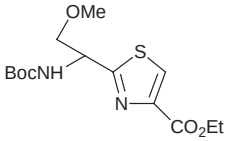
condensation of amino acid esters with tetrahydro-2,5-dimethoxyfuran {giving 1-(1H-pyrrolyl) derivatives}[801] and with 2-(formylmethyl)cyclohexanone.[802] Racemization to the extent of 9 - 18% occurs during the formation of the pyrroles, when acetic acid is used as reaction medium, probably because of N-acetylation and side-reactions that generate the oxazol-5(4H)-one, but problems can be avoided by the use of other solvents.

The photolysis of α-N-phthalimido acids (see Vol. 28, p. 63) has been reviewed.[803] Radical attack has been established to occur at nitrogen using ferrate(V) species formed from K_2FeO_4 and ethanol at pH 10, in contrast to the behaviour of hydroxyl radicals, which preferentially attack side-chain functions in methionine and phenylalanine.[804]

6.2.2 Reactions at the carboxy group – The compliant character of acid halides derived from suitably N-protected amino acids is now clearly established, and Fmoc-L-amino acid chlorides and fluorides have been reviewed from the points of view of their preparations and uses.[805] The fluorides of severely hindered amino acids have been prepared and used in peptide synthesis;[806] diethylaminosulfur tetrafluoride shows superior characteristics as reagent for their preparation.[807]

N-Trityl-O-methyl D- or L-serine has been converted into the corresponding thiazole (110) with preservation of stereochemistry, changing from carboxy group *via* ester, aldehyde and alkanol functions; these steps illustrate standard interconversions that are applicable to all N-protected amino acids.[808] The route is a reversal of a newly-developing standard synthesis of amino acids in which C-2 of a thiazole becomes the carboxy group of the target amino acid (see Ref. 186). The 2-aminothiazol-5-yl analogue has been prepared *via* a diazoketone.[809] N-Benzyl imino acid esters condense with cysteamine in the presence of Bu^i_3Al to give 2-(α-iminoalkyl)thiazolines.[810]

Decarboxylation is a stage of the classic colour-forming reaction of amino



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acids that employs ninhydrin as oxidant. Certain solvents (DMSO; methyl Cellosolverm) enhance the colour intensity more than variations in other reaction parameters (pH, temperature, presence of hydrindantin or ascorbic acid).[811] Decarbonylation of N,N-disubstituted-αα-disubstituted glycines through thermal elimination of their acid chlorides (- CO, - HCl) has been illustrated with a pipecolic acid derivative, and offers a route to enamides *via* acyliminium ions.[812] A more involved approach to the replacement of the carboxy group of an amino

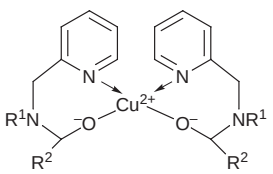
acid (>$CHCO_2H$ →>$CMeCONH_2$→>CMeNHR) has been illustrated with 3-carboxypiperidine (nipecotic acid).[813]

Thiol esters may be prepared in high yields from acids and thiols using ethyl 3-(dimethylamino)propylcarbodiimide on a solid support.[814] Amino acid 'active esters' (still in favour as aminoacylating agents) include pentafluorophenyl esters, prepared from unprotected amino acids using pentafluorophenyl trifluoroacetate, acetate, or corresponding carbamates, and simultaneously providing N-protection,[815] and hydroxybenzotriazolyl esters, used after isolation of their stable rearranged amides for the preparation of tert-butyl esters of N-tritylamino acids, and β-lactones of serine and threonine.[816] N-Benzhydrylglycolamide esters proposed for carboxy group protection, can be cleaved using either Bu_4NF in MeCN or DMF, or by hydrolysis by K_2CO_3 in aqueous DMF.[817] The 3-methylpent-3-yl grouping has been advocated for side-chain carboxy group protection of aspartic acid (*cf.* Vol. 28, p.69).[818]

Conversion of N-protected α-amino phosphonic acids into monoesters is efficiently brought about using peptide synthesis coupling agents BroP or TPyClU and an alkanol.[819]

Reactions of amino acid esters include conversion of alkyl esters of tyrosine and o-tyrosine into oximes through sodium tungstate-H_2O_2 or dimethyldioxirane oxidation, for use in the synthesis of marine sponge metabolites.[820] Conventional ammonolysis in toluene or water converts methyl esters of the familiar aromatic amino acids into amides;[821] this apparently simple process has usually been considered to be open to serious losses through side-reactions, and no doubt a later paper will give further details on this aspect.

Non-enzymic enantioselective hydrolysis studies of N-protected DL-amino acid p-nitrophenyl esters are of interest in a number of contexts, with a search for solutions to mechanistic problems being a predominant theme in studies of cationic micelles, containing copper(II)-complexes of the homochiral N-(pyrid-2-ylmethyl)-β-aminoethanols (111),[822] or various hexapeptides.[823] Transition metal salts promote the hydrolysis of alanine decyl ester in a reversed micelle environment.[824] Novel water-soluble crosslinked polymers imprinted with the transition state analogue (±)-phenyl 1-benzyloxycarbonylamino-3-methylpentyl phosphonate, but rendered capable of enantioselection through carrying L-histidine moieties and quaternary tetra-alkylammonium groups, have been shown to favour the L-enantiomer when presented with p-nitrophenyl N-benzyloxycarbonyl-DL-leucinate.[825]



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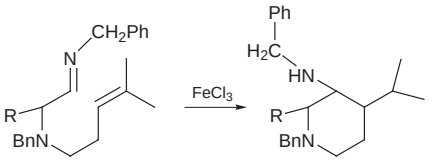
Preparative aspects, linking to the essential role of ester cleavage as a stage of selective deprotection of amino acids in organic synthesis, are represented in the use of bis(tributyltin)oxide for cleavage of methyl, benzyl, and phenacyl esters linked to resins,[826] and $CF_3SO_3SiMe_3$ for cleavage of tert-butyl esters while leaving tert-butyl ethers unaffected.[827]

Glycine dimethylacetal has had a rare use, providing access to the enol ether AcNHCH=CHOMe used as dienophile for the synthesis of novel pyran-based β-amino acids as enzyme inhibitors.[828]

α-Amino aldehydes are well established starting materials in broad areas of synthesis, and the preparation of this sensitive species is now a matter of routine, *e.g.* by $LiAlH_4$ reduction of Weinreb amides (preceding Horner-Emmons homologation to $=CHCO_2Et$ then on to 'aminoalkoxyoxiranes'[829]) Preparation of argininals from Boc N^{γ}-nitro-L-arginine, then to $BocNHCHR^1CONMeOMe$ and reduction, is another example.[830] Aldol reactions of the products are useful for carbon chain extension (see Section 4.19), and under some reaction conditions, pyrroles are at the end of the reaction path;[831] the 2-vinylpyrrolidine formed from ethyl Z-L-prolinate *via* the aldehyde has been elaborated by bromofluorination (NBS/Bu_4NF/HF) and dehydrobromination into the 1-fluorovinyl analogue.[832] A formal hetero-ene reaction is illustrated in the Lewis acid-catalysed cyclization of N-[2-(3-pentenylaminoethylidene] benzeneamines of aminoaldehydes prepared from methyl alaninate, leucinate, and phenylalaninate (112) into 3-amino-2,4-dialkylpiperidines (113).[833] Vinyl magnesium bromide



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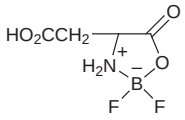
generates vinyl ketones that have been elaborated into corresponding oxiranes in a route to novel protease inhibitors.[834] The aldol reactions of α-amino aldehydes with nitroalkanes mediated by Bu_4NF proceed with high anti-anti diastereoselectivity to provide useful trifunctional compounds $Bn_2NCHR^1CH(OH)$-CHR^2NO_2.[835] Reduction of the carboxy group of an acylated proline is very straightforward (conversion into ester, then DIBALH reduction) as illustrated in conversions of the aldehyde function into the diethyl dithioacetal and (less believably) into the thioaldehyde.[836] Reduction to the aldehyde *via* the primary alcohol has been the preferred route for some workers; BH_3-SMe_2 then $COCl_2$ - DMSO and NEt_3, of Boc-L-proline or -pipecolic acid;[837] BH_3-SMe_2 then py/SO_3 for Boc-L-proline;[838] $LiAlH_4$ then $COCl_2$ - DMSO and NEt_3 for N,N-dibenzyl-L-phenylalanine.[839] Zinc borohydride is needed in only stoichiometric amounts for reduction of amino acids to 2-aminoalkanols, though reflux in THF during 5 hours is required.[840] α-Amino ketones have become favoured starting materials

for chain extension through Horner-Emmons alkenation (see also Ref. 829), with some control over the Z/E-isomer ratio being available through choice of reagent.[841] β-Ketoesters $BocNHCHRCOCH_2CO_2Et$ may be prepared from a Boc-amino acid and the lithium salt of ethyl acetate, through the involvement of carbonyl di-imidazole,[842] and γ-amino-β-ketophosphonates $Bn_2NCHRCOCH_2$-$P(O)(OMe)_2$ are formed analogously from the benzyl ester and the lithium salt of dimethyl methanephosphonate.[843]

Sodium or lithium borohydride reduction of the carboxy group of the common amino acids, after esterification, leads to primary alcohols. Serine presents an annoying problem insofar as loss of chirality is ensured unless the side-chain hydroxy group is derivatized; the TBDMS group or oxazoline formation fulfil this role satisfactorily.[844] Reduction of Boc-amino acid esters by $LiBH_4$ gives the corresponding alcohols; conversions from derived methanesulfonates ($CH_2OMs \rightarrow CH_2Cl \rightarrow CH_2SO_3^-Na^+$) gives 2-substituted taurines.[845] Good yields of alkanols are obtained through reduction of mixed carbonic anhydrides with $NaBH_4$ and for the conversion of these into aldehydes through periodinane oxidation (*e.g.*[846] β-benzyl N-Boc-D-aspartate; see also syntheses of ADDA, Refs. 444-446, 510).

The natural protein kinase inhibitor balanol contains the β-amino alcohol moiety, and combinatorial solid-phase synthesis of simple analogues from Fmoc-β-amino alcohols has been reported.[847] Prolinol and pyroglutaminol have been used in a pyrrolizidine synthesis [(-)-heliotridane].[848] A wide variety of established uses of these compounds in synthesis is extended by condensation of homochiral examples with dimethyl acetylenedicarboxylate to give, after ozonolysis, morpholine 2,3-diones of potential value in asymmetric synthesis.[849] Phosphinamides prepared from L-prolinol are effective partners in asymmetric reductions of ketones by borane.[850]

6.2.3 Reactions involving both amino and carboxy groups – The easy selective esterification of side-chain carboxy groups in aspartic and glutamic acids has been used as a demonstration of the usefulness of B,B-difluoroboroxazolidinones (114) for protecting both amino and α-carboxy groups (Vol. 26, p. 62); side-chain tert-butyl esters of these amino acids were obtained through H_3PO_4-catalysed addition to isobutene after mixing the amino acid with BF_3-Et_2O.[851] Condensation of amino acids with hexafluoroacetone achieves the same outcome (*i.e.* masking both amino and carboxy groups of an amino acid in the form of an easily-cleaved heterocycle), and also usefully activates the carboxy group so that aminoacylation can be brought about (of amines, *e.g.* for an aspartame synthesis).[852] N-(9-Phenylfluoren-9-yl)amino acid oxazolidinones give



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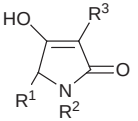
aminoketones by reaction with organolithium reagents, with negligible racemization.[853]

The powerful acylation reactivity shown by N-carboxyanhydrides (NCAs) was established in the earliest days of polymer chemistry, and their uses in peptide synthesis were recognized from the 1960s but were resisted by most practitioners. A new synthesis of NCAs under mild conditions *via* an α-isocyanato acid involves nitrosation (NO + O_2) of N-carbamyl-L- or D-amino acids.[854] Preparations and uses of N-alkoxycarbonyl-NCAs (UNCAs; now accepted as having overcome some of the problems associated with uses of NCAs in synthesis) have been reviewed,[855] and new results include the preparation of γ-amino-β-ketoesters through condensation of UNCAs with the lithium enolate of ethyl acetate,[856] alkanolysis to give N-protected amino acid esters,[857] and the formation of N-urethane-protected 3,5-dialkyl-3-aminopyrrolidin-2,4-diones under the influence of base.[858] Acyl analogues of these compounds have been known for more than 50 years as self-acylation products of 2-alkyl- or aryl-oxazol-5(4H)-ones, and an independent study providing the same information has been published.[859]

Uses of amino acids in the preparation of heterocyclic compounds,[860] and for the preparation of chiral auxiliaries for use in asymmetric synthesis,[861] have been reviewed.

More familiar heterocyclic structures are represented in studies leading to novel reaction products; these include 5-(tert-butyloxycarbonyl)oxy-oxazoles formed from N-acylamino acids and $(Boc)_2O$,[862] and 2-alkyloxy-oxazol-5(4H)-ones formed through milder cyclization conditions.[863] A Pictet-Spengler-like reaction, with interesting stereochemical details, of a 4-arylideneoxazol-5(4H)-one (an 'azlactone') with trans-(2-aminocycloalkyl)indoles opens up a route to tetrahydro-β-carbolines;[864] photodecarbonylation of 'azlactones' leads to trappable ketenimines, though this is wavelength-dependent, mild conditions causing only Z/E-isomerization.[865] Several Diels-Alder reactions have been reported for the chiral 'azlactone' (25; Refs. 180, 181), including the Danishefsky diene as a substrate, and uses for the resulting 1-aminocyclohex-2-ene 1-carboxylic acid derivatives.[866] N-Substituted 4-(aminomethylene)oxazol-5(4H)-ones provide 3-aminopyridino-4-ones when condensed with cyclic ketones,[867] and a distantly-related intramolecular cyclization *via* a mesoionic oxazol-5(4H)-one provides 4-oxo-4,5,6,7-tetrahydroindoles.[868] Homochiral tetramic acids (115) are formed through the condensation of succinimido esters of N-alkoxycarbonyl-L-amino acids with active methylene compounds.[869] The long-standing acceptance of another structure for the L-tryptophan - pyrroloquinolinequinone condensation product appears to have been put to rest (see also Ref. 615), with an imidazolo-



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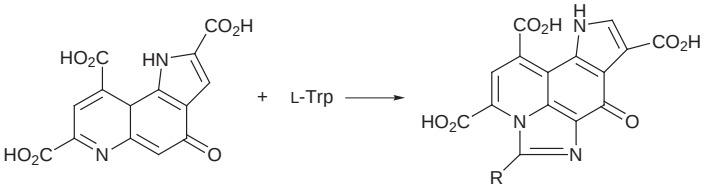


(116) R = indol-3-yl-$(CH_2)_n$; n = 0, 1

pyrroloquinoline structure (116) now assigned, and discovery of another two co-products (116; R = indol-3-yl and indol-3-ylmethyl).[870]

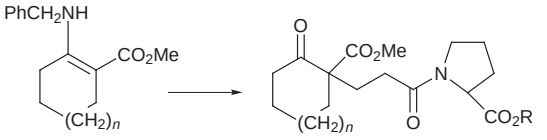
Hydantoins have been prepared by attaching an amino acid to a solid-phase benzyloxycarbonylation reagent, followed by amidation and cyclization causing detachment from the resin.[871] Adducts are formed between C_{60} and amino acid derivatives at 110-120°, but further details are not given in the *Chem. Abstr.* source of this citation;[872] however, the results no doubt correspond with reports of the formation of fulleroprolines *via* azomethine ylides[873] (also formed from glycine ethyl ester, thermally, whereas the 5-alkoxycarbonylproline analogue is formed photochemically[874]); fulleropyrrolidines have been obtained from amino acid Schiff bases *via* azomethine ylides.[875] 4-Hydroxypyrrolidin-2-ones formed from N-protected amino acids and Meldrum's acid give corresponding Δ^3-pyrrolin-2-ones as main products when preparation of O-Boc derivatives was attempted.[876] Pyrazino[2,3-e]-1,4-diazepines have been prepared through an intramolecular aza-Wittig reaction following condensation of 3-aminopyrazine-2-carboxylic acid with an α-amino acid ester, then generation of an iminophosphorane intermediate.[877]

Common L-α-amino acids have been incorporated with $NbCl_5$ or $TaCl_5$ to generate Diels-Alder catalysts, with a 2:1-ratio successfully bringing about good enantioselectivity in representative examples.[878]

The other main topic covering reactions in which both amino and carboxy groups are involved is the polymerization of amino acids to give oligo- and polypeptides. The process can be strongly accelerated by heterogeneous surfaces, *e.g.* clay minerals,[879,880] and by less subtle means, *e.g.* thermal condensation of aspartic acid - proline mixtures.[881] These studies continue a long tradition relating to interests in putative prebiotic processes, and since the product distribution resulting from thermal polymerization of amino acid mixtures is often less-than-random, these are anything but routine studies.

6.2.4 Reactions at the α-Carbon Atom of α-Amino Acids – Electrochemical α-methoxylation is one of the few synthetically feasible operations under this heading, and a thorough review has appeared[882] that also covers anodic methoxylative decarboxylation of amino acid derivatives (the Hofer–Moest reaction) to give homochiral building blocks for amino acid synthesis through diastereoselective amidoalkylation (see also Section 4.7). Boc-L-Proline methyl ester has been α′-methoxylated in this way with simple equipment[883] (see also Ref. 268).

6.2.5 Reactions Specific to β- and Higher Homologous Amino Acids – Excluding papers describing reactions at amino or carboxy groups, that are performed with higher homologues in just the same way as for the α-amino acids, the particular interest under this heading lies with uses of β-lactams and β-lactones for the synthesis of other amino acids. Uses of β-lactams for asymmetric synthesis of non-protein α-amino acids have been reviewed,[884] and further examples of Baeyer-Villiger oxidation of α-hydroxy-β-lactams to NCAs have been described[885] (see also Ref. 224). The last-mentioned study describes one-pot TEMPO conversions giving members of the threonine and azathreonine families. $TiCl_4$-Catalysed alkylation of enamines (117) by N-acryloyl-L-proline esters proceeds with high diastereoselectivity.[886] The conversion of the homologue $PhCH_2OCH_2CH(NHZ)CH{=}CHCO_2Me$, formed from L-serine,[887] into threo-aminoalcohols by iodine-mediated cyclocarbamation has been described,[888] and an analogue tethered to a solid phase has been converted into 2-oxotetrahydropiperazines.[889]



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Some reactions of β- and higher homologous amino acids fall into this Section on the basis of reactions at amino and carboxy groups, and other papers are discussed in the next Section. An example is (R)-4-trimethylammonio-3-chlorobutanoic acid, uneventfully prepared starting from (S)-carnitine (an otherwise useless industrial by-product) and converted into the corresponding enantiomerically-pure β-lactone, from which substituted (R)-carnitines have been prepared through aminolysis (see also Ref. 486).[890]

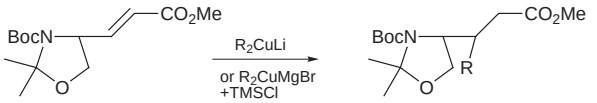
6.3 Specific Reactions of Amino Acids – As usual, this Section covers reactions of side-chains of the common amino acids, and therefore implicitly includes the use of one amino acid to synthesize another.

An extraordinary observation, that aromatic hydroxylation may be achieved through directing a blowtorch at the surface of an aqueous solution of phenylglycine or a homologue, is considered to be consistent with the intermediacy of hydroxy radicals.[891]

ω-Halogenoalkyl side-chains can be generated by direct halogenation of the common aliphatic amino acids, but reliable samples are usually prepared in other ways, *e.g.* β-halogenoalanines from serine (represented by the organozinc reagent $BocNHCH(CO_2Me)CH_2ZnI$, whose use in synthesis is illustrated by coupling with 4-iodoquinoline[892] - see also Refs. 924, 925). However, β-bromo-L-valine is accessible from L-valine, and has been used for S_N2-reaction studies to reveal neighbouring group participation by an amide

function, favouring substitution to give the β-hydroxy compound at the expense of E_2 elimination.[893]

β-Bromination of protected dehydroamino acids can be accomplished using N-bromosuccinimide, and β-acetoxylation occurs with lead tetra-acetate; the products appear to be quite stable and amenable to controlled use in synthesis, and hydrolysis and oxazolone formation, and other manipulations, have been described.[894] Bromination of methyl N-formyl-αβ-dehydroalaninate, $HCONHC(=CH_2)CO_2Me$, in this way is Z-selective.[895] Cyclization of N-(α-halogenoacetyl)-αβ-dehydroalanines with Bu_3SnH in boiling toluene gives pyroglutamic acid through a radical cyclization mechanism, best results being secured with dichloro- and trichloroacetamides,[896] and iminophosphoranes of αβ-dehydrotryptophans are easily cyclized with benzylglyoxal and decarboxylated to give eudistomins T and S, and xestomanzamine A.[897] Irradiation (>280 nm) of N-acetyl αβ-dehydro-(4-chlorophenyl)alanine esters yields 1-azetidine carboxylic acid amides and isoquinolines through 1,5-acetyl shifts.[898] 1,3-Butadiene and a chiral 2-acetamidoacrylate provide (1S,3R)-1-amino-3-hydroxycyclohexane-1-carboxylic acid through Diels-Alder addition followed by a directed hydroxylation.[899] Elaboration of the unsaturated grouping in chiral γ-amino-αβ-unsaturated esters (118) with dialkyl or diaryl cuprates leads to the corresponding β-substituted γ-amino esters with high syn-stereoselectivity, and further reactions (potassium enolate + trisyl azide; oxaziridine oxidation; Mitsunobu inversion) have been performed with clear stereochemical control.[900] Optimized procedures have been established (choice of N-protecting group; optimum Sharpless catalyst) for asymmetric dihydroxylation of chiral γ-amino-αβ-unsaturated esters.[901] N-Toluene-p-sulfinyl cis-(Z)-2-phenyl-1-(2-methoxycarbonylethenyl)aziridines exposed to $Pd(PPh_3)_4$ give the (E)-isomers through Pd-mediated ring opening.[902]



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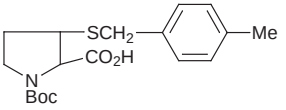
Methyl vinylglycinate and allylglycinate undergo chemo- and regioselective hydroboration by diorganoboranes, leading to homoserines, δ-hydroxynorvaline, and corresponding 2-amino-4- and 5-boronoalkanoic acids.[903] β-Acetoxyallylglycine esters undergo Pd(II)-catalysed rearrangement into δ-hydroxy-βγ-unsaturated analogues.[904] Where the side-chain C=C grouping is several atoms removed from the amino and carboxy groups of an α-amino acid, its reactions are more typical of a simple alkene, as illustrated in $OsCl_3$-catalysed oxidation to α-ketols $R^1R^2C(OH)CO(CH_2)_nCH(NHR^3)CO_2R^4$, a process used in a synthesis of (S)-5-hydroxy-4-oxonorvaline.[905] Allenic side-chains are dihydroxylated with OsO_4 to give regioisomeric α-ketols (erroneously formulated in this paper).[906] The cyclic anhydride of 4-methyleneglutamic acid yields Diels-Alder adducts with 2,3-dimethylbuta-1,3-diene,[907] and cyclopropane construction on to protected 3,4-

dehydro-L-prolines can be brought about through rhodium(II) acetate-catalysed reaction with dimethyl diazomalonate.[908]

The first synthesis of homochiral βγ-alkynylglycines $H_2NCH(C(CR)CO_2H$ has been described,[909] through elaboration of the CH_2OH function illustrating one of an increasing number of uses for synthons based on L-serine. tert-Butyldimethylsilyl ethers of N-protected serine esters can be cleaved selectively using 1% I_2 in methanol.[910] N-Fmoc-O-[(benzyloxy)hydroxyphosphinyl]-L-serine and threonine have been prepared using a new phosphite-forming reagent, $^{i}Pr_2NP(OBn)(OCH_2CCl_3)$.[911] N-Acetylation of most amino acids in the presence of an alkali metal cyanate (the Schlack-Kumpf reaction) leads to 2-acetylaminohydantoins, and with serine and threonine gives the N-acetylthiohydantoins of cysteine and β-methylcysteine respectively when ammonium thiocyanate is used.[912] This finding is the latest in a long history of the use of β-hydroxy-α-amino acids for the synthesis of their sulfur analogues, and needs rigorous proof of stereochemical details for the case of threonine. Conversion of the TBDMS ether of cis-3-hydroxy-L-prolinol into N-Boc-3-(4-methylbenzyl)thio-L-proline (119) with inversion of configuration follows established mechanistic principles.[913] N-Thioalkanoyl and alkanoyl-α-amino β-hydroxy acids have been cyclized to thiazolines and oxazolines respectively using poly-(ethyleneglycol)-supported Burgess reagent.[914] Chiral bis-oxazolines have been prepared from Z-D-serine methyl ester through carboxy group manipulation ($CO_2Me \rightarrow CMe_2OH \rightarrow CMe_2OTMS$) and N-deprotection and bis-N-acylation with dimethylmalonyl chloride, and used as ligands for incorporation into copper(I) triflate catalysts in the crucial asymmetric intramolecular cyclopropanation step in a phorbol synthesis.[915] Conversion of L-serine into enantiomers of 3-hydroxy-4-nitroprolinols, as potential enzyme inhibitors, involves distinctive routes with routine functional group transformations preceding tandem Michael-Henry condensation with nitroethene.[916]



(119)

A convenient preparation of allo-L-threonine from Boc-L-threonine based on traditional Walden inversion chemistry has been described.[917] Substitution of the hydroxy group of β-hydroxy-α-amino acids can be accomplished in numerous ways, additional to those just described, Mitsunobu sulfonation with inversion being an effective way of converting hydroxy-L-proline (as its N-benzoyl methyl ester) into its non-natural stereoisomer.[918] Intramolecular Mitsunobu lactonization of N-trityl trans-4-hydroxy-L-proline and ring opening with methanol gives cis-4-hydroxy-L-proline methyl ester,[919] while N-toluene-p-sulfonylserine tert-butyl ester gives tert-butyl aziridine-2-carboxylate through Mitsunobu processing.[920] Mitsunobu reactions with L-serine esters are free from side-reactions leading to dehydroalanine derivatives when phenylfluorenyl- and trityl-N-protec-

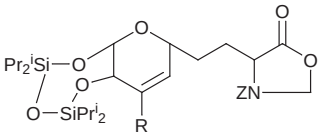
tion is used.[921] Side-chain protection (TBDMS) of (2S,3S)-3-hydroxyleucine is appropriate when it is used as its N-Fmoc acid chloride in synthesis of an A83586C fragment.[922]

N-Trityl-3-iodoalanine methyl ester, prepared from serine, can be used to prepare γ-functionalized amino acids through substitution of the halogen by malonate nucleophiles.[923] Aziridinecarboxylates are major side-products of this process, to the extent of 5 - 50%. The derived alkylzinc iodide BocNHCH(CH_2-ZnI)CO_2Me has been coupled with 1-iodoferrocene, catalysed by palladium(0) species.[924] C-3 and C-1 Adducts (*e.g.* 120) formed by β-face attack, have been obtained through the reaction with protected glucals of the alkylzinc iodide reagent derived from butyrine.[925]

N-Boc-(2S,3S,4R,4E)-2-Amino-3-hydroxy-4-methylocten-6-oic acid and N-Boc-(2S,4R)-2-amino-4-methylhexanoic acid have been prepared from the acetonide of D-serine aldehyde, which functions as a formylglycine equivalent, through $TiCl_4$-catalysed alkylation with (E)-crotylsilane and elaboration of the ethene grouping.[926] The enantiomeric Garner aldehyde derived from L-serine (121) continues to prove its worth in broad areas of organic synthesis, though the topics covered here are mostly syntheses of amino acids and near relatives. The corresponding methyl ester reacts with lithiated diethyl difluoromethylphosphonate to give β-(αα-difluoromethyl)phosphonate analogues of L-phosphoserine, L-phosphothreonine, and L-phospho-allothreonine after converting the β-hydroxy function into H,[927] and with N-benzoylglycine methyl ester, followed by hydrogenation of the resulting dehydroamino acid derivative, to give meso-2,4-diaminoglutaric acid.[928] ω-Aminosphingosine analogues have been obtained through aldolization of (121),[929] and another sphingosine synthesis from a masked form of serine, *viz* an aziridinecarboxylic acid, has been described; (2R,3R)-N-toluene-p-sulfinyl 2-alken-1-ylaziridinecarboxylate esters subjected to hydrolysis cause threo ring-opening, while, remarkably, TFAA gives the natural D-erythro-sphingosine.[930]

Homoserine has been used for a reliable L-vinylglycine synthesis ($NaBH_4$/PhSeSePh for Z-L-homoserine lactone cleavage;[931] and photoelimination from N-phthaloyl esters of homoserine and other γ-functionalized α-amino acids[932]), and in a versatile synthesis of α-substituted β-amino acids (the hydroxyethyl side-chain becomes the eventual carboxymethyl moiety).[933]

4-Hydroxy-L-proline has been used for the preparation of N-α-benzoyl-cis-4-amino-L-proline through standard steps ($OMs \rightarrow N_3 \rightarrow NH_2$);[934] the 4-mercapto analogue has been prepared through intramolecular cyclization of the same intermediate mesylate after conversion of the carboxy group into thiolcarboxylate.[935] Substitution with retention of configuration has been explored for the synthesis of acromelic acid analogues from 4-toluene-p-sulfonyloxyproline using lithium diaryl cuprates,[936] while (2S,3R,4S)-3-carboxymethyl-4-phenylproline is best prepared *via* 3,4-dehydroproline through manganese(III) acetate-catalysed oxidative radical addition of a monoalkyl malonate.[937] 4-Hydroxyproline starts the first synthesis of lycoperdic acid (122) through samarium iodide-mediated alkylation with methyl acrylate to give the proline-based spiro-γ-lactone as a mixture of epimers, followed by RuO_4-$NaIO_4$ oxidation to the pyroglutamate



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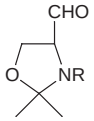

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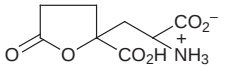


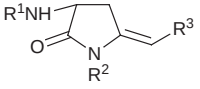
(122)

and ring cleavage.[938] Decarboxylation of 4-hydroxy-L-proline (cyclohexanol, cyclohex-2-en-1-one, 160°C, 4 h) gives (R)-3-hydroxypyrrolidine for use in a (-)-retronecanol synthesis.[939]

4-Oxo-L-proline undergoes regioselective enolization after N-phenylfluorenylation, permitting alkylation leading to β-alkylprolines,[940] and Bucherer-Bergs synthesis (see also Ref. 98) and other elaboration steps to give (2R,4S)-4-amino-4-carboxy-2-alkylpyrrolidines.[941] Chiral pyrrolidines are also accessible from 5-oxo-L-prolines (*i.e.* L-pyroglutamates) in a similar way.[942]

The di-anion of (3S,2R)-3-hydroxyproline ethyl ester has been alkylated with a variety of alkyl halides with net retention of configuration to give the α-alkylated hydroxyprolines, contributing part of the structure of microbial metabolites paraherquamide A and lactacystin.[943]

Aspartic and glutamic acid derivatives rival serine analogues for their potential applicability in amino acid synthesis, and this topic is reviewed here and also in Section 4.19. Of course, these compounds are also useful in organic synthesis outside the amino acid field, but no attempt is made here to cover this wider area in a thorough manner. Bulky esters have proved useful as β-protecting groups of aspartic acid, in minimizing the commonly-observed side reaction for aspartic acid synthons that leads to aspartimides, and 2,4-dimethyl-3-pentyl esters have been successful in this context.[944] The aspartimide derivative (123) has been used for the synthesis of 2-amino-4-oxoalkanoic acids.[945] α-Methyl Z-L-asparaginate and the glutamine analogue can be selectively hydrolysed at the amide group by tert-butyl nitrite in refluxing MeCN,[946] and used in a synthesis of pipecolic acid. Aspartic acid derivatives are involved in syntheses of the natural β-amino acid, ADDA (see Refs. 444-446), one of a number of fragments required for a convergent synthesis of microcystins and nodularins (a new approach to β-methyl-L-aspartic acid derivatives is also included in this study).[947] These preceding examples, like other β-amino acid syntheses from aspartic acid covered in Section 4.19, exploit the fact that aspartic acid is both an α- and a β-amino acid. L-Asparagine has been used to prepare the oxazolidinone (124) for use in



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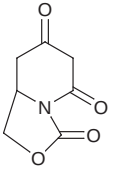
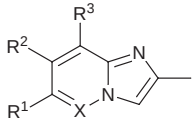


(124)

the preparation of substituted pipecolic acids (better prepared from lysine; see later),[948] and to prepare the tetrahydropyrimidinone (93) that provides a starting material for a synthesis of (R)- and (S)-α-alkyl-β-amino acids.[949] (R)-Isoserine, prepared from D-asparagine, leads to the analogue of the Garner aldehyde (121; $Me_3SnCH{=}CH$- in place of CHO) for syntheses of Caramel Colour III derivatives as potential immunomodulators.[950] Since many further applications of this type can be expected, simple modifications to the aspartyl side-chain will extend the range of synthetic targets that can be addressed. N-Protected (S)- and (R)-2,3-diaminopropanals have been prepared from L- and D-aspartic acids, respectively.[951] The elusive L-aspartic acid semialdehyde is best prepared through hydrolysis of its enol ether, (S)-2-amino-4-methoxybut-3-enoic acid.[952] The equally problematical L-aspartic acid β-chloride has been prepared in the form of its 2,2-difluoromethyloxazolidinone (*cf* Scheme 26; in other words, aspartic acid doubly protected by condensation with hexafluoroacetone), and used through Stille coupling to prepare γ-oxo-α-amino acids (4-oxo-ornithine, and 5-hydroxy-4-oxo-L-norvaline).[953] A preparation of 2-amino-4-oxobutanoic acid, from which both enantiomers of 2-amino-4,4-dichlorobutanoic acid (armentomycin) and its fluorine analogues have been prepared, has used similarly-protected aspartic acid intermediates.[954] The novel electrophilic sulfenylating agent, 2,4-dimethoxybenzyl p-toluenethiolsulfonate, has been used to generate thiols bearing an acid-labile S-protecting group, as illustrated in the preparation of (2R,3R)-3-mercaptoaspartic acid through attack by the side-chain anion of protected L-aspartic acid.[955] The heterocyclic side-chains (125) and (126) have been built on to the β-carboxy group of phthaloyl DL-aspartic acid.[956]



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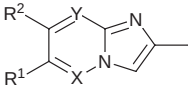
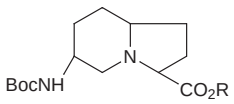


(126)

Glutamates employed in similar ways are the starting point for syntheses of 5-tert-butyl-L-proline (from γ-methyl 9-phenylfluorenyl-L-glutamate after γ-lithiation and reaction with pivaloyl chloride, then cyclization);[957] of indolizidine amino acids (127; through a similar cyclization of glutamate-derived β-keto-esters);[958] of γ-benzyl-substituted L-pyroglutamates [through palladium(0)-catalysed cross-coupling of ethyl (2S,4S)-4-(4-bromobenzyl) N-Boc-pyroglutamate with various organostannanes];[959] of δ-substituted prolines, including the δ-phosphonalkyl derivative (128), viewed as a conformationally-restricted analogue



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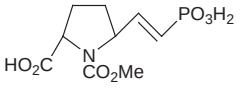
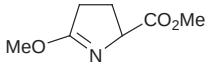


(128)

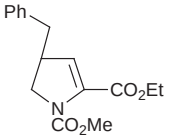
of (R)-2-amino-7-phosphonoheptanoic acid;[960] of condensed 5-arylpyrrolidin-2-ones [from the acyliminium salt of N-(N′-acetyl-N′-arylaminomethyl)pyroglutamyl chlorides formed under Friedel-Crafts conditions];[961] of substituted pipecolic acids *en route* to homopipecolic acids including streptolutine;[962] and of L-indospicine, (S)-6-amidino-2-aminohexanoic acid.[963]

For the preparation of apparently simple pyroglutamate derivatives, unusual measures are sometimes needed for best results; thus, it is claimed to be advisable to start with the imino ether (129) in order to prepare N-(4-nitrobenzyl)pyroglutamate.[964] The straightforward N-alkylation procedure with alkyl chlorides in THF is satisfactory for pyroglutamate esters, however, if a strong base (NaH) is used.[965] An unusual use as chiral auxiliary is shown in Diels-Alder additions of N-alka-1,3-dienyl-L-pyroglutamates with nitroso compounds.[966]



(129)

The ring carbonyl function plays a role in a group of uses of pyroglutamates; the protected dehydroprolinate (130; see Vol. 28, p.66), prepared from pyroglutamic acid, has been used for highly diastereoselective Michael reactions leading to all-trans-3,4-disubstituted prolines.[967] 4-Substituted 2,3-methanoprolines have also been prepared in this study, from the same starting material. 4,4-Dialkylation can be accomplished *via* the pyroglutaminol aminal (131).[968] The pyroglutamate synthon (132) derived from L-glutamic acid, and the D-serine-derived synthon (133), have been used for a synthesis of the four stereoisomers of β-benzylglutamic acid, and their β-methallyl and β-isobutyl analogues.[969]



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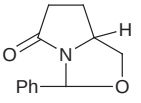

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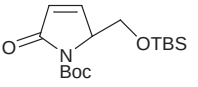

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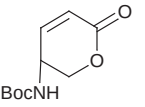


(133)

α-Acyl-N-acylglycinamides tethered to a solid phase provide a library of imidazoles through treatment with ammonium acetate.[970] The side-chain aldehyde function in methyl (1S,2R)-2-formyl-1-benzoylaminocyclopropane-1-carboxylate has been extended through standard Horner-Emmons chain extension to provide a glutamic acid homologue ($CHO \rightarrow CH_2CH_2CO_2H$),[971] and the sulfur analogue (121; S in place of ring O) of the Garner aldehyde, prepared from L-cysteine in four steps, has given segments needed for a total synthesis of curacin A through a similar chain extension.[972]

S-Nitroso L-cysteine is of enhanced interest due to the roles established for nitric oxide (though is clear that S-nitrosothiols cannot serve as *in vivo* carriers of

NO species[973]). Its formation from the two fragments is catalysed by iron(0) or by iron(II) ions,[974] more plausibly the latter species, under anaerobic conditions at neutral pH.[975] S-Nitroso-L-cysteine is stable in aqueous media within the pH range 1 - 5, and indeed is considerably more stable in higher pH solutions than previously assumed;[976] the mechanism of fragmentation into its constituents crucially involves catalysis by copper(I) species,[977] and catalysis by transition metal ions more generally.[978] Peroxynitrite ions, formed *in vivo* by reaction between superoxide and nitric oxide free radicals, can bring about cleavage of DNA and can cause protein peroxidation. The sulfur amino acids offer protection by intercepting the oxidant, but more effective still in this role are selenocysteine and selenomethionine[979] (rapidly oxidized by peroxynitrite, but only to the selenoxide[980]). In contrast with the well-known outcome for methionine, oxidation of selenomethionine with H_2O_2 in the presence of cyanogen bromide gives 2-amino-4-butyrolactone through C-Se cleavage of an unusual intermediate, Se,Se-dihydroxyselenomethionine, releasing either methaneseleninic acid or MeSeCN.[981] Fe(III)-Catalyzed oxidation of S-(aminoethyl)cysteine ketimine, a compound of long-standing interest as the product of deamination of S-aminoethylcysteine, generates radical intermediates *en route* to known products.[982]

Sulfoxides from protected S-(p-tolyl)-L-cysteine undergo highly stereospecific S_N2-type displacement of sulfinyl by OH under Pummerer reactions conditions to give threonine derivatives,[983] and protected L-methionine sulfoxides and sulfones have been cyclized with sure stereochemistry to give amino ketones and related compounds after Ramberg-Backlund rearrangement or Raney nickel desulfurization of intermediates.[984]

More conventional studies, invoking the high nucleophilicity of the thiol side-chain function, have focussed on the formation of S-α-glycosides of N-phthaloyl-L-cysteine esters using simple glycosylating agents.[985] Caesium carbonate-catalysed Michael addition of allyl Fmoc-L-cysteinate to methyl Boc-dehydroalaninate gives a protected lanthionine,[986] and analogous additions to dopaminequinone lead mainly to C-5 adducts with C-2 adducts as minor side-products.[987] Mixed disulfide formation can be achieved using $MeSO_2SCH_2CH_2OH$ as sulfenylating agent.[988] Substitution of the methionine side-chain function by bromine gives a valuable synthesis intermediate that has been used in reactions with purines (adenine, *etc.*) to give α-(β-purinylethyl)-α-amino acids.[989] Rearrangement of S-(purin-6-yl)cysteine gives the N-substituted amino acid.[990] Conversion of the side-chain of α-allyl-L-methionine (constructed *via* an oxazolidinone) into the sulfonium salt is a crucial step in a synthesis of α-aminolactams.[991]

Lysine derivatives give fluorescent 2-hydroxy-1,2-dihydropyrrolin-3-ones with aliphatic aldehydes and peroxides (Vol. 25, p.5), but so do simple aliphatic amines; therefore, the generation of fluorescence cannot be used as a reliable marker for protein degradation caused by ageing.[992] The generation of a 4-methylimidazolium salt by reaction of 1,3-di-N^{α}-hippuryl-L-lysine with methylglyoxal has been established; this must now be considered to be a potential protein modification reaction[993] (see also Vol.28, p.66). The crosslinking amino

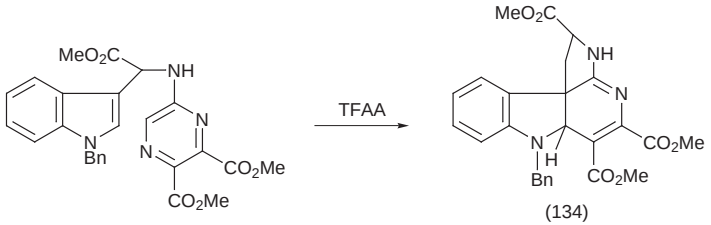
acid pentosidine is formed in mixtures of ribose, lysine and arginine, together with '$penK_2$', a crosslink found in ribose-treated proteins and formed by characteristic Maillard processing,[994] identical with a protein constituent reported earlier.[995] The lysine side-chain is implicated in the formation of an imidazolium crosslink with stoichiometry 1:2, through model reactions of N-protected amino acids with methylglyoxal, though arginine forms a pyrimidine with this compound, in a 1:1-addition process.[996] Also in the context of cell reactions, L-lysine α-aminotransferase has been shown to catalyse the first of two steps involved in the conversion of L-lysine into L-ε-aminoadipic acid.[997] Hofmann rearrangement of resin-bound aminoacyl-L-glutamine gave the corresponding (S)-2,4-diaminobutanoic acid derivative which on guanylation gave L-norarginine in the form of its dioxopiperazine (for its use as catalyst for asymmetric Strecker reactions, see Ref. 131).[998] Guanidinylation of the ornithine side-chain contained in a macrocyclic peptide has been described.[999]

The chemistry of N^{ω}-hydroxy-L-arginine, topically interesting in view of the search for nitric oxide sources *in vivo*, has been reviewed.[1000] The kinetics of degradation, under physiological conditions, of the nitric oxide synthase inhibitors N^{G}-nitro-L-arginine and its methyl ester have been determined, establishing the first step for the degradation of the ester to be conversion into the free acid.[1001]

Crystallization of N^{α}-Z-L-histidine hydrazide from water gives 2-oxo-1,3,7-triazabicyclo[4.3.0]nona-6,8-diene-4-carboxyhydrazide,[1002] through a well-known reaction of the histidine side-chain. This example is based on a surprising cyclization involving the N-protecting group, and the compound contains a ring system that is amenable to alkylation followed by ring-opening, to give 1′-alkyl-L-histidines.[1003] Isolation from proteins of amino acids modified only at histidine indicates that 4-hydroxynon-2-enal (known to react with this side-chain; see Vol. 28, p. 72) reacts solely at this site.[1004] 3-Methylhistidine can be isolated from acid-hydrolysed urine proteins but decomposition is significant if the temperature exceeds 120°.[1005]

Cyclic tautomers of tryptophan have been useful in syntheses of analogues, and their preparation and behaviour have been reviewed.[1006] Epimerization in acid, of one of the best known of these tautomers (represented by cis-1,3-disubstituted N^{β}-benzyl-1,2,3,4-tetrahydro-β-carbolines), has been shown to involve C-1 - N-2 bond scission.[1007] Studies of oxidation and inhibition of oxidation have been prominent with tryptophan, and copper chelation has been shown to protect the amino acid from the effects of pro-oxidant systems containing copper ascorbate.[1008] 5-Hydroxytryptophan gives the 2-bromo compound through electrophilic bromination,[1009] and electro-oxidation in the presence of glutathione gives not only 4-S-glutathioninyl-5-hydroxytryptophan but also 7-S-glutathioninyltryptophan-4,5-dione, the latter being formed through nucleophilic addition of thiolate to the oxidized amino acid.[1010] Schiff bases of L-tryptophan give enaminones through Mannich-Michael condensation with electron-rich siloxydienes, *en route* to highly functionalized indoloquinolizines (as in yohimbine and reserpine alkaloids).[1011] Ergot alkaloids are constructed by linking through C-4 of the indole portion of tryptophan to the glycine moiety,

and the Heck reaction has supplied the crucial means of accomplishing this in a synthesis of chanoclavine-I from 4-bromotryptophan.[1012] Intramolecular cycloaddition of an N-triazinyl-L-tryptophan employing trifluoroacetic anhydride provides a single diastereoisomer (134),[1013] while the more familiar cyclization involving aldehydes in the Pictet-Spengler reaction gives β-carbolines from abrine (N-methyl-L-tryptophan).[1014]



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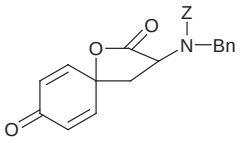
Protection of the indole grouping of tryptophan, to avoid ring-substituted side-products in synthesis, has been studied for a range of substituents in place of the N^{in}-proton, with cyclohexyloxycarbonyl proving the best in terms of stability and removeability.[1015]

Standard aromatic substitution reactions shown by L-tyrosine (see also Section 4.12) include iodination by I_2 to give the expected 3-iodotyrosine, using a liquid membrane system (I_2, KI in an aqueous phase separated from aqueous tyrosine by a solution of a crown ether in dichloroethane).[1016] Alkoxylation (BF_3/MeOH) of the L-tyrosine synthon (135) gives the 2,4-dimethoxyphenylalanine.[1017] L-Phenylalanine, p-substituted with a vicinal tricarbonyl moiety (-$COCOCO_2R$), has been prepared from Z-L-tyrosine benzyl ester through routine steps after substitution of the derived triflate with tert-butyl acrylate;[1018] this triflate also features in preparations of 4-carboxy- and 4-methoxycarbonyl-L-phenylalanines through Pd(0)-catalysed carbonylation reactions,[1019] and analogous diphenylphosphinylation.[1020] Aldol addition to p-formyl-L-phenylalanine starts a satisfactory route to L- and D-(p-phosphonofluoromethyl)-phenylalanines.[1021] Conversion of protected 3-chloro-L-tyrosine into vancomycin diaryl ether sub-units has been worked out through mild Ru-complex catalysis[1022] as an alternative to thallium(III) nitrate oxidation to form the oxygen bridge within a dipeptide formed from modified L-tyrosines.[1023] 4-Nitrophenylalanine has been used to prepare the 4-(oxomalonylamino) analogue,[1024] and to prepare 4-bis(2-hydroxyethyl)amino-L-phenylalanine for N-aminoacylation (at the latest possible stage) to allow construction of N-aminoacyl-melphalans through



(135)

replacement of OH by Cl.[1025] Pd-Mediated tert-butylthiolysis of N-Boc-4-iodo-L-phenylalanine gives a protected L-thiotyrosine.[1026] Hydrogenation of L-phenylalanine (H_2/Adams catalyst) starts a route to (S)-2-(N,N-dibenzylamino)-3-cyclohexan-1-ol, chain extension giving a core mimetic for a classic renin inhibitor.[1027]

4′-O-(Carboxymethyl)ation of trichloroethyl N-Boc-L-tyrosinate,[1028] 4′-O-(laevulinoyl)ation (then tethering to a solid phase, $NaBH_4$ deprotection and incorporation into oligonucleotides),[1029] and 4′-O-(2-fluoromalonyl)ation,[1030] have been accomplished.

6.4 Effects of Electromagnetic Radiation on Amino Acids – Mycosporin-like amino acids (*e.g.* shinorine) present in eggs of the sea urchin *Strongylocentrotus deoebachiensis* are found to exert a photoprotective role against UV damage of cell constituents;[1031] the opposite approach is seen in a study of common UV stabilizers as inhibitors of the photodegradation of tryptophan.[1032] Yet another type of study under this heading is represented by a study of the effect of isoleucine on the fluorescence of aqueous solutions of Schiff bases of pyridoxal and pyridoxal-5′-phosphate.[1033]

The riboflavin-sensitized photolysis of tryptophan and tyrosine has been reviewed;[1034] the process involves radical intermediates and leads to complex mixtures, or to dityrosines, respectively. The discovery that triplet state Rose Bengal inefficiently produces superoxide in aqueous media[1035] will have to be taken into account in the interpretation of the results of many previous studies, *e.g.* factors affecting reaction rates of Rose Bengal-promoted photo-oxidation of alkyl esters of tryptophan.[1036]

Fluorescence of N-acetyl-L-tryptophanamide is a topic of continuing interest (840 nm laser excitation has been investigated[1037]), especially fluorescence decay in reverse micelles (Vol.28, p.74),[1038] similarly for N-acetyl-β-homotyrosine methylamide[1039] and analogous N-acetyl-N′-(ω-diethylaminoalkyl)amides (with consideration of quenching by intramolecular electron transfer).[1040] A sophisticated approach to assessing triplet-state properties of tryptophan, 5-hydroxytryptophan and 7-aza-tryptophan uses optically-detected magnetic resonance in zero applied magnetic field.[1041]

Irradiation of aqueous 5-aminolaevulinic acid at 37°C leads to at least two products, 2,5-(β-carboxyethyl)dihydropyrazine and the corresponding pyrazine.[1042] Some further insights may be gained of the mode of action of this amino acid, widely-used as a photosensitising agent for cancer photochemotherapy[1043] (irradiation at 628 nm[1044]) through laboratory-based studies. Photosubstitution of N-acetyl-DL-2-chlorotyrosine in methanol gives 2-methoxylation (45%), accompanied by intramolecular cyclization to methyl 1-acetyl-6-hydroxyindoline-2-carboxylic acid (35%).[1045] Products of UV irradiation of solid lysine have been separated by gel permeation chromatography and analysed by GLC-MS (the Abstracts source of this citation does not give specific information on the reaction products).[1046]

7 Analytical Methods

7.1 Introduction – A recent text gives background information on the analysis of enantiomer mixtures in amino acid samples.[1047] Specific analytical techniques appropriate for samples from extraterrestrial and deep sea environments, relevant to theories of prebiotic chemical evolution, have been reviewed.[1048] The HPLC analysis of urine for free and total crosslinking amino acids containing the pyridinium grouping (see also Ref. 656) has been reviewed.[1049]

7.2 Gas-Liquid Chromatography – Generally applicable protocols for amino acid analysis by GLC involve derivatization and separation, followed by quantitation of the GLC effluent by conventional detectors, or by MS. More sophistication usually relates to the detection stage, and there is little to report that is particularly new this year. Derivatization of amino acid mixtures as N(O)-ethoxycarbonyl ethyl esters takes less than 10 minutes;[1050] similarly for N(O,S)-isobutoxycarbonyl methyl esters.[1051] N-Acetyl propyl esters have been used for determination of the ^{13}C-content of amino acids,[1052] and TBDMS derivatives for ^{15}N-analysis for wheat protein hydrolysates.[1053] Derivatization as N-benzyloxycarbonyl 2,2,2-trifluoroethyl esters has been advocated for the determination of enantiomer ratios for amino acid samples through separation over Chirasil-Val;[1054] derivatization is not needed for N,N-dimethylamino acids from peptide alkaloid hydrolysates[1055] but N-trifluoroacetyl esters are appropriate for enantiomeric analysis of common amino acids[1056] by GC using modified cyclodextrin CSPs (see also Refs. 741, 744, 745).

Analysis of particular amino acids has been addressed, for studies that aim to solve mechanistic problems or provide physiological information. 5-Hydroxy-2-aminovaleric acid has been proposed as a specific marker for oxidative attack at arginine and proline residues in proteins;[1057] it is formed by reduction of the γ-glutamylsemialdehyde liberated by hydrolysis of oxidized proteins, in trace amounts calling for GLC-MS-SIM analysis. Routine GLC-MS instrumentation supports attention given as in previous years, to the analysis of N-acetyl-aspartic and glutamic acids,[1058] N^{τ}-methylhistidine,[1059] S-(2-aminoethyl)-L-cysteine and other metabolites developed in cystathioninuria,[1060] S-(aminoethyl)cysteine ketimine,[1061] and S-nitroso-L-cysteine.[1062]

7.3 Ion-exchange Chromatography – Standardized protocols continue to be reported but need no discussion here; however, a thoughtful assessment of corrections for losses (or ways of avoiding losses) during sample preparation of amino acid mixtures for ion-exchange analysis, has been published.[1063]

7.4 Thin-layer Chromatography – Though this section is small enough, there are more papers than usual, perhaps reflecting a growing abandonment of snobbery when faced with a choice of this simple technique, applicable to free amino acids, rather than a more esoteric and time-consuming analytical method.

Standard practice is illustrated for the determination of proline and hydro-

xyproline in biological samples,[1064] while more sophistication is needed for fluorescence quantitation of tryptophan, 5-hydroxytryptophan, and their metabolites at 10 - 100 ng levels.[1065] Separation of arginine from citrulline has been described,[1066] and quantitation by densitometry has been applied to a variety of amino acid mixtures.[1067] Derivatization of amino acid mixtures is usually undertaken to facilitate separation and quantitation, and dabsylation is favoured for the estimation of phosphoserine, phosphothreonine, and phosphotyrosine in phosphoprotein hydrolysates,[1068] and for assessment of a multiple-development TLC procedure with dabsylated mixtures of common amino acids.[1069] Separation by TLC of dimethylaminonaphthylazobenzene-thiohydantoins prepared from amino acids has been reported.[1070]

TLC has been used for assessing binding between amino acids and a surfactant (Ref. 690).

7.5 High-performance Liquid Chromatography – Numerous amino acid analysis examples are given in a new monograph.[1071] A review of HPLC methods for amino acid analysis stresses the need for careful sample treatment and precise derivatization protocols.[1072]

In some cases, the analysis is directed at a particular amino acid that can be detected without derivatization, because of some inherent property such as radioactivity {[^{11}C-methyl]-L-methionine[1073]}, fluorescence (3-methylhistidine; λ_{excit} 260nm; $\lambda_{emission}$ 455nm;[1074] tryptophan[1075]), or UV absorption (crosslinking amino acids pentosidine - a marker for diabetes and uremia, though naturally increasing with age,[1076] pyridinoline and deoxypyridinoline in serum,[1077] elastin crosslinks aldosine and cyclopentenosine,[1078] the collagen crosslink lysylpyridinoline in urine;[1079] copper(II) complexes of amino acids at 255 nm;[1080] and 3-nitro-L-tyrosine, formed from tyrosine with NO + superoxide[1081]). Conductivity detection has been chosen for HPLC analysis of meso-alanopine and D-strombine in invertebrate muscle extracts,[1082] and for a phosphoserine assay.[1083]

Overlapping of peaks in the separation of free amino acids can be lessened by ion-pairing additives, and a dual-mode gradient ion-pair HPLC approach has been advocated that includes sodium dodecanesulfonate and perchloric acid in the eluent.[1084] Crown ether-containing phases have been applied for HPLC analysis of neurotransmitter amino acids.[1085]

Pre-column derivatization continues to be the favoured approach for general purpose amino acid analysis (also for MS analyses; see Section 5.4). The o-phthaldialdehyde - mercaptoethanol reagent system has been used with automated equipment for analysis of the glycine/aspartic acid/glutamic acid/taurine/GABA group of neurotransmitters,[1086,1087] and for enantiomer ratio determinations using OPA with a homochiral thiol.[1088] The finding that Beer's Law is obeyed by these OPA-derived isoindoles goes against received wisdom, which states that reproducible fluorescence intensities are dependent upon following rigid protocols.[1089] The more sensitive naphthalenedialdehyde - cyanide equivalent of the OPA protocol has been used for arginine analysis.[1090] Comparison of o-phthaldialdehyde - 3-mercaptopropionic acid with the Fmoc chloride reagent in the automated equipment context has led to indecisiveness in favouring either

one or the other.[1091] 6-Aminoquinolinyl derivatives show similar lowest limits to the OPA or Fmoc analogues.[1092] The OPA procedure continues to be used for clearing primary amines from samples when imino acids are the prime analytical target [*e.g.* proline and hydroxyproline[1093] analysed as their fluorescent 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl derivatives, λ_{excit} 315 nm; $\lambda_{emission}$ 385 nm].

The N-phenylthiocarbamoyl derivatives formed from amino acids with phenyl isothiocyanate through the PTA derivatization procedure are claimed to be inferior to benzylthiocarbamoyl analogues, which give better separation characteristics when tested with mixtures of up to 22 amino acids.[1094]

Dabsylation continues to deserve the confidence of long-standing advocates and newcomers, with a use described for the estimation of levels of dityrosine formed through enzyme-catalysed oxidation of tyrosine.[1095] An example of its sensitivity is shown in an application to hydrolysates of electroblotted proteins.[1096] The structurally-related N-(4-phenylazobenzyloxcarbonyl)amino acids have been recommended.[1097]

Dansylamino acids separated efficiently after establishing an appropriate micellar mobile phase, have been detected on the basis of their fluorescence[1098] or chemiluminescence [$Ru(Py)_3^{2+}$/oxalate].[1099] Like fluorescence yield, chemiluminescence intensity is subject to inhibition (the fact that amino acids have this effect on lucigenin[1100] implies that careful assessment of any proposed procedure is necessary). New reagents for amino acid derivatization have been established, indicating a growth point in sensitive fluorimetric amino acid analysis: 1,2-naphthoquinone-4-sulfonic acid,[1101] 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid[1102,1103] (the homocysteine derivative was found to be highly unstable to light)[1104] and 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (for a sensitive assay of 3-nitrotyrosine in human plasma)[1105] have been advocated. Improved protocols for HPLC of Fmoc-amino acids have been worked out,[1106] and a solid-phase 6-aminoquinolinecarbamate has been developed for derivatization of amino acids.[1107] L-Carnitine and its acyl derivatives have provided a test for HPLC analysis using an unusual derivatization protocol through the carboxy group, by dicyclohexylcarbodi-imide coupling to 1-aminoanthracene.[1108]

The determination of enantiomer ratios through HPLC over a chiral stationary phase (CSP; see also Section 5.6) has been developed vigorously (see also Refs. 741, 744, 745), and new commercial products, *e.g.* the β-cyclodextrin-based CSPs Chiradex and Cyclobond I[1109] have appeared. CSPs derived from (S)-1-(1-naphthyl)ethylamine have been evaluated, in cis- and trans-isomeric forms with the trans-form showing best performance.[1110] A CSP carrying L-cysteine has been used for the resolution of dansyl-DL-amino acids with a copper(II) salt in the mobile phase.[1111] The chiral selectivity of N-(tert-butylaminocarbonyl)-(S)-valyl- and (R)-1-(α-naphthyl)ethylaminocarbonylglycyl-aminopropylsilica towards derivatized L- and D-amino acids has been calculated and the results compared with experimental data.[1112]

An alternative approach to the determination of enantiomer ratios, employing chiral derivatizing reagents and HPLC separation of the resulting diastereoisomers, forms the basis of estimations for erythro- and threo-β-methyl-phenyl-

alanine, -tyrosine, and -1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid; comparisons of results obtained with Marfey's reagent, 1-fluoro-2,4-dinitrophenyl-5-alanine amide, with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate are presented.[1113] All amino acids present in Micropeptin 90 are of the L-configuration based on derivatization with Marfey's reagent, or with 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide, and HPLC,[1114] and the same technique has been used to establish the absolute configuration of 3-amino-6-hydroxypiperidin-2-one within aeruginopeptin 228-A.[1115] A new chiral reagent (S)-(+)-2-tert-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid, gives fluorescent derivatives wth amino acids (λ_{excit} 310nm; $\lambda_{emission}$ 380 nm),[1116] and a further example of a chiral isothiocyanate, (R)-(-)-4-(3-isothiocyanopyrrolidin-1-yl)-7-(NN-dimethylaminosulfonyl)-benz-2-oxa-1,3-diazole, has been advocated for the estimation of D-amino acid content in hydrolysed peptides.[1117] The ligand exchange principle applied to the determination of D:L-ratios for free amino acids is illustrated for HPLC over Zorbax with aqueous L-arginine - copper(II) as eluent,[1118] or the equivalent use of (+)-monoethyl N-(1′-hydroxymethyl)propyl-α-aminobenzylphosphonic acid or (-)-(R)-2-aminobutan-1-ol.[1119]

7.6 Fluorimetric Analysis – In view of the title of this Section, several citations that have been placed elsewhere in this Chapter, particularly from the preceding and next Sections, could have been located here instead. The relative fluorescence yields of members of a series of dansylamino acids have been determined, data that will assist the verification of analytical results employing these derivatives.[1120]

7.7 Capillary Zone Electrophoresis and Other Analytical Methods – The inexorable rise to preeminence of capillary zone electrophoresis (CZE) and related analytical separation techniques over the past decade, justifies its explicit mention in the title of this Section. Applications and sample preparation protocols for HPCE of amino acid mixtures are very much on a par with those used for HPLC, but substantial benefits associated in particular with sensitivity and resolution are available. A review of CZE including amino acid applications has appeared.[1121]

Typical procedures exploring new detection methods are illustrated in CZE analyses of underivatized amino acids: N-nitroso-L-arginine,[1122] identification of amino acids in the haemolymph of the fairy shrimp (UV-laser fluorescence),[1123] and pulsed electrochemical detection.[1124] Derivatives include the phenylthiohydantoin of 3-methylhistidine (UV; more sensitive than the equivalent HPLC protocol),[1125] and quantitative *in vivo* monitoring for aspartic and glutamic acids and other neuroactive amino acids as OPA-β-mercaptoethanol derivatives (laser-induced fluorescence).[1126,1127] CZE Analysis of N-acetyl derivatives of aspartic acid and of α-aminoadipic acid has been described.[1128]

Ligand exchange differentiation permits the separation of a mixture of 11 dansylamino acids by micellar electrokinetic chromatography (MEKC) when copper(II) and NN-di-decyl-β-alanine are part of the running buffer.[1129] N-Dodecanoyloxycarbonyl-L-valine serves as chiral selector for MEKC of 3,5-

dinitrobenzoyl-DL-amino acids.[1130] CZE and MEKC of protected amino acids have been reviewed.[1131]

Exploitation of indirect chemiluminescence generated by copper(II) complexes of amino acids for their detection and quantitation has not proved satisfactory, since copper(II) ions are poor catalysts for the generation of chemiluminescence from the H_2O_2 - luminol reagent; precision is no better than 3 - 6%.[1132]

Resolution of underivatized amino acid enantiomers is achieved by ligand exchange with copper(II) - L-proline or hydroxy-L-proline in the CZE buffer,[1133] and similar use of dextrin 10-sulfopropyl ether as a novel chiral buffer additive has been explored [the CZE basis of the methods is replaced by the MEKC mode with this type of additive].[1134] The detection of 0.1% L-tryptophan in a sample of the D-amino acid has been claimed through the use of a triethanolamine - H_3PO_4 buffer containing α-cyclodextrin.[1135] In a reversal of this protocol, N-p-tert-butylcalix[4]arene tetrakis(acyl-L-alanine tert-butyl ester) is effective for CZE resolution of a non-amino acid racemate.[1136] However, N-derivatization is most commonly employed for such studies and the following have been reported: resolution of a mixture of 12 dansylamino acids by CZE using a β-cyclodextrin (formamide or N-methylformamide is superior to use of a water-based eluent),[1137] of 13 N-{2-(9-anthryl)ethyloxycarbonyl}amino acids using cyclodextrins, by CZE (or, better, by MEKC; only γ-cyclodextrin offered any benefit for the CZE technique),[1138] and for the separation of 2-methyltaurine enantiomers.[1139] n-Octyl β-D-glucopyranoside has been used as pseudo-stationary phase in HPCE resolution studies with N-(alkoxycarbonyl)-DL-amino acids.[1140] A crop of papers has appeared, describing the exploration of a variety of homochiral compounds as chiral selectors for CZE resolution of N-derivatized amino acids. These studies exploit, particularly, the high purity of easily-available clinical products, including vancomycin and ristocetin A,[1141] digitonin or β-escin (resolution of PTHs).[1142] Vancomycin performs better than any cyclodextrin in this respect, when applied to the separation of aminoquinolinylcarbamates prepared from amino acids.[1143]

A range of non-chromatographic electrometric methods offers valuable opportunities for particular situations, often with superior sensitivity and reproducibility compared to established analytical procedures. Thus, a 5 fg limit has been put on electrochemical detection of neurotransmitters,[1144] and 8 ppb using gold ultramicroelectrodes,[1145] and similar results for a nickel-chromium alloy electrode,[1146] for amperometric detection of glycine and other free amino acids in a flowing electrolyte. Amino acids derivatized with 4-chloro-7-nitrobenzofuran have been subjected to cathodic stripping square wave voltammetric analysis,[1147] and the polarographic response given by amino acids has been assessed as $<1 \mu g\ mL^{-1}$.[1148]

7.8 Assays for Specific Amino Acids – Following the sophistication of the results described in preceding sections, instrumentation covered here is often rudimentary and traditional. However, the specificity often compensates for the loss of sensitivity, such as choramine T conversion of hydroxyproline into pyrrole and its quantitation through Ehrlich reagent colorimetry at 550 nm.[1149] Assay methods have been reviewed for L-arginine and its metabolites, since the central

role of this amino acid in the nitric oxide story has become a matter of special interest.[1150] A silver(I)-based electrode serves the purpose of estimation of cysteine in the presence of cystine,[1151] and an unusual principle has been exploited for the same purpose (inhibition by cysteine of the oxidation of 4-methoxy-1,2-diaminobenzene by H_2O_2/Fe^{3+}).[1152]

The assay methods depending on enzyme specificity have formed the dominant part of this section over the years, and biosensor applications continue to attract new ideas. Tetrathiofulvene-mediated enzyme electrodes (glutamate oxidase + glutaminase)[1153] and a similar L-glutamate assay system (microdialysis probe based on an immobilized enzyme reactor and coated platinum electrode)[1154] represent the advancing field, and ammonium ion-responsive electrodes that have been enzymically sensitized for the analysis of arginine or creatine (immobilized urease, and arginase or creatinase respectively) illustrate the continuing theme of this type assay based on electrochemical measurements.[1155] A system introduced earlier for the analysis of L-phenylalanine and phenylpyruvic acid, using coupled phenylalanine dehydrogenase and glutamine transaminase K, has been rendered 10 times more sensitive for the determination of phenylalanine levels in blood.[1156] Another strand is illustrated by quantitation based on the spectrophotometric determination of NAD that is released through deamination, catalysed by leucine dehydrogenase, of the three branched chain aliphatic amino acids involved in ribosomal protein synthesis.[1157]

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2
Peptide Synthesis

BY DON T. ELMORE

1 Introduction

This review cites fewer references than its predecessor[1] and at least part of the decrease is in the methodological sections. The majority of the reviews cited here[2–28] relate to Section 2 of this report; the remainder[29–46] are more akin to papers cited in the appendix to this report. The title of section 2.6 has been changed to reflect the interest in syntheses on soluble macromolecular supports and to indicate where combinatorial methods are discussed. The latter area continues to expand. For those who have not yet come to terms with the new philosophy in organic chemistry that it is sometimes desirable to produce a library of compounds in one synthetic exercise rather than making compounds singly, there are plenty of reviews[11–25] to provide an introduction.

2 Methods

2.1 Amino-group Protection – Protection of α-amino groups with the Z-group can be effected using inverse-phase transfer catalysis with ZCl as the reagent.[47] When Boc_2O is used in aqueous solution to block hindered amino acids, some reagent is hydrolysed because of the low speed of acylation. By using a lipophilic base such as $Me_4N^+OH^-$ to solubilize the amino acid in MeCN, an aqueous solution is not required.[48] Yields are very good. For the more general case of α-amino group protection using Boc_2O, ZOSu or FmocOSu, $CHONMe_2$ containing a strong acid (e.g. CF_3CO_2H, HBF_4, $4\text{-}MeC_6H_4SO_3H$) with an excess of a tertiary base with pK_a α 6 (e.g. pyridine) is recommended as solvent.[49] The same type of solvent is also recommended for peptide couplings involving Pfp esters. $SnCl_4$ rapidly removes Boc groups at room temperature;[50,51] one group[51] recommends adding phenol as scavenger. The acid-sensitive thionopeptide bond if present is unaffected by this treatment.[50] For orthogonal protection of α-amino groups and hydroxyl groups, Boc is recommended for the former and Bu^tMe_2Si for the latter.[52] The Boc group can be selectively removed with HCl/EtOAc at 25 °C. Pentafluorophenyl esters such as $CF_3COOPfp$ and FmocOPfp are recommended for the *N*-protection of amino acids.[53] As reported earlier, the use of morpholine for the removal of Fmoc groups is inclined to be slow and incomplete.[54] Piperidine functions more

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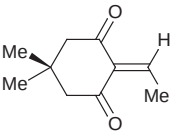
quickly and causes no β-elimination or enantiomerization of chiral centres in glycopeptides.

The 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl group (Dde) (1) is proposed as a selective and orthogonal protecting group for primary amino groups in solid-phase peptide synthesis (SPPS) using a 2-chlorotrityl resin.[55] The *N*-phthaloyl group can be removed in a two-step process, the second of which is enzyme catalysed.[56] The 5-membered ring can easily be opened in 0.2 M buffer (pH 8.0) in aqueous MeCN (Scheme 1). The second step is effected using a phthaloylamidase isolated from *Xanthobacter agilis* which had been cloned into *Streptomyces lividans*. This mild procedure could be useful, for example, in the assembly of two-chain peptides containing Lys residues when three orthogonal groups could be required. *N*-Alkylation of amino acids and their esters is not really concerned with reversible protection, but it is convenient to mention it here. Fmoc amino acids can be reductively methylated in a one-pot process.[57] The Fmoc amino acid is reacted with HCHO and the product is reduced with Et_3SiH. Reductive amination of aldehydes is reported to be satisfactory using sodium cyanoborohydride, but replacement of the latter by sodium triacetoxyborohydride gives improved yields which are usually >70%.[58] The Arndt-Eistert reaction has been proposed as a mild and selective method for protecting α-amino groups[59], but a mild method for deprotection is required before it is likely to be attractive in peptide synthesis. The amino group of phenylglycine has been protected by either of two new reagents for peptide synthesis.[60] Benzothiazole-2-sulfonyl chloride (betsyl chloride) and 5-methyl-1,3,4-thiadiazole-2-sulfonyl chloride (thisyl chloride) are attractive because their amino acid derivatives readily form the acyl chlorides with $SOCl_2$ and these couple with amino acid esters rapidly with minimal enantiomerization. The betsyl and thisyl groups can be removed reductively by a variety of methods but preferably using either 50% H_3PO_2 in aqueous tetrahydrofuran at 50-65 °C or Zn/AcOH-EtOH. Finally, a large-scale preparation of α-Boc-amino-α-Fmoc-glycine in two steps from



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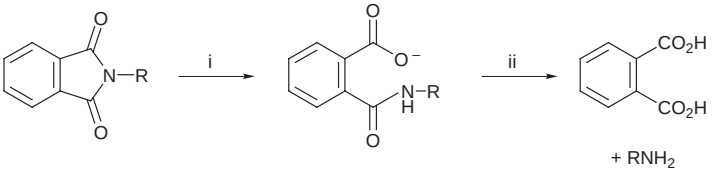
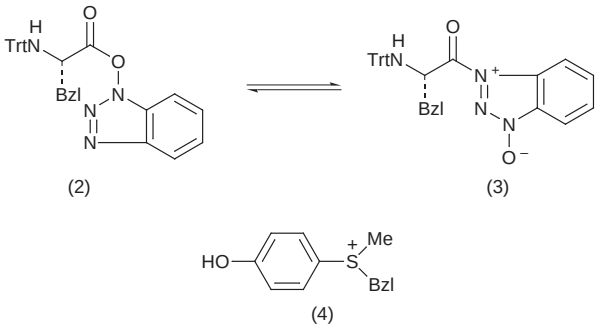


Reagents: i, 0.2M Buffer (pH 8.0), $MeCN/H_2O$; ii, Phthaloylamidase

Scheme 1

$FmOCONH_2$, $CHOCO_2H$ and $BocNH_2$ has been described.[61] The product is a useful starting material for peptide synthesis using Fmoc chemistry.

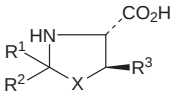
2.2 Carboxyl-group Protection – Reaction of *N*-trityl amino acids with BOP in presence of tertiary base gives a mixture of two products (2, 3) which can be separated chromatographically.[62] When (3) is reacted with 2 equivalents of $KOBu^t$ at 0 °C, the Bu^t esters of *N*-Trt amino acids generally result. Trt-Ser-OH and Trt-Thr-OH, however, afford the corresponding β-lactones. t-Butyl esters of Boc amino acids can be synthesized[63] from *N*-Boc-*N*-carboxyanhydrides by treatment with Bu^tOH in the presence of K_2CO_3 at 45 °C. This method also works with *N*-Z-*N*-carboxy-anhydrides. *N*-Acylamino acids and *N*-acylpeptides are converted into benzyl esters without racemization or enantiomerization[64] by reaction with (4-hydroxyphenyl)benzylmethylsulfonium salts (4) in the presence of K_2CO_3 in CH_2Cl_2. Since the reaction involves the formation of a benzyl cation, it is not surprising that derivatives of His, Tyr and Trp give rather lower yields of desired product. Prop-2-ynyl esters have been proposed[65] as convenient intermediates for peptide synthesis since they are easily deprotected by reaction with benzyltriethylammonium tetrathiomolybdate. A speculative mechanism is depicted in Scheme 2. 1-Adamantyl esters of unprotected amino acids can be obtained from the reaction of amino acid tosylates with 1-adamantanol and dimethyl sulfite in boiling toluene.[66] It was shown that Boc-Leu-Ala-Val-OAd could be *N*-deprotected with 4M-HCl in dioxan for 25 min at 20 °C then *C*-



Scheme 2

deprotected with CF_3CO_2H for 60 min at 20 °C. Bu^t esters can be be selectively cleaved in the presence of Bu^t ethers[67] using $CF_3SO_3SiMe_3$. Several ester groups related to Bu^t were examined as protecting groups for the β-CO_2H moiety of Asp in SPPS with a view to minimizing base-catalysed aspartimide formation[68,69]. The best results were obtained using either 3-methyl- or 2,4-dimethyl-3-pentyl esters. Phenacyl, methyl and benzyl esters of *N*-protected amino acids and dipeptides can be deesterified, and *N*-protected amino acids linked to PAM or Wang resins, but not Merrifield resin, can be liberated by heating with bis(tributyltin) oxide in $CHCl_3$, benzene or toluene.[70] Tetrabutylammonium fluoride (0.1 M) in $CHONMe_2$ is an alternative to aqueous K_2CO_3 for removal of *N*-benzhydryl-glycolamide ester groups in the solution synthesis of peptides.[71] Faster deprotection is obtained with a freshly prepared solution of reagent. *O*-Glycosylated amino acid and peptide (methoxy-ethoxy)ethyl esters can be deesterified at pH 6.6 and 37 °C using papain or lipase M (from *Mucor javanicus*).[72] Additionally, *O*-acetyl groups can be detached from the saccharide moiety by lipase WG (from wheat germ).

2.3 Side-chain Protection – The side chains of the Pfp esters of *N*-Fmoc- or N-Z-derivatives of Ser, Thr and Tyr can be protected by Bu^t groups by subjecting them to acid-catalysed reaction with isobutene.[73] The Pfp group is stable under these conditions. Boc groups attached to nitrogen on an aromatic ring or in a heterocyclic ring can be removed after adsorption on silica by subjecting the substrate to heating at ~180 °C and 0.2 mmHg.[74] This method might be useful if Boc is used to protect the indole nitrogen atom of Trp. Boc derivatives of aliphatic amines are unaffected by this treatment. ψ-Proline groups (5; X = O,S) can serve as useful protecting groups for the side chains of Ser, Thr and Cys since they tend to disrupt any tendency to form β-sheets which interferes with chain elongation in peptide synthesis.[75]

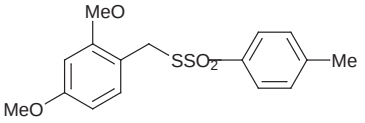


(5)

The amide nitrogen atom of Asn and Gln can be blocked by Trt groups in SPPS where Bpoc protection of amino groups is used.[76] Less than 0.1% of Trt group is removed during the standard removal of Bpoc group during 15 min in 0.5% CF_3CO_2H. A further advantage is the high solubility of Bpoc-Asn(Trt)-OPfp and the corresponding Gln derivative in CH_2Cl_2. For SPPS using Fmoc chemistry, several xanthenyl groups have been used to protect the amide groups of Asn and Gln.[77] *N*-9H-Xanthen-9-yl (Xan), *N*-2-methoxy-9H-xanthen-9-yl (2-Moxan) and *N*-3-methoxy-9H-xanthen-9-yl (3-Moxan) derivatives of α-Fmoc Asn and Gln have been prepared and used in peptide synthesis. The amide protecting groups are removed during acidolytic removal of a peptide from the support.

As an extension of their work on the protection of the β-CO_2H of Asp,[68,69] Karlström and Undén have designed and tested the N^{im}-(2,4,dimethylpent-3-yloxycarbonyl) (Doc) group for protecting the side chain of His.[78] The τ-*N*-atom is the site of substitution. It is resistant to nucleophiles but readily removed by HF. Perhaps more promising is the discovery that the 1-adamantyloxymethyl group, which is introduced via the corresponding chloride, blocks the π-*N*-atom.[79,80] It is easily removed by CF_3CO_2H, but is stable to 20% piperidine so it could be useful in SPPS involving Fmoc chemistry. The Doc group can be used to protect the indole ring of Trp. The group is introduced by reaction of Boc-Trp-OBzl with Doc-Cl in presence of DMAP or $KOBu^t$. The former base effects reaction without racemization but inconveniently slowly. Reaction is rapid in presence of $KOBu^t$ but there is some loss of chiral purity. The group is stable to 20% piperidine, somewhat labile to 50% CF_3CO_2H and rapidly cleaved by a cocktail containing CF_3CO_2H and CF_3SO_3H. The Doc^{in} group provides excellent protection against alkylation during removal of Bu^t groups. The N^{in}-allyloxycarbonyl group may be marginally more useful for protection of the indole ring[82] since it is orthogonal to both the Fmoc and Boc groups. It is introduced into Boc-Trp-OBu^t using allyl chloroformate in presence of DBU and a little DMAP. The N^{in}-Alloc group is removed with $Pd^0(Ph_3P)_4$. The successful synthesis of dynorphin A(1-13) and phosphorylated sleep inducing peptide suggest that it is worthy of a wider and more searching test. The N^{in}-cyclo-hexyloxycarbonyl group is another group for the protection of the Trp side chain.[83] It is stable during SPPS with Boc chemistry but is cleaved with HF.

A new electrophilic sulfenylating reagent, (2,4-dimethoxy-benzylthio)-4-methylbenzenesulfonate (6), has the novelty that it introduces a thiol group bearing an acid-labile protecting group.[84] The Asp derivative (7) is an example and this was incorporated into a tripeptide required in a study of the enzyme isopenicillin N synthase. The introduction of a thiol group in this way could provide a site for introducing a reporter group or a powerful metal-ligating group in synthetic peptides. The 4-methoxytrityl group (Mmt) has been used to protect the thiol group of Fmoc-Cys-OH[85] for SPPS. The Mmt group is considerably more acid-labile than the Trt group being removed with 0.5-1% CF_3CO_2H. This procedure was used to synthesize a somatostatin analogue.



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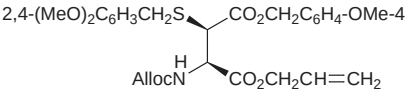


(7)

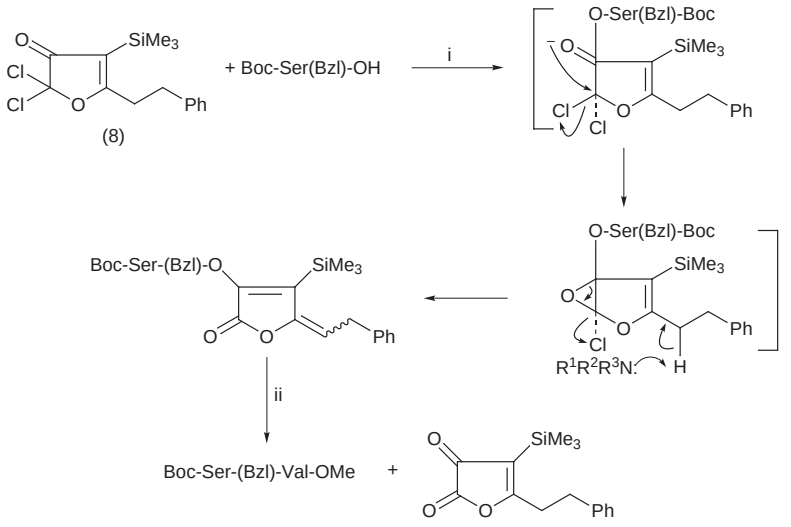
2.4 Disulfide Bond Formation – The oxidation of a nonapeptide containing two cysteine residues with $K_3Fe(CN)_6$ at pH 8 gave mainly a mixture of parallel and antiparallel dimers in aqueous solution but when MeOH was incorporated in the solvent, the cyclic monomer predominated.[86] Temporary blockage of the thiol group in cysteine peptides using 2,2′-dithiobis(5-nitropyridine) gives an inter-

mediate unsymmetrical disulfide that can react with another cysteine derivative to give an unsymmetrical cystine peptide.[87] An undecapeptide sequence of HIV-2 gp41 was produced[88] by oxidation of the bis-Acm peptide using I_2/CH_3CO_2H. Cystine peptides have been obtained directly from protected peptidyl resins using $Me_3SiCl/Me_2SO/CF_3CO_2H$. This treatment simultaneously removed protecting groups, formed disulfide bonds and detached the peptide from the resin.[89] A peptide containing a cystinyl-cystinyl-cystine motif has been synthesized in order to study the conformation.[90] The dithiasuccinoyl (Dts) group was proposed some time ago as a protecting group for α-amino groups. Dts-Gly has now been used as an oxidising agent to promote the formation of intramolecular disulfide bonds.[91]

2.5 Peptide Bond Formation – Diethylaminosulfur trifluoride (DAST) is preferable to other fluorinating agents such as cyanuric fluoride for the preparation of Fmoc amino acid fluorides[92], since the isolation of product is simpler. Fmoc amino acid fluorides couple rather slowly with extremely hindered amino acid derivatives, but this problem can be overcome[93] using a previously published method in which the free amino group is silylated with, e.g., *N,O*-bis(trimethylsilyl)acetamide before attempted coupling. This procedure also prevents the slow loss of Fmoc group.

The use of a symmetrical anhydride such as Boc_2O in the presence of NH_4HCO_3 and pyridine converts a carboxylic acid into its amide.[94] The synthesis of dipeptide derivatives from 4-nitrophenyl esters is reported[95] to proceed faster in frozen dioxan at −18 °C than at 40 °C. It would be interesting to know if more reactive components such as Pfp esters or acyl fluorides behave similarly and if the acceleration merits the extra step of freezing. *N*-Hydroxypyridine-2(1H)-thione esters couple with amino acid esters or the Nps derivatives of the latter to give good yields of peptides.[96] Coupling with Nps derivatives is preferred since neutral conditions are used.

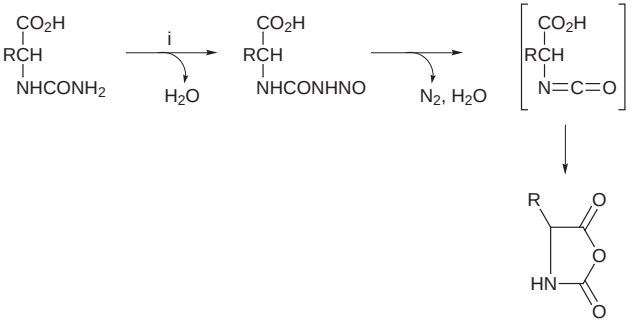
The use of highly hindered tertiary bases in couplings using onium reagents has been thoroughly studied[97]; 2,6-di-t-butyl-4-dimethylaminopyridine is now the preferred teriary base to use. *O*-(7-Azabenzotriazol-1-yl)-*N*-phenyl-*N,N′*,N′-trimethyluronium hexafluorophosphate has been synthesized as a potentially useful coupling uronium-type reagent[98] but a thorough assessment of its performance is required. 1-(2-Naphthylsulfonyloxy)-6-nitrobenzotriazole has been used earlier for solution-phase synthesis and has now been successfully employed in SPPS.[99] 1-Cyano-1,2,4-triazole has been used to couple phthaloylglycine and glycine methyl ester[100], but syntheses are required with chiral components in order to assess its potential value. BOP-Cl in the presence of Boc-aminomonothioacids has been used for the synthesis of thionopeptides.[101] An intriguing new peptide coupling reagent, 2,2-dichloro-5-(2-phenylethyl)-4-(trimethylsilyl)-3-furanone (8) (Scheme 3) has been reported.[102] There are three interesting points about this reagent: (a) couplings can be carried out in aqueous solvents, (b) no loss of chirality was observed during coupling and (c) dipeptides can be made directly from free amino acids.



Reagents: i, $R^1R^2R^3N$; ii, H–Val–OMe

Scheme 3

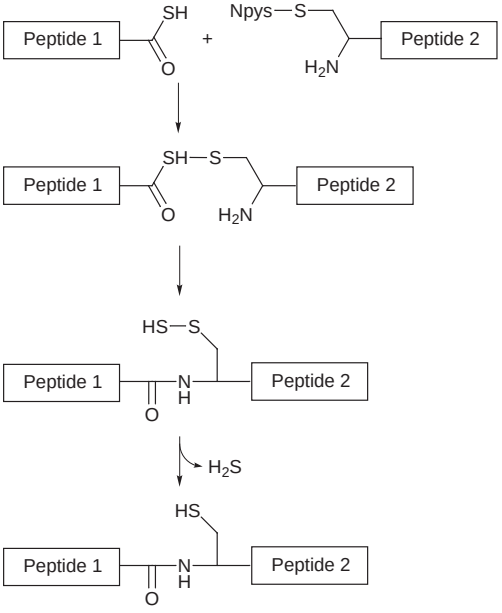
A new synthesis[103] of chirally pure oxazolid-2,5-diones (*N*-carboxyanhydrides) involves nitrosation of α-*N*-carbamoylamino acids with a mixture of NO and O_2 (Scheme 4). A less versatile synthesis starts from α-hydroxy-β-lactams in which C_4 is quaternary.[104] Nevertheless, this provides a simple method of synthesizing conformationally restricted peptides. Seventy Fmoc, Z and Boc *N*-substituted *N*-carboxyanhydrides have been synthesized and characterized.[105] Pyridine is recommended as the base to be used if Boc_2O is used to *N*-acylate a carboxyanhydride, whereas *N*-methylmorpholine is recommended for acylations involving chloro-formates.



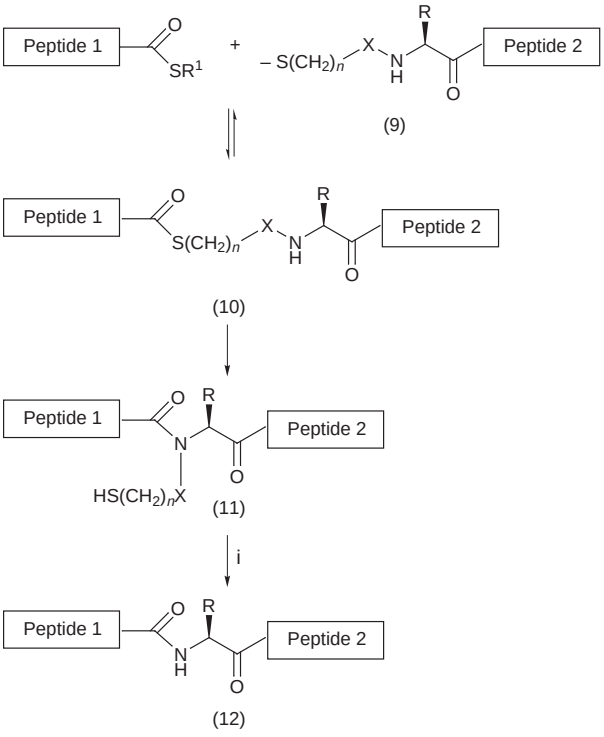
Reagents: i, NO, O_2

Scheme 4

Capillary electrophoresis in presence of 18-crown-6-tetra-carboxylic acid is a sensitive method for determining the rate of enantiomerization during peptide coupling reactions.[106] In the Spot method of multiple peptide synthesis, diisopropylcarbodiimide/HOBt is recommended; yields are better than with Pfp esters and no enantiomerization was detected.[107] Further support for carbodiimide coupling has been accumulated from a study of fragment coupling.[108] The test system involved Fmoc-Asp(OBu^t)-Phe-OH and H-Lys(Boc)-Pepsyn KA. Best results were again obtained with diisopropylcarbodiimide/HOBt. The outcome was underlined by the successful synthesis of a 29-residue peptide from a 13-residue *N*-terminal fragment and a 16-residue *C*-terminal fragment. A detailed quantitative study of the degree of enantiomerization resulting from the coupling of Z-Ala-Xaa-OH with H-Val-OMe has been carried out.[109] In studies mainly with *N*-cyclohexyl-*N*′-(2-morpholinoethyl)carbodiimide methosulfate, it was shown that enantiomerization of the *O*-acylisourea or oxazol-5(4H)-one can both occur. With Z-Ala-Xaa-OH (Xaa = Trp, Lys(Z) or Met) enantiomerization occurs mainly through the oxazolone, whereas the converse is true when Xaa = Cys(Bzl), Ser(Bzl), Ser, Asn or Gln. Workers in the peptide synthetic field could benefit from the accumulation of more of this kind of data, for example examining the effect of solvent, structure of carbodiimide and structures of both protected dipeptide and free amino ester. In order to couple Fmoc-Cys(Trt)-OH in SPPS with minimal enantiomerization, the symmetrical anhydride procedure is recommended.[110] Detection of loss of chiral purity in cysteine residues during peptide assembly can be



Scheme 5



Reagent: i, Zn/H^+

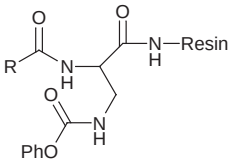
Scheme 6

achieved by reaction of the thiol group with 4-vinylpyridine. After acid hydrolysis of a sample of peptide, the cysteine-containing enantiomers can be separated by capillary electrophoresis in presence of crown ethers.[111] Coupling of a thioester of Boc-Ala-Ala-OH with H-Gly-OBzl in the presence of *N*-hydroxysuccinimide and *N*-methyl-morpholine is accompanied by a disappointing degree of enantiomerization even in tetrahydrofuran as solvent.[112] The proclivity of Asp and Asn residues to undergo enantiomerization via succinimide intermediates has been explained theoretically from an *ab initio* quantum mechanical study. The enhanced acidity of the imide >NH group is the cause of the problem.[113] A method of fragment coupling which involves the formation of an intermediate *S*-acyldisulfide[114] has been described (Scheme 5). The latter undergoes intramolecular rearrangement to form a peptide bond and this second intermediate is reduced to the required peptide with loss of H_2S. This method requires the availability of peptides containing an *N*-terminal β-thiol-α-amino acid and the work by Baldwin's group[84] described above offers an appropriate route. A similar method[115] (Scheme 6) is an extension of an earlier publication from Kent's group and is slightly more restrictive in the

choice of terminal residues, but potentially more versatile in another sense since it could give rise to peptides with *N*-substituents on the newly formed peptide bond. A peptide α-thiol ester reacts via a thiol exchange reaction with either an α-*N*-(ethanethiol)peptide (9; X = CH_2) or an α-*N*-(oxyethanethiol)-peptide (9; X = O) to give a ligation product (10). This rearranges spontaneously to give (11). If X = S, (11) can be reduced to (12).

2.6 Peptide Synthesis on Macromolecular Supports and Methods of Combinatorial Synthesis – New supports for peptide synthesis continue to be examined. Chitosan has high swelling ability and a high content of free amino groups.[116] Moreover, it has passed the classic test for the synthesis of ACP(65-74).[117] In this latter paper, it is reported that coupling yields can be increased by working at 55 °C rather than room temperature. A large-pore polydimethylacrylamide resin has been produced by the copolymerization of *NN*-dimethylacrylamide, *NN′*-bisacryloyl-1,3-diaminopropane and *N*-methacryloyl-1,3-diaminopropane;[118] the capacity was 0.66 mmoles/g. A new polymethylacrylamide resin with high loading capacity has also been produced.[119] The realization that increasing the hydrophilicity of supports is one way of diminishing the difficulties associated with the synthesis of hydrophobic peptides has resulted in the synthesis of polymers which incorporate glycols[120] or are based on polyethyleneglycol (PEG).[121–123] Some supports are designed to be used in the liquid phase.[124,125] One kind of support has been designed which has high fluidity and high loading capacity.[126] This support is of special interest because it is possible to monitor the stepwise coupling of amino acids by ^{13}C NMR spectroscopy.

The Dpr(Phoc) linker (13) has been examined[127] using Fmoc chemistry. Difficulties were encountered when piperidine was used to remove Fmoc groups, since amide formation occurred at the Phoc group. It was important to retain the Phoc group until the *C*-terminal tripeptide had been assembled in order to avoid diketopiperazine formation. The test peptide had a sequence of 6 Pro residues at the *C*-terminus so the latter was a real danger. Morpholine was used to remove the Fmoc group after the first two coupling stages then the Phoc group was cyclized to (14) using PhONa/PhOH in $CHONMe_2$. Thereafter, standard Fmoc SPPS was used. The 4-carboxy-trityl[128] and 4-carboxy-4′-cyanotrityl[129] linkers have been used. The latter has the advantage that the linker has better acid stability than the linker lacking the nitrile group. Thymosin β_{10} was synthesized using Fmoc chemistry. Xanthenyl-amide linkers (15) (n = 1,4) permit the synthesis of *C*-terminal amides under very mild conditions.[130] Thus, peptides could be detached using 1-5% (v/v) CF_3CO_2H while most protecting groups were



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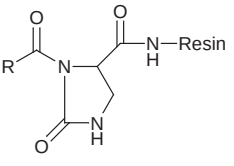
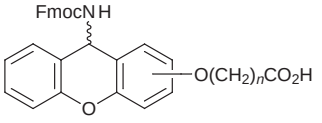


(14)

retained and Trp residues were not alkylated. *N*-Aminoalkyl- amides have been obtained using an allyl linker (16).[131] After coupling to the resin, the Trt group is removed and the free hydroxyl group is esterified with 4-nitrophenyl chloroformate. The 4-nitrophenyl group is reacted with the monotrityl derivative of a diamino alkane. The Trt group is removed and a peptide is assembled on the amino group. The peptide derivative is detached using $(Ph_3P)_2PdCl_2$/$[Me(CH_2)_3]_3SnH$. *N*-Sulfonamido hydroxamic acids have been prepared[132] by first modifying the Wang resin by the Mitsunobu reaction to give a resin-bound *O*-hydroxylamine and then coupling *N*-blocked amino acids on the amino group. An old safety-catch method involving the alkylation of a sulfonamido group due to Kenner and Sheppard has been adapted and improved for SPPS.[133] A photolabile linker with a safety catch (17) has been designed.[134] Photolysis does not occur until the 1,3-dithian has been hydrolysed to the benzoin derivative. Another photolabile linker has been used for assembling combinatorial libraries of carboxylic acids[135] but not, so far, libraries of peptides.



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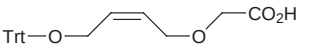

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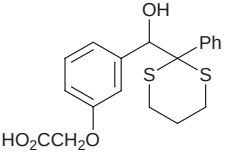

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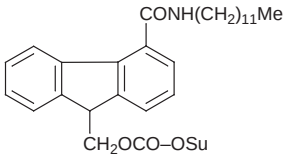
The Hmb group continues to be studied and exploited. This group is now clearly a most important development in SPPS. A method for introducing the Hmb group consists of the reductive alkylation of the amino group of a resin-bound amino acid or peptide using 2-hydroxy-4-methoxybenzaldehyde.[136] This facilitates the introduction of the Hmb group at any point in a peptide synthesis. The choice of the location of Hmb groups is important. Thus, in the synthesis of a subunit of human $\alpha_{1E\text{-}3}$ calcium channel protein[137] (Ac-NSLMVSRGSGLAGGLDEADTC-NH_2), protection of Gly^8 and Leu^{15} gave unsatisfactory results, whereas protection of Gly^{10} and Leu^{15} afforded success. For the synthesis of HIV-1_{Bru}*tat* (1-72), 5 fragments containing 11-17 residues were prepared with AcHmb groups in selected places[138] and the fragments were chosen so that Gly was *C*-terminal in 4 of them. The Hmb group has also been used in the synthesis of phosphopeptides via post-assembly global phosphorylation.[139] It was necessary to protect the hydroxyl group of the Hmb moiety to prevent its phosphorylation and the resultant irreversible stabilization to acidolysis. Following the phosphorylation, the protecting group on the Hmb moiety

(Ac or Alloc) was removed by hydrazinolysis or Pd-catalysed cleavage to restore the acid lability of the Hmb group. The AcHmb group has been used to protect aspartyl bonds in order to prevent aspartimide formation during assembly of a peptide and also during attachment of a glycoside moiety.[140] An alternative approach to overcoming the difficulties associated with the synthesis of hydrophobic peptides employs a hydrophilic tail between the resin and the hydrophobic peptide during assembly and subsequent detachment of this hydrophilic tail.[141] For example, in the synthesis of H-$(Ala)_{12}$-OH, the sequence $HOCH_2CO$-[Gly-Arg(Tos)]$_4$-Gly- was assembled on PAM resin followed by the attachment of 12 Ala residues. Deprotection and detachment of the depsipeptide was followed by mild basic hydrolysis to release the dodecapeptide. A control synthesis of H-$(Ala)_8$-Gly-PAM resin showed difficult coupling began at the stage of attaching the sixth Ala residue. This approach was also used to synthesize a fragment of a chemotactic protein. It has been shown that SPPS of peptides by the fragment condensation method is successful if side chains of Lys and Arg residues are unprotected provided that a crown ether is present to form a noncovalent complex with the latter.[142] Thus, an excellent yield of product was obtained from the coupling of Fmoc-Phe-Arg-Ala-Lys-Ala-Gly-OH and H-Pro-Asp(OBu^t)-Leu-Tyr(Bu^t)-resin in the presence of 18-crown-6. Some new points concerning coupling methodology in SPPS have been reported.[143] In order to avoid formation of diketopiperazine during attachment of the third residue on a Wang type resin, it is suggested that Trt be used for amino group protection before attachment of the third residue since it can be removed by very mild acid treatment. Subsequently, using Fmoc chemistry, coupling can be carried out without neutralization using 7-azabenzotriazol-1-yloxytris-(pyrrolidino)phosphonium hexafluorophosphate (PyAOP). Coupling difficulties encountered with α-*C*-methylamino acids can be alleviated if the amino function on the resin is first silylated using Me_3SiCl and coupling is effected with either HATU or DCCI/HOBt.[144] A drastic decrease in the volume of solvent used in the coupling stage is recommended.[145] Only the volume of solvent included in the swollen resin is required. As a result, coupling is accelerated, economy of solvent use is achieved and multiple peptide synthesizers are possible with effectively no reaction vessels. A protocol has been proposed for the synthesis of small proteins which permits a one-step purification pro-cedure.[146] Boc chemistry with HBTU/HOBt coupling is recommended. On completion of assembly, the lipophilic moiety (18) is attached to act as a chromatographic probe and the protein is purified by a fast one-step reverse-phase chromatography. A chaperonin (101 residues) was synthesized by this technique. The solvation properties of resins and various peptide-resins



(18)

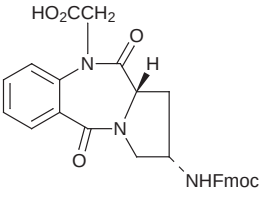
correlated better with the sum of solvent electron-acceptor and electron-donor numbers than with other other solvent polarity parameters.[147] Plots of resin swelling versus the sum of electron acceptor and donor numbers allowed the determination of maximum solvation region characteristic for each class of resin. The Ugi 4-component method of peptide bond synthesis has been applied to the assembly of a library of 96 amino acid amides and dipeptides.[148]

There is little new to report on deprotection and detachment procedures. The C=N bond of resin-bound peptide-oximes can be selectively reduced with concomitant deprotection and detachment using trialkylsilanes in CF_3CO_2H.[149] Acidolytic cleavage of peptides bearing a *C*-terminal Cys(Acm) residue from Wang resin can lead to removal of the Acm group and subsequent formation of a disulfide bond.[150] Best results were obtained with little or no water and with an added scavenger. When detaching bromo- or chloro-acetylated peptides from resins, the haloacetyl group remains intact using HF containing 3-cresol and one of the following: thiophenol, 3-thiocresol or 1,2-ethanedithiol.[151] The preferred method for detaching peptides from a photolabile nitro-resin involves treatment with HI/CF_3SO_3H followed by 0.2M-NaOH/90% EtOH.[152]

Some improved hardware, software and analytical methods have been described. Using commercial hardware, software was modified to permit resin mixing by bubbling nitrogen through it.[153] A multiple automated robotic synthesizer has been constructed which can synthesize 5-15 peptides consecutively.[154] A robotic synthesizer for assembling peptide and pseudopeptide libraries has been designed.[155] The infrared spectra of single resin beads have greatly improved resolution if the bead is flattened.[156] Particular attention was paid to differentiating νC=O modes of resin-bound compounds with minor structural differences. Several resins were examined by this method. High resolution ^{1}H NMR spectra can be obtained on peptides bound to polystyrene resins by the use of magic-angle spinning combined with high-resolution magic-angle spinning probes.[157] Alternatively, peptides bound to a resin through a photolabile linker can be analysed by MALDI mass spectrometry.[158] Time-of-flight secondary ion mass spectrometry of peptides on resin beads can distinguish between positive ions emanating from the *N*-terminus and negative ions characteristic of the *C*-terminal region.[159]

Examples of the application of SPPS to the combinatorial assembly of peptide libraries are given next. A library of peptides containing one of three possible 3-phosphino-2-amino acids was constructed and Rh was complexed to the phosphine ligands on the resin and screened for ability to catalyse the hydrogenation of methyl 2-acetamidoacrylate.[160] An aminomethyl resin was derivatized with three linkers which, after combinatorial synthesis, permitted stepwise detachment of peptides in media of increasing acidity. One of the qualitatively identical liberated portions of the library could be used for Edman sequencing while the other two could be screened for potential pharmacological properties.[161] A pentapeptide library was constructed and screened for peptides that bound a nonapeptide segment of the CNS dopamine D2 receptor.[162] Similarly, peptide libraries were screened for potential ligands of elastase, renin and thermolysin.[163] The converse of this approach involved the assembly of large libraries of potential

ligands for peptides.[164] Each component of the potential ligand library contained a dyestuff to facilitate examination of interaction with peptides. By linking the β-carboxyl group of Boc-Asp-OFm as a benzyl ester to an insoluble support, libraries of cyclic peptides of various sizes have been constructed.[165] The pharmacophores (19 and 20) have been incorporated into peptide libraries.[166] A library of 57500 compounds of the general type $NH_2CHR^1CONR^2CHR^3$-$CONHR^4$ has been assembled by solid-phase methodology in combination with *N*-alkylation.[167] A commercial, hydrophilic, polyhydroxylated methacrylate resin has been used as the basis for the assembly of a peptide library.[168] An amino group was introduced by coupling $NH_2CH_2CH_2NH_2$ to free carboxyl groups and a commercial linker, 4-[(R,S)-α-[1-(9H-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]phenoxyacetic acid, was coupled using PyBOP and *N*-methylmorpholine. In a novel approach to the construction and coding of a peptide library, peptide substrates of proteinases were attached to PEG-polystyrene beads. Those substrate molecules near the surface are accessible to enzyme and are cleaved, whereas those in the interior are not. Subsequent syntheses with Boc and Fmoc protecting groups allows the generation of two structures on the same bead. Those molecules near the surface are available for receptor interactions while those in the interior are not but are available for coding.[169] A library of 10^5 peptides was assembled and good ligands were found for proteins such as anti-β-endorphin antibody, streptavidin and thrombin. Molecular tags containing stable isotopes can be incorporated into the resin beads.[170] The tag can contain a single isotopic atom or can be quite complex. The tag can be read by mass spectrometry after cleavage from the resin. A similar approach uses deuterium-labelled protecting groups.[171] Calculation of the first-derivative C-D stretching absorbances relative to the resin backbone derivative absorbances permits the determination of chemical yields in solid-phase reactions involving changes in deuterium content. In view of the gradual increase in interest in soluble-phase syntheses on macromolecular supports, it is not surprising that this principle has been applied to the assembly of peptide libraries.[172] The molecules (21 and 22) can be ring-



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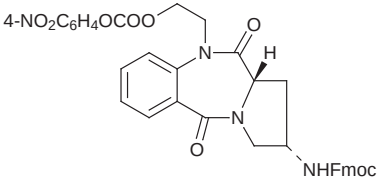

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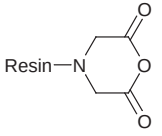

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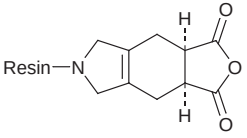

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opened by various nucleophiles and a library of peptides can be constructed making use of the liberated carboxyl group. Finally, a theoretical strategy has been developed[173] to arrive at optimal molecular leads with the generation of rather small libraries. This philosophy was used to identify stromelysin inhibitors and is likely to be exploited extensively in the future.

There are some miscellaneous aspects including side reactions of SPPS that should be mentioned. When Fmoc-Asp-OAll was used in the attempted synthesis of a cyclopeptide, a further example of aspartimide formation was encountered leading to a linear peptide with the same molecular weight as the desired cyclic peptide.[174] Transesterification of the benzyl ester group was observed in the attachment of Boc-Glu(OBzl)-OH to resin using $Me_4{}^+NOH^-$ as catalyst.[175] Further, capping with $(CH_3CO)_2O$ of a resin containing an Arg(NO_2) residue led to formation of Arg(Ac) in the product and formylation of His and Lys side chains can occur during detachment of a peptide from resin using hydrogenation catalysed by $Pd(OAc)_2$ in $CHONMe_2$. This is a salutary tale for those who claim that SPPS has no further surprises or difficulties to present. If Boc-Asp-OFm is attached to a resin via the β-carboxyl group in order to prepare cyclopeptides, enantiomerization of the Asp residue can occur.[176] It is recommended that coupling of the Asp derivative to the 4-bromomethylphenylacetyl derivative of MBHA resin should be effected in presence of either $CsHCO_3$ at pH 7 in $CHONMe_2$ at 50 °C overnight or in presence of $ZnCO_3$ in $CHONMe_2$ at room temperature during 45 min.

Several papers extend the possibilities of SPPS. Thiono-peptides can be synthesized from *N*-protected aminomonothio acids using 6-nitrobenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate.[177] A pentaaza-analogue of Leu enkephalin has been synthesized on a PEG support.[178] Unnatural amino acids can be introduced into peptides by *C*-alkylation of an *N*-terminal Gly residue on a resin when the Gly residue has been converted into a Schiff base using benzophenone.[179] The nonionic organic bases ('Schwesinger bases') are recommended for the alkylation step. The Schiff base must be hydrolysed after the alkylation step and before the next amino acid is coupled. The SPPS of peptides containing a Phe residue bearing a 4-$COCOCO_2R$ substituent has been described.[180] β-Sulfonopeptides can be prepared, although the reaction conditions must be carefully optimized.[181,182] 1-(1′-Adamantyl)-1-methylethoxycarbonyl is recommended for protecting the ε-amino group of Lys because it is more easily removed than Boc; premature detachment of the peptide from the resin is thus avoided.[183]

2.7 Enzyme-mediated Synthesis and Semisynthesis – Immobilization and insolubilization of enzymes continues to attract attention. Although α-chymotrypsin which has been crosslinked with glutaraldehyde is much less active than the native enzyme, it is still popular because it is more stable in high concentrations of organic solvents.[184] Alternatively, chymotrypsin can be supported on polyamide-based resins and used in solvents such as MeCN or CH_3CO_2Et.[185] In a more novel approach, chymotrypsin can be electrochemically adsorbed on a Pt electrode and then covered by a thin membrane of polypyrrole.[186] The enzyme is

still active and K_{eq} shifts towards synthesis by 25% due to the hydrophobic environment. Using Ac-Phe-OEt and H-Ala-NH_2 as substrates, a positive potential (0.4-0.8 V versus Ag/AgCl) was applied to the electrode. This enhanced both the synthetic and hydrolytic activities of the immobilized enzyme with the former being most affected. With 0.2 M substrates, a 73% yield of peptide was obtained. This technique will surely attract considerable attention. Thermolysin has been immobilized on dextran which contains a small number of carboxyl groups.[187] The enzyme was used to catalyse the synthesis of Boc-Asp-Phe-OMe in a 2-phase system containing PEG and dextran to force phase separation. The effects exerted by the medium have been further defined. The synthesis of H-Gly-Gly-Phe-NH_2 using papain in saline media is influenced by enzyme denaturation, changes in kinetic control and increasing salting-out power as salt concentration is increased.[188] The relationship between product yield and nature of anion and cation parallels the Hofmeister lyotropic series. Further evidence has been adduced supporting the concept that soluble or immobilized proteinases afford excellent yields of small peptides in water-miscible solvents containing small amounts of water.[189–191] Organic solvents were suitable for the synthesis of dipeptide fragments of CCK-8[192] and for the acylation of amino acids by aminoacylase.[193] The consequences of adding very small quantities of certain anionic detergents has been further studied.[194] Sodium dodecyl sulfate and Aerosol OT solubilize proteinases in organic solvents. For example, complexes of α-chymotrypsin with Aerosol OT are about 1000 times more active than enzyme which has been simply suspended in EtOH. Somewhat surprisingly, pepsin catalysed the synthesis of several hydrophobic octa- and deca-peptides in 4.3 M urea solution containing 27% $CHONMe_2$ at pH 4.5.[195] Pepsin also afforded high yields of product from the coupling of Z-Ala-Ala-Phe-OH and H-Trp-Ala-Leu-Ala-Phe-OMe in 6 M guanidine hydrochloride or 0.5% sodium dodecyl sulfate. The use of frozen solutions during peptide synthesis continues to be intriguing as well as practically useful. For example, coupling of Ac-Tyr-OEt and H-Arg-OPr gave 88% of product, but this does not appear to be explained by a freeze-concentration model because there is a simultaneous, dramatic change in substrate specificity at the S′ site.[196] Syntheses using $Na_2CO_3.10H_2O$ as the only source of water look promising. Leu-enkephalin has been synthesized[197] using thermolysin, chymotrypsin and papain to catalyse peptide bond formation and subtilisin to de-esterify a tripeptide intermediate. The choice of species for the source of an enzyme is a variable that has received little attention. Thus, only 9 out of 25 commercial lipases studied can hydrolyse ethyl L-2-amino-4-phenylbutyrate without affecting the D-ester.[198] The effect of species source of proteinases on peptide bond formation deserves examination.

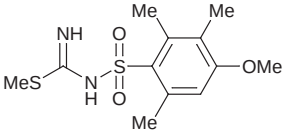
The substrate specificity of proteinases in peptide bond formation continues to be studied. *C*-Allylglycine (Ag) is recognized by subtilisin since H-Ag-Phe-Phe-Ag-OEt gives rise to the dimeric linear octamer.[199] Subtilisin also accepts unnatural L-amino acids in coupling reactions with chiral amines to give (S,S)-alkylamides.[200] Since chymotrypsin operates via the Ping Pong mechanism, it is to expected that the use of a powerful acylating agent will favour peptide bond formation. Improved yields are obtained by using 2,2,2-trifluoroethyl esters as

acylating agents. Thus, Z-Phe-OCH_2CF_3 and H-Leu-NH_2 gave a 98% yield of the dipeptide amide in presence of chymotrypsin,[201] whereas Z-Phe-OMe gave only 31% under similar conditions. In a related paper,[202] it has been reported that papain and lipase P catalyse stereoselective transesterification of *N*-protected amino acid or peptide alkyl esters in the presence of an oxime to give an oxime ester which can then suffer nucleophilic attack to form a new peptide bond. Clearly, reaction is favoured by the formation of the more reactive intermediate ester. The mild conditions used in enzyme-catalysed peptide synthesis enable rather sensitive substrates to be used without risk of degradation. Several dipeptide derivatives of γ-carboxyglutamic acid have been produced in high yield by papain-catalysed coupling reactions.[203] The use of the so-called inverse substrates such 4-guanidinophenyl esters in trypsin-catalysed reactions has been extended.[204,205] The method is useful for making peptides of D-amino acids. Nonproteolytic enzymes can be useful in peptide synthesis, especially for removing suitable protecting groups. Thus penicillin G acylase can remove *N*-protecting groups such as *N*-phenylacetyl[206] or *N*-(4-phenylacetoxy)benzyloxycarbonyl[207] without affecting peptide bonds, *C*-terminal ester groups or phosphate groups.

There are some relevant kinetic studies to report. The coupling of Z-Asp-OH and H-Phe-OMe catalysed by thermolysin in homogeneous solution kinetically accords with the Theorell-Chance mechanism.[208] The kinetics of coupling of Z-Leu-OH or Z-Phe-OH with H-Gly-NH_2 in aqueous organic solvent in the presence of thermolysin or carboxypeptidase Y were studied taking into account the solvent-induced deactivation of the enzyme.[209] Lineweaver-Burk plots for the thermolysin-catalysed synthesis of dipeptide amides in aqueous MeCN are linear for the carboxyl component.[210] Similar plots for the amino components, however, showed apparent substrate inhibition when the concentration of MeCN reached 70% (v/v). Analysis of K_m and k_{cat} for both components indicated that the mechanism is Random Bi Bi at 40% (v/v) MeCN. A useful kinetic variable for enzyme-catalysed peptide synthesis in partly aqueous solution is the *p* value which is defined as the nucleophile concentration at which the rates of aminolysis and hydrolysis of the intermediate acyl enzyme are equal.[211] The variation of *p* with temperature was studied for the synthesis of various dipeptide amides in the presence of chymo-trypsin. Crown ethers exert an interesting kinetic effect on chymotrypsin and subtilisin. Transesterification is accelerated by crown ethers in organic solvents and this effect is even more noticeable if the enzymes are freeze-dried in the presence of crown ether.[212] When chymotrypsin is conjugated to PEG, the relative positions of the 'ar', 'am' and 'h' subsites are unaltered.[213] Finally, the double mutant of subtilisin, subtiloligase (P221C,P225A), has been further studied as a catalyst for peptide synthesis.[214,215]

2.8 Miscellaneous Reactions Related to Peptide Synthesis – Peptide bonds involving the carbonyl group derived from an *N*-alkyl amino acid residue are reported to be labile to CF_3CO_2H during SPPS.[216] During the SPPS of peptides with a protected Cys residue at the *C*-terminus, the use of piperidine to remove an Fmoc group on the *N*-terminus can cause a base-catalysed β-elimination

giving a dehydroalanine residue.[217] The latter can react with piperidine to give a residue of 3-(1-piperidinyl)-alanine. The problem of δ-lactam formation when synthesizing Arg dipeptides has been examined in some detail.[218] Various groups such as Tos, Boc_2 and Pmc were used to protect the guanidino group. It was shown that the use of the Boc_2 protection and coupling by means of unsymmetrical anhydrides resulted in the greatest amount of δ-lactam, but no protecting group or coupling process completely suppresses this side reaction. With this information to hand, it is pertinent to ask the question would enzymic coupling with trypsin or post-coupling guanidination of synthetic Orn peptides offer better prospects of obtaining higher yields of products that could be more easily purified? The latter possibility is perhaps more relevant than hitherto since two new reagents (23, 24) have been designed[219] for the conversion of amino groups into guanidine derivatives. The results were generally satisfactory, but it appears that no attempt was made to guanidinate peptides that were still attached to the resin following SPPS. There is some ambivalence about the tendency for Asp residues to form aminosuccinyl residues. Mostly it is an annoying side reaction, especially since some enantiomerization can occur. On the other hand, the amino succinyl residues can give rise to analogues of naturally occurring peptides which may be of pharmacological interest. Experiments have been reported that help to clarify these side reactions.[220] A general method for synthesizing peptides containing α,β-diamino acids has been described[221] (Scheme 7). A method for the synthesis of amides and peptides from carboxylic and azides involving two redox reactions has been further studied.[222] The observation that urethane-protected *N*-carboxyanhydrides can undergo base-catalysed dimerization in aprotic solvents could undermine a method of peptide synthesis that has hitherto looked attractive.[223] Finally, when Fmoc-Tyr(PO_3H_2) is used in SPPS, intramolecular formation of pyrophosphate can occur, but only if two such residues are adjacent.[224] This result could have been anticipated, since Todd's group showed over 40 years ago that nucleoside-5′-phosphates formed symmetrical pyrophosphates when treated with carbodiimides or $(CF_3CO)_2O$.



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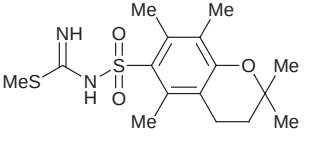

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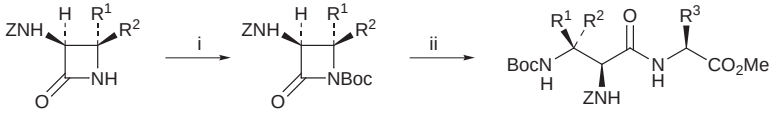

Reagents: i, $(Boc)_2O$, DMAP in MeCN; ii, (*S*)-$NH_2CHR^3CO_2Me$

Scheme 7

3 Appendix: A List of Syntheses Reported Mainly in 1996

Peptide/protein *Ref.*

3.1 Natural Peptides, Proteins and Partial Sequences

ACTH
- Linear and cyclic *N*-terminal sequence 225

Adipokinetic hormone
- Synthesis of (^{3}H-pGlu^{1})-AKH-1 226

Adrenomedullin
- Fragments (1-19 and 20-52) and their coupling 227

Amyloid protein (chromosome 20)
- *N*-Terminal decapeptide sequences 228
- SPPS of β-amyloid (1-42) 229

Angiotensin
- Cyclic analogues of angiotensin II 230
- Lipopeptide analogues of angiotensin II 231

Antibacterial peptides
- Amphipathic peptides based on Leu, Lys and Ala 232
- Gramicidin S analogues 233–235
- Bacitracin A 236
- *C*-Terminal pentapeptide from efrapeptin C 237
- 11-Residue basic fragment of bovine lactoferrin 238

Antitumour peptides/proteins
- Octapeptides related to DNA contacting loop of p53 protein 239
- Distamycin derivatives 240
- QSAR of tallimustine derivatives 241
- Sarcolysin antitumour peptide 242

Apolipoprotein
- Human apolipoprotein A-II fragment (18–30) 243

Axinastatins
- Linear precursors and their cyclization 244

Bacterial proteins
- Peptides from *E. coli* pilus proteins 245

Blood coagulation factors/inhibitors
- Bovine prothrombin fragment (35–62) 246
- Tick anticoagulant peptide (TAP) 247

Bombyxin
- Zwitterhormon derivatives 248

Bradykinin/Kallidin
- Bradykinin receptor antagonists 249–251
- Kallidin analogues 252

Brazzein
- Sweet protein from *pentadiplandra brazzein* 253

3.2 Sequential Oligo- and Poly-peptides

3.3 Enzyme Substrates and Inhibitors

3.4 Conformations of Synthetic Peptides

3.5 Glycopeptides

3.6 Phosphopeptides and Related Compounds

3.7 Immunogenic Peptides

3.8 Nucleopeptides, PNAs

3.9 Miscellaneous Peptides

3.10 Purification Methods

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3
Analogue and Conformational Studies on Peptide 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] Most of the publications covered in this chapter were published in 1996. However, some of the 1995 publications not covered last year have been included. No work published in patents or in unrefereed form (such as conference proceedings) has been included. The section on biologically active peptides has been expanded to cover non-peptide ligands acting on the peptide receptors. This is becoming an important aspect of peptide research because in the recent years many non-peptide compounds have been shown to act as antagonists of the endogenous ligand. In some cases (e.g. angiotensin II and CCK), the non-peptide ligands have also been shown to have agonist properties. 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. A final section dealing with the advances in formulation and delivery technology has been included. Throughout this chapter, amino acids are referred 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

To increase the metabolic stability and to alter the conformational preferences of peptides, synthetic routes to various peptide bond replacement moieties have been reported.[2–14] In addition, a number of heterocyclic ring systems have been described which can lead to conformationally restricted peptides with increased metabolic stability. Some of these residues, if suitably substituted, can act as di- or tri-peptide replacements. In other cases, the non-peptide moiety acts to induce the types of conformations (e.g. β- and γ-turns and β-bends) which a flexible peptide can attain due to the presence of certain amino acid residues. Syntheses of 2-isoxazoline, indolizidinone, oxazole, thiazole, diketopiperazine, lactams and substituted proline derivatives have been reported.[12–15]

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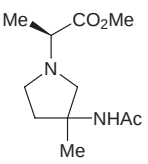
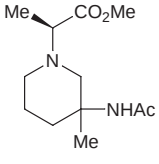
2.1 Ψ[CH_2NH]-Aminomethylene, Ψ[CH(CN)NH]-Cyanomethyleneamino and Ψ[CH_2O]-Ether Analogues – Synthetic routes to conformationally restricted dipeptide isosteres of Ala-Ala [(**1**) and (**2**)] containing a reduced peptide bond (CH_2NH) are reported. As shown in structures (**1**) and (**2**), the conformation of the dipeptide was restricted by linking the α-carbon of the amino acid to the NH group by two or three methylene groups.[2] Aminomethylene pseudopeptide analogues of a histone peptide [Cys-Gly-Gly-Ile-Arg-Gly-Glu-Arg-Ala] have been reported.[3] To assess the effect of peptide bond replacement on the antigenic and immunogenic properties of peptides, each of the peptide bonds was replaced in turn by a Ψ[CH_2NH] group. The reduced amide bonds were incorporated in the peptides by reductive amination of the required Boc-amino aldehydes in the presence of $NaBH_3CN$. Three of the analogues, Cys-Gly-Gly-IleΨ[CH_2NH]Arg-Gly-Glu-Arg-Ala, Cys-Gly-Gly-Ile-ArgΨ[CH_2NH]Gly-Glu-Arg-Ala and Cys-Gly-Gly-Ile-Arg-GlyΨ[CH_2NH]Glu-Arg-Ala, cross-reacted strongly with the antibodies generated against the parent peptide or protein H3. The remaining two peptides, Cys-Gly-Gly-Ile-Arg-Gly-GluΨ[CH_2NH]Arg-Ala and Cys-Gly-Gly-Ile-Arg-Gly-Glu-ArgΨ[CH_2NH]Ala did not react with any of the antibodies. The aminomethylene analogues were much more stable to trypsin than the parent peptide.

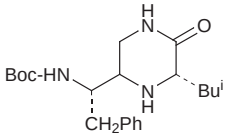


(1)

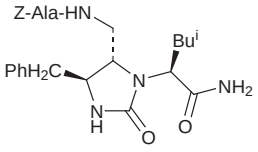


(2)

The synthetic routes to cyanomethyleneamino Ψ[CH(CN)NH] pseudopeptides have been reported in the past. These pseudopeptides can be reduced to give Ψ[$CH(CH_2NH_2)NH$] derivatives.[4] The newly generated amino group can be used to synthesise branched chain peptides or cyclic peptides. Conformationally restricted scaffolds like 2-oxopiperazines (**3**) and 2-oxoimidazolidines (**4**) were also synthesised from the ψ[$CH(CH_2NH_2)NH$] containing peptides.



(3)

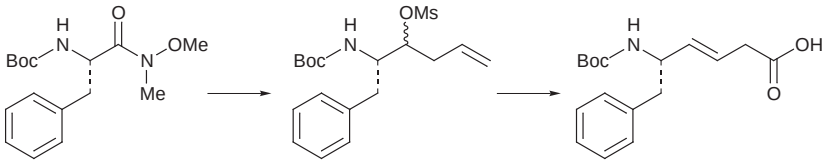


(4)

A new synthesis of the ψ[CH_2O] pseudopeptides from N-protected-5-substituted morpholin-3-ones has been reported.[5] The morpholin-3-ones were prepared in two steps from the corresponding amino alcohols by treatment with ethyl

chloroacetate, followed by protection of the amide by *p*-methoxybenzyl group. The morpholin-3-one was alkylated using activated (benzyl bromide and allyl bromide) as well as unactivated (cyclohexylmethyl bromide and phenethyl bromide) alkyl halides. Removal of the protecting group followed by acid hydrolysis gave the Ψ[CH_2O] pseudopeptides. If methanol was added to the acidic reaction mixture before concentration, the corresponding methyl esters of the Ψ[CH_2O] pseudopeptides were obtained.

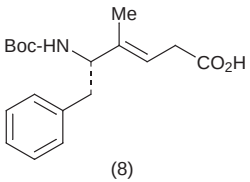
2.2 Ψ[CH=CH]-Isosteres and Related Analogues – Various routes for the synthesis of Ψ[CH=CH] and Ψ[C≡C] analogues have been reported. Synthesis of Boc-PheΨ[E-CH=CH]Gly-OH is described starting from Boc-Phe (Scheme 1).[6] Using propylphosphinic anhydride, Boc-Phe was converted into the N-methoxy-N-methyl derivative (**5**) which was then converted into the corresponding unsaturated homoallylic ketone by reacting with allyl magnesium bromide. Subsequent reduction gave the homoallylic alcohol which was converted into mesylate (**6**). β-Elimination of the mesyloxy (MsO) group of Boc-NHCH(CH_2Ph)CH(OMs)CH_2CH=CH_2 gave a conjugated diene. The resulting diene was converted into the corresponding PheΨ[E-CH=CH]Gly isostere (**7**) by subsequent hydroboration and Jones oxidation.



Scheme 1 Synthesis of Boc-Pheψ[*E*-CH═CH]Gly-OH

γ-Methyl-E-olefin isostere (**8**) of Phe-Gly dipeptide was synthesised starting from Boc-Phe.[7] The methylketone derivative of Boc-Phe, [Boc-NH-CH(CH_2Ph)-CO-CH_3] on reaction with diethyl-(3-trimethylsilyl-2-propynyl)-phosphonate gave a mixture of E/Z enyne (4:1) which was separated by HPLC. Hydroboration of the E enyne with dicyclohexylborane followed by oxidation using H_2O_2 gave the required pseudopeptide isostere Boc-PheΨ[E-CMe=CH]Gly-OH. Racemisation occurred during the olefination reaction.

Synthesis of a 5-amino-3-pentynoic acid derivative for incorporation into



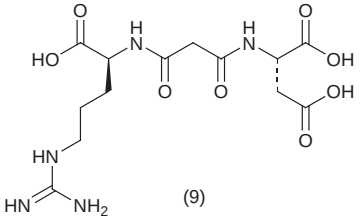
peptides, Z-NH-CH_2-C≡C-CH_2-COOH, as a Ψ[C≡C] analogue of Gly-Gly has been reported.[8] Activation of Z-NH-CH_2-C≡C-CH_2-COOH by the mixed isobutyl carbonic anhydride method only gave poor yields of the peptides. However, use of EDCI coupling method gave satisfactory yields. Using this procedure, the acetylenic dipeptide mimic was incorporated in a hexapeptide derivative Z-Tyr(3-I)-Thr-Leu-NH-CH_2-C≡C-CH_2-CO-Phe-Ala-Val-OMe.

Analogues of bombesin(6-14)nonapeptide containing Ψ[E-CH=CH] isosteres have been reported.[9] One of these analogues containing a LeuΨ[E-CH=CH]Leu isostere at the C-terminus, D-Phe-Gln-Trp-Ala-Val-Gly-His-LeuΨ[E-CH=CH]Leu-NH_2, did not show any agonist activity (amylase release from rat dispersed pancreatic acini) up to a concentration of 100 nM. However, the nonapeptide derivative inhibited amylase release in a dose-dependent manner (IC_{50} 4.6 nM). Three other pseudopeptide analogues, D-Phe-Gln-Trp-Ala-Val-Gly-His-LeuΨ[E-CH=CH]D-Leu-NH_2, D-Phe-Gln-Trp-Ala-Val-Gly-His-ValΨ[E-CH=CH]Leu-NH_2, and D-Phe-Gln-Trp-Ala-Val-Gly-His-ValΨ[E-CH=CH]D-Leu-NH_2, were moderate to weak agonists in the *in vitro* assay (EC_{50} values 7.7, 15.4 and >30 nM, respectively). In addition to demonstrating agonist activity, the pseudopeptide D-Phe-Gln-Trp-Ala-Val-Gly-His-ValΨ[E-CH=CH]Leu-NH_2 also antagonised amylase release (IC_{50} 100 nM). The D-Leu containing pseudopeptides D-Phe-Gln-Trp-Ala-Val-Gly-His-LeuΨ[E-CH=CH]-D-Leu-NH_2 and D-Phe-Gln-Trp-Ala-Val-Gly-His-ValΨ[E-CH=CH]D-Leu-NH_2 were very weak antagonists (IC_{50} values 10 μM and 1mM, respectively).

2.3 Ψ[$COCH_2$]-Ketomethylene, α-Hydroxy Ketomethylene and Ketovinyl Isosteres – A stereoselective route for the synthesis of ketomethylene dipeptide isosteres containing an aspartic acid residue, Boc-NH-CH(R)-$COCH_2$-CH(CH_2COO*t*-Bu)-$COOCH_2$Ph (R = CMe_3, $CHMe_2$, CH_2CHMe_2, CH_2Ph, Me), has been reported.[10] Boc-amino acid methyl esters were first converted into the ketophosphonates, Boc-NH-CH(R)-$COCH_2$-P(O)$(OMe)_2$, by treatment with lithium dimethyl methylphosphonate and then reacted with benzyl glyoxylate to give Boc-NH-CH(R)-CO-CH=CH-$COOCH_2$Ph. Stereoselective addition of allyl *tert*-butyl malonate to the Michael acceptors yielded the suitably protected malonate adducts. Deallylation followed by decarboxylation of the malonate derivatives gave the desired dipeptide isosteres. Synthetic routes to ketovinyl, (E)-H_2N-CH_2-CO-CH=CH-CO_2H, and α-hydroxy ketomethylene dipeptide isosteres have been described.[11,12] Conformational preferences of ketovinyl dipeptide isostere, investigated by *ab initio* calculations of model compound (E)-H_2NCH_2COCH=$CHCO_2H$, indicated that the S-cis,S-cis conformer is the most stable, suggesting that these isostere can be used as building blocks for β-sheet structures.[11]

2.4 Retro and Retro-inverso Pseudo Peptides – Retro and retro-inverso analogue of the platelet aggregation inhibitory peptide Arg-Gly-Asp have been reported.[13] The two peptides [(**9**) and (**10**)] were synthesised by reacting $Asp(OBzl)_2$ and Arg(Mts)-OBzl sequentially with malonic acid using diphenylphosphorylazide. Compared to the tetrapeptide Arg-Gly-Asp-Ser (100% inhibi-

tion at 1 mg/ml), the modified peptides (**9**) and (**10**) were less potent (57 and 53% inhibition) inhibitors of ADP-induced platelet aggregation. However, the modified peptides were more potent than the tetrapeptide in inhibiting lung metastasis induced by B16-BL6 melanoma cells.



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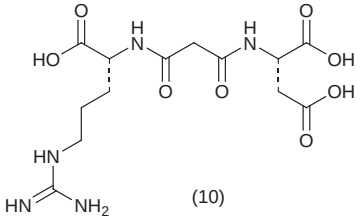

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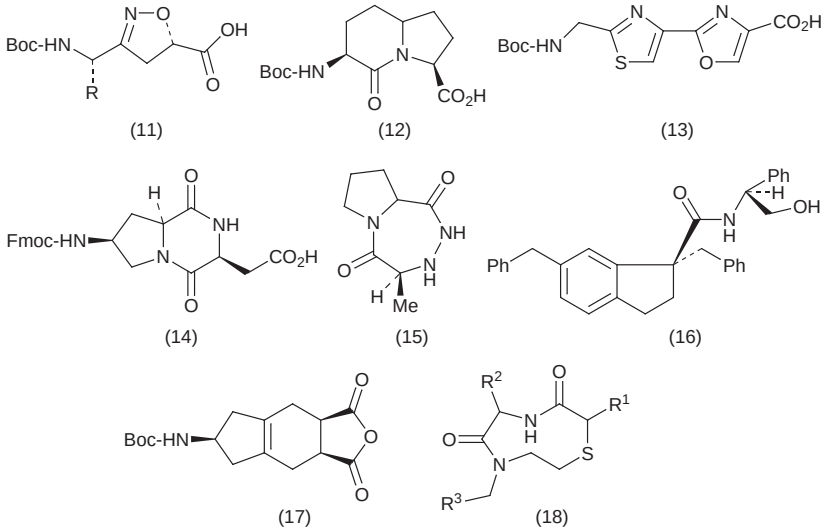
2.5 Azapeptides – Using the routes established in the literature, protected hydrazine derivatives, Boc-NH-NH(R)-OPfp (pentafluorophenyl esters), were synthesised and coupled on a poly(ethylene glycol) monomethyl ether support to generate peptides containing α-aza-amino acids.[14] As an example, an all-aza [Leu[5]]enkephalin analogue, AzTyr-AzGly-AzGly-AzPhe-NH-NH-$CH_2CH(CH_3)_2$, was synthesised. The all-aza-peptide hydrazide did not compete with the natural ligand for binding to a monoclonal antibody 3-E7 which was generated against β-endorphin and shown to bind [Leu]enkephalin. One of the reasons for the lack of activity may be the altered conformation of the peptide. [Leu]enkephalin has been reported to have a β-turn and the aza-peptide is expected to have an extended conformation.

2.6 Rigid Di-, Tri-peptide and Turn Mimetics – In addition to the peptide bond replacements mentioned above, a number of heterocyclic systems have been described which could be inserted within the peptide chain to alter the conformations of the biologically active peptides. Some of these residues, if suitably substituted, can act as di- or tri-peptide replacements. In other cases, the non-peptide moiety acts to induce the types of conformations (e.g. β- and γ-bends and α-helical turns) which a flexible peptide can attain due to the presence of certain amino acid residues. Some aspects of peptidomimetic research have recently been reviewed.[15–17] Syntheses of a number of ring systems (**11-18**) suitable for incorporation into peptides, e.g. 2-isoxazoline (**11**) and indolizidinone (**12**), oxazole, thiazole, bisthiazole, oxazolyl-thiazole, thiazolyl-oxazole (**13**), diketopiperazine (**14**), 1,2,5-triazepine-3,6-diones (**15**), 1,1,6-trisubstituted indanes (**16**) and lactam derivatives have been reported.[12,18–27] A number of these derivatives were synthesised starting from amino acid derivatives. For example, indolizidinone derivative (**12**) was synthesised starting from glutamic acid and oxazole- and thiazole-containing derivatives were prepared starting from glycinamide. Template **17** is an anhydride and, as such does not require any orthogonal protecting group. After reaction with an amine, it can be used as an amino acid derivative.[21]

The diketopiperazine template (**14**) was used to synthesise c(Ala[1]-Asn[2]-Pro[3]-



Asn[4]-Ala[5]-Ala[6]-template) and c(Ala[1]-Arg[2]-Gly[3]-Asp[4]-template). The conformational properties of both these peptides were investigated by NMR in aqueous solution. A highly populated type-I β-turn conformation within the Asn-Pro-Asn-Ala motif was observed in the case of c(Ala[1]-Asn[2]-Pro[3]-Asn[4]-Ala[5]-Ala[6]-template) peptide. The Arg-Gly-Asp peptide displayed modest antagonistic activity towards both the integrin αIIb/β3 and αv/β3 receptors. The 1,1,6-trisubstituted indane derivative (**16**) was designed as an α-helix mimic containing benzyl and indolyl groups in appropriate positions required for neurokinin antagonist activity.[27] The X-ray crystallographic data indicated the presence of an α-helical conformation. However, compound (**16**) showed only a modest binding affinity at the NK_1 receptor (IC_{50} 3.6 μM). The binding affinity was comparable to the linear dipeptide derivative Z-Trp-Phe-NH_2. Synthesis of a cyclic peptide containing a β-turn mimetic (3S,6S,9R)-2-oxo-3-amino-7-thia-1-azabicyclo[4.3.0]nonane-9-carboxylic acid reported earlier, c(Gly-Leu-Asp-Val-BTD), has been published.[28,29] The solution structure of c(Gly-Leu-Asp-Val-BTD) was determined by two-dimensional ^{1}H-NMR and systematic conformational searching combined with molecular dynamics studies. The peptide was shown to contain two hydrogen bonds between the Gly and Val residues, and a type I β-turn with Leu and Asp at the (i + 1) and (i + 2) positions of the turn. The cyclic peptide was found to be a modest inhibitor of VCAM-1/VLA-4 (integrin $\alpha_4\beta_1$) interaction.

3 Cyclic Peptides

Conformationally restricted cyclic peptide analogues of biologically active peptides are included in the sections dealing with individual peptides (section 4).

Sequences of naturally occurring cyclic peptides, their biological activities and some of the conformational studies using cyclic peptides are mentioned below.

3.1 Conformational Studies – Conformational studies using various spectroscopic techniques (e.g. NMR and X-ray) and theoretical methods have been reported on side chain to C-terminal,[30] N- to C-terminal,[30–34] and side chain to side chain cyclic[35–37] and disulfide bridge containing peptides.[38,39] In the case of N- to C-terminally linked compounds, a combination of 1D NMR and molecular mechanics calculation techniques was shown to reproduce the results obtained previously using other techniques.[30] The five cyclic peptides used in the study were an endothelin antagonist [c(Leu-D-Trp-D-Asp-Pro-D-Val)], three α-amylase inhibitors [c(Phe-Ala-Trp-Arg-Tyr-Pro), c(Ala-Ser-Trp-Arg-Tyr-Pro) and c(Ala-Trp-Arg-Tyr-BTD)] and a renin inhibitor [2-naphthylpropionyl-Glu-Sta-Leu-NH-CH_2-C_6H_4-CH_2NH-] containing a linking group between the side chain carboxyl of the Glu residue and the C-terminal Leu residue.

X-ray diffraction and NMR methods were used to study the conformational preferences in peptides containing Aib, β-alanine and δ-aminovaleric acid residues. In the solid state, the Aib containing cyclic peptide c(Gly-Aib-Gly-Gly-Aib-Gly) adopted a conformation with one β-turn (type I) and its mirror image at the other side of the ring.[31] The β-alanine containing cyclic pentapeptide c(Pro-Phe-Phe-β-Ala-β-Ala) adopted a conformation characterised by a *cis* β-Ala^5-pro^1 peptide bond in the solid state. In solution, the peptide was present as two slowly inter-converting conformers, characterised by a *cis-trans* isomerism around the β-Ala^5-Pro^1 peptide bond.[32,33] The crystal structure of δ-aminovaleric acid containing peptide c[(δ-Ava-Gly-Pro-Thr(tBu)-Gly] showed the presence of a type I β-turn.[34]

In a systematic study to investigate the effect of side chain to side chain cyclisation (i to i+3), conformational preferences of a series of peptides, Ac-Xxx-Pro-Gly-Zzz-NH_2 [Xaa = Lys, Orn, Dab or Dap; Zzz = Glu or Asp], were investigated using various modelling and CD and NMR spectroscopic techniques.[35] The Pro-Gly sequence has been shown to exist previously in type II β-turns. The results suggested that in DMSO-d_6 solution these peptide adopt a variety of conformations that can be related to type II β-turns and γ-turns, but never to type I β-turns. The peptide backbone conformation, in this closely related series of peptides, was shown to be a function of the composition of the side chain but not the ring size. In a series of amphipathic α-helical tetradecapeptides, Ac-Glu-Xaa-Glu^3-Ala-Leu-Lys-Lys^7-Glu-Xaa-Glu^{10}-Ala-Leu-Lys-Lys^{14}-NH_2 (Xaa = Ile, Val, Ala), lactam bridges spaced between i to i+4 residues were formed by linking the side chains of Glu^3 and Lys^7 and Glu^{10} and Lys^{14}. All the lactam-bridged peptides were substantially more helical than the corresponding linear peptides.[36] Size-exclusion chromatography indicated that the Ala-based cyclic peptides existed as monomers, whereas the Ile-based peptides were dimeric (interchain association) in nature. Another approach leading to helical peptides involved linking the Glu i and i+7 side chains by a -NH-$(CH_2)_4$-NH- linking group.[37] Ac-Ala-Glu-Xaa-Ala-Ala-Ala-Lys-Phe-Leu-Xaa-Ala-His-Ala-NH_2 (Xaa = Glu) analogue containing a -NH-$(CH_2)_4$-NH- link between the two

glutamic residues maintained helicity. The corresponding -NH-$(CH_2)_3$-NH- analogue maintained helical conformation but showed significant bending of the helix axis.

Conformational preferences for some disulfide bridge containing peptides, e.g. Cys-Leu-Pro-Arg-Glu-Pro-Gly-Leu-Cys, $(Ac\text{-}Cys\text{-}X\text{-}Cys\text{-}NHMe)_2$ and $(Ac\text{-}Cys\text{-}X\text{-}X\text{-}Cys\text{-}NHMe)_2$ (X = Ala, Val), have been reported.[38,39] NMR study showed that in the case of the cyclic nonapeptide Cys-Leu-Pro-Arg-Glu-Pro-Gly-Leu-Cys at least three conformations of the peptide are present in solution - a major form which is the all-*trans* conformer and two minor forms where the Leu-Pro or Glu-Pro peptide bonds are in the *cis* conformation.

3.2 Naturally Occurring Cyclic Peptides – A number of cyclic peptides containing unusual features (unnatural amino acids and linkages) have been isolated from different sources. Some of this work has been reviewed.[40] The isolated peptides have been used as a source for new biologically active peptides. In some cases, conformational studies have also been carried out. The cyclic peptides containing unusual linkages include aciculitins A-C,[41] nostocyclin,[42] lyciumin A,[43] and kapakahines A-D.[44] In addition to several other unusual features, aciculitins contain a direct bond between the imidazole and phenyl rings of the His and Tyr side chains. The aciculitins A-C, isolated from the lithistid sponge *Aciculites orientalis*, inhibited the growth of *Candida albicans* and were cytotoxic toward the HCT-116 cell line. Nostocyclin, a 3-amino-6-hydroxy-2-piperidone containing depsipeptide, isolated from a hepatotoxic strain of cyanobacterium *Nostac* sp., inhibited protein phosphatase-1 activity at high concentrations. An angiotensin converting enzyme inhibitor lyciumin A (Pyr-Pro-Tyr-Gly-Val-Gly-Ser-Trp) contains a direct bond between the indole nitrogen atom of Trp and the α-carbon of Gly^4 residues. The cyclic part of the peptide contains a type II β-turn-like conformation between the Val and Gly residues.[43] In kapakahines, the ring is closed by a bond from the indole nitrogen of Trp^1 to the β-carbon of Trp^2.

Structural and biological studies on various other cyclic peptides containing natural and unnatural amino acids in the sequence have been reported.[45–58] Some of these cyclic peptides are listed in Table 1. Only a limited amount of biological data has been reported for these peptides. The antibacterial peptide loloatin inhibited the growth of methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus* sp., and penicillin resistant *Staphylococcus pneumoniae* with minimum inhibitory concentrations of 1-2 μg/ml. The plasmin inhibitory peptide agardhipeptin A, c(His-Gly-Trp-Pro-Trp-Gly-Leu), and agardhipeptin B did not inhibit thrombin, trypsin, chymotrypsin, elastase and papain at a concentration of 100 μg/ml. In the case of astin peptides 1,2-*cis* dichlorinated proline residues was shown to be important for the antitumour activity.[51]

4 Biologically Active Peptides

4.1 Peptides Involved in Alzheimer's Disease – Alzheimer's disease (progressive loss of memory, cognition, and behavioural stability) is associated with the

Table 1. Cyclic peptides isolated from natural sources (no biological data given in some cases)

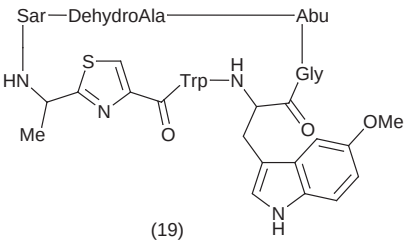
Peptide	Biological Activity	Ref.
Agardhipeptin A: c(His-Gly-Trp-Pro-Trp-Gly-Leu)	Plasmin inhibitor	45
Agardhipeptin B: c(Trp-Leu-Pro-Trp-Ala-Pro-Trp-Val)		45
c(D-Val-L-Leu-D-alloIle-L-Tyr-D-Arg)	Stimulated U937 cell-mediated	46
c(D-Val-L-Leu-D-Leu-L-Tyr-D-Arg)	Degradation. of ^{125}I-fibrin	
c(D-Val-L-Leu-D-alloIle-L-Phe-D-Arg)	(IC_{50} 7.5-32 μM)	
c(D-Val-L-Leu-D-Leu-L-Phe-D-Arg)		
Loloatin B: c(Trp-Val-Orn-Leu-D-Tyr-Pro-Phe-Phe-Asn-Asp	Antibacterial (Gram-positive)	47
Yunnanin C: c(Gly-Ile-Gly-Phe-Tyr-Ser-Pro)	Inhibitor of P-388 lymphocytic	48,
Yunnanin D: c(Gly-Ile-Ser-Phe-Arg-Phe-Pro)	Leukaemia cell growth (IC_{50} 2.2 μg/ml)	49
Cycloleonuripeptide A: c(Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Pro-Met-Ile)	Inhibitors of P-388 lymphocytic	50
Cycloleonuripeptide B: c(Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile)	Leukaemia cell growth (IC_{50} 3.7-6 μg/ml)	
Cyclonuripeptide C: c(Gly-Pro-Pro-Pro-Pro-Tyr-Pro-Pro-Met((O)-Ile)		
Astin A; c(β-Phe-Thr-Pro(Cl_2)-Abu-Ser)	Antitumour (Sarcoma 180A)	51
Astin B; c(β-Phe-Abu-Pro(Cl_2)-Thr-Ser)		
Astin C; c(β-Phe-Abu-Pro(Cl_2)-Abu-Ser)		
Dichotomin A: c(Gly-Thr-Phe-Leu-Tyr-Val)		52
Dichotomin B: c(Gly-Thr-Phe-Leu-Tyr-Thr)		
Dichotomin C: c(Gly-Thr-Phe-Leu-Tyr-Ala)		
Dichotomin D: c(Gly-Val-Gly-Phe-Tyr-Ile)		
Dichotomin E: c(Gly-Tyr-Ala-Phe-Ala)		
Pseudostellarin D: c(Gly-Tyr-Gly-Pro-Leu-Ile-Leu)		53
Podacycline A: c(Gly-Leu-Leu-Gly-Ala-Val-Trp-Ala-Gly-Gly)		54
Podacycline B: c(Phe-Ala-Gly-Thr-Ile-Phe-Gly-Phe)		

development of intraneuronal and extracellular filamentous lesions in the limbic and cerebral cortices. Amyloid β protein is one of the several different proteins that can accumulate excessively in the extracellular spaces of various tissues and produce distinct human diseases.[59] In addition, a 35 amino acid peptide -Glu^{61}-Gln-Val-Thr-Asn-Val-Gly-Gly-Ala-Val-Val-Thr-Gly-Val-Thr-Ala-Val-Ala-Gln-Lys-Thr-Val-Glu-Gly-Ala-Gly-Ser-Ile-Ala-Ala-Ala-Thr-Gly-Phe-Val^{95}- (derived from a heat-stable 140 amino acid precursor protein NACP) has been shown to be a minor component (10% of the level of Aβ) of the Alzheimer's neuritic plaque.[60] A short peptide incorporating an Aβ fragment (Lys-Leu-Val-Phe-Phe; Aβ$^{16–20}$) was shown to bind full-length Aβ and prevent its assembly into amyloid fibrils when incubated with the pentapeptide derivative Ac-Gln-Lys-Leu-Val-Phe-Phe-NH_2 at equimolar concentrations. Through alanine substitution, it was demonstrated that Lys^{16}, Leu^{17}, and Phe^{20} are critical for binding to Aβ fibril formation.[61] In addition to the pentapeptide derivative, a 14-amino acid peptide, Val-Leu-Gly-Gly-Gly-Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala (fragment of the ac-

tivity-dependent neurotrophic factor), was shown to potently and completely prevent neuronal cell death associated with NMDA, GP120 and β-amyloid peptide treatment.[62] A homologous peptide from hsp60, Val-Leu-Gly-Gly-Gly-Cys-Ala-Leu-Leu-Arg-Cys-Ile-Pro-Ala, was about 100,000-fold less potent.

Processing of the β-amyloid precursor protein by various enzymes to generate β-amyloid(1-40) is also being investigated. This work may result in blocking the production of this amyloidogenic peptide. The enzyme activities responsible for these cleavages have been termed β and γ-secretase (not yet identified). Using various substrates and identification of the cleavage products, it has been concluded that cathepsin D is unlikely to function as γ-secretase.[63]

4.2 Antimicrobial Peptides – Thiazole containing antimicrobial peptides A21459 A and B were isolated from actinomycetes.[64,65] The cyclic peptides A21459 A (**19**) and B (Abu replaced by Ala) contained several unnatural amino acids like methoxytryptophan, sarcosine, dehydroalanine and α-aminobutyric acid and inhibited protein synthesis in bacteria. The peptides were most potent against Gram-negative organisms (*Clostridium difficile* and *Neisseria caviae*) (MICs of 0.03-0.13 μg/ml). The potency against a number of other organisms was more modest (MICs of 8-64 μg/ml).



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To investigate the biological role of the δ-amino groups of the Orn residues in gramicidin S [c(Val-Orn-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro)], four analogues, [Ser^{2}]-, [$Ser^{2,2'}$]-, [Glu^{2}]- and [$Glu^{2,2'}$]-gramicidin S, were synthesised and evaluated against several microorganisms. Except for [$Glu^{2,2'}$]-gramicidin S (inactive at 50 μg/ml), the remaining three peptides did show some antibacterial activity. However, the peptides were much less potent than gramicidin S (MICs 3-50 μg/ml). NMR and CD studies of these analogues indicated that the peptides possessed four intramolecular hydrogen bonds between the Val and Leu residues similar to those of gramicidin.[66] Analogues of naturally occurring cationic amphipathic α-helical peptides have been synthesised as antibacterial agents. In addition to their antibacterial properties, these peptides (isolated from various mammalian and non-mammalian sources) have been shown to be cytotoxic towards normal mammalian cells. Thus one of the aims of this work has been to design more selective antibacterial agents. Pardaxin, a 33-amino-acid pore-forming polypeptide toxin (isolated from the Red Sea Moses sole *Pardachirus marmoratus*), was found to be comparable to that of other known native

antibacterial peptides such as magainins and cecropins.[67] Eight truncated and modified pardaxin analogues were synthesised with a free carboxylic acid or an -$NH(CH_2)_2$-NH_2 group at its C-terminus. [$Des^{23–33}$]-pardaxin, [$des^{23–33}$]-pardaxin-$NH(CH_2)_2$-NH_2 and Lys-[$des^{23–33}$]-pardaxin-$NH(CH_2)_2$-NH_2 [Lys-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-$NH(CH_2)_2$-NH_2] were comparable in potency to pardaxin as antibacterial agents with considerably reduced haemolytic activity.[67] Analogues of pardaxin containing D-amino acid residues, [D-Pro^7]-, [D-$Leu^{18,19}$]- and [D-Pro^7, D-$Leu^{18,19}$]-pardaxin, retained most of the antibacterial activity, but did not show haemolytic activity up to a concentration of 50 μM.[68] The D-amino acid-containing analogues do not retain the alpha-helical structure, thus indicating that the helical structure is less important for the antibacterial activity and more important for cytotoxic effects in mammalian cells.

Another cationic family of antibacterial peptides (cathelicidins) has recently been identified in myeloid cells. The C-terminal domain of this precursor protein demonstrates broad spectrum antibacterial properties.[69,70] Amino acid sequences of the two bovine cathelicidin-derived antimicrobial peptides have been reported.[71] The two peptides, Gly-Arg-Phe-Lys-Arg-Phe-Arg-Lys-Lys-Phe-Lys-Lys-Leu-Phe-Lys-Lys-Leu-Ser-Pro-Val-Ile-Pro-Leu-Leu-His-Leu-Gly and Gly-Gly-Leu-Arg-Ser-Leu-Gly-Arg-Lys-Ile-Leu-Arg-Ala-Trp-Lys-Lys-Tyr-Gly-Pro-Ile-Ile-Val-Pro-Ile-Ile-Arg-Ile-Gly, exert a potent antimicrobial activity against Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, and fungi. Both peptides were also cytotoxic to human erythrocytes and neutrophils, although at higher than microbiocidal concentrations. As in the case of pardaxin analogues mentioned above, the N-terminal 18 amino acid peptide fragments of the above peptides retained all the antibacterial activity but the cytotoxic effects on erythrocytes were greatly reduced.[71]

In addition to the fragments of the naturally occurring antibacterial peptides mentioned above, some cationic/hydrophobic peptides have also been obtained by synthetic approaches (including combinatorial) and have been shown to possess antibacterial properties.[72-74] The 7, 14 and 21 amino acid peptides, H-(Lys-Leu-Ala-Lys-Lys-Leu-Ala)$_n$-NH_2, H-(Lys-Leu-Ala-Lys-Leu-Ala-Lys)$_n$-NH_2 (n = 1, 2, 3), H-(Lys-Ala-Leu-Lys-Ala-Leu-Lys)$_3$-NH_2, H-(Lys-Leu-Gly-Lys-Lys-Leu-Gly)$_n$-NH_2, and H-(Lys-Ala-Ala-Lys-Lys-Ala-Ala)$_n$-NH_2 (n = 2, 3), were evaluated for bacteriocidal and cytotoxic activities. The Leu/Ala-containing 21-mers were bacteriostatic at 3-8 μM and cytotoxic to 3T3 cells at about 10 μM concentrations. The Leu/Ala- or Leu/Gly-containing 14-mers and the Leu/Gly 21-mer were bacteriostatic at 6-22 μM but had much lower cytotoxicity toward 3T3 cells and higher selectivities than the natural antimicrobial peptides magainin 2 amide and cecropin B amide. The 7-mer peptides were devoid of biological activity. Examples of other antibacterial peptides include Tyr-Lys-Leu-Leu-Lys-Leu-Leu-Leu-Pro-Lys-Leu-Lys-Gly-Leu-Leu-Phe-Lys-Leu-NH_2 and Lys-Aib-Aib-Lys-Lys-Aib-Aib-Lys-Aib-Aib-Lys-Lys Aib-Aib-NH_2.

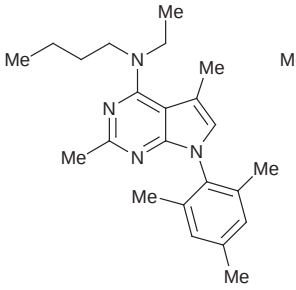
4.3 ACTH/CRF Peptides – Recent evidence that corticotrophin releasing factor (CRF) may be involved in stress and stress-related disorders has created

new interest in the field and attempts are being made to design new peptide and non-peptide antagonists of ACTH. In order to assess the importance of the amino acid side chains in ovine CRF [Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2] with respect to receptor binding and activation, each of the amino acid residues was replaced by a similar amino acid.[75] The Thr^{1}, Asn^{2}, Asp^{3}, $MeAla^{4}$, Ala^{17}, Nle^{21}, Arg^{23}, Leu^{24}, Asn^{26}, Leu^{28}, Ala^{32}, Arg^{36} and Nle^{37} analogues were similar to the parent peptide in the binding assay (80 - 120%). The $MeAla^{5}$, Trp^{6}, Leu^{6}, Thr^{7}, Nle^{8}, Glu^{9}, Nle^{10}, Ser^{11}, Leu^{12}, Ala^{13}, Glu^{13}, Nle^{14}, Nle^{15}, Lys^{16}, Asp^{17}, Nle^{19}, Asp^{20}, Ser^{22}, Nle^{27}, Asn^{29}, Asn^{30}, Leu^{31}, Gln^{34}, Lys^{35}, Glu^{39} and Leu^{41} analogues were less potent than o-CRF (1 - 70%). Only the Trp^{12}, Leu^{18}, Glu^{25}, Thr^{33}, Nle^{38} and Leu^{40} analogues were more potent than o-CRF (160 - 500%). However, in releasing ACTH from anterior pituitary cells, only Trp^{12} analogue was more potent than o-CRF. All the other analogues with increased binding affinity were comparable to o-CRF in releasing ACTH. Those analogues showing decreased binding affinity also exhibited decreased activity in the ACTH release assay. The results indicate that the N-terminal 1-4 and residues 21-29, 32-33, and 36-41 can be replaced without significant loss in binding affinity. Amino acid residues 5-17 were much more sensitive to minor changes.

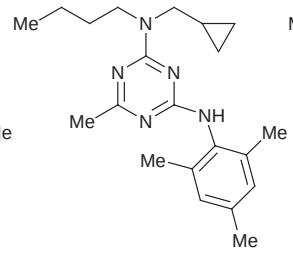
ACTH(1-39) and several N- and C-terminally truncated analogues of ACTH were studied for their ability to stimulate cAMP generation and to displace bound ^{125}I-ACTH from cloned mouse ACTH receptor. Only three of the peptides tested, ACTH(1-24), ACTH(1-39), and ACTH(1-17), were found to have agonist activity with EC_{50} values of 7.5, 57, and 49×10^{-12} M respectively. Two peptides, ACTH(11-24) and ACTH(7-39), were devoid of agonist activity but had substantial competitive antagonist activity with IC_{50} values of approximately 1 nM. In binding studies, ACTH(1-39) and ACTH(1-24) were able to fully displace bound ligand, and Scatchard analysis indicated a dissociation constant (K_D) of 0.84 and 0.94 nM for the two peptides, respectively. ACTH(I-17), ACTH(11-24), and ACTH(7-39) were only capable of displacing 60-70% of bound ligand. A three-site model for the interaction of ACTH and its receptor is proposed on the basis of these findings.[76]

Using various synthetic and random screening approaches, a number of non-peptide antagonists of ACTH have been discovered.[77–80] The early reported examples include compounds (**20**) and (**21**). SAR studies on compound (**21**) (K_i 57 nM) showed that replacement of the N-propyl group by N-ethyl or N-butyl resulted in a 40-fold reduction in potency. Changing the methyl group at position 2 of the triazine nucleus by an ethyl group gave an inactive compound. Removal of the 1- or 5-nitrogen from the triazine derivative (**21**) gave pyrimidine derivatives which also displayed ACTH antagonist activity. The most potent compound of the series was (**22**) (K_i 2.3 nM).[80] Compound (**20**) (CP-154,526) binds with high affinity to CRF receptors in IMR32 cells (a human neuroblastoma cell line (K_i 2.7 nM) and showed similar high affinity in cerebral cortical and pituitary sites labelled by ^{125}I-oCRF in multiple species (IC_{50} <10 nM). The selectivity of CP-154,526 for the CRF receptor subtypes was examined on CRF_1

and CRF_2 receptors expressed in Chinese hamster ovary cells. The compound competed for ^{125}I-oCRF binding to the CRF_1 receptor subtype with a K_i of 2.7 nM. In contrast, the K_i for inhibition of binding by ^{125}I-sauvigine to CRF_2 receptors was >10 μM. CP-154,526 blocked CRF-stimulated adenylate cyclase activity in membranes prepared from rat cortex and pituitary. Systemically administered CP-154,526 antagonised the stimulatory effects of exogenous CRF (4 μg/kg o-CRF, i.v.) on plasma ACTH (ID_{50} 13 ± 1.5 mg/kg), locus coeruleus neuronal firing and startle response amplitude. Potential anxiolytic activity of CP-154,526 was revealed in a fear-potentiated acoustic startle paradigm.[77,78]



(20) (CP-154,526)



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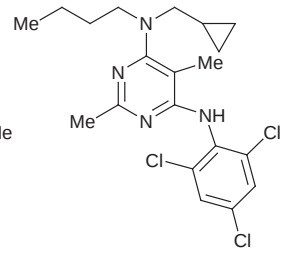

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4.4 Angiotensin II Analogues and Non-peptide Angiotensin II Receptor Ligands

Due to the importance of angiotensin II antagonists in cardiovascular diseases (e.g. hypertension), most of the work published this year on angiotensin has been on the non-peptide antagonists. The discovery, development and clinical evaluation aspects of non-peptide AT_1, AT_2 and mixed (AT_1/AT_2) inhibitors have recently been reviewed.[81] In the peptide area, to investigate the biologically active conformation of angiotensin II, cyclic peptide analogues were synthesised.[82] The amino acid residues known to be important for the agonist activity (Tyr, His, Phe) and the C-terminal carboxyl group were left unchanged and the side chains of the amino acid residues in positions 5 and 7 were linked by a disulfide bridge or an amide bond (Table 2). Most of the compounds (Apt = 4-amino-*trans*-proline, Mpt = 4-mercapto-*trans*-proline and Mpc = 4-mercapto-*cis*-proline) were much less potent than angiotensin II in the receptor binding assay. The most potent analogue of the series, c[Sar^1, hCys^5, Mpc^7]angiotensin II, was about 10-fold less potent than the parent peptide in the binding assay. In an *in vitro* assay (contraction of the isolated rabbit aorta), c[Sar^1, hCys^5, Mpc^7]angiotensin II was about 400-fold less potent than angiotensin II. Modifications of the C-terminal dipeptide (-Pro^7-Phe^8) of [Sar^1, Val^5]angiotensin II with constrained aromatic (Tic) and hydrophobic (Oic) amino acids has led to analogues with negligible affinity for the AT_1 receptor, but nanomolar affinity for the AT_2 receptor.[83] Several of the analogues, Sar-Arg-Val-Tyr-Val-His-Phe-Phe, Sar-Arg-Val-Tyr-Val-His-D-Tic-Phe, Sar-Arg-Val-Tyr-Val-His-Phe-Oic, Sar-Arg-Val-Tyr-Val-His-D-Phe-Oic and Sar-Arg-Val-Tyr-Val-His-Tic-Oic were >50-fold more selec-

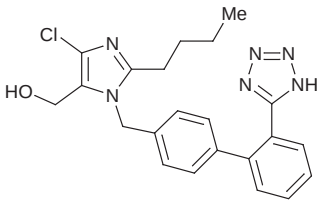
Table 2. Analogues of angiotensin II

Angiotensin analogue	K_D(nM) (Relative affinity, AII = 100)
Angiotensin II	2.0 (100)
c[Sar^1, Cys^5, Pen^7] Angiotensin II	>10000
c[Sar^1, Asp^5, Apt^7] Angiotensin II	>10000
c[Sar^1, Glu^5, Apt^7] Angiotensin II	>10000
c[Sar^1, Cys^5, Mpt^7] Angiotensin II	>10000
c[Sar^1, Cys^5, Mpc^7] Angiotensin II	2300 $\pm$ 150 (0.09)
c[Sar^1, hCys^5, Mpt^7] Angiotensin II	750 $\pm$ 150 (0.27)
c[Sar^1, hCys^5, Mps^7] Angiotensin II	20 $\pm$ 8 (10)

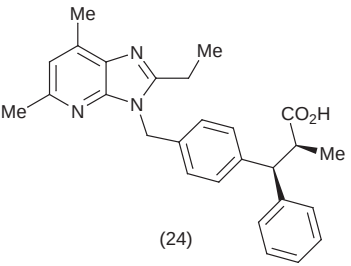
tive at the AT_2 receptors. The most potent and AT_2-selective analogue of the series was Sar-Arg-Val-Tyr-Val-His-Phe-Oic (IC_{50} 240 and 0.51 nM, respectively, at the AT_1 and AT_2 receptors.

The discovery of DuP753 (losartan) (**23**), based on a weak lead reported much earlier, opened the way to non-peptide antagonists of angiotensin II. Attempts are being made to synthesise novel, more potent, selective (acting either AT_1 or AT_2 receptors) or non-selective analogues.[84–89] The synthetic approaches have been successful in identifying various replacements for the imidazole and the biphenyl tetrazole groups. Recent examples of angiotensin antagonists containing new biphenyl tetrazole replacements include compounds (**24**) and (**25**). The 3,3-diphenylpropionic acid analogue (**24**) was a potent AT_1-selective antagonist comparable in potency (*in vitro* and *in vivo*) to losartan.[84] The 5-oxo-1,2,4-thiadiazole derivative (**25**) produced a dose-dependent inhibition of angiotensin II (*in vivo*), which lasted for 24 hours when administered at a dose of 0.01-1 mg/kg.[85] Examples of antagonists modified at the imidazole end include compounds (**26**) and (**27**). The acyliminothiadiazoline derivative (**27**) (KRH-594) was about 10-fold more potent than losartan and inhibited angiotensin II induced pressor response in conscious normotensive rats at 6 and 24 hours after oral administration (0.1 to 3 mg/kg). Replacement of the acetyl group in (**27**) by various other groups, e.g. cyclopropylcarbonyl, benzoyl, *ortho*-chlorobenzoyl, *ortho*-carboxybenzoyl, led to some loss in *in vivo* potency.[87] Incorporation of a *n*-butyl sulfonyl carbamate and an alkyl group in the biphenyl moiety into MK-996 (a potent AT_1 antagonist) resulted in a non-selective antagonist L-162,389 (**28**) (AT_1 and AT_2 binding affinities 2.1 and 3.8 nM, respectively). Other AT_1/AT_2 antagonists have been made by similar changes and conformational restriction.[88,89] Macrocyclic analogue (**29**) bound primarily to the AT_1 receptor (AT_1 and AT_2 receptor IC_{50}s 23 and 4000 nM, respectively) whereas compound (**30**) bound to both the receptors with similar affinity (IC_{50}s 20-30 nM).

4.5 Bombesin/Neuromedin Analogues – Bombesin/neuromedin and a number of other peptides have been claimed as growth factors in various forms of cancers.[90,91] Various aspects of some of the peptide growth factors have been reviewed.[92] A few analogues, D-Phe-Gln-Trp-Ala-Val-Gly-His-NH-CH(CH_2-



(23) losartan (DuP 753)



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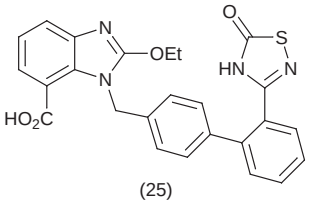

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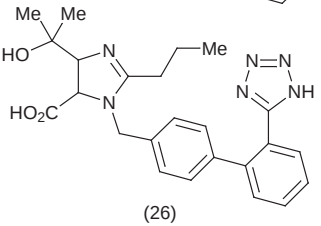

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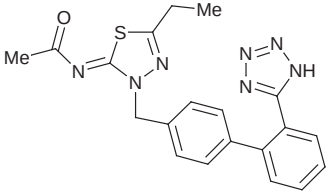

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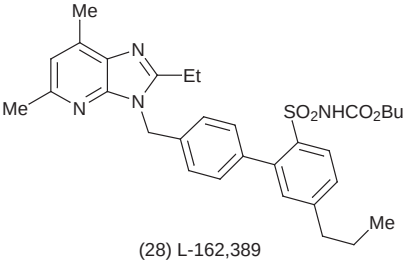
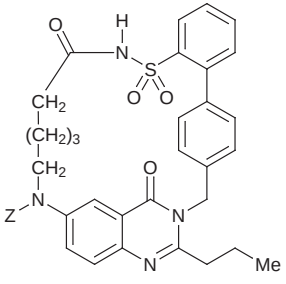


(28) L-162,389



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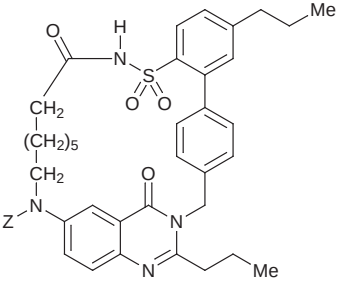

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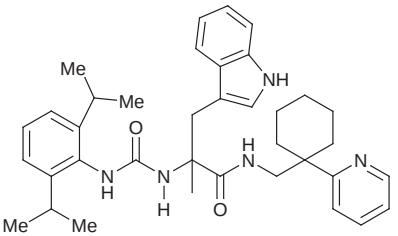
$CH(CH_3)_2$)-CHOH-$(CH_2)_3$-CH_3 and 3-hydroxyphenylpropanoyl-His-Trp-Ala-Val-D-Ala-His-ProΨ(CH_2NH)Phe-NH_2, have been tested in various SCLC models.[93,94] A new human bombesin receptor subtype (subtype 3) was cloned and the C-terminal octapeptide of neuromedin B [Ac-Leu-Trp-Ala-Thr-Gly-His-Phe-Met-NH_2], [D-Phe^6]BN(6-13) propyl amide (EC_{50} 84 nM), and [D-Phe^6-Phe^{13}]BN(6-13) propyl amide (EC_{50} 5 nM) were shown to be the most potent agonist analogues acting at this receptor subtype.[95] A number of other analogues previously reported to be agonists and antagonists of bombesin, 4-pyridylcar-

bonyl-His[7], D-Ala[11], LysαCO-CH_2-CH_2-Ph)[12]]-BN(7-13)-OMe, [D-Phe[6]]BN(6-13)-OMe, [D-pentafluoroPhe[6], D-Ala[11]]BN(6-13)-OMe, [Leu[13]Ψ[CH_2NH]-Leu[14]]BN and [desaminoPhe[6], His[7], D-Ala[11], D-Pro[13]Ψ[CH_2NH]Phe[14]]-BN(6-14), did not show agonist or antagonist activity up to a concentration of 10 μM.

SAR studies in bombesin(7-14)-octapeptide, Ac-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2, have been carried out using Ala replacements and peptide bond modifications.[96] Ala replacements indicated that Trp and Leu residues were most important for the biological activity. [Ala[2]]bombesin(7-14)-NH_2 and [Ala[13]]bombesin(7-14)-NH_2 were 3000 to >10,000-fold less potent than the parent peptide at the cloned neuromedin B and GRP receptors. [Ala[10]]bombesin(7-14)-NH_2 and [Ala[12]]bombesin(7-14)-NH_2 were 200-1000-fold less potent. In the amide bond replacement series of compounds, [Trp[8]Ψ[CH_2NH]Ala[9], Leu[14]]-bombesin, [Gly[11]Ψ[CH_2NH]His[12], Leu[14]]-bombesin and [His[12]Ψ[CH_2NH]Leu[13], Leu[14]]-bombesin, were >500-fold less potent than bombesin and [Leu[14]]bombesin in stimulating amylase release from dispersed pancreatic acinii. Two other compounds, [Ala[9]Ψ[CH_2NH]Val[10], Leu[14]]bombesin and [Leu[13]Ψ[CH_2NH]Leu[14]]-bombesin, were antagonists of bombesin. Except [MeHis[12]]bombesin(7-14)-octapeptide which had binding affinity similar to bombesin(7-14)-octapeptide, all the other N-methyl amino acid substituted analogues were much less potent. [MeAla[9]]-, [MeVal[10]]- and [MeMet[14]]-octapeptides were the least potent compounds. Based on the above results, various derivatives of Trp, Val and Leu were screened and chemically modified to generate non-peptide neuromedin B receptor selective antagonists. N-Methyltryptophan derivative (**31**) (PD165929) was one of the more potent antagonists.[97]



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4.6 Bradykinin Analogues – Some aspects of bradykinin receptors and receptor ligands have been reviewed.[98] Analogues of bradykinin (BK) were synthesised to generate more information on the bioactive conformation.[99] The synthetic analogues, [D-MePhe[7]]BK, [Hyp[3], Thi[5], D-MePhe[7]]BK, D-Arg-[Hyp[3], Thi[5], D-MePhe[7]]BK, D-Arg-[Hyp[3], D-MePhe[7], MePhe[8]]BK, D-Arg-[Hyp[3], D-MePhe[7], MePhe[5,8]]BK, [MePhe[7]]BK, D-Arg-[MePhe[7]]BK, [MePhe[2]]BK, [D-MePhe[2]]BK, [MePhe[3]]BK, and [D-MePhe[3]]BK, were all agonists in the *in vitro* rat uterus and guinea pig ileum preparations. Except [D-MePhe[2]]BK, [MePhe[3]]BK, and [D-MePhe[3]]BK, which were equipotent in both the tissue preparations (0.02-0.15% of BK), all the other compounds were relatively much more potent in the rat

uterus preparation (0.2-136% of BK) than in the ileum preparation (0.005-4% of BK). the most potent agonist analogues in the rat uterus preparation were [D-MePhe^7]BK, [Hyp^3, Thi^5, D-MePhe^7]BK and D-Arg-[Hyp^3, Thi^5, D-MePhe^7]BK (136, 89 and 86% BK, respectively. The three analogues were 300, 4000 and 10,000-fold, respectively, less potent in the ileum preparation. One of the MePhe analogues, [MePhe^2]BK, was a weak antagonist of bradykinin in the guinea pig lung strip preparation.

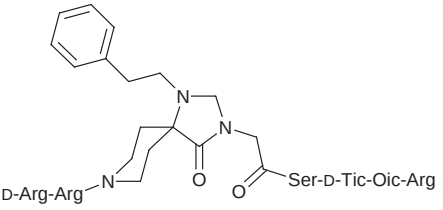
Small linear and cyclic peptides derived from the five C-terminal amino acid residues of a bradykinin B_2 receptor antagonist [D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-Oic-Arg] (HOE 140) have been reported.[100] A number of the linear peptides, X-Y-D-Tic-Oic-Arg [X-Y = Phe-Ser, Gly-Phe, Abu-Thi, Abu-Phe, 5-guanidino-$(CH_2)_5$CO-Thi, 4-guanidino-$(CH_2)_4$CO-Thi, Arg-Thi and aminodecanoyl-Thi] were found to be antagonists (pA_2 values 5.4-6.4) of bradykinin-induced contractions in rabbit jugular vein preparation (B_2 receptor). No activity was observed in the rabbit aorta preparation which only contains B_1 receptors. Slightly more potent B_1 receptor antagonists were obtained in the cyclic peptide series of compounds, c(X-Y-Z-D-Tic-Oic-Arg) [X-Y-Z = Abu-Thi-Ser, β-Ala-Thi-Ser, Abu-Tic-Ser, Abu-Phg-Ser, Abu-Thi-Ala, Abu-Ala-Ser, Abu-Trp-Ala, Gly-Ala, Gly-Phe, Abu-Thi, Gly-Thi, Sar-Thi, Lys-Thi or Arg-Thi) (pA_2 values 6.2-7.4). The conformation of the most potent cyclic peptide c(Gly-Thi-D-Tic-Oic-Arg) was investigated by NMR studies and the peptide was found to adopt a β-turn structure similar to that predicted for the high affinity antagonists and the C-terminal tetrapeptides like Ser-D-Tic-Oic-Arg and Ser-D-Phe-Oic-Arg.

In an effort to synthesise more potent non-selective (B_1/B_2) antagonists, positions 7 and 8 of D-Arg^0-Arg-Pro-Hyp-Gly-Thi-Ser-Xxx^7-Yyy^8-Arg^9 were modified to include several unnatural amino acids [Xxx-Yyy = D-Tic-N-Cyclohexylglycyl, D-Tic-N-Cyclopentylglycyl, D-Tic-N-Phenylglycyl, D-Tic-N-(methylcyclohexyl)glycyl, N-benzylglycyl-Tic, N-phenethylglycyl-Tic, N-benzylglycyl-N-cyclohexylglycyl, D-Phe-N-Cyclohexylglycyl, D-Phe-N-Benzylglycyl]. The D-Tic-N-Cyclohexylglycyl analogue, D-Arg^0-Arg-Pro-Hyp-Gly-Thi-Ser-D-Tic-N-Cyclohexylglycyl-Arg^9 and the corresponding 5-guanidinopentanoyl^0 analogue were potent and selective B_2 receptor antagonists (pA_2 9.3 and 9.09, respectively, rat uterus). All the other analogues were much less potent at the B_1 and B_2 receptors.[101]

A series of pseudopeptides containing alkyl-, cycloalkyl-, aryl-, and aralkyl-substituted 1,3,8-triazaspiro[4,5]decan-4-one-3-acetic acids as amino acid surrogates to replace the Pro^2-Pro-Gly-Phe^5 section of the B_2 receptor antagonist D-Arg-Arg-Pro-Pro-Gly-Phe-Ser-D-Tic-Oic-Arg were synthesised and their binding affinities for the B_2 receptors were evaluated.[102] The highest affinity compound (**32**, NPC 18521) (K_i 0.15 nM) contained the largest substituent (phenethyl) at position 1 of the spirocyclic mimetic. Two other compounds containing a phenyl or cyclohexyl substituents in this position were somewhat less potent (K_i 7.7 and 0.57 nM, respectively). The phenyl and cyclohexyl analogues were active in an *in vivo* model in blocking bradykinin-induced hypotension in rats (ED_{50} 400 and 600 mg/kg, respectively) and rabbits (ED_{50} 22 and 8.6 mg/kg, respectively). NPC 18521 (10 and 30 nmol/kg, ip), given 30 minutes prior, produced significant and

long-lasting inhibition of rat paw oedema induced by bradykinin (3 nmol/paw) and carrageenin (300 μg/paw), without affecting the oedema induced by the selective bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, in rats pre-treated with *E. coli* endotoxin.[103]

Non-peptide antagonists of bradykinin have been published.[104,105] One such compound (FR173657) (**33**) was a potent and selective B_2 receptor antagonist. *In vivo*, oral administration of FR173657 in anaesthetised guinea-pigs inhibited bradykinin-induced bronchoconstriction in a dose dependent manner (ED_{50} 0.075 mg/kg), but did not inhibit histamine-mediated bronchoconstriction even at 1 mg/kg. FR173657 also inhibited carrageenin-induced paw oedema with an ED_{50} of 6.8 mg/kg 2h after the carrageenin injection in rats.



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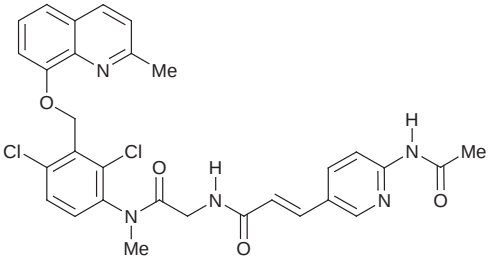


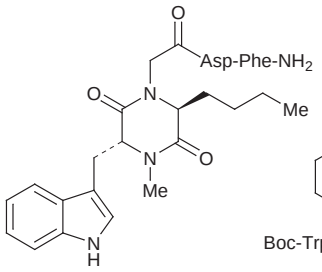
(33)

4.7 Cholecystokinin Analogues – Biological aspects of CCK work have been reviewed.[106,107] Chemically, most of the effort has been directed at the rational design of CCK peptidomimetics and non-peptide analogues. One of the more interesting aspects of the CCK research has been the discovery of non-peptide agonists. Although a large number of non-peptide antagonists of various peptides have been reported, only a few agonist analogues (e.g. angiotensin II agonists) have been reported in the past. In terms of the peptide analogues of CCK, further modifications of an octapeptide analogue Asp-Tyr-D-Phe-Gly-Trp-MeNle-Asp-Phe-NH_2 (SNF 9007; previously claimed to interact with the CCK-B and μ–opiate receptors) by replacing the Trp residue by β-MeTrp residues have been reported.[108] Two of the analogues, Asp-Tyr-D-Phe-Gly-(2S,3R)-β-MeTrp-MeNle-Asp-Phe-NH_2 and Asp-Tyr-D-Phe-Gly-(2S,3S)-β-MeTrp-MeNle-Asp-Phe-NH_2, showed significant δ–opiate receptor affinity (2-4-fold less potent than

SNF 9007). Both these compounds were >1500-fold less potent than SNF 9007 at the CCK-B receptors. Two other analogues, Asp-Tyr-D-Phe-Gly-(2R,3R)-β-MeTrp-MeNle-Asp-Phe-NH_2 and Asp-Tyr-D-Phe-Gly-(2R,3S)-β-MeTrp-MeNle-Asp-Phe-NH_2, were less potent than SNF 9007 at both the CCK-B and opiate receptors.

Peptidomimetic analogues have been synthesised by starting from the C-terminal tetrapeptide Boc-Trp-Met-Asp-Phe-NH_2 of CCK/gastrin. This approach has led to agonist and antagonist analogues.[109–111] In one series of compounds, based on a proposed bioactive conformation of a CCK_B agonist Boc-Trp-MeNle-Asp-Phe-NH_2, cyclic CCK-4 analogues were synthesised by replacing the Trp-Met dipeptide by a diketopiperazine moiety (resulting from a cyclisation between Nle and N-substituted D-Trp residues). The Asp-Phe-NH_2 was linked to the diketopiperazine ring. The most potent ligand of the series (**34**) [K_i 77 and >10,000 nM, at CCK_B and CCK_A receptors, respectively] exhibited potent and full CCK_B receptor agonist properties in promoting the hydrolysis of inositol phosphates (EC_{50} = 8 nM) in CHO cells, stably transfected with the rat brain CCK_B receptor. This compound was also shown to be a potent selective CCK_B/gastrin receptor agonist since it increased gastric acid secretion measured in anaesthetised rats on iv administration.[110] In another series, 3-oxoindolizidine ring was used as a template to arrange the Trp, Asp and Phe side chains. The resulting compounds (**35**) and (**36**) were weak CCK_A and CCK_B receptor antagonists.[111] N-Methyl-D-Trp and 1,5-benzodiazepine derivatives like (**37**)-(**39**) were also shown to be agonists of CCK_A and CCK_B. The discovery was based on the optimisation of random screening leads. The MeTrp analogue (**37**, PD 149164) (IC_{50} CCK_A 75 nM, CCK_B 0.083 nM) was a full agonist at the CCK_A receptor and an antagonist at the CCK_B receptor.[112] Like CCK-8, the MeTrp analogue (at a dose 1000 times higher than that of CCK-8) increased the flow of pancreatic juice and protein output when administered intravenously. Agonist/antagonist properties were also observed in the 1,5-benzodiazepine series of compounds.[113–115] In compound (**38**), the substitution pattern at the anilinoacetamide nitrogen played an important role for the activity. While compounds with a hydrogen or methyl substituent were weak antagonists of CCK-8 on the isolated guinea pig gallbladder, the ethyl, *n*-propyl (**38**), *n*-butyl and cyanoethyl derivatives were agonists. Hydrophilic substituents at the anilinoacetamide nitrogen (-CH_2-COOH, -CH_2COOEt, -CH_2-CH_2-NH_2, -CH_2-CH_2-NH-Z) or the phenyl ring resulted in weak agonists or antagonists. The most potent compound (**38**) displayed 86% CCK-8 functional activity in the guinea pig gallbladder assay at 30 μM (CCK-8 = 100% at 1μM) and showed similar affinity for CCK_A and CCK_B receptors. Compound (**38**) was not active following oral administration. However, in a mouse gallbladder emptying assay, (**39**) (GW7854, mixed CCK_A agonist/CCK_B antagonist) was active when administered intraperitoneally or orally.[115]

In addition to the agonist and mixed agonist/antagonist analogues mentioned above, a number of CCK_A and CCK_B receptor antagonists based on the previously known structural types[116–121] and some dual histamine (H_2)/gastrin antagonists have been reported.[122,123] Examples of some structurally different



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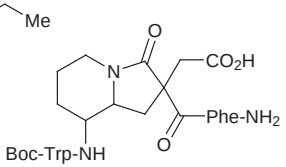

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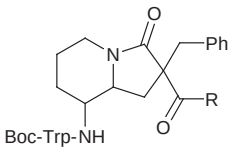

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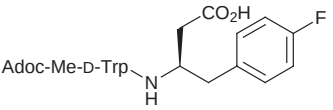
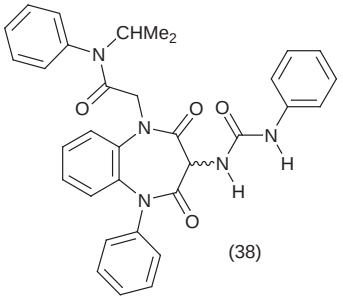


(37) PD 149164



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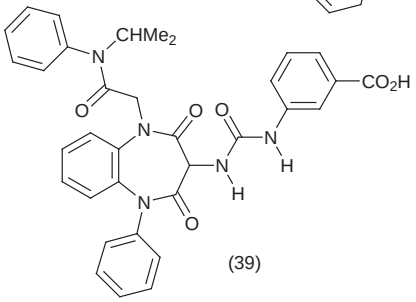
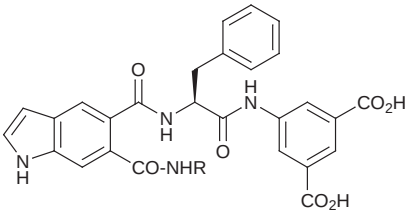


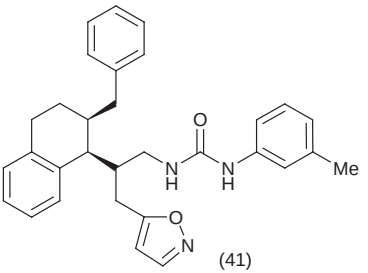
(39)

classes of compounds include (**40**)-(**42**). Compounds (**40**) and the glutamic acid derivative (**42**) (potent antagonists of CCK_B/gastrin receptor) inhibited pentagastrin-stimulated acid secretion in anaesthetised rats and gastric fistula dogs.

4.8 Endothelin Analogues – Information on endothelin receptors, receptor ligands, and potential therapeutic targets has been reviewed.[124] Truncated (linear and cyclic) analogues of endothelin were synthesised by linking segment 3-11 of ET-1 to carboxyl-terminal fragments of various lengths (16-21, 17-21, 21) with an aliphatic spacer [aminocaproic acid (Aca)].[125] In the rat aorta (ET_A receptor preparation), all of the analogues were inactive. However, in the lung parenchyma (ET_B receptor preparation), [$Cys(Acm)^{3,11}$ $Trp(For)^{21}$]-(3-11)-Aca-(17-21)ET, [$Cys(Acm)^{3,11}$, $Trp(For)^{21}$]-(3-11)-Aca-(18-21)ET, [$Cys(Acm)^{3,11}$, $Trp(For)^{21}$]-(3-11)-Aca-(19-21)ET, [$Trp(For)^{21}$]-(3-11)-Aca-(16-21)ET and (3-11)-



(40) [R = 1-adamantylmethyl]



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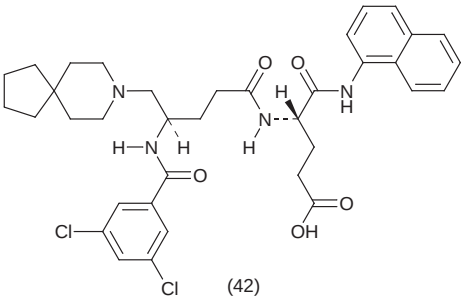
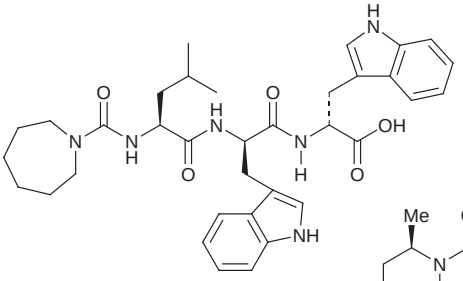


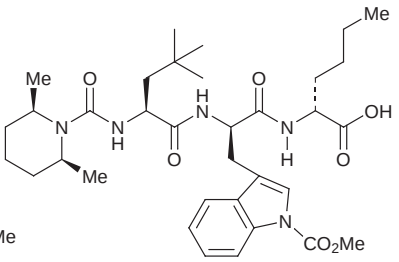
(42)

Aca-(16-21)ET displayed agonist activity (EC_{50} 3-30 nM). Molecular modelling studies indicated different conformations for the formylated and the nonformylated analogues. A cyclic disulfide bridge containing analogue, Ac-Cys-His-Leu-Asp-Cys-Ile-Trp-OH, showed no agonist activity in rabbit bronchus and aorta preparations up to 15 μM concentration.[126]

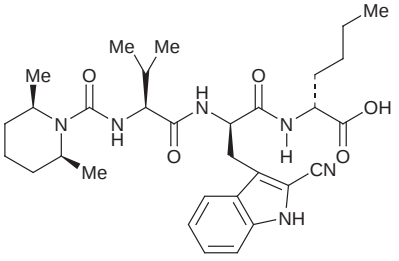
A number of peptide and non-peptide antagonists of endothelin selective for the ET_A or ET_B receptors have been reported. The design of peptidic antagonists has been based on the naturally occurring antagonists of endothelin. RES-701-1, an ET_B receptor-specific antagonist of endothelin (ET_A IC_{50} >1000 nM, ET_B IC_{50} 10 nM) was obtained by screening natural products of microbial origin. This novel peptide consists of a 16 amino acid cyclic peptide, Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp, in which the N-terminal amino group of the glycine residue is linked to the β-carboxyl group of the Asp residue by an amide bond. Structure-activity studies indicated that the C-terminal Trp residue was not important for the activity. Des-Trp^{16}, Ala^{16}, Gly^{16}, Phe^{16}, Tyr^{16}, Nal^{16}, D-Trp^{16}, L-Trp^{17} and D-Trp^{16}-Trp^{17} were all ET_B selective endothelin antagonists (ET_B IC_{50} 6.3 to 450 nM). Phe^{16}, Nal^{16} and D-Trp^{16} were the most potent analogues (ET_B IC_{50} 12, 6.3 and 7.3 nM, respectively).[127] Additional ET_A and ET_B receptor selective antagonists of endothelin have been prepared based on cyclic pentapeptides of microbial origin.[128-130] Examples of potent, ET_A-receptor selective cyclic pentapeptides include c(D-Val-Leu-D-Trp-D-Asp-Pro) (BQ 123). Linear tripeptide derivatives were subsequently developed as ET_A [(**43**), BQ-485] or ET_B [(**44**, BQ-788) and (**45**, BQ-017)] receptor selective or non-selective (**46**, BQ-928) antagonists of endothelin. SAR around this series of tripeptide derivatives indicated that the substituents present in the D-Trp residue



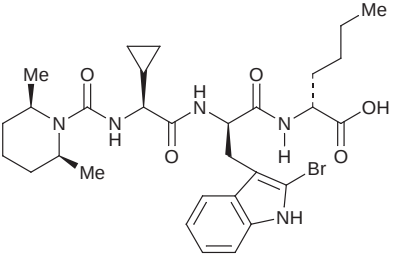
(43) BQ-485



(44) BQ-788



(45) BQ-017



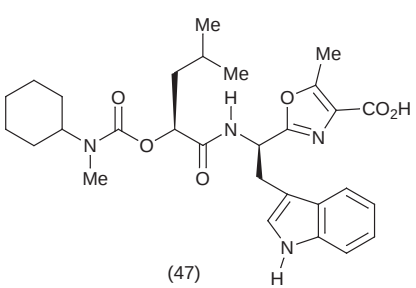
(46) BQ-928

were important for the affinity and selectivity. For example, the D-Trp analogue BQ-485 was ET_A-selective and N^{in}-methoxycarbonyl-D-Trp and 2-cyano-D-Trp analogues (BQ-788 and BQ-017) were ET_B selective. In comparison to 2-cyano-D-Trp analogue, the 2-bromo-D-Trp analogue was a non-selective endothelin antagonist.

In the cyclic pentapeptide [c(D-Val-Leu-D-Trp-D-Asp-Pro) (BQ-123)] series of compounds, amino acid replacements converted the ET_A selective antagonist BQ-123 (ET_A IC_{50} 22 nM and ET_B IC_{50} 18000 nM) into ET_B selective and non-selective antagonists. For example, c(D-*t*-Leu-Leu-2-chloro-D-Trp-D-Asp-Pro), c(D-*t*-Leu-Leu-2-bromo-D-Trp-D-Asp-Pro) and c(D-Pen(Me)-Leu-2-bromo-D-Trp-D-Asp-Pro) were nearly equipotent at both the receptors and c(D-Pen(Me)-Leu-2-cyano-D-Trp-D-Asp-Pro) was much more potent at the ET_B receptor (ET_A IC_{50} 23000 nM, ET_B IC_{50} 22 nM). In the *cis*-(2,6-dimethylpiperidino)carbonyl-Leu-D-Trp-D-Nle series of analogues, all replacements of the N-terminal residue led to a decrease in affinity for both ET_A and ET_B receptors. The 2-bromo-D-Trp (ET_A IC_{50} 5.6 nM, ET_B IC_{50} 2.5 nM), 2-chloro-D-Trp (ET_A IC_{50} 15 nM, ET_B IC_{50} 5.6 nM) and 2-methyl-D-Trp analogues were potent antagonists of endothelin at both the receptors. In comparison, 2-cyano-D-Trp (ET_A IC_{50} 470 nM, ET_B IC_{50} 1.6 nM) and 2-ethyl-D-Trp (ET_A IC_{50} 180 nM, ET_B IC_{50} 5

nM) analogues were much more potent at the ET_B receptor. Amino acid replacements for the other two amino acid residues in the tripeptide derivatives did not change the selectivity pattern of the analogues.[128] Incorporation of heterocyclic groups at the C-terminal end of the D-Trp containing dipeptide endothelin antagonists resulted in compounds with somewhat improved pharmacokinetic profile.[131–133] For example, compound (**47**), a highly-selective ET_A antagonist containing a substituted azole group at the C-terminus, gave 13.5% oral bioavailability when administered at a dose of 10 mg/kg.

Antagonists similar to compounds (**43**)-(**47**) were also discovered by using a rational approach starting from the endothelin C-terminal dodecapeptide derivative, succinyl-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp (IRL 1543) (ET_B K_i 0.077 nM).[134] Replacing each of the amino acid, in turn, with glycine indicated that Phe^{14}, $Ile^{19,20}$ and Trp^{21} were the most important residues. Based on this evidence, a series of compounds having an aromatic moiety attached through a spacer to the amino group of the Trp residue were synthesised. One such compound, N-*trans*-2-phenylcyclopropanoyl-Trp (IRL 1722), showed weak inhibitory activity (K_i 16 μM). Further work around this lead, including chemical features from a tachykinin antagonist (also shown to be an endothelin antagonist), resulted in a potent ET_B-selective endothelin antagonist IRL 1841 (ET_B K_i 36 nM, ET_A K_i 11,000 nM). A further chemical optimisation provided a more potent analogue (**48**) (IRL 2500, ET_B K_i 1 nM and ET_A K_i 440 nM). IRL 2500 antagonised the ET_B-mediated contraction of the guinea pig tracheal tissue induced by ET-3. ET_A-selective antagonist BQ-123 was inactive in this preparation up to a concentration of 10 μM. The ET-1-induced contraction of the rat aorta was not affected by IRL 2500 up to a concentration of 30 μM. Another rational design approach based on using *gluco* and *allo*-carbohydrate derivatives as scaffolds for arranging amino acid side chains (Leu, Phe, Asp and Trp) present in BQ123 did not lead to endothelin antagonists.[135]



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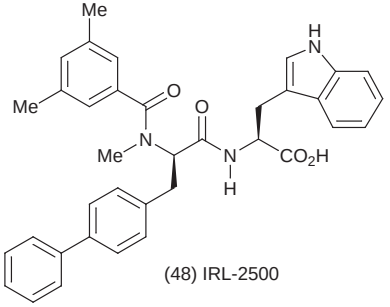
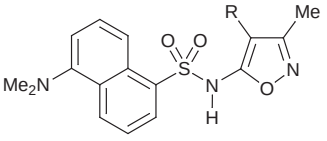


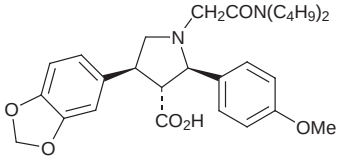
(48) IRL-2500

Non-peptide antagonists of endothelin have been discovered by random screening approaches. 9-Substituted acridine derivatives, benzene-, naphthalene- and thiophene-sulfonamides, 1-Benzyl-3-thioaryl-2-carboxyindoles, 1,3-Diaryl-2-carboxyindoles and 2,4-Diarylpyrrolidine-3-carboxylic acid derivatives have been reported as endothelin ET_A or ET_A/ET_B antagonists.[136–142] In addition, dual

antagonists of endothelin and angiotensin II have also been reported.[143] Examples of some of the ET_A antagonists and ET_A/angiotensin II antagonists include compounds (**49**)-(**52**). A comparison of compounds (**51**) and (**52**) demonstrates that it is possible to obtain selective and non-selective compounds in the same series by chemical modifications. 1,3-Diaryl-2-carboxyindole derivative (**51**) was about 100-fold more selective ET_A receptor antagonist (ET_A IC_{50} 47 nM, ET_B IC_{50} 5.6 μM) and compound (**52**) was a non-selective antagonist (ET_A IC_{50} 30 nM, ET_B IC_{50} 50 nM).[139]



(49) R = Me, BMS-182874; R = Br, TBC-10616



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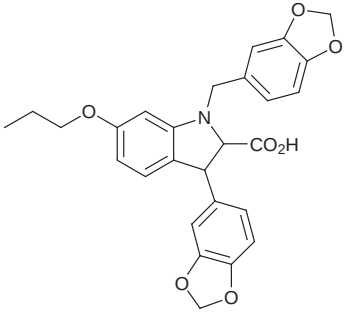

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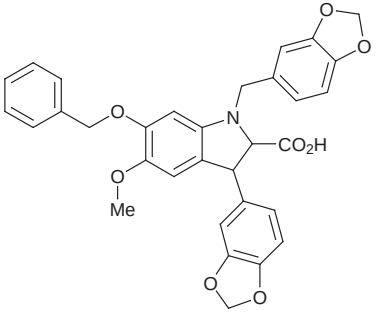


(52)

4.9 Growth Hormone-releasing Peptide and Non-peptide Analogues – Potent peptides which release growth hormone *in vitro* and *in vivo* have been known for some time. Examples of such peptides include His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2, Ala-His-D-Nal(2)-Ala-Trp-D-Phe-Lys-NH_2, D-Ala-D-Nal(2)-Ala-Trp-D-Phe-Lys-NH_2 and His-D-2-methyl-Trp-Ala-Trp-D-Phe-Lys-NH_2 (hexarelin). These peptides have no structural homology with growth hormone or growth hormone releasing hormone, and act *via* specific receptors present either at the pituitary or at the hypothalamic level both in humans and in animals.[144] Further work in the field has resulted in the discovery of more peptide and non-peptide growth hormone secretagogues.[145–152] A number of these peptide and non-peptide analogues have demonstrated potent growth hormone releasing activity in various animal species including man.[153–156]

In a series of tri- to hexapeptide derivatives, a number of analogues, His-D-2-MeTrp-Ala-Trp-D-Phe-Lys-NH_2, D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2, isonipecotinyl-D-βNal-Trp-Phe-Lys-NH_2, isonipecotinyl-D-βNal-D-βNal-Phe-Lys-NH_2, imidazolylacetyl-D-2-MeTrp-D-Trp-Phe-Lys-NH_2, imidazolylacetyl-

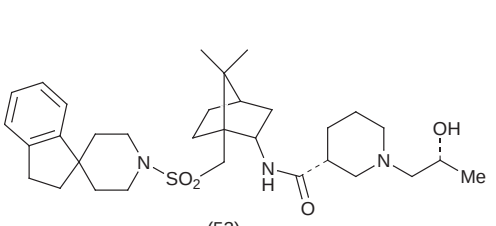
Table 3. Grwoth hormone releasing peptides

Compound	GH release EC_{50} (nM)
GHRP-6	6.2 ± 1.5
Inip-D-Nal(2)-D-Nal(2)-Phe-Lys-NH_2(G7039), (Inip = isonipecotic acid)	0.18 ± 0.04
Inip-D-Nal(2)-D-Trp-Phe-Lys-NH_2	1.1 ± 0.4
Inip-D-Nal(2)-D-Nal(2)-Phe-NH_2	0.28 ± 0.07
Inip-D-Nal(2)-D-Nal(2)-NH$(CH_2)_4NH_2$	17 ± 3
Inip-D-Nal(2)-N-Me-D-Nal(2)-NH$(CH_2)_4NH_2$	0.38 ± 0.08
Inip-D-Nal(2)-D-Nal(2)-ol	1.8 ± 0.8
Inip-D-Nal(2)-D-Trp-ol (G7502)	10.6 ± 6.2
Inip-D-Nal(2)-D-Nal(2)-N$(CH_2$-CH_2-Ph)-CH_2CONH_2 (G7143)	0.34 ± 0.2
c(D-Lys-D-Nal(2)-Ala-Trp-D-Phe-Glu-Lys-NH_2 [Amide bond between Lys^1 and Glu^6 side chains) (G7203)]	0.43 ± 0.11
c(D-Aad-D-Nal(2)-Ala-Trp-D-Phe-Orn-Lys-NH_2 [Amide bond between Aad^1 and Orn^6 side chains)	44 ± 9
c(D-Orn-D-Nal(2)-Ala-Trp-D-Phe-Aad-Lys-NH_2 [Amide bond between Orn^1 and Aad^6 side chains)	> 100
c(D-Orn-D-Nal(2)-Ala-Trp-D-Phe-Glu-Lys-NH_2 [Amide bond between Orn^1 and Aad^6 side chains)	28 ± 10
c(D-Lys-D-Nal(2)-Ala-Trp-D-Phe-Asp-Lys-NH_2 [Amide bond between Lys^1 and Glu^6 side chains)	84 ± 10
GHRP-6	6.2 ± 1.5
His-D-Nal(2)-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	0.6 ± 0.3
His-D-Trp-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	6.2 ± 1.5
$NH_2(CH_2)_5CO$-D-Nal(2)-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	0.20 ± 0.03
His-D-Nal(2)-D-Trp-Phe-Lys-NH_2	6.8 ± 1.2
His-D-Trp-D-Trp-Phe-Lys-NH_2	26 ± 4
Tyr-D-Trp-Ala-Trp-D-Phe-NH_2	>1000
$NH_2(CH_2)_5CO$-D-Nal(2)-D-Trp-Phe-Lys-NH_2	4.6 ± 1.5
$NH_2(CH_2)_5CO$-D-Nal(2)-D-Nal(2)-Phe-Lys-NH_2	2.8 ± 0.4
His-D-Trp-D-Trp-Phe-NH_2	>1000
Tyr-D-Trp-D-Trp-Phe-NH_2	>1000

D-βNal-Phe-Lys-NH_2, isonipecotinyl-D-2-MeTrp-D-Trp-Phe-Lys-NH_2, isonipecotinyl-D-2-MeTrp-D-βNal-Phe-Lys-NH_2, 4-(aminomethyl)cyclohexanecarbonyl-D-2-MeTrp-D-Trp-Phe-Lys-NH_2, γ-aminobutyryl-D-2-MeTrp-D-Trp-Phe-Lys-NH_2, and γ-aminobutyryl-D-2-MeTrp-D-βNal-Phe-Lys-NH_2 (tetrarelin) significantly increased plasma GH levels (60-198 ng/ml; control levels 15 ng/ml) in 10 day-old rats when administered subcutaneously at a dose of 300 μg/kg.[145] Starting from the linear hexapeptide derivative D-Ala-D-Nal(2)-Ala-Trp-D-Phe-Lys-NH_2 as a lead, additional linear and cyclic peptides were synthesised (Table 3). The most potent cyclic heptapeptide c(D-Lys-D-Nal(2)-Ala-Trp-D-Phe-Glu-Lys-NH_2 (amide bond between Lys^1 and Glu^6 side chains) was about 10-fold more potent than the linear hexapeptide GHRP-6. Minor variations to this cyclic peptide, e.g. reducing the ring size or altering the direction of the side chain amide bond, resulted in less potent compounds.[146] Two of the more potent linear peptides, Inip-D-Nal(2)-D-Trp-Phe-Lys-NH_2 and Inip-D-Nal(2)-D-Trp-ol (*in vivo* growth hormone release EC_{50} 0.07 and

0.80 μg, respectively), were shown to be more potent than a non-peptide GH secretagogue L-692,585.

As in the case of many other peptides, non-peptide growth hormone secretagogues were discovered by random screening. Examples of the non-peptide growth hormone secretagogues include compounds (**53**)-(**55**). Replacement of the D-tryptophan residue in (**54**) by D-Nal (α or β) or D-Phe gave less potent compounds.[150] However, replacing the benzyl side chain of D-Phe by Ph-CH_2-CH_2-, Ph-CH_2-CH_2-CH_2- or Ph-CH_2-O-CH_2- groups gave compounds comparable in potency to L-162,752. Replacement of the Aib residue in (**54**) by Gly, Ala, D-Ala, Ser and a number of other unnatural amino acid residue also gave much less potent compounds (EC_{50} 48 - 2100 nM).[151]



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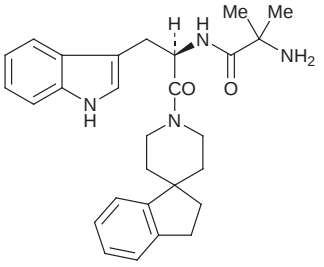
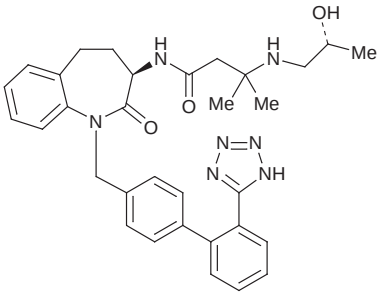


(54) (L-162,752)

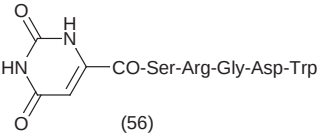


(55) L-692,585

4.10 Integrin Related Peptide and Non-peptide Analogues – In addition to the well-known examples of IIb/IIIa and VLA-4/VCAM-1, work is also progressing on other integrins, e.g. α_v β_5 and α_v β_6, and α_4 β_7, because of the possible involvement of these integrins in a number of disease processes.[157–164] Exogenously added bone sialoprotein peptides, e.g. Glu-Pro-Arg-Gly-Asp-Asn-Tyr-Arg, containing an Arg-Gly-Asp sequence in their backbone structure, but not the more common fibronectin-derived Gly-Arg-Gly-Asp-Ser peptide, strongly inhibited breast cancer cell (MDA-MB-231) adhesion to extracellular bone matrix at micromolar concentrations. Cyclic peptide derivatives with the Glu-Pro-Arg-Gly-Asp-Asn-Tyr-Arg sequence were more effective inhibitors of tumour cell adhesion to bone than their linear equivalents. The Arg-Gly-Glu-analogue Glu-Pro-Arg-Gly-Glu-Asn-Tyr-Arg was somewhat less potent in inhibiting breast cancer

cell adhesion to bone.[158] The cyclic pentapeptide c(Arg-Gly-Asp-D-Phe-Val), a retro-inverso analogue of c(D-Arg-Gly-Asp-Phe-Val), c(Arg-Gly-Asp-Arg-Gly-Asp) and c(Arg-Gly-Asp-Arg-Gly-D-Asp) were shown to be a highly potent and selective inhibitors for the $\alpha_v\beta_3$ integrin.[160,161] Cyclic S-S disulfide bridge compounds c(Cys-Leu-Asp-Thr-Cys) and c(Cys-Leu-Asp-Thr-Ser-Cys), (IC_{50} 200-400 μM) were shown to be active in the $\alpha_4\beta_7$-mediated adhesion assays.[162] At higher concentrations, the more potent peptide, c(Cys-Leu-Asp-Thr-Cys), also inhibited $\alpha_4\beta_1$ mediated binding to VCAM-1, although at a much lower efficiency than inhibition of $\alpha_4\beta_7$/MAdCAM-1 interaction. Other modifications of the Leu-Asp-Thr-Ser-Leu sequence (part of the MAdCAM sequence) also resulted in inhibitors of $\alpha_4\beta_7$/MAdCAM-1 interaction. Ac-Leu-Asp-Thr-Thr-Leu-NH_2 completely inhibited (100%) $\alpha_4\beta_7$/MAdCAM-1 interaction at 500 μM. Replacement of the N-terminal acetyl group by various aromatic groups led to more potent compounds (IC_{50} 4-20 μM).[164]

Further work on Arg-Gly-Asp analogues (peptide and Non-peptide) has continued in the hope of improving potency, selectivity and oral activity. Various synthetic studies, including peptide bond replacements and cyclic peptides, and conformational studies have been reported.[165–171] Cyclic disulfide bridge containing compounds, c(H-Cys-Arg-Gly-Asp-Phe-Cys-NH_2), c(H-Cys-Arg-Gly-Asp-Phe-Cys-Gly-NH_2), c(H-Cys-Arg-Gly-Asp-Cys-NH_2) and c(H-Cys-Arg-Gly-Asp-Cys-Gly-NH_2) were much more potent in a platelet aggregation assay (90-100% inhibition at 100 μM) than the linear tripeptide Arg-Gly-Asp-NH_2 (16% inhibition at 100 μM).[169] N-terminal modifications of the linear peptides gave more potent compounds.[171] In comparison to Ac-Ser-Arg-Gly-Asp-Trp-NH_2 (IC_{50} 74 μM), phenylacetyl-Ser-Arg-Gly-Asp-Trp-NH_2 types of compounds did not lead to a significant improvement in activity. 3-Indolylacetyl- and 3-indolylbutyl-Ser-Arg-Gly-Asp-Trp derivatives were about 4-6-fold more potent. Compounds containing heterocyclic groups at the N-terminus were about 15-20-fold more potent than Ac-Ser-Arg-Gly-Asp-Trp-NH_2. Compound (**56**) (NSL-9403) containing an orotyl group at the N-terminus showed antithrombotic activity in an *in vivo* guinea pig model.



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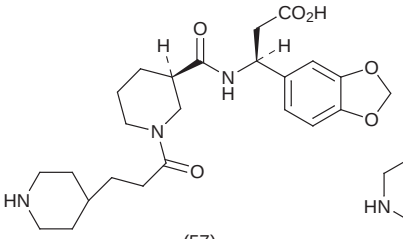
The design of non-peptide analogues in the IIb/IIIa field has been based on the structure activity studies on Arg-Gly-Asp based peptides and random screening approaches. Structure-activity relationship studies of the existing peptide and non-peptide antagonists of IIb/IIIa have indicated that a basic functional group that mimics the side chain of the arginine residue in the Arg-Gly-Asp-Phe type of tetrapeptides and a carboxylic acid group mimicking the Asp side chain are critical to the receptor binding and platelet aggregation activities of these compounds. In addition, a lipophilic group near the carboxylic acid function

enhances the potency of the antagonists. The remainder of the molecule appears to serve as a template to hold the important functional groups in the proper spatial arrangements. A number of different templates have been utilised along with different basic and hydrophobic groups in the design of new compounds. All these different approaches have resulted in potent, orally active platelet aggregation inhibitors.[172–187] Examples of some different structural types are shown in structures (**57**)-(**64**). In the L700,462 (**59**) series of compounds, replacement of the $SO_2C_4H_9$ group by SO_2CH_3, $SO_2(CH_2)_3CH_3$, $SO_2(CH_2)_4CH_3$, $SO_2CH_2CH(CH_3)_2$, $SO_2(CH_2)_2OCH_2CH_3$, $CO(CH_2)_4CH_3$, $CONH(CH_2)_3CH_3$, $CONHCH_2C_6H_5$, $SO_2NH(CH_2)_3CH_3$, $SO_2C_6H_5$, SO_2-2-thienyl, SO_2-3-pyridyl, $SO_2CH_2C_6H_5$, SO_2-4-(COOH)C_6H_4 and SO_2-2-(COOH)C_6H_4 groups gave potent compounds. When given orally, the SO_2-3-pyridyl analogue inhibited *ex vivo* platelet aggregation ((60%) at a dose of 0.1 mg/kg for a period of 200 minutes.[177] Replacement of the benzolactam core in (**60**) with other bicyclic heterocycles did not lead to enhanced potency.[178] When administered orally to dogs, L-767,679 (**60**) inhibited (*ex vivo*) ADP-induced platelet aggregation at a dose of 0.3 mg/kg. The ethyl ester analogue of L-767,679 showed better duration of action (>8 hours) when administered orally at a dose of 0.5 mg/kg. Replacement of the amidinophenyl or piperidinylethyl Arg mimetics commonly used in a number of IIb/IIIa antagonists by tetrahydrothienopyridine moiety was used to design new IIb/IIIa antagonists (**63**). Size of the tetrahydropyridine ring in (**63**) could be altered to a 7-membered ring without any significant effect on the activity. Introduction of an alkyl group at the β position of thiophene ring resulted in a considerable loss in potency. Both the oxyacetic acid groups were important for the activity; removal of any of these resulted in some loss in potency.[184] ME3277 did not effect vitronectin and fibronectin receptors at 100 μM. In the case of compound (**64**), a number of derivatives [R = n-C_4H_9, n-C_8H_{17}, phenyl, 1-naphthyl, 2-naphthyl, benzyl, *p*-n-propyl phenyl and 4-biphenyl] were potent inhibitors of IIb/IIIa. The n-butyl sulfonyl derivative was shown to be active (1mg/kg, iv) in a canine model of coronary artery occlusion.[185]

In addition to the synthetic inhibitors of the protein-protein interaction mentioned above, another approach to interfere with the integrins would be to prevent the expression of such proteins. One such naturally occurring cyclic peptide (**65**, HUN-7293, a fungal metabolite) has been isolated.[188] The peptide contains a number of unnatural amino acids including D-2-hydroxy-4-cyanobutyric acid and D-propylleucine. HUN-7293 suppressed cytokine-induced expression of VCAM-1 on human endothelial cells. 3D structure of the cyclic peptide was elucidated both in solution and single crystals by using NMR spectroscopy and X-ray diffraction techniques, respectively.

4.11 LHRH Analogues – Very few analogue and conformational studies of LHRH have been reported this year.[189–191] Betidamino acids, N′-monoacylated (optionally, N′-monoacylated and N-mono- or N,N′-dialkylated) aminoglycine derivatives, in which each N′-acyl/alkyl group may mimic naturally occurring amino acid side chains, were incorporated in positions 1, 3 and 10 in an LHRH antagonist Ac-D-2-Nal-D-4-Cpa-D-3-Pal-Ser-4-Aph(Ac)-D-4-Aph(Ac)-Leu-Lys-



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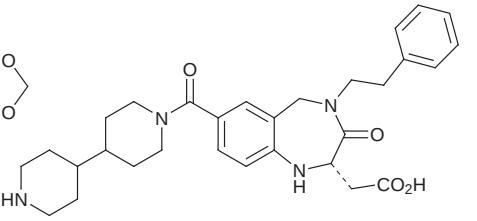
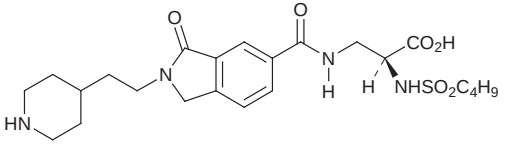
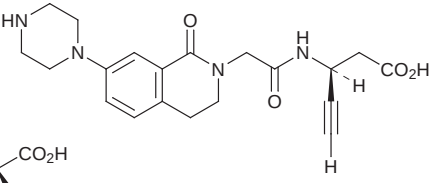


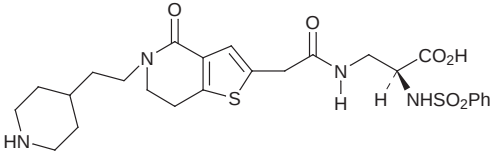
(58) (SB-214857)



(59) (L700,462)



(60) (L-767,679)



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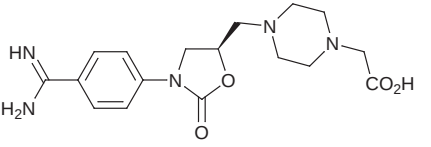
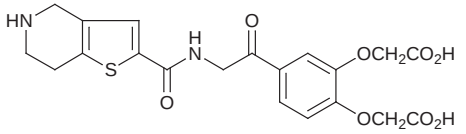
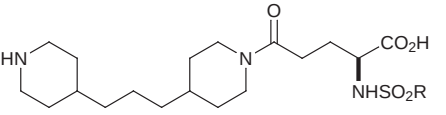


(62) (EMD 76,344)



(63) (ME3277)



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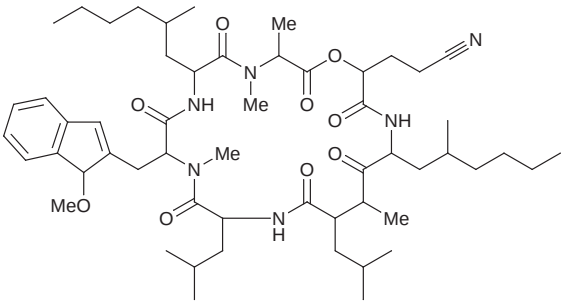
Pro-D-Ala-NH_2 [Acyline; 2-Nal = 2-naphthylalanine, 4-Cpa = 4-chlorophenylalanine, 3-Pal = 3-pyridylalanine, Aph = 4-aminophenylalanine, and Ilys = N^{ε}isopropyllysine]. Most betide diastereomers were equipotent with Acyline.[189] Biological results indicated small differences in relative potencies between the D and L nonalkylated betidamino acid-containing Acyline derivatives. In an attempt to correlate structure and observed potency, Ramachandran-type plots were calculated for a series of betidamino acids and their methylated homologues. According to these calculations, betidamino acids have access to a more limited



(65) HUN-7293

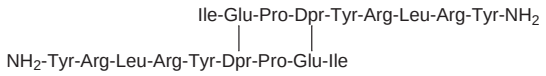
number of conformational states (including those associated with α-helices, β-sheets, or turn structures), with deeper minima than those observed for natural amino acids.

4.12 Neuropeptide Y (NPY) Analogues – Neuropeptide Y, an amidated 36-amino acid peptide, is widely distributed in the central and peripheral nervous system. Physiological responses of NPY, including cardiovascular effects and feeding behaviour, are mediated by six receptor (Y_1-Y_4 and two Y_5 types) subtypes.[192,193] Conformational studies on a C-terminal methionine analogue have suggested an unstructured or extended conformation from Tyr^1 to Ala^{12} connected to a well-defined amphipathic α-helix from Pro^{13} to Arg^{35}. The assignment was confirmed by comparison of nuclear Overhauser effect based two-dimensional ^{1}H-NMR spectroscopy.[194] The C-terminal Tyr-NH_2 of NPY appears to be very important for the biological activity. NPY(1-35)-NH_2, NPY(1-35)-OH, NPY(1-36)-OH, NPY(1-35)-Oet, NPY(1-35)-OBzl, NPY(1-35)-OCH_2-CH_2-Ph, NPY(1-35)-benzyl amide and substituted benzyl amides showed very poor affinity for the Y_1 receptors (IC_{50} 5000 - > 10,000 nM). A few other analogues, e.g. NPY(1-35)-NH-CH_2-CH_2-Ph(4-OH), NPY(1-35)-NH-CH_2-CH_2-Ph(4-OMe), NPY(1-35)-NH-CH_2-CH_2-Ph(4-NH_2), NPY(1-35)-NH-CH_2-CH_2-Ph(4-Br), NPY(1-35)-NH-CH_2-CH_2-Ph(4-Me), were somewhat more potent (IC_{50} 150 - 400 nM). NPY(1-36)-thioamide was also >40-times less potent than NPY.[195] One of the C-terminally modified analogues, NPY(1-35)-NH-CH_2-CH_2-Ph(4-OH), showed no intrinsic activity in human SK-N-MC cells (Ca^{2+} release) but was able to antagonise NPY.

In contrast to the C-terminal Tyr-NH_2 group, various amino acid residues from the N-terminus or the central part of the sequence could be eliminated with retention of biological activity. N-Terminal truncation gave several series of interesting compounds. Y_2 receptor (porcine hippocampal membranes) binding studies in [Xaa^{11}]NPY(11-36), [Xaa^{12}]NPY(12-36), and [Xaa^{13}]NPY(13-36) [Xaa^{11} = Lys, Arg, Asp, Glu, Asn, Gln, Thr, Ser; Xaa^{12} = Ala, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys, Thr, Ser, Tyr, Gly, Val, Phe, Trp; Xaa^{13} = Ala, Lys, Arg, Asp, Glu, Pro, Asn, Gln, Thr, Ser] series of compounds revealed that

[Xaa^{11}]NPY(11-36) analogues were considerably more potent (IC_{50} values 0.49-9.3 nM) than [Xaa^{12}]NPY(12-36) (IC_{50} values 5-100 nM) and [Xaa^{13}]NPY(13-36) (IC_{50} values 12-50 nM) analogues. Upon acetylation or succinylation of the N-terminal amino group, the binding affinity of [Xaa^{12}]NPY(12-36) recovered to a level similar to that of [Xaa^{11}]NPY(11-36). No significant difference was observed between the increases caused by acetylation and those caused by succinylation.[196] Peptide analogues of neuropeptide Y with a Tyr^{32} and Leu^{34} replacement resulted in a decapeptide Tyr-Ile-Asn-Leu-Ile-Tyr-Arg-Leu-Arg-Tyr-NH_2 with 3700-fold improvement in Y_2 receptor affinity (rat brain; IC_{50} = 8.2 $\pm$ 3 nM) when compared to the native NPY(27-36) fragment.[197] The decapeptide was an agonist at the Y_1 receptor (human erythroleukemia cell; ED_{50} = 8.8 $\pm$ 0.5 nM) with potency comparable to that of NPY(1-36) (ED_{50} = 5 nM). The replacement of Leu with Pro at position 4 of the decapeptide afforded an antagonist of NPY in PILL cells (Tyr-Ile-Asn-Pro-Ile-Tyr-Arg-Leu-Arg-Tyr-NH_2; IC_{50} = 100 $\pm$ 5 nM). Deletion of the N-terminal tyrosine of the Pro^4 decapeptide resulted in a 10-fold improvement in antagonistic activity with a parallel 4-fold decrease in Y_2 affinity. Antagonists of NPY were also obtained by modifications in positions 28, 29 and 32 in the C-terminal 27-36 region. One such compounds, des-Asn^{29}(D-Trp^{28,32})NPY(27-36), bound to both Y_1 (SK-N-MC, K_i 10 (M) and Y_2 (SK-N-BE2, K_i = 1.01 $\pm$ 0.03 μM) receptors. This peptide did not exhibit any agonist activity at Y_1 receptors, and exhibited comparable potencies in antagonising the effects of NPY on the synthesis of cAMP and mobilisation of Ca^{2+} in HEL cells. Further SAR studies resulted in the development of [desAsn^{29}, D-Trp^{28,32}, Nva^{34}]-NPY(27-36) (K_i 328 nM) and [desAsn^{29}, Trp^{28,32}, Nva^{34}]-NPY(27-36) (K_i 359 nM) as weak NPY Y_1 receptor antagonists.[198]

A cyclic C-terminal dodecapeptide of NPY, obtained by replacing Leu^{28} and Thr^{32} by Lys and Glu, and linking the two side chains by lactamisation, was shown to be >100-fold more potent than the corresponding linear peptide in a Y_2 receptor preparation (human neuroblastoma cells SMS-KAN). Signal transduction was investigated by measuring Ca^{2+} current inhibition in human SH-SY5Y cells and cyclic Ac-[Lys^{28}-Glu^{32}] NPY(25-36)-NH_2 and NPY were shown to be equipotent in this assay.[199] Dimerisation of the C-terminal peptides, either by disulfide bridge formation or amide bond formation between the two peptide chains, also led to Y_1 receptor antagonists.[200-202] For example, bis(31/31′){[Cys^{31}, Nva^{34}]NPY(27-36)}, bis(31/31′){[des-Asn^{29}, Cys^{31}, Nva^{34}]NPY(27-36)}, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}]NPY(27-36)}, bis(31/31′){[des-Asn^{29}, Cys^{31}, Trp^{32}, Nva^{34}]NPY(27-36)}, bis(31/31′){[des-Asn^{29}, Cys^{31}, Trp^{28,32}, Nva^{34}]NPY(27-36)} and bis(31/31′){[des-Asn^{29}, Cys^{31}, D-Trp^{28,32}, Nva^{34}]NPY(27-36)} were about 10-100-fold more potent antagonist at the Y_1 receptor. The most potent and selective analogue was a much smaller peptide, bis(31/31′){[Cys^{31}, Trp^{32}, Nva^{34}]NPY(31-36)}, (K_i 46 nM at Y_1 receptor and >10,000 nM at Y_2 receptors). The most potent dimeric peptide containing amide bond links between the two peptide chains (**66**) was much more potent (rat brain IC_{50} 0.021 nM and SK-N-MC cell IC_{50} 0.20 nM) than the linear nonapeptide (rat brain IC_{50} 10 nM). Intracerebroventricular injection of (**66**), (1229U91) (30 μg) into male rats completely inhibited NPY (5 μg)-induced food intake without any other behavioural



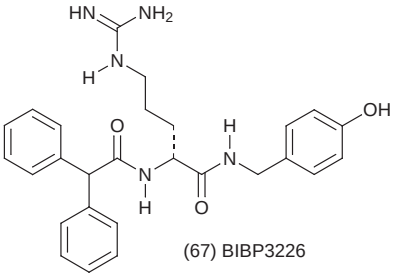
(66)

changes. Furthermore, icv injections of 1229U91 significantly suppressed physiological feeding behaviour after overnight fasting.

In the centrally truncated series of linear and cyclic peptides, des-AA$^{6\text{-}24}$[Aoc^5]NPY, des-AA$^{6\text{-}24}$[Aoc^5, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[Aoc^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[D-Ala^5, Aoc^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[MeAla^5, Aoc^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}17}$[MeAla^5, Aoc^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[D-Ala^5, Gly^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[Aoc^6, D-Trp^{5,32}]NPY, c(2/26)des-AA$^{8\text{-}24}$[Glu^2-D-Ala^6, Aoc^7, Lys^{26}, D-Trp^{32}]NPY and c(2/27)des-AA$^{7\text{-}24}$[Asp^2-D-Ala^6, D-Lys^{27}, D-Trp^{32}]NPY were >1000-fold less potent than NPY at Y_1 and Y_2 receptors.[203] However, des-AA$^{7\text{-}24}$[D-Ala^5, Aoc^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[MeAla^5, Aoc^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[D-Ala^5, Gly^6, D-Trp^{32}]NPY, des-AA$^{7\text{-}24}$[Aoc^6, D-Trp^{5,32}]NPY and c(2/26)des-AA$^{8\text{-}24}$[Glu^2-D-Ala^6, Aoc^7, Lys^{26}, D-Trp^{32}]NPY were weak antagonists of NPY. Des-AA$^{7\text{-}24}$[D-Ala^5, Aoc^6, D-Trp^{32}]NPY antagonised NPY-induced hypertension in rats. Histamine releasing activities of some centrally truncated analogues have been reported.[204]

Non-peptide antagonists of NPY (**67**)-(**69**) have been reported.[205–210] The first NPY non-peptide antagonist, N-diphenylacetyl-D-Arg derivative (**67**), displaced I^{125}-labelled neuropeptide Y with high affinity (K_i = 7 nM) from the human NPY Y_1 receptor and proved to be highly selective. It displayed potent antagonistic properties both in *in vitro* and *in vivo* models. In the pithed rat model, (**67**) alone had no effect on blood pressure but inhibited NPY-induced increase in blood pressure in a dose-dependent manner (ED50 = 0.11 ± 0.03 mg/kg iv).[207] Random screening provided a nitroquinoline series of non-peptide antagonists (**68**). Most of the chemical modifications of (**68**) (PD9262, Y_1 receptor K_i 282 nM and Y_2 receptor K_i >10,000 nM) resulted in much less potent compounds (K_i values 250 - >10, 000 nM at the Y_1 receptor). Only the replacement of the amino group of the arylsulfonyl moiety by 2-chloro, 2-fluoro or 2-isopropyl groups gave 2-5-fold more potent compounds in the binding assay.[209] Non-peptide antagonist (**69**) (SR 120819A) demonstrated oral activity.[210] When given orally, it blocked the presser response of [Leu^{31}, Pro^{34}]NPY (5 μg/kg iv) with a long duration of action (> 4 h at 5 mg/kg po).

4.13 Opioid (Enkephalin, β-Casomorphin, Morphiceptin, Deltorphin and Dynorphin) Peptides – Various aspects of opiate peptide receptors (classification and structure-function) and structural studies (X-ray) have been reviewed.[211–213] Analogues of enkephalin containing a sulfonamide group in the side chain or in the main chain have been reported.[214,215] The side chain modified Cys^2 sulfonamide analogues of [Leu^5]- and [Met^5]enkephalin were compared with [Leu^5]- and [Met^5]enkephalins and Tyr-D-Ala-Gly-Phe-Leu-Arg (dalargin) in the guinea pig ileum (μ and κ receptors) and mouse vas deferens (predominantly δ receptors) preparations. In comparison to [Leu^5]-enkephalin and dalargin (IC_{50} values 138



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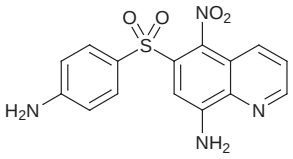

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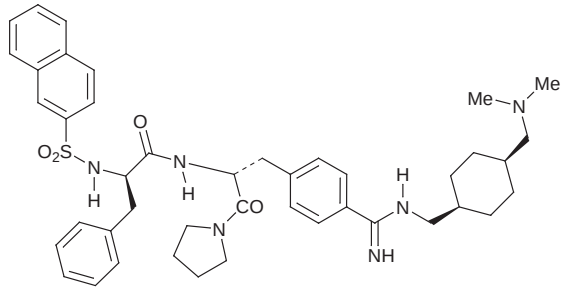
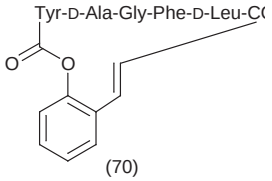


(69)

and 9.5 nM, respectively), the [Cys(O_2NH_2)^2, Leu^5]enkephalin was much less potent (IC_{50} 7980 nM) in the guinea pig ileum preparation. Similarly, [Cys(O_2NH_2)^2, Met^5]enkephalin was much less potent than [Met^5]enkephalin (IC_{50} values 4200 and 40 nM, respectively). However, in the mouse vas deferens preparation, [Cys(O_2NH_2)^2, Leu^5]enkephalin was 300-fold more potent than [Leu^5]enkephalin and [Cys(O_2NH_2)^2, Met^5]enkephalin was 10-fold more potent than [Met^5]enkephalin. Thus the two sulfonamide analogues were much more selective for the δ receptors.[214] The backbone modified analogues, Tyr-Gly-Gly-Phe-LeuΨ[CH_2SO_2]-NH_2, Tyr-Gly-Gly-PheΨ[CH_2SO_2NH]Leu-NH_2, Tyr-Gly-Gly-PheΨ[CH_2SO_2NH]Leu, Tyr-Gly-GlyΨ[CH_2SO_2NH]Phe-Leu-NH_2 and Tyr-Gly-GlyΨ[CH_2SO_2NH]Phe-Leu were evaluated in a binding assay. The C-terminal sulfonamide analogue Tyr-Gly-Gly-Phe-LeuΨ[CH_2SO_2]-NH_2 was similar in potency to Leu-enkephalin or Leu-enkephalinamide. Replacement of the Phe^4-Leu^5 amide bond resulted in 10-50 fold less potent compounds. The rest of the analogues were much less potent (> 800-fold).[215]

Conformational and hydrophobicity measurement studies on cyclic D-Pen^2, D-Pen^5 analogue have been reported.[216,217] Reasonably good correlation was obtained between lipid membrane permeability and hydrophobicity in a set of position 3 modified (Gly, Ala, Leu, Phe) analogues. However, Phe residue added on the N-terminal end of the peptide (position 0) resulted in lower permeability compared to the Phe^3 analogue. More hydrophobic analogues of [D-Ala^2, D-Leu^5]enkephalin (Tyr-D-Ala-Gly-Phe-D-Leu) containing a 1,4-dihydrotrigonellyl group linked at the N-terminus and a bulky, lipophilic ester group (cholesteryl or 1-adamantaneethyl ester) group at the C-terminus were synthesised as prodrugs

for the brain-targeted delivery of the peptide. Intravenous injection of the compounds produced a significant and long-lasting response in rats monitored by the tail-flick latency measurements.[218] A γ-glutamyl-transpeptidase-sensitive prodrug of dermorphin has been reported. The prodrug, γ-glutamyldermorphin, showed little affinity for opioid receptors but showed antinociceptive activity when injected intrathecally.[219] A coumarin-based esterase-sensitive cyclic prodrug of [D-Ala^2, D-Leu^5]enkephalin has also been reported.[220] When incubated in a phosphate buffer in the presence of porcine liver esterase, the cyclic prodrug (**70**) released the parent peptide ($t_{1/2}$ 761 min).



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Conformationally restricted analogues of the N-terminal undecapeptide of dynorphin A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln) were obtained by incorporating side chain to side chain lactam bridges between the i and i + 4 positions to stabilise α-helical conformations.[221] Cyclic peptides c(Asp^2, Lys^6)Dyn A(1-11)-NH_2, c(Asp^3, Lys^7)Dyn A(1-11)-NH_2, c(Lys^2, Asp^6)Dyn A(1-11)-NH_2, c(Lys^5, D-Asp^9)Dyn A(1-11)-NH_2, [Asp^2, Lys^6]Dyn A(1-11)-NH_2, [Asp^3, Lys^7)Dyn A(1-11)-NH_2, [D-Asp^3, Lys^7)Dyn A(1-11)-NH_2, [Lys^2, Asp^9)Dyn A(1-11)-NH_2 and [Lys^5, D-Asp^9)Dyn A(1-11)-NH_2 were much less potent than the parent dynorphin(1-11) analogue (IC_{50} 0.58 nM κ receptor, 9.9 nM μ receptor and 26 nM δ receptor). Cyclisation between D-Asp^2 and Lys^6 side chains in c(D-Asp^2, Lys^6)Dyn A(1-11)-NH_2 led to an analogue with potency and selectivity enhancement for the μ opioid receptor (IC_{50} 110 nM κ receptor, 3.4 nM μ receptor and 12 nM δ receptor), whereas cyclisation between D-Asp^3 and Lys^7 side chains in c(D-Asp^3, Lys^7)Dyn A(1-11)-NH_2 led to a potent ligand (IC_{50} 4.9 nM κ receptor, 310 nM μ receptor and 130 nM δ receptor) with κ receptor selectivity. In comparison to the cyclic peptides, the linear peptide, [D-Asp^2, Lys^6]Dyn A(1-11)-NH_2, was much more potent (IC_{50} 1.5 nM κ receptor, 0.08 nM μ receptor and 1.15 nM δ receptor) than the corresponding cyclic peptide c(D-Asp^2, Lys^6)Dyn A(1-11)-NH_2 and other cyclic peptides.

Deltorphin analogues (δ and μ receptor ligands) were obtained by incorporating a series of achiral $C^{\alpha,\alpha}$-dialkyl cyclic α-amino acids, 1-aminocyclopropane-1-carboxylic acid (Ac_3c), 1-aminocyclopentane-1-carboxylic acid (Ac_5c) and 1-aminocyclohexane-1-carboxylic acid (Ac_6c), in position 2, 3, 4, or 2 and 3 in deltorphin C, and in position 2 in (Ac_6c^2, des-Phe^3)deltorphin C hexapeptide.[222] The synthetic analogues, Tyr-Ac_6c-Phe-Asp-Val-Val-Gly-NH_2, Tyr-D-Ala-Ac_6c-Asp-Val-Val-Gly-NH_2, Tyr-D-Ala-Phe-Ac_6c-Val-Val-Gly-NH_2, Tyr-Ac_6c-Ac_6c-Asp-Val-Val-Gly-NH_2, Tyr-Ac_6c-Asp-Val-Val-Gly-NH_2, Tyr-D-Ala-Ac_5c-Asp-

Val-Val-Gly-NH_2, Tyr-D-Ala-Phe-Ac_5c-Val-Val-Gly-NH_2, Tyr-D-Ala-Ac_3c-Asp-Val-Val-Gly-NH_2 and Tyr-D-Ala-Phe-Ac_3c-Val-Val-Gly-NH_2, were compared with the parent peptide Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 in δ and μ receptor binding assays and *in vitro* tissue preparations. Tyr-D-Ala-Phe-Ac_6c-Val-Val-Gly-NH_2, Tyr-D-Ala-Phe-Ac_5c-Val-Val-Gly-NH_2 and Tyr-D-Ala-Phe-Ac_3c-Val-Val-Gly-NH_2 were the most potent analogues in the δ and μ receptor binding assays and *in vitro* tissue preparations.

Several cyclic peptide analogues of β-casomorphin-5 (Tyr-Pro-Phe-Pro-Gly) and morphiceptin (Tyr-Pro-Phe-Pro-NH_2) have been reported.[223-227] In Tyr-c(D-Xaa-Phe-Pro-Gly-) and Tyr-c(D-Xaa-Phe-D-Pro-Gly-) (Xaa = Abu, Orn, Lys) series of side chain to C-terminally linked cyclic peptides, the D-Xaa^2 containing compounds bound to the μ and δ opioid receptors. The corresponding L-Xaa containing analogues were inactive in both the receptor preparations.[223] Position 3 modified analogues of Tyr-c(D-Orn-2-Nal-D-Pro-Gly-), were synthesised by replacing the 2-naphthylalanine residue by benzothienylalanine, His(Bzl), Tyr(Bzl), 4'-benzoylphenylalanine, 4'-benzylphenylalanine, thyronine, thyroxine, 4'-biphenylalanine, 4'-biphenylglycine and 3,3-diphenylalanine residues. In the guinea pig ileum preparation, only benzothienylalanine analogue was 50-fold more potent than the parent peptide. Most of the other analogues were partial agonists. The 4'-benzylphenylalanine and 4'-biphenylalanine containing peptides were antagonists in the guinea pig ileum preparation. In the mouse vas deferens preparation, except benzothienylalanine analogue, the rest of the analogues were either partial agonists or antagonists.[224] Conformational properties of a number of cyclic β-casomorphin analogues, including Tyr-c(D-Orn-2-Nal-D-Pro-Xaa-) (Xaa =D-Ala, Sar or MeAla), Tyr-c[D-Orn-2-Nal-Pro-Gly-] and Tyr-c[D-Orn-Phe-D-Pro-Gly-] were investigated by NMR and molecular modelling techniques.[223,225,226] Significant conformational differences were observed not only in the orientation of the two aromatic side chains but in the backbone conformations of the ring part. Several preferred conformations contained *cis* peptide bonds. For example, Tyr-c[D-Orn-2-Nal-Pro-Gly-] was shown to exist in two conformations, a major one (90%) characterised by a *cis* amide bond between 2-Nal^3 and Pro^4, and a minor one (10%) showing *cis* amide bonds both between D-Orn^2 and 2-Nal^3 and 2-Nal^3 and Pro^4 residues. A number of other publications leading to pharmacophore models for various receptor subtypes have been published.[228–231]

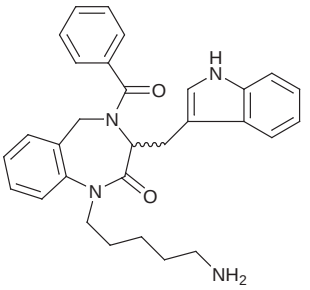
Cyclic (monomeric and dimeric) and linear peptide analogues of morphiceptin are reported.[227] In comparison to morphiceptin (guinea pig ileum IC_{50} 318 nM, mouse vas deferens IC_{50} 4800 nM), only side chain to C-terminally linked peptides Tyr-c(D-Lys-Phe-D-Pro-Gly-), Tyr-c(D-Orn-Phe-D-Pro-Gly-), Tyr-c(D-Lys-Phe-D-Pro-) and Tyr-c(D-Orn-Phe-D-Pro-) were more potent (guinea pig ileum IC_{50} 2.1-5.4 nM, mouse vas deferens IC_{50} 4.8-60 nM), than the parent peptide. The dimeric analogue $[Tyr\text{-}c(D\text{-}Abu\text{-}Phe\text{-}D\text{-}Pro\text{-})]_2$ was only slightly more potent (guinea pig ileum IC_{50} 158 nM, mouse vas deferens IC_{50} 306 nM). All the other linear and cyclic peptide analogues, Tyr-D-Lys-Phe-Pro, Tyr-D-Orn-Phe-Pro, Tyr-D-Abu-Phe-Pro, Tyr-D-Lys-Phe-D-Pro, Tyr-D-Orn-Phe-D-Pro, Tyr-D-Abu-Phe-D-Pro, $[Tyr\text{-}c(D\text{-}Lys\text{-}Phe\text{-}Pro\text{-})]_2$, $[Tyr\text{-}c(D\text{-}Orn\text{-}Phe\text{-}Pro\text{-})]_2$

and [Tyr-c(D-Abu-Phe-Pro-)]$_2$ were much less potent in both the receptor preparations. None of the peptides was any more selective for the μ receptor than Tyr-Pro-Phe-Pro-NH_2.

Peptidomimetic analogues of opiate peptides have been reported.[232,233] In one case, using conformational preferences of a δ-receptor selective ligand [Tmt^1, D-Pen^2, D-Pen^5]enkephalin (Tmt = 2',6'-dimethyl-β-methyltyrosine) and pharmacophore (two aromatic moieties and an amino group) searching approach, two compounds (benztropine mesylate and phentolamine mesylate) were found to be active in μ, δ, and κ receptor opiate binding assays (IC_{50} 3.4-13.2 μM).

4.14 Somatostatin Analogues – The diverse biological effects of somatostatin (SST) are mediated through a family of G protein coupled receptors of which 5 members (SST_1-SST_5) have been recently identified by molecular cloning.[234] In human neuroblastoma cells, somatostatin-14 binds primarily to somatostatin receptor SST_2 subtype.[235] Reports on the effects of somatostatin analogues on various forms of cancer have been published.[236–238] Synthesis of a technetium-99m containing analogue of somatostatin for use in radioisotopic imaging studies has been reported.[239] Other analogues of somatostatin have been synthesised to investigate the role of various receptor subtypes.[240] The design of these analogues has been based on the previously reported cyclic octapeptide analogues. The cyclic disulfide bridge containing analogues, Phe(*p*-NO_2)-c[Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2, Phe(*p*-NO_2)-c[D-Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2, Phe(*p*-NO_2)-c[Cys-Tyr-D-Trp-Lys-Thr-Cys]-Tyr-NH_2, Phe(*p*-NO_2)-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-Tyr-NH_2 and D-Phe(*p*-NO_2)-c[D-Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2, displaced ^{125}I-Tyr^{11}-SS-14 from SST_2 and SST_5 receptors but not from SST_1 and SST_3 receptor subtypes. Two of the cyclic peptides containing a D-Cys residue, Phe(*p*-NO_2)-c[D-Cys-Tyr-D-Trp-Lys-Val-Cys]-Tyr-NH_2 and Phe(*p*-NO_2)-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-Tyr-NH_2, behaved as somatostatin antagonists at the SST_2 receptor subtype. The corresponding L-Cys compounds were agonists. Non-peptide mimetics of somatostatin, based on sugars as templates carrying the side chains of Trp, Phe and Lys residues (important for the biological activity) in somatostatin have been reported in previous years. The approach has been extended to other scaffolds to generate peptidomimetics of somatostatin (**71**)-(**74**)[241,242] The 1,4-benzodiazepin-2-one derivative (**71**) displaced [^{125}I-Tyr^3]octreotide with an IC_{50} of 7 μM. Similarly, the other three analogues (**72**)-(**74**) were also poor ligands at the somatostatin receptor (IC_{50} values between 10-15 μM).

4.15 Tachykinin (Substance P and Neurokinins) Analogues – Most of the work in the tachykinin area has been directed towards discovering non-peptide antagonists at NK_1, NK_2 or NK_3 receptors.[243] Only a limited number of SAR (linear and cyclic peptides), lipid membrane binding and receptor interaction studies on the peptide analogues have been reported.[244–247] In order to investigate the effects of glycosylation on tachykinin antagonists, glyco analogues of NK_2 antagonists, MEN 10376 (Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2) and MEN 10414 (Asp-Tyr-D-Trp-Val-D-Trp-NH_2), were synthesised.[247]



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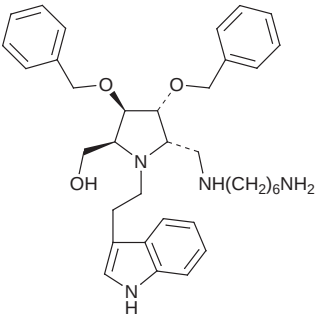

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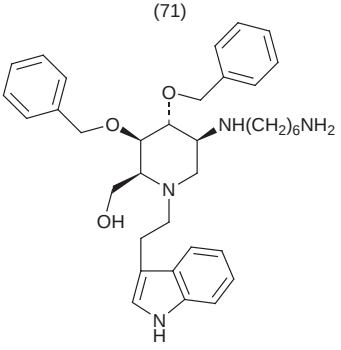

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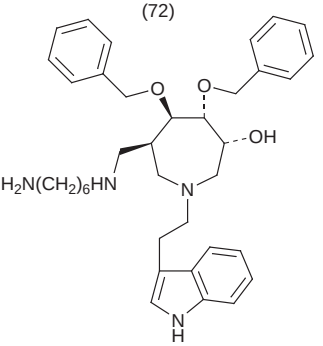

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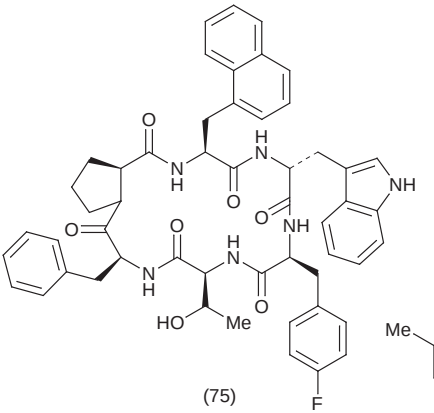
In comparison to the non-glycosylated parent peptides, the glycosylated peptides [Asn(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2, Asp-Tyr(Glc)-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2, Tyr(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2, Ser(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-D-Trp-Lys-NH_2, Asp-Tyr(Glc)-D-Trp-Val-D-Trp-NH_2 and Ser(Glc)-Asp-Tyr-D-Trp-Val-D-Trp-NH_2] were much less potent. Like the parent peptides, the glycosylated peptides were more potent in the isolated rabbit pulmonary artery preparation than in hamster tracheal preparation.

Monocyclic (**75**) and bicyclic (**76**) peptide antagonists of neurokinins have been reported.[248-251] The design of the monocyclic antagonist (**75**) was based on an earlier reported somatostatin receptor agonist L-363,301 (**77**) which did not bind to substance P receptor (IC_{50} >1 μM for NK_1 receptor) and, some sugar derivatives binding to both somatostatin and substance P receptors. Chemical modifications derived from the SAR in non-peptide substance P antagonists led to the replacement of the Lys side chain by hydrophobic residues (e.g. Nal). The Ala, Tyr and Leu analogues were much less potent ligands at the NK_1 receptor (IC_{50} >1 μM) but the Phe analogue was moderately potent (IC_{50} > 95 nM). Additional replacements led to a potent NK_1 receptor antagonist (**75**) (IC_{50} 2 nM for NK_1 receptor) which did not show any agonist activity.[248] Analogues of the NK_1 antagonist (**75**) containing Phe in place of Phe(*p*-F) and Phe, Ser, Asp, D-Pro, Ala, Trp, D-Phe, D-hPhe and Cha in place of Nal were less potent (IC_{50} >



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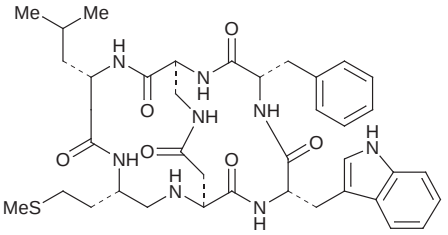

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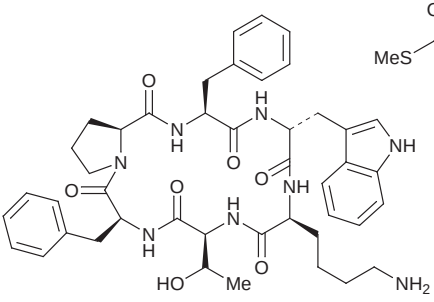

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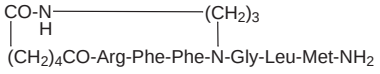
35-1389 nM). The bicyclic peptide (**76**) was an NK_2 receptor antagonist (pK_B NK_1 6.1, NK_2 10.1, NK_3 inactive at 10 μM).[249]

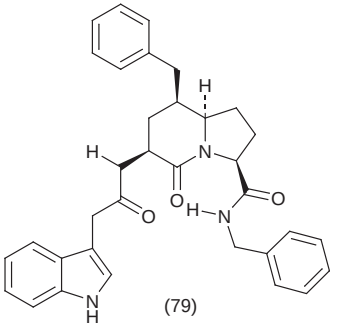
NK_1 selective agonists were obtained in another series of cyclic peptides in which the N-terminus of the peptide was linked to the backbone.[250,251] The design of these backbone to N-terminal cyclic peptides with the general formula c[-$(CH_2)_m$-NHCO-$(CH_2)_n$-Arg-Phe-Phe-N-]CH_2CO-Leu-Met-NH_2 (n = 2, 3, 6 and m = 2, 3, 4) was based on the NMR and molecular modelling data on an NK_1 selective analogue, Ac-Arg-Phe-Phe-Pro-Leu-Met-NH_2 (EC_{50} NK_1 3 nM, NK_2 >200,000 nM, NK_3 0.5 nM). Two of the cyclic peptides, c[-$(CH_2)_3$-NHCO-$(CH_2)_3$-Arg-Phe-Phe-N-]CH_2CO-Leu-Met-NH_2 and c[-$(CH_2)_4$-NHCO-$(CH_2)_3$-Arg-Phe-Phe-N-]CH_2CO-Leu-Met-NH_2 (**78**; EC_{50} NK_1 5 nM, NK_2 >50,000 nM, NK_3 >10,000 nM) were the most potent and NK_1 selective analogues of the series. Two



(78)

of the larger ring compounds, c[-$(CH_2)_2$-NHCO-$(CH_2)_6$-Arg-Phe-Phe-N-]CH_2CO-Leu-Met-NH_2 and c[-$(CH_2)_3$-NHCO-$(CH_2)_6$-Arg-Phe-Phe-N-]CH_2CO-Leu-Met-NH_2, were weak ligands at the NK_1 receptor (EC_{50} values 160 and 60 nM, respectively), but showed some activity at the NK_3 receptors (EC_{50} 1000 nM). All the other compounds, except c[-$(CH_2)_2$-NHCO-$(CH_2)_2$-Gly-Arg-Phe-Phe-N-]CH_2CO-Leu-Met-NH_2 [inactive in all the three receptor preparations (EC_{50} values >10,000 nM], were weak agonists at the NK_1 receptor (EC_{50} values 180-4000 nM) and inactive at NK_2 and NK_3 receptors (EC_{50} values > 10,000 nM).

Non-peptide antagonists of neurokinins have been obtained by random screening and semi-rational approaches (starting from the peptide antagonists or based on the Phe^7-Phe^8 residues of substance P). In a 'semi-rational' approach used to design non-peptide tachykinin antagonists, various scaffolds were used to orient the important amino acid side chains in the appropriate positions.[252,253] In one case, based on one of the earlier reported NK_2 cyclic peptide antagonist, c(Gln-Trp-Phe-Gly-Leu-Met), and conformational analysis of a similar peptide, c(Gln-Trp-Phe-Gly-LeuΨ[CH_2NH]Met) which indicated the presence of a β-bend, a conformationally restricted lactam derivative was used as a scaffold. However, compound (**79**) and similar analogues were weak antagonists at the NK_2 receptor (IC_{50} ~1-3 μM). Other compounds using piperazinone and 4-aminopiperidine (**80**; IC_{50} 12 nM) as scaffolds gave NK_1 receptor antagonists.[253] Weak NK_1 and NK_3 antagonists were obtained using 2,4-disubstituted morpholin-3-one skeleton.[254] Compound (**81**) was inactive at the NK_1 receptor (< 10% inhibition at 10 μM) and a weak antagonist at the NK_3 receptor (IC_{50} 4.09 μM). In comparison, compound (**82**) was a weak antagonist at the NK_1 receptor (IC_{50} 3.5 μM) and inactive at the NK_3 receptor (< 10% inhibition at 10 μM).



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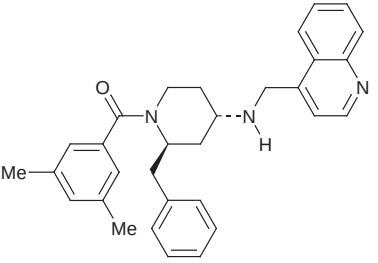

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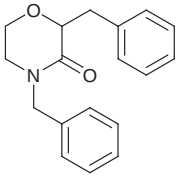

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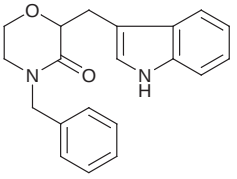
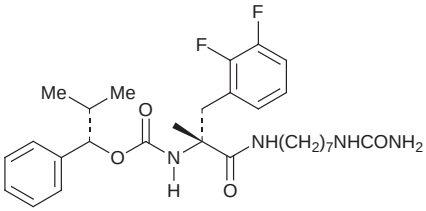


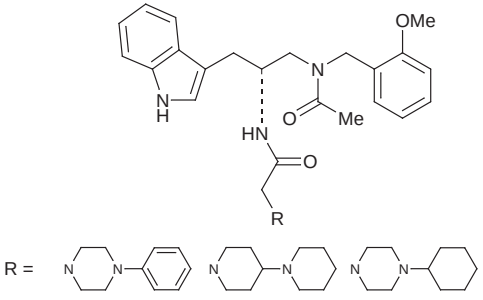
(82)

In another 'semi-rational' approach, the use of a dipeptide library as the source of a micromolar chemical lead compound for the human tachykinin NK_3 receptor was investigated.[255] The screening of a dipeptide library through a cloned human NK_3 receptor binding assay resulted in the identification of Boc-Phe-Phe-NH_2, Boc-Trp-Phe-NH_2, Boc-Phe-Trp-NH_2, Boc-Phe-Tyr-NH_2, $(CH_3)_2CH(CH_2)_2CO$-Trp-Trp-NH_2, and $(CH_3)_2CH(CH_2)_2CO$-Phe-Trp-NH_2 as weak ligands for the human NK_3 receptor (IC_{50} 1760-9610 nM). α-Methyl substitutions in Boc-Phe-Phe-NH_2 gave less potent compounds except Boc-Phe-α-MePhe-NH_2, which retained potency. Optimisation of the N- and C-terminal groups and changes in the Phe position gave a potent NK_3 antagonist. The structure-activity relationship of the C-terminal portion of this dipeptide lead was first explored and led to the identification of the urea derivative Boc-Phe-D-α-MePhe-$NH(CH_2)_7NHCONH_2$ (PD157672). This modified dipeptide has a Ke of 7 nM in blocking senktide-induced increases in intracellular calcium levels in human NK_3 receptors stably expressed in CHO cells. Subsequent optimisation of the N-terminal Boc-Phe group and the α-MePhe residue side chain of PD157672 led to the identification of (**83**), (PD161182), a non-peptide NK_3 receptor selective antagonist. Compound (**83**) blocks the senktide-evoked increases in intracellular calcium levels in cloned human NK_3 receptors stably expressed in CHO cells with Ke of 0.9 nM (NK_3 IC_{50} = 7.3 nM; NK_2 IC_{50} = 790 nM; NK_1 IC_{50} = 3000 nM).[255]



(83) PD161182

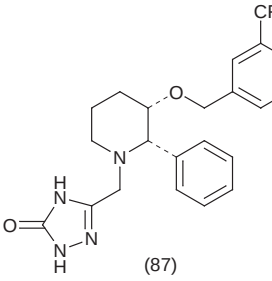
A number of the tachykinin antagonists are tryptophan or phenylalanine derivatives.[256-259] Examples of the tryptophan and phenylalanine based neurokinin antagonists include compounds (**84**)-(**86**). The three antagonists (**84**)-(**86**)



(84) LY303241 (85) LY303870 (86) LY306740

inhibited both the vasodepressor and salivary responses to substance P in a dose-dependent manner. LY303241 and LY306740 were more potent in inhibiting the vascular response to substance P while LY303870 was more potent in inhibiting the salivary response.[259]

A number of non-peptide antagonists of neurokinins, obtained by medicinal chemistry around random screening leads, have shown activity in a number of *in vitro* and *in vivo* test systems.[260–267] Many of these show good oral bioavailability. Examples of potent and selective antagonists include compounds (**87**)-(**91**). The triazolone (**87**) was a potent, orally active (46% oral bioavailability in the rat and 24% in rhesus monkey) NK_1 receptor antagonist (IC_{50} 0.05 nM; NK_2 and NK_3 IC_{50}s >1000 nM). It blocked SP-induced dermal extravasation in the guinea pig. It also inhibited cisplatin-induced emesis in the ferret.[260,261] Similarly, another NK_1 receptor antagonist (**88**) demonstrated a wide spectrum of anti-emetic activity, inhibiting emesis in ferret induced by cisplatin, cyclophospamide, morphine, etc. Replacement of the trifluoromethyl group in (**88**) by Me, Et, *n*-propyl, *cyclo*-propyl, Ph, SMe, SO_2Me, NH_2, $N(Et)_2$, NHAc and CF_3 gave compounds with similar potency in the *in vitro* NK_1 receptor binding assay. However, in the *in vivo* antiemetic test in ferrets, the most potent compound was the trifluoromethyl analogue (**88**) (ED_{90} 0.03 mg/kg).[263,264] Compound (**89**) was an NK_3 selective antagonist (Human NK_3 K_i 1.2 nM, NK_2 K_i 40.3 nM, NK_1 K_i 1300 nM). The non-peptide (**89**) was also active in a hNK_3 receptor expressing (HEK 293) cellular assay. At 1-1000 nM concentration, the compound produced a concentration-dependent antagonism of NK_B-induced Ca^{2+} mobilisation with a



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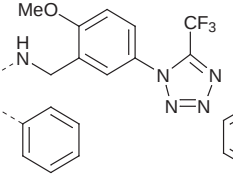
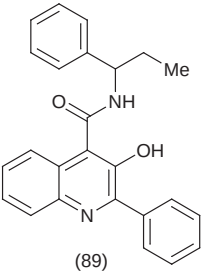


(88) GR205171



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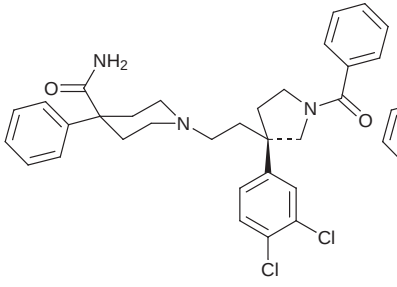
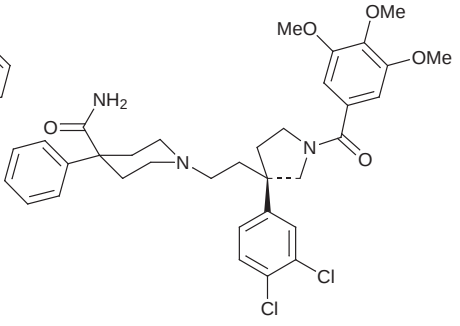


(90) MDL 103,220



(91) MDL 105,212

K_b of 3.0 nM.[262] MDL 103,220 (**90**) was an NK_2 receptor-selective antagonist (NK_2 receptor IC_{50} 2.25 $\pm$ 0.20 nM; NK_1 receptor IC_{50} 161 $\pm$ 34.9 nM). The affinity at the NK_1 receptor was improved by replacing the benzoyl group by 2-methoxy- or 3-methoxy-benzoyl groups (IC_{50} 73 and 107 nM, respectively). The corresponding 3,4,5-trimethoxy (**91**) and 3,4,5-triethoxy analogues were nearly equipotent at the NK_1 and NK_2 receptors but the trimethoxy analogue was about 10-fold more potent (NK_2 receptor IC_{50} 18.8 $\pm$ 2.23 nM; NK_1 receptor IC_{50} 6.19 $\pm$ 0.85 nM) than the triethoxy analogue.[267]

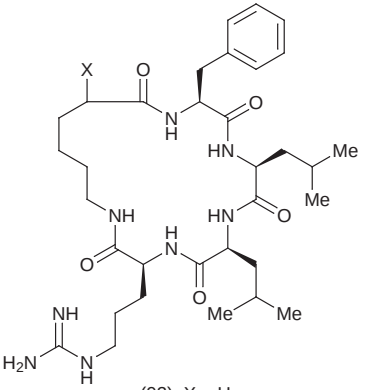
4.16 Thrombin Receptor Peptides – In recent years, thrombin has been shown to have a number of cellular actions that are mediated by proteolytic activation of a specific cell surface receptor known as the thrombin receptor. Activation of the receptor occurs by thrombin cleavage of an extracellular N-terminal domain thereby exposing a new N-terminus that intramolecularly binds to the receptor. Thrombin receptors (a member of G protein-coupled family of receptors) are present on a variety of cells, including platelets, endothelial cells, vascular smooth muscle cells, fibroblasts and some macrophages and white cells. Considerable evidence has accumulated which indicates that locally generated thrombin, followed by thrombin receptor activation, plays a central role in the development of atherosclerosis and in the restenosis of coronary arteries following angioplasty.[268–270] Thus antagonists of thrombin receptor may have therapeutic potential in various diseases. Various aspects of thrombin receptor research have been reviewed.[271–274]

Based on the human thrombin receptor 'tethered ligand' sequence Ser^{42}-Phe-Leu-Leu-Arg-Asn-Pro-Asn-Asp-Lys-Tyr-Glu-Pro-$Phe^{55}$and its fragments, e.g. (Ser^{42}-Phe-Leu-Leu-Arg^{46}), a number of agonist and antagonist analogues have been reported.[275–278] SAR studies on Ser-Phe-Leu-Leu-Arg pentapeptide were carried out by replacing various amino acid residues.[277] Replacement of Phe^2 by Phe(*p*-F) gave 3-fold improvement in potency. $Phe(OCH_3)^2$ analogue was 2-fold less potent. All the other analogues, Phe(*p*-I)^2, Phe(*p*-NH_2)^2, Tic^2, Phe(*p*-guanidino)^2 and $hPhe^2$, were much less potent (EC_{50} = 9->300 μM). Replacement of Phe^3 in Ser-Phe(*p*-F)-Phe-Leu-Arg-NH_2 pentapeptide by Phe(*p*-F)^3, Phe(*p*-guanidino)^3, 2-Nal^3, 1-Nal^3 and Tic^3 gave only minor differences in potency (EC_{50} values 40-470 nM). Ser-Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2, was the most potent peptide (EC_{50} = 40 nM) and Ser-Phe(*p*-F)-Tic-Leu-Arg-NH_2, was the least potent pentapeptide (EC_{50} = 470 nM) in stimulating platelet aggregation. N-Terminal modifications of the agonist pentapeptide, Ser-Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2, by introducing groups like (2-thiophene)acetyl-, (3-thiophene)acetyl-, N-acetyl-2-aminobenzoyl-, phenylacetyl-, (2-thiophene)sulfonyl-, (2-flurophenyl)acetyl-, (3-fluorophenyl)acetyl-, (4-fluorophenyl)acetyl-, (3-chlorophenyl)acetyl- and (3-indole)acetyl- did not lead to improvement in potency.

In comparison to the above pentapeptide derivatives, N-terminal modifications of the tetrapeptide Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2 resulted in peptides which inhibited Ser-Phe-Phe-Leu-Arg-Arg-NH_2 and Ser-Phe-Leu-Leu-Arg-Asn-Pro-NH_2 stimulated platelet aggregation.[277] *Trans*-cinnamoyl-Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2 was the most potent compound of the series (IC_{50}s 7.8

and 200 nM against the two agonists). Additional substitutions in *trans*-cinnamoyl-Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2 by $hPhe^2$, $Phe(p\text{-}NO_2)^2$, $Phe(p\text{-}Cl)^2$, $Phe(p\text{-}OMe)^2$, Phe^2, Cit^2, His^2, Pal^2, Orn^2, Arg^2, $hArg^2$, $Arg(tetramethyl)^2$, $hArg(tetramethyl)^2$ and Lys^2 residues did not lead to further improvement in potency. A number of the tetrapeptide derivatives, e.g. N-acetyl-4-aminobutyryl-, 2-thiopheneoyl-, 3-thiopheneoyl-, 3-furanoyl-, 2-indolyl- and 4-chlorobenzoyl-Phe(*p*-F)-Phe(*p*-guanidino)-Leu-Arg-NH_2 and *trans*-cinnamoyl-Phe(*p*-F)-Lys-Leu-Arg-NH_2, *trans*-cinnamoyl-Phe(*p*-F)-hArg-Leu-Arg-NH_2 and *trans*-cinnamoyl-Phe(*p*-F)-hArg(tetramethyl)-Leu-Arg-NH_2, were partial agonists.

The minimal peptide sequence Phe-Leu-Leu-Arg of the N-terminal thrombin receptor peptide has also been used to synthesise cyclic peptides as thrombin receptor agonist/antagonist peptides.[278] Three cyclic peptide analogues, c(Phe-Leu-Leu-Arg-Aca) (Aca = aminocaproic acid) (**92**), c(Phe-Leu-Leu-Arg-N^{ε}Lys) (**93**), and c(Phe-Leu-Leu-Arg-Gly) were synthesised and evaluated in a gastric smooth muscle strip assay. Compound (**93**), wherein the ε-amino group of lysine was coupled to the α-carboxyl of arginine, exhibited a contractile activity comparable to that of the linear pentapeptide Ser-Phe-Leu-Leu-Arg-NH_2. However compound (**92**), wherein the aminocaproic linker group yielded a ring size the same as for compound (**93**) but without a primary amino group, exhibited a contractile activity 600-1000-fold lower than compound (**93**).

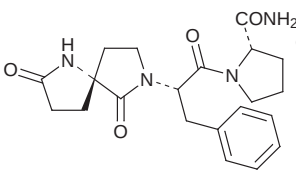


(92) X = H
(93) X = NH_2

In addition to the thrombin receptor related peptides mentioned above, several other inhibitors of thrombin-stimulated platelet aggregation and angiogenesis have been reported recently.[279–281] For example, a heptapeptide Leu^{271}-Ala^{277} (Leu-Asn-Ala-Glu-Asn-Asn-Ala) from kininogen domain 3 inhibited thrombin-induced aggregation of platelets with an IC_{50} of 65 μM. The effect was specific for the activation of thrombin but not ADP or collagen. The synthetic peptide did not inhibit the active site of thrombin at a concentration below 500 μM.[280] Similarly, plasma kininogens were found to be selective inhibitors of alpha-thrombin activation of platelets and endothelial cells. Bradykinin and an

analogue, Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Ser-Ser-Arg-Ile-Gly, inhibited α-thrombin-induced platelet aggregation and secretion with an IC_{50} of 0.25 and 1 mmol/L and of 0.23 and 0.5 mmol/L, respectively.[281]

4.17 Thyrotropin-releasing Hormone Analogues – In addition to controlling the release of pituitary hormones (e.g. thyrotropin releasing hormone and prolactin), TRH has been shown previously to have CNS effects. Conformationally restricted analogues of TRH (Pyr-His-Pro-NH_2) (**94**, **95**) and a related dipeptide (**96**) have been reported.[282–285] The spirocyclic analogue (**94**) was three times less potent than the corresponding linear peptide, [Phe^2]TRH, in the binding and functional assays. Both these compounds (IC_{50}s in the binding assay 1500 nM and 4400 nM, respectively) were >150 times less potent than TRH (IC_{50} 10 nM).[282] The 6,5-bicyclic lactam ring containing analogues of [Cha^2]TRH (**95**) showed widely differing potencies. One of the diastereomers (S at the δ carbon of the Pro ring) was 1200 times less potent than the corresponding R isomer. The more potent analogue of the two was about 3-fold more potent than [Cha^2]TRH in the binding and the functional assays, but >150-fold less potent than TRH.[283] Computer simulations predicted that the positions of the Cha^2 and Pro-NH_2 moieties relative to the glutamate were different in the two analogues and that the conformation of the higher affinity analogue was different from that of *trans*-TRH in solution but was superimposable on that of *trans*-TRH found in a model of the TRH/TRH receptor complex.[284] The diketopiperazine derivative (**96**) completely reversed electroconvulsive shock-induced amnesia at doses of 0.1 and 1.0 mg/kg and scopolamine-induced amnesia (83%) at a dose of 1 mg/kg.[285] CNS profile of an earlier reported TRH analogue (NS-33) has been described.[286,287]



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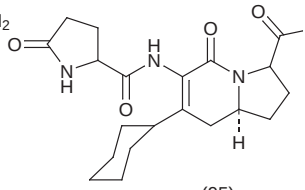

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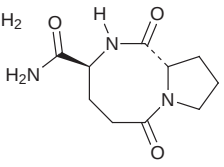


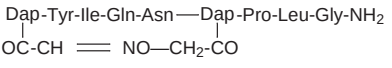
(96)

4.17 Vasopressin and Oxytocin Analogues – Very few analogues of oxytocin and vasopressin have been reported this year.[288–291] In addition, some studies directed towards the receptor ligand interactions and metabolism of vasopressin and oxytocin analogues are reported.[292,293] [2-Nal^3]-Arg vasopressin was about 6-fold less potent than Arg-vasopressin as a pressor agent (V_1-receptors), but was much more potent than Arg vasopressin as an antidiuretic agent.[288] [2-Nal,[3] D-Arg^8]-Vasopressin was a potent V_1 receptor antagonist (pA_2 8.65) of vasopressin. Replacement of Asn^5 by diaminopropionic acid (Dap) or diaminobutyric acid (Dab) in oxytocin and vasopressin antagonists, d$(CH_2)_5$[$Tyr(Me)^2$]AVP, d$(CH_2)_5$[$Tyr(Me)^2$ Thr^4,Tyr-$NH_2{}^9$]OVT, desGly-NH_2, d$(CH_2)_5$[D-Tyr^2, Thr^4]OVT, desGly-NH_2, d$(CH_2)_5$[D-Phe^2, Thr^4]OVT and desGly-NH_2,

d$(CH_2)_5$[D-Trp^2, Thr^4]OVT, have been reported. Although the compounds were less potent than the parent peptides, the antagonists were more selective than the Asn^5 analogues.[289] Analogues of the linear vasopressin antagonist D-Tyr(Et)^2-Phe^3-Gln^4-Asn^5-Arg^6-Pro^7-Arg^8-Tyr^9-NH_2 substituted with L-, or D-pyroglutamate at position 1, Asn or Val at position 4 and Arg or Met at position 6 have been reported.[290] All of these peptides bound to the V_{1a} vasopressin receptor with affinities ranging 33.6-5470 nM. Of this series, only two peptides, [LpGlu^1, Val^4, Arg^6, Tyr^9]AVP-NH_2 (K_d = 48.4 nM) and [D-pGlu^1, Val^4, Arg^6, Tyr(NH$_2$)^9]AVP (K_d = 691 nM), bound to the V_2 vasopressin receptor. One of the D-pGlu analogues, D-pGlu-D-Tyr(Et)-Phe-Val-Asn-Arg-Pro-Arg-Tyr-NH_2, exhibited the highest V_{1a}/OTR selectivity [V_{1a}VPR K_d = 82 nM; OTR no binding at 10 μM). Simultaneous replacements in positions 3, 5 and 8 gave less potent oxytocin analogues, [Aib^3, Thr^5]OT, [Aib^3, Thr(OMe)^5]OT, [Aib^3, Orn^8]OT, [Thr(OMe)^5,Orn^8]OT and [Phe^2, Thr(OMe)^5, Orn^8]OT.[291]

Chimeric peptides obtained by linking vasopressin and bradykinin analogues have been reported.[294] Single-chain peptides linking vasopressin and bradykinin either directly or via an aminohexanoic acid linker resulted in compounds, [Arg-vasopressin(1-9)-bradykinin(1-9) and Arg-vasopressin(1-9)-ε-Ahx-bradykinin(1-9)], which were more potent at the bradykinin B_{2a} receptor (K_d 52-61 nM) and much weaker at the vasopressin V_{1a} and V_2 receptors (K_d 710-5300 nM). [Arg^6]Arg-vasopressin(4-8)-ε-Ahx-[D-Arg^0, Hyp^3, D-Phe^7, Leu^8]bradykinin(1-9)] was the most selective analogue at the B_{2a} receptor (K_d 21.4 nM at the B_{2a} receptor and >10000 at the V_{1a} and V_2 receptors) and [phenylacetyl-D-Tyr(Et)^2, Arg^6-Lys(N$^{\varepsilon}$D-Arg^0, Hyp^3, D-Phe^7, Leu^8-bradykinin)^9]-Arg vasopressin was the most potent analogue at the V_{1a} receptors (K_d 1060 nM at the B_{2a} receptor, 0.12 nM at the V_{1a} and >10,000 nM at the V_2 receptors). Two other chimeric analogues, [phenylacetyl-D-Tyr(Et)^2, Arg^6]AVP(1-8)-εAhx-D-Arg^0-[Hyp^3, D-Phe^7, Leu^8]bradykinin(1-9) and [phenylacetyl-D-Tyr(Et)^2, Arg^6]AVP(1-7)-D-Arg^0-[Hyp^3, D-Phe^7, Leu^8]bradykinin(1-9), bound to all three receptors (K_d 4.81 and 12.3 nM at the B_{2a} receptor, 0.31 and 0.32 nM at the V_{1a} and 29.5 and 500 nM at the V_2 receptors).

Analogues of oxytocin replacing the disulfide bridge by an oxime bond have been obtained by the reaction of an aldehyde and amino-oxy side chain in the presence of the remaining unprotected side chains.[295] No biological data is reported for the two analogues (**97**) and (**98**).



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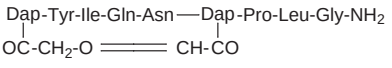
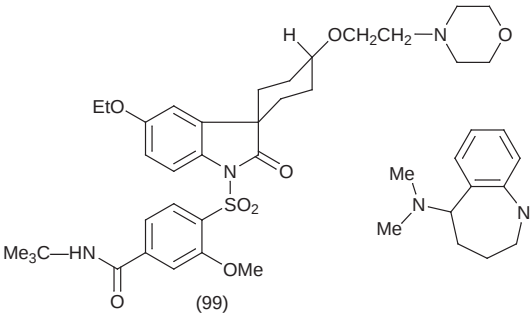


(98)

Non-peptide antagonists of vasopressin have been described.[296–298] Compounds (**99**) (OPC-31260) and (**100**) (SR121463A) are potent and selective, orally active, vasopressin V_2 receptor antagonists. Non-peptide (**99**) antagonised Arg-vasopressin-stimulated adenylyl cyclase activity (K_i = 0.26 ± 0.04 nM) without any intrinsic agonist effect. In normally hydrated conscious rats, (**99**) induced

aquaresis after iv (0.003-0.3 mg/kg) or oral (0.03-10 mg/kg) administration. The effect was dose-dependent and lasted about 6 hours at a dose of 3 mg/kg po. Na^+ and K^+ excretions were unchanged. SR 121463A did not show antidiuretic effects in vasopressin-deficient Brattleboro rats. In alcohol-anaesthetised rats, (**100**) increased urine flow after oral administration at a dose of 1-30 mg/kg.



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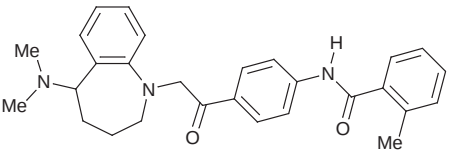

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5 Enzyme Inhibitors

Most of the work this year has been on converting enzyme, HIV protease, farnesyltransferase, various matrix metalloproteases and thrombin inhibitors. Only a limited amount of work has been published on the inhibitors of renin, elastase, calpain and cathepsin D.

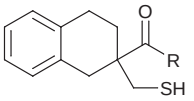
5.1 Converting Enzyme [Angiotensin (ACE), Neutral Endopeptidase (NEP), Endothelin and Interleukin-1β (ICE)] Inhibitors – Many peptides are obtained from their precursors by the action of converting enzymes. For example, angiotensin converting enzyme cleaves a dipeptide from the C-terminus of angiotensin I to generate the pressor peptide angiotensin II. In addition, some of the biologically active peptides (e.g. bradykinin and atrial natriuretic factor) are degraded by the converting enzymes. Thus many physiological and pathological processes are controlled by converting enzymes. Inhibitors of angiotensin, neutral endopeptidase, endothelin and interleukin-1β have been reported.

5.1.1 Angiotensin Converting Enzyme and Neutral Endopeptidase Inhibitors – Many inhibitors of these enzymes, containing a thiol, carboxyalkyl or a phosphinic acid group as a zinc binding ligand, have been described in the past. Further modifications have been attempted to generate more potent and dual inhibitors of ACE and NEP. Peptide-based inhibitors of the two enzymes include mercaptoacyldipeptides, N-(1-carboxy-3-phenylpropyl)- derivatives and retro-inverso analogues of angiotensin I and bradykinin fragments.[299–301] Mercaproacyldipeptides of the general formula $HSCH(R_1)CO\text{-}NHCH(R_1')CO\text{-}N(R)CH(R_2')COOH$, e.g. N-[(2S)-2-mercapto-3-methylbutanoyl]-Ile-Tyr and N-[(2S)-2-mercapto-3-phenylbutanoyl]-Ala-Pro inhibited neutral endopeptidase and

ACE (IC_{50} 1.4-3.8 nM NEP and 0.26-0.3 nM ACE). Both the compounds were active when administered orally.[299] In the N-(1-(R,S)-carboxy-3-phenylpropyl)-Ala-Ala-Tyr-p-aminobenzoate (K_i = 16 nM) series of NEP inhibitors, replacement of the second alanine by D-Ala or Asp abolished inhibitory activity, while Val, Ser and Leu substituted analogues retained activity. A relative potency order of Ala> Val> Ser> Leu was observed.[300] In comparison to Bz-Phe-Val-Trp, Bz-Gly-Val-Trp and Bz-Phe-Gly-Trp (ACE inhibition K_i 100-180 nM and concentration for 2-fold potentiation of BK 28-36 μM), retro-inverso analogues Bz-gGly-D-Val-mTrp, An-mPhe-Gly-Trp, Bz-gPhe-mGly-Trp, Bz-Phe-gGly-mTrp, Bz-gGly-Arg-mTrp and Bz-gGly-D-Arg-mTrp were much weaker inhibitors in both the assays.[301] The most potent peptide in the ACE inhibition assay was An-mPhe-Gly-Trp (K_i 1.2 μM).

In the mercaptoacyldipeptide series of compounds, conformationally restricted analogues of thiorphan have been prepared and in other cases interactions in the P' positions have been optimised. Restricting the phenyl ring in thiorphan by a methylene or ethylene bridge led to potent inhibitors. The ethylene bridge compounds, e.g. (**101**) [R = Gly-OBzl, αAla-OMe, -NH$(CH_2)_6$COOH, Ala-OBzl, Val-OBzl, Leu-OBzl, Phe-OBzl, Tyr-OBzl], were comparable in potency to thiorphan.[302,303] Mono-, di- and tri-cyclic azepinones have been incorporated in the mercaptoacyldipeptide analogues (mimicking an Ala-Pro dipeptide framework) to improve binding interactions to the S_1', S_2' and S_3' subsites.[304–307] Examples of such compounds include (**102**)-(**104**). The monocyclic azepinone analogue (**102**) demonstrated a high level of activity versus ACE (IC_{50} 5 nM) and NEP (IC_{50} 17 nM) both *in vitro* and *in vivo*. Compound (**102**) inhibited angiotensin I-induced pressor response in normotensive rats (ED_{50} 0.12 μmol/kg iv) and showed oral activity.[304] SAR studies in the tricyclic azepinone compound (**104**) (CGS 28106) (ACE IC_{50} 40 nM and NEP IC_{50} 48 nM) indicated that replacement of the phenyl group by a methyl, isopropyl or a biphenyl group reduced the potency against ACE by <4-fold. However, the potency against NEP was reduced by >20-fold. Some other changes, e.g. replacement of the mercaptoacyl group by HS-C$(CH_3)_2$-CO-, resulted in loss of potency against both the enzymes (IC_{50} >1000 nM). CGS 28106 (**104**) displayed good oral bioavailability. In conscious rats, the compound (10 mg/kg) inhibited pressor responses induced by angiotensin II and prevented the degradation of exogenously administered atrial natriuretic peptide. Administration of the compound by oral or intravenous routes gave a similar profile in both the tests.[306,307] Other examples of thiol containing di- and tri-peptide inhibitors of ACE/NEP include compounds (**105**) (K_i ACE 0.35 nM, NEP 1.6 nM) and (**106**). Both the compounds demonstrated oral activity.[308,309]

In the carboxyalkyl series of inhibitors, glutaric acid derivatives of the type (**107**) and (**108**) were inhibitors of NEP. Replacement of the Gly residue in (**107**) by Ala or β-Ala gave slightly less potent compounds (IC_{50} 2.8-20 nM). The C-terminal Met and Pro analogues were much less potent (IC_{50} 101 and >1000 nM, respectively). Biphenyl group replacement also resulted in much less potent analogues.[310] However, replacement of the P_2' substituent with a series of amino-heterocycles which might have hydrogen bond interactions with the enzyme gave



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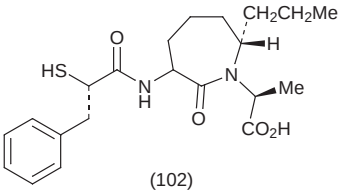

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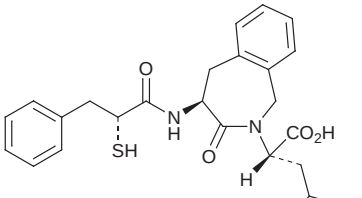

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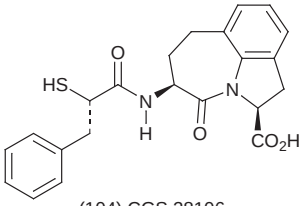
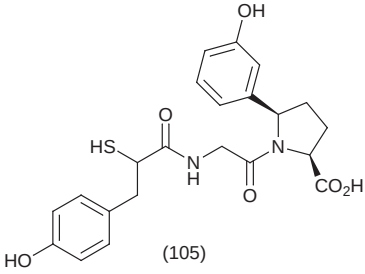


(104) CGS 28106



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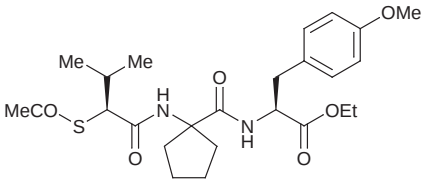
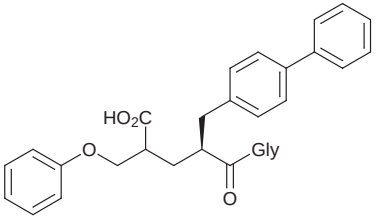


(106)

more potent compounds. For example the aminothiazolyl analogue (**108**) was about 5-fold more potent (NEP IC_{50} 0.69 nM) than compound (**107**). In comparison, aminothiadiazolyl and aminoimidazolyl analogues were much less potent (IC_{50} values 92 and 218 nM, respectively). The phenoxymethyl group in (**108**) was also important for the enzyme inhibitory activity. Its replacement by a H, CH_3, or n-butyl group gave less potent compounds (IC_{50} values 122, 58 and 9.8 nM, respectively).[311]



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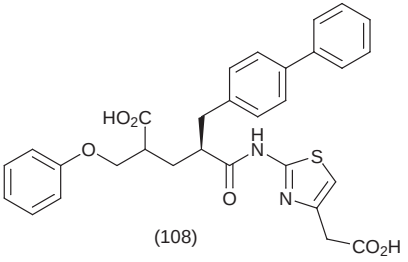

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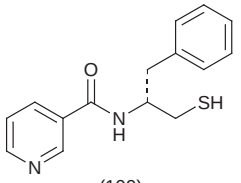
5.1.2 Endothelin Converting Enzyme Inhibitors – The identity of the enzyme responsible for converting endothelin precursor to endothelin has not yet been established with certainty. Enzymes with neutral metalloprotease, aspartyl protease and cysteine protease activities have been claimed to process big endothelin to endothelin-1. The design of inhibitors has been based around the naturally occurring inhibitor of metalloproteases, phosphoramidon, and other inhibitors of ACE and NEP mentioned above. Random screening of zinc metalloprotease library resulted in the discovery of amino acid derivatives like (**109**) and (**110**) as weak inhibitors of ECE. Further replacements of both the pyridyl and phenyl groups in (**109**) (IC_{50} 7.8 μM) by a number of other aromatic substituents led to compound (**111**) (IC_{50} 1.7 μM) which was 2-fold more potent than phosphoramidon.[312] A number of analogues of (**110**) in which Ar^1 is phenyl, 4-benzyloxy-Ph, 4-phenyl-Ph, 3-phenyl-Ph, 4-Br-Ph or 3-Br-Ph and Ar^2 is indole-3-yl, Ph 4-(OH)-Ph, 4-(OCH_2Ph)-Ph or 4-(2-thienyl)-Ph, were similar in potency to phosphoramidon.[313]



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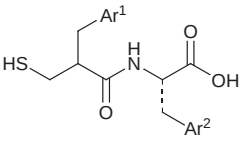

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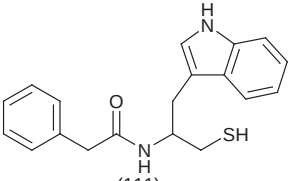

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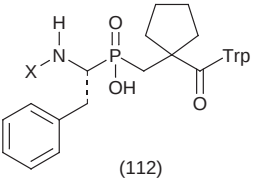
Phosphinic acid derivatives like (**112**) (X = N-$MeSO_2$-Lys or *p*-nitrobenzoyl) inhibited ECE (IC_{50} 50-70 nM), ACE (IC_{50} 1.5-2.5 nM) and NEP (IC_{50} 55-90 nM). Replacement of N-$MeSO_2$-Lys or *p*-nitrobenzoyl residues in (**112**) by benzyloxycarbonyl, acetyl, *t*-butylcarbonyl and benzoyl groups led to reduction in the enzyme (ECE) inhibitory activity (IC_{50} values 250-600 nM).[314] Other examples of phosphinic acid based ECE inhibitors include compounds like (**113**) and (**114**). Analogues of (**113**) containing Thr, Ile, Gln, Tyr or Arg residue at the C-terminal end were poor inhibitors of ECE (IC_{50} <10 μM). The most potent compound of the series containing a Trp residue in this position was comparable in potency to phosphoramidon (IC_{50} ~1 μM). All of these compounds also inhibited NEP (IC_{50} >1 μM).[315] Phosphinic acid derivative (**114**) was a more potent (IC_{50} 25 nM) and selective (13% inhibition of NEP at 300 nM) inhibitor of ECE. Replacement of the N-terminal Lys in (**114**) by a Z-group and the Nal residue by a Phe or Leu residue resulted in much less potent compounds.[316]

5.1.3 Interleukin-1β Converting Enzyme Inhibitors – Interleukin-1β converting enzyme (ICE) is a cysteine protease found primarily in monocytic cells. The enzyme cleaves precursor interleukin-1β to generate biologically active mature interleukin-1β, a cytokine which elicits an inflammatory response *in vivo*. The substrate specificity of interleukin-1β converting enzyme was defined by using a combinatorial positional scanning approach. Ac-X-X-X-



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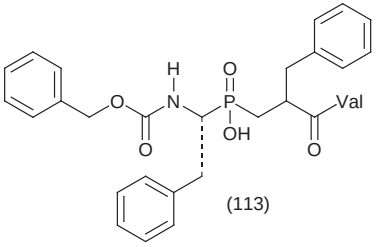

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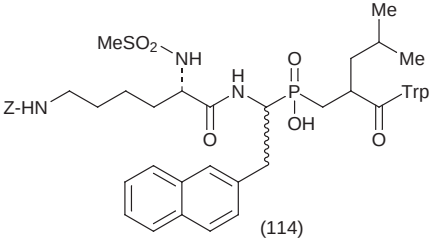
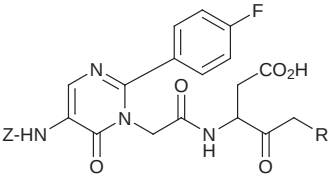


(114)

Asp-aminomethylcoumarin derivatives were synthesised and evaluated as substrates. Ac-Trp-Glu-His-Asp-aminomethylcoumarin was the best substrate. Hydrophobic amino acids were preferred in S_4 (Trp> Tyr> Nle> Leu) position.[317] Z-Val-Ala-Asp derivatives have been reported as inhibitors of ICE. Replacements of the Val-Ala dipeptide (P_3-P_2 binding region) by a substituted pyrimidine derivative led to compounds (**115**)-(**117**) which were similar in potency to the corresponding Val-Ala analogues.[318]



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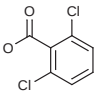

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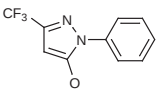

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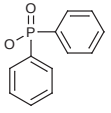

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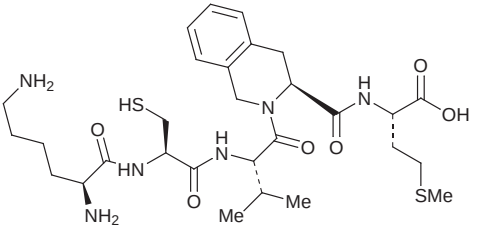
5.2 Ras Protein Farnesyltransferase Inhibitors – Cysteine farnesylation of the ras oncogene product Ras is required for its transforming activity and is catalysed by farnesyltransferase. Protein farnesyltransferase catalyses the transfer of a farnesyl group from farnesyl diphosphate to a cysteine residue of a protein substrate such as Ras.[319] The enzyme recognises a tetrapeptide sequence CAAX

(C is cysteine, A is an aliphatic amino acid and X is the C-terminal amino acid, which is usually Met, Ser, Ala, Cys, or Gln] at the C-terminus of the protein. A closely related enzyme, geranylgeranyltransferase, recognises the CAAX motif when X is either Leu or Phe, but transfers a geranylgeranyl group from geranylgeranyl diphosphate. Inhibition of farnesyl protein transferase represents a possible method for preventing association of Ras p21 to the cell membrane, thereby blocking its cell-transforming capabilities. Such inhibitors may have therapeutic potential as anticancer agents.[320]

Initial approaches for the design of farnesyltransferase inhibitors were based on the tetrapeptide Cys-Val-Phe-Met.[321] Conformationally restricted analogues obtained by replacing the Val-Phe residues in Lys-Cys-Val-Phe-Met pentapeptide by MeVal-Phe, t-butylglycyl-Phe, MeVal-Phg and Pro-Tic dipeptides were similar in potency to the parent peptide (IC_{50} 1-2 μM). A number of other analogues containing Val-D-Tic, D-Pro-Tic, Pro-D-Tic, D-Val-Tic and D-Pro-D-Tic dipeptides were much less potent (IC_{50} 15-100 μM). Most potent enzyme inhibitors were Val-Tic (**118**), MeVal-Tic and Tbg-Tic containing compounds (IC_{50} 5-40 nM). Peptide bond replacements in the conformationally restricted analogues led to compound (**119**) which was effective in prolonging the survival time in athymic mice implanted intraperitoneally with H-*ras*-transformed RAT-1 tumour cells when administered ip twice a day (45 mg/kg per injection) for 11 consecutive days.[322] Replacement of the Val-Phe dipeptide by *p*-aminobenzoic acid or 3-(aminomethyl)benzoic acid gave moderately potent inhibitors like Cys-NH-C_6H_4CO-Met. Further modifications led to compounds like (**120**) (IC_{50} 40 nM).[323] Removal of the carboxylic acid in (**120**) results in a 10-fold loss of



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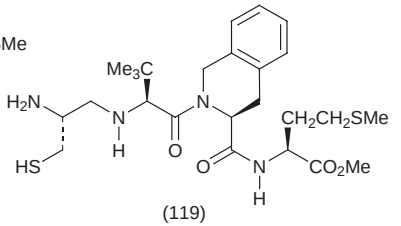

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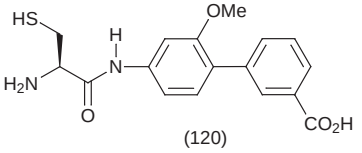

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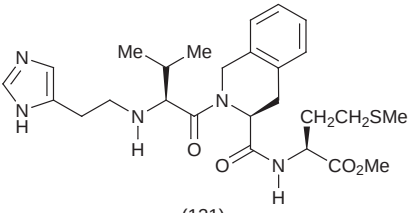
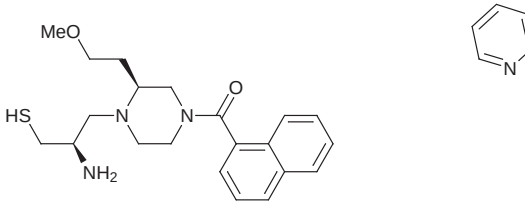


(121)

inhibitory activity. Substitution of the cysteine residue by 4-imidazolyl group, linked *via* 1-, 2-, or 3-carbon alkyl or alkanoyl spacers, in the conformationally restricted Val-Tic-Met or tLeu-Tic-Gln analogues gave potent inhibitors which were active in the cell-based assays.[324] One of the more potent analogues, (**121**) (BMS-193269) (IC_{50} 0.79 nM), inhibited the growth of H-*ras*-transformed NIH 3T3 cells in soft agar (IC_{50} 5 μM).

Non-peptide inhibitors of farnesyltransferase have been reported.[325,326] One of the 2-substituted piperazine derivatives (**122**) was a potent inhibitor of the enzyme (IC_{50} 3 nM) and inhibited post-translational processing of ras protein in RAT1 cells transformed with viral Ha-*ras*. Compound (**122**) inhibited the growth of tumours arising from Ha-*ras*-transfected RAT1 cells implanted in nude mice (45 mg/kg per day, ip or twice a day orally).[325] In the aminoacyl series of non-peptide inhibitors (**123**, X = CO), replacement of the N-phthaloylglycine residue by a number of other protected amino acid residues, e.g. phthaloyl-Leu, His, Ala, Met or Phe, and Z-Gly, Z-Tyr, Boc-Cys(4-methoxybenzyl) and Boc-glycine residues gave less potent compounds (IC_{50} 1.2-10 μM). In the sulfonylamide series of compounds (**123**, X = SO_2), vinyl and benzyl sulfonamide derivatives were most potent (IC_{50} 1.0 μM). Replacement of the phenyl group by *p*-nitrophenyl, naphthyl or thiophenyl groups gave less potent compounds.[326] Farnesyl phosphate and hydroxamic acid derivatives of amino acids and peptides have been reported as inhibitors.[327,328] The most potent inhibitors of the series include the phenylalanine derivative (**124**) (IC_{50} 80 nM) and Val-Leu-Met derivative (**125**).



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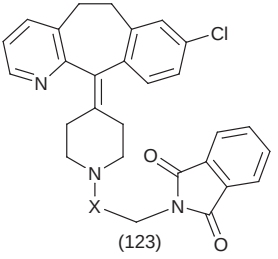

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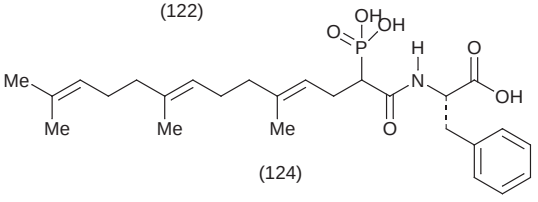

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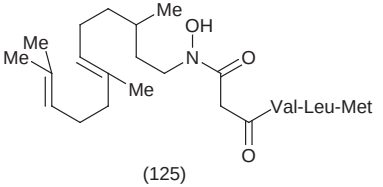

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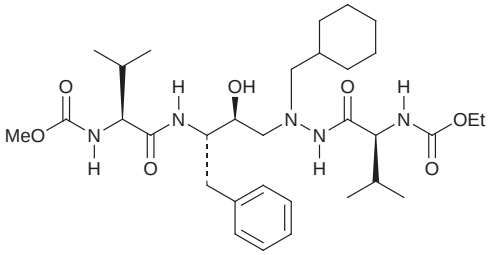
5.3 HIV Protease Inhibitors – This has been one of the most active area of research in the field of enzyme inhibitors. The design of HIV protease (an aspartyl protease) inhibitors has been based on the leads discovered in the earlier years. Work on inhibitors containing the hydroxymethylcarbonyl isostere has been reviewed.[329] A series of aza-peptide analogues with a (hydroxyethyl)hydrazine isostere has been reported.[330] In compounds like (**126**) (IC_{50} 56 nM, EC_{90} 100 nM in MT-2 cells), small modifications of the P_2P_3 and $P_2'P_3'$ substituents had little effect on enzyme inhibition but greatly influenced the pharmacokinetic profile. In the P_1' position, replacement of the cyclohexyl group by other substituents, e.g. isopropyl, isobutyl, 4-MeO-phenyl, 4-F-phenyl, 4-CN-phenyl, 4-OH-phenyl, 4-tolyl, 4-CF_3-phenyl, 2,3,4-MeO-phenyl and 2-thienyl, resulted only in minor changes in *in vitro* activity (IC_{50}s 16 to 84 μM). The N- and C-terminal substituents were very important for the *in vivo* activity. Compounds containing one or two ethyl carbamate groups produced a substantial increase in plasma concentrations upon oral application in mice.



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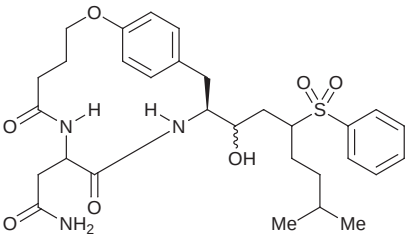
Conformationally constrained macrocyclic inhibitors based on the linear peptide inhibitors of HIV protease, Ac-Leu-Val-PheΨ[$CHOHCH_2$]Phe-Ile-Val-NH_2 and Ac-Leu-Asn-PheΨ[(S)-CHOH-CH_2]-Pro-Ile-Val-NH_2, have been reported.[331-335] The tripeptide units in the inhibitors were replaced by conformationally rigid 15 or 16-membered macrocyclic structures to generate compounds like (**127**) and (**128**). The selection of the macrocyclic structure was based on the X-ray structure of the linear peptide bound to the enzyme. Inhibitor (**127**) was about 5-fold more potent (K_i 0.6 nM) than the linear peptide. The hydroxyamide analogue (**128**) (IC_{50} 19 nM, whole cell assay IC_{50} 76 nM), was more potent than the corresponding hydroxyethylamine derivative. However, compared to the corresponding hydroxyamide acyclic derivative the cyclic peptide was about 20-fold less potent. To increase the solubility of the macrocyclic inhibitors, the P1' to P3' residues were incorporated into a macrocyclic oligo ethylene glycol ether linkages. Compounds with a ring size of 13-20 atoms showed HIV inhibitory potency in the range of 6-20 nM. However, the compounds like (**129**) showed poor oral bioavailability at high doses (125 mg/kg).[335]

SAR studies around Ro-31-8959, a potent inhibitor of HIV protease reported several years ago, have continued in the hope of improving potency and oral



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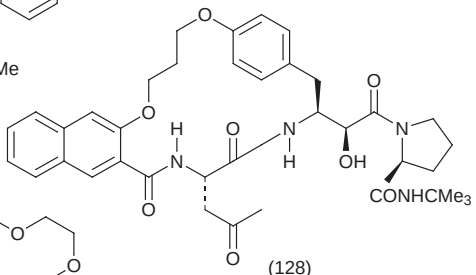

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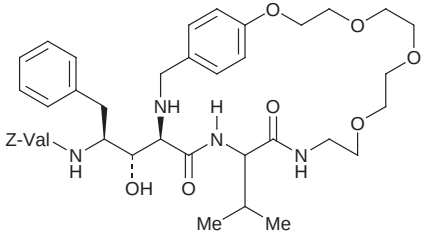
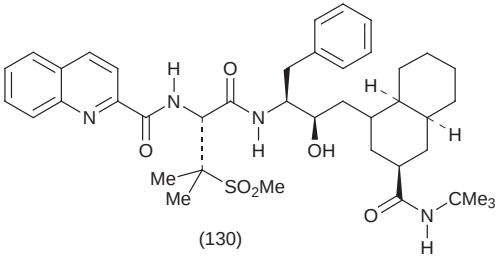


(129)

bioavailability.[336–340] Various substituents have been incorporated in the P_2 (Asn in Ro 31-8959) and P_3 sites to give compounds like (**130**)-(**132**). Compound (**130**) (LB-71206) and the corresponding β-methylsulfonylalanine analogue were found to be about 2 to 3-fold more potent than Ro 31-8959. However, in the whole cell



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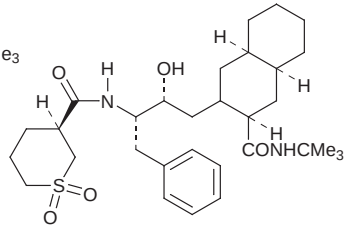

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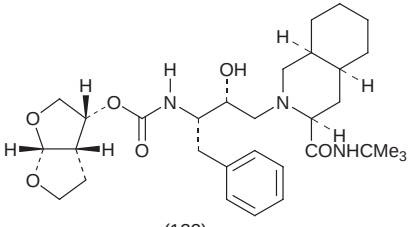
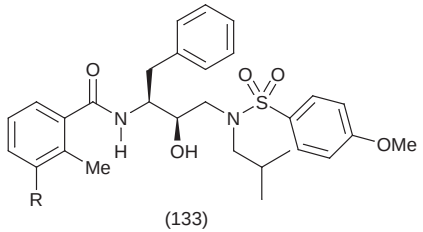


(132)

assay (**130**) was found to be more potent (IC_{50} 4.9 nM) than the β-methylsulfonylalanine analogue (IC_{50} 22 nM), thus indicating that the two methyl groups in the β-methylsulfonylvaline residue were more important for the entry of the compound in the cells.[336–340]

In addition to the above peptide based analogues, a number of other analogues modified in P_2/P_3 and P_2'/P_3' positions and C_2 symmetric inhibitors (**133**)-(**137**) have been reported. The more potent (R)-(Hydroxyethyl)sulfonamide derivatives [**133**, R = OH or NH_2] (IC_{50} 3 and 6 nM, respectively) showed oral activity (bioavailability 12 and 54%, respectively).[341-347] The hydroxyindane analogue (**134**) also showed good (47%) oral bioavailability.[342] SAR studies in the case of (**136**) led to weak inhibitors (X = H, OH, OCH_3, OBu^i, OCH_2OCH_3, SCH_3, SO_2CH_3, SO_2NH_2, and Y = H, OCH_3 or OPh) of the enzyme (IC_{50} 45-1600 nM). Two of the compounds (X = SO_2NHBu^t, Y = H and X = $CONHBu^t$ and Y = CH_3) were somewhat more potent (IC_{50} 6 and 1.7 nM, respectively). In the whole cell assay the more potent compound (**136**, X = $CONHBu^t$ and Y = CH_3) showed poor activity (IC_{90} = 400 nM).[346] SAR studies aimed at improving the antiviral potency and reducing the high binding to human plasma proteins observed in the case of an earlier reported HIV protease inhibitor (ritonavir) led to (**137**). Compound (**137**) was 2-fold more potent than ritonavir in MT4 cells in



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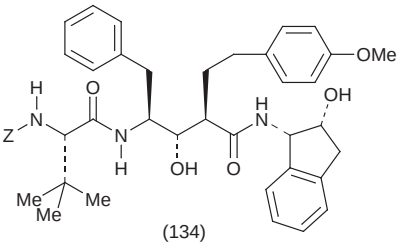

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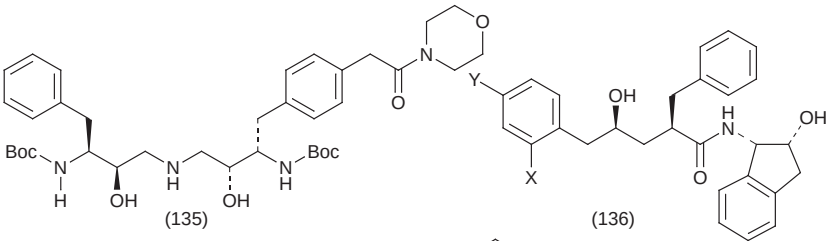

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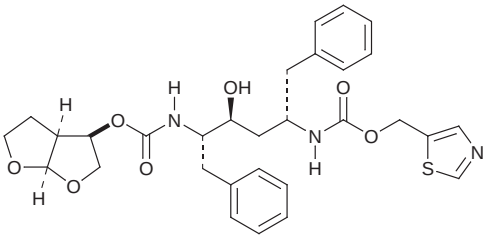
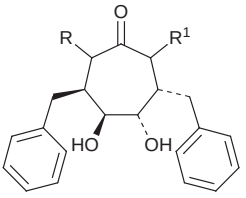


(137)

the presence of human plasma and exhibited 41% oral bioavailability in the rat at a dose of 10 mg/kg.[347]

Non-peptide inhibitors of HIV protease have been obtained by a combination of random screening, molecular modelling, X-ray data and medicinal chemical approaches. Cyclic urea derivatives (symmetrical or non-symmetrical) of the general formula (**138**) have been identified as HIV protease inhibitors.[348–354] Compounds with various substituents (R and R1 positions) with hydrogen bond donor/acceptor functionalities show potent *in vitro* enzyme inhibition activity. Although a number of compounds, e.g. (**139**) (A-98881), show potent inhibitory activity in MT-4 cell culture assay, in general, the compounds showed poor pharmacokinetic profile and low oral bioavailability. In comparison, a non-peptidic cyclic sulfone derivative (**140**) demonstrated 74% oral bioavailability when administered at a dose of 10 mg/kg in dogs. The duration that the plasma drug concentration remained 40 times above EC_{90} exceeded 12 hours.[355]



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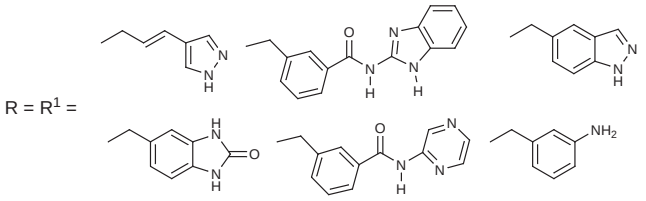

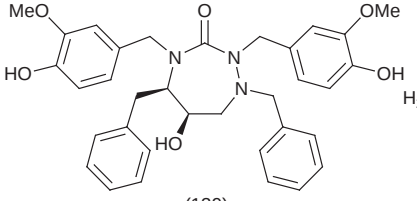

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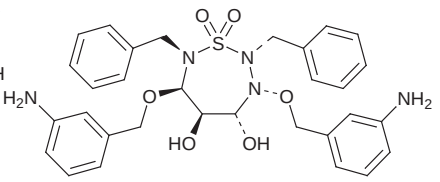
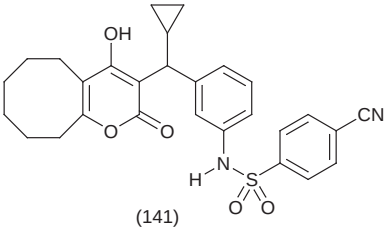


(140)

Another series of non-peptide inhibitors are based on hydroxycoumarin derivatives. The original lead, obtained by random screening, was modified to generate potent inhibitors.[356–361] Compounds (**141**)-(**143**) were potent inhibitors of HIV protease in *in vitro* and *in vivo* assays. Compound (**141**) and the corresponding *p*-fluorosulfonamide derivative displayed good pharmacokinetic profile after oral administration at 90, 180, 360 or 720 mg/kg. However, both



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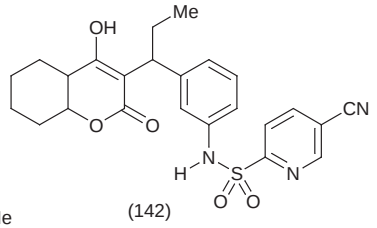

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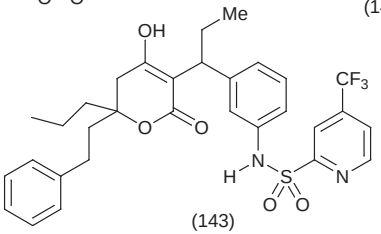


(143)

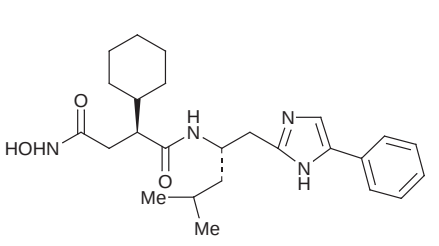
compounds showed severe toxic signs (including death) at 360 and 720 mg/kg level.[357]

A number of other publications on non-peptide inhibitors and peptide-based irreversible inhibitors of HIV protease have appeared.[362–364] Examples of irreversible inhibitors include Z-PheΨ[(R,S)-*cis*-epoxide]Gly-Val (K_i 1.32 μM), 2-quinolinecarbonyl-Asn-PheΨ[(R,S)-*cis*-epoxide]Gly-Ile (K_i 0.018 μM) and 2-quinolinecarbonyl-Asn-PheΨ[(R,S)-*cis*-epoxide]Gly-NH-CH(isopropyl)$_2$ (K_i 0.001 μM). A number of other enzymes like chymotrypsin, papain and human renin were not inactivated by the most potent compound 2-quinolinecarbonyl-Asn-PheΨ[(R,S)-*cis*-epoxide]Gly-NH-CH(isopropyl)$_2$, but human cathepsin D was inactivated by this compound (IC_{50} 12.5 μM).[364]

5.4 Matrix Metalloproteinase Inhibitors – Matrix metalloproteinases (e.g. collagenases, stromelysins and gelatinases) are a family of zinc-containing proteinases involved in extracellular remodelling and degradation. These enzymes have been implicated in a wide variety of biological processes and diseases such as rheumatoid arthritis, osteoarthritis, tumour metastasis and multiple sclerosis. Based on the information generated in the case of converting enzyme inhibitors, a number of inhibitors of matrix metalloproteinases containing hydroxamate, thiol, N-carboxyalkyl and phosphorus groups involved in chelating the essential zinc metal and other peptidic and non-peptidic groups involved binding to various binding pockets (S_1, S_1'-S_3') in the enzymes, have been reported. Various aspects of matrix metalloproteinases have been reviewed.[365-367]

Modifications in the P_2'-P_3' region of the hydroxamate-based inhibitors have resulted in compounds like (**144**) and (**145**). Compounds containing benzimidazole and imidazole (**144**) heterocycles as amide bond isosteres or a heteroatom-based modifications of the P_1' group (**145**) were moderately potent inhibitors and showed some selectivity. For example, (**144**) was a more potent inhibitor of matrilysin (IC_{50} 3.1 nM) and a much weaker inhibitor of human fibroblast

stromelysin and collagenase (IC_{50}s 5900 and 480 nM, respectively).[368,369] The C-terminal methyl group of (**145**) could be replaced by -$(CH_2)_2$-1-pyrrolidino, -$(CH_2)_3$-1-morpholino and -$(CH_2)_3$-COOH groups. In the case of phosphinic acid-based inhibitors, the phosphinic acid analogues, e.g. (**146**), were more potent than the corresponding phosphonate and phosphonamidate analogues. The phenethyl (hPhe) compounds (P_1' position) were more potent than the corresponding benzyl (Phe) analogues.[370] Phthalimidobutyl substituent was much better for binding in the P_1-P_3 sites of MMP-3 than many other substituents, e.g. $PhCH_2CONH(CH_2)_4$-, $C_2H_5NHCONH(CH_2)_4$-, $PhCH_2NHCONH(CH_2)_4$- and Bzl-Pro-NH$(CH_2)_4$-. The Bzl-Pro-NH$(CH_2)_4$- analogue was about 10-fold more potent than the phthalimidobutyl analogue in inhibiting MMP-2.



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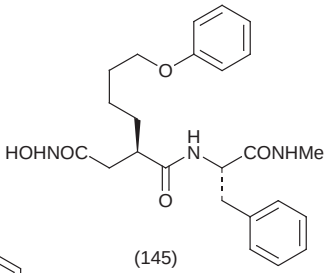

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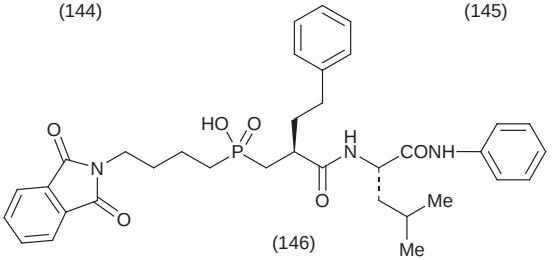
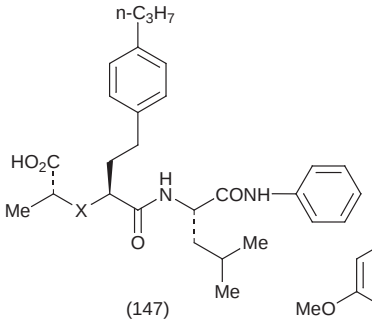


(146)

SAR studies in the N- or C-carboxyalkyl series of inhibitors (**147**) (X = NH or CH_2) indicated that potent MMP-3 inhibitors could be obtained in both series of compounds.[371] However, in general, C-carboxyalkyl analogues were less potent. For example, the X = NH compound [K_i 5900 nM (MMP-1), 3.5 nM (MMP-2) and 18 nM (MMP-3) was more potent than the C-carboxyalkyl (X = CH_2) analogue (K_i >10,000 nM (MMP-1), 310 nM (MMP-2) and 68 nM (MMP-3). In an *in vivo* assay, the C-carboxyalkyl analogue, despite being a 3-fold less potent inhibitor of MMP-3 *in vitro*, was 2-fold more potent than the N-carboxyalkyl analogue (ED_{50} values 32 and 65 mg/kg, respectively) when administered orally. Substitutions in the P_1 residue of (**147**) also gave MMP-3 (stromelysin) inhibitors. The 4-methoxysulfonamide derivative (**148**) (IC_{50} 50 nM) was similar in potency to the corresponding *p*-toluenesulfonamide analogue. Replacement of the C-terminal Leu amide moiety by various heterocyclic groups gave much less potent analogues (IC_{50} values >20 μM).[372]

Inhibitors of MMP-1 (collagenase) were also obtained in the C- and N-



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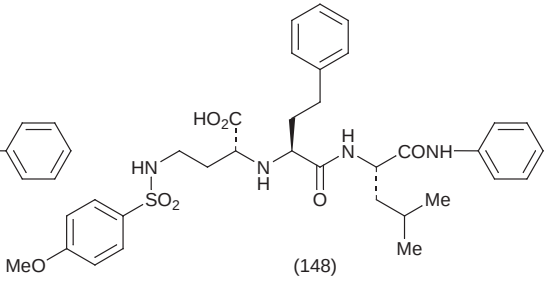

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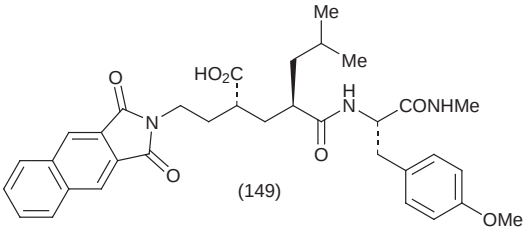
carboxyalkyl series (e.g. **149**). The P_1' position in the arylimidoethyl derivatives (**149**) could be modified to include cyclohexyl or cyclopentyl side chain (in place of the Leu side chain) and the P_2' position could be Phe-NHMe or Tyr(Me)-NHMe. In general the C-carboxyalkyl analogues were either equipotent or somewhat more potent than the N-carboxyalkyl analogues.[373]

The effects of P_1 substitution on the inhibitory activity and selectivity can be illustrated in a series of compounds like (**150**). N-Carboxyalkyl analogues of this type were found to be inhibitors of MMP-1 (collagenase), MMP-2 (gelatinase A) and MMP-3 (stromelysin).[374] Compound (**150**), containing a phthalimidobutyl substituent at P_1 position, inhibited collagenase (IC_{50} 720 nM), gelatinase A (IC_{50} 86 nM and stromelysin (IC_{50} 8 nM). The corresponding Phth-N-CH_2-CH_2-analogue was more potent at inhibiting gelatinase A (IC_{50} 20 nM) but much less potent in inhibiting collagenase (IC_{50} 2090 nM) and stromelysin (IC_{50} 33 nM). Replacement of the phthalimidobutyl substituent by Ph-$(CH_2)_2$-CONH-$(CH_2)_3$-



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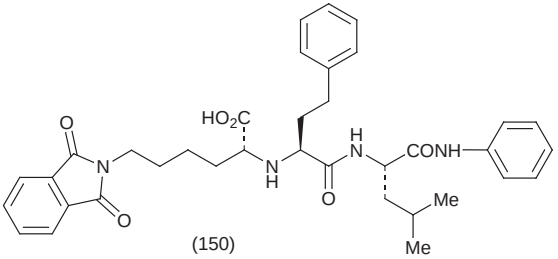
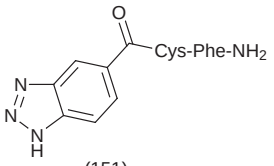


(150)

resulted in a more potent and selective inhibitor of stromelysin [IC_{50} 9.5 nM (stromelysin), 2200 nM (collagenase), gelatinase 87% inhibition at 1000 nM)]. The Ph-$(CH_2)_3$-CONH-$(CH_2)_3$- was a selective but much less potent inhibitor of stromelysin [IC_{50} 120 nM (stromelysin), collagenase 36% inhibition at 10 μM, gelatinase 70% inhibition at 1000 nM)].[374]

Some dipeptide derivatives have also been reported as inhibitors of various metalloproteinases.[375] Trifluoroacetyl-Cys-Phe-OMe and trifluoroacetyl-Cys-Phe-NH_2 were identified as selective inhibitors of collagenase (IC_{50} 40-63 nM; gelatinase IC_{50} >1000 nM). Acetyl-Cys-Phe-NH_2 was 1000-fold less potent than the trifluoroacetyl analogue against collagenase (IC_{50} >100 μM). Replacement of the phenylalanine residue by Leu, Trp and Pal(3) residues in the acetyl-Cys-Phe-NH_2 series of compounds resulted in improved potency against gelatinase (IC_{50}s 4-7 μM). N-terminal modifications in the acetyl-Cys-Phe-NH_2 series of compounds gave potent inhibitors against both the enzymes. For example, (**151**) inhibited collagenase and gelatinase (IC_{50} 8 and 24 nM, respectively). The corresponding pyridylalanine and thiophenylalanine (Phe replacement) analogues were less potent.

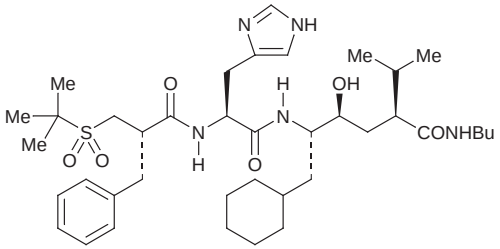


(151)

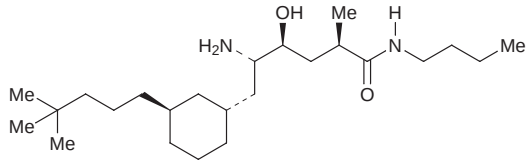
5.5 Inhibitors of Renin (Aspartyl Protease) – Mechanism of action of aspartyl proteases has recently been discussed.[376] Potent inhibitors of renin have been reported in the past. Although, these inhibitors demonstrated activity in various *in vitro* and *in vivo* tests when administered intravenously, the bioavailability of the compounds was very poor when administered orally. Therefore, one of the most important objective in the design of renin inhibitors has been to identify compounds which could be used as antihypertensive agents by oral administration. Various physicochemical parameters which may help to improve oral bioavailability have been evaluated using the existing inhibitors.[377,378] In general, the results were consistent with the information already available in the literature. For example, in a series of inhibitors containing 2-amino-1-cyclohexyl-6-methylheptane-3,4-diol fragment, more hydrophobic compounds were better absorbed in rats than the compounds with ionisable functionality (intestinal absorption in a single-pass perfusion model) and small molecular weight, neutral compounds were better absorbed than the large molecular weight compounds. All compounds were rapidly taken up into the liver regardless of log *P* or molecular weight.[377] Similar conclusions have been drawn from experiments using radiolabelled compounds. In this series, rate of appearance in bile was dependent on the molecular weight of the compounds.[378]

In an attempt to design small molecular weight compounds, conformational

analysis of the binding mode of CGP 38560 (**152**) was carried out. This indicated that the S_1 and S_3 pockets constitute a large contiguous, hydrophobic binding site accommodating the P_1 cyclohexyl and the P_3 phenyl groups in close proximity to each other. This led to the synthesis of δ-amino hydroxyethylene dipeptide isosteres lacking the P_4-P_2 peptide backbone. The most active compounds (**153**) and (**154**) inhibited human renin in the submicromolar range (IC_{50} 300 and 700 nM, respectively).[379] Replacement of the His residue (P_2 position) in an inhibitor of renin (KRI-1314) by (2R,3S)- and (2S,3S)-2-amino-3-(1,3-dithiolan-2-yl)-3-hydroxypropanoic acid resulted in >10-fold reduction in potency.[380]



(152) CGP38560



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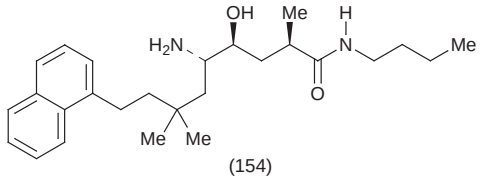

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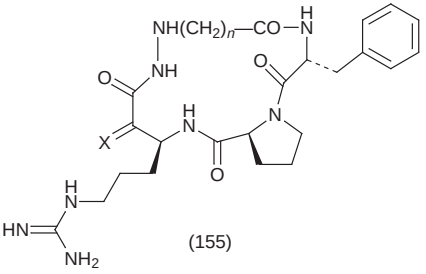
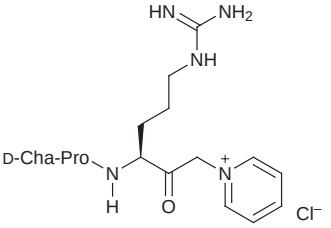
5.6 Inhibitors of Thrombin (Serine Protease) – Biological and pharmacological aspects of thrombin inhibitors have been reviewed and comparisons have been made between thrombin and factor Xa inhibitors.[381–383] Thrombin inhibitors like D-Phe-Pro-Arg aldehyde have been known from a long time. One of the main problem with these agents has been the lack of oral activity. Further work has, therefore, continued to obtain inhibitors with oral activity and improved pharmacokinetic profile. The tripeptide D-Phe-Pro-Arg aldehyde has been used as a starting lead for the design of all new inhibitors. Macrocyclic tripeptides containing an α-keto amide linkage have been designed as thrombin inhibitors based on a naturally occurring inhibitor theonamide.[384] The most potent peptide (**155**; n = 5, X = O) inhibited both thrombin (K_i 3.1 ± 0.4 nM) and trypsin (K_i 5.2 ± 1.2 nM). A number of other similar compounds (n = 5,6,7,9 or 10 and X = O) without a benzyl side chain were poor inhibitors of thrombin (K_i 15-1800 nM). Elimination of the keto amide functionality resulted in a compound (n = 7



(155)

and X = H/OH) which did not inhibit thrombin at a concentration of 50 μM. Other divalent thrombin inhibitors containing an α-keto-amide transition-state mimetic have been reported.[385]

In addition to an α-keto-amide transition-state mimetic, a number of other C-terminal modifications include compounds like (**156**) and (**157**). The most potent compound of the tripeptidyl pyridinium methyl ketone series was (**156**) (K_i = 0.19 ± 0.06 nM). N-Terminal acetyl derivative of (**156**), Ac-D-Cha-Pro-Arg-CH_2-$^+NC_5H_5$, and the corresponding D-Phe analogue, Ac-D-Phe-Pro-Arg-CH_2-$^+NC_5H_5$, were about 100- to 1000-fold less potent than (**156**). Modifications at the C-terminal end also gave less potent compounds. Both D-Cha-Pro-Arg-CH_3 and D-Cha-Pro-Arg-CH_2-C_6H_5 were about 250-fold less potent.[386] Peptidoyl heterocycles of the type (**157**) (based on elastase inhibitors) inhibited both thrombin (K_i 0.19 nM) and trypsin (K_i 3.1 nM).[387] The selectivity was enhanced by replacing the 2-benzothiazole group of (**157**) by N-Me-2-benzimidazole (thrombin K_i 8.1 nM, trypsin K_i 290 nM) and N-Me-2-imidazole (thrombin K_i 50 nM, trypsin K_i 4400 nM) groups, but the two compounds were less potent inhibitors of thrombin than (**157**).



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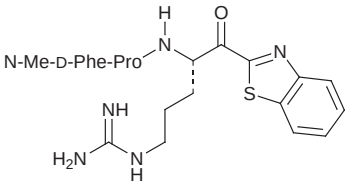
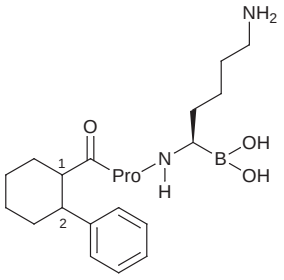


(157)

Starting from the Ac-D-Phe-Pro-BoroArg (DuP714) and Ac-D-Phe-Pro-boroLys analogues (reported earlier) (K_i values for human thrombin 0.041 and 0.24 nM, respectively), additional boronic acid derivatives have been designed as thrombin inhibitors.[388–390] Initially, (3-phenylpropionyl)-Pro-boroLys and (3-trifluoromethyl)phenylpropionyl-Pro-boroLys, were identified as potent, orally active inhibitors of thrombin. An X-ray crystal structure analysis of the complex of (3-phenylpropionyl)-Pro-boroLys bound to thrombin was used to modify the

compounds further by restricting the conformation of the 3-phenylpropionyl part of the inhibitors. Cyclohexane, pyrrolidine and substituted benzoyl rings were used as constraints to restrict the available positions of the phenyl ring. Compounds (**158**) (1R, 2R) and (**159**) (3S, 4R) were comparable in potency (K_i 0.46 and 0.32 nM, respectively) to (3-phenylpropionyl)-Pro-boroLys (K_i 0.80 nM). Most of the analogues of (**160**) [X = CH_2, O, S, or SO_2 and trifluoromethyl group replaced by 2-CH_3, -SCH_3, 3-CF_3, 4-CF_3, 2-Br, 3-F, 2-OCH_3, 3-OCH_3] were potent inhibitors of thrombin (K_i values 0.07 to 0.8 nM).[389] Additional modifications for the 3-phenylpropanoyl group and the proline residue led to C_6H_5-CH_2-CH_2-CO-Sar-boroArg and C_6H_5-CH_2-CH_2-CO-Sar-boroLys as moderately potent (4-8-fold less potent than the D-Phe-Pro analogues) analogues. Non-peptidic replacements for D-Phe-Pro led to (**161**) (about 10-fold less potent in the *in vitro* assays) which demonstrated oral activity when administered at a dose of 5 mg/kg.[390]



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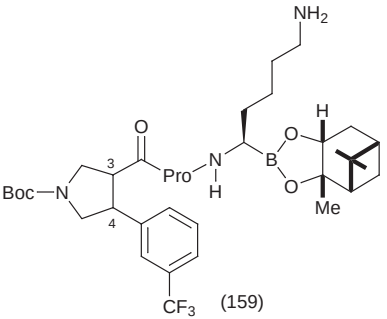

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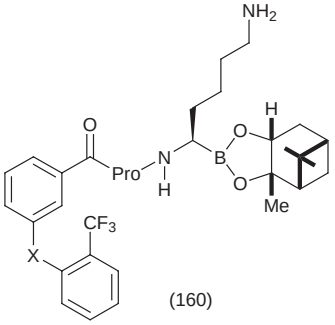

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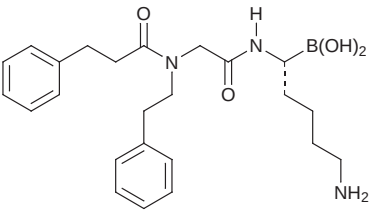

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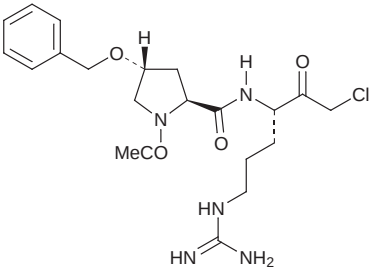
Modifications in the D-Phe-Pro region in a chloromethylketone series of compounds led to a selective inhibitor (**162**) of thrombin.[391] Hydroxyproline derivative (**162**) (IC_{50} 0.9 nM) was about a 1000-fold less potent inhibitor of factor Xa and a 250-fold poorer inhibitor of plasmin. Replacements of the acetyl group by CH_3-CH_2-, CH_3CH_2CO- and CH_3SO_2- groups led to a significant reduction in potency (25-8000-fold). Modifications of the benzyloxy group also led to decreases in potency. Cyclohexylmethyloxy analogue was about 5-fold less potent but the phenethyl analogue was about 40-fold less potent (IC_{50} 40 nM). *In*

vivo, the phenethyl analogue was active in a rat carotid artery thrombosis model when administered as an iv bolus (3 mg/kg) plus a continuous infusion (0.3 mg/kg per min.).

Replacement of the C-terminal arginine aldehyde by other arginine mimetics has also resulted in potent thrombin inhibitors.[392–394] Substitution of the arginine aldehyde moiety by *p*-amidinobenzylamine gave a potent and selective inhibitor of thrombin which was comparable in potency to the transition-state aldehyde analogue. The corresponding *m*-amidinobenzylamine, *p*-amidinophenethylamine and *m*-amidinophenethylamine derivatives were at least 100-fold less potent. The most potent *p*-amidinobenzylamine analogue (**163**) was much less potent inhibitor of trypsin (130-fold), plasmin (26,000-fold), tissue plasminogen activator (170,000-fold) and urokinase (400,000-fold).[392] Incorporation of L-3-piperidyl(N-guanidino)alaninal residue in the P_1 position as a conformationally restricted analogue of arginine along with a six- or a seven-membered lactam sulfonamide moiety at P_3 to P_4 positions gave potent inhibitors like (**164**) which showed much more selectivity against other serine proteases like factor Xa and trypsin. Some of the compounds also showed oral activity.[393,394] Like (**164**), P_1-arginal derivatives incorporating P_3-P_4 lactam sulfonamide derivatives were also potent inhibitors of thrombin.[394]



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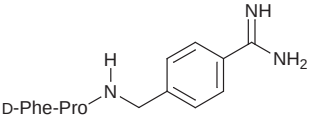

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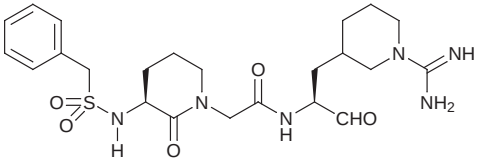


(164)

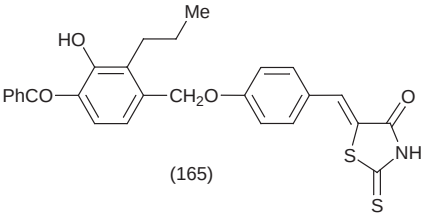
A novel class of α-thrombin inhibitors (based on hirudin) designed to interact through their N-terminal end with the α-thrombin active site in a nonsubstrate mode and to specifically bind the fibrinogen recognition exosite have been developed.[395] An appropriate spacer peptide that is able to properly orient the inhibitors at the two sites was used to link the required amino acid residues. This spacer allowed the size of the inhibitors to be reduced to about one-third of the amino acid residues in the hirudin sequence. The most active compounds of the

series, Chg-Arg-Nal(2)-Thr-Asp-D-Ala-Gly-β-Ala-Pro-Glu-Ser-His-hPhe-Gly-Gly-Asp-Tyr-Glu-Glu-Ile-Pro-Aib-Aib-Tyr-Cha-D-Glu and Chg-Val-Nal(2)-Thr-Asp-D-Ala-Gly-β-Ala-Pro-Glu-Ser-His-hPhe-Gly-Gly-Asp-Tyr-Glu-Glu-Ile-Pro-Aib-Aib-Tyr-Cha-D-Glu, inhibited α-thrombin catalysed hydrolysis of Tos-Gly-Pro-Arg-*p*-nitroanilide with K_i = 0.09 and 0.21 nM, respectively.

5.7 Miscellaneous (Calpain, Cathepsin B, Cathepsin D, Elastase, Protein-tyrosine Kinase, Protein-tyrosine Phosphatase and Virus Ribonucleotide Reductase) Inhibitors – A series of dipeptidyl α-ketoamides of the general structure R_1-Leu-D,L-AA-CONH-R_2 (AA = Abu, Phe, Nva) were reported as inhibitors of cysteine proteases calpain I, calpain II and cathepsin B.[396] Z-Leu-Abu-NH-Et inhibited calpain I, calpain II and cathepsin B with similar potency levels (K_i 250, 210 and 240 μM, respectively). Some of the analogues, e.g. Z-Leu-Abu-CO-NH-CH_2-CH(OH)-C_6F_5, Z-Leu-Abu-CO-NH-CH_2-CH(OH)-C_6H_4(4-OPh), Z-Leu-Abu-CO-NH-CH_2-CH(OH)-C_6H_4-3-OC_6H_4(3-CF_3), Z-Leu-Abu-CO-NH-CH_2-2-quinolinyl, Z-Leu-Phe-CO-NH-$(CH_2)_3$-4-morpholinyl and Z-Leu-Nva-CO-NH-CH_2-2-pyridyl, were more potent against calpain I. More potent inhibitors of calpain II were Z-Leu-Abu-CO-NH-$(CH_2)_2$-OH, Z-Leu-Abu-CO-NH-$(CH_2)_5$-OH, Z-Leu-Abu-CO-NH-C_6H_{11}, Z-Leu-Abu-CO-NH-$(CH_2)_2$-Ph, Z-Leu-Abu-CO-NH-$(CH_2)_3$-Ph, Z-Leu-Abu-CO-NH-$CH_2C_6H_3$(3,5-$(OCH_3)_2$), Z-Leu-Abu-CO-NH-CH_2-CH(OH)-Ph, Z-Leu-Abu-CO-NH-CH_2-2-furyl, Z-Leu-Abu-CO-NH-CH_2-2-tetrahydrofuryl, Z-Leu-Abu-CO-NH-CH_2-2-pyridyl, Z-Leu-Abu-CO-NH-$(CH_2)_3$-4-morpholinyl, Z-Leu-Phe-CO-NH-Et, Z-Leu-Phe-CO-NH-CH_2-CH_2-CH_3, Z-Leu-Phe-CO-NH-CH_2-CH(OH)-Ph, Z-Leu-Phe-CO-NH-CH_2-CH(OH)-C_6H_4-3-OCH3(3,4-Cl_2), Z-Leu-Phe-CO-NH-CH_2-2-pyridyl, Z-Leu-Phe-CO-NH-CH_2-2-quinolinyl, Ph-CH_2-CH_2-CO-Leu-Abu-CONHEt, Ph_2-CH-CO-Leu-Abu-CO-NH-CH_2-2-pyridyl, Ph_2-CH-CO-Leu-Abu-CO-NH-$(CH_2)_3$-morpholinyl and Ph_2-CH-CO-Leu-Phe-CO-NH-$(CH_2)_3$-morpholinyl. Most potent cathepsin B inhibitors include Z-Leu-Abu-CO-NH-CH_2-CH(OH)-C_6H_4[4-$N(CH_3)_2$], Z-Leu-Abu-CO-NH-$(CH_2)_2$-3-indolyl, 2-quinolinyl-CO-Leu-Abu-CO-NH-Et and Ph_2-CH-CO-Leu-Abu-CO-NH-Et. In addition to the above peptides, aldehyde and ketoamide derivatives of xanthen-9-yl-Leu-Leu, e.g. xanthen-9-yl-Leu-Leu-al and xanthen-9-yl-Leu-Leu-CO-NH-Et, inhibited calpain I with IC_{50} values in the range of 25-130 nM.[397] Z-Leu-Leu-heterocycles (imidazole, thiazole, and tetrazole derivatives) were weak inhibitors of calpain I (< 77% inhibition at 10 μM).[398]

Random screening and chemical modifications have led to non-peptide inhibitors of cathepsin D (aspartyl endopeptidase) (**165**, IC_{50} 210 nM) and elastase (**166**, **167**).[399–401]

Heptapeptides Tyr-Ile-Tyr-Gly-Ser-Phe-Lys and Glu-Phe-Glu-Tyr-Ala-Phe-Phe have been reported as substrates for p60-c-src protein tyrosine kinase.[402,403] In the case of Tyr-Ile-Tyr-Gly-Ser-Phe-Lys heptapeptide, a hydrophobic L-amino acid in position 2 and a basic amino acid in position 7 proved crucial for activity as a substrate. In addition, the L-tyrosine residue at position 3 was critical as the phosphorylation site and was found to be stereospecific, as substitution with the D-enantiomer at this position rendered the peptide inactive. A number of protein



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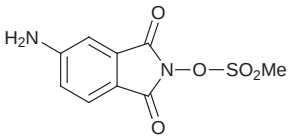

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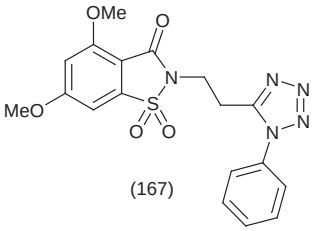
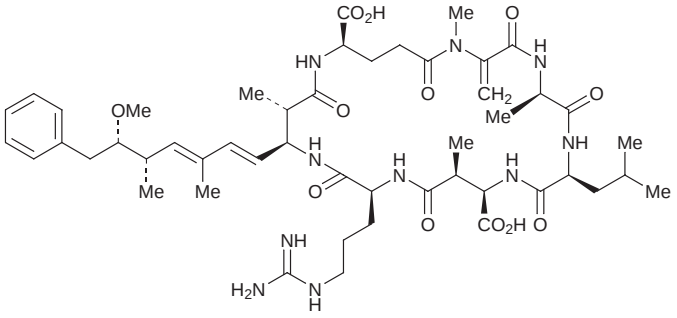


(167)

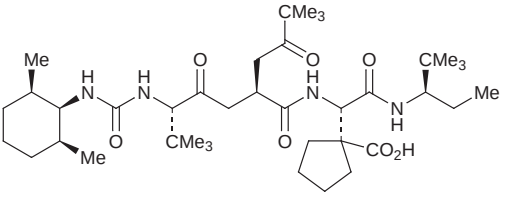
phosphatase inhibitors have been reported.[404–407] Several synthetic analogues of microcystin-LR (**168**), e.g. c(D-Ala-Leu-D-isoAsp-Ala-β-Ala-D-isoGlu-Ala), c(D-Ala-Leu-D-Asp-Ala-Cys-D-Glu-Asp), c(D-Ala-Leu-D-Asp-Ala-β-Ala-D-Glu-Asp), c(D-Ala-Leu-D-isoAsp-Ala-Cys-D-isoGlu-Asp) and c(D-Ala-Leu-D-isoAsp-Ala-β-Ala-D-isoGlu-Asp), inhibited protein phosphatase. Microcystin-LR inhibited the dephosphorylation of [^{32}P]phosphorylase by protein phosphatase 2A with an IC_{50} value of 0.2 nM. In comparison the cyclic peptides and a number of linear peptides inhibited the enzyme (4-86%) at 1 mM concentration. The heptapeptide c(D-Ala-Leu-D-Asp-Ala-Cys-D-Glu-Asp) was the most potent analogue (IC_{50} 500 μM).



(168) Microcystin-LR

Further modifications of the earlier reported inhibitors of viral ribonucleotide reductase (an enzyme required for efficient replication of viral DNA) gave more

potent compounds like (**169**) and (**170**). Both the compounds inhibited herpes simplex virus-1-induced keratitis in a dose-dependent manner.[408]



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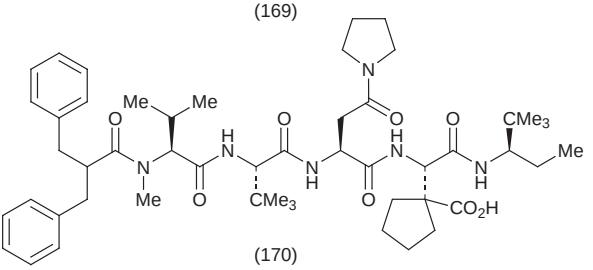

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6 Advances in Formulation/Delivery Technology

Progress is continuously being made in the delivery of peptides.[409,410] The most recent development being the needle-free injection technology which allows the administration of solid compounds across the skin under helium pressure. This is likely to be a convenient method for self-administration of peptide-based drugs. Additional improvements have been made in the delivery of peptides by the subcutaneous,[411–414] pulmonary,[415–419] ocular[420] and oral routes.[421–432] A considerable amount of work on slow release depot formulations using biodegradable polymeric systems, e.g. poly(lactic-co-glycolic) acid copolymers, has been carried out.[433–435] Noninvasive magnetic resonance techniques, electron paramagnetic resonance (EPR) and magnetic resonance imaging (MRI) have been developed to characterise drug release and polymer degradation *in vitro* and *in vivo*. MRI makes it possible to monitor water content, tablet shape and response of the biological system such as oedema and encapsulation.[413]

Slow release formulations of a somatostatin analogue, lanreotide, allowing the drug to be administered two or three times a month, have been developed for the treatment of acromegaly and for suppressing GH and IGF-I levels.[411,412] In an extended clinical trial (1-3 years), slow release lanreotide was administered every 14 days or every 10 days. At the 6-month visit, mean GH values were 5 μg/L or less in 68% and 2.5 μg/L or less in 27% of patients, and these results remained unchanged during the 1-3 year follow-up period. A significant decrease of the pituitary tumour volume was observed in 3 (13%) patients.

Moderate bioavailabilities have been achieved by intranasal administration of

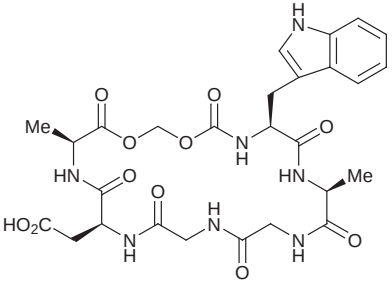
peptides and proteins. In a rat model, the bioavailabilities of calcitonin, insulin, epidermal growth factor, interferon-γ, erythropoietin and interleukin 1-receptor, having molecular weights ranging from 3400 to 60,000 were found to be 18, 9.1, 59.1, 36.8, 17.8 and 6.8%, respectively, following intratracheal administration.[415] In the case of a large protein, growth hormone, the absolute bioavailability following sc relative to iv administration was 49.5%. However, the bioavailabilities of the nasal doses were: 7.8% (0.05 IU), 8.9% (0.10 IU) and 3.8% (0.20 IU).[416]

Although some success has been achieved (e.g. with cyclosporine), successful development of oral peptide formulations remains a significant challenge. Poor membrane permeability, enzymatic instability, and large molecular size are three main factors that have remained major hurdles for the oral absorption of peptides. Current efforts are directed towards understanding some of these problems.[424] Absorption-enhancing agents that have been effective, at least in research environments, with smaller drug candidates, have resulted only in a fairly low peptide absorption (<10%) and have also resulted in significant alterations in the normal cellular morphology of the gastrointestinal tract, at least on a transient basis.[421] For example, intestinal absorption of recombinant human erythropoietin encapsulated in liposomes was 0.74-31% and 3.3-30% as evaluated by circulating reticulocyte counts and percentage circulating reticulocytes of erythrocytes, respectively, in comparison to those for intravenous administration of the same dose.[423] A high level of radiolabel uptake was observed in the blood, liver, spleen, kidneys, small intestine and large intestine after oral administration of TRH and LHRH conjugated to lipoamino acids and lipopeptides. In general, the uptake of tripeptide TRH analogues was higher than the decapeptide LHRH analogues. Within the same series, conjugates with two lipidic moieties showed higher uptake than the conjugates with one lipidic unit The novel conjugates developed have been absorbed and detected after oral administration and appear to be stable for a considerable time *in vivo*.[428]

The role of N-methylation in transporting peptides across Caco-2 cells (a cell culture model of the human intestinal epithelium) was investigated using a set of Phe and MePhe containing peptides [Phe-Gly, Phe-Phe-Gly, Phe-Phe-Phe-Gly, Ac-Phe-NH_2, Ac-Phe-Phe-NH_2, Ac-Phe-Phe-Phe-NH_2, Ac-Phe-Phe-MePhe-NH_2, Ac-Phe-MePhe-MePhe-NH_2, Ac-MePhe-MePhe-MePhe-NH_2 and Ac-MePhe-MePhe-MePhe-NHMe]. No obvious relationship of permeability with the octanol-water partition coefficient was found. However, there was a dependence on the total number of unsubstituted amide nitrogens in the peptide backbone. As the amide bond content increased, permeability decreased despite significant increases in the octanol-water partition coefficient. As the amide bonds were methylated, permeability increased with concomitantly slight changes in partition coefficient. It is suggested that the principal determinant of transport is the energy required to desolvate the polar amide bonds in the peptide for it to enter and diffuse across the cell membrane. The effect of N-methylation is to remove a hydrogen bonding donor group from the peptide and effectively reduce the transfer energy from water into the cell membrane.[431]

A cyclic acyloxyalkoxycarbamate prodrug approach to enhance the membrane

permeability and metabolic stability of peptides has been reported. A model linear hexapeptide (H-Trp-Ala-Gly-Gly-Asp-Ala-OH) was converted into the acyloxyalkoxy derivative Ala-Gly-Gly-Asp-Ala-O-CH_2-O-CO-Trp and cyclised to yield (**171**). Transport and metabolism characteristics of (**171**) were assessed using the Caco-2 cell culture model. When applied to the apical side of Caco-2 cell monolayers, the cyclic prodrug ($t_{1/2}$ = 282 $\pm$ 25 min) was significantly more stable than the hexapeptide ($t_{1/2}$ = 14 min) and at least 76-fold more able to permeate than the parent peptide.[425,432]



(171)

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4
Cyclic, Modified and Conjugated Peptides

BY JOHN S. DAVIES

1 Introduction

In recent years this Chapter has become a sanctuary for about 200 papers, with the general theme covering the structural elucidation, synthesis and conformational aspects of naturally occurring cyclic molecules. Post-translationally modified peptides have also been an increasing section. However, the year 1996, saw a significant increase in the published papers included under these topics, and required access to a broader number of Journals to retrieve the information. In a minority of cases, some Journals were not readily available, so reporting had to be based on summary abstracts, but hopefully still represents the essence of the original work.

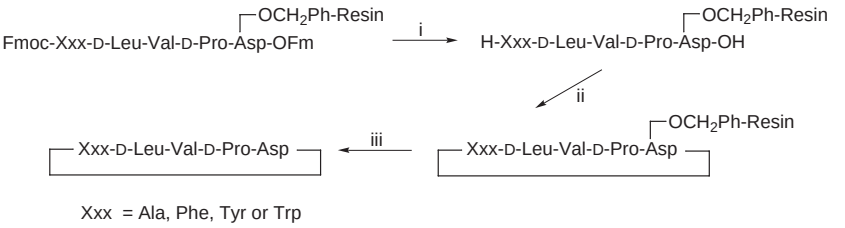
It is remarkable to see annually the wealth of complicated structures that Nature produces. To quote from a recent review[1] 'Nature has used a library approach to constructing ligands for specific receptors and enzymes by combining a limited functional diversity of 20 amino acid side-chains with a small array of secondary structure motifs - reverse turns, α-helices and β-strands'. Many of the molecules reviewed become prototypes and multipurpose tools[2] in pharmacology and biotechnology. To achieve a structural elucidation requires a multifaceted approach of X-ray determinations, high field NMR techniques and molecular mechanics, some chemical degradation and a chiral determination of the component units. The subtleties of these techniques are no longer elaborated upon in this Report, and is recognition that 'state of the art' technology has become almost routine in proving novel structures.

The major source of the papers reviewed here was CA Selects[3] on Amino Acids, Peptides and Proteins (up to Issue 12, June 1997 was scanned) but title pages of mainstream Journals were also checked to retrieve a minority of titles which seem to evade key-word abstracting. Proceedings of the 14th American Peptide Symposium[4] was available and those[5] of the 24th European Peptide Symposium at Edinburgh were in press, during the gestation period of this Chapter, but no formal reporting from these sources has been made. For the first time, electronic conferencing[6] covered subject matter of interest to this Chapter, but adhering to the policy of this publication not to review conference proceedings or patents, it is sufficient to note the existence of this new development.

Amino Acids, Peptides and Proteins, Volume 29

2 Cyclic Peptides

2.1 General Considerations – To be able to choose or predict a successful coupling agent for a particular cyclisation of a linear peptide still remains an elusive goal, although many high yielding cyclisations have been reported this year. So when a particular coupling reagent works for one sequence, its success in another context is never guaranteed. Thus a 'try it and see' policy still prevails, although the recently developed HOAt-based series of reagents are emerging with good credits. Yet in many papers, during this reporting period there have been examples quoted of racemisation at C-terminal residues resulting in extra diastereoisomers being identified in the product mixtures. Reduction of epimerisation and increased efficiency is highly desirable in producing cyclic peptide libraries. In the synthesis[7] of four analogues of the endothelin antagonist cyclo(D-Trp-D-Asp-Pro-D-Val-Leu) according to Scheme 1, where Xxx in cyclo(Xxx-D-Leu-Val-D-Pro-Asp) was exchanged in turn for Ala, Phe, Tyr and



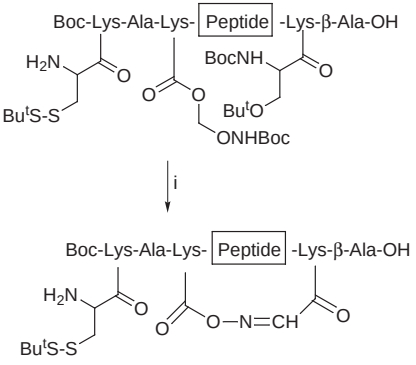
Reagents: i, 20% piperidine; ii, BOP or HOAt; iii, HF

Scheme 1

Trp, two problems were noted - long cyclisation times with BOP/HOBt and unacceptable levels of racemisation at the aspartyl residue. Incomplete removal of piperidine during washing cycles was identified as the reason for piperidide formation which affected cyclisation times, while racemisation of the aspartyl residue could be reduced by using pentafluorophenyl esters or reducing the amount of base during couplings. Several conditions for the attachment of the β-carboxyl group of aspartic acid to the resin have been explored.[8] The most successful route involved the caesium or zinc salts of Boc-Asp(OH)-OFm. Since many cyclic peptides include N-alkylated amides in their backbones, it is salutary to reflect on a report[9] that, even in the presence of trifluoroacetic acid, N-methyl amides, bearing carbonyl groups on the N-terminal side, can be cleaved both in the solid and solution phases. The mechanism is believed to involve an oxazolone-like intermediate in which equilibration of the chiral centre of the N-alkylated residue occurs. One of the leading groups in the field of cyclic peptide conformations has proposed[10] that spatial screening is a useful concept in the design of selectivity and superactivity in bioactive sequences. The spatial orientation of pharmacophoric groups on a distinct backbone conformation can be

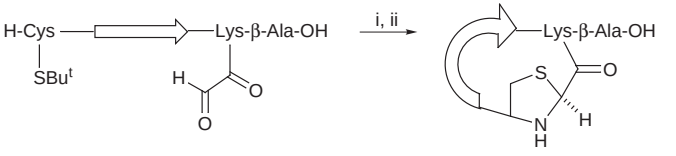
systematically screened, by shifting one (or more) D-amino acid residue around a distinct scaffold.

The assembly of non-protected linear peptides into cyclic products requires linkages with coupling potential outside the interference of reactive amino acid side chains. Schemes 2[11] and 3[12] outline two approaches which are useful for further applications[13] for the production of multiple cyclic antigen peptides.



Reagents: i, $NaIO_4$, pH 7.0 for 1 min

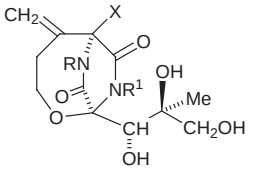
Scheme 2



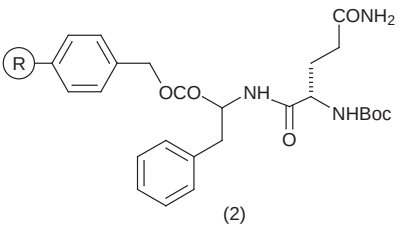
Reagents: i, $NaIO_4$, pH 6.8; ii, tris (carboxyethyl)phosphine, pH 4.2

Scheme 3

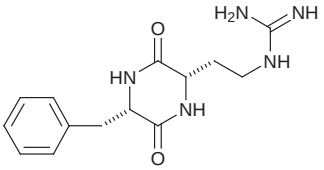
2.2 Dioxopiperazines (Cyclic Dipeptides) – Cyclo(-Trp-Phe) has been identified[14] in an anonymous *Penicillium* fungus, and has been shown to have plant growth regulating activity. The dioxopiperazine ring in bicyclomycin (1) (R = R^1 = H, X = OH) adopts a twist-boat conformation. Synthesis of analogues[15] has shown that changing the piperazinedione unit in bicyclomycin, *e.g.*, where R = Me, or when X is replaced by OMe, OEt, OCH_2CF_3, $OCHMe_2$, OAc, *etc.* led to pronounced changes in biochemical and biological activities. Strong binding interaction between the dioxopiperazine ring and the binding site in rho has been implicated by the results. Analogues[16] of the chitinase inhibitor cyclo-(-L-Arg-D-Pro) from marine bacteria have been tested against two yeasts,



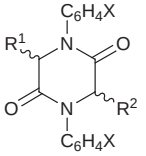
(1)



(2)

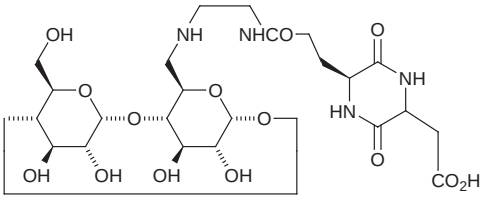


(3)



(4) *cis*-(*R*,*R*), R^1 = Me, R^2 = Me

(5) *trans*-(*R*,*S*), R^1 = Me, R^2 = Me



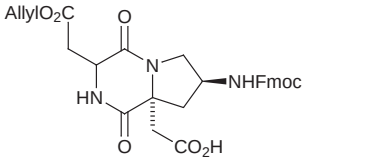
(6)

Saccharomyces cerevisiae and *Candida albicans*. Cyclo(-L-Arg-L-Pro) and cyclo(-D-Arg-L-Pro) were synthesised for the study.

All reactions in the four-step sequence in converting (2) into (3), including a Hofmann rearrangement have been performed[17] on a Merrifield resin. Cyclisation was carried out under acid catalysis, and the product (3) has recently been shown to be an enantioselective catalyst for the Strecker synthesis (*S*)-phenylglycine derivatives. Stereoselective synthesis[18] of N,N′diaryl-2,5-dioxopiperazines (4) and (5) is successful by starting either with a single enantiomer or a racemate of 2-bromopropananilides. Two S_N2 N-alkylation reactions are implied as an explanation for the stereoselectivity. Cyclo(-Glu-Glu) has been attached[19] *via* a linker to a β-cyclodextrin using TBTU/HOBt. High field NMR studies at 500, 600 and 700 MHz on the resulting derivative (6) have given complete assignments for the unmodified glucopyranose units, while showing that the dioxopiperazine ring is in a flagpole boat conformation perched above the cyclodextrin structure. The undesired side reaction of dioxopiperazine formation during solid phase peptide synthesis can be suppressed[20] by choosing a trityl group for N-protection of the second residue in a sequence. Its mild deprotection (0.2% TFA - 1% H_2O in dichloromethane), and the direct activation of the next residue with PyAOP without a neutralisation, assist the suppression.

The use of dioxopiperazines as asymmetric catalysts continues to demand synthetic and mechanistic interest. Asymmetric hydrocyanation of aldehydes by cyclo[-(*S*)Phe-(*S*)His] has been the subject of interest for many years, but it is now suggested[21] that the solid phase characteristics of the dioxopiperazine should be considered. Kinetic measurements (2nd order reaction) indicate that two different imidazole bases bound to a dioxopiperazine polymer grid must participate in the catalysis. Aryl modifications[22] have also been carried out on cyclo[-Phe-His]. The norarginine dioxopiperazine (3), the first reported Strecker synthesis catalyst, has been applied[23] to the catalytic synthesis of optically pure arylglycines.

β-Turn mimetics for peptide and protein loops continue to be of significant interest. Three variations of mimetic templates based on dioxopiperazines have been developed and incorporated into cyclic environments. Thus (7)[24], synthesised from 4-HOPro and L-aspartic acid, has been inserted into a cyclic sequence involving Ala-Asn-Pro-Asn-Ala-Ala, which is the immunodominant epitope on the surface protein of the malaria parasite *Plasmodium falciparum*. Stabilisation of a stable type I β-turn has been confirmed by NMR studies. A similar stabilisation of the β-turn in this motif can be achieved[25] by incorporation of (8). However, when (8) was incorporated as X into cyclo(-Ala-Arg-Gly-Asp-X), the tetrapeptide loop appears by NMR to undergo rapid conformational averaging. Tricyclic variant (9) of the template[26] when incorporated as Y into cyclo(-Ala-Asn-Pro-Asn-Ala-Ala-Y) resulted in an extended backbone at residues Asn-Pro-Asn and a type I β-turn at Asn-Ala-Ala-Y. Although not strictly homodetic cyclic dipeptides, reverse turn mimetics (10) and (11) have been provided[27] by one-step electrochemical cyclisation of Boc-(*S*)Ser-Pro-OMe and Boc-Hse-Pro-OMe respectively.



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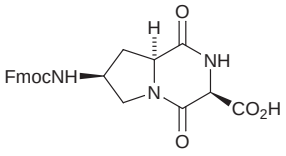

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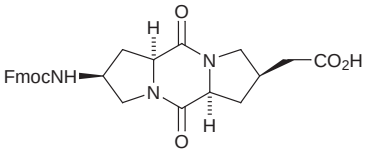

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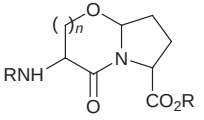
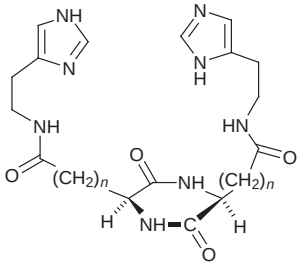


(10) $n = 1$
(11) $n = 2$

Dioxopiperazines (12) and (13) are the result[28] of condensing histamine with cyclo(-Asp-Asp) and cyclo(-Glu-Glu) respectively. The I_{50} values for the Cu(II) bis complexes are amongst the highest available and are only 6 times lower than that determined for superoxide dismutase. The major collision-induced fragmentations[29] of the $M-H^-$ ions of a number of symmetrically and unsymmetrically

substituted dioxopiperazines can be attributed to side-chain losses, which are useful for identification of the compounds. Loss of RCHO due to migration of a ring substituent to the CO group is also prevalent. ^{1}H NMR saturation transfer and T_1 experiments[30] have been used to compare H-exchange rates on the amide protons of dioxopiperazines as compared with 2-piperidone. Acid-catalysed exchange rate constants are similar, but base-catalysed exchange rate constants k_{OH} are 740-fold larger for the dioxopiperazine.



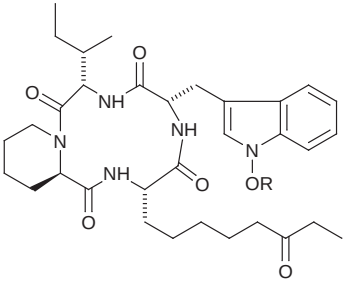
(12) $n = 1$
(13) $n = 2$

Cyclo(-Ala-Ser)·H_2O and cyclo(-Gly-Ser) have had their X-ray crystal structures compared.[31] There is little change in the backbone structure, but the three-dimensional structures of H-bonds involving the hydroxy groups differ. X-ray studies and NMR measurements have been reported[32] on N,N′-ethylene-bridged -Ala-Ala and -Phe-Phe respectively.

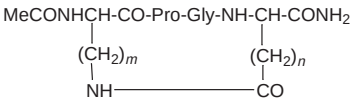
2.3 Cyclotripeptides – Only one report[33] of interest in this constrained ring system has been noted this year, and it relates to the development of cyclic β casomorphin-5 analogues. The tetrapeptide Boc-Tyr(Bu^t)-D-Xaa-Phe-Yyy-OH (Xaa = Lys, Orn, A_2bu; Yaa = Pro in L or D configuration) when cyclised between Xaa side-chain and the C-terminal Yyy-OH gave mostly the dimers, *e.g.* in [H-Tyr-cyclo-D-Lys-Phe-Pro)]$_2$ where L-Pro is present, no monomer could be detected. If D-Pro was inserted the cyclic tripeptides/cyclic hexapeptide ratio was 20:80. The cyclic tripeptide monomers showed high opioid activity while the cyclic dimers were 2-3 orders of magnitude less active.

2.4 Cyclotetrapeptides – Apicidin (14) and its congener apicidin A (15) have been characterised[34] from *Fusarium pallidoroseum*. Apicidin is a potent inhibitor of apicomplexan histone deacetylase (IC_{50} 1-2 nm) and has shown *in vivo* efficacy against *Plasmodium berghei* malaria. The conformation[35] of synthetic cyclo(-D-Phe-Pro-Sar-Gly) has been determined in solution and in the solid state. Only the *cis-trans-cis-trans* conformer could be detected in the X-ray determination of the crystal, but in solution another conformer, *trans-cis-trans-cis*, was also found. No intramolecular H-bonds could be detected. NMR studies[36] in d_6DMSO solution of H-Tyr-cyclo(D-Orn-Phe-Pro-Gly) and H-Tyr-cyclo(Orn-Phe-Pro-Gly) have

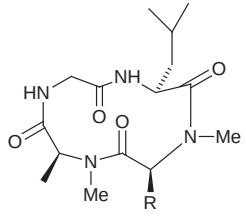
shown only one preferred conformer present for the D-Orn analogue while its L-Orn partner had two or more preferred conformers. Solution structures of a model series[37] (16) have been investigated to examine the influence of side-chain lactamisation on backbone flexibility. Most of the results confirm that the cyclopeptides can adopt a variety of conformations relating to type II β-turns and γ-turns, but never to type I β-turns. It was somewhat surprising that a β-turn was absent in the relatively flexible 18-membered ring compound (16) m = 4, n = 2, while it was observed in (16) m = 4, n = 1. One conclusion drawn is that the exact conformation depends on the specific conformation of the ring and does not correlate with ring size. Cyclo(-Leu-Sar-D-MeAla-Gly) has been synthesised[38] to provide a starting point for alkylation studies to explore the notion that cyclisation of a simple backbone followed by introduction of substituents at a later stage might have advantages. The cyclisation step to the cyclopeptide was carried out by activation of the Gly residue using pentafluorophenyl esters. Li^+ or phosphazenium enolates provided the nucleophiles to react with reactive electrophiles, and the series (17) of compounds confirmed that alkylation took place on the S_i face. Benzylation reactions were also investigated with the hope that the N-benzylated groups could be removed later, but unfortunately the ring system broke down at the debenzylation stage.



(14) R = Me
(15) R = H



(16) $m = 4$ to $m = 1$
$n = 2$ to $n = 1$



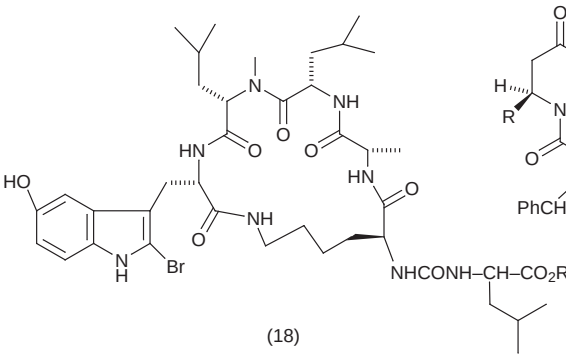
(17) R = Me, $CH_2{=}CHCH_2$, EtCHOH, PhCHOH, *etc.*

2.5 Cyclopentapeptides – This size of ring system continues to be one of the favourite templates for investigations. Nature's contribution comes from the

discovery of the novel plactins A, B, C and D isolated[39] from the fungal strain F165 of *Agonomycetales*, and found to have the structures:

A: cyclo(-D-Val-Leu-D-aIle-Tyr-D-Arg)
B: cyclo(-D-Val-Leu-D-Leu-Tyr-D-Arg)
C: cyclo(-D-Val-Leu-D-aIle-Phe-D-Arg)
D: cyclo(-D-Val-Leu-D-Leu-Phe-D-Arg)

Fmoc chemistry on a 2-chlorotrityl resin using PyBOP/HOBt was the basis for assembling the linear precursors which were cyclised with PyBOP/HOBt and N-methylmorpholine. The plactins stimulate U937 cell-mediated degradation of ^{125}I-fibrin plates by 50% at the 7.5 – 32 μM level. A synthetic protocol,[40] compatible with the later formation of 2-bromo-5-HOTrp, and following a biomimetic route to the calmodulin antagonist konbamide (18), has revealed that the synthesised products containing either D- or L-2-bromo-5-hydroxytryptophan show slight variation in physical properties as compared to the natural compound, although the mass spectra are the same. Some fine tuning of the structure is obviously required. Analogues[41] of nodularin, an inhibitor of the catalytic sub-unit of mammalian Ser/Thr protein phosphatase PP1 and PP2A,



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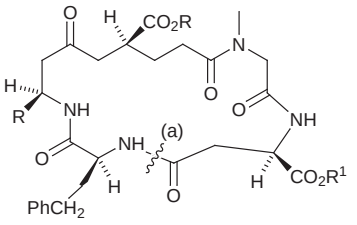

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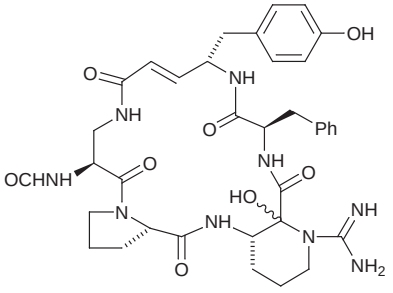

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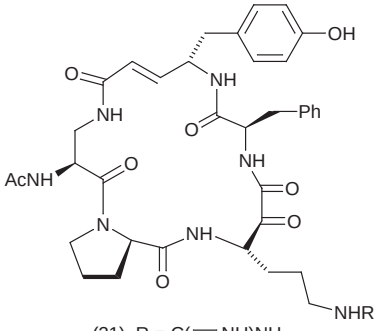


(21) R = C(=NH)NH$_2$
(22) R = H

Table 1. *Comparison of coupling reagents for three thymopentin-derived sequences*

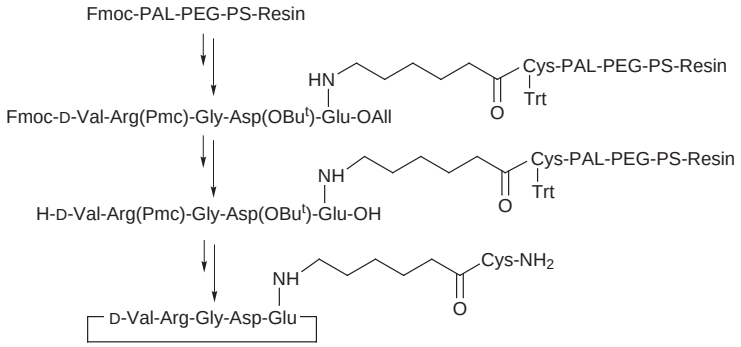
Linear Peptide	Reagent	% Cyclo-monomer	% Cyclo-dimer	% D-Tyr (epimer)
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	HAPyU	55	25	8.8
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	PyAOP	56	20	10.9
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	HATU	53	22	8.3
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	TBTU	50	22	15.1
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	BOP	38	13	20.2
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	PyBOP	52	17	13
H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	DPPA	23	7	19.8
H-Arg(Tos)-Lys(Z)-Asp(OBn)-Val-Tyr(Bn)-OH	HAPyU	53	25	6.2
H-Val-Arg(H^+)-Lys(Ac)-Ala-Val-Tyr-OH	HAPyU	25	42	7.0
H-Arg(H^+)-Lys(Z)-Asp(OBn)-Val-Tyr-OH	HAPyU	43	33	7.0
H-Arg(H^+)-Lys(Ac)-Asn-Val-Tyr-OH	HAPyU	70	16	7.0
H-Arg(H^+)-Lys(Ac)-MeGly-Val-Tyr-OH	HAPyU	80	n.d.	8.3
H-Arg(H^+)-Lys(Ac)-(Hmb)Ala-Val-Tyr-OH	HAPyU	64	n.d.	8.6

have been synthesised to probe interactions between the active sites and the nodularin macrocycle. The analogues (19), where R = H, or CH_2OH and R^1 = H or CH_3, were synthesised by cyclisation of the pentafluorophenyl ester of the linear precursor at position (a) in the structure. X-Ray analysis has been applied[42] in quite an interesting way to confirm the structure of a complex generated between a fragment of cyclotheonamide A from alkaline hydrolysis, and α-thrombin. Structure (20) has been suggested for the hydrolysate which corresponds to cleavage at the α-keto amide bond of the cyclotheonamide backbone, and rearrangement at the argininyl residue. A new synthesis[43] of the thrombin inhibitor cyclotheonamide B (21) has been reported, which utilises guanidination of an ornithine residue in (22), using N,N′-di-(t-Boc)thiourea. Activation of the prolyl carboxyl group of the linear precursor with pentafluorophenyl diphenylphosphinate was the key step in macrolactamisation.

A true assessment of cyclisation reagents and conditions can only be obtained if the cyclisation yields, degree of cyclodimerisation and C-terminal epimerisation are compared for a number of reagents used on the same peptides. Three demanding thymopentin-derived sequences, H-Arg(NO_2)-Lys(Z)-Asp(OBn)-Val-Tyr-OH, H-Arg(Tos)-Lys(Z)-Asp(OBn)-Val-Tyr(Bn)-OH, and H-Val-Arg(H^+)-Lys(Ac)-Ala-Val-Tyr-OH, have been the focus of a detailed survey[44] to compare HOAt-derived coupling agents with other established reagents. Table 1 summarises a representative set of results.

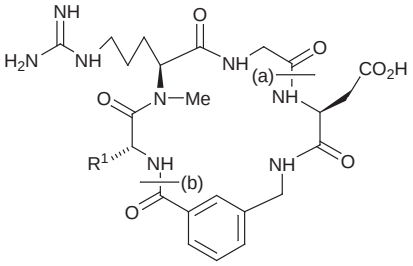
For the HOAt-derived onium salts examined, reactions were extremely rapid and C-terminal epimerisation could be kept below 10%, in contrast to an earlier report that *e.g.* DCC/DMAP gave complete epimerisation to D-Tyr during cyclisation. A serious side-reaction[45] has been found when cyclisation has been carried out on a resin where Fmoc-Asp-O-Allyl was incorporated into the sequence at the N-terminus of 6-aminohexanoic acid (ε-Ahoc). In the synthesis, the product produced along with cyclo[-D-Val-Arg-Gly-Asp-Asp(ε-Ahoc-Cys-

NH_2)] was an aspartimidyl linear peptide, whose free amino terminus produced further side reactions. On-line cyclisation on a resin has also been tested,[46] with a modified side-chain tail, which will allow grafting onto different supports for new applications to cell-adhesion assays and affinity chromatography. An example of this approach is summarised in Scheme 4.

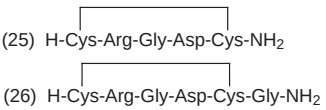


Scheme 4

Last year's report of the first synthesis of the antithrombotic cyclic RGD peptide (23) has now been followed[47] by a large scale synthesis in 9 steps from commercially available starting materials, without chromatographic purification. Cyclisation was carried out at point (a) in (23) using HBTU/DIEA, but not under high dilution conditions. This cyclopentapeptide backbone has also been the basis[48] for attachment of Tc-99m co-ordinating ligands to aid in the detection of thrombi. Chelating ligands bearing Tc-99m can be attached through 6-aminohexanoic acid linkers at R in (24). Formation of the cyclic compounds in this case involved activation with TBTU, to give cyclisation at position (b). When incorporated into rapidly growing thrombi in canine models chelated (24) showed clearly visible images after 50 mins. Based on the information that the sequence Ser-Phe-Leu-Leu-Arg is a minimal requirement of the tethered receptor activity of the so-called thrombin releasing-activating peptides (TRAP's), a number of cyclic analogues have been assembled[49] on solid phase (2-chlorotrityl resin). Thus, cyclo(Phe-Leu-Leu-Arg-Acp), cyclo(Phe-Leu-Leu-Arg-ε-Lys) and cyclo(Phe-Leu-Leu-Arg-Gly) were synthesised from Leu as C-terminus using TBTU, BOP/HOBt for cyclisation and Pmc as an Arg protecting group. The spatial proximity of the Arg/Phe side-chains, deduced by NMR, may be important in recognition. Other variations[50] on the sequence included replacing Phe and Arg by their D-analogues. Five amino acid residues can also be tied back into a cycle by disulfide links, which now can be formed while the sequence is still linked to the solid support.[51] S-Acetamidomethylcysteine residues on the resin can be oxidised directly with iodine as in the synthesis of (25) and (26) and their hexapeptide analogues. The synthesis and conformational characteristics of cyclo[Gly-Pro-Asp(OcHex)-Gly-NH$(CH_2)_4$CO], cyclo[Gly-Pro-Asn-Gly-



(23) R = H, R^1 = Et
(24) R = $NH_2(CH_2)_5CONH$, R^1 = Me_2CH



(25) H-Cys-Arg-Gly-Asp-Cys-NH_2
(26) H-Cys-Arg-Gly-Asp-Cys-Gly-NH_2

$NH(CH_2)_4$-CO] and cyclo[Gly-Pro-Asn(GlcNAc)-Gly-$NH(CH_2)_4$-CO] have been reported.[52]

Numerous cyclic peptides containing RGD sequences have been designed as antiaggregatory molecules to inhibit the binding with the integrin GPIIb/IIa. More recently the integrin $\alpha_V\beta_3$ has been recognised as a potentially important pharmaceutical target involved in neovascularization, which also recognises RGD but it appears that the Arg/Asp side-chains need to be much closer. This hypothesis has been supported[53] utilising earlier conformational studies on a family of peptides based on the sequence cyclo(D-Xxx-MeArg-Gly-Asp-Mamb) where Mamb is *meta*-aminomethyl benzoic acid. Previous work had shown that replacement of D-Xxx-MeArg with L-Arg-L-Arg resulted in a reversal in selectivity in favour of binding to $\alpha_v\beta_3$. Conformationally this amounted to a change from a type II′ turn to a type I turn, which has now[53] been proven with the discovery that cyclo(Arg-Gly-Asp-Pro-Arg) adopts a type I β-turn and is the most active in its affinity for $\alpha_v\beta_3$. The influence of the residues flanking RGD in cyclo(-Arg^1-Gly^2-Asp^3-D-Phe^4-Val^5) and cyclo(-Arg^1-Gly^2-Asp^3-Phe^4-D-Val^5) has also been investigated.[54] Detailed NMR and biological data on four classes of cyclic pentapeptides able to sustain βII′/γ-turns in various positions revealed that the residue in position 4 and the proton of the amide bond between 3 and 4 are essential for high biological activity towards $\alpha_v\beta_3$. By contrast residue 5 has no influence on activity. The receptor model to summarise the result appears in Figure 1. When a series of β-turn mimetics have been inserted[55] into the βII′/γ-turn arrangements of cyclo(RGDX), where X represents the dipeptide equivalents (27), (28) and (29) instead of D-Phe-Val, the mimetic residues are reluctant to adopt the desired β-turn position. They do so more readily in cyclohexapeptides. The compound containing (27) as the insert proved to be the most active in inhibiting the binding of vitronectin to the $\alpha_v\beta_3$ receptor. The need for a proton at the amide bond between Asp^3 and the following residue is supported by these results. The β-turn as a selectivity switch in cyclic RGD peptides has been reviewed.[56]

The highly potent endothelin antagonist BQ-123 [cyclo(D-Trp-D-Asp-Pro-D-Val-Leu)] continues to be a template for analogue studies. Two analogues synthesised,[57] cyclo(D-Leu-D-Val-Pro-D-Asp-Trp) (IP-147) and cyclo(D-Trp-D-

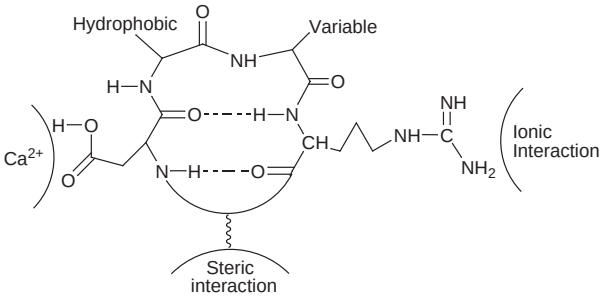
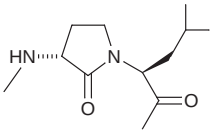
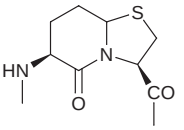


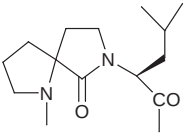
Figure 1



(27) (*R*)-ANC



(28) BTD

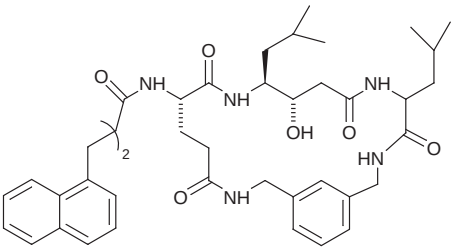


(29) (*S,S*)-*spiro*-Pro-Leu

Asp-Ac_3c-D-Val-Leu) (IPI-725), show different activities. IPI-725 proved to be a strong endothelin ET_A antagonist, while IP-147 was weak. Both molecules were synthesised on solid phase using an oxime resin for cyclisation on the resin. An explanation to the variance in the activity of the two cyclopentapeptides has been provided by conformational studies which suggest that the hydrophobic patch made up of Val and Pro is important for activity. Mimicking the Leu-Trp-Asp residues may not be sufficient. Longer acting analogues[58] of BQ-123 are also required due to its poor oral absorption and rapid breakdown in plasma. Replacement of the Pro residue with hydroxyprolyl allowed further derivatisation at the hydroxyl position and has provided the series cyclo(D-Trp-D-Asp-ROPro-D-Val-Leu) where R is Gly, Orn, Lys, Arg, His, *etc*., to maximise the chances of involvement of the hepatic anion transport system. Cyclisation of linear precursors was carried out between Asp(OBzl) as C-terminal and RO-Pro at the N-terminal position using water soluble carbodiimide/HOBt. All analogues exhibited potent ET_A receptor binding affinity similar to BQ-123 and a longer retention time in plasma.

BQ-123, cyclopentapeptide α-amylase inhibitors such as cyclo(Phe-Ala-Trp-Arg-Tyr-Pro) and renin inhibitor (30) have been used[59] as test compounds for a simplified approach to conformational work utilising ^{1}H NMR and solution phase modelling techniques. Only information derived from reported $^3J_{NH\text{-}H\alpha}$ coupling constants and temperature dependence of the amide protons were used in conjunction with Amber and GB/SA solvation modelling. Conformations of four out of the five compounds matched previously published data. The fifth underwent too much conformational averaging for the simplified approach. Five conformers in slow exchange were observed[60] in 2D-NMR and distance geometry

calculations on cyclo(Ser-D-Leu-Asp-Val-Pro-). The major conformer (66%) contained *cis* proline as part of a type V1a2 β-turn. The molecule inhibits the interaction between the integrin, very-late antigen-4 (VLA-4; $\alpha_4\beta_1$) and vascular cell adhesion mol-1 (VCAM1). The cyclic pentapeptide was synthesised on a Rink acid-sensitive resin using a Fmoc/Bu^t regime, and the linear precursor cyclised using TBTU/DMAP. Cyclic bradykinin antagonists showing pA_2 values around 5-7 in bradykinin-induced smooth muscle contractions in rabbit jugular vein have been synthesised,[61] and a type II′ β-turn structure confirmed as a typical conformation. The best analogue turned out to be (31).



(30) (31) Thi = thienylglycine

Low-mode searching (LMOD) for the exhaustive exploration of the potential energy hypersurface of molecules has been applied[62] and proved successful for defining the conformation of BQ-123, cyclo(D-Trp-D-Asp-Pro-D-Val-Leu). The unusual conformational preferences in cyclo(Pro-Phe-Phe-β-Ala-β-Ala) have been explored[63] using X-ray methods, NMR and restrained molecular dynamics. In the solid state the Phe^3 and β-Ala^4 are at the corners of a β-turn, with the β-Ala^5-Pro bond showing up as *cis*. In the solution phase the *cis* β-Ala^5-Pro bond also exists as its *trans* rotamer. In both CD_3CN and DMSO the molecule exists as two slowly interconverting conformers.

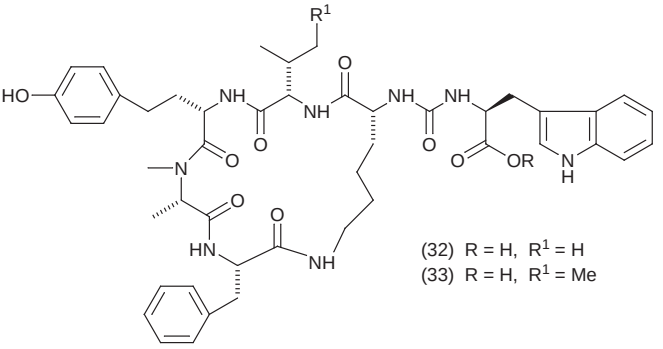
2.6 Cyclohexapeptides – Ferintoic acids A (32) and B (33) represent two additional members of a new class of cyclic peptides isolated[64] from microcystin producing cyanobacteria, *Microcystis aeruginosa*. Neither (32) or (33) showed any inhibition of chymotrypsin activity. The sponge *Ircinia dendroides* has yielded[65] a new cyclic hexapeptide, waiakeamide (34). It is the first time that methionine sulfoxides have been found in a marine source. As part of a continuing study[66] in search of new bioactive cyclic peptides in plants, four cyclohexapeptides, dichotomins A-D, and a cyclopentapeptide, dichotomin E [cyclo-(Gly-Tyr-Ala-Phe-Ala)] have been characterised from the roots of *Stellaria dichotoma* L var *lanceolata Bge*. Dichotomins A-D were found to have the structures:

A cyclo(Gly-Thr-Phe-Leu-Tyr-Val)
B cyclo(Gly-Thr-Phe-Leu-Tyr-Thr)

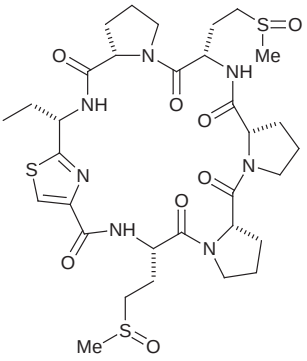
C cyclo(Gly-Thr-Phe-Leu-Tyr-Ala)
D cyclo(Gly-Val-Gly-Phe-Tyr-Ile)

The clinical interest in RAVII (35) as an anti-tumour agent continues to be an impetus to studying the nature of its pharmacophore. The conformation of the *cis* N-methylamide bond between Tyr^5 and Tyr^6 has been deemed to be critical, so a *trans* form produced[67] *via* the analogue (36) has been investigated. Produced from a minor congener RA-V which has a hydroxyl group at position Y in (35), the analogue (36) had an IC_{50} value only 7 times less toxic than (35). The authors conclude that the *cisoid* conformation of the N-methylamide between Tyr^5 and Tyr^6 is not essential. Thiono analogues,[68] [$Tyr^6\Psi$[CSNH]-D-Ala^1]RAVII, [D-$Ala^1\Psi$[CSNH]-Ala^2; $Tyr^3\Psi$[CSNH]Ala^4]RAVII and [$Ala^2\Psi$[CSNH]-Tyr^3; $Tyr^3\Psi$ CSNH]Ala^4]RAVII, have been synthesised using Davy's reagent (37), in addition to known thionoamide analogues. The analogue [$Tyr^3\Psi$[CSNH]Ala^4]RAVII has been reduced by nickel borohydride to [$Tyr^3\Psi$[CH_2NH]Ala^4]RAVII, and an X-ray determination of this showed a different conformation of the 18-membered ring, which might account for the loss of activity in the analogue. The treatment of RAVII (38) with boron tribromide[69] removed both OMe groups on the two tyrosines, but partial re O-methylation with diazo(trimethylsilyl)methane afforded analogues with alkyl substituents on tyrosyl 3. In this way analogues (38) - (41) were produced, and although active *in vitro* and *in vivo* they did not exceed the activity of (35). In a very interesting discussion between the Japanese[70] and the American[71] groups who have totally synthesised RAVII, it has emerged[70] that the latter group's original intermediate (42) must have been the DL-N,N-dimethylcyclodityrosine. A re-investigation[71] has confirmed the observation, and has revealed that the DL-form must undergo a re-epimerisation at the C-9 centre during the macrolactamisation process using $(PhO)_2P(O)N_3$, to re-form the natural S-configuration in the final RAVII.

High field NMR conformational studies[72] have shown that both cyclo(Gly-Tyr-Val-Pro-Met-Leu) and its phosphotyrosyl analogue, related to the SH2 domain of the PH85 subunit of phosphatidylinositol-3-OH kinase, exhibit similar conformational isomers. A 2:1 mixture of *trans:cis* Pro conformers exists in 75% CD_3OD/D_2O which changes to 1:1 in the respective pure solvents. Incorporation[73] of the β-turn mimetic BTD in a LDV cyclic peptide (43) has preserved the 2 H-bonds in the β-turn structure. The molecule inhibits the interaction between the integrin $\alpha_4\beta_1$ and vascular cell adhesion molecule (VCAM-1). TBTU/DMAP were used to cyclise the linear precursor between the BTD carboxyl and the glycyl amino group. The highly active somatostatin cyclic hexapeptide cyclo(Phe-Pro-Phe-D-Trp-Lys-Thr) has been used[74] as a template to study the conformational constraints offered by sugar molecules. Various NMR techniques, in combination with distance geometry calculations, show in general that inserts of the type shown in (44) favour β-turns, yet as inserted in (45) the sugar mimics a γ-turn. Compound (44) had an IC_{50} value of 0.15 μM for inhibition of release of growth hormone. Cyclo(Arg-Gly-Asp-Arg-Gly-Asp) and cyclo[Arg-Gly-Asp-Arg-Gly-D-Asp] have been prepared[75] on the solid phase by side-chain attachment of an Asp residue to a Wang Resin with a 2.4-dimethoxybenzyl ester protection for the



(32) R = H, R^1 = H
(33) R = H, R^1 = Me



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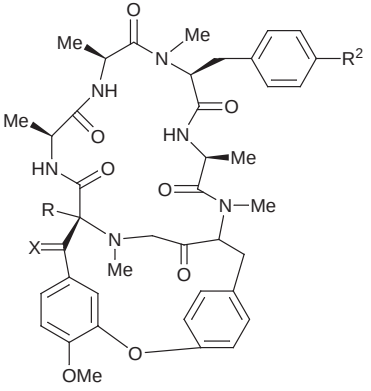
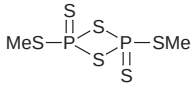
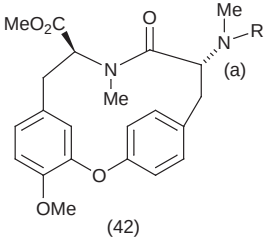


(35) X = H_2, R = H, R^2 = MeO
(36) X = O, R = CH_2–CH=CH_2, R^2 = MeO
(38) X = H_2, R = H, R^2 = Et
(39) X = H_2, R = H, R^2 = allyl
(40) X = H_2, R = H, R^2 = Me
(41) X = H_2, R = H, R^2 = vinyl



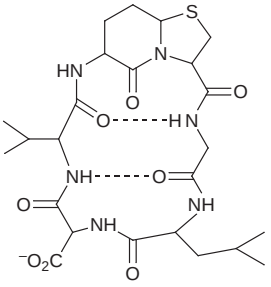
(37)



(42)

α-carboxyl of the attached Asp residue. Both the cyclic compounds showed modest activities in a binding assay based on $\alpha II_b/\beta 3$ fibrinogen and $\alpha V\beta 3$ vitronectin interactions. A relatively short distance between Asp and Arg side-chains was implied from the type I β-turn conformation deduced to be present.

NMR and molecular dynamics have been applied[76] to bridged analogues of substance P such as (46) and (47). The more flexible (47) proved to be more active than (46) while the analogue (48) was found[77] to show selectivity to the NK-1 neurokinin receptor. A minilibrary approach[78] has been used, based on earlier studies on β-glucose scaffolds devoid of amide bonds, to transform a peptidal somatostatin receptor ligand into an NK-1 receptor ligand. The best NK-1 receptor antagonist (49) achieved by such an approach had an IC_{50} of 2.0 ± 0.4 nM. Synthesis was carried out on a trityl linked solid support where four amino acids (Ala, Tyr, Leu and Phe) were simultaneously introduced as Lys replacements (Scheme 5).



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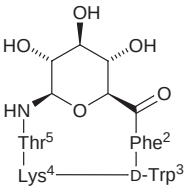
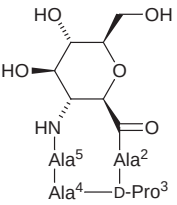
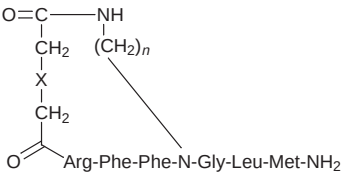


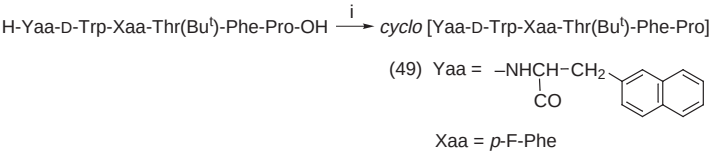
(44)



(45)



(46) X = -(Me)═CH–, *n* = 3
(47) X = CH_2, *n* = 4
(48) X = (CH_2) *n* = 3

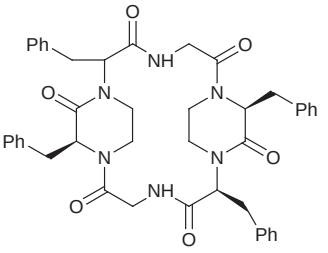


Reagents: i, DPPA/$NaHCO_3$/DMF

Scheme 5

Cyclic hexapeptide libraries (each containing 5832 cyclohexapeptides) dissolved in electrolyte solutions have been used[79] as chiral selectors in capillary electrophoresis to separate derivatised amino acid enantiomers. Cyclo(Gly-Aib-Gly)$_2$ has been synthesised[80] and crystallised. In the crystal it adopts a conformation

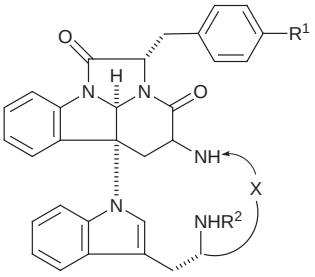
with a β-turn (type I) at one end and its mirror image on the other side of the ring. Semi-empirical calculations have been performed[81] on published conformations of cyclo(Gly-Pro-Gly)$_2$ using different force fields (DISCOVER cvff and cff 91, AMBER and CHARMM). The structure most heavily weighted contained at least one type I β-turn. Cyclisation of melanotropin fragments, Gly-His-Phe-Arg-Trp-Gly and its D-Phe derivative, has resulted[82] in cyclic analogues with diminished biological activity. By virtue of the presence of six amide bonds in its backbone, the X-ray determined[83] conformational form of (50) can be included in this sub-section. Both piperazine-2-one rings have 'nearly-chair' forms. A crystal structure[84] of cyclo(Adm-Cyst)$_2$ where Adm = 1,3-adamantanedicarbonyl and Cyst = cystine di-methyl ester shows a 2-fold rotation axis, for the molecule in the crystal, that passes through one adamantyl group and through the centre of the opposing S-S bond.



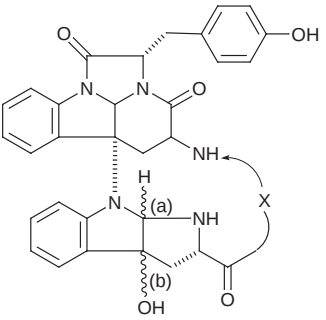
(50)

2.7 Cycloheptapeptides – The cyanobacterium *Oscillatoria-agardhii* (NIES 204) has been shown[85] to be not only a source of a cycloheptapeptide agardiheptin A, cyclo-(His-Gly-Trp-Pro-Trp-Gly-Leu), but also an octapeptide analogue of agardiheptin B, cyclo(-Trp-Leu-Pro-Trp-Ala-Pro-Trp-Val). Only agardiheptin (A) with an IC_{50} of 65 μg/mL inhibited plasmin. The marine sponge *Cribrochalina olemda* has yielded[86] two pairs of kapakahines, having the structures (51) and (52) for kapakahines A and B respectively and (53) and (54) for the related C and D stereoisomers. Both pairs carry the unique feature of an amide linkage between two Trp residues. Further investigation[87] of the Okinawan sponge *Hymeniacidon* sp., which has already been a source of the cycloheptapeptides hymenamides A-E and cyclooctapeptide hymenamides G, H, J and K, has yielded another cycloheptapeptide, hymenamide F [cyclo(Leu-Arg-Pro-Pro-Ala-Val-Met)]. The Met residue was analysed as the S-oxide, and the compound in MeOH showed two β-turns (type I at Met(oxide)-Leu and type VI(a) at Pro-Pro) and 3 transannular H-bonds at NH(Ala)/CO(Arg), NH(Val)/CO(Arg) and NH(Arg)/CO(Val).

Higher plants continue to be investigated as a source of cyclic peptides. Thus seeds of *Vaccaria segetalis* have yielded[88] segetalin E, cyclo(Gly-Tyr-Val-Pro-Leu-Trp-Pro), while the roots of *Stellaria yunnaensis* are a source[89] of yunnanin C, cyclo(Gly-Ile-Gly-Phe-Tyr-Ser-Pro) which shows inhibitory activity



(51) R^1 = OH, R^2 = H, X = Ile-Pro-Val-Pro-Ile
(52) R^1 = H, R^2 = Phe, X = Ala-Leu

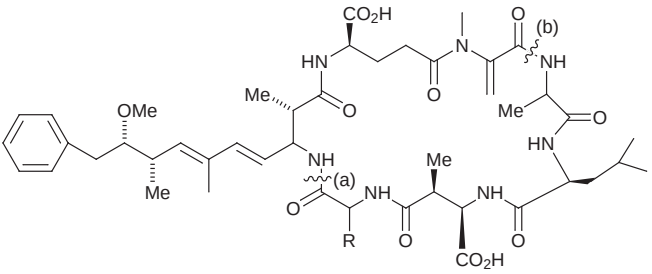


(53) X = Ile-Pro-Val-Pro-Ile
a = H, b = OH
(54) X = Ile-Pro-Val-Pro-Ile
a = H, b = OH

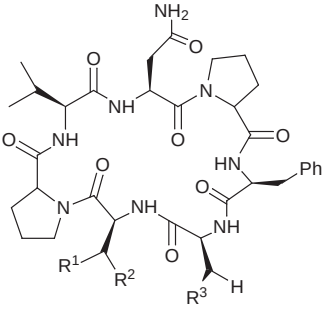
against P-388 leukaemia cells (IC_{50} 2.2 μg/mL). Cyclogossine A from the latex of *Jatropha gossypifolia* L. (Euphorbiaceae) has been proven[90] by 2D NMR and associated techniques to be cyclo(Leu-Ala-Thr-Trp-Leu-Gly-Val). The latex of *Jatropha podagrica* has produced[91] both podacycline B, cyclo(-Phe-Ala-Gly-Thr-Ile-Phe-Gly), and a nonapeptide, podacycline A, having the structure cyclo(Gly-Leu-Leu-Gly-Ala-Val-Trp-Ala-Gly).

The first total synthesis[92] of microcystin LA (55), a serine-threonine phosphatase inhibitor relies heavily on the reagent HATU, both for difficult couplings and for the final cyclisation step at position (a). Several analogues of the cyclic backbone of (56) (without the Adda side-chain) have been investigated[93] as test models for solid phase and solution phase synthesis *via* sites (a) and (b). Only at site (b) did an analogue synthesis prove possible in the solution phase. Solid phase techniques based on anchoring the side-chain of Asp to an MBHA resin with the α-COOH protected as a fluorenylmethyl group proved the most successful at sites (a) and (b). Unexpectedly[94] these analogues lacking an Adda unit had some inhibitory activity against the phosphatase PP2A. A cautionary concern[95] about the biological activity of some extracted natural cyclic peptides has resulted from the synthesis of axinastatins 2 and 3, (57) and (58) respectively. The biological activities of the synthesised compounds were 10-100-fold less than the natural products, suggesting that the natural extract is complexed with the exceptionally potent antineoplastic compounds, halichondrins/halistatins. Synthesis was accomplished in the solution phase using t-butyl esters for C-protection, Fmoc for N-protection and diethylphosphorocyanidate DEPC (or the nitrophenyl ester) for coupling. Final cyclisation was carried out using BOP-Cl. Similar worries about contaminated specimens have been made[96] after the synthesis of the marine sponge stylopeptide 1, cyclo(Leu-Ile-Phe-Ser-Pro-Ile-Pro), using Fmoc/Bu^t ester protocols in the solution phase. TBTU (67% yield) and BOP-Cl (13% yield) were used for cyclisation and the synthesised product proved identical with the natural product, except in its biological activity.

An infra-red and circular dichroic study[97] has revealed a β-turn conformational

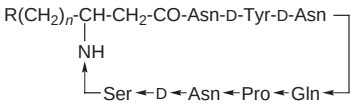


(55) R = Me
(56) R = $(CH_2)_3$–NH–C(=NH)–NH_2



(57) R^1 = Pr^i, R^2 = H, R^3 = Me
(58) R^1 = Pr^i, R^2 = H, R^3 = Et

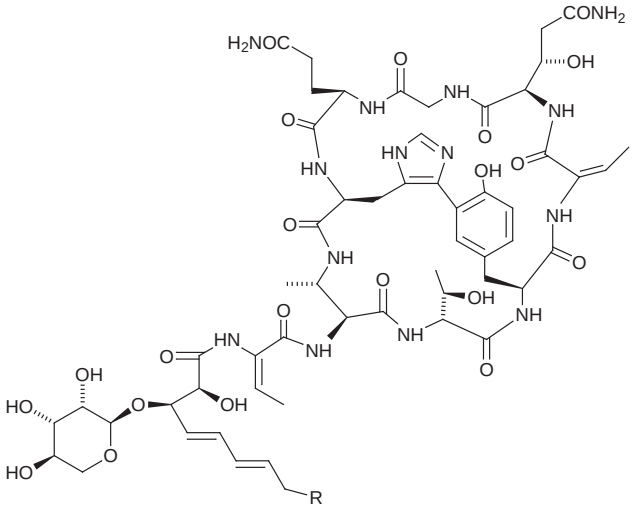
pattern in iturin A (59) similar to previously published NMR data. A cyclic heptapeptide, from a library constructed[98] by head to tail cyclisations of side-chain resin-bound linear sequences, proved to be an effective host for Ca^{2+} ions. The binding to Ca^{2+} varied from Xxx = MeAla > Gly $\simeq$ Sar in the mixture cyclo[Gly-Asp-D-Pro-Xxx-Asp-D-Pro-Asp(Aca-PheNH$_2$)].



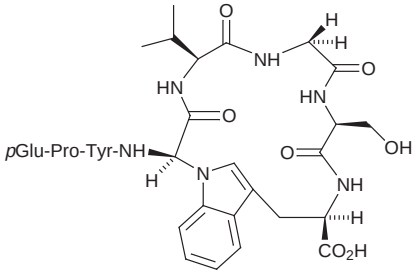
(59)

2.8 Cyclooctapeptides – The lithistid sponge *Aciculites orientalis* has been shown[99] to contain three cyclic peptides, aciculitins A (60), B (61) and C (62). The structures contain the unusual histidine-tyrosine bridge. The aciculitins inhibited the growth of *Candida albicans* and were cytotoxic towards the HCT-116 cell line. Lyciumin (A) (63) has been included under this sub-section by

default, because it had been listed[100] in its original paper as a cyclooctapeptide. It is really a cyclic pentapeptide, with a tripeptide side-chain, and from 600 MHz NMR studies combined with molecular dynamics calculations, an unambiguous assignment of an *R* configuration to the C_α glycine substituent could be made. During the cleavage[101] of a tetrapeptide precursor off a benzophenone resin the cyclooctapeptide cyclo-[-Arg(Tos)-Sar-Asp(cHex)-Phg-]$_2$ was produced where Phg = phenylglycyl. The ratio of cyclic tetramer to cyclic octamer was 41:59 in the example cited but varied depending on the sequence. In the solution phase, the best cyclisation conditions used BOP/HOBt, giving cyclo(Arg-Sar-Asp-Phg)$_2$ which inhibits platelet aggregation with an IC_{50} of 0.36 μmol dm^{-3}. Some restricted conformational forms with H-bonds across two



(60) R = C_5H_{11}
(61) R = C_6H_{13}
(62) R = C_7H_{15}

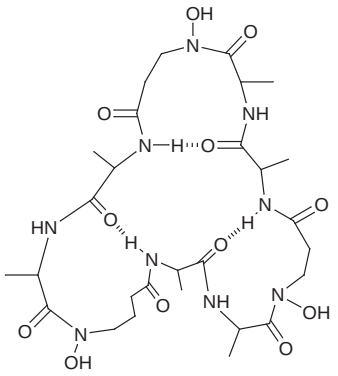


(63)

pairs of NHAsp/COPhg were observed in NMR and CD spectra. The catalytic triad (Ser)(His)(Glu) of serine proteases has been incorporated[102] into disulfide-bridged cyclic octapeptide Ace-Ala-Cys-Ser-Pro-Gly-His-Cys-Glu-O^-, supporting a β-turn. Its catalytic activity, nine times faster than histidine in the hydrolysis of p-nitrophenylacetate, is far short of Nature's enzymic power. *Stellaria delavayi* has been shown to be the source[103] of a further two new cyclopeptides characterised as stelladelin C, cyclo(-Val-Pro-Tyr-Pro-Pro-Phe-Tyr-Ser), and stelladelin B, cyclo(-Gly-Ile-Pro-Pro-Ala-Tyr-Asp-Leu).

2.9 Cyclononapeptides and Cyclodecapeptides – The higher plant *Leonurus heterophyllus* has yielded[104] in its fruits (used as a Chinese drug for invigorating blood circulation) three new proline-rich cycloleonuripeptides (A-C) which have been identified as cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Pro-Met-Ile), cyclo(-Gly-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile) and cyclo(-Gly-Pro-Pro-Pro-Pro-Tyr-Pro-Pro-Met(O)-Ile-). The cyclic nonapeptide cyclo[-Ala-Ala-β(HO)Ala]$_3$, synthesised[105] in the solution phase, and cyclised under high dilution with BOP/HOBt/NMM, forms hexadentate octahedral complexes with Fe(III) in aqueous solution. The NMR data suggests that the solution structure can be interpreted as (64). A brief review[106] has appeared on the synthesis and conformation of cyclic decapeptides. A marine worm collected on Papua New Guinea has yielded[107] a potent Gram positive antibiotic having the structure cyclo(-Val-Orn-Leu-Tyr-Pro-Phe-Phe-Asn-Asp-Trp). In preliminary tests this compound, related to the tyrocidines, inhibited the growth of methicillin resistant *Staphylococcus aureus*, vancomycin resistant *Enterococcus* sp. and penicillin resistant *Streptococcus pneumoniae* with MIC values of 1-2 μg/mL. The cyclic analogue[108] of $ACTH_{1\text{-}10}$, cyclo(-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly), has been synthesised in the solution phase using a pentafluorophenyl ester at C-terminal glycine for cyclisation, and also after solid phase precursor synthesis, the compound was obtained by BOP-catalysed cyclisation. The cyclic $ACTH_{1\text{-}10}$ analogue contains the catalytic triad residues of chymotrypsin, but it gave a poor catalytic rate of hydrolysis of phenylalanine esters, probably due to the flexibility of the cyclic structure. Cyclic decapeptides[109] restrained *via* disulfide links typified by (65) have been synthesised by solid phase with the final -S-S- link being achieved by I_2 oxidation of the two Cys(Acm)-containing peptides on the solid support. Analogues of kallidin (Lys-Bradikynin) have been cyclised[110] either *via* TBTU/HOBt or by DPPA-catalysed conditions. The former reagents appeared to be the faster but, consistent with previous findings, preliminary pharmacological experiments on rat duodenum showed the cyclic analogues to be significantly less potent than the linear ones.

Based on a previous report that two well resolved peaks in the HPLC of (66) might be due to a slow conformational equilibrium, an NMR study[111] has now shown the existence of at least three conformations in solution. It is concluded that *cis-trans* isomerism at the Pro^3 residue determines conformation, and the first of the HPLC peaks was shown to contain the *trans-trans*, *trans-cis* forms of the peptide, while the second peak contained the *cis-trans* form. A cyclodecapeptide, cyclo(-Pro-Phe-Phe-Aib-Leu)$_2$, related to the cyclolinopeptide A has been



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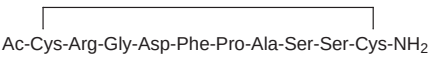


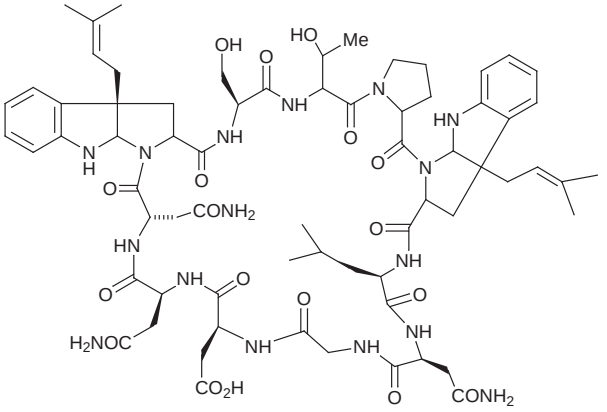
(65)

analysed[112] by X-ray diffraction and by NMR, and a perfectly symmetrical structure can be seen using both technologies. Six intramolecular NH···CO H-bonds with formation of four turns (3 type I and one type III) represent the best intrepretation of the data. Comparisons have been made[113] between linear and cyclic decapeptides using CD spectroscopy. The three cyclic peptides used were cyclo[-Glu(OBzl)-Pro-Gly-Glu(OBzl)-Gly]$_2$, cyclo[-Lys(Z)-Pro-Gly-Lys(Z)-Gly]$_2$ and cyclo[-Phe-Lys(Z)-Pro-Gly-Lys(Z)-Pro]$_2$, which were studied in different solvents, chloroform, trifluoroethanol, and acetonitrile. Solvent polarity had more influence on linear peptide conformations than their cyclic analogues. βI Turns, and γ-turns could be detected in the conformation of the peptides.

(66)

2.10 Higher Cyclic Peptides – Cyanobacterium, *Microcystis-aureginosa* (NIES-88), already known to be a source of the microcystins has also yielded[114] kawaguchipeptin A, whose structure (67) was worked out using a combination of physical techniques. As part of the search for a somatostatin antagonist, the effect of introducing conformational distortion has been researched through the synthesis[115] of a homodetic tricyclic peptide (68). The task necessitated the use of five dimensional orthogonal amino protection and three carboxyl protecting groups. Scheme 6 summarises the key strategy. The tricyclic peptide failed to completely inhibit ^{125}I-labelled somatostatin binding.

A number of gramicidin S analogues have again been investigated. Replacement of the L-Orn residue by L-Ser or L-Glu has been carried out[116] using solution phase synthesis utilising azide method or N-hydroxysuccinimide esters for cyclisation. The activities of the analogues were weaker than the parent compound, which could indicate an involvement of the δ-amino group of Orn in



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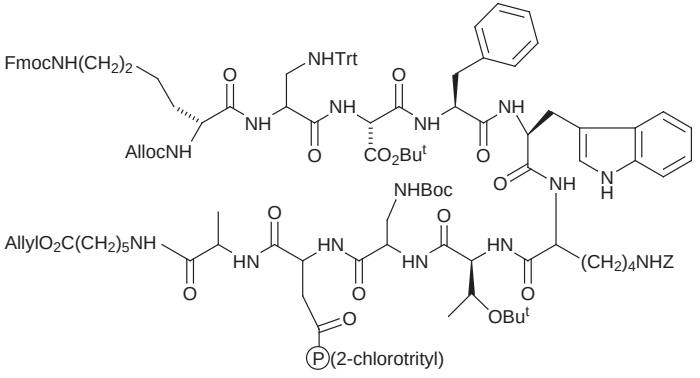
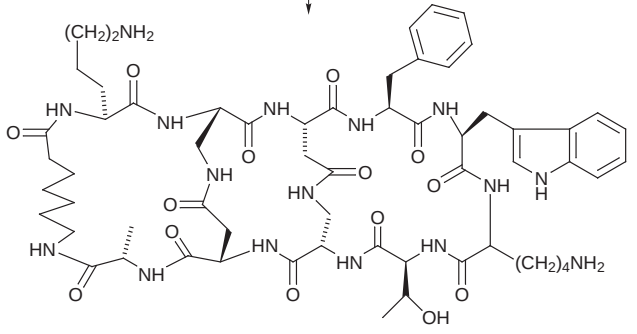


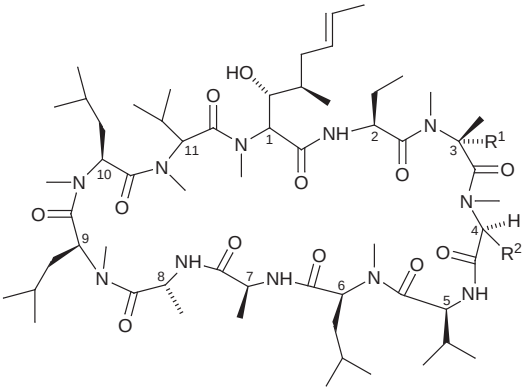


(68)

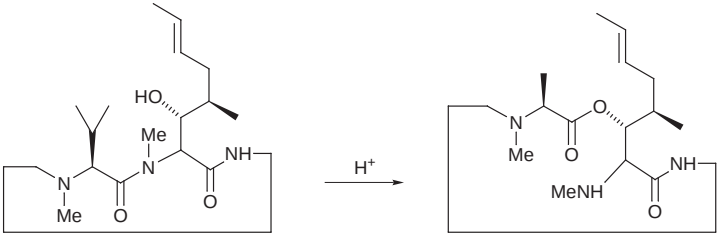
Scheme 6

the biological interactions. Replacement[117] of the D-Phe residues in gramicidin S with either D-His, D-Ser, D-Tyr or D-Asn, followed by subsequent testing against Gram-positive and Gram-negative bacteria, have revealed the need to re-evaluate the role that gramicidin S and analogues could play as broad spectrum antibiotics. [Hexafluorovalyl[1,1]]-Gramicidin S has been synthesised[118] on a benzophenone-oxime resin. The conformation of the analogue was the same as the parent compound but was somewhat weaker in its biological activity. A judicious choice of cyclisation point for the synthesis[119] of gramicidin S and analogues has made it possible to cyclise linear precursors with Lys or Orn side-chains unprotected. The protocol was simply to react the linear precursor with DCC/HOBt/DIEA at room temperature for less than 6 hrs.

The improved hydrophilic properties[120] of [D-MeSer[3], 4(OH)MeLeu[4]] cyclosporin A (70) has resulted in a higher affinity for cyclophilin A. Its lack of immunosuppressing activity has given analogue (70) interesting anti HIV-1 activity. It was synthesised from cyclosporin A (69), by reaction with butyllithium (-78 °C in THF) and trapping the hexa-anion with solid paraformaldehyde



(69) CSA, R^1 = H, R^2 = CH_2CHMe_2
(70) R^1 = CH_2OH, R^2 = $CH_2C(OH)Me_2$
(71) R^1 = CH_2OH, R^2 = CH_2CHMe_2



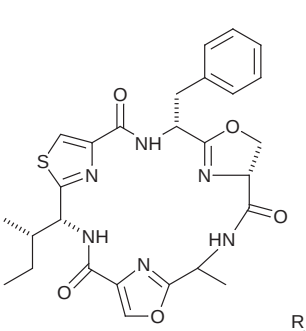
Cyclosporin A Isocyclosporin

Scheme 7

yielding (71) which was enzymatically hydroxylated to (70). Two further novel cyclosporins, [Thr^2, Leu^5, Ala^{10}]cyclosporin and [Thr^2, Ile^5]cyclosporin, have been described[121] from fungi classified as *Acremonium luzulae* (Fuckel) W. Gams and Leptostroma anamorph of *Hypoderma eucalyptii* Cooke & Harkn. An X-ray analysis[122] of the di-Me isosorbide solvate of cyclosporin A (69) revealed a new orientation for the MeBmt^1 side-chain which allowed H-bond contact at MeBmt^{1}OH - Sar^{3}CO. Isocyclosporin, formed according to Scheme 7, appears[123] to be devoid of regular secondary structure. X-Ray data show a *cis* amide bond between residues 2 and 3, and 3 and 4, respectively and transannular H-bonds between NHMeLeu^4 / COMeLeu^{10} and Val^{5}CO / Ala^{7}NH. Alkali treatment of cyclosporin A (69) destroys the MeBmt residue,[124] and gives rise to anhydro-MeBmt-cyclosporins, as well as [Sar^1] cyclosporin. A new asymmetric synthesis[125] has been reported of β-hydroxy α-amino acids, including MeBmt.

Another total synthesis[126] of the immunosuppressant FK506 has been presented. The top and bottom halves of the molecule are coupled by addition of a vinyl cuprate with a spiroenone.

2.11 Peptides Containing Thiazole/Oxazole Rings – Cultured cyanobacterium *Oscillatoria raoi* De Toni has yielded[127] two structurally related cyclic hexapeptides, raocyclamide A (72) and B, where the oxazoline ring is replaced by a serine residue. Both exhibit a moderate cytotoxicity against sea urchin embryos. NMR studies[128] have yielded data that confirm the structures of sulfomycins II and III to be (73) and (74) respectively. The genus *Lissoclinum* continues to reveal a rich source of biologically active molecules. A full account[129] has now been given of



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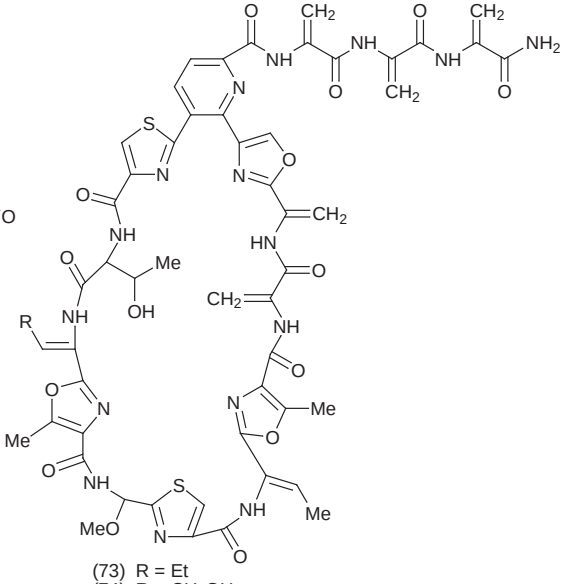
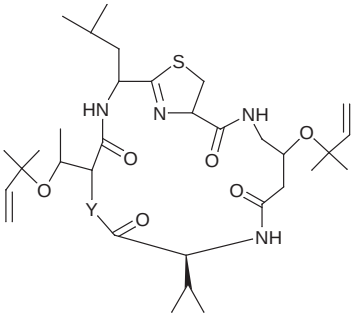
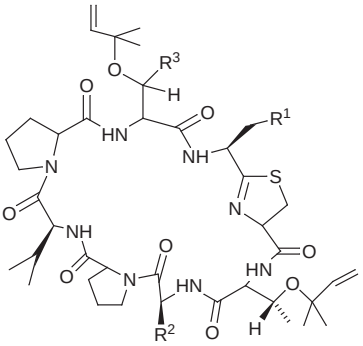


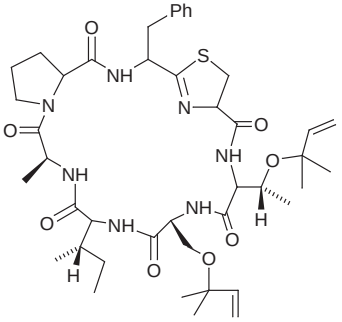
(73) R = Et
(74) R = CH_2OH



(75) Y =  (76) Y = 



(77) $R^1 = R^2 = CH_2CHMe_2$, $R^3 = Me$
(78) $R^1 = PhCH_2$, $R^2 = Me_2CH$, $R^3 = H$
(79) $R^1 = Me_2CH$, $R^2 = PhCH_2$, $R^3 = Me$



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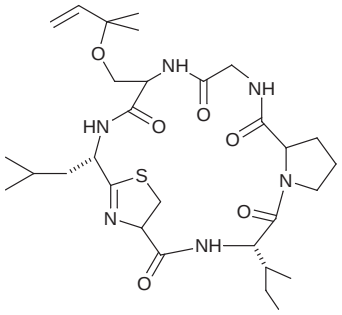
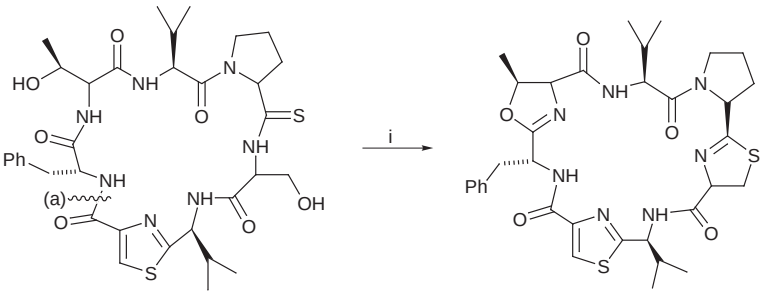
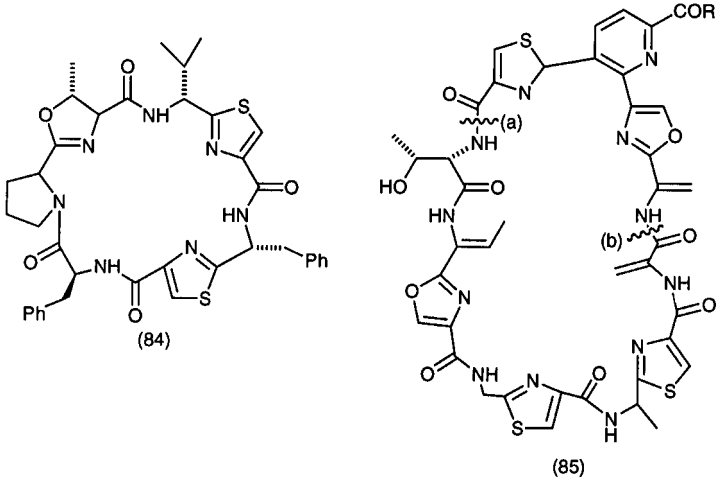


(81)

the cyclic hexa- and octa-peptides isolated from *Lissoclinum patella* from Fiji. Patellins 1 and 2 have been assigned structures (75) and (76), while patellins 3 and 4 has been given the same sequence (77) but need to be assigned different stereochemistries. Structures (78) and (79) have been determined for patellins 5 and 6 while trunkamide A (80) represents a new cycloheptapeptide. Similar biosynthetic origins seem plausible for keenamide (81) from the notaspidean mollusk *Pleurobranchus forskalii*.[130] Keenamide exhibited significant activity against P-388, A-549, MEL-20 and HT-29 tumour cell lines. Scheme 8 chronicles the later stages[131] of the synthesis of cyclodidernamide (83), showing the sequential formation of thiazoline and oxazoline rings. The initial cyclic backbone was created at position (a) in (82), with protected Ser and Thr side-chains, using DPPA/i-Pr_2NEt at 0 °C which gave a 74% yield. Twenty-three steps and an overall yield of 4.4% highlights the struggle to synthesise[132] lissoclinamide 7 (84), which was achieved by a novel strategy combining the use of the Burgess reagent for simultaneous oxazoline and thiazoline formation and an oxazoline to thiazoline interconversion. A practical synthesis[133] of the main skeleton of thiostrepton

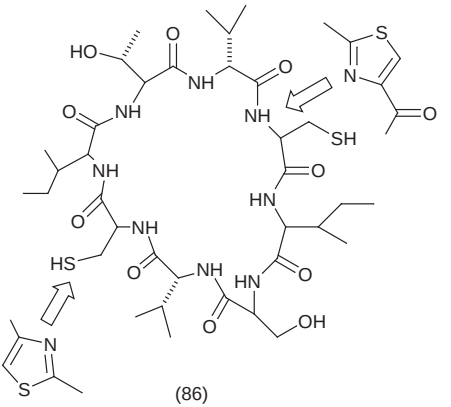


Scheme 8



peptide antibiotics A 10255G and J, as represented in (85), has been reported. Fragments corresponding to coupling at (a) and (b) were constructed as key precursors for the synthesis.

Conformational comparisons[134] between Thr and Cys-containing macrocycles and their thiazole/oxazoline counterparts has revealed how Nature has adopted these five-membered rings to impose severe conformational restrictions on cyclic octapeptides. Thus the interchanges, depicted in (86) and monitored by 2D NMR, showed that D-(Val)-Thz units induce three intramolecular H-bonds to form within the structure. Further conversions of the Thr units in (86) to oxazoline rings (Oxn) produced a highly constrained pseudoboat or saddle-shaped macrocycle, cyclo[Ile(Oxn)-D-(Val)Thz-Ile(Oxn)-D-Val(Thz)-].



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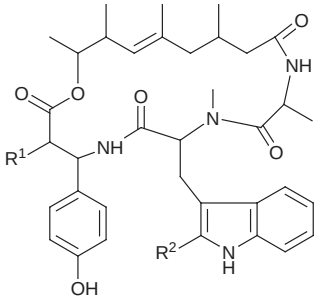
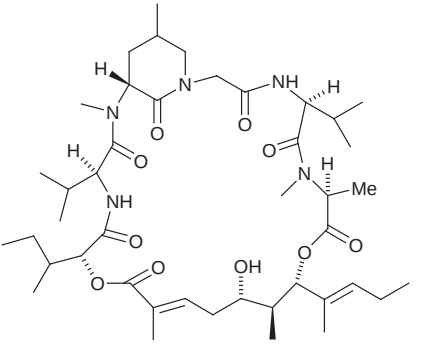


(87) R^1 = OMe, R^2 = H
(88) R^1 = OMe, R^2 = Cl
(89) R^1 = H, R^2 = H
(90) R^1 = H, R^2 = Cl



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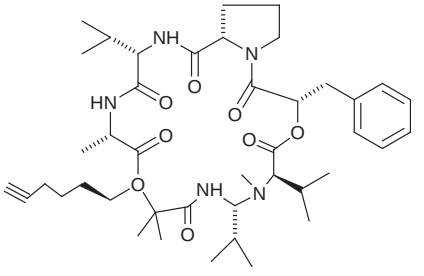
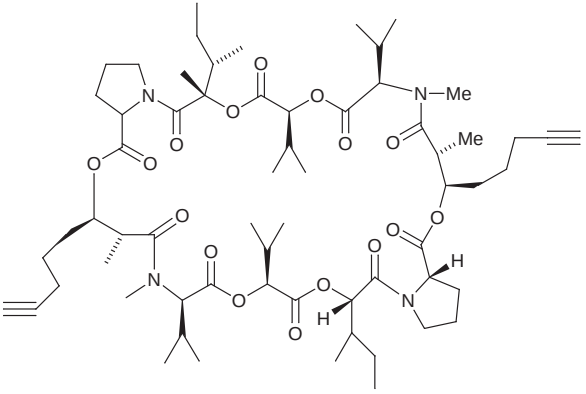


(92)

2.12 Cyclodepsipeptides – This sub-section has again retained its status as the largest compilation of the Chapter and continues to be the depository for listing Nature's most ambitious structures. Many new structures have come from extended screenings of previous sources. Thus additional members to the family of jaspamides, geodiamolides and neosiphoniamolides have been found[135] in the myxobacteria *Chondromyces-crocatus*, which has yielded the chondramides A (87) - D (90), new antifungal agents. Aurilide (91) has been identified[136] from extracts of the Japanese sea hare *Dolabella auricularia*, while a sea mollusc *Philinopsis speciosa* has yielded[137] kulolide (92), which is active against L-1210 and P388 leukaemia cells at IC_{50} values of 0.7 and 2.1 μg/mL respectively. The novel octynoic residue in kulolide (92) is almost repeated in onchidin B (93) from the mollusk *Onchidium* sp. which contains[138] a 3-hydroxy-2-methyloct-7-ynoic acid residue. Similarities to other marine cyclodepsipeptides such as the didemnins, discordemins and theonella peptolides have been found[139] in the structure (94) of callipeltin A from the New Caledonian lithisda sponge *Callipelta sp.* Cells infected by the HIV virus seem to be protected by callipeltin A. Two minor components, callipeltin B (95) and the acyclic form of callipeltin A, have also been identified.[140] Activity against AIDS opportunistic infections, amongst other bioassays, have been reported[141] for six new kahalilides from a marine mollusc, *Elysia rufescens*. Only the previously reported kahalide F (96) exhibited note-



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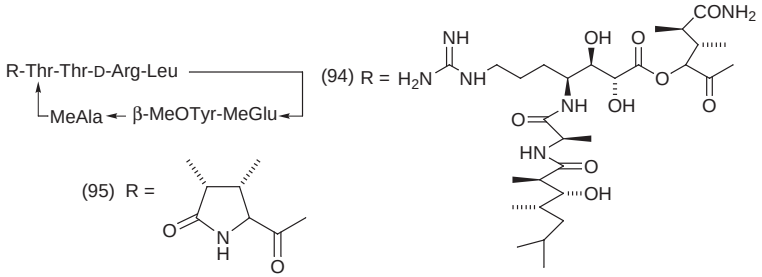
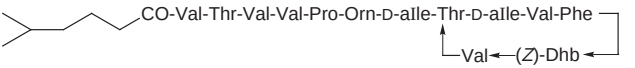
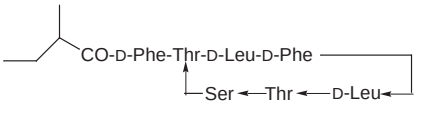




(96)

Dhb = dehydrobutyric acid



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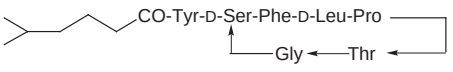

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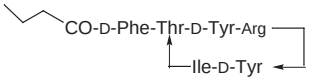

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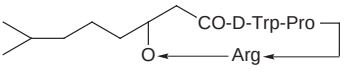

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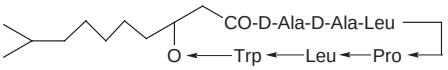

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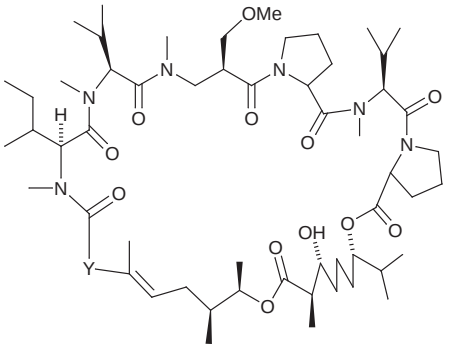
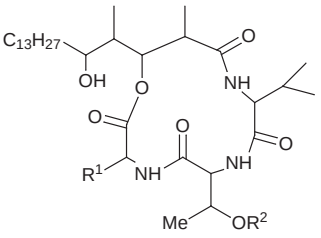
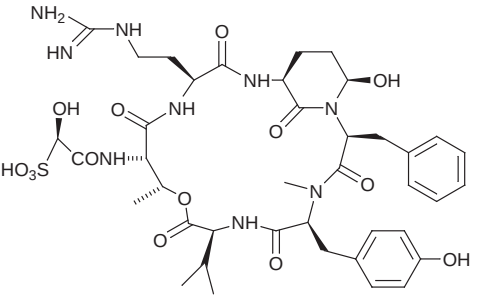


(102) Y = ═COMe
(103) Y = CH_2CO

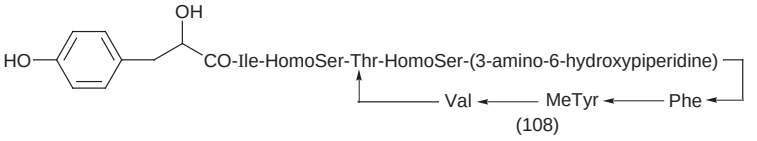
worthy biological activity. However, smaller quantities of the kahalides [A-E have structures (97) - (101)] have been elaborated also in the green alga *Bryopsis* sp. on which the mollusc feeds. Biological activity in this series seems to be lost if the ester bonds are hydrolysed. Extracts from *Dolabella auricularia* have been subjected[142] to further fractionation to give dolastatin G (102) and nordolastatin G (103), the latter being the product of the acid hydrolysis of (102). Three novel stevastelin congeners A3 (104), D3 (105) and E3 (106) have been isolated[143] from the culture broth of a *Penicillium* mutant, and the absolute stereochemistry of micropeptin 90, first isolated in 1995, has been confirmed[144] as (107) using chemical degradation and NMR techniques. The 3-amino-6-hydroxypiperidone in (107) residue has also been found[145] in nostrocyclin (108) from the cyanobacterium *Nostoc* sp. The cyclodepsipeptide is non-toxic but inhibits protein



(104) $R^1 = MeCO_2CH_2$═, $R^2 = SO_3H$
(105) $R^1 = CH_2$═, $R^2 = SO_3H$
(106) $R^1 = CH_2$═, $R^2 = H$



(107)



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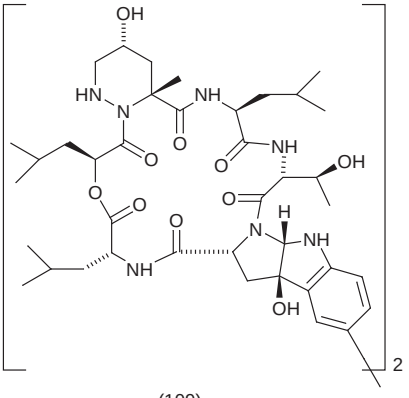

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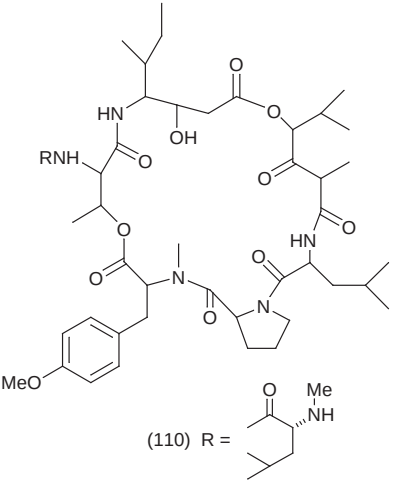
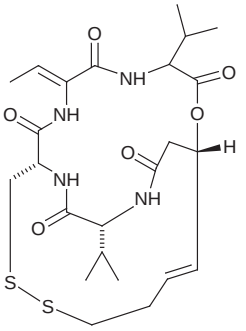


(110) R =

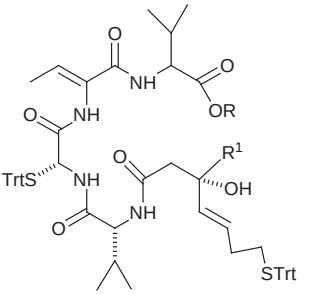
phosphatase *in vitro*. The prize for the year's most exotic newcomer goes to himastatin, a new antitumour antibiotic[146] from *Streptomyces hygroscipus III*. The structure of himostatin (109) is a unique dimer joined through a biphenyl linkage between two oxidised tryptophan units. A bent figure-eight best represents the conformation of didemnin A (110) as determined by X-ray crystallography.[147] This structure is similar to didemnin B whose conformation has already been determined, although there seem to be conformational differences in the macrocycle at the isostatine depside link.

Nature's complicated cyclodepsipeptide structures provide a real challenge to

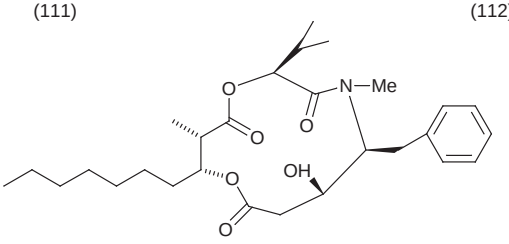
the world's synthetic research groups. However, the biological activity is often sufficiently attractive for the effort to be justified. The first total synthesis[148] of the antitumour depsipeptide FR-901,228 (111) has been achieved in fourteen steps with an overall 18% yield. The linear precursor (112) was cyclised by the Mitsunobu reaction or using water-soluble carbodiimide, followed by iodine oxidation for the formation of the disulfide ring. To augment the synthesis reported in last year's Report, other research groups have reported their synthesis of hapalosin (113) this year. Its multidrug resistance reversing activity has prompted a synthetic approach[149] which would differentiate the activities of *cis* and *trans* N-Me amide forms. In trial runs a N-demethylated analogue showed no activity and was not amenable to direct N-methylation, so the final step, a



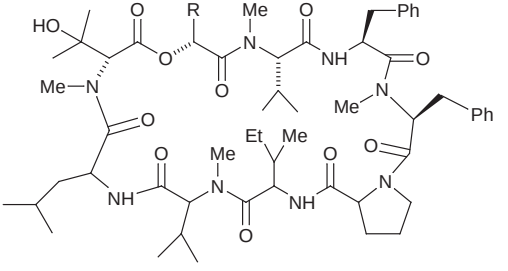
(111)



(112)

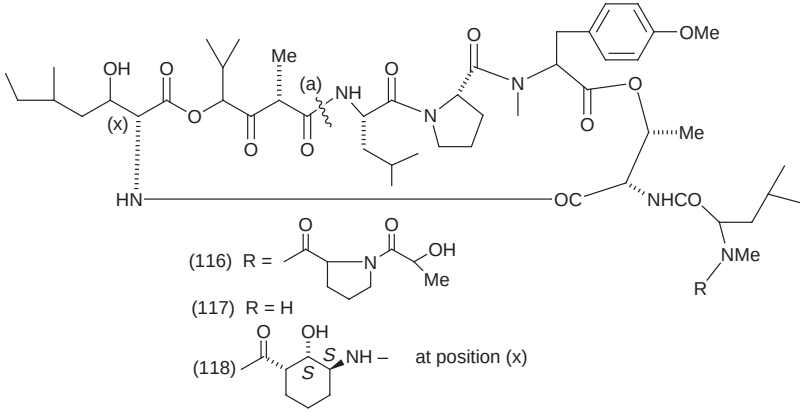


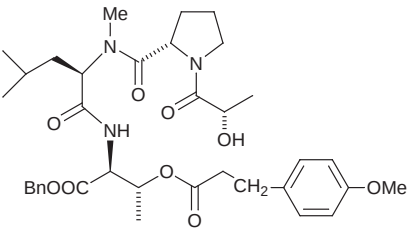
(113)



(114) R = EtCH(Me)CH_2
(115) R = cyclohexyl

macrocyclisation between the N-MePhe residue and the isovaleric acid carboxyl using DPPA, proved successful. The *cis* N-methyl amide conformation was found to be crucial for activity. The final cyclisation strategy utilising DPPA was also used in another synthesis,[150] with the N-methylated function being introduced *via* formylation to give an oxazolidine which was reduced with $NaBH_3CN$. A full report of a synthesis[151] of hapalosin, first published in a note last year, has appeared, together with a synthesis of the des N-methyl analogue. Again confirmation that the *cis* N-methylamide predominates in hapalosin has been obtained. Another strategy[152] involves cyclisation between N-MePhe hydroxyisovaleric acid using EDC/HOBt/Et_3N giving a 37% yield, and using methoxy methyl ether to protect the hydroxyl group. N-Alkylated residues have been the focus of special synthetic interest also in two separate syntheses of aureobasidin A (114). Trial runs[153] with cyclisation at the depside link proved unsuccessful. Links involving N-methylated residues were best synthesised using PyBroP, and the final cyclisation between the prolyl and allo-isoleucyl residues was carried out using PyBroP in 45% yield. (About 7% of racemisation at the Pro residue occurred.) A Boc/Bzl protecting protocol, N-methylated amino acids produced by the reaction of MeI/NaH with Boc-amino acids, and a mixed anhydride coupling regime summarise the main features of another synthesis[154] of (114). However, the mixed anhydride coupling gave extensive racemisation of N-methyl





(119)

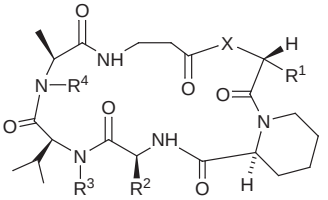
amino acid residues, so the BOP reagent was chosen for cyclisation at the MeVal/Phe bond. Analogues (115) and (114) were obtained in 44% and 21% yield respectively at the cyclisation stage. When treated[155] with HF at room temperature aureobasidin A cleaved specifically at this MeVal/Phe bond and could be recyclised using HATU in 12% yield.

A decade's intensive surveys[156] of metabolites of marine tunicates *Tridemnum solidum* and *Alidium albicans*, together with synthetic and semisynthetic analogues have provided 42 didemnin congeners for structure-activity study. It is concluded that simple N-acylation of the N-terminus of didemnin A (117) enhanced activities in antitumour, antiviral and immunosuppressive activities and that the original stereocentres and functional groups in didemnins B (116) and A (117) are essential for all bioactivities. A constrained ring analogue (118) has also been synthesised[157] to give a new macrocyclic core in didemnin B. Final cyclisation was at point (a) with HBTU giving a cyclisation yield of 50%. Three new side-chain β-turn analogues of didemnin B have also been synthsised,[158] but only (119) was weakly active.

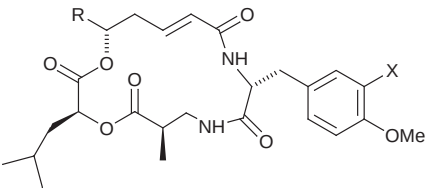
In a continuing search for anti-hepatitis B virus agents, extracts from the fungus *Metarhizium anisopliae* have yielded[159] a new cyclodepsipeptide destruxin E2 (120). It showed a lower suppressive activity compared with other destruxins, whose SAR have also been studied in the same report. Main conclusions drawn are: the side-chain of the first and third residue has to be hydrophobic; the N-Me residue at 4 plays an important role; the lactone part seems essential. Conclusive evidence for the requirement for a depside bond has been shown[160] by the fact that the all-amide destruxin analogues (121) - (123) are inactive. A succession of N-methylated residues seem to be easily hydrolysable under the acid conditions required to remove Boc protection, so Z-protection had to be used. DPPA appeared to be of considerable use for the ring closures.

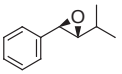
Earlier in this sub-section the origins[142] of dolastatin G (102) and nordolastatin G (103) were discussed, but with IC_{50} values of 1.0 and 5.3 μg/mL against $HeLaS_3$ cells, synthetic interest[161] in the structure was inevitable. Lactonisation, forming the depside at the C-terminal of a Pro residue, only occurred in 3% yield for (103) and 29% for (102) using 2,4,5-trichlorobenzoyl chloride/DMAP under reflux. Cryptophycin (A) (124) and its analogues, isolated from blue green alga have shown excellent activity against solid tumours in mice. The benzylic epoxide moiety in their structure is required for biological activity but confers instability on the molecule. To explore the role of the epoxide enones, (125) and (126) have been synthesised.[162] Fragment condensation using trichlorobenzoyl chloride brought about cyclisation as well, and was applied also to the syntheses of cryptophycins A-D. Epoxidation was carried out using m-chloroperbenzoic acid.

The expanding crisis in antibiotic resistance of bacterial pathogens has spurred synthetic interest in members of the virginiamycin family, well known for their extreme pH sensitivity. Three separate syntheses have adopted three separate cyclisation strategies to link up the macrocycle. Thus in the synthesis[163] of (-)-virginiamycin M_2 (127), the final cyclisation was made between the oxazole carboxyl and prolyl amine group using the Mukaiyama method of amide coupling (2-chloro-1-methylpyridinium iodide/n-Bu_3N). Cyclisation at point (a)



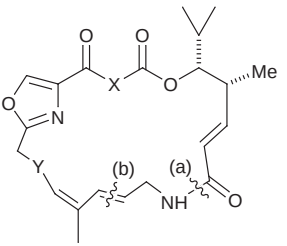
(120) $R^1 = CH_2CH(OH)CH_2Cl$, $R^2 = Pr^i$, $R^3 = Me$, $R^4 = Me$, X = O
(121) $R^1 = Me$ (D and L), $R^2 = Et$, $R^3 = Me$, $R^4 = Me$, X = NMe
(122) $R^1 = MeCH_2CH_2$ (D and L), $R^2 = Et$, $R^3 = Me$, $R^4 = Me$, X = NMe
(123) $R^1 = MeCH_2CH_2$ (D and L), $R^2 = Et$, $R^3 = Me$, $R^4 = Me$, X = NH



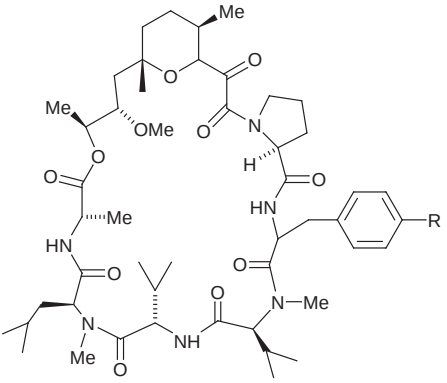
(124) X = Cl, R = 
(125) X = Cl, R = PhCH=CHCO
(126) X = H, R = PhCH=CHCO

using BOP-Cl, with the β-hydroxyketone masked as an acetal, was the final link in the total synthesis[164] of madumycin II (128). A Stille type coupling, ($Pd_2(dba)_3$ and Ph_3As) involving tributylstannate and vinylygous bromide termini at position (b) in the structure was key to the synthesis[165] of 14,15-anhydropristinamycin II_B (129). Progress is being maintained[166] on the development of fragments suitable for the total synthesis of the azinothricin family member, the antibiotic A83586C. An asymmetric synthesis of the 'northern' sector side-chain has been accomplished, and the C(1) to C(47) backbone has been produced for cyclisation between the threonyl residue and the β-hydroxyleucine residue. Rapamycin-peptide hybrids such as (130) have been synthesised[167] as part of a study of the optimum length of peptide which can be incorporated into the macrolide, so as to maintain FKBP affinity. Cyclodepsipeptide (130) did not give a dramatic improvement in binding. A check on the chirality of the 3-hydroxytetradecanoic acid of natural surfactin B_2 (131) through the syntheses[168] of both diastereoisomers confirmed that the D-form was identical to the natural material. The depside link was made through the use of CDI and the macrocyclisation carried out between the hydroxy acid and the amino group of Glu, using a succinimide ester.

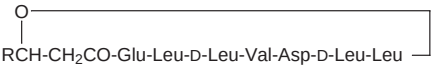
No annual compilation goes by without interest being shown in ionophoric cyclodepsipeptides. Macrocyclisation[169] using BOP-Cl (11% yield) at the Ala



(127) X = Pro, Y = $COCH_2CH(OH)$
(128) X = Ala, Y = $CH(OH)CH_2CH(OH)$
(129) X = Pro, Y = COCH=CH



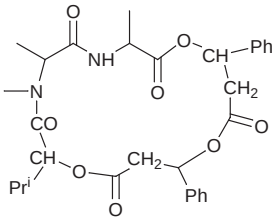
(130)



(131) R = $CH_3(CH_2)_{10}$

residue has completed a route to the synthesis of the ionophore pithomycolide (132) from the fungus *Pithomyces charterum*. Three different conformations of a valinomycin, cyclo[-Val-D-Hyiv-D-Val-L-Lac)$_3$, have been found[170] in anhydrous crystals, hydrated crystals grown in dimethyl sulfoxide and in crystals grown from dioxan. Each of the conformations suggest different mechanisms of ion capture. The former conformation could capture potassium by disruption of the 5 ← 1 H-bond, and the conformation in crystals from DMSO could be pictured as the complexation closing up three petals of a 'flower' arrangement around the desolvating ion. In the third form, water molecules reside in the centre of a bracelet with six 4 ← 1 H-bonds, and the suggestion of a channel being formed is made. Varying the size of the valinomycin affects complexation, contraction in ring size gives loss of ion transport while expansion permits larger ions to complex. Three reports[171] on aspects of the synthesis of valinomycin and analogues have been published. *In situ* deprotection of the Z group, as in Scheme 9, became a favoured approach, and for analogues such as cyclo(-D-Phe-Hyiv-Phe-D-Lac)$_3$, cyclo(-D-Ala-L-Hyhc-L-Val-D-Hyhc)$_3$ and cyclo[(-D-Phe-Hyiv-Phe-D-Lac)$_2$-D-Phe-Hyiv-Tyr-D-Lac]. None of the new substituents introduced affected the ion-binding properties, although the cyclisation yields varied depending upon the sequence.

A convergent Fmoc-solid phase synthesis with side-chain to side-chain cyclisation, a chemoselective cleavage step and segment condensation allows[172] two combinational libraries of actinomycin analogues to be set up. Relationships[173] between pyridine nitrogen basicity and steric crowding in pristinamycins have been modelled by using substituted picolineamides. Increasing the bulkiness at amide nitrogen led to enhanced reduction of the pyridyl ring.



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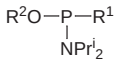
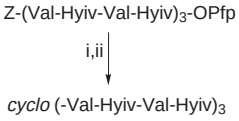


(133) R^1 = Me, R^2 = *p*-MeOBzl
(134) R^1 = C_8H_{17}, R^2 = *p*-MeOBzl



Reagents: i, H_2/Pd; ii, DMAP/dioxan/EtOH 90 °C

Scheme 9

3 Modified and Conjugated Peptides

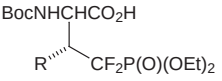
This section contains reports on peptides which have non-peptidic conjugates attached to their side-chains. This year again a great deal of interest has been generated in this sector.

3.1 Phosphopeptides – Syntheses of phosphopeptides continue to be dominated by two main approaches, the 'global' phosphorylation approach where side-chain hydroxy groups are phosphorylated after the peptide chain has been assembled, and the 'building block' approach where suitably protected phosphate-bearing units are built into the chain assembling process. Both methods can be hampered by β-elimination of Ser and Thr derivatives at the deprotection stage but this can be reduced[174] by using bis-(pentafluorophenyl)phosphate triesters. The 'global' approach has been extended[175] to be compatible with oxidation-sensitive amino acids such as Trp, Met and Cys. Instead of using I_2 or m-chloroperbenzoic acid to form the phosphotriesters, conditions such as I_2/lutidine/THF/water seem to eliminate side reactions. A subtle modification to the 'global' protocol is the 'on-line' phosphorylation where an N-terminal Fmoc-Tyr-peptide on the resin is phosphorylated with di-t-butyl-N,N-diethylphosphoramidite/1H-tetrazole, oxidised with m-chloroperoxybenzoic acid, and then peptide synthesis is continued under usual conditions.[176] The $F_{c\gamma}$ receptor peptide EAENITITY(P)SLLKH-PEAL was synthesised in this manner.[176] Similarly the same approach[177] was effective in the synthesis of phosphotyrosine peptide Stat 91 (695-708). The relatively slow deblocking of benzyl phosphate derivatives has been a stimulus[178] to global protection using the more labile p-methoxybenzyl N,N-diisopropyl aminomethylphosphonamidite (133) and its octyl analogue (134).

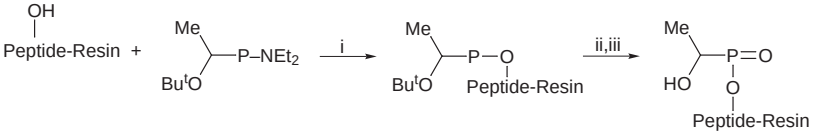
It has been reported[179] that incorporation of protected phosphotyrosine as a building block is better for combinatorial work, and Fmoc-phosphotyrosine devoid of phosphate protection is satisfactory. Fmoc-Tyr(PO_3H_2)-OH for this purpose has been synthesised *via* the TMSI/BSTFA cleavage of Fmoc-Tyr-($PO(OEt)_2$)-OH. When Fmoc-Tyr(PO_3H_2)-OH is used[180] to incorporate consecutive phosphotyrosine residues, a pyrophosphate can form between two adjacent Tyr residues. This is more severe with increased coupling times and for repetitions of coupling. Minimisation[181] of pyrophosphate formation is possible by using Fmoc-Tyr(PO_3H_2)-OH with TBTU/HOBt/N-Me morpholine and 0.4M LiCl in N-Me pyrrolidinone. Alloc-Ser[$PO(OAllyl)_2$]-OH, prepared in a one-pot procedure for Alloc-Ser-OH, has been introduced[182] at the N-terminus of a sequence on solid phase. Global cleavage of the allyl ester groups followed by coupling of a Lys-Ile-Gly fragment gave H-Lys-Ile-Gly-Ser(PO_3H_2)-Thr-Glu-Asn-Leu-Lys-His-OH, an important epitope of tau phosphoprotein in Alzheimer's disease. A segment condensation developed[183] for the synthesis of large domains of human p53 protein allows for introduction of phosphorylated and non-phosphorylated fragments. An N-terminal phosphorylated segment p53 (303-334) was prepared by introducing phosphorylated Ser at 315 as Boc-Ser(PO_3Bzl_2)-OH. Serine 392 in the C-terminal segment was phosphorylated by the global phosphitylation approach. CD spectroscopy revealed that phosphorylation at Ser 315 seemed to increase α-helical content, which was abolished when Ser 392 was phosphorylated. Fmoc-Tyr[$PO(NHR)_2$]-OH where R = n-Pr or i-Pr have been obtained[184] crystalline from their Z-Tyr protected precursors. The P-N bonds are stable towards 20% piperidine/DMF and were completely cleaved by 95% TFA. Prevention of β-elimination in allyl protected phosphate derivatives is possible if amino-groups are protected with phenylacetyl groups which can be removed[185] enzymatically (penicillin G acylase at pH 6.5).

There has been much interest in developing non-hydrolysable phosphotyrosine mimics, such as phosphonomethyl analogues. Insertion of an αα-difluoro group next to the phosphonic acid lowers the pK_a value of the group, and this has been deemed advantageous. Thus new chiral syntheses[186] of αα-difluoroalkyl phosphonate analogues of phosphoserine, phosphoallothreonine and phosphothreonine, giving derivatives such as (135), have been reported. A commercially available chiral auxiliary has facilitated[187] the efficient synthesis of Fmoc-4-(phosphonodifluoromethyl)-L-phenylalanine. H- and Methyl-phosphono peptides can be prepared[188] on a solid phase using phosphoramidite with tetrazole as catalyst. Scheme 10 summarises the stages for the methylphosphonate analogue. The H-phosphonate is obtained using $(Bu^tO)_2P\text{-}NEt_2$ as the reagent. To add[189] to the choice of methylenephosphonate analogues of phosphorylated tyrosine available, mono-hydroxylated (136) and mono-fluorinated (137) methylene phosphonate derivatives have been synthesised. C-Methyl substitution at the methylenephosphonate group, as well as substitution at the α and β positions of tyrosine, have also been successful.[190] When racemates of Fmoc-4-phosphonomethyl-DL-Phe-OEt, with the side chain phosphonate protected with *tert*-butyl or methyl groups, are subject to resolution, only the dimethylphosphono derivative could be resolved[191] by Carlsberg esterase. On the basis of modelling studies,[192] the

analogues (138) and (139) have been added as N-terminal residues on to Glu-Glu-Ile-Glu and Glu-D-Trp-NH_2 and tested for binding affinity to pp50^{src} SH2. Values of IC_{50} in the micromolar range were reported. Replacement[193] of the CH_2SH part of the Cys residue in glutathione with $P{=}O(OR)_2$, where R ranged from methyl, ethyl, diisopropyl to dibutyl esters, has given a new class of glutathione-S-transferase inhibitors giving IC_{50} values of 291, 139, 64 and 21 μM for each ester respectively. As part of ongoing research to control the solubility of peptides, O-phosphorylated serine ester has been linked[194] to poly(t-butylacrylate) or poly(acrylic acid) using the derivative (140) whose protecting group could be removed by H_2/Pd/C. It has been shown[195] that crystals of O-phospho-DL-threonine and its L-enantiomer are zwitterions having the structure $HO_3^-POCHMeCH(NH)O_2H$.

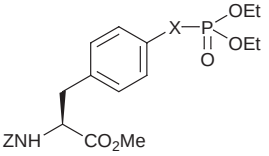


(135) R = H, R = (*R*)-Me, R = (*S*)-Me

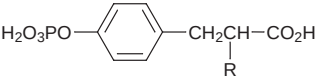


Reagents: i, tetrazole; ii, *m*–chloroperbenzoic acid; iii, TFA

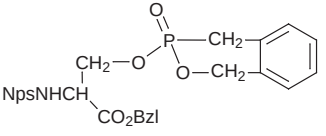
Scheme 10



(136) X = CHOH
(137) X = CHF



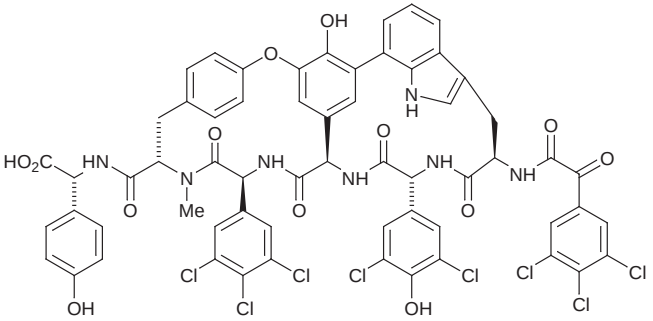
(138) R = Ph
(139) R = NHCOMe



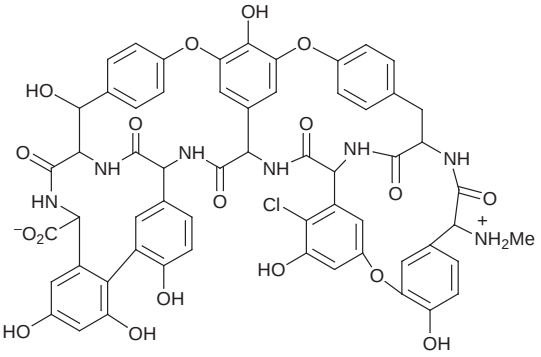
(140)

3.2 Glycopeptide Antibiotics – An excellent overview[196] of this important field, especially the mechanism of action, has been written by a leading authority in this area. The hurdles that remain in the total synthesis of the vancomycins have also been reviewed[197] briefly.

The crystal structure[198] of vancomycin has been reported, which confirms that it exists as an asymmetric dimer which allows the docking of two D-Ala-D-Ala units in opposite directions. It is proposed that the asparagine side-chain may hold the binding pocket in a suitable conformation for peptide docking, swinging away when the peptide enters. The previously reported planar structure of (-)-chloropeptin has now been augmented[199] with a complete stereostructure (141), elucidated by NMR and molecular dynamics techniques. Structural differences between the aglycone (142) of antibiotic A40926 and the recently published X-ray structure of ureido-balhimycin have been revealed[200] by an X-ray analysis of A40926, a member of the ristocetin group. The small changes in the structure of A40926 affect the H-bonding characteristics, and it shows less of a tendency to form tight dimer formation.



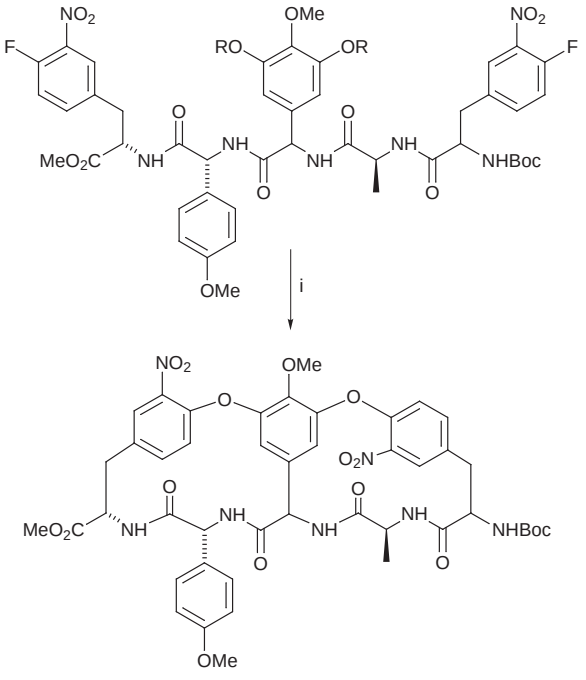
(141)



(142)

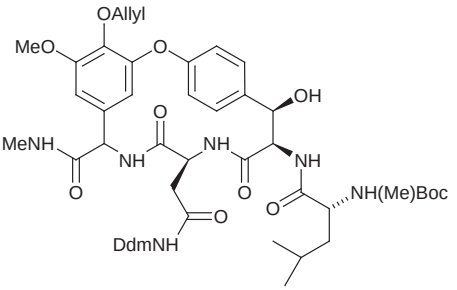
Three main aromatic coupling methods dominate the race to a total synthesis of the vancomycin group. The nucleophilic aromatic substitution strategy, involving displacement of fluorine is well demonstrated[201] by the synthesis of a model bicyclic C-O-D-O-E ring according to Scheme 11. The same strategy has been used[202] to make the macrocyclic ether subunit (143) of orienticin. Results on nucleophilic aromatic substitution in the vancomycin series have spurred on[203] the development of a facile route to cycloisodityrosine derivative (144) from o-nitro-fluorinated phenyl precursors. The D-O-E-G ring system (145) of teicoplanin, which forms the binding pocket for the carboxylate region of D-Ala-D-Ala of the bacterial cell wall, has also been synthesised[204] by K_2CO_3/DMF catalysed displacement of a fluoride to form the diaryl ether link.

The 16-membered cyclic peptido aryl ether subunits have also been constructed using the cyclopentadienylruthenium (RuDCp) moiety to activate chlorarenes towards aromatic substitution. Thus a model for the B-C-F ring of ristocetin A and teicoplanin has been synthesised[205] using chemistry summarised in Scheme 12 to give (146). The same chemistry has also been extended[206] to displace another chlorine, ending up with the model triaryl diether (147). The construction of the diaryl ether linkage in (148) was achieved[207] using arene-

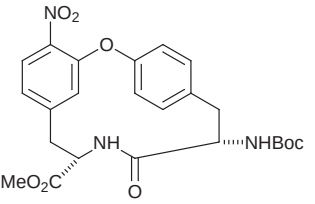


Reagents: i, CsF in DMF,–5 °C

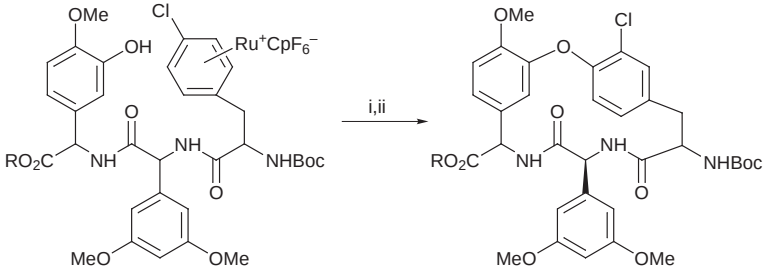
Scheme 11



(143)



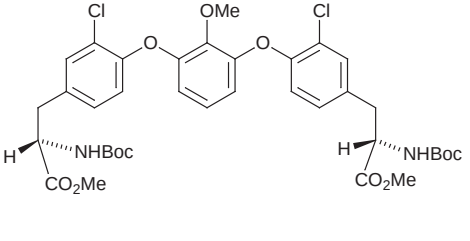
(144)



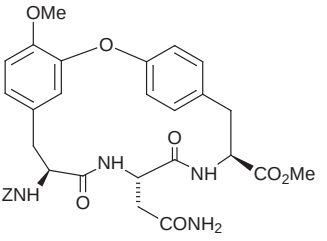
(145) R = Me
(146) R = Et

Reagents: i, Na, 2,6-dibutylphenoxide; ii, sunlamp

Scheme 12



(147)

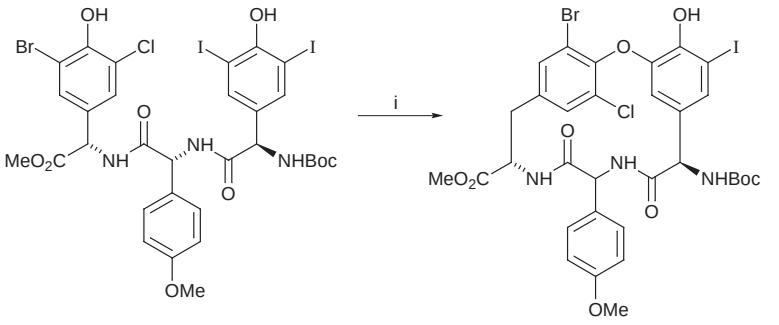


(148)

ruthenium chemistry, while macrocyclisation on to the amino group of asparagine was carried out using DPPA/$NaHCO_3$.

The third method for aromatic ether formation has been applied[208] to mixed halogenated phenols using thallium(III) oxidation followed by selective reduction as summarised in Scheme 13.

Analogue studies on the vancomycin series have often been led by the pressure to develop increased activity against resistant bacteria and to increase the affinity

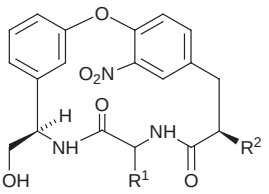


Reagent: i, thallium trinitrate

Scheme 13

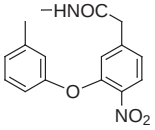
towards the D-Ala-D-Ala. In order to assess[209] the effect dimers of vancomycin would have on molecular recognition and antibacterial properties, head to head linked dimers have been prepared using either $NH(CH_2)_6NH$, $NH(CH_2)_3SS(CH_2)_3NH$, $NH(CH_2)_2SS(CH_2)_2NH$ or $NH(CH_2)_2NH(CH_2)_2$-$H(CH_2)_2NH$. The dimers showed enhanced potency against vancomycin-resistant enterococci, and had a higher affinity for Ac-Lys(Ac)-D-Ala-D-Lac-OH. Designed as molecules to enhance affinities through having an extra primary hydroxyl group, and synthesised *via* the S_NAr approach to ether formation, analogues (149) and (150) represent modified carboxyl binding pockets.[210] Reaction of teicoplanin (151) with $NaBH_4$ produced[211] open pentapeptide derivatives due to reductive hydrolysis of the amide bond between residues 2 and 3, and the carboxyl group of amino acid 2 was reduced to a primary alcohol. Other glycopeptides, such as vancomycin, ristocetin and A-40,926, underwent selective cleavage at the same position. Teicoplanin-derived hydrolysed sequences retained residual antibacterial properties, and seem to be key intermediates for the synthesis of new family members. From the products of reductive hydrolysis useful derivatives of teicoplanin have been produced,[212] where residues 1 and 3 have been completely removed by double Edman degradation. N-Alkylation of the disaccharide amino group of antibiotic A82846 using aldehydes in the presence of $NaBH_3CN$ has produced[213] most potent derivatives against resistant enterococci. Attempts[214] to form cycloisodityrosines by alkylation of di- and triglycine derivatives containing imidazolidinones using $LiNR_2$ reagents have failed. The aglycone part of teicoplanin (151) has been linked[215] to a solid support for solid phase synthesis of combinatorial libraries. The central amino acid of vancomycin, *R*-(4-MeO-3,5-di(OH)phenyl)glycine, has been synthesised[216] *via* a Sharpless asymmetric dihydroxylation of substituted styrene.

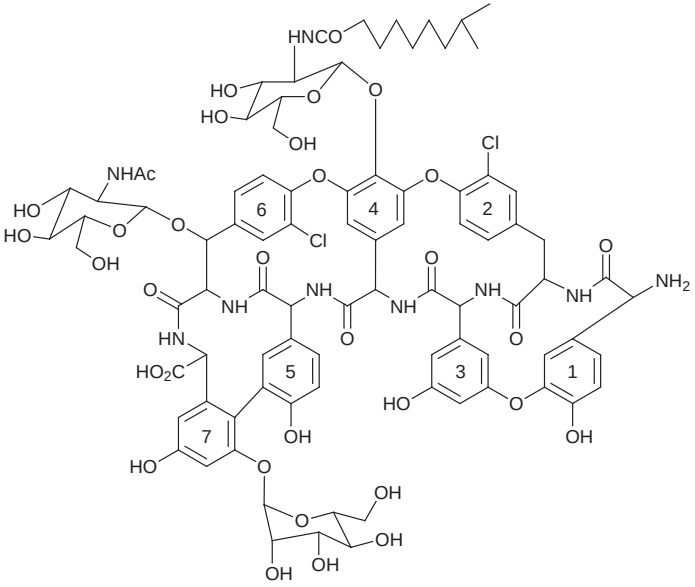
The hydrogen-bonded model for vancomycin group interaction with bacterial cell wall analogues (a monomeric version is depicted in Figure 2) has been accepted as a template from which further modifications of the antibiotics in order to enhance binding can be made. The binding properties of two synthetic



(149) R^1 = Me, R^2 = $NHCOCH(CH_2CHMe_2)\overset{+}{N}H_2Me$

(150) R^1 —— R^2 =





(151)

analogues of vancomycin have been discussed,[217] using the features of Figure 2 as a model. The exchange of C-terminal Ala to a lactic acid caused no change in the donor-acceptor relationships of H-bonding towards the model vancomycins. Molecular modelling, however, indicated that in the D-Ala-L-Lac case the depside link might be flexible enough to reduce interaction at the acetyl end of the model. The vancomycins bind as dimers, where the dimerisation constants vary in the range 10 to 10^6 dm^3 mol^{-1}, but ristocetin A and the related eremomycin form asymmetric dimers. Using ^{13}C-labelled N-Ac-D-Ala-D-Ala as a probe molecule for ^{13}C NMR studies[218] it has been shown that the two distinct binding

sites of the ristocetin A asymmetric dimer have different affinities whereas those of eremomycin show no detectable difference. Using UV spectroscopy and NMR NOESY experiments it has been demonstrated[219] that chloroeremomycin binds di-N-Ac-Lys-D-Ala-D-Ala (X = NH in Figure 2), albeit with a reduced binding constant. A good correlation has been observed[220] between overall ligand binding energy (ΔG°) and amide NH chemical shifts in the binding pockets of glycopeptide antibiotics. In this way binding affinities for eremomycin and chloroeremomycin by N-Ac-Lys-D-Ala-D-Lac have been worked out.

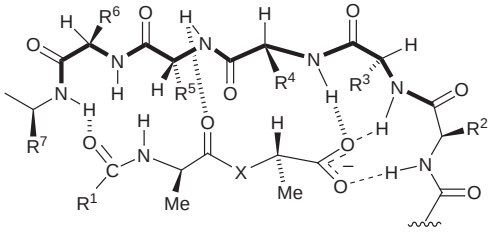
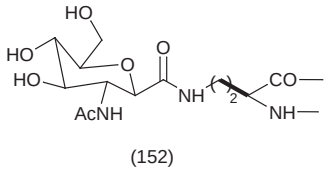


Figure 2

3.3 Glycopeptides – There was still sufficient activity in this area to once again warrant sub-division of this section to O-glycopeptides and N-glycopeptides. But certain reports are relevant to both sectors and will be considered first. Although not readable to the Reporter, Chinese readers can benefit from a 98-reference review[221] on recent developments in the synthesis of glycopeptides. The effect of glycosylation on the conformation of peptides has been debated for years. NMR work[222] on the effects of glycosylation on the C-terminal pentapeptide interactions are transient in nature and do not greatly influence the secondary structure of the peptide. In contrast to this observation, for C-glycoside analogues[223] of cyclo(D-Pro-Phe-Ala-CGaa-Phe-Phe) where CGaa is (152), NMR and molecular dynamics techniques proved that the CGaa side-chains interact with the BI/βII′ conformations of the cyclic peptide. The unit (152), as its tribenzyl ether derivative, was compatible with linear precursor assembly on 2-chlorotrityl resin using TBTU/HOBt for coupling, followed by cyclisation with DPPA. Amongst more than 3000 LHRH analogues already prepared can now be included[224] the S-glycopeptide (154) derivative of the agonist buserelin (153) and the C-glycoside derivative (155). These derivatives were synthesised by segment condensation in solution. Analogue (154) exhibited similar bioactivity to buserelin while (155) was only 25% as active. A general strategy[225] for linking unprotected peptides to aldehydic lipids or carbohydrates by formation of oxime bonds in 60-75% yield has been reported.

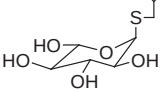
O-Glycopeptides – Synthesis was by far the most popular activity in this subsection with three approaches being prevalent: the use of protected glycosylated

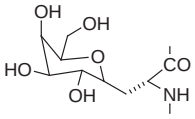


(152)

p-Glu-His-Trp-Ser-Tyr-X-Leu-Arg-ProNHEt

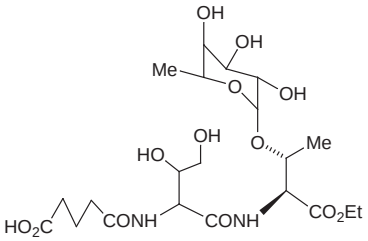
(153) X = D-Ser(Bu^t) —NH CO—

(154) X = 

(155) X = 

amino acids as building blocks; the glycosylation of residues already formed into peptides; a combination chemoenzymatic techniques.

Building blocks for solid phase glycopeptide synthesis can be built up[226] using diphenyl ketone Schiff bases as amino protecting groups for Ser and Thr. A key step[227] in making several fucopeptides such as (156) as sialyl Lewis^x mimetics was the conversion of L-fucose into tribenzylfucosyl phosphite which was then coupled to Boc-L-Thr-OEt with trifluoromethane sulfonic acid. Mimetic (156) had the same anti-inflammatory activity as sialyl Lewis^x in the E-selection binding assay. Analogue (158) has been synthesised[228] as an analogue of the immunodominant T cell epitope (157) on type Ii collagen. An Fmoc-5-hydroxyvaline disaccharide derivative with silyl protecting groups proved important to the synthesis. The protected disaccharide unit (159) proved to be a significant building block[229] for the synthesis of Thomsen-Friedenrich (TF) antigen and α-sialyl(2-6)TF. Solid phase synthesis and conformational studies of helper T cell immunogenic peptides have been elaborated[230] using polyacetylated building block (160) for the glycosylated unit. NMR studies showed random conformations which were not influenced by the glycosylation at different positions. Fmoc-Ser/Thr(Ac_3GalNAcα)-OH have been used[231] for the solid phase synthesis of glycopeptides related to HIVgp120 and mucins, and in these syntheses piperidine deprotection was much better than morpholine, with no β-elimination experienced. A single step synthesis[232] utilising an efficient HPLC purification stage has proved successful in the formation of O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl/lucopyranosyl) N-α-Fmoc-hydroxyproline, which is used for the preparation of antinociceptive neoglycopeptides. Fmoc-Ser(α-D-GalN_3)-OH and its Thr analogue were incorporated[233] as building blocks into short peptide sequences derived from salivary mucins followed by transformation of the azide group into the acetamido group on the resins. The two glycopeptide sequences produced were APPETT(α-D-GalNAc)AAP-OMe and PAPPSS(α-D-GalNAc)SAP-OMe. A simple polymerisation[234] of the repeating unit (Ala-Ala-Thr*) where each Thr* is attached to (Galβ→3GalNAcα) has yielded an analogue (n = 10-12) antifreeze glycoprotein which showed antifreeze properties *in vitro*. Fmoc-Thrβ-D-Ac_4Gal(1→3)α-D-Ac_2GalNAc-1)-OH activated with TBTU was incorporated[235] satisfactorily into a solid phase protocol for the synthesis of drosocin. Both the synthetic product and the natural product from *Drosophila* had the same antibacterial activity. Benzylated glycosyl derivatives of Fmoc-Ser-OH were



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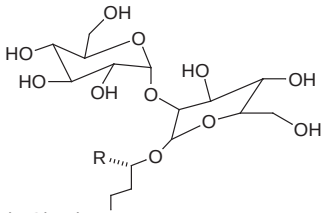
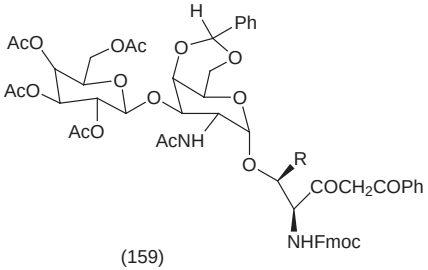
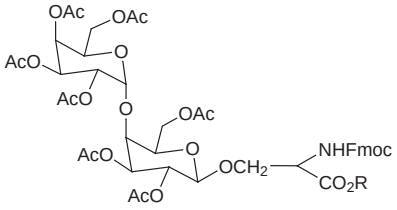


Gly^{256}-Glu-Hyp-Gly-Ile-Ala-Gly-Phe-**NH**-CO-Gly-Glu-Gln-Gln-Gly-Pro-AA^{270}

(157) R = CH_2NH_2 and AA^{270} = α-D-Glcp(1→2) β-D-Galp-Hyl
(158) R = H, AA^{270} = Lys



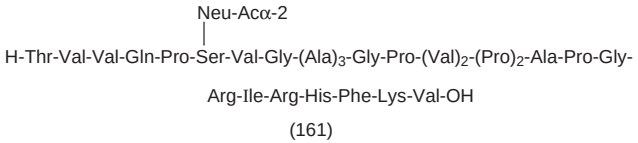
(159)



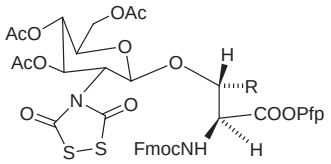
(160)

successfully utilised[236] in an automated peptide synthesiser to form (161) (asialo-[Ala^{18}]-B-chain of human α2HS glycoprotein) after removal of benzyl groups by hydrogenation. The N-dithiasuccinoyl group in (162) is a successful precursor of O-β-D-GlcpNAc because it can be quantitatively converted into an N-Ac derivative using thiolysis as demonstrated[237] in the synthesis of H-Ser-Ala-Val-Ser(Ac_3β-D-OGlcpNAc)-Ser-Ala-NH_2, a fragment from serum response factor.

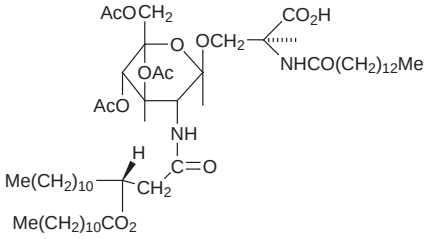
Trisaccharide imidates [$(RCNHCCl_3)$] in the presence of TMSOTf or BF_3-OEt_2 can be glycosylated[238] with tri- and tetra-saccharide donors before coupling to serine derivatives. In the same way[239] trisaccharide, 2,3,4,6-Ac_4 β-D-Glp(1→6)-2,3,4-Ac_3α-D-Man-(1→6)-2,3,4-Ac_3α-D-Man trichloroimidate will couple to Fmoc-Ser-Pro-OBzl or its Z-analogue using BF_3-OEt_2. A mimic (163) of the glycopeptide sequence Ala-Ser(β-D-Xyl)-Gly-Ala found in human bone and cartilage linker region has been synthesised[240] using a convergent approach.



(161)



(162) R = H, Me

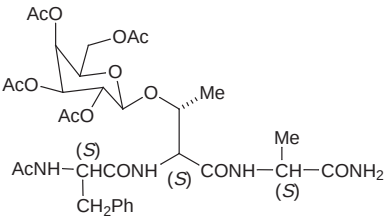


(163)

The general synthetic strategy[241] for synthesis of lipid A (163) was to link the protected amino acid onto a protected sugar unit, then derivatise it with a fatty acid derivative. Hexa- and nona-glycosyl hexapeptides with phytoalexin properties have been prepared[242] by convergent block synthesis. N-Terminal fragments already linked to glycosides were protected with Fmoc, the C-terminal units as methyl esters, and coupling was carried out using EEDQ.

Model tripeptide (164) has been used[243] in a systematic investigation of the degree of epimerisation and β-elimination that occurs during the deacylation of O-acylated glycopeptides. There was no evidence (< 1%) of epimerisation or β-elimination under various conditions used to deacylate, but some (< 5%) did accompany the more severe conditions required to remove benzoyl groups. High energy collision induced dissociation[244] of O-linked glycopeptides produces sufficient information to identify the peptide sequence and to determine the glycosylated sites. 2D-NMR studies have shown[245] that glycosylated serine attached to the imino group of proline does not appear to stabilise any *cis*-conformation at the proline link.

Side-chain glycosidic residues can be enlarged[246] under the catalysis of yeast β-galactosidase. Thus, Galβ1→4GlcNAcβ-O(N-Z)-Ser-OEt was obtained from GlcNAc-β-O-(N-Z)-SerOEt as acceptor, and lactose. β-D-Galactosidase was also the catalyst for the synthesis[247] of Gal(β1→3)Gal(β1→4)Xyl(β)-L-Ser, prepared by stepwise enzymic transglycosidation starting with xylopyranosyl p-nitrophenyl



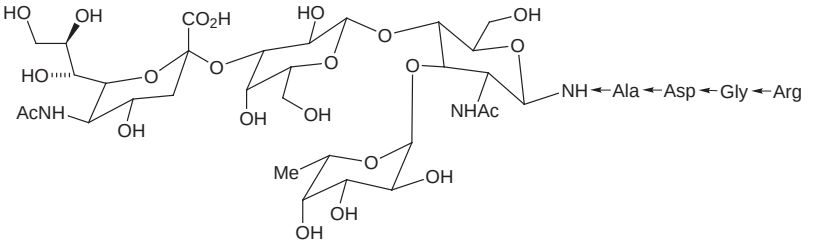
(164)

derivative. The link on to serine was *via* the coupling of glycosyl trichloroacetamidate on to Z-Ser-OBzl in the presence of trimethylsilyl triflate. In a slightly different enzymic synthesis, glycosylated peptide fragments have been assembled[248] by using the mild conditions of removing p-phenylacetoxybenzyloxycarbonyl urethane N-protecting groups with penicillin G acylase. The advantages of the methodology have been exemplified by the preparation of the sensitive peptide conjugate H-Ser(PO_3H_2)-Pro-Thr-Ser(GlcNHAc)-Pro-SerGlcNHAc)-OH a repeat sequence in mammalian RNA polymerase II.

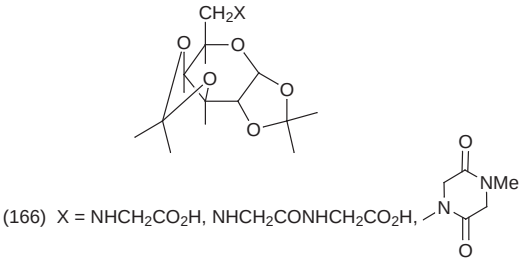
N-Glycopeptides – A set of accessible transferases has also aided[249] the activation of donor sugars to allow rapid assembly of oligovalent sialyl Lewis^x conjugates. The core glycopeptide was assembled using EDCI or DCC/HOBt and the side chains elaborated to the tetrasaccharide stage by the enzymes. Novel sialyl Lewis^x conjugates such as (165) have been synthesised[250] as inhibitors of E- and P-selectin mediated cell adhesion. X-Ray crystallography and NMR studies[251] have been used on model compounds (166) to study H-bonding patterns after N-glycation, while a complete signal assignment[252] in ^1H and ^{13}C spectra of a 21-amino acid glycopeptide from human serum transferrin has enabled a complete characterisation to be made. NMR studies[253] have also shown the presence of long range ^1H-^1H NOEs between sugar and peptide residues in Ac-Asn-(β1-NGlcNAc)-Leu-Thr-NH_2 and Ac-Glu(β1-NGlcNAc)-Leu-Thr-NH_2. In organic solvents N-glycosylation stabilises a main-chain to side-chain turn, where the Thr^3 NH hydrogen bonds to Asn^1γ-carboxamide, and the C-terminal amide is within H-bonding distance to the GlcNAc endocyclic oxygen. Non-natural bivalent N-linked glycopeptides[254] of general structure (167) as probes for selectin recognition have been synthesised *via* the coupling of aspartyl residue side chains to glycosiding amines using HBTU/HOBt.

Peptidoglycan monomers for the generation of combinatorial libraries have been synthesised equally well on polyethylene glycol and polystyrene resins, but the efficiency of coupling was in the order HATU > HBTU > PyBOP > EEDQ.[255] A typical unit synthesised was (168).

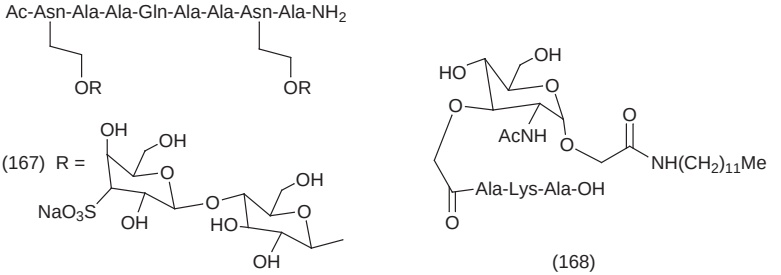
3.4 Lipopeptides – Iturin A2 (169), the antifungal metabolite from *Bacillus subtilis*, with its unique β-amino acid iturinic acid, has been a demanding unit to synthesise enantiomerically pure. It has now been accomplished[256] by synthesis of iturinic acid by lithium diorganocuprate addition to the tosylate derivative of the



(165)

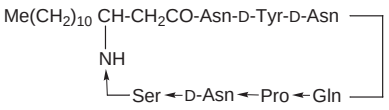


(166) X = $NHCH_2CO_2H$, $NHCH_2CONHCH_2CO_2H$,



(168)

α-carboxyl-reduced analogue of aspartic acid. The linear precursor to (169) for cyclisation at the Ser carboxyl group with DPPA (73% yield) was synthesised on solid phase. Pheromone analogues[257] with varied amounts of lipoconjugation at the C-terminal of two allelic forms have been synthesised to study antagonists for *Ustilago mayadis*. C-Termini modifications of alleles GRDNGSPIGYSS-X and NR-GQPGYY-X included X represented as Cys(farnesyl)OMe, Cys(farnesyl)-OH, Cys(n-dodecyl)-OMe, and the unnatural aminodecanoic acid and N-hexadecylglycine. There was an increase in biological activity with increasing length of lipophilic anchor, but no evidence is seen of additional structure-inducing or receptor-binding effects. With the aim of making more bio available peptides, novel N-farnesyl(Frn) amino acids have been synthesised[258] by alkylation of the amino acid esters. Analogues included *e.g.* Cys-(N-Frn)Val-OBz, Phe(N-Frn)Val-OBz, Phe(N-Frn)MetOMe and a model, Cys-Val-Phe-(N-Frn)MetOMe, as potential inhibitor of human p21ras-farnesyl transferase. For enhanced solubility in enzyme-catalysed construction of lipopeptides it has been shown[259]



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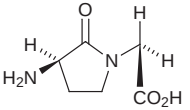

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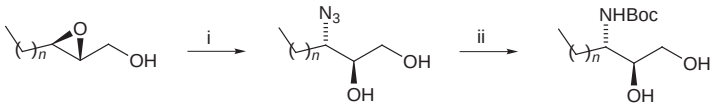
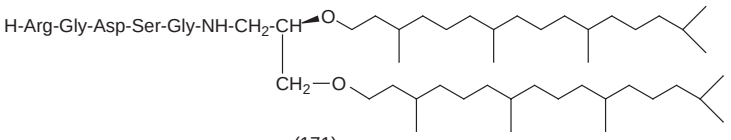


Reagents: NaN_3 ii, $H_2/Pd/C$

Scheme 14

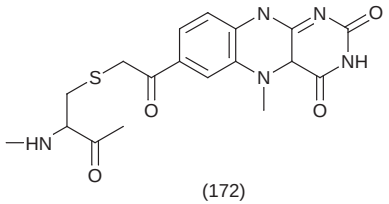


(171)

that choline esters of the peptides have advantages. The centre of the pheromone YIIKGVFWDPACS-farnesyl)-OCH_3 has been conformationally restricted[260] by inserting (170) in place of either Lys^4-Gly^5 or Gly^5-Val^6. Only by replacing Lys^4-Gly^5 did a super-active (32-fold higher) agonist materialise, suggesting a bioactive reverse turn was a requirement.

A general approach[261] to enantiomeric synthesis of lipidic amino acids and peptides has been reported and summarised in Scheme 14. A number of amides and esters of α-amino-acids with linear side-chains have been tested[262] against secreted humal platelet phospholipase A_2, with the amides of amino acids with long-chain amines giving the highest *in vitro* activity. As part of a study[263] in using thermostable lipids as drug carriers, the cell adhesion motif Arg-Gly-Asp-Ser has been attached to 2,3-diphytanoxypropaneamine, via a thioester activated by Ag^+ to give (171).

3.5 Miscellaneous Conjugates – In the synthesis[264] of phytosulfokine-α and -β having structures, H-Tyr(SO_3H)-Ile-Tyr(OSO_3H)-Thr-Gln-OH and H-Tyr-(SO_3H)-Ile-Tyr(OSO_3H)-Thr-OH respectively, the sulfate group was added using p-nitrophenyl sulfate in the presence of aryl sulfotransferase, after assembly of the peptides on solid phase. Flavin unit (172) has been introduced[265] on the side chains of Cys at the 6th, 7th and 8th position of each α-helical 14-peptide, in order to undertake a CD and cyclic voltammetry study. The flavin redox probe provided significant information about the assembly and function of the α-helix peptides on a gold electrode surface. Two peptide oligonucleotide conjugates, (173) and (174), comprising the active site mimic of ribonuclease A and the



(172)

′5d[CACCGACGGCGC]$^{3'}$-O-$(CH_2)_a$-CO-[X-Gly-His]-OH

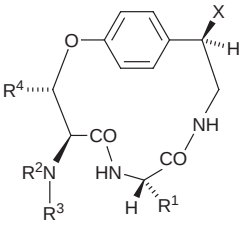
(173) X = $^{\varepsilon}$Lys
(174) X = His

Cu(II) complexing metallopeptide respectively have been synthesised[266] on solid phase using pentafluorophenyl esters. The linker DMT-O$(CH_2)_9CO_2$Pfp was then introduced to create a link with the oligonucleotide sequence.

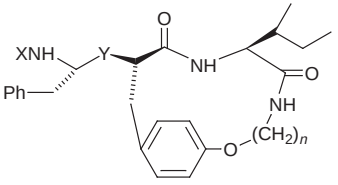
4 Miscellaneous Structures

Not every molecule sits comfortably in the above sub-divisions of this Chapter, so in this section we draw together a miscellany of structures no less important than ones reviewed above.

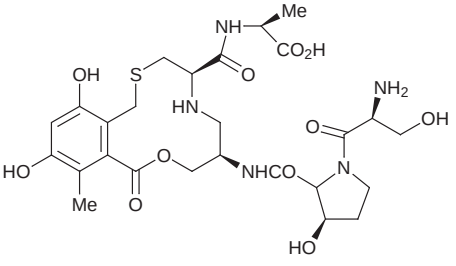
A new cyclopeptide alkaloid, sanjoinine-G1, has been isolated[267] from the seeds of *Zizyphus vulgaris* var. *spinosus* and given the structure and absolute configuration in (175). As an alternative to macrolactamisation, aryl alkyl ether bond formation using nucleophilic displacement of a fluoride has been a key strategy[268] in the synthesis of analogues (176) related to the pandamine group of alkaloids. Cyclopeptidemimetics[269] (177) based on imitating the Phe-Ile-Val tripeptide C-terminus of an inhibitor of HIV-1 protease have been prepared, and molecular modelling and X-ray studies reveal that they have a unique enzyme binding mode. A total synthesis[270] of cyclothialidine (178), a DNA gyrase inhibitor from *Streptomyces filipinensis* has been described. The final macrolactamisation was made using the Mitsunobu conditions utilising the aromatic carboxyl group and the side-chain of Ser. Side-chains were then added using a Boc strategy. A single step synthesis[271] utilising the condensation of 1,3-adamantanediarbonyl chloride with L-Cys di-methyl ester was all that was needed to make a series of cyclo(Adm-Cyst)$_n$ structures n = 2,3,4 or 5. When n = 2-4 the molecules transported Na^+ and K^+ ions across model membranes; when n = 5 negligible transport properties were seen. Transannular H-bonding has been shown[272] by NMR CD and X-ray diffraction to stabilise a left-handed double helix conformation in the tryptophan-derived molecule (179, $R^1 = CO_2Me$, $R^2 = H$) whereas the analogous tryptamine-derived macrocycle (179, $R^1 = H$, $R^2 = Si(CHMe_2)_3$) shows less tendency to form a helix in the crystal.



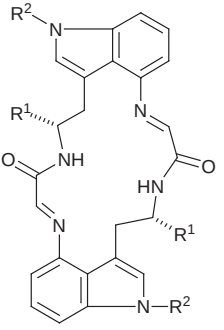
(175) X = OH, $R^1 = CH_2CHMe_2$, R^2 = H, $R^3 = COCH(NMe_2)CH_2Ph$, $R^4 = CHMe_2$, R^5 = H
(176) X = H, $R^1 = CH_2Ph$, R^2, R^3, benzyl, allyl, R^4 = H, $R^5 = NO_2$, NH_2, H



(177) n = 3, 4, X *e.g.* = Ac-Leu-Val
Y = $CH(OH)CH_2N$ or CH_2NH



(178)



(179)

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5
Metal Complexes of Amino Acids and Peptides

BY E. FARKAS AND I. SÓVÁGÓ

1 Introduction

This chapter deals with the synthesis, structures, reactivity and solution equilibria of metal ion- amino acid and metal ion-peptide complexes, including the most important synthetic derivatives of the naturally occurring ligands. The review covers the papers published in 1995 and 1996. A new international journal: 'Journal of Biological Inorganic Chemistry' sponsored by the Society of Biological Inorganic Chemistry started in early 1996.[1] The journal is primarily concerned with the advances in the understanding of the systems involving one or more metal ion set in a biological matrix, but synthetic analogues mimicking function and structure are also of interest to the Journal.

Four Volumes of the 'Handbook of Metal Ligand Interactions in Biological Fluids'[2] appeared in 1995 and several chapters of Volume 1 cover the topics of this review.[3–9] These topics involve an overview on the most important results obtained for amino acid complexes[3] with special emphasis on stereoselectivity[4] and on sulfur-containing amino acids.[5] Three chapters deal with the complexes of peptides[6] with special emphasis on the peptide hormones[7] and sulfhydryl-containing peptides.[8] Metal complexes of aminophosphonates which are amongst the most important derivatives of these ligands are described.[9] In the continuation of the series on the critical survey on the stability constants of the metal complexes, reviews concerning the equilibrium data of metal complexes of amino acids with polar side chains[10] and those with positively charged side chains[11] were published.

The interest in metal complexes of amino acids arose a long time ago. During the early decades, $3d^5$-$3d^{10}$ metal ions, especially copper(II) ion were the focus of the investigations. Copper(II)-amino acid/amino acid derived systems have had special interest because of several reasons; e.g. as models (i) to collect more information about the structure and function of copper proteins and (ii) to study the distortion of the copper(II) coordination polyhedron. Since the interest arose quite a long time ago the main features of simple copper(II)-amino acid systems have already been clarified. For this reason binary systems are rarely studied nowadays, unless different derivatives of amino acids are the ligands. On the other hand, there is now a trend to perform molecular mechanics calculations for modelling the thermodynamic and structural properties of these complexes, but

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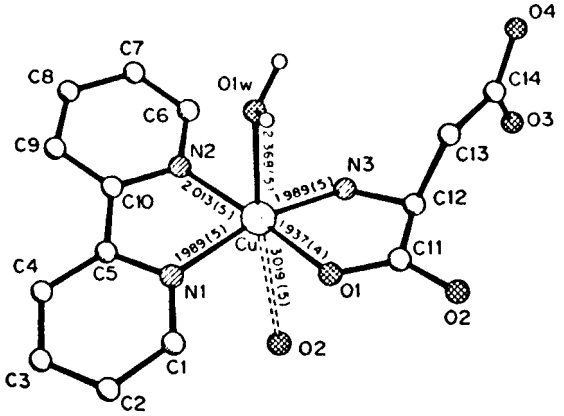
most of the studied copper(II)-containing systems are ternary ones. Different therapeutic uses have accelerated studies on amino acid complexes with several other metal ions (e.g. platinum, palladium, tin, rhenium, ruthenium and several lanthanides) in recent years. In these cases, there are several unsolved problems also for binary systems and numerous papers have been published on these subjects.

Investigations of the metal complexes of peptides have received increased attention. Among them the peptides containing strongly coordinating side chain donor groups and the oligopeptide segments of various peptide hormones and metalloproteins are the most interesting. Concerning the metal ions a huge number of studies have focused on complexation with copper(II), but several other transition elements, including the various oxidation states of vanadium and platinum, are also widely studied. On the other hand, more and more examples of metal ion- promoted deprotonation and coordination of amide nitrogen have been published. The studies on metal complexes of peptide molecules mainly describe the speciation and structures of the peptide complexes in solution, but the amount of X-Ray crystallographic data for the solid state is also increasing.

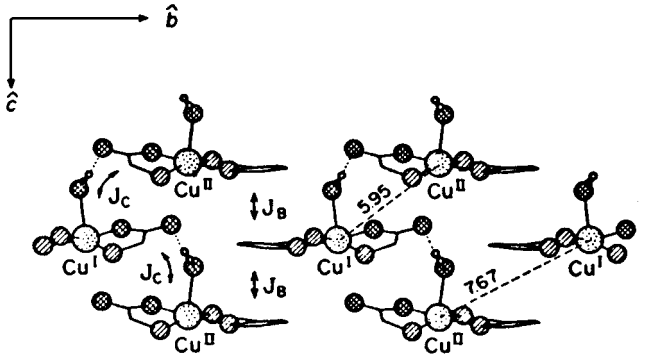
2 Amino Acid Complexes

2.1 Synthesis and Structural Studies – Metal ion-amino acid-2,2'-bipyridine (bpy)/or 1,10-phenanthroline (phen) ternary complexes have traditionally been favoured as simple models to understand enzyme-metal ion-substrate complexes. The Cu(II)-Asp-bpy-trihydrate complex was found to have orthorhombic geometry.[12] The arrangement of Asp and bpy around the metal ion is five-coordinated distorted square pyramidal. The two nitrogens of bpy, amino-N and carboxylate-O of Asp are in the equatorial plane **(1)** and an oxygen from a water



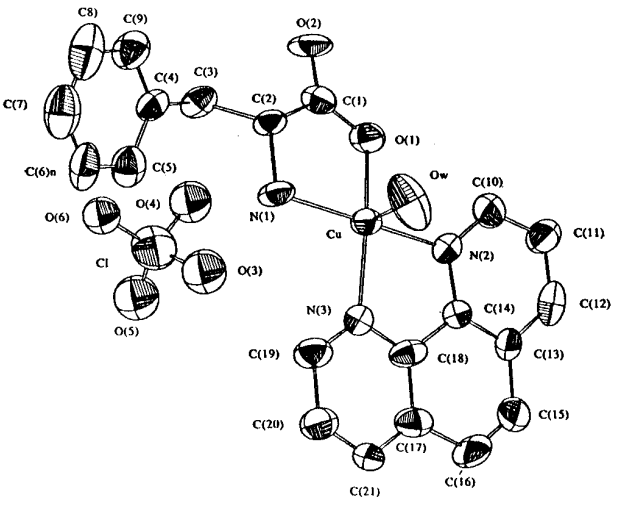
(1)

molecule occupies an apical position (0.237 nm). The sixth position is occupied by a side-chain (β) carboxylate-O from a neighbouring Asp molecule but this is not considered to be a structural bond (the length of Cu-O in this case is over 0.3 nm). The crystal lattice **(2)** shows that the compound exhibits a layered structure for the copper ions. Within a layer the ions are connected by a chemical path involving a carboxylate bridge, a hydrogen bond and also stacking of aromatic rings of bpy ligands. This layered structure provides the possibility of magnetic interactions between copper(II) ions.



(2)

An X-Ray crystal structure was determined for the copper(II)-phen-L-Phe ternary complex, $[Cu(H_2O)(phen)(L\text{-}Phe)]\cdot ClO_4$ **(3)**.[13] In this complex the metal



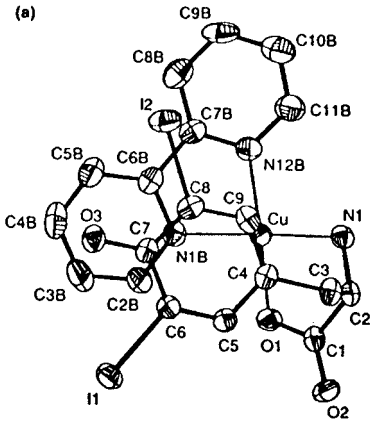
(3)

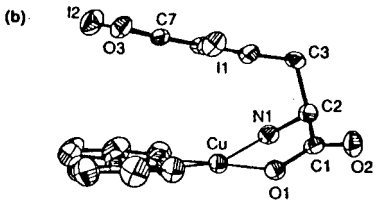
ion sits in a square pyramidal arrangement. The two phen nitrogens and the carboxylate oxygen as well as the amino nitrogen of the Phe occupy the basal plane while the apical position is occupied by a water molecule. Formation constants for the cobalt(II)-, nickel(II)-, copper(II)-bpy, phen or imidazole(Im) and L-Phe ternary complexes were also determined in the same work.

Thyroid hormones are iodinated derivatives of the amino acid Tyr, namely 3,5,3'-triiodo-L-thyronine and L-thyroxine. It is not yet clear whether metal ions are involved in the receptor binding and functioning of thyroid hormones but iodo groups are essential for the hormone-activity. Owing to the bulkiness and polarizability of iodo groups, their important role in the hormone-receptor binding through noncovalent interactions was assumed. The copper(II)-3,5-diiodo-L-tyrosine-bpy ternary system was studied to gain some more information about weak interactions between diiodo side chain and aromatic rings of coordinated ligands. Two types of complexes formed in this system; $[Cu(bpy)(I_2TyrO^-)]\cdot 2H_2O$ **(4a)** and $[Cu(bpy)(I_2TyrOH)(NO_3)]CH_3OH$ **(5a)** were prepared.[14] Both complexes have similar X-Ray structure with distorted five-coordinate, square pyramidal geometry. The two nitrogens of bpy and nitrogen and oxygen atoms of I_2Tyr are situated in the equatorial positions while the axial position is occupied by a water molecule **(4a)** or a nitrate ion **(5a)**. The copper(II) ions lie above the basal planes by 0.016 nm and 0.023 nm, respectively. Side views (**4b**) and **(5b)** clearly show the stacking interaction between bpy and the side chain aromatic ring of I_2Tyr. In addition a weak interaction between the iodine substituent and one of pyridine rings was found. It was also found that the deprotonation of the phenolic-OH moiety had only a small effect on stacking. This was explained by the hindered hydration of the phenolate oxygen which is sandwiched by the two bulky iodines and weakly coordinated to a pyridine ring. Based on these X-Ray results and also on the solution equilibrium studies it was concluded that iodo groups of thyroid hormones may promote the hormone-receptor binding by the interaction between the hydrophobic side groups and aromatic rings of the receptor site.

Copper(II) complexes of reduced Schiff base ligands **(6)** of salicylaldehyde with glycine (R= -H), alanine (R= $-CH_3$), leucine (R= $-CH_2CH(CH_3)_2$), isoleucine (R= $-CH(CH_3)-CH_2CH_3$), phenylalanine (R= $-CH_2C_6H_5$) and glycine methyl ester have been studied and X-Ray structures of the ternary adducts formed between the copper(II)-alanine derivative and phen **(7)** or Im (**8**) have been determined.[15] In compound **(7)**, the basal positions of the square pyramid are occupied by the three donor atoms of N-(2-hydroxybenzyl)-D,L-alanine **(6)** and by one of the phen-N atoms. The other phen-N binds at the axial position. Compound **(8)** has square planar geometry with Im-N occupying the fourth planar position. The average in-plane bonding distance in **(8)** is significantly shorter than that in **(7)**. The above complexes, for which molecular mechanics calculations were also made, are thought to serve as models for the intermediate species in the biological racemization and transamination reactions of amino acids.

The phenolate oxygen of tyrosine residues in transferrins has a special biological role. To know more about the metal binding capability of this donor the copper(II) complexes formed with 2-hydroxyhippuric acid(2OHhip) in binary





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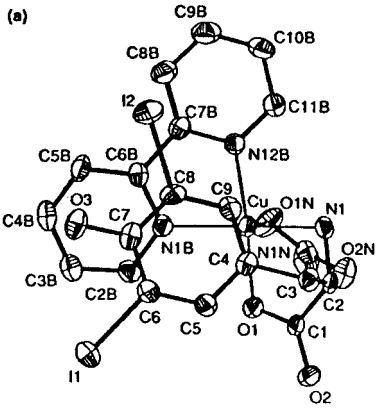

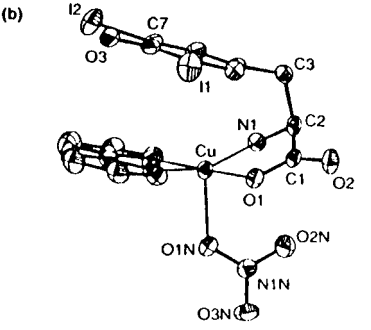

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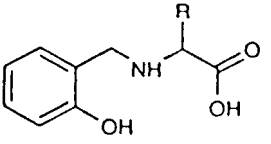
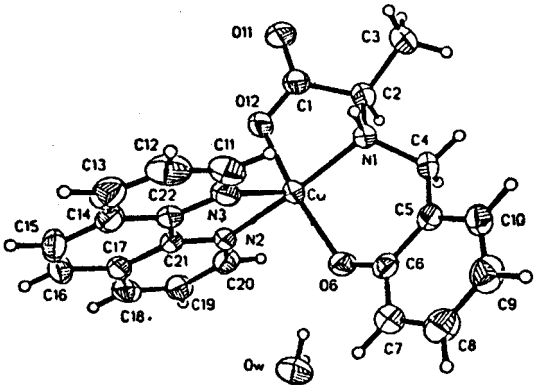


(6)

and ternary systems have been studied,[16] and the X-Ray structure of [Cu(bpy)(2-OHhip)]$_2$·8H_2O has been determined **(9)**. In this dimeric species each copper(II) has a distorted square pyramidal geometry with the two nitrogens of bpy and the carbonyl and phenolate oxygens of 2OHhip occupying the four equatorial positions. The fifth position is occupied by the centrosymmetric phenolate oxygen, which is an equatorial ligand for the second copper(II). The carboxylato group is uncoordinated. Solution equilibrium studies were also reported in the same study.[16]



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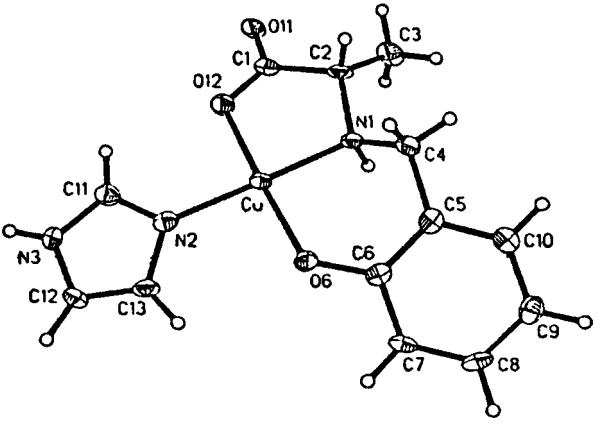
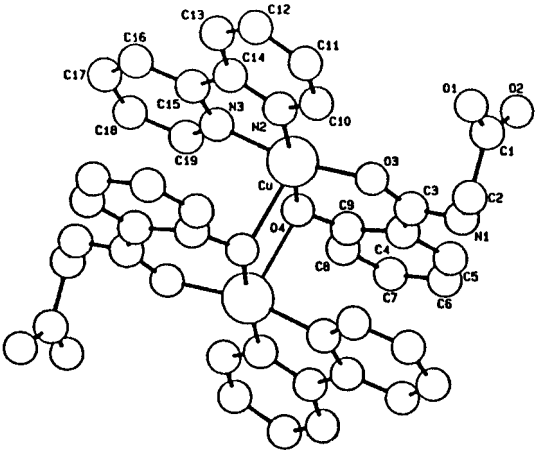


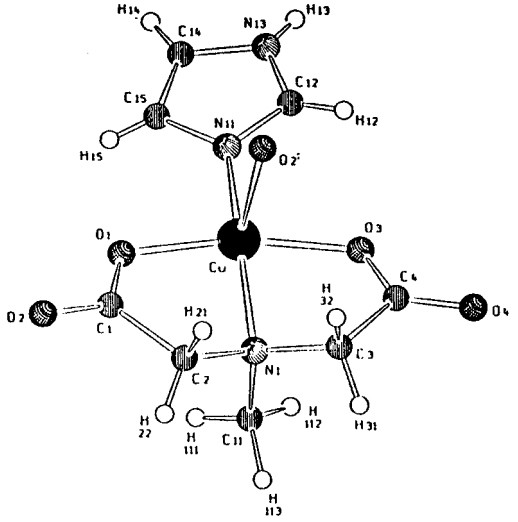
(8)

As models for the coordinating role of the imidazole ring of histidine in copper proteins and enzymes, the copper(II)-N-methyl-iminodiacetato (MIDA)-Im and copper(II)-iminodiacetato (IDA)-N-methyl-imidazole (1MIm) complexes [Cu-(MIDA)(Im)] **(10)** and [Cu(IDA)(1MIm)$(H_2O)_2]\cdot H_2O$ **(11)** were prepared and characterized.[17] The MIDA ligand was found to coordinate as a tridentate ligand via the atoms N(1), O(1) and O(3). These donors and the N(11) atom of Im define a distorted square base coordination plane **(10)**, out of which the copper(II) is displaced by 0.011 nm toward the fifth donor atom, an oxygen of the adjacent MIDA. Compound **(10)** consists of polymeric zig-zag chains **(12)**. In the case of [Cu(IDA)(1MIm)$(H_2O)_2]\cdot H_2O$ **(11)** the N(11) of MIm and N(1),O(1) and O(3) atoms of an IDA were found define a square coordination. Two oxygens (O(10) and O(20)) of weakly coordinated water molecules complete the unsymmetrical



(9)

elongated octahedral polyhedron around the copper(II) atom. In crystals of this compound the stability is reinforced by an extended hydrogen bond system. Comparison of (**10**) and **(11)** showed that N-methylation of IDA gave an anhydrous compound in which the N-H of Im is involved in inter-chain hydrogen bonds. However, N-methylation of Im (1MIm) resulted in a hydrated compound with a remarkable hydrogen bonding network.



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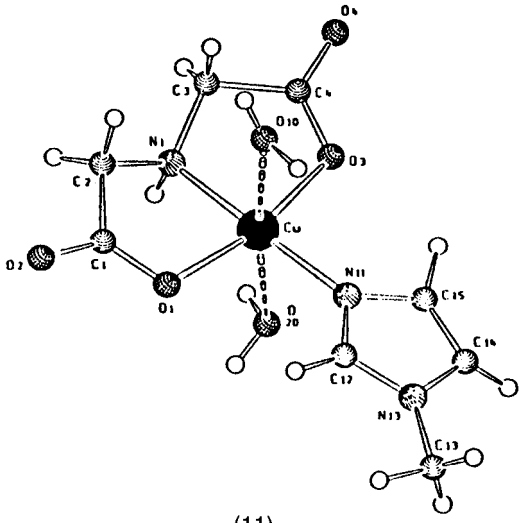

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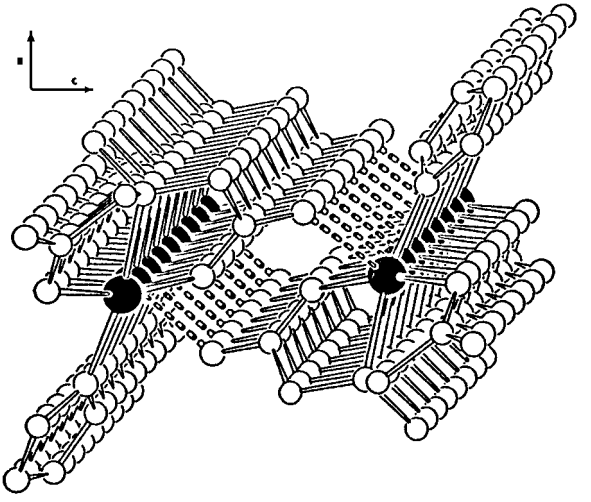
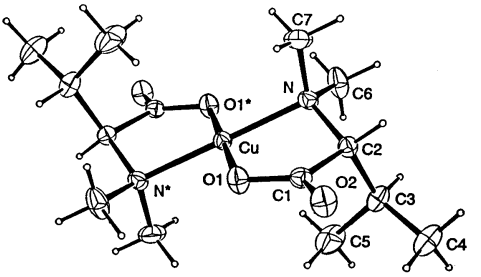


(12)

Alkylated and dialkylated α-amino acids, due to the extensive steric strain caused by the bulky groups, are thought to be good models to study the copper(II) coordination polyhedron. Generally three kinds of crystal modifications are formed between copper(II) and N-alkylated amino acids: (i) square-planar coordination around the metal (red), (ii) with water molecule(s) in one or

two apical position(s) (blue) and (iii) cage (acetato-like) structures (green) which are very rare. Structure **(13)** shows the irregular square-planar geometry with oxygens and nitrogens in trans positions of red bis(D,L-N,N-dimethyl-valinato)-copper(II).[18] However, the blue complex modification with the L isomer of this alkylated amino acid was found[19] and its structure is shown in **(14)**. The geometry of this latter complex is an irregular square pyramid. The copper(II) atom, as is general in the case of crystal modification (ii), is situated somewhat above (0.021 nm) the equatorial plane towards the fifth donor (water oxygen) in the apical position. The blue modification was also found in the case of aquabis(L-N-benzylalaninato)copper(II)-monohydrate.[20]



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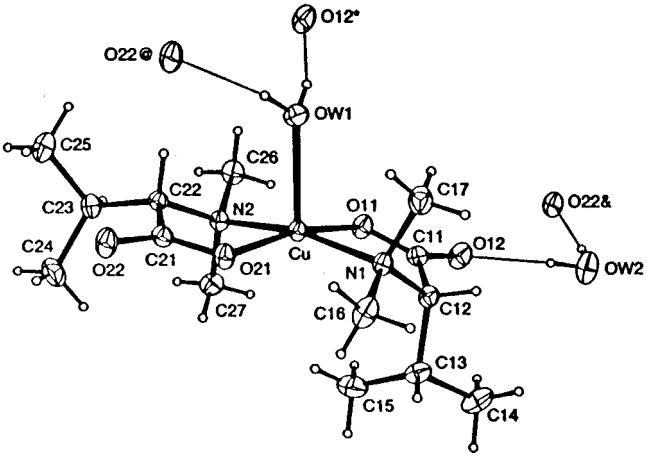

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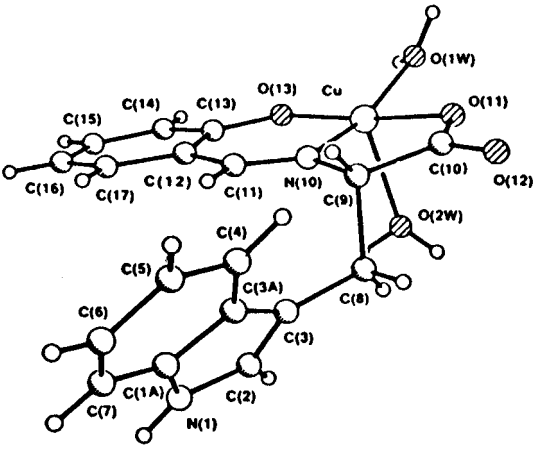

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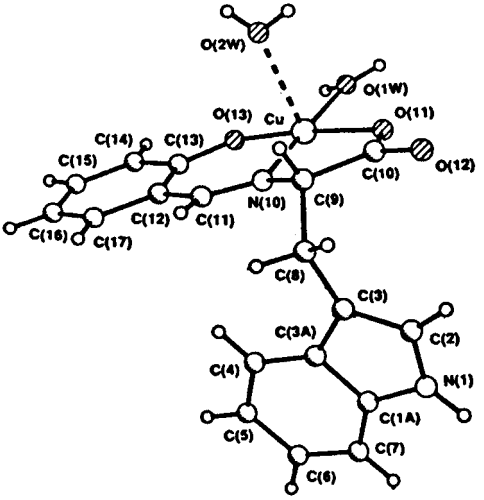
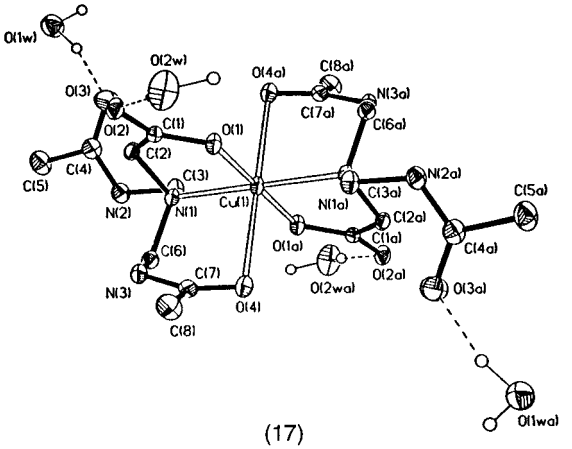


(16)

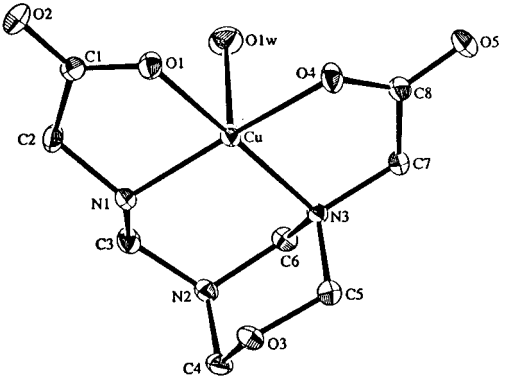
Two isomeric complexes **(15)** and **(16)** have been prepared in the case of the N-salicylidene-tryptophanato-copper(II) system.[21] The coordination geometry is approximately square pyramidal in both complexes. The Schiff base ligand is tridentate and an oxygen atom of a water molecule occupies the fourth position of a square. The coordination sphere is completed by an axial water molecule at the same side as the indole ring in the erythro isomer (**15**) or at an anti position in the threo isomer **(16)**. Copper(II) ion catalysed Mannich-type condensation

reactions between chelated amino acidates and formaldehyde have received special interest in recent years. cis-$Cu(Gly)_2 \cdot H_2O$ was found to undergo a Mannich type reaction with formaldehyde and acetamide.[22] Each Gly was found to react with two molecules of acetamide and in the final product the two Gly residues were trans with respect to each other. The X-Ray crystal structure of bis[(N,N-di-N'-methyl-acetamido)-Gly]Cu(II)$\cdot 2H_2O$ **(17)** containing copper(II) in a distorted octahedral environment was studied. The two Gly chelates and copper(II) determine the equatorial plane while two carbonyl oxygens - one from each of the two N-methylacetamido groups - occupy the axial positions. Mannich reaction between cis-$Cu(Gly)_2$, formaldehyde and ammonia resulted in the product [3N,5N-methylamino(1,3,5-oxadiazacyclohexyl)diacetato]copper-(II)$\cdot H_2O$.[23] The structure of this compound **(18)** contains the two amino nitrogen atoms of the chelated Gly moieties, N(1) and N(3) bridged by a dimethylene-amine group. The nitrogen atom N(2) is linked to N(3) by a dimethylene ether group forming an interesting C_3N_2O ring. The geometry of the complex is distorted square pyramidal, the copper being five-coordinate. The basal plane is defined by the donors of Gly moieties and the apical position is occupied by a water molecule. Condensation reactions of zinc(II)-, cobalt(II)-, copper(II)- and nickel(II)-4-hydroxy-L-prolinato complexes with formaldehyde led to the formation of the respective metal complexes of N,N-methylenedi(4-hydroxy-L-proline)-M(II) in which the amino-N atoms were linked by a methylene bridge.[24] X-Ray structures for the cobalt(II) and zinc(II) complexes showed the distorted octahedral geometry of the complexes defined by the two N and two O donors of the chelated ligand and two water molecules.



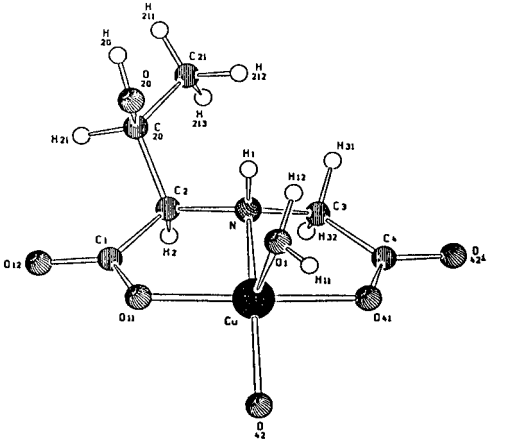
(17)

The copper(II)-N-carboxymethyl-D,L-threoninato complex was studied as a possible model for copper(II)-IDA-H_2O/-Im/-byp complexes.[25] The structure of complex **(19)** showed that the geometry is slightly distorted square pyramidal. One ligand coordinates to the copper(II) in a tridentate manner and the O(42)



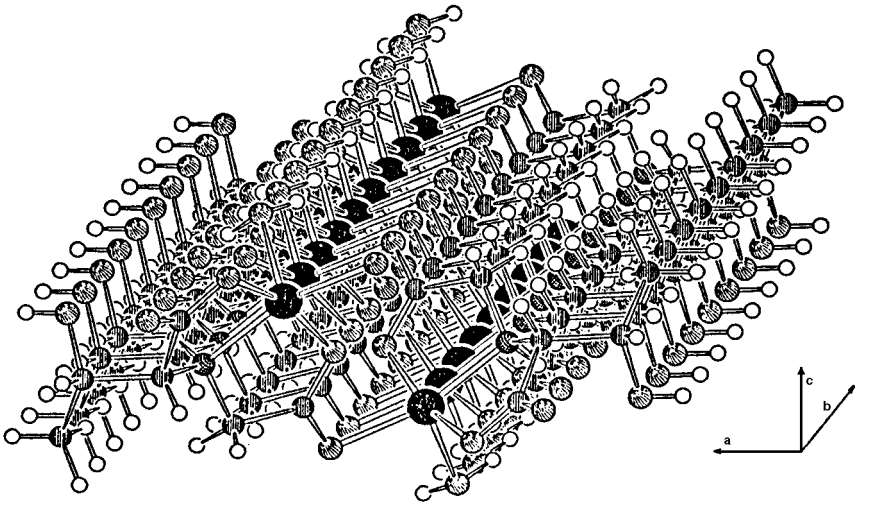
(18)

atom from the ligand of an adjacent complex unit completes the basal plane. The copper(II) is displaced (0.027 nm) from the mean plane towards the aqua ligand in the apical position. An additional weak interaction (sixth position) with an oxygen atom belonging to the same carboxylate group as O(42) in **(19)** was found. The compound exhibits a hydrogen bonded polymeric zig-zag chain structure **(20)**. Stability constants for the species formed in aqueous solution are also published in this paper.[25]



(19)

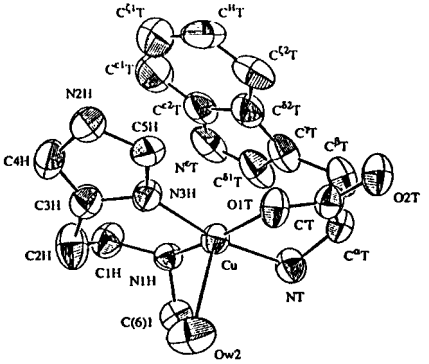
There is currently great interest in studying the possibilities and effects of noncovalent weak interactions in metal complexes of amino acids and their derivatives. Results of such a study have been published on some metallocyclo-



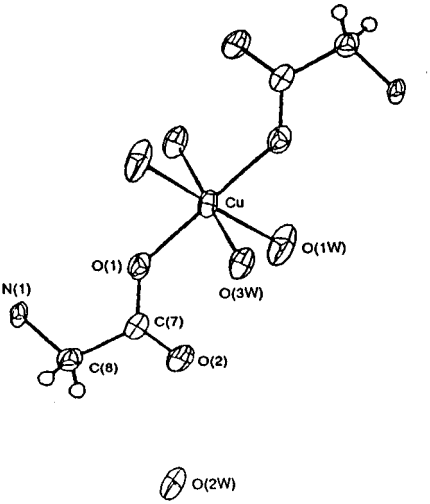
(20)

dextrin amino acid ternary systems. The X-Ray structure of the copper(II)-6-deoxy-6-[(2-(4-imidazolyl)ethyl)amino]cyclomaltoheptaose-L-Trp complex has been determined.[26] In this complex the histamine moiety, the L-Trp and copper(II) are outside the β-cyclodextrin (βCD) cavity and the primary hydroxyl groups of βCD are not coordinated directly to the metal ion. Two nitrogens of the histamino moiety and nitrogen and oxygen of Trp are coordinated to the copper(II) ion, resulting in a square-pyramidal structure. The fifth position is occupied by a water molecule. As is typical in this type of structure, the copper(II) is displaced out of the main square plane (0.017 nm) **(21)**. The relative orientation of the side chain ring of L-Trp with respect to the imidazole ring of the histamine moiety and the copper(II) coordination plane is very interesting, showing contact, d-π interaction, between the side chain of Trp and copper(II) and stacking between the rings (imidazole ring of the histamine moiety and indole residue of Trp). The common coordination mode of natural α-amino acids involves the chelation of the ligands through the amino-N and α-carboxylate-O donors. An unusual coordination mode of Gly was found in $[Cu(Gly)_2(H_2O)_4][OC_6H_2(NO_2)_3]_2 \cdot 2H_2O$, **(22)**,[27] the structure of which is a distorted octahedron. The copper(II) ion is coordinated by two water molecules and two unidentate Gly ligands bonded via single carboxylate oxygens. Identical ligands are in trans positions to each other. The octahedral sphere is completed by two more distant water molecules. The amino groups are present in protonated form. The picric acid moiety was found to serve merely to stabilize the lattice. Copper(II) complexes of compounds obtained by the reaction between ethyl-α-ketocyclo-pentylcarboxylate and D,L-Phe or D,L-Val were synthesized and characterized by FT-IR, UV-VIS, MS and thermal analysis.[28] The coordination

of ester carbonyl was found and the results were consistent with a square pyramidal environment around the copper(II) ion.

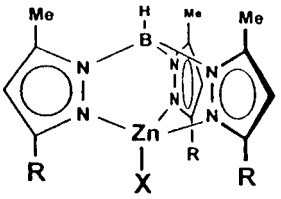


(21)



(22)

In addition to determining X-Ray crystal structures, in some cases, molecular mechanics calculations were also made and the results were compared.[15,18–20] Two molecular mechanics models were used to calculate the geometries of nine copper(II) complexes of α-amino acids and their N-alkylated derivatives and also the diastereoselectivity in the copper(II)-N,N-dimethylvaline system.[29] Structures of some interesting zinc(II) complexes which model the chemical environment of zinc(II) in biomolecules have been determined.[30–32] Cys(thiolate) or His(imidazole) are frequently found in the coordination sphere of zinc(II). This is why

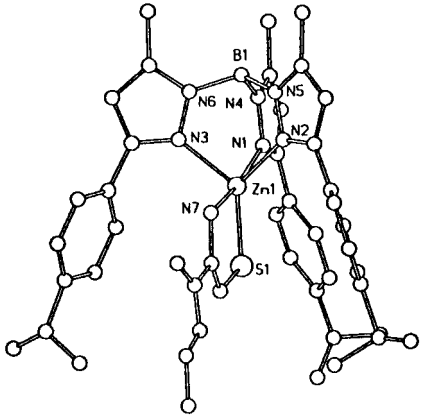


R = tert.C_4H_9: $Tp^{tBu,Me}$Zn-X

R = p-iPr-C_6H_4: $Tp^{Cum,Me}$Zn-X

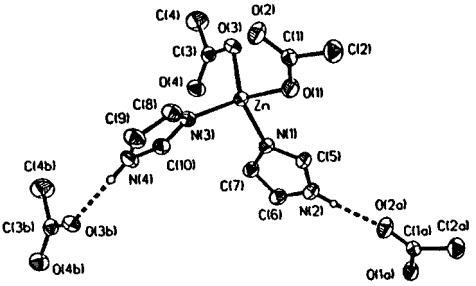
R = C_6H_5: $Tp^{Ph,Me}$Zn-X

(23)



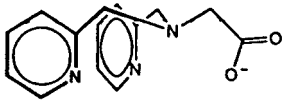
(24)

numerous compounds containing the zinc(II) ion encapsulated by a pyrazolylborate unit **(23)** were prepared.[30] The coligands were different N- and C-protected cysteines or histidines with intended monodentate coordination via their thiolate or imidazole groups. Monodentate thiolate coordination was readily achieved but not imidazole coordination. When the coligand was cysteine ethyl ester, i.e. the N terminus was not protected, a five-membered N,S chelate was formed resulting in a complex with coordination number five having trigonal-bipyramidal geometry **(24)**. Monodentate coordination of the imidazole to zinc(II) and also to cobalt(II) was achieved, however, in the monomeric complexes, $M(Im)_2(acetate)_2$, where M = Zn(II) or Co(II) which were synthesized and characterized by X-Ray crystallography.[31] The complexes formed with the two metal ions are isostructural. The structure of the zinc(II) complex shown in **(25)** has the metal ion coordinated by a pair of monodentate imidazoles and a pair of syn monodentate acetates, forming distorted tetrahedral geometry. The most interesting finding in the crystal structures of these complexes is the carboxylate-imidazole-metal system which strikingly resembles the triad systems in some zinc(II) enzymes and cobalt(II)-substituted enzymes. In these systems, the non-coordinated nitrogens of the imidazoles participate in intermolecular hydrogen bonds with carboxylate groups from adjacent molecules.



(25)

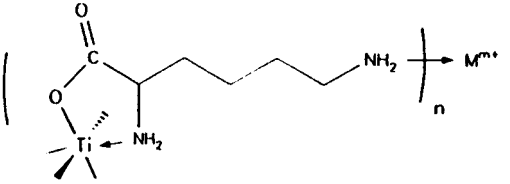
The structure and function of zinc enzymes in which the zinc(II) is coordinated by N,N,O donors was modelled by complexes containing the N,N-bis(2-picolyl)-glycine ligand (26), which had to be synthesized indirectly.[32] The coordination of imidazole, 2-methylimidazole and diphenylphosphate to this complex was studied. Some of the binary and all of the ternary complexes were prepared and analysed by X-Ray crystallography which confirmed monodentate coordination of imidazole-N or phosphate-O in the solid state. Solution NMR studies and conductivity measurement revealed various states of dissociation and solvation of the ternary complexes.



(26)

Synthetic work on nickel(II)-amino acid/-amino acid derivative complexes has been carried out in very few cases.[33–35] Stereoselectivity of aldol reactions between chiral nickel(II) complexes of non-racemic Schiff bases of Gly and aliphatic aldehydes were found.[33] An asymmetric dinuclear nickel(II) complex as a possible model for the active site of urease was synthesized using the novel asymmetric ligand 1-[bis(2-pyridylmethyl)amino]-3-[2-(2-pyridyl)ethoxy]-2-hydroxypropane.[34] Possible industrial applications could stimulate work on synthesis and isolation of titanum alkoxide-amino acid (Gly, Lys) complexes.[35] In the case of Lys the non-coordinated side chain amino group could take part in intermolecular coordination to other metal ions, e.g. nickel(II) ion, and this is schematically shown in **Scheme 1**.

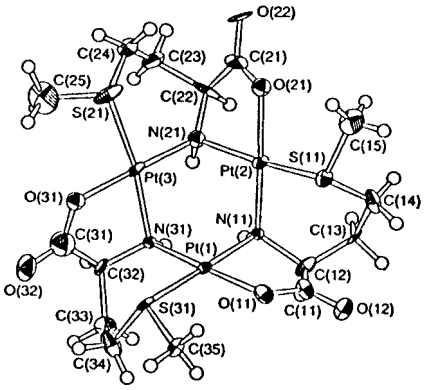
The great interest in platinum complexes is mostly based on their biological importance. As is well known, cisplatin, cis-$[Pt(NH_3)_2Cl_2]$ is one of the most widely used anticancer drugs, and is believed to bind DNA. The *in vitro* experiments demonstrating that metallation occurs through nucleobases have significantly increased the interest in studies on platinum - nucleobase systems. The discovery of a platinum-methionine adduct in the urine of patients under-



Scheme 1

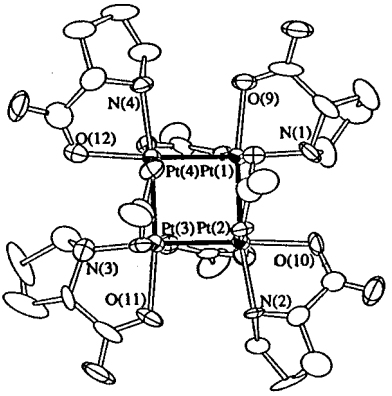
going cisplatin therapy accelerated the studies of platinum(II) methionine as well as other sulfur-containing amino acid and peptide complexes. In many cases, the very inert platinum(II) is modelled by palladium(II) in the studies.

Several different platinum(II) and palladium(II) complexes containing amino acids or derivatives have been synthesized during the past two years,[36–44] but X-Ray structures have not been determined in all cases. An interesting cyclic trinuclear platinum(II) complex, $[Pt_3(L\text{-}Met)_3]\cdot H_2O$ **(27)**, was characterized.[36] In this compound tridentate coordination by each Met can be seen and the metal ions are bridged by the N-donors of the methionines.



(27)

In a tetranuclear platinum(II) complex prepared from the well-known octaacetato platinum(II) cluster, the metal ions are bridged by four acetato ligands while the other four are replaced by L-Pro, chelating the metal ions by the N,O mode **(28)**.[37] This cluster, having a chiral environment around each platinum, is expected to be of use in asymmetric reactions. Mononuclear complexes were found to be formed between the alkyl (Me, Et, iPr) ester derivatives of methionine (or histidine) and platinum(II) or palladium(II).[38] The complexes were prepared and characterized. The X-Ray structure was determined in the case of $[PdCl_2(L\text{-}MetOMe)]$, showing the distorted square-planar coordination of the metal ion. Two chlorines and nitrogen plus sulfur atoms of L-MetOMe are coordinated. The chlorines, but not the amino acid esters, in $[PdCl_2(L\text{-}MetOMe)]$, $[PtCl_2(L\text{-}MetOMe)]$ and $[PdCl_2(L\text{-}HisOMe)]$ could be replaced by 5'GMP or 5'CMP. If



(28)

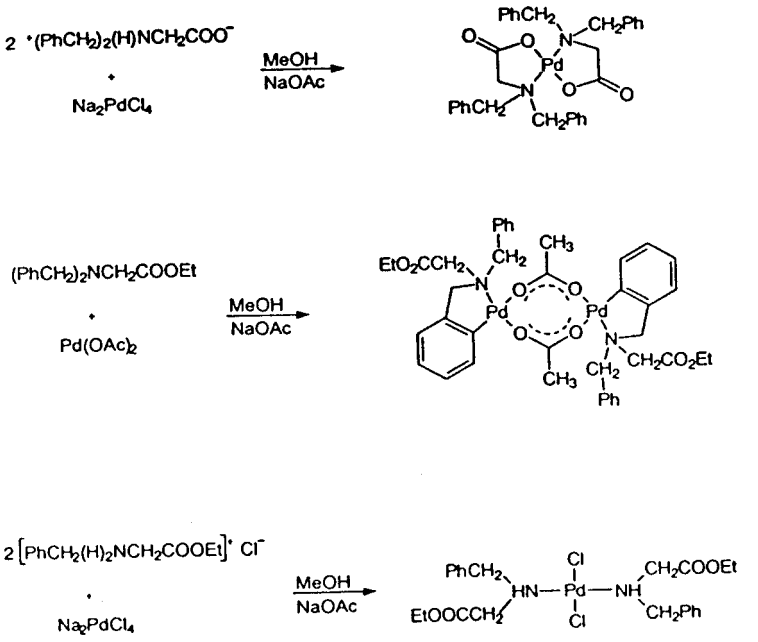
the amino acid or amino acid derivative has no side chain donor, which is able to coordinate to platinum(II) or palladium(II), normal bidentate coordination of the amino acid via amino nitrogen and carboxylate oxygen atoms occurs.[39,40] However, when the cis-[$(NH_3)_2$Pt(Gly)] complex containing the Gly in N,O chelated form was reacted with 1-MeU, a ternary species, [$(NH_3)_2$Pt(Gly)(1-MeU)] with monodentate coordination of Gly was formed.[40] X-Ray studies showed that the Gly was coordinated through its amino nitrogen only and the fourth position was occupied by the N-3 of the 1-MeU in this ternary complex.

Different N-modified glycine derivatives (N,N-dibenzylglycine, O-ethyl-N-benzylglycinate and O-ethyl-N,N-dibenzylglycinate) were used to synthesize palladium complexes,[41] as shown in **Scheme 2**. As can be seen, N,O type chelates are exclusively formed with amino acid anions, while monodentate coordination via the nitrogen occurs if Na_2PdCl_4 is reacted with the ester derivative and the use of $Pd(OAc)_2$ leads to orthopalladation. The X-Ray structures of the N,O chelated and monodentate coordinated compounds were determined for some complexes and those formed with N,N-dibenzylglycine and the ethyl ester of N-monobenzylglycine are shown in **(29)** and **(30)**, respectively.

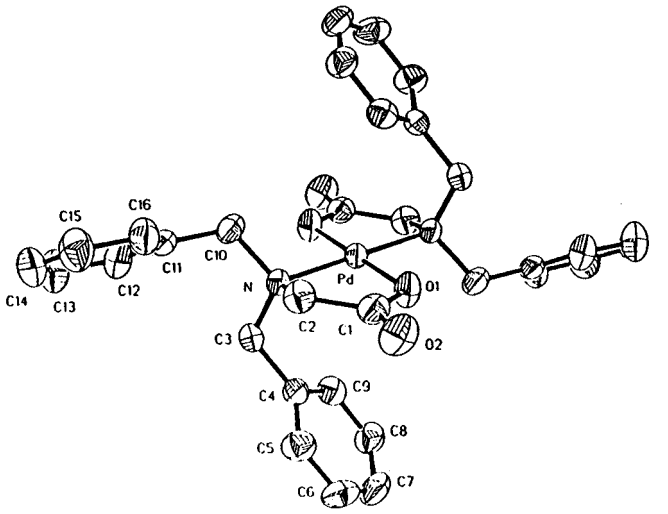
Interesting spontaneous oxidation to a diplatinum(III) complex was found if the dimerization of trans-[$Pt(NH_3)$(1-MeC-N3)$(H_2O)_2$]$^{2+}$ was carried out in presence of Gly.[42] The X-Ray structure of the diplatinum(III) complex [$Pt_2(NH_3)_2$(1-MeC-N3,N4)$_2$(Gly-N,O)$_2$]$(NO_3)_2 \cdot 3H_2O$ **(31)** showed the head-tail orientation of the two methylcytosinato rings.

Many ternary complexes between Pd(II)-2-[(dimethylamino)methyl]phenyl-C,^{1}N and different α-amino acids were synthesized.[43] The compounds were found to show characteristic CD spectra for the absolute configuration of the C_α-carbon of amino acids.

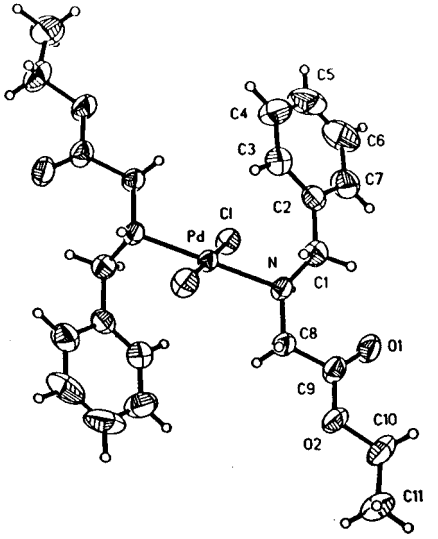
A synthetic amino acid, aziridine-2-carboxylic acid was used as a ligand to synthesize different metal (including palladium(II), platinum(II)) complexes[44] and the the synthesis of different 2-hydroxyiminocarboxylate-Pt(II) or -Pd(II) complexes was also reported.[45] The reactivity of some synthesized platinum(II)


2 *(PhCH2)2(H)NCH2COO-
Na2PdCl4
MeOH
NaOAc
PhCH2
CH2Ph
Pd
(PhCH2)2NCH2COOEt
Pd(OAc)2
MeOH
NaOAc
Ph
CH2
CH3
EtO2CCH2
CH2CO2Et
2[PhCH2(H)2NCH2COOEt]+ Cl-
Na2PdCl4
MeOH
NaOAc
Cl
HN
NH
CH2COOEt
EtOOCCH2
CH2Ph

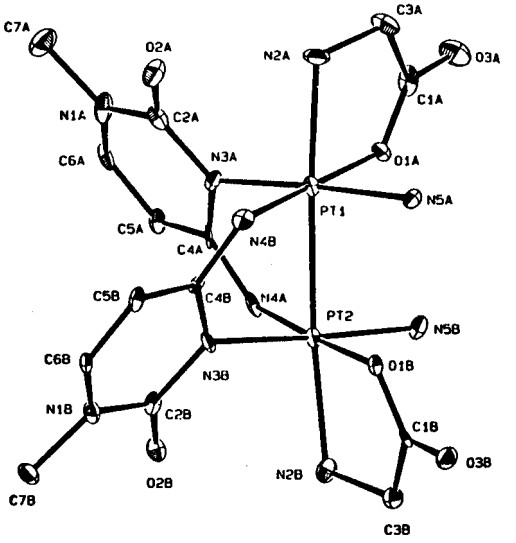
Scheme 2


C15
C16
C10
C11
C12
C14
C13
N
Pd
O1
C2
C1
O2
C3
C4
C9
C8
C5
C6
C7

(29)



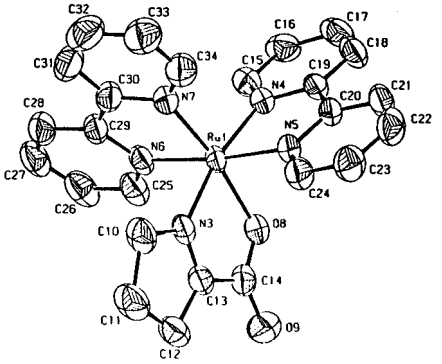
(30)



(31)

and palladium(II) complexes formed with amino acid derivatives has been studied in some cases.[46–48] The accelerated conversion of nitrile group into oxazoline group in the side chain of amino acid coordinated to platinum(II) was

found[46] as was the efficient promotion of ester cleavage in N-((tert-butyloxy)-carbonyl)-L-methionine p-nitrophenyl ester in certain platinum(II), palladium(II) complexes.[47] Palladium(II) complexes of N-trienylidene-L/D-methionine methyl ester were synthesized and characterized and their reactivity toward CO insertion was studied.[48]



(32)

α-Amino acids are oxidatively deaminated to keto acids in biological systems. To mimic this process, anodic oxidation at constant potential of different Ru(α-amino aciditato)(phen)$_2$ or (bpy)$_2$ complexes was studied. This resulted in the dehydrogenation of the coordinated α-amino acids[49] and the products were the corresponding (α-imino acidato)Ru(bpy)$_2$ or (α-imino acidato)Ru(phen)$_2$ complexes. The crystal structure of [Ru(Pro-H$_2$)(bpy)$_2$]ClO$_4$·3H$_2$O was determined and is shown in **(32)**. Several other metal (iridium(III), cobalt(III) and ruthenium(II)) complexes with different α-iminocarboxylates were also synthesized and characterized.[50] Great interest in photoredox-active compounds has stimulated studies on different ruthenium(II)-polypyridyl-type compounds. New bidentate ligands derived from Lys and Cys have been prepared, **(Figure 1)**, and using these, ternary complexes with bis(bpy)ruthenium(II) have been synthesized.[51] The coordination modes were characterized by ^{1}H NMR. The compound [Ru(bpy)$_2$-(BocLysCH$_2$bpy)$_2$]$^{2+}$ was found to show interesting redox behaviour. N-Salicylaldiminate derivatives of Gly or Phe were used to synthesize oxovanadium(V) ternary complexes having quinolin-8-olate[52] or ethane-1,2-diol[53] as co-ligands. The X-Ray structure of the complex formed with the Phe derivative and ethane-1,2-diol is shown in **(33)**. This complex shows tridentate coordination of the amino acid derivative and bidentate coordination of the diol. One oxo atom occupies the sixth position of the severely distorted octahedron.

Due to the facile oxidation of Cys by oxidized metal ions, the preparation of complexes containing high oxidation state metal and Cys is usually difficult. Reaction of Cr(VI) with different -SH-containing compounds, i.e. L-Cys, L-Cys-ethyl ester or mercaptopropanediol, resulted in the formation of corresponding Cr(III) products.[54] Taking the above into account, the complex [OsH$_2$(N-ac-L-

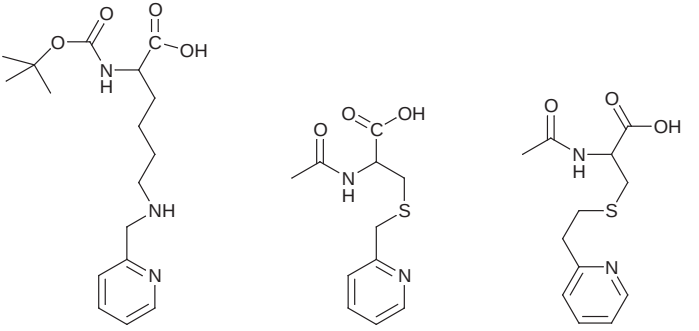
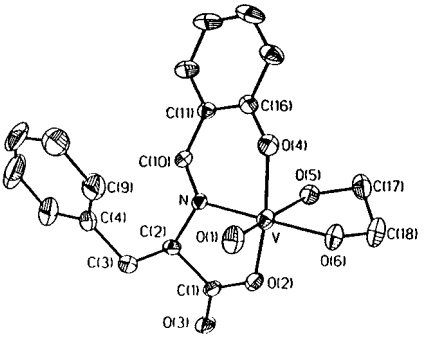


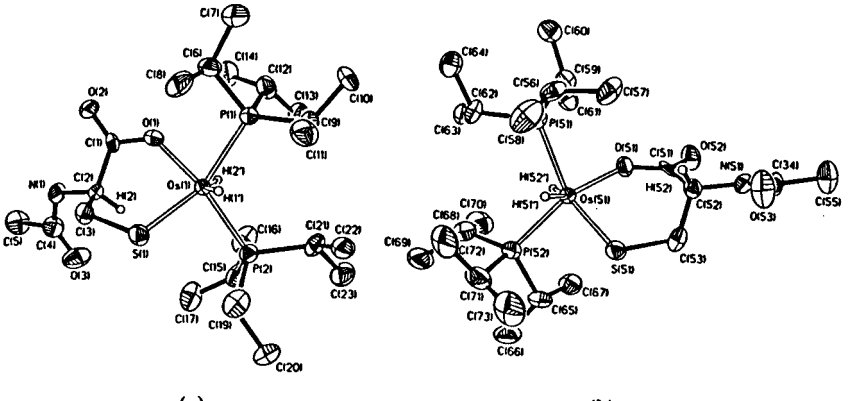
Figure 1



(33)

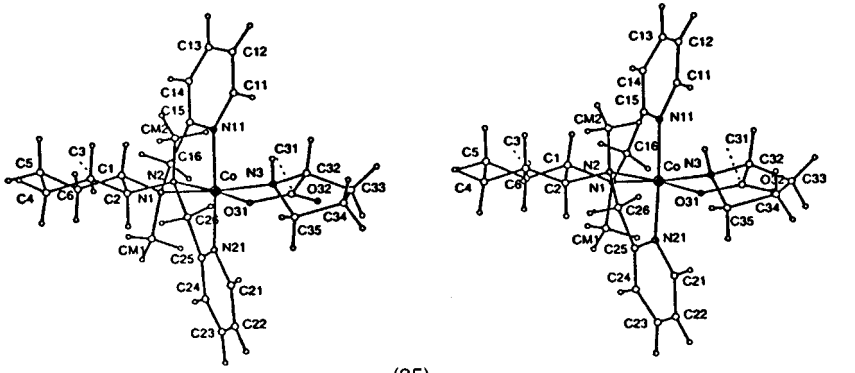
cys)$_2$(PPr$_3$)$_2$] is unusual.[55] According to X-Ray analysis, the structure has two independent molecules in the asymmetric unit **(34)**. The N-acetyl-L-Cys ligands coordinate via their O and S donors, while two positions are occupied by two phosphorus atoms and there are two vacant coordination sites in each molecule. FAB-MS was used to study α-amino acid-Cr(III) complexes, which in some cases are monomers and in other cases dimers.[56]

Many cobalt(III) complexes are known to form geometrical isomers. Coordination of the flexible tetradentate ligand N,N'-diethylethylenediamine-N,N'-di-α-butyrate which occupies four of the coordination sites around a cobalt(III) ion, theoretically allows the formation of three geometrical isomers in different ternary complexes. When Gly, L-Ala or L-Phe were used as co-ligands, one isomer with remarkable selectivity was found to form.[57] If Λ-α-[Co(III)-(N,N'-dimethyl-N,N'-di(2-picolyl)-1S,2S-diaminocyclohexane)Cl$_2$]$^+$ was reacted with α-amino acids, such as Pro, Ala or 2-amino-2-methylpropanedioic acid, the two chlorides were replaced by N,O chelates of the co-ligands with retention of configuration at the metal centre. Enantiomeric preference of S-Pro was found from a racemic mixture, but enantiospecific discrimination was not found with the less bulky Ala. The crystal structure of the ternary complex formed with S-



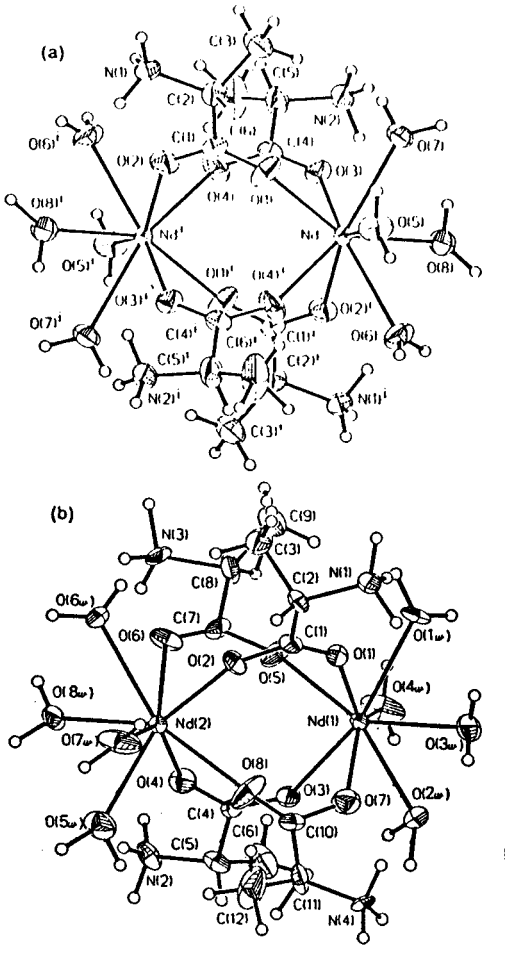
(34)

Pro, **(35)**, was determined.[58] Several dihydrido-Ir(III)-α-amino acidato complexes were synthesized and their reactions with phenylacetylene were studied.[59] Studies on iron-amino acid sytems are quite rare, despite the fact that proteins containing different iron-sulfur cores are known. Binding of iron-sulfur clusters to the protein backbone generally occurs via Cys. Compounds formed between the Fe-S cluster, $[Fe_2S_2(SBu)_4]^{2-}$ and L-Cys ethyl ester hydrochloride or L-Tyr methyl ester were synthesized and cluster interconversion reactions were evaluated.[60]



(35)

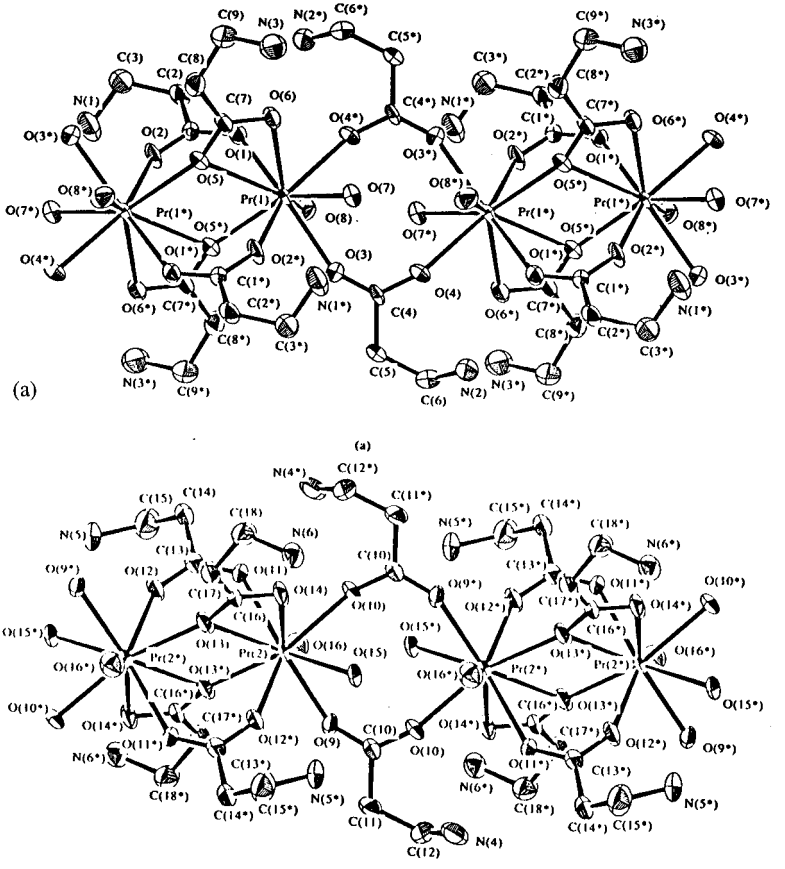
L-Piperidine-3-carboxylic acid was used as a model for the amino acid Pro, and the X-Ray structure of its cadmium(II) complex was determined.[61] X-Ray evidence was given for the effect of ligand chirality on the structure of the dimeric neodymium-L-Ala and neodymium-D,L-Ala complexes, $[Nd_2(Ala)_4(H_2O)_8](ClO_4)_6$.[62] In both cases, the neodymium cations were eight-coordinated



(36)

by four water molecules and four oxygens of the Ala residues while the two neodymium ions were linked by four bridging carboxyl groups. If DL-Ala molecules were the ligands, the dimer was centrosymmetric **(36a)**, but in case of L-Ala it was noncentrosymmetric **(36b)**. The same composition and coordination mode was found for $[Nd_2(Ile)_4(H_2O)_8](ClO_4)_6$.[63] The crystal structure of the praseodymium-β-Ala complex $[Pr_2(\beta\text{-Ala})_6(H_2O)_4](ClO_4)_6H_2O$ was found to comprise two kinds (very similar) of polymer chains, both with dimeric repeating units, **37a** and **37b**. In each dimeric unit, two praseodymium ions are connected by different types of carboxyl bridges of four β-Ala molecules. These dimeric units are linked to one another by two simple bridging carboxyl groups to form

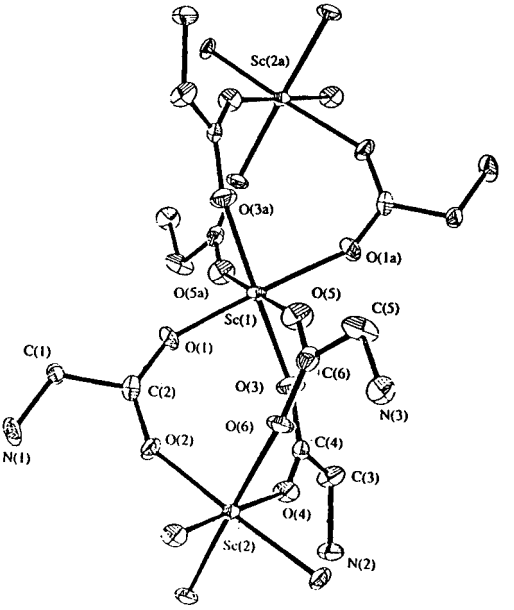
an infinite polymer chain. With two coordinated water molecules the metal ions are nine-coordinated.[64]



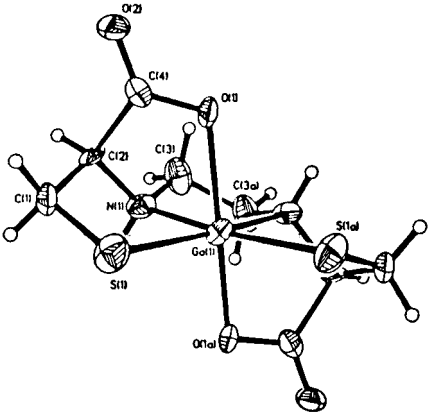
(37)

The crystal structure of the scandium(III)-Gly complex $[Sc_2(Gly)_6(ClO_4)_6]_n$ shows a great difference in coordination number compared to other lanthanide-amino acidato complexes.[65] The smaller ionic radius of the scandium(III) has resulted in a significantly smaller coordination number (6) and the geometry around the scandium(III) ion is octahedral in this infinite chain structure, built from dimeric units **(38)**.

The synthesis and characterization of the Gly complexes, $La[Fe(CN)_6]$-$3Gly{\cdot}2H_2O$ and $La[Co(CN)_6]3Gly.2H_2O$ have been reported in a paper.[66] Lanthanum(III) is coordinated by some of the CN ligands, the carboxylates of the Gly molecules and one of the waters. Indium(III) and gallium(III) complexes

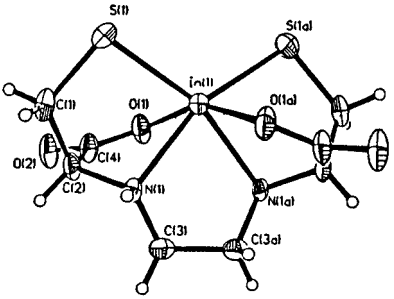


(38)



(39)

(39) and **(40)**. of N,N'-ethylenedi-L-Cys were characterized by X-Ray crystallography.[67] In both complexes the metal ions are coordinated by two sulfur atoms and two nitrogen atoms in the equatorial plane and the axial positions are occupied by two carboxylate oxygens.



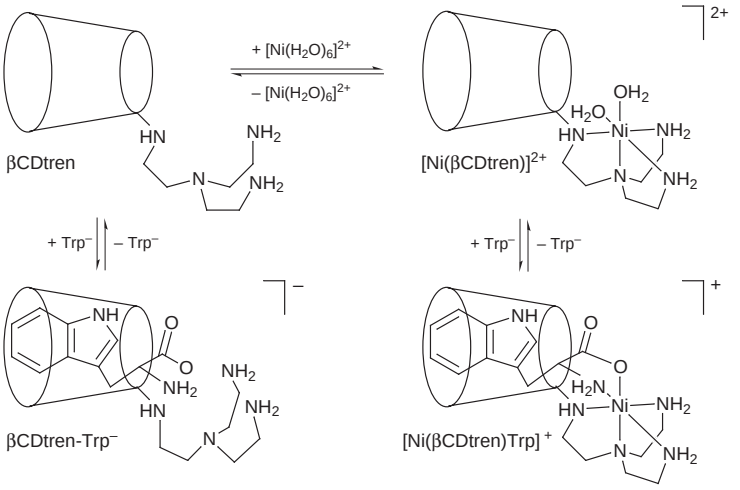
(40)

2.2 Solution Studies – A variety of spectroscopic methods, e.g. UV-VIS, ESR, NMR was used to determine the stoichiometry, stability and bonding mode of species formed in solution. Speciation studies on metal ion-amino acid systems have also used pH-potentiometry. In several cases complexes formed both in solution and in the solid state were studied. As in the solid state, copper(II) complexes were studied in many cases and the main interest focused on ternary systems during the period of coverage.

The effect of water dissolved in organic solvents such as methylene chloride, deuterated acetone, dioxan and isopropanol on the coordination sphere of copper(II) complexes containing different dialkyl amino acids was studied by ESR.[68] It was found that water dissolved in methylene chloride or dioxan caused the broadening of the copper(II) hyperfine lines, suggesting slower motion of the complex. The effect was really significant if the amino acid derivatives had bulky side chains, as in Val and Ile. Water coordination and hydrogen bond formation between the carboxylate oxygens and the coordinated water molecule was assumed. The effect of amino acid residues on the binding and the reaction of copper(II) with DNA was studied by ESR.[69] Stereospecific binding of complexes formed with L-Thr or L-Ser to the B form of DNA fibres was found but this was not the case with Gly, Pro, His, Lys or Arg, clearly indicating the essential role of the hydroxyl groups in regulating the stereospecific binding of the complexes. The precise nature of the binding remains to be clarified.

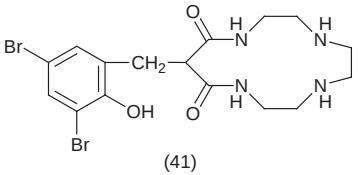
The role of non-covalent interactions in ternary complex formation was also evaluated in the complexation between metallo-β-cyclodextrin derivatives and amino acids which was the subject of several studies.[26,70,71] The βCDtren-Trp-metal ion (Ni(II), Cu(II), Zn(II)) systems were studied[70] and **Scheme 3** shows the possible interactions in the βCDtren-Trp-Ni(II) system. The results were compared with those for βCD and βCDpn and the following conclusions were made: a comparison of the stability constants for ternary complexes formed with Trp showed a significant increased stability in the order βCD < βCDpn < βCDtren. The particularly high stability of Trp-βCDtren was explained by the existence of stronger non-covalent interactions in this complex. Also the stabilities of $[M(\beta CDtren)]^{2+}$ complexes were substantially greater than those of $[M(\beta CDpn)]^{2+}$, indicating the terdentate nature of βCDtren. Moreover, the

stepwise stability constants for the formation of the ternary metallocyclodextrin, complexes $[M(\beta CDtren)\text{-}(R)/(S)Trp]^{+}$ were significantly greater than those of the analogous complexes formed with βCDpn. Looking at Scheme 3, one can conclude that the coordination of all the possible donors in a ternary system, especially to copper(II) ion, cannot be expected.



Scheme 3

In agreement with expectation, stability constants for copper(II)-diethylenetriamine/dipropylenetriamine-Gly/β-Ala complexes show that the formation of mixed ligand species in these systems, where the tridentate ligands occupy three equatorial positions, around the metal ion and Gly or β-Ala has to occupy one equatorial and one axial position, is unfavoured.[72] If, however, the tridentate ligand is the alkyl-substituted derivative of diethylenetriamine, N,N,N,N',N'-pentamethyldiethylenetriamine, the formation of ternary complexes of copper(II) with different amino acids is somewhat more favoured.[73] The higher stability of these ternary complexes compared with the corresponding dien complexes was explained by the poorer σ-donating property of tertiary amino groups of alkyl-substituted dien. The effects of the N- or C-substituents of amino acids on the stabilities of ternary complexes were also evaluated. In other works using calorimetry the four equatorial positions around copper(II) ion were blocked by nitrogen donors of a macrocyclic ligand **(41)** allowing only axial coordination of other ligands (including amino acids).[74,75] By blocking only two coordination sites of copper(II) ion by strong donors, the remaining two equatorial positions allow the formation of the five-membered 'amino acid type chelate' in the equatorial plane. This was found in the case of copper(II)-ciprofloxacin **(41)** -Gly or -Tyr systems,[76] in which two oxygens of the ciprofloxacin and amino-acid type N,O chelate are coordinated in the equatorial positions. Most probably hydrophobic interactions between the coordinated ligands made the formation of the



(41)

ternary complex more favoured in the case of Tyr. Ternary, copper(II)-containing complexes were assumed to form in the copper(II)-mediated transport of α-amino acids across a bulk chloroform membrane.[77]

In the case of many metal ions, complex formation with amino acids has been much less studied and understood than with copper(II). In some such cases binary and ternary complexes have recently been studied. Complexes formed between L-His derivatives or related ligands and oxovanadium(IV) were studied by spectroscopic measurements.[78] As per Figure 2, 3-Methyl-L-histidine (2.2), 1-methyl-L-histidine (2.3), L-histidine methyl ester (2.4), L-histidinol (2.5), N-α-acetyl-L-histidine (2.6) and histamine (2.7) were used as ligands.[78] Systematic modification of these different moieties of L-His resulted in the following conclusions: 'Histidine-like' tridentate coordination via amino-N, imidazole-1N and carboxylato-O was found in case of the 3-methyl derivative (2.2). Blocking of imidazole-1N (2.3) reduced the complex-forming ability of the ligand, so that VO^{2+} hydrolysis became favoured. The same effect of substitution on the amino nitrogen (2.6) was found. When the carboxylate moiety was modified or eliminated as in compounds (2.4), (2.5) and (2.7), bidentate coordination through amino-N and imidazole-1N donors was detected. The equilibrium results on dioxovanadium(V)-serine and -threonine systems showed that only coordination of carboxylate moieties to the metal ion occurred.[79]

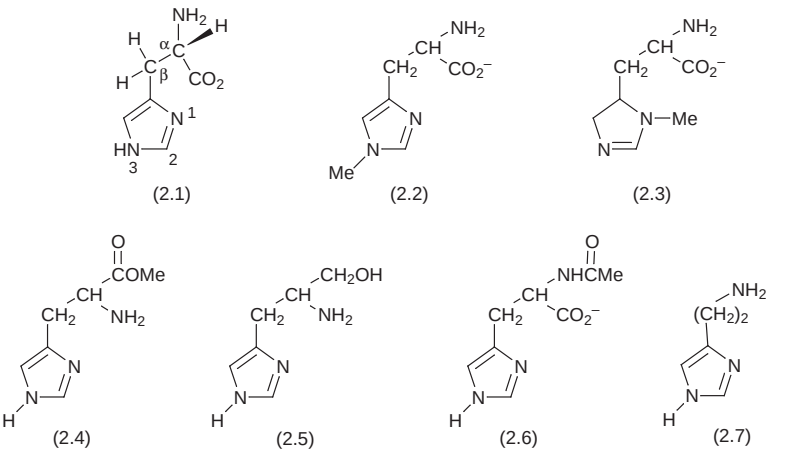


Figure 2

Solution equilibrium results for cadmium(II)-amino acid systems have been published in a few cases. Potentiometric titrations were carried out for more than twenty cadmium(II)-N-(2-acetamido) iminodiacetic acid-amino acid ternary systems and the stability constants were determined.[80]

The CD spectrum of the optically active complex, Λ-fac-[Rh(III)-S-Ala] was reported and discussed.[81] The solubilization of fac-tris(amino acidato)cobalt(III) by sulfuric acid and sodium sulfate was investigated.[82] It was found that the solubility of tris-complex involving L-Phe decreased with increasing concentration of sulfuric acid but that the solubility of fac-$Co(Ala)_3$ increased.

Iron(III) complexes of a DOPA (3,4-dihydroxyphenyl-L-alanine)-containing adhesive protein from *Mytilus edulis* were studied.[83] The protein was found to form complexes very similar to low molecular weight catecholates. Depending on the protein-iron(III) ratio two different types of complexes were observed at physiological pH: a mononuclear, ESR-active tris(catecholato) species at high ratios, and an ESR-silent μ-oxo- or μ-hydroxo-bridged cluster, involving bis(catecholato) coordination of ferric ions at low ratios.

N,N,N',N'-tetrakis(carboxymethyl)-L-Orn is a member of the known polymethylenediaminotetraacetic acid derivatives containing an α-carboxy substituent. Its complex formation with nickel(II) ion was studied[84] and the stability constants for the different species, including dimeric and trimeric ones, were determined. Determination of the absolute configuration at the α-carbon atom of substituted Gly using 2D NOESY spectra is the subject of an NMR study on nickel(II) complexes of Schiff bases of (S)-2-(N-benzylpropyl)aminobenzophenone and α-monosubstituted Gly.[85]

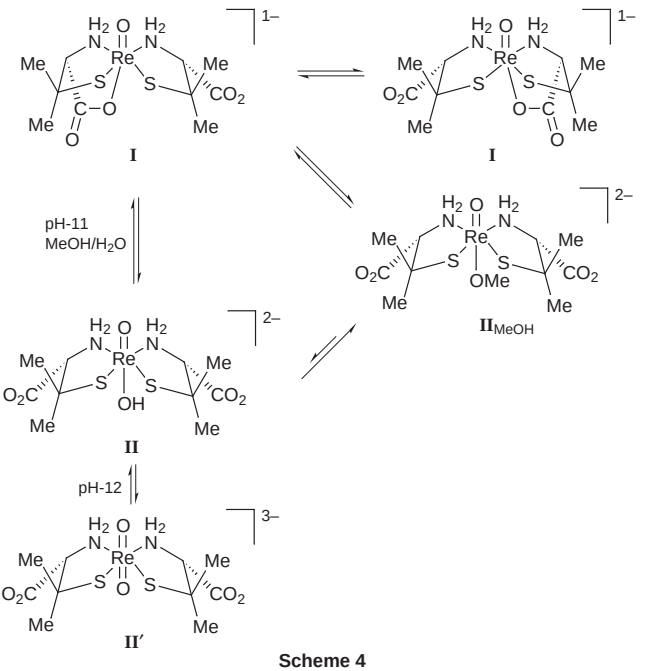
The N,N'-ethylenedi-L-Cys multidentate ligand is a very effective chelator as was concluded from the crystal structures of its gallium(III) **(39)** and indium(III) **(40)** complexes and also from the stability constants of its complexes with cobalt(II), lead(II), cadmium(II), zinc(II) and nickel(II) ions.[67] The stability constants of these complexes are significantly higher than the overall constants of bis-complexes containing Cys.

Equilibrium studies on dimethyl- and diethyltin(IV) complexes of some amino acids were carried out by a potentiometric method.[86] N,O Chelation of simple amino acids was assumed and the effects of different side chain donors were discussed. Evidence for bidentate coordination of Ala, Phe, Trp and Val to dimethyltin(IV) was also obtained from other potentiometric work.[87] Stability constants for complexes formed in the tin(II)-Gly system below pH 4.5 were determined by potentiometry,[88] and evidence for bidentate coordination of Gly was obtained. The precipitate formed in this system at pH ca. 5 was also characterized.

Lipophilic lanthanide complexes co-ordinated by fluorinated β-diketone ligands were found to be efficient carriers for extraction and rapid transport of potassium benzyloxycarbonylamino acidates.[89,90] The formation of 1:1 complexes between the lanthanide complexes and benzyloxycarbonylamino acidates via carboxylates was confirmed by ^{13}C NMR. Terdentate coordination of different diaminocarboxylates and bidentate coordination of different sulfur-containing amino ligands (L-Cys, L-Phe and L-cysteic acid) was assumed on

the basis of pH-metric measurements in ternary complexes formed with nickel(II).[91]

Rhenium(V) is known to be stabilized in the $[M^{V}O]^{3+}$ core of complexes formed by ligands containing N,S,O donors. This is why N and S donors are commonly employed in complexes used in radiopharmaceutical chemistry. Solution studies on oxo(anti-D-Phen)(syn-D-Phen)rhenium(V)[92] and oxo(D-Phen)(L-Phen)rhenium(V)[93] were performed. The effect of base on the solution behaviour of oxo(D-Phen)(L-Phen)rhenium(V)[93] was evaluated and the results, showing the formation of the dioxo complex at pH ca. 12, are summarized in **Scheme 4**.



Scheme 4

Because of the biological interest in interactions between metal ions and hydroxamic acid or phosphonic (phosphinic) acid derivatives of amino acids, several papers have been published.[9,94–101] Modification of the carboxylic group to phosphonic (-PO(OH)$_2$) or phosphinic ones (-PRO(OH)) changes the size, the charge and acidity of coordinating groups. A comparison of the complexing properties of amino acids and their phosphonic or phosphinic derivatives was made during the cited period.[9] In addition to the review, cobalt(II), nickel(II) and copper(II) complexes of phosphinic analogues of Gly,[94] zinc(II) complexes of phosphonic analogues of Glu[95] and copper(II) complexes of citric acid derivatives[96] were studied. In the case of the copper(II)-Gly analogue complexes not only solution equilibrium but also solid state studies were carried out and X-Ray structures are reported for complexes of two derivatives.[94]

Modifying the carboxylic moiety of an amino acid to a hydroxamic acid group (-CONHOH) makes significant changes in the coordination character of the molecule. Typical hydroxamate type O,O chelates formed with iron(III), aluminium(III) and gallium(III)[99] containing the amino group in the NH_3^+ form. Moreover, the electron withdrawing effect of NH_3^+ results in the lower stability of O,O chelates and more favoured hydrolysis of the complexes. If the metal ion is nickel(II), complexes formed by coordination of amino-N and hydroxamate-N atoms were exclusively found.[97] The comparable stabilities of the two types of chelates resulted in the formation of dinuclear species in the pH range 3-6 in the case of copper(II). Above this pH nitrogen-coordinated monomer exists. Coordination of side chain donors of Lys, Arg and Met derivatives was not observed in any case. Ternary complexes in copper(II)-glycinehydroxamic acid-vitamin B_6 compounds (pyridoxol, pyridoxal, pyridoxamine)[101] and nickel(II)-/copper(II)-/zinc(II)-/cadmium(II)-α-alaninehydroxamic acid-ethylenediamine[100] systems were also studied.

The sulfonic group is known to be a poor ligand for metal ions. However, its coordinating ability was found to be enhanced by incorporating an amino-nitrogen in a chelatable position to the sulfonic group.[102] Ternary complexes formed in the copper(II)-L-His-aminomethane-sulfonic acid or 2-aminobenzene sulfonic acid systems contained the aminesulfonates as bidentate ligands. The formation of ternary complexes was found to be favoured in both systems but only in the acidic pH-range. At physiological pH only the $Cu(Hist)_2$ binary species exists in measurable concentration. Recent interest in the nutritional and clinical role of selenomethionine (SeMet) and its metal complexes initiated the potentiometric and spectroscopic studies on copper(II)-SeMet and zinc(II)-SeMet systems.[103] The coordinating behaviour of sugar amino acids toward different transition metal ions was found to be similar than that of other N-protected derivatives.[104,105] In the case of amino sugars the presence and position of the amino group was found to be crucial in the complex formation.[106]

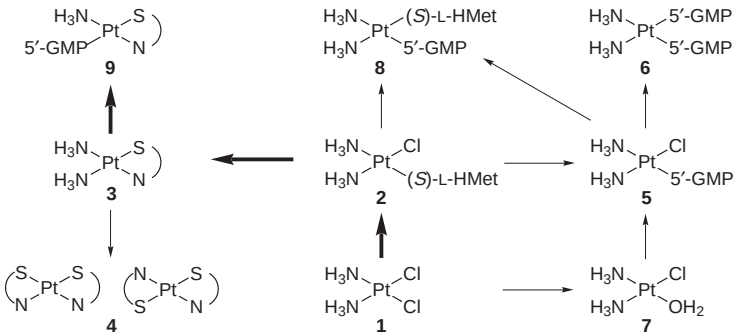
The only work referring to copper(I) amino acid complexes was in the gas phase and the relative copper(I) ion affinities of 20 natural amino acids were determined by a kinetic method.[107] The following affinity order was found: Gly < Ala < Ser < Val < Leu < Ile < Thr < Pro < Asp < Asn < Glu < Phe < Tyr < Cys < Gln < Met < Trp < His < Lys < Arg, showing more favoured interaction if the ligand had bigger alkyl side chain and especially if it had soft side chain donors such as a sulfur atom or aromatic π-electrons.

2.3 Kinetic Studies – Most of the kinetic work on metal ion-amino acid/derivative complexes deals with catalytic effects and oxidation of amino acids (especially sulfur-containing) by high oxidation state metal ions.

The main goal of studies involving platinum and palladium complexes was to collect more information about the interaction of cisplatin and related complexes with DNA and its constituents.

The reaction between cisplatin, $[PtCl_2(NH_3)_2]$, 5'-GMP and/or L-Met was studied at pH 7 by ^1H and ^{15}N NMR spectroscopy.[108] The surprising result of this study was that the reaction of 5'-GMP with cisplatin was faster in the

presence of L-Met than in its absence. The reason for this is that a major species formed in this system is $[Pt(L\text{-}Met\text{-}S,N)(5'\text{-}GMP\text{-}N^7)(NH_3)]^+$ (**Scheme 5**, species **5.9)** in which the ammine trans to the sulfur is replaced by the 5'-GMP. The favoured formation of this was explained by the labilization of NH_3 trans to S in intermediate species, **(Scheme 5** species **5.2** and **5.3)** providing alternative pathways for 5'-GMP binding. If, however, the two positions in the coordination sphere of platinum(II) were occupied by en (ethylenediamine) the coordination of 5'-GMP was not found. The same result was observed in the case of the Pt(dien)-cysteine (or glutathione)-5'-GMP system.[109]



Scheme 5

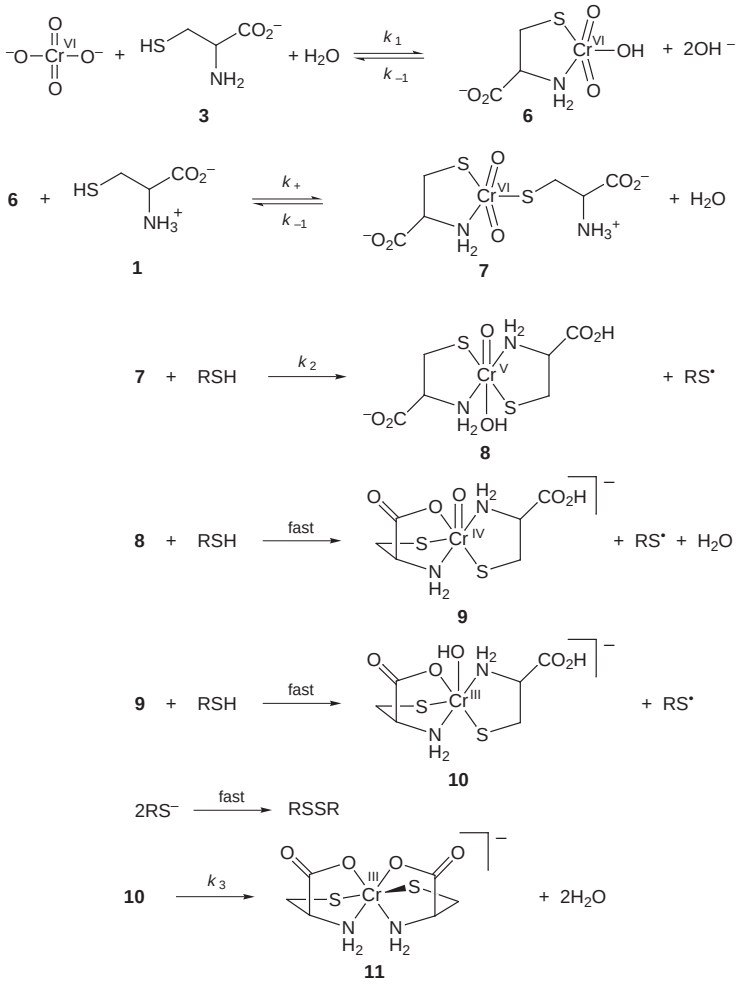
Kinetic studies[110] on complex formation and chelation of cis-$[Pd(R_2NCH_2CH_2NR_2)Cl_2]$ (R = H, Me or Et) with L-Met and S-methyl-L-Cys showed that reactions proceed via the formation of a reactive intermediate, $[Pd(R_2NCH_2CH_2NR_2)Cl(H_2O)]^+$. At high Met concentration, when R = H, a direct pathway involving $[Pd(en)Cl_2]$ was also observed. This reaction, however, was found to be supressed by steric hindrance, i.e. when R = Me or Et. Following monodentate coordination via the S-donor, a slower ring-closure (N,S) reaction of Met was observed, which also slowed down significantly on increasing the steric hindrance (H>Me>Et). In the case of S-methyl-Cys nucleophilic attack of a second molecule was found to compete with either a solvolysis or a ring-closure reaction.

Significant differences between the complex-forming behaviour of L-Met, L-Cys, D,L-homo-Cys and glutathione in reactions with the cisplatin analogue compound [meso-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine] dichloroplatinum(II) were also observed.[111] All the sulfur-containing compounds initially reacted with the Pt-complex forming ternary complexes. However, while the ternary complex was found to be the final product with Met, the thiol-containing ligands completely replaced the diamine derivative, resulting in bis-chelated binary complexes as final products. When complex formation reactions of the Met-containing complex of palladium(II) $[Pd(L\text{-}Met)Cl_2]$ with inosine or 5'-IMP was studied and the rate constants compared with those for the corresponding reactions of analogous ethylenediamine-/or ethylenediamine derived complexes, a

marked increase in reactivity of the former complex was found.[112] This lability was explained by the known high trans-influence of the S-donor and the labilization of the donor trans to this sulfur.

Interesting NMR results supporting the existence of five-coordinated intermediates in reactions of a platinum(II)-nitrolotriacetate system at pH~3 are reported.[113] All three 'glycinato arms' are coordinated in this case but ^{195}Pt NMR data suggest quite weak coordination of the axial carboxylate, for which the chemical shift is -1309 ppm compared to -1317 ppm for an authentic four-coordinate analogue.

Studies on platinum(IV) complexes are rare.[114,115] Reduction of platinum(IV)



Scheme 6

antitumour drugs by thiol-containing molecules prior to the interaction between platinum(II) and DNA was modelled by the reaction of trans-dichloro-tetracyanoplatinate(IV) with thioglycolic acid, L-Cys, DL-Pen and glutathione.

The kinetics and mechanism of Cr(VI) reduction by L-Cys were studied in neutral aqueous solution. A sequence of three pseudo-first-order processes was necessary to explain the observed spectral changes: (i) formation of a Cr(VI)-Cys bis-complex, (ii) its conversion into a precursor Cr(III) complex by sequential one-electron reduction with three Cys and (iii) the rearrangement of the precursor to the final product. The details of the proposed mechanism are shown in **Scheme 6**.[116]

A comparison of the results for complex formation reactions between iron(III) and Cys or penicillamine showed that much faster oxidation of Cys to the corresponding disulfide occurred. In addition to the determination of rate constants for the complexation reactions and for the oxidation reactions the initial absorbances were used to calculate equilibrium constants for the iron(III) complexes.[117] The kinetics and mechanism of substitution reaction between ruthenium(III)-edta and Cys were studied by the stopped-flow technique. The substitution was found to be first-order for both reactants. The rate constant showed a maximum in the pH-region 7.8-8.0.[118]

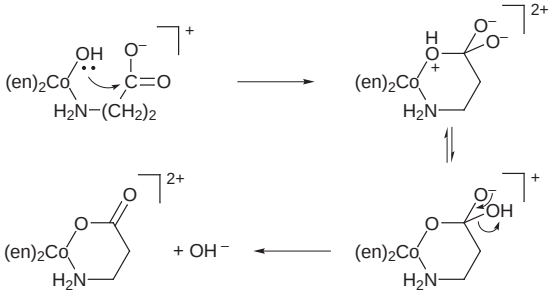
The reduction of cobalt(II) complexes of the amino acids Gly, Ala, Asn, Asp and Glu was studied.[119] The species were reduced on a mercury electrode at different pH values. Except for Asp, all the amino acids were found to coordinate to the metal ion as bidentate ligands and a mechanism for their reduction was suggested. Values of electrode reaction rate constants and dissociation rate constants for the different species were calculated. Stability constants for the complexes CoL, CoL_2 and CoL_3 with Gly, Ala, Asn and CoL and CoL_2 with Asp, Glu were also calculated and compared with literature data.

Base hydrolysis and mercury(II)-catalysed aquation of cis-$[Co(en)_2(\beta\text{-Ala})Cl]^{2+}$ and cis-$[Co(en)_2(\beta\text{-Ala-OMe})Cl]^{2+}$ were studied using UV-VIS, potentiometry and stopped-flow techniques.[120] In the initial compounds four nitrogens of en molecules, amino-N of β-Ala or β-Ala-OMe and chloride were coordinated. The following single kinetic process was found for base hydrolysis of cis-$[Co(en)_2(\beta\text{-Ala})Cl]^{2+}$:

$$\text{cis-}[Co(en)_2(\beta\text{-Ala})Cl]^{2+} + OH^- \rightarrow \text{cis-}[Co(en)_2(\beta\text{-Ala})OH]^{2+} + Cl^- \qquad (1)$$

In the case of cis-$[Co(en)_2(\beta\text{-Ala-OMe})Cl]$,$^{2+}$ containing the methyl ester derivative of β-Ala the situation is more complicated as not only was hydrolysis of the chloride observed but also the hydrolysis of the ester group resulting in the consumption of two moles of base per mole of complex. It was also observed that after the base consumption the pH of the solution began to rise with time due to the ring closure reaction shown in **Scheme 7**. Racemization reactions of fac-$[Cr(L\text{-Val})_3]$ complexes in DMF were studied by UV-VIS and optical rotation measurements.[121]

The $Cu(His)_2$ complex can catalyse the oxidation of ascorbate.[122] A kinetic and spectroscopic study of this system showed (i) a saturation effect of the oxidation



Scheme 7

rate at increasing O_2, ascorbate and $Cu(His)_2$ concentrations, (ii) negligible reduction of the metal ion to copper(I), (iii) the formation of an intermediate copper(II)$(His)_2$-ascorbate ternary complex. A reaction scheme involving the formation of intermediate complex, O_2 - catalyst - substrate was assumed.

The oxidation reactions of DOPA is the subject of an interesting study. Copper(II)- and nickel(II)-L-DOPA complexes were studied by CD and UV measurements using phosphate buffered solutions.[123] The mechanism of complex formation and oxidation of the coordinated ligand in the absence of and in the presence of the known catalyst poly-L-Lys are reported.

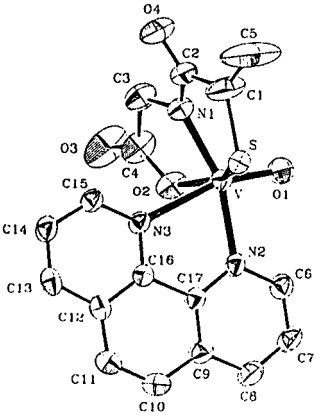
3 Peptide Complexes

3.1 Synthesis and Structures of Peptide Complexes – Metal binding sites of simple dipeptide molecules are generally characterized by the simultaneous coordination of the terminal amino and deprotonated amide nitrogens and carboxylate oxygen donor atoms. The structural parameters of the various peptide complexes are, however, significantly influenced by the presence of the coordinating and even non-coordinating side chain residues. As a consequence, the coordination chemistry of peptide molecules largely depends on both the nature of metal ions and the amino acid sequences of the peptide molecules.

Copper(II) complexes of L-Leu-L-Phe and L-Leu-D-Phe have been prepared and structurally characterized.[124] The coordination geometry of copper(II) is described as a distorted square pyramid with the dipeptide occupying three equatorial positions (N(amino), N(amide) and O(carboxylate)), the fourth being occupied by a carboxyl oxygen of an adjacent molecule. A water molecule and a peptide oxygen of another complex complete the coordination sphere of copper(II) in apical positions for L-Leu-D-Phe and L-Leu-L-Phe, respectively. Thus, the dipeptides act also as bridging ligands and both compounds exhibit a polymeric structure in the solid state. Dissolution in water results in breaking of the polymeric structure and an interaction between the metal ion and phenyl residue of L-Leu-L-Phe was found. The origin of the stacking interaction

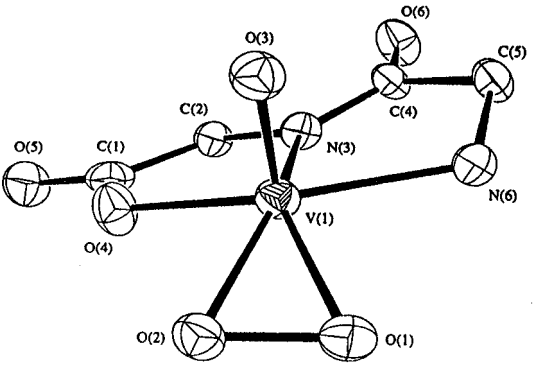
between the copper centre and the aromatic ring was also evaluated by molecular orbital calculations.

Vanadium is a trace element that produces significant physiological effects, including the insulin-like properties of both vanadate and various oxovanadium species. As a consequence, several peptide complexes of vanadium have been prepared and structurally characterized. The first dipeptide complexes of oxovanadium(IV) were obtained in ternary systems containing phenanthroline as second ligand.[125] The structure **(42)** of the anionic species $[VO(phen)L]^-$ (L stands for the trianion of N-(2-mercaptopropionylglycine)) shows a severely distorted octahedral geometry for VO(IV) and the dipeptide is coordinated via the thiol(S), deprotonated amide(N1) and carboxylate(O2) donor atoms. The ternary species [VO(phen)(GlyGly)] and [VO(phen)(GlyAla)] were also prepared and coordination of the terminal amino(N), deprotonated amide(N) and one of the carboxylate(O) donor atoms was concluded.[125] A crystalline glycylglycine complex of monoperoxovanadate has been obtained in another study[126] and its X-Ray structure determined **(43)**. The coordination of vanadium(V) is pentagonal bipyramidal with a peroxo group and a tridentate (amino(N), amide(N), carboxylate(O)) peptide molecule in the equatorial positions. The axial sites are occupied by the oxo group and one oxygen of the adjacent anion. The dimeric complex dissociates to monomeric units in aqueous solution, which were proposed to be responsible for the inhibition of the vanadium-catalysed decomposition of hydrogen-peroxide by glycylglycine.



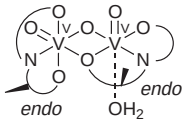
(42)

In case of L-salicylidene-L-valinate (Sal-L-Val) a dinuclear vanadium(V) complex **(44)** with the stoichiometry of $[V_2O_3(Sal\text{-}L\text{-}Val)_2(H_2O)]$ has been obtained in solid state.[127] The compound contains a double bridge formed by the oxygen donors of the oxo and carboxylate groups and is one of the rare examples where the two VO(V) groups make an angle near 90° to each other. A vanadium(IV) complex $[VO(Sal\text{-}Gly\text{-}Gly)(H_2O)_n]$ has been isolated from rela-



(43)

tively concentrated solutions containing oxovanadium(IV)-sulfate, glycylglycine and salicylaldehyde.[128] On the basis of EPR and IR spectral parameters of the complex the deprotonation and coordination of amide nitrogens were again shown. Vanadium(IV) complexes of the active-site peptides of the enzymes protein tyrosine phosphatases (PTPs) have been studied by EPR measurements.[129] The coordination of the nitrogen (N3) donor atom of the side chain imidazole of Val-His-Cys-Ser-Ala-Gly-NH_2 was shown below pH 6, while a switch to sulfur coordination of the cysteinyl residue was proposed in slightly basic media. The solution structure and rate of formation of the vanadium(V)-dipeptide complex of Gly-Tyr have been examined with EPR and multinuclear NMR measurements.[130] The coordination of the terminal amino(N), deprotonated amide(N) and carboxylate (O) donor atoms of the dipeptide was again shown and slow formation kinetics of the vanadium(V)-dipeptide interaction have been discussed.



(44)

Transition metal complexes of oligopeptides containing histidyl residues received increasing attention. Zinc(II) complexes of nine dipeptides and one tripeptide involving histidyl residues were prepared in the solid state and stoichiometric compositions and binding modes of the complexes were classified into several categories:[131] (a) ZnL_2 monomeric species were obtained in the case of His-Gly, His-Phe, His-His, His-Gly-NH_2 and His-Met-NH_2, in which only the N-terminal histidyl residues are coordinated via the amino and Im(N3) nitrogen donor atoms; (b) 1:1 complexes in polymeric structures were obtained in the case of Ala-His and β-Ala-His with the coordination of the amino and amide nitrogen

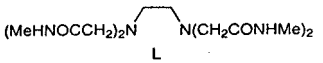
and the carboxylate oxygen donor atoms and with bridging imidazole residues; (c) the formation of 1:1 species was also characteristic of His-Asp and His-Gly-Gly, but in this case it was explained by charge neutralization and by further amide coordination, respectively; (d) the $Zn(Gly\text{-}His)_2$ bis complex was identified as a coordination polymer in the solid state containing octahedral zinc(II) ions with ZnN_4O_2 coordination pattern. The zinc(II) complexes of a series of tri-, tetra- and pentapeptides with His-$(X)_n$-His sequences were prepared in the continuation of the previous work.[132] Only 1:1 complexes were obtained in most cases and the coordination of both imidazole residues and the polymeric nature of the complexes were concluded. The only bis complex was obtained in the reaction of $Zn(ClO_4)_2$ with His-Gly-Gly-His and the coordination of four imidazole nitrogen donor atoms was proposed. The complexation of zinc(II) with Gly-L-His-Gly was followed by ^1H NMR spectroscopy in another study and the formation of the 3N-coordinated (amino, amide and Im(N3)) monomeric complex as the main species was concluded.[133]

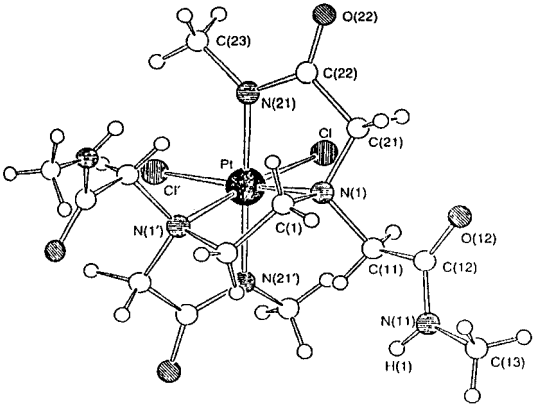
It has already been well-identified that carnosine (β-Ala-L-His) forms a stable dimeric complex with copper(II). The spectroscopic, magnetic and electrochemical behaviour of the dimeric species $[Cu_2(carnosine)_2(H_2O)_2]\cdot 2H_2O$ was studied recently and very high redox stability and very low superoxide dismutase (SOD) activity of the complex was observed.[134] On the other hand, based upon the results obtained from spectroscopic studies on the ternary systems containing copper(II), imidazole and glycylglycine the existence of some monomeric species in the copper(II)-carnosine system was proposed in solution.[135]

It is widely accepted that stable metal ion coordination of small peptide molecules is linked to the involvement of deprotonated amide nitrogens in metal binding. Several new examples of the metal ion promoted amide coordination have been reported and structurally characterized, including the various peptide or amide complexes of lead(II), platinum(II and IV), dialkyltin(IV), oxorhenium(V) and oxotechnetium(V). The binary and ternary (with bpy) complexes of lead(II) formed with N-tosylglycine, N-tosyl-β-alanine and N-benzoylglycine were investigated in aqueous solution by NMR spectroscopy and some of them were also characterized crystallographically.[136] It was found that the ligands behave as simple carboxylates at acidic pH, but they switch to dianionic bidentate (N,O)-chelating ligands around neutrality due to the involvement of the deprotonated amide nitrogen as an additional donor site. The results obtained for the N-benzoylglycine system are particularly interesting, because lead(II) is the only metal ion found so far to substitute the proton of the -CONH- moiety in binary complexes with N-carbonyl amino acids. Stability constants of the various lead(II) complexes have also been determined and it is noteworthy that the log β values obtained for PbL complexes of N-tosylglycine and N-tosyl-β-alanine are not affected by the chelate ring size.[136]

The reaction of the ligand 1,2-diaminoethane-N,N,N',N'-tetra(N-methylacetamide) (L) with $K_2[PtCl_4]$ resulted in the formation of a platinum(IV) complex cis-$[Pt(LH_{-2})Cl_2]$, which is the first reported structure of a platinum(IV) species containing deprotonated amide nitrogen.[137] The method of the formation of the complex is also novel, because it appears that the presence of the deprotonated

amide groups results in facile oxidation of platinum(II) to platinum(IV) in the presence of air, a reaction that normally requires the addition of oxidizing agents. The X-Ray structure of the complex **(45)** revealed that LH_{-2} is a tetradentate ligand coordinated to the metal through the amino groups and two deprotonated amide nitrogen donor atoms. The complex contains an octahedral platinum(IV) with chloride ligands cis and amide groups trans to each other. The Pt-N(amide) bond distance is a bit shorter than the Pt-N(amine) distance, consistent with the fact that it is a better σ-donor. On the other hand, the platinum(II) complex of glycylglycine, trans-$[PtCl_2(Gly\text{-}GlyOH)_2]$, containing monodentate dipeptide ligands has also been synthesized, but the attempts to oxidize this to a platinum(IV) complex resulted in the hydrolysis of the peptide ligand and in the formation of the amino acid complex trans-$[Pt^{IV}Cl_2(GlyO)_2]$.[138]



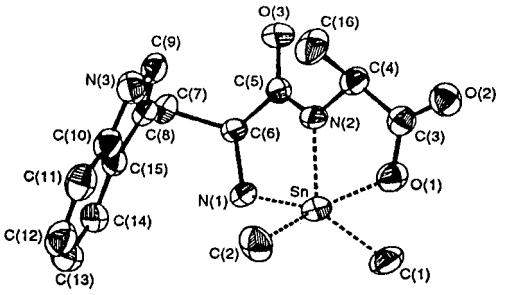


(45)

Platinum(II) and palladium(II) complexes of several other ligands containing the amide groups have also been studied. Deprotonation and coordination of the amide groups were reported to occur in the bis complexes of platinum(II) and palladium(II) with the amide and N-methylamide deivatives of 2-hydroxyimino-propionic acid and with the oximes of pyruvoylglycine and pyruvoyl-L-phenylalanine.[45] Platinum(II) complexes of several dipeptides, including X-Gly and Gly-X (where X stands for Gly, Ala, Val and Leu), have been prepared and structurally characterized.[139] The complexes were identified as anionic species with the stoichiometry of K[Pt(X-Gly)Cl] or K[Pt(Gly-X)Cl] containing square planar platinum(II) coordinated via the characteristic amino(N), amide(N) and carboxylate(O) binding sites and with a halide ion at the fourth equatorial position. The antitumour activity of the complexes K[Pt(Ala-Gly)Cl] and

K[Pt(Gly-Ala)Cl] against methylcholantrene-induced Meth A fibrosarcoma has also been measured and a slight growth inhibition was observed. Cisplatin, cis-[diammindichloro-platinum(II)], did not show any antitumour activity under these conditions and the dipeptide complexes were reported to be 1000 times less toxic than cisplatin.[139] Imidazole nitrogens of histidyl residues are among the preferred binding sites of soft elements including platinum(II). As a consequence, the ternary systems containing cis-[diamminediaquaplatinum(II)]$^{2+}$ cation with histidine and its peptide derivatives have been thoroughly studied by ^{15}N and ^{195}Pt NMR spectroscopy.[140] The formation of carboxylate bonded species was detected initially at low pH values (2-3) and they were transformed into an amino(N), carboxylate(O)-bonded chelate upon standing. Increasing the pH to 8-9 resulted in the formation of another chelate via Im(N3), amide(N)-coordination. From these data it was concluded that the nitrogen atom of an exposed imidazole ring away from either terminus of a peptide chain will readily coordinate to platinum(II) and this coordination can be considered as an anchor for the formation of the 6-membered Im(N3), amide(N)-chelate ring.[140]

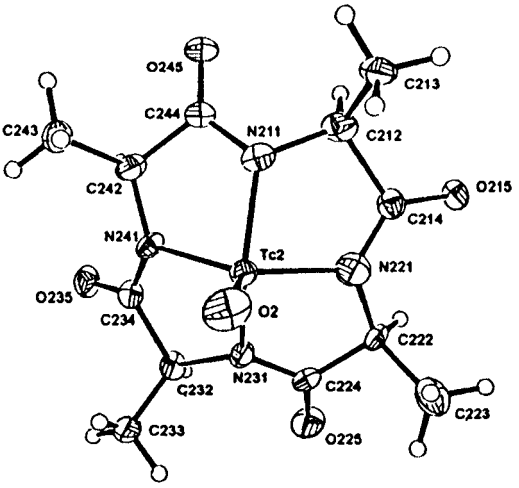
The crystal and molecular structure of dimethyl(L-tryptophyl-L-alaninato)-tin(IV)-methanol(1/1) **(46)** has been determined by X-Ray crystallography and the molecular dynamics of three dipeptide complexes of $R_2Sn(IV)$ have been investigated by variable-tempereture ^{119}Sn Mössbauer spectroscopy.[141] A five-coordinated trigonal bipyramidal environment of tin(IV) was proposed in the complex **(46)**, two methyl carbons, the terminal amino nitrogen, deprotonated peptide nitrogen and terminal carboxylate oxygen donor atoms being the metal binding sites. The monomeric structures of the complexes were interconnected through hydrogen bonds, which appear to be much stronger in Me_2Sn(Trp-Ala) and Me_2Sn(Trp-Tyr) than in Ph_2Sn(Trp-Ala). Similar tridentate binding of dipeptides was reported in the $R_2Sn(IV)$ (R = Me or Ph) complexes of Trp-Ala, Trp-Tyr, Trp-Trp and His-Tyr,[142] while monodentate binding of N-acetyl amino acids and N-acetyl di- or tripeptides was concluded in the interactions of the ligands with triphenyltin(IV).[143]



(46)

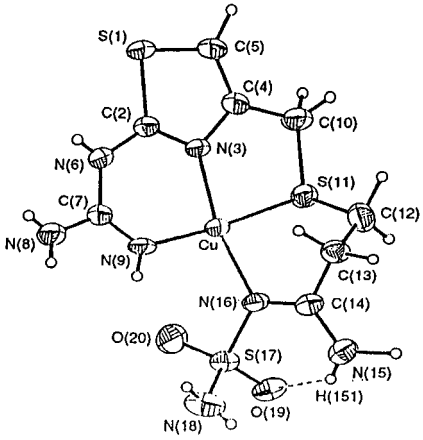
The coordination of amide nitrogen atoms was also reported in the rhenium(V) and technetium(V) complexes of various peptide molecules. In the interaction of

oxorhenium(V) with mercaptoacetylglycine ethyl ester the formation of a bis complex was concluded containing S(thiol), N(amide) and S(thiol), O(carbonyl) bidentate ligands at the equatorial sites.[144] The oxorhenium(V) complex of mercapto acetyl-triglycine $Na_2[ReO(MAG_3)]\cdot 5H_2O$ has been structurally characterized.[145] The dianionic complex was described as a distorted square pyramid with one sulfur and three nitrogen donor atoms forming the base and the oxo ligand at the apex. In case of oxotechnetium(V) the coordination of 4N donors (amino and three deprotonated amides) were reported in the complexes of several tetrapeptides.[146] The formation of similar species was identified in the initial interaction of oxotechnetium(V) with tetraalanine, but this converted into a complex of cyclic tetraalanine, as shown in structure **(47)**.[147]



(47)

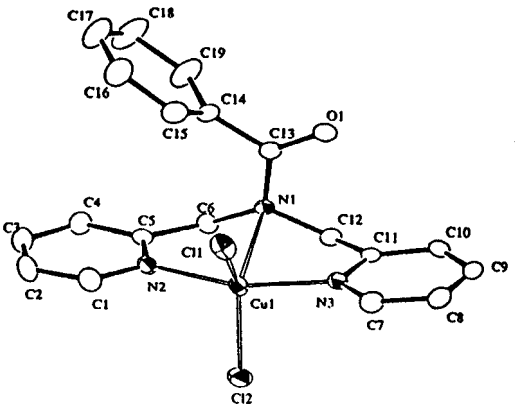
Several oligopeptides and derivatives are directly used in clinical therapy or they represent the active sites of metalloenzymes or peptide hormones. Metal complexes of these ligands are particularly interesting and have been widely studied both in solution and in the solid state. Famotidine (3-{[2-(diaminomethyleneamino)-thiazol-4-yl]-methylsulfanyl}-N^2-sulfamoyl-propionamidine) is an efficient anti-ulcer drug having many metal binding sites, including the amide type nitrogens. Remarkable metal-binding ability of the ligand was shown and structure **(48)** of the complex $[CuL](ClO_4)_2$ (L is famotidine) has been determined X-Ray crystallographically.[148] The binding sites of neutral famotidine involves the thioether sulfur (S11), the thiazole ring nitrogen (N3), guanidine nitrogen (N9) and amidine terminal nitrogen (N16) in nearly planar geometry. The copper(II)-famotidine-histidine ternary system was studied in solution by potentiometric and spectroscopic measurements and it was proposed that famotidine is a very competitive chelating agent even in the presence of strongly coordinating amino acids.



(48)

Methionine enkephalin is a pentapeptide (Tyr-Gly-Gly-Phe-Met), which can form rather stable complexes with transition metal ions if the amide nitrogens are involved in metal binding. A ^{1}H NMR study on the complexes of barium(II), calcium(II), zinc(II), lanthanum(III), magnesium(II), strontium(II) and cadmium(II) with methionine enkephalin, however, indicated only a weak interaction between the metal ions and the peptide ligand, supporting that neither the terminal amino nor the amide nitrogen donor atoms take part in metal ion coordination.[149] Internal or C-terminal prolyl residues of peptides containing the secondary amide groups are generally considered as 'break-points' for the metal ion coordination of peptide amide nitrogen atoms. As a consequence, captopril (1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline) behaves as a monodentate thiol-containing ligand forming polymeric species with gold(I). However, a comparison with various other gold(I) antiarthritic drugs shows that Au(I)-captopril forms polymers of much higher degree than other thiols.[150] In contrast with the previous findings on the nonbonding nature of secondary amides, copper(II) was reported to catalyse amide isomerization and it was explained by the involvement of secondary amide nitrogens in metal binding.[151] The existence of this type of coordination was proved by the X-Ray structure determination of the copper(II) complex of a secondary amide ligand shown in structure **(49)**. A direct copper(II)-N(secondary amide) bond is shown in this complex with a Cu-N distance of 0.249 nm, which is significantly higher than the Cu-N(pyridine) distances of 0.204 and 0.202 nm.[151]

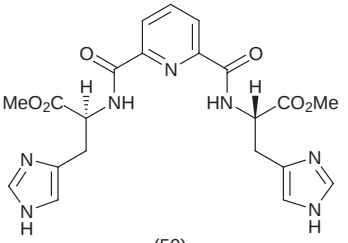
It has already been well documented that conformation of peptide molecules is significantly influenced by metal ion coordination. The structure of the mercury(II) complexes of -Cys-Pro-Leu-Cys- peptide sequences were studied by various spectroscopic techniques.[152,153] It was concluded that linear coordination of mercury to the thiol sulfur atoms regulates the Cys^{1}-Pro-Leu-Cys^{4} units to



(49)

form a new type of turn structure with two hydrogen bonds between Cys[1](S)-Leu(NH) and Pro(CO)-Cys[4](NH).

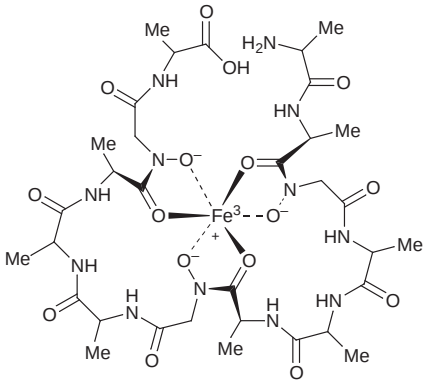
Several papers deal with the synthesis and metal ion coordination of peptide derivatives containing unnatural amino acids. It has been proved that metal binding ability of peptides can be significantly increased if powerful bidentate ligands are incorporated in the molecules. Amino acid derivatives containing chelating side chains (2,2'-bipyridine or phenanthroline) at the β-carbon atom were prepared and involved in several hexapeptides with natural amino acids.[154] It was concluded that the novel chelating amino acids generally bind metal cations with higher affinity than the naturally occurring residues due to the bidentate nature of the side chain functionality. A novel artificial peptide named HPH-pep comprising a pyridine and two histidine units **(50)**, was synthesized. Its copper(II) complex has a unique pentacoordinated structure, in which the pyridine nitrogen, two deprotonated peptide nitrogens and two imidazole bind to copper(II).[155] Concerning the superoxide-quenching profile of Cu(II)-HPH-pep it was found that it did not scavange hydrogen peroxide or hydroxyl radicals and hence the scavanging activity was specific to superoxide, and the complex did not generate hydrogen-peroxide upon scavanging superoxide.



(50)

A pair of linear and cyclic peptide based trihydroxamate ligands have been prepared through fragment condensation of suitably protected Ala-Ala-β(OH)Ala units. Hexadentate octahedral complexes were formed with iron(III), but the metal ion-holding capacity of these complexes is still lower than that of ferrioxamine-B.[156] The iron(III) complex of a dodecapeptide trihydroxamic acid of an Ala-Ala-(OH)Gly-Ala sequence was prepared in another study.[157] The complex has a well-defined peptide loop structure **(51)** and exhibited siderophore activity for a test microorganism.

Two cyclic-branched peptides (Azu and Pla) were synthesized as models of the blue copper proteins azurin and plastocyanin, respectively. Spectroscopic measurements clearly indicate the interaction of copper(II) with both peptides; however, their coordination geometry is still uncertain.[158] The CD titration of Azu with mercury(II) ion indicates the existence of two species, $[AzuHg]^{+}$ and $[AzuHg_2]^{3+}$.



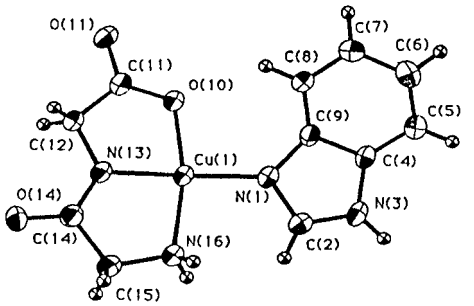
(51)

It has already been exemplified by the previous references and by earlier literature studies that the metal ion coordination of deprotonated amide nitrogens generally requires the presence of a primary ligating group or 'anchor', which is in a chelating position with the nitrogen donor atom of the amide group. In the case of peptides the terminal amino group is the most common anchor, but various side chain residues can have the same effect. In the copper(II) complexes of salicylglycine the deprotonated phenolate-O donor atom was reported to behave as an anchor. The species $[Cu(LH_{-1})]\cdot 2H_2O\cdot MeOH$ was prepared under basic condition in the reaction of copper(II) with salicylglycine, in which the metal ion is coordinated via the carboxylate and phenolate oxygen and the deprotonated peptide nitrogen donor atoms. In contrast with the results of the copper(II)-containing systems, complexes of composition $M(HL)_2$ were obtained in case of nickel(II) and cobalt(II) and the coordination of only the carboxylate oxygen donor atom was concluded.[159]

N-Acetyl amino acids and peptides are generally monodentate ligands, unless an effective side chain donor atom promotes the deprotonation and coordination

of the amide groups. The formation of S(thiol)-bonded dimeric complexes was reported in the interaction of platinum(II) with N-acetyl-L-cysteine,[160,161] while bidentate coordination (S(thioether,N(amide)) of N-acetyl-L-methionine was described in the reaction with the anticancer drug carboplatin.[161]

Several papers have been published on the ternary complexes of transition elements containing peptide or amide ligands. Tridentately bonded dipeptide complexes of transition elements were prepared in one group of these studies and their interactions with various monodentate and bidentate ligands were characterized.[162-165] A new ternary complex of glycylglycine with copper(II) and benzimidazole (bzim) [Cu(Gly-Gly)(bzim]·$3H_2O$ **(52)** has been prepared with an approximately square planar geometry of copper(II) via the coordination of a tridentate (NH_2, N(amide),O(carboxylate)) dipeptide and N(1) of benzimidazole ligand.[162]

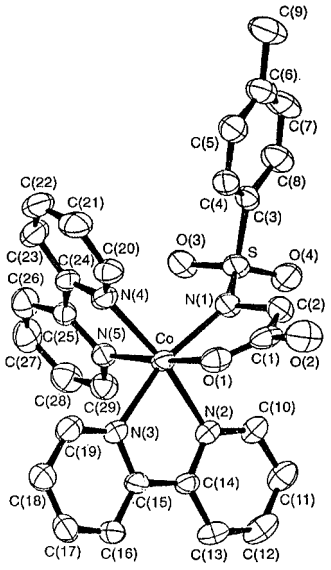


(52)

Glycyl-DL-methionine forms stable square planar complexes with platinum(II) and palladium(II) through the coordination of terminal amino and deprotonated amide nitrogen and thioether sulfur donor atoms. The fourth coordination sites of the metal ions can easily interact with nitrogen donors of various nucleobases, which was studied by spectroscopic techniques.[163] In case of copper(II) the ternary complexes of [Cu(dipeptide)$(nucl)_2$]$Cl_2.H_2O$ (where dipeptide stands for Gly-Gly,Gly-Ala,Gly-Val and Gly-Leu; nucl=inosine and guanosine) were obtained and (NH_2,CO)- and (NH_2,N(amide))-coordination of the dipeptides were proposed at low and at high pH values, respectively.[164] The presence of 5-coordinated copper(II) was concluded in the ternary system containing copper(II), glycylglycine and phenanthroline, the latter bidentate ligand occupying an equatorial and an axial coordination site.[165]

In another group of ternary systems the coordination of N-acetyl amino acids and peptides were studied in the presence of stable chelating ligands.[166-168] In the case of N-acetyl-phenylglycine only monodentate (carboxylate) coordination of the ligand was observed in the cobalt(II), nickel(II) and copper(II) complexes of various nitrogen donors,[167] while in the case of sulfonamides deprotonation and coordination of the amide groups was proposed in the nickel(II) and cobalt(II) complexes containing 2,2'-bipyridine as the second ligand.[168] The crystal and

molecular structure of the species $[Co(bpy)_2L]\cdot 2H_2O$ (L = N-p-tosylsulfonylglycinate dianion) shows an octahedral cobalt ion in complex **(53)** and N(amide),O-(carboxylate)-coordination of the sulfonamide. The pK values for amide deprotonation were 7.8 and 7.4 in the cobalt(II) and nickel(II) complexes, respectively, but in the case of N-tosylsulfonyl-β-alanine and N-benzoylglycine the hydrolysis of the metal ions prevented the formation of the deprotonated complexes.[168]



(53)

For mixed hydroxo complexes of cobalt(III) which also contain peptide ligands, the preparation and characterization of a series of dihydroxo bridged dinuclear complexes have been described.[169]

Mass spectrometry is a widely used technique to study the metal binding pattern of peptide complexes in the gas phase.[170–175] These studies revealed that cysteinyl thiol[170] or histidyl imidazole nitrogen donor atoms[171,172] are generally the main metal binding sites. However, the involvement of the C-termini and other oxygen donor atoms and the deprotonated amide nitrogen in metal binding was also concluded and it was shown that the non-coordinated side chain residues also have a significant impact on the fragmentation of peptide complexes.[173–175]

3.2 Reactivity - Metal-ion-assisted Transformations of Peptide Molecules – Metal ions generally have a significant influence on the the reactivity of peptide molecules, including the formation, oxidation, decarboxylation and hydrolysis of peptides. Thiol-containing peptides, especially glutathione (γ-L-glutamyl-L-cysteinylglycine) are readily oxidized by various metal ions. Among them the

chromium(VI)- and copper(II)-catalysed oxidations seem to be the most interesting and have been studied recently.

Chromium(VI) has been proven to be an extremely toxic and carcinogenic substance. This is explained by the reduction of Cr(VI) to various Cr(V) or Cr(IV) species by cellular components including peptides containing thiol groups, like glutathione. The kinetics of the oxidation of D-penicillamine and glutathione by potassium chromate was studied under basic conditions.[176] In the case of glutathione a biphasic reaction was observed and explained by the formation of an $RS\text{-}CrO_3^-$ intermediate followed by its slow decomposition. The various steps of the reduction of chromium(VI) are represented by equations (2-5), where R stands for glutathione:

$$RSH + HOCrO_3^- \rightleftharpoons RS\text{-}CrO_3^- + H_2O \quad (2)$$

$$RSH + RS\text{-}CrO_3^- \rightarrow Cr(IV) + R\text{-}S\text{-}S\text{-}R \quad (3)$$

$$RS\text{-}CrO_3^- \rightarrow Cr(V) + RS^{\cdot} \quad (4)$$

$$H^+ + RS\text{-}CrO_3^- \rightarrow Cr(V) + RS^{\cdot} \quad (5)$$

The formation of similar $RS\text{-}Cr^{VI}O_3^-$ species was identified in the reaction of $Cr_2O_7^{2-}$ with γ-glutamylcysteine, N-acetylcysteine, cysteine and the methyl ester of N-acetylcysteine.[177] The chromium(VI)-thiolate species were reported to be more stable in DMF than in aqueous solution and their stability towards reduction in aqueous solution followed the order: cysteine < N-acetylcysteine < γ-glutamylcysteine < glutathione. The exclusive formation of a chromium(IV) intermediate was concluded in another study[178] on the reaction of chromium(VI) with glutathione in the presence of glycine in acidic media. The intermediate reacts with the tripeptide, resulting in the formation of the final products, chromium(III) and oxidized glutathione.

Copper(II) is known to oxidize thiols spontaneously, which results in the formation of disulfides and copper(I) complexes. The role of dioxygen in the copper-catalysed autooxidation of cysteine in presence of glycylglycine-phosphate buffer was followed spectrophotometrically.[179] It was found that in this case cysteine is oxidized not only by the copper(II) species, but also by a copper(I)-O_2 adduct and the oxidation step catalysed by the latter species was proposed to be the rate-determining. Stable ternary complexes containing Cu(II)-S(thiol) bonds were obtained in another study.[180] Tetradentately coordinated (NH_2,N,N,COO) copper(II) complexes of tripeptides (Gly-Gly-X, X = Gly, Leu and β-Ala) were reacted with N-acetyl-L-cysteine and L-cysteine. Substitution of carboxylate of tripeptides by S(thiol)-coordination was proposed in both cases, followed by bidentate (NH_2,S)-coordination of the amino acid instead of the second amide nitrogen of the tripeptide.[180]

Selective cleavage of peptides and proteins is one of the most common and most important procedures in biochemistry. More and more examples show that various metal ions (especially palladium(II) and probably copper(II)) can have a catalytic role in that respect. Palladium(II) complexes are generally anchored by a heteroatom of a side chain residue of the peptide and catalyse hydrolysis of the amide bond involving the adjacent carbonyl group. The thioether group of

methionine is the best studied anchor and hydrolysis of MetAla[181] and N-acetyl-MetGly[182] have been studied in the presence of various palladium(II) complexes. It was found that both coordination complexes and organometallic palladium-containing species can promote selective hydrolysis of peptide bonds and can do so even in solvents containing little water. In the comparison of the catalytic activity of differently bonded palladium(II) complexes the order of (N,N) > (S,N) > (S,S) has been concluded and the importance of polynuclear complexes in the hydrolytic cleavage of peptides discussed.[182]

The imidazole nitrogen atom of a histidyl residue can be considered as another anchor for the palladium(II)-catalysed hydrolytic cleavage of peptides.[183,184] The reaction of $[Pd(en)(H_2O)_2]^{2+}$ with His-Gly and its derivatives revealed that only the catalyst bound to the N3 of imidazole can effect the hydrolysis of the amide bond of His-Gly.[183] The regioselectivity of this cleavage was also reported, because in the reaction of $[Pd(en)(H_2O)_2]^{2+}$ with various dipeptides and Ac-Gly-His-Gly only the amide bond involving the carboxylic group of histidine is cleaved, while the amide involving amino group of His is not affected.[184]

The catalytic activity of copper(II) in the hydrolytic cleavage of peptides has also been reported.[185] It was found that copper(II) cleaves with moderate specificity the oligopeptides containing internal -Ser-His- or -Thr-His- sequences at the N-terminal site of Ser or Thr. The activity is about 100-fold lower for peptides lacking histidine.

Metal ions can influence not only hydrolysis, but formation of peptide molecules and it is especially interesting under possible prebiotic conditions.[186,187] The copper(II) induced peptide formation was studied in systems containing glycine and α- or β-alanine and α- or β-aminobutyric acid and the formation of peptides resulting from all possible amino acid combination was observed, but α-amino acids were preferably linked to glycine when competing with their β-analogues.[186] The effect of exchangeable cations (alkali, alkaline and aluminium ions) on montmorillonite-catalysed peptide formation was investigated in another study.[187] The role of cobalt(III)-ligated peptides as acyl acceptors in peptide synthesis was also reported.[188] It was concluded that positively charged metallopeptides were more efficient nucleophiles than the corresponding amides or free peptides.

The kinetics of the oxidation of Gly-Gly by electrolytically generated manganese(III) was investigated in aqueous sulfuric acid[189] and the following stoichiometry was proposed:

$$4Mn(III) + NH_2\text{-}CH_2CONH\text{-}CH_2\text{-}COOH + 3H_2O \rightarrow \\ \rightarrow 4Mn(II) + 2HCHO + 2CO_2 + 2NH_4^+ + 2H^+ \qquad (6)$$

The reaction shows a first order dependence on manganese(III) and the reduced product, manganese(II), has no significant effect on the rate.

Doubly deprotonated copper(III) complexes of several tripeptides including C-terminal histidine or histamine were prepared and structurally characterized.[190] The presence of histidine resulted in a decrease of the pK values of coordinated amino groups as compared to those of other tripeptide complexes and very rapid

oxidative decarboxylation of the complexes was observed. The kinetics of the substitution reactions between the deprotonated Gly-Gly complex of copper(II) and various tetra-aza macrocycles has been studied by stopped-flow techniques[191] and it was concluded that the more thermodinamically stable complexes react more slowly.

The oxidase activity of copper(II) complexes containing coordinated histidyl peptides was studied via the autooxidation of ascorbic acid. The oligopeptides His-$(His)_n$-Gly, N-Ac-His-$(His)_n$-Gly (n = 3,8 and 18) and N-Ac-His-$(His)_n$-His-$NH\text{-}CH_3$ (n = 2,3 and 4) were prepared and catalytic activity of copper was evaluated in terms of the dependence on structure of peptides and metal ion to ligand ratio.[192]

3.3 Solution Equilibria - Stability Constants of Metal-ion–Peptide Complexes – The stoichiometry of the complexes formed in the reaction of copper(II) with simple tri- or tetrapeptides are generally characterized by the existence of only monomeric 1:1 complexes in solution. A recent potentiometric and spectroscopic study on the copper(II) complexes of oligoglycines and several related ligands revealed that several bis complexes are also present at intermediate pH values.[193] Namely, in the case of tetraglycine the complexes $[CuL],^+$ $[CuLH_{-1}]$, $[CuLH_{-2}]^-$ and $[CuLH_{-3}]^{2-}$ were identified as the main species in equimolar solutions, which correspond to the (NH_2,CO)-, (NH_2,N,CO)-, (NH_2,N,N,CO)- and (NH_2,N,N,N)-coordinated complexes, respectively. In the presence of excess tetraglycine the 4N-coordinated species $[CuLH_{-3}]^{2-}$ is again the main component at high pH values, but several bis-complexes were detected in the pH-range 5 to 9. The formation of these species was explained by the unsaturated coordination sphere of $[CuLH_{-1}]$ and $[CuLH_{-2}],^-$ which can easily bind a second tetraglycine via (NH_2,CO)-coordination resulting in the stoichiometries $[CuL_2H_{-1}]^-$ and $[CuL_2H_{-2}].^{2-}$ The stability constants and the characteristic EPR and UV-visible spectral parameters of the oligoglycine complexes are reported and the effect of the size of chelate rings on the spectral parameters of copper(II) complexes is also discussed.[193] The UV-circular dichroism spectral map of the positions of the charge transfer bands involving donor groups of peptides and other biologically important ligands was reported in another study on various copper(II) complexes.[194]

The origin of thermodynamic stereoselectivity of proton and copper(II) complexes with diastereomeric dipeptides containing aromatic side chains (L-Ala-L/D-X, X = Ala, Phe, Trp) was studied by potentiometric, calorimetric and spectroscopic measurements.[195] It was found that protonation constants of the amino groups are higher for the (L,D)-diastereomers than for the (L,L)-isomers, while the opposite is true for the carboxylate groups and it was concluded that protonation of the amino group is favoured enthalpically, while the protonation of carboxylate is favoured entropically. The complex $[CuLH_{-1}]$ was the main species in all cases, but the (L,L)-isomers formed more stable complexes than the (L,D)-ones.

The involvement of α,β-dehydro-amino acids in peptide sequences significantly influences the metal binding ability of the ligands. The copper complexes of

dipeptides Pro-Δ-X (X = Ala, Abu, Val, Phe) containing the double bond or trigonal carbon at the C-termini and their saturated counterparts were studied by potentiometric and several spectroscopic techniques.[196] On the one hand, it was concluded that the stability constants of the complexes with dehydro-peptides are higher than those of the saturated analogoues. However, the formation of bis complexes $[CuL_2H_{-2}]^{2-}$ with 4N-coordination in slightly basic solution was reported to occur in the systems containing Pro-Δ-X (where X stands for Abu, Val and Phe), but the formation of this species was excluded in the case of dipeptides with C-terminal Δ-Ala.

Most of the solution studies on the peptide complexes deal with peptides containing side chain residues, which are, at least in principle, able to coordinate strongly the metal ions. Among them the peptides of histidine are probably the most interesting and the best studied. Complex formation between copper(II) and the peptide hippuryl-L-histidyl-L-leucine was investigated in aqueous solution by means of potentiometric, UV-VIS and CD measurements.[197] The complexes $[CuL],^{+}$ $[CuLH_{-2}]^{-}$ and $[CuLH_{-3}]^{2-}$ were identified as the main species with increasing pH, and these contain N3(Im)-monodentate, (2N(amide) + N3(Im))-tridentate and (2N(amide) + N3(Im) + N(amide)-axial)-tetradentate ligands, respectively. The system can be considered as a model for the coordination properties of internal chain proteins. Copper(II) complexes of Gly-Sar-L-His-Gly were studied potentiometrically and spectroscopically.[198] The results confirmed the previous findings that internal sarcosyl (and prolyl) residues work as a 'break-point' for amide coordination. The presence of a histidyl residue, however, increased the metal-binding ability of the tetrapeptide and $[CuL]^{+}$ and $[CuLH_{-1}]$ were described as the main species. The coordination of the terminal amino nitrogen and the carbonyl oxygen donor atoms of the Gly-Sar bond and the Im(N3) of His was suggested for $[CuL],^{+}$ while $[CuLH_{-1}]$ is described as a $Cu(N_3O)$-chromophore, in which the amino group, the deprotonated amide of the Sar-His bond and the Im(N3) nitrogen atoms are the metal binding sites. The hydrolysis of the peptide at Gly-Sar bond in the presence of copper(II) was also reported.[198]

The previous studies revealed that in addition to the terminal amino group the side chain imidazole nitrogen donor atoms can act as an anchor for amide coordination. This finding was confirmed by the results obtained on the copper(II) complexes of angiotensin II fragments.[199] Complex formation between copper(II) and the hexapeptide Asp-Arg-Val-Tyr-Ile-His and the corresponding N-terminally shortened pentapeptide was studied by potentiometric and spectroscopic techniques and the competition between the amino and imidazole nitrogen donor atoms in anchoring the metal ions was discussed as a function of pH and the distance of the competing residues.

Zinc(II) complexes of eight dipeptides containing His and Cys residues and N- and C-protected derivatives were studied via potentiometric titrations.[200] The 1:1 complexes were the dominant species in all cases and the formation of bis complexes was reported only in the case of protected peptides. The complexes were also obtained in solid state, with the composition of [LZnX] (X = halide ion) or $[L_4Zn_5X_2]$ containing tetrahedral zinc(II) ions and at least tridentately

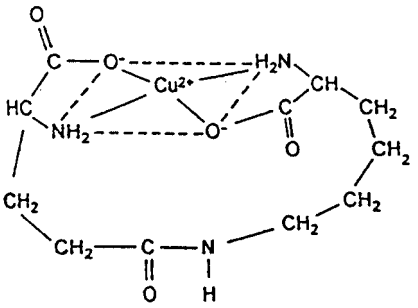
bonded dipeptides. It was also proposed that the complexes are monomers in solution and thiolate- bridged polymers in the solid state. The results obtained on the zinc(II) complexes of dipeptides containing C- or N-terminal His or Cys residues were reported in another study.[201] The formation of seven- to ten-membered chelate rings was assumed to explain the remarkable stability of the zinc(II) complexes.

Transition metal complexes of di- and tripeptides containing histamine instead of the C-terminal histidyl residues were studied by the combined application of potentiometric and spectroscopic techniques.[202,203] Stability constants and spectral parameters of cobalt(II) and nickel(II) complexes of glycylhistamine and sarcosylhistamine were compared to those of glycylhistidine. The formation of a similar 3N-complex (amino(N), amide(N) and Im(N3)-coordination) as the main species was concluded, but the stability constants for the mono complexes of the histamine-containing peptides were slightly lower than those of Gly-His, while the bis complexes were formed in higher concentrations. The existence of polynuclear species via (N1,N3)-imidazole bridging was reported in the nickel(II)-containing systems at pH>9. In the case of glycylhistamine NMR studies show the existence of tetrameric units, while the steric hindrance of the N-CH_3 group in sarcosylhistamine produces a series of coexisting oligomers with n>4.[202] Copper(II), nickel(II) and cobalt(II) complexes of the tripeptide glycylglycylhistamine were studied by potentiometric, EPR and NMR spectroscopic methods. In case of copper(II) and nickel(II) the species $[MLH_{-2}]$ predominates around physiological pH. The coordination of four nitrogen donor atoms (amino(N), 2 amide(N) and Im(N3)) was proved by single crystal X-Ray analysis of the copper(II) complex. However, in the case of cobalt(II) the species $[CoL]^{2+}$ predominates in the same pH range, in which the terminal amino and imidazole nitrogen donor atoms are coordinated in the form of a macrochelate. The 4N-species of cobalt(II) was formed at much higher pH values. Further deprotonations in basic media (formation of $[MLH_{-3}]^-$) was assigned to N1H deprotonation of imidazole without metal ion coordination of this nitrogen atom.[203]

The role of the extra carboxylate groups of aspartyl and glutamyl residues in the coordination chemistry of peptides was studied in another group of reports.[204–206] The complex formation reactions of nickel(II) and zinc (II) with the tripeptide Asp-Asp-Asp and a series of tetrapeptides containing one or two Asp or one Glu residues were studied by potentiometric and various spectroscopic methods.[204] It was concluded that the glutamyl residue has no significant effect on the complex formation processes of these peptide molecules, although the increase in stability of the [ML] species is higher for nickel(II) than was reported for copper(II) complexes, suggesting that the γ-carboxylate group has the most pronounced effect on the stability of the 1N-species. On the other hand, the β-carboxylate of the Asp residue stabilizes the complexes significantly, particularly when present as the N-terminal residue. As a consequence, deprotonation and coordination of subsequent amide nitrogens are suppressed in the case of nickel(II) and this process does not take place at all in the case of zinc(II). Two new functionalized dipeptides cyclo(-L-aspartyl-L-aspartyl)bis(histamine) and its glutamyl analogue were synthesized and their copper(II) complexes and SOD-

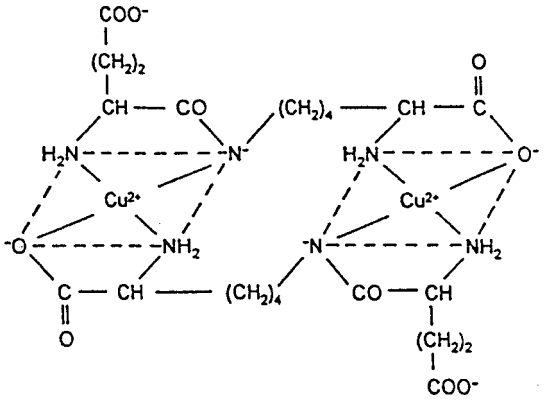
activity studied.[205] It was concluded that the imidazole(N3) nitrogen and the carbonyl oxygen donor atoms were coordinated (species $[CuL]^{2+}$) in equimolar solution and below physiological pH, while the species $[CuLH_{-2}]$ with 4N-coordination (2 Im(N3) + 2 amide(N)) predominates in alkaline solution. Another 4N-complex, $[CuL_2]$,$^{2+}$ with four imidazole nitrogen coordination was formed in the presence of excess of ligand and it was the most active species against superoxide.

In the case of monoamino-dicarboxylic acids (e.g. glutamic acid) and diamino-monocarboxylic acids (e.g. lysine) different part of the molecules (α- and γ- or α- and ϵ-sites) can be involved in the peptide bond. Copper(II) and nickel(II) complexes of L-γ-Glu-L-ϵ-Lys and L-Glu-L-ϵ-Lys were studied by means of potentiometric and spectroscopic measurements.[206] In the case of the derivatives of γ-Glu the amino acid residues and the amide bonds are too far from each other and coordination of the amide nitrogens is not favoured. The species [ML] predominates in both cases containing bis(N,O)-bonded metal ions and a 14-membered loop around the metal ions **(54)**. In L-glutamyl-L-ϵ-lysine the amino group of Glu is in the position to form a chelate with the amide nitrogen and this type of coordination is characteristic of both copper(II) and nickel(II). Solution equilibria of these systems are, however, rather complicated, because various polynuclear species can be formed involving the $[CuL_2H_{-2}]$ dimeric species **(55)**.



(54)

Oxytocin and vasopressin are well characterized peptide hormones of neurohypophysis consisting of nine amino acids with an internal disulfide bridge between Cys^1 and Cys^6 residues. The copper(II) complexes of oxytocin and the derivatives 5-Asp-oxytocin, 4-Glu-oxytocin and Gly-Gly-Gly-Lys^8-vasopressin were studied by potentiometric and spectroscopic measurements.[207] The formation of 4N-coordinated (amino(N) + 3 amide(N)) complexes was characteristic of all ligands and this type of coordination is especially favoured in the case of oxytocin, probably due to the specific conformation of the ring coupled by the disulfide moiety. In the case of 4-Glu-oxytocin the involvement of the γ-carboxylate of the Glu residue in metal binding of the 2N-complex was proposed, which



(55)

resulted in the enhanced stability of the corresponding species. A moderately intense charge transfer band (λ = 380 nm) developed in the copper(II)-Gly-Gly-Gly-Lys[8]-vasopressin system in parallel with the formation of the 2N-complex and it was explained by a weak S(disulfide)→Cu(II) interaction in solution.

α-Hydroxymethylserine (Hmser) has been identified as the N-terminal amino acid residue in several antibiotics. The copper(II) complexes of the dipeptides containing this residue were studied by potentiometric and spectroscopic measurements.[208] The coordination mode of the dipeptides containing Hmser residues is almost the same as Gly-Ala or Gly-Ser, but the formation of the species $[CuLH_{-3}]^{2-}$ was also detected at high pH values. It was proposed that the weakly bonded carboxylate is replaced by a deprotonated alcoholate group of a Hmser residue and the formation of this species was characteristic only of the dipeptides containing Hmser residue at the C-termini.

Leucine enkephalin is a pentapeptide (Tyr-Gly-Gly-Phe-Leu) and one of the most important endogenous opioid peptides. Copper(II) complexes of the amide derivatives of the pentapeptide (Leu-OH→Leu-NH_2) including the sarcosyl (Gly^3→Sar) derivatives were recently studied.[209] The successive formation of 1N, 2N, 3N and 4N-coordinated complexes by increasing pH was reported, similar to other pentapeptide complexes, but the thermodynamic stability of the species were higher than those of methionine enkephalin. The enhancement of stability was particularly pronounced in the case of the 2N- and 3N-complexes. It was pointed out that the internal sarcosyl residue works as a 'break point' to the formation of 3N- or 4N-complexes and the existence of dimeric species was detected in this case, containing tyrosyl bridging residues. Metal ion coordination of the deprotonated phenolate group of tyrosyl residue was demonstrated by the appearence of the characteristic O(phenolate)→Cu(II) charge transfer band at λ = 368 nm.[209] The copper(II) complexes of tyrosine and its dipeptide derivatives were studied by fluorescence spectroscopy and it was concluded that fluorescence

quenching is a promising method for the investigation of metal complexes of ligands containing Tyr or Trp residues.[210]

Phosphonic acid derivatives of amino acids and dipeptides are receiving increasing attention. Cobalt(II) nickel(II), copper(II) and zinc(II) complexes of dipeptides containing Gly, Ala, Leu or Phe residues at the N-termini and 1-aminoethylphosphonic acid or 1-aminomethylphosphonic acid at the other termini were studied by potentiometric and several spectroscopic techniques.[211,212] The metal ion speciation of the systems are very similar to those of simple dipeptides, but deprotonation and coordination of the amide nitrogen was confirmed only in the complexes of copper(II).

N-Pyruvoylamino acid oximes are structural analogues of dipeptides containing an oxime moiety C(=NOH) in place of the terminal amino group. The results on the proton and copper(II) complexes of N-pyruvoyl amino acid oximes and amino acid amide oximes have been reported recently.[213] The exceptional stability of the copper(II) complexes was explained by the presence of the two alternating donor centres (N and O) in the ligands, which both have high affinity for copper(II), but they cannot coordinate to the same metal ion, which results in extensive formation of dimeric complexes above pH 5.

The metal-binding ability and coordination chemistry of various amides and derivatives including N-acetyl amino acids and sulfonamides were also studied in solution. In case of N-acetyl aspartic acid only the coordination of the carboxylate groups was reported to occur in the complexation reactions with calcium(II) and lanthanide(III) ions,[214] and similar conclusions were reached in the interaction of lanthanide(III) ions with Gly-L-Leu.[215] On the other hand, N-p-amino- and N-p-nitro-phenylsulfonyl (denoted as aps and nps, respectively) derivatives of amino acids and dipeptides proved to be very effective ligands for copper(II).[216,217] Potentiometric and spectroscopic studies revealed that copper(II) ions are able to deprotonate and bind sulfonamide nitrogen donor atoms below pH 5 via the formation of an $(N,^{-}COO^{-})$-chelate. It was concluded that the basicity of the sulfonamide nitrogen atom is lower than the peptidamide nitrogen and, therefore, no distinct anchoring group is necessary to promote amide deprotonation and coordination. As a consequence, the coordination equilibria of the metal complexes of phenylsulfonyl peptides could be significantly different from those of the parent peptide molecules. This is represented by the copper(II) complexes of nps-Ala-His and aps-Ala-His, where the extensive formation of an imidazole-bridged dimeric species $[Cu_2L_2H_{-2}]$ was detected.[217]

The interaction of the mixed metal complex, trans-$[(CH_3NH_2)_2Pt(Mecyt)_2PdCl]^{+}$ (Mecyt stands for methylcytosinate anion) with various N-acetyl amino acids and dipeptides was studied by potentiometric and ^{1}H NMR measurements.[218] The palladium(II) ion of the mixed metal complex is capable of monodentate binding via the substitution of chloride ion and the study is one of the very few reports in which heavy transition or main group elements are involved in equilibrium studies. It was concluded that only side chain donor atoms of the ligands take part in coordination with palladium(II) and that the amide nitrogen donor atoms of N-acetyl methionine, -histidine and -lysine and dipeptides (Gly-Met and Gly-Lys) are not involved in binding to palladium.

However, the formation of dimeric species was detected in the case of N-acetyl-histidine and dipeptides, containing the imidazole (N1/N3) and side chain donor groups as bridging residues, respectively.

N-D-Gluconyl amino acids can be considered as N-protected amino acids with additional donor groups in the sugar moiety. Their reaction with diethyltin(IV) showed that in case of the derivatives of α-amino acids the metal ion is coordinated through the carboxylate, deprotonated amide and C(2)-hydroxy group, while in the case of β-alanine only oxygen donors are involved in metal binding.[219] In the reaction of diethyltin(IV) with derivatives of L-cysteine, including N-acetyl-L-cysteine, the coordination of sulfur and oxygen donors was concluded and metal binding of amide group was excluded at all pH values.[220]

Similar to the solid state studies cited in Section 3.1 there are several reports dealing with the possible involvement of amides and dipeptides in metal binding in solutions of ternary systems. Equilibrium studies on the copper(II) complexes of N-benzoylglycine and N-(4-aminobenzoyl)glycine revealed that the ligands have a low affinity for the metal ion, but in the presence of a second ligand containing aromatic nitrogen donors (e.g. histidine or bpy) deprotonation and coordination of the amide nitrogen donor atoms occurred.[221] Stability constants of various mixed ligand complexes of copper(II) and nickel(II) with dipeptides on one side and ATP,[222] aromatic amines[223] and derivatives of imidazole[224] on the other side were determined by pH-metric titrations in aqueous solution and the relationship between thermodynamic stability and structure has been discussed. Significant stereoselectivity was reported in the equilibrium parameters of ternary copper(II) complexes of (S)-amino acid amides and (R/S)-histidine or tyrosine and the results were explained on the basis of hydrophobic stacking interactions.[225]

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6
Current Trends in Protein Research

BY JENNIFER A. LITTLECHILD

1 Introduction

This review has attempted to cover some of the advances made in protein research during the year 1996. It would be impossible to cover all aspects of this subject and only selected areas have been covered.

With the complete sequencing of the genome of several organisms taking place a huge amount of information regarding the primary structure of proteins will become available. How the individual proteins fold from information within this primary sequence is still not clearly understood and the study of protein folding pathways has continued to be an important area of research.

2 Protein Folding

2.1 New Methods – A basic two-state folding model of small proteins has been described where a new term 'foldon' has been used to refer to basic folding units.[1] Site-directed mutagenesis is used as a tool to mutate specific amino acids that cause small changes in a protein. The kinetics and equilibrium of folding is measured for the mutant proteins. Complete analysis has been carried out for several small proteins of which two have been described recently.[2,3] The folding event is thought to occur by a nucleation-condensation phenomenon where certain conserved residues play important roles.[4] The nucleus is considered to be those residues that are best formed in the transition state and replacement of aminoacid residues within the nucleus by ones that lower the stability does not prevent folding but slows it down.[5] If one nucleation site is destroyed an alternative nucleation site can arise as has been shown for an SH3 domain where different folding transition states can give rise to the same native structure.[6] The folding mechanism of larger proteins is more complex since they are considered to be built up of 'foldons' that fold separately in nucleation-condensation reactions and then dock together.[7] Sometimes the 'foldons' can fold as stable units and other times their folding is dependent on each other. NMR is a powerful technique to monitor protein folding.[8] Hydrogen exchange labelling has been used to study early protein folding intermediates of hen lysozyme and ubiquitin.[9] The structure of the acid state of ribonuclease HI has also been characterised by hydrogen exchange and 2D NMR.[10] Houry and Scheraga have

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used pulsed hydrogen exchange combined with a double-jump transient unfolding procedure to measure the protection of amide protons in an early intermediate on the folding pathway of ribonuclease A.[11]

Monitoring of protein folding has been helped by the recent development of experimentation to follow events on a submillisecond time scale. A review on the advances in this area has been published in 1996 by Eaton *et al.*[12] The protein cytochrome c has been used in a study by Pascher *et al.*[13] where folding has been triggered by electron transfer providing microsecond time resolution. Other studies with cytochrome c have been reported by Sosnick *et al.*[14] where molecular collapse is considered the rate limiting step in folding and by Colon *et al.*[15] where the side chain packing of the N and C terminal helices play a critical role in the folding kinetics. Fast events in the folding of cytochrome c have been followed by using nanosecond photodissociation of carbon monoxide in the unfolded state.[16] A direct observation of fast protein folding has been made using apomyoglobin from the cold denatured state using nanosecond laser temperature jump techniques.[17] Photolysis of the carbon monoxide complex of myoglobin has been followed by time-resolved crystallographic techniques.[18] All of these techniques are allowing data to be obtained on some of the elementary folding processes occurring in these heme containing proteins.

The technique of stopped flow circular dichroism has been used to study protein folding of staphylococcal nuclease[19] and ribonuclease A.[20] In this latter study double jump stopped-flow circular dichroism was used to measure the recovery of signal at 222 and 275 nm indicating the presence of significant secondary and tertiary structure in early intermediates in the folding pathway.

The importance of protein folding in relation to disease was highlighted in 1996 with the article by Will *et al.*[21], describing a new variant of Creutzfeldt-Jakob disease in the UK. It is thought that a fundamental change in the fold of the prion protein is the fundamental event. This is the result of a loss of α helical content and a gain in β sheet structure of the protein. The first three dimensional structure of a mouse prion protein domain PrP(121-231) was reported by the group of Wuthrich in 1996.[22] This fragment of the protein does not however contain PrP(109-121), which in other investigations appears to be a key region for the α to β secondary structure conversion during PrPSc formation.

2.2 Chaperones and Protein Folding – In the cell, proteins are thought to fold with the help of other proteins called molecular chaperones. Previous chapters in this series have discussed this work. An important paper in 1996 described the crystal structure of the peptide binding domain of Dnak in the presence of a bound synthetic.[23] The protein consists of two subdomains which are a β sandwich and an extended structure of α helices (Figure 1). The peptide binds to the chaperone in an extended conformation into a channel of 5 Å by 7 Å formed by loops of the β sandwich. The peptide with aminoacid sequence of NRLLLTG interacts with Dnak by van der Waals contacts and hydrogen bonds. The side chain interactions are hydrophobic in nature and centre on the central leucine residue which is found to be bound in a deep hydrophobic pocket. The helical domain appears to form a removable lid on the substrate complex and two

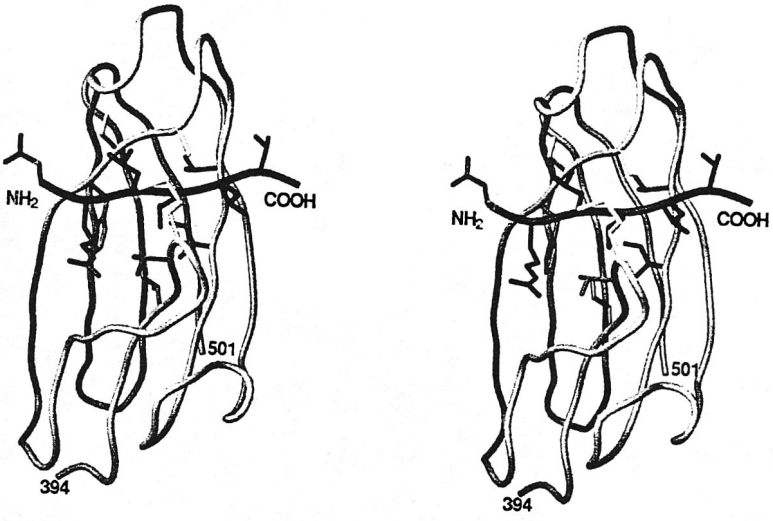

Figure 1. *Stereo view of the peptide binding to the β-subdomain of Dnak. Three leucines in the peptide interact with the protein. Reproduced with permission from reference 23.*

different crystal forms have been described where the lid is open or closed. It is thought that the binding and release of substrate is induced by conformational changes in the ATPase domain of the protein. The precise mechanism will only be understood when structural information is available for the whole protein and with different peptides bound. Sometimes this is difficult to achieve and information needs to be built up from other biophysical techniques. One of these is the use of low angle X-ray scattering which indicates that the ATPase and peptide binding domains of Dnak are connected by a short hinge region or are just in contact with each other.[24] It is observed that ATP binding causes a conformational change in the protein.

NMR structures have been reported for the human Hsp40 J-domain[25] and for the J domain and the Gly/Phe-rich region of the *Escherichia coli* DnaJ chaperone.[26] DnaJ has been shown to have a zinc finger-like domain which is involved in binding to denatured protein substrates.[27,28] This publication presents evidence that full length DnaJ can co-operate with DnaK and GrpE in the refolding of denatured protein, luciferase. The larger chaperone system GroEL/GroES has seen a great increase in the understanding of how it refolds proteins. The crystal structure of the GroES co-chaperonin has been reported at 2.8 Å.[29] It is shown to be a flexible dome-shaped heptamer composed of β sheets (Figure 2). An 8 Å orifice in the roof is surrounded by negatively charged residues. Flexible loop regions which make contact with GroEL protrude from the base of the dome. The structure of the equivalent protein from *Mycrobacterium leprae* was also reported and is similar.[30] Other studies have shown that protein folding does occur in the central cavity of the GroEL-GroES complex[31] and active

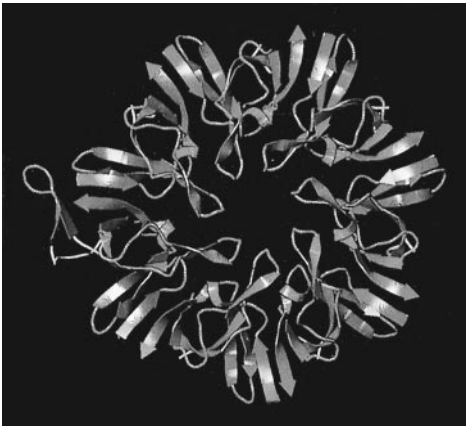

Figure 2. *Structure of the GroES heptamer. Reproduced with permission from reference 29.*

intermediates of the folding reaction of rhodanese and green fluorescent protein have been characterised by fluorescence measurements.[32] Another valuable technique to be put alongside other structural analysis is cryo electron microscopy. This has been elegantly used by the group of H. Sabil to show the dynamic movement of the chaeronin ATPase cycle. Three dimensional reconstructions of GroEL and GroEL-GroES complexes with difference nucleotide-bound states have allowed the visualisation of rotations in the apical GroEL subunit domains induced by nucleotide and GroES binding.[33] A crystal structure at 2.4 Å resolution of GroEL complexed with ATPγS[34] shows the ATP binding site in the GroEL domains (see Amino Acids, Peptides and Proteins Vol. 26) but does not show the long range conformational changes in GroEL as seen by cryo electron microscopy (Figure 3). The combined techniques of X-ray and cryo EM will be used increasingly in the future to study large macromolecular assemblies where often only parts of the assembly can be crystallised.

Amide protein exchange has been used to examine the folding of barnase by GroEL.[35] Hydrogen exchange labelling and electrospray ionisation mass spectrometry has been used to look at the conformational properties of dihydrofolate reductase (DHFR) bound to GroEL .[36] About 20 hydrogens are protected in DHFR bound to GroEL. The technique of electrospray mass spectrometry has found many new applications in monitoring protein folding. Determination of regions of GroEL that interact with DHFR have also been reported by Clark *et al.*[37]

A limitation in protein folding is the isomerisation of proline residues. Two recent papers identify a so called trigger factor in *E. coli* that is a prolyl isomerase and is found with nascent polypeptide chains.[38,39] An interesting paper by Patino *et al.*[40] supports the fact that a cytoplasmically inherited genetic element in yeast is shown to be a prion-like aggregate of the cellular protein Sup35. Aggregation of this protein depends on the functional state of the chaperone protein Hsp104.

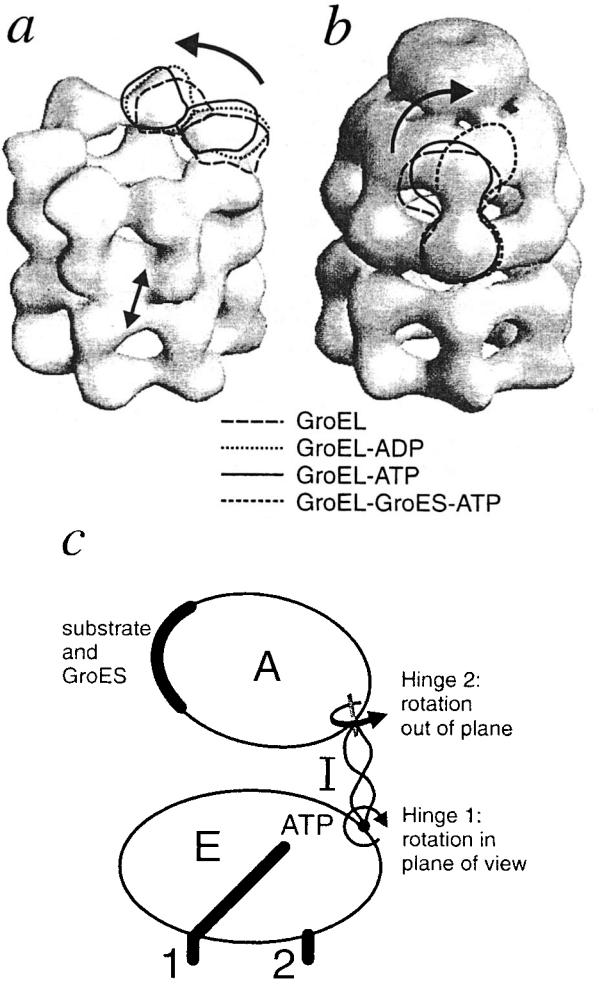

Figure 3. *Domain movements as seen by cryoelectron microscopy during the GroEL functional cycle. (a) Diagram showing the displacement of the two adjacent apical domains in GroEL, GroEL-ADP and GroEL-ATP outlined on the GroEL-ADP structure. (b) Outline of one GroEL subunit in GroEL, GroEL-ATP and GroEl-GroES-ATP. (c) Allosteric transmission from nucleotide binding to changes in hinge rotation and inferring contacts. Reproduced with permission from reference 33.* © *Cell Press.*

This paper makes a direct connection between a prion phenomenon and the function of a molecular chaperone.

3 Techniques – Mass Spectrometry

Electrospray mass spectrometry is a technique that is finding increasing applications in protein research. The use of this technique has been reviewed in 1996 by Banks and Whitehouse.[41] Proteins up to 150 kDa can be analysed and the sensitivity can be high – for example the molecular weight of a protein such as carbonic anhydrase (28,780 Da) can be determined to 1 Da using 9×10 moles of enzyme from a crude preparation of red blood cells.[42] By the technique of secondary ion generation it is now possible to obtain protein sequence information although this technique is still undergoing development. Two other reviews in 1996 in Methods in Enzymology address the use of mass spectrometry in protein and peptide analysis.[43,44] Several other uses of mass spectrometry have recently emerged. Apart from the obvious analysis of covalent modification of proteins such as chemical modification and post-transcriptional modification such as phosphorylation or glycosylation, the technique has been used to monitor enzyme substrate/enzyme inhibitor interactions, and protein folding. The binding of acyl CoA to acyl CoA binding protein and the differences caused by modifications to both protein and cofactor have been studied by the group of Dobson.[45] Complex formation of bovine pancreatic trypsin inhibitor and trypsin have been studied by Kraunsoe *et al.*[46] The screening of combinatorial libraries of peptides and their ability to bind to carbonic anhydrase has been monitored by mass spectrometry by the group of Whitesides.[47] The binding of oligonucleotides to proteins has also been monitored.[48] Many studies have now used mass spectrometry to monitor protein folding. This is followed by changes in the charge state distribution or deuterium incorporation. The technique is often combined with NMR experiments. The rate of deuterium exchange and the higher number of protons protected from exchange for β lactamase bound to the chaperone GroEL, compared to the uncomplexed protein, has been used to show that a significant amount of native structure remains in the GroEL β-lactamase complex at temperatures of 48 °C.[49]

4 Extremophilic Proteins

An area that is increasing in interest is the study of proteins isolated from organisms that live in extreme conditions of high or low temperature, acidic or basic conditions, high salt and high pressures. Many of the proteins of these organisms are adapted to these conditions and the manner of how they achieve this is of interest in order to understand protein stability. Many of the organisms fall into the class of the archaea, which have features of both prokaryotes and eukaryotes and are thought to be some of the earliest forms of life. Some

organisms are known to contain small molecules or other proteins that can help stabilise proteins inside the cell.

4.1 Antifreeze Proteins – The antifreeze proteins can inhibit the growth of large ice crystals at the expense of small ones – a process called recrystallisation inhibition. They are found in many organisms that live in cold environments such as marine fish, insects, plants and bacteria. The antifreeze proteins (AFP) isolated to date can be divided into different types and further subdivisions are emerging. An example of type I AFP was reported in 1996 by Chao *et al.*[50] These proteins appear to be long single α helices which exist in solution as 3-5 kDa monomers. This fish protein is build up of 11 amino acid repeats, TXXNXXXXXXX, where X is mainly alanine and N is sometimes aspartate or threonine. Two different types of type I AFP's have been found in winter flounder, one found in the liver which is similar to the repetitive type described above and the other found in the skin, scales, *etc* which is non-repetitive and is more amphipatic.[51]

The high resolution X-ray structure of a type I AFP from winter flounder has recently been described[52] at 4 °C and −180 °C. This structure is a 37 residue protein that forms a slightly curved helix which is stabilised by pair-wise side chain interactions and by N and C terminal capping structures. All of the non-alanine residues have a role in ice binding or helix stabilisation. Circular dichroism studies have shown this protein to have 100% helix at 0 °C and only 50% at 22 °C. The four threonine/aspartic acid or threonine/asparagine ice binding motifs are regularly spaced along one flat surface which matches the water molecule spacings along the 01-12 direction on the 20-21 plane of ice. NMR studies in solution show that the putative ice-binding threonines on the helical AFP are free to sample all rotamer positions[53] whereas the X-ray structure shows that they have restricted confirmations due to binding to ice.

A sophisticated computer modelling study of the non-repetitive type I AFP binding to the ice surface has been identified by etching techniques.[54] This short-horn sculpin antifreeze protein shows sterospecific binding to 2-10 faces of ice.

The type II AFP's are larger, 14-24 kDa and are homologues of the carbohydrate recognition domain of calcium dependent lectins. Recently the structure of a type II AFP from herring has been modelled on the basis of homology to the Ca^{2+} dependent lectins.[55] This group can be further subdivided due to their dependence on Ca^{2+} for activity. In some cases Ca^{2+} is required and other divalent metals such as Ba^{2+}, Zn^{2+}, Mg^{2+} result in decrease in activity and alterations in the ice crystal morphology. Other members of this group do not require Ca^{2+}. A related protein of the type II class is lithostathine (pancreatic stone protein). The structure of this protein has been determined by both crystallography and NMR in 1996.[56,57]

The structure of a type III AFP has been described by Jia *et al.*[58] This protein is from eel pout. This structure is shown in Figure 4 and shows a flat amphipathic ice-binding face for ice binding. Five residues on this face form hydrogen bonds that match two ranks of oxygen on the 10-10 ice prism plane in the 0001 direction of ice. Another important asparagine residue is important in the binding of the protein to the interface between ice prism and basal planes. A recent solution

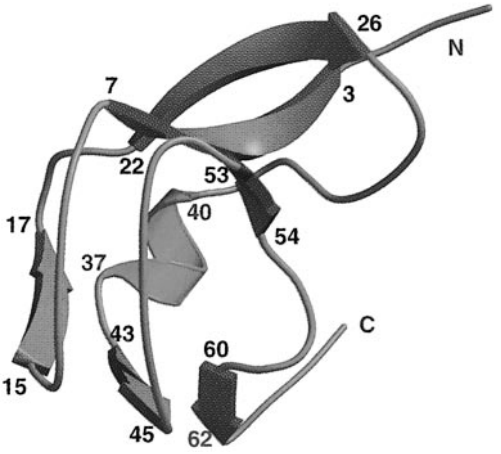

Figure 4. *Ribbon diagram of AFP, with secondary structure elements labelled by amino acid numbering. Reproduced with permission from reference 58.*

structure of this protein[59] challenges the dominance of the hydrogen-bonding contribution to the energetics of AFP binding to ice and suggests that van der Waals interactions and entropic effects also play an important role.

A further group of AFP's of the type VI 12.3 kDa type have been reported that have 22% sequence identity with low density lipoprotein receptor binding doman of human apolipoprotein E3 which have been shown to have a four-helix bundle motif. Structural information on this group of proteins will be forthcoming.

It appears that nature has adapted several different classes of protein to have the ability to bind to ice and therefore reduce the effect that ice formation would have on organisms and tissues of organisms adapted to exist in cold environments. An understanding of this mechanism could prove useful for development of proteins of this type for commercial use.

4.2 Thermophilic Proteins – *4.2.1 Elongation Factor Ts (EF-TS) from Thermus thermophilus.* – This is a protein factor which interacts with another factor EF-Tu during bacterial protein synthesis. The structure of EF-Ts from this hyperthermophilic bacterium forms a dimeric structure.[60] The dimer contacts comprise a core of hydrophobic amino acid side chains that pack closely and that are surrounded by a hydrogen bonding network. In addition a cysteine residue from one subunit forms a disulfide bond with the corresponding cysteine from the other subunit. This is thought to increase the thermal stability of the dimer. It is not usual to find disulfide bridges in cytoplasmic proteins. However in a complex structure of EF-Tu/EF-Ts from the mesophile *E. coli* the EF-Ts forms a dimer.[61]

4.2.2 DNA Polymerase/DNA Complex Thermus aquaticus – This bacterial polymerase has revolutionised molecular biology, since it was due to its

thermostability that it has been used so effectively for gene application in the so called Polymerase Chain Reaction (PCR). The structure of this enzyme has been determined by X-ray methods[62] and is almost identical to the non-homologous Klenow fragment from *E. coli* DNA polymerase. This enzyme is in a complex with DNA and provides structural evidence that the primer template approaches the polymerase from the 3′-5′ exonuclease side. The DNA is part A form and part B form.

4.2.3 Ribosomal Protein L1 (Thermus thermophilus) – It has been necessary to use thermophilic sources for the production of ribosomal proteins that can form good crystals for structural analysis. Ribosomal protein L1 is one of the largest of the 50 found in the bacterial ribosome. It binds to 23SrRNA and regulates its own expression by binding to mRNA. The protein is a two domain αβ protein which is thought to be relatively flexible. It is thought to become stabilised by an induced fit mechanism on binding of RNA at the interface of the two domains.[63]

4.2.4 Ribosomal Protein L14 (Bacillus stearothermophilus) – Another ribosomal protein from the large subunit is L14. The structure has been described by Davies *et al.*[64] The protein which is a single domain composed of a five stranded β barrel and a C terminal loop containing two α helices and a β ribbon extension. The protein is located on the surface of the 50S subunit at the 30S-50S interface and between the centres of activity for peptidyl transferase and GTPase. The structure shown in Figure 5 indicates potential binding sites for RNA and other proteins.

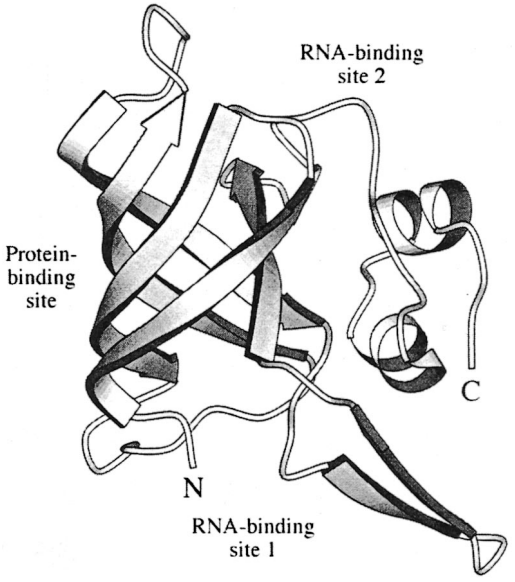

Figure 5. *Ribbon representation of ribosomal protein L14 indicating the location of each of the three putative binding regions for RNA and protein. Reproduced with permission from reference 64.*

4.2.5 Ribosomal Protein S8 (Bacillus stearothermophilus) – Another protein structure, this time from the small ribosomal subunit, is that described for S8.[65] This protein plays a crucial role in the folding of 16SrRNA and specifically binds to an extended loop structure in the RNA. This protein also binds to mRNA controlling the transcription of an operon coding for a group of ribosomal proteins. The S8 protein has two domains and the sites where another ribosomal protein S5 and RNA are known to chemically crosslink can now be mapped on the overall protein structure.

4.2.6 D-Glyceraldehyde-3-phosphate Dehydrogenase (Thermus aquaticus) – The structure of this glycolytic enzyme is similar to the structure of the *Bacillus stearothermophilus* enzyme but it is more thermostable. This is attributed to an increased number of hydrogen bonds and intra-subunit salt-links and a decreased surface to volume ratio, with respect to other species.[66]

4.2.7 Methanothermus fervidus Histone Protein – The archaea are a separate kingdom of life that have features of both prokaryotes and eukaryotes. The DNA 'machinery' is thought to be more eukaryotic like and this is confirmed by the description of a protein with a histone-like fold from this hyperthermophilic archaea. The structure of HMfB has been determined by NMR methods.[67] HMfB does not possess a long disordered N terminus and it has been suggested that this absence contributes to its thermostability.

4.2.8 [3Fe-4S] Ferredoxin from Sulfolobus – The *Sulfolobus* species is a thermoacidophilic archaeon. The structure of the ferredoxin from this organism has been studied by X-ray methods.[68] The structure can be divided into two parts. A core region composed of a $(\beta\alpha\beta)_2$ fold that contains the two buried [3Fe-4S] clusters and an N-terminal extension. A novel zinc-binding site is found located at the interface of the N-terminal extension and the core which is thought to play a stabilising role. It is proposed that this feature is common to all thermoacidophilic archaeal ferredoxins.

5 Proteins of Medical Interest

The last few years have seen an increase in structural information on proteins of direct medical interest. Many of these proteins can now be produced in reasonably large amounts for both structural characterisation and in many cases drug therapy.

5.1 Tissue-type Plasminogen Activator – This protein plays an important role in fibrinolysis. It is involved in the cleavage of a specific arginine/valine peptide bond that converts plasminogen to plasmin. Plasmin solubilises cross-linked fibrin networks and results in dissolution of blood clots. Tissue-type plasminogen activator is secreted by endothelial cells into the blood as a 527 residue glycoprotein. The protein consists of five distinct domains which makes it difficult

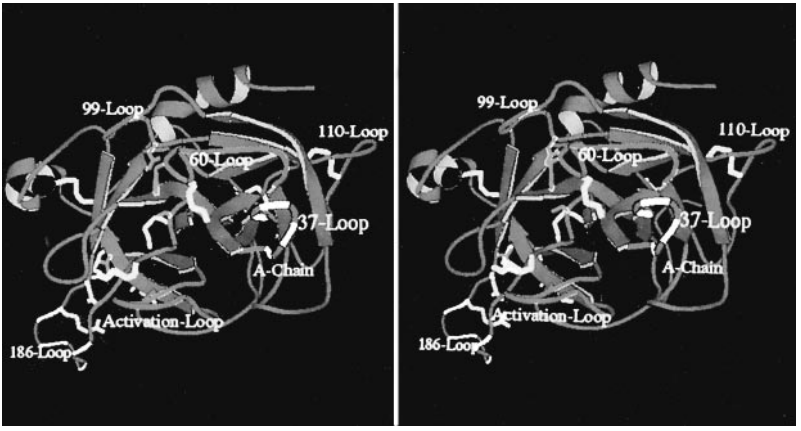

Figure 6. *Stereo ribbon plot of the catalytic domain of human tissue plasminogen activator. The active site residues Ser, His, Asp are shown as are the five disulfide bridges. Some surface loops around the active site are labelled. Reproduced from* J. Mol. Biol., *1996,* **258***, 117 by permission of the publisher Academic Press.*

to crystallise for structural determination. The domains are a finger domain, an epidermal growth factor-like domain, two kringle domains and a C-terminal trypsin like proteolytic domain. In 1996 the group of Bode[69] determined the crystal structure of the catalytic domain of recombinant two-chain human tissue-type plasminogen activator. The structure shown in Figure 6 allows conclusions to be made on specificity, plasminogen cleavage site geometry and kringle-2 domain attachment. This proteolytic domain consists of two six-stranded β barrels and possesses the architecture of the chymotrypsin like serine proteinases. The catalytic residues are located at the junction of both barrels. The catalytic triad of serine, histidine, aspartic acid are arranged as for trypsin. The active site of tissue-type plasminogen activator is shaped and narrowed by surface loops which modify its binding properties. Mutagenesis studies have shown that mutation of a specific histidine residue to an aspartate and glutamate is involved in converting tissue-type plasminogen activator into a zymogen.[70] Variants of tissue-type plasminogen activator are being used in cases of acute myocardial infarction and other thromboembolic disorders.

5.2 Bcl-X_L – Programmed cell death in multicellular eukaryotes or so called apoptosis is inhibited by the protein Bcl-X_L. Bcl-X_L is localised in long-lived human postmitotic tissue such as neural tissue. The structure of this protein has been determined by both NMR and X-ray methods.[71] The protein as shown in Figure 7 consists of two central hydrophobic helices about 30 Å long, flanked by five amphipathic helices on either side. The structure reveals a resemblance to the membrane insertion domains of some bacterial toxins such as diphtheria toxin and the colicins. These toxin domains are thought to dimerise and form pH

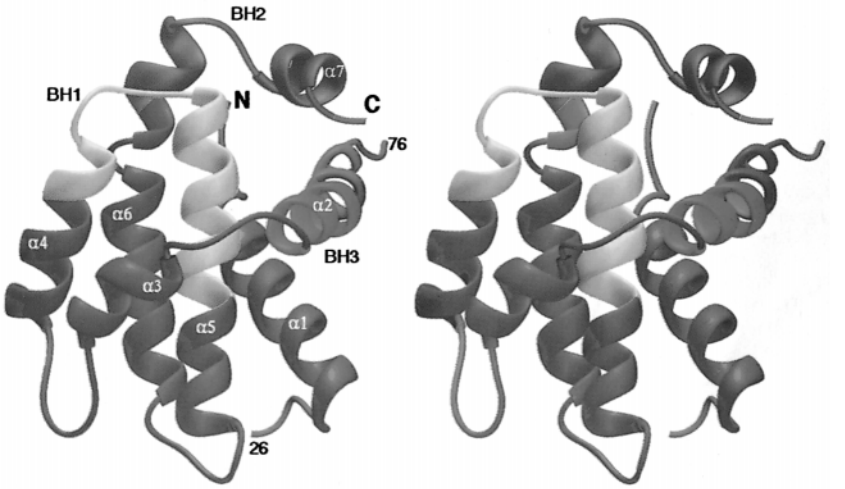

Figure 7. *Figure of the averaged, minimised NMR structure of BcL-X_L. Reproduced with permission from reference 71.*

dependent pores in the membrane. It is thought that Bcl-X_L may act in a similar way promoting cell death either by upsetting electrochemical gradients essential for homeostasis or inhibited cell death by eliminating a chemical imbalance.

5.3 ADP Ribosyl Cyclase – This enzyme catalyses the synthesis of cyclic ADP ribose from the cofactor NAD^+. It is involved in cyclising NAD^+ by linking the N1 position of the adenine ring to the Cl^1 of the terminal ribose, displacing the nicotinamide group. Cyclic ADP ribose regulates calcium induced calcium release in sea urchin eggs and mammalian cells. The enzyme has sequence homology to some other interesting proteins such as lymphocyte differentiation antigen CD38 and a membrane-anchored protein in the bone marrow of stromal cells. The structure of the ADP ribosyl cyclase from Aplysia has been solved in 1996.[72] The enzyme is an interesting dimer in which each monomer folds into two domains separated by a deep cleft. The enzyme contains two distinct pockets that are composed mainly of sequence conserved residues. It is thought that these recognise either nicotinamide or adenine, with the intersubunit cleft binding the ADP ribose intermediate.

5.4 Cadherins – Cadherins mediate cell adhesion and play a role in normal cellular development. They are involved with cell-cell contacts, cell polarity and tissue morphology. Reduced levels of epithelial cadherin is associated with increased invasiveness of human tumours. These proteins are composed of five tandemly repeated extracellular domains, a single membrane-spanning segment and a cytoplasmic region. The N-terminal extracellular domains are involved with calcium dependent cell-cell contact. Calcium induces a conformational change in this region. A structure of the mouse E-cadherin provides information

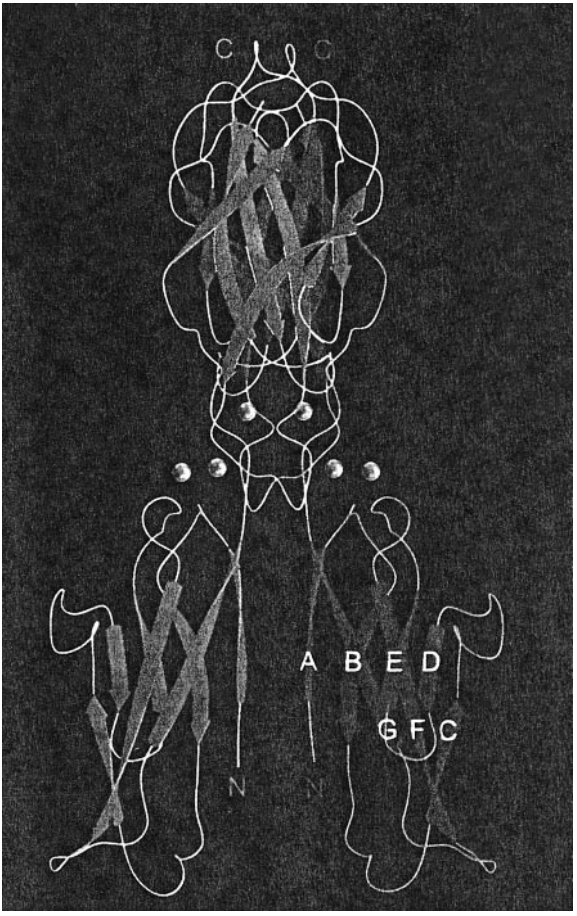

Figure 8. *The E-cadherin dimer showing the cluster of three calcium ions that bind in the linker region connecting the N and C terminal domains in each monomer. Reproduced with permission from reference 73.*

on this calcium induced rigidification and dimerisation.[73] Two N-terminal extracellular E-cadherin domains form a calcium mediated symmetrical dimer. The monomers are composed of two seven-stranded β-barrel domains connected by a 10 residue linker and bridged by a novel arrangement of three calcium ions as shown in Figure 8.

5.5 Cartilage Oligomeric Protein – This protein is a pentameric glycoprotein of the thrombospondin family that is found in cartilage and tendons. These proteins are formed from α helical bundles and the crystal structure of a five stranded coiled coil of cartilage oligomeric protein was described by Malashkevich *et al.*[74]

The mainly α helical chains form a left handed superhelix with 140 residues/turn and a supercoiled radius of 8.6 Å. The structure is stabilised by complementary hydrophobic interactions between neighbouring helices. The structure shows some similarity to models of the acetylcholine receptor and other ion channels. Ions are thought to move through these channels in a hydrated form.

5.6 Cyclin-dependent Kinase 2/CksHs1 Complex – The structures of cyclin-dependent kinase 2 and CkSHs1 (human cell cycle protein) have previously been reported. Cyclin-dependent kinase (CDK) 2 belongs to a group of protein kinases that regulate the cell cycle in eukaryotes. CksHs1 is one of two isoforms of the human cell cycle regulatory protein Cks that is essential for the cell cycle. Cks proteins are important proteins since they control the timing and mechanics of cell division by binding to the catalytic subunit of CDK. A complex showing the interaction of these two proteins has been reported by Bourne *et al.*[75] The catalytic domain of CDK2 is in the C-terminus of the protein and CksHs1 binds using all four of its β-strands. Changes occur at the interface of the two proteins when they bind to each other. This interface is made up by both hydrophobic and polar interactions. The binding site is different to that of other proteins that bind to CDK and it is proposed that CksHs1 is targeting CDK2 to other proteins to form macromolecular assemblies.

5.7 Cyclin-dependent Kinase/Cyclin-dependent Kinase Inhibitor Complex – Proteins have been found that bind to cyclin-dependent kinase/cyclin A complexes and inhibit their activity. These inhibitors, called CK1's induce cell-cycle progression by their redistribution between different cyclin-CDK complexes. The structure of this complex has recently been described[76] and helps to understand the complex interactions between different protein molecules involved in cell cycle control. The inhibition $P27^{kip}$ fills the catalytic cleft of CDK2 where the cofactor ATP would bind.

5.8 Phosphoinositide 3-Kinase p85α Subunit, Breakpoint Cluster Region Homology Domain – This kinase plays a role in signal transduction from a number of membrane receptors. The breakpoint cluster region-homology (BH) domain from the adaptor subunit of the kinase interacts with Cdc42Hs and Rac proteins. This binding causes activation of the catalytic kinase subunit. The crystal structure of the BH domain of this kinase has been described by Musacchio *et al.*[77] The domain is all α helical with seven main helices separated by variable length loops. The structure of a four helix bundle/three helix projection domain is thought to be a previously undescribed protein fold.

5.9 Mammalian Phosphoinositide-specific Phospholipase Cδ – This enzyme is also involved in signal transduction generating two second messengers, D-myo-inositol-1,4,5 triphosphate ($InsP_3$) and sn-1,2-diacylglycerol by catalysing the hydrolysis of phosphatidylinositol-4,5-bisphosphate. The crystal structure of the protein described by Essen *et al.*[78] is composed of three domains. An N-terminal domain has four EF-hand motifs, which is very similar to calmodulin. The

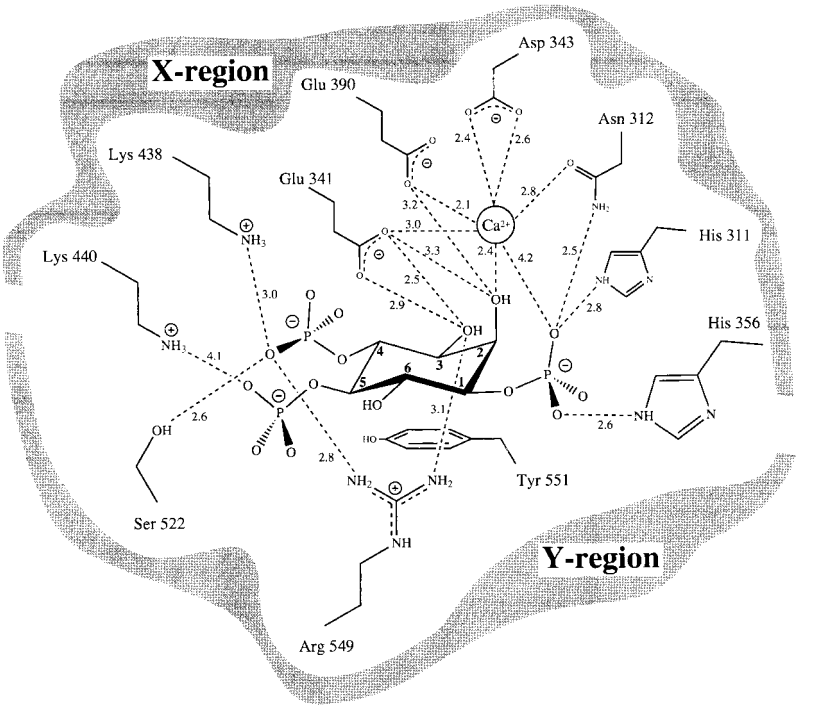

Figure 9. *Schematic diagram of the network of $InsP_3/CA^{2+}$ interactions in the active site of phosphoinositide-specific phospholipase Cδ. Reproduced with permission from reference 78.*

catalytic domain is a distorted TIM barrel structure. The active site is located at the C-terminal end of the barrel. The C2 domain is an eight stranded antiparallel β sandwich which has a similar structure to another C2 domain, synaptotagmin I. Detailed interactions of the $InsP_3/Ca^{2+}$ at the active site of the enzyme is shown in Figure 9. The conformational changes that occur in the C2 domain of phospholipase C-δ1 have been investigated by Grobler *et al.*[79]

5.10 Proliferating Cell Nuclear Antigen (Human)$p21^{WAF1/C1P1}$, C-Terminal Fragment Complex – The cell cycle checkpoint protein $p21^{WAF1/C1P1}$ inhibits the cyclin-dependent kinases that control the initiation of the S-phase of the cell cycle and subsequent DNA replication. Elevation of nuclear levels of the tumour suppressor protein p53 occurs upon detection of DNA damage, stimulating the transcription of $p21^{WAF1/C1P1}$ causing arrest of DNA replication. The proliferating cell nuclear antigen (PCNA) described in 1994 by the group of Kuriyan is a ring shaped molecule that encircles DNA. The structure of the complex reported in 1996 by the same group suggests that $p21^{WAF1/C1P1}$ inhibits PCNA by masking elements that are required for the binding of other components of the polymerase machinery.[80]

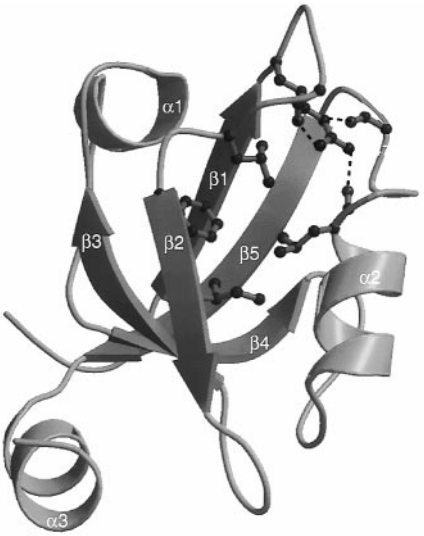

Figure 10. *Ribbon diagram of the PDZ domain with secondary structure elements and N and C termini labelled. The side chains forming the hydrophobic pocket are shown. Reproduced with permission from reference 81.*

5.11 DIG (Disc-large Protein), PDZ Domain – This protein domain belongs to a group also referred to as DHR or GLGF repeats, that are found in ion-channel and receptor clustering proteins. They have a 90 amino acid residue repeat and are protein recognition modules. Many proteins have been found to be composed of repeating domains and the approach is to solve the crystal structure of individual domains by NMR or X-ray since the whole protein would not form crystals and be too large for NMR solution. PDZ domains recognise proteins containing a consensus C-terminal tripeptide motif S/TXV that is found in neuronal ion channels and synaptic receptors. A human PDZ domain from DIG is a five stranded antiparallel β barrel flanked by three α helices.[81] A cavity large enough for binding of the tripeptide has been found which includes a hydrophobic pocket (see Figure 10) and a groove that runs over the surface of the domain.

5.12 Fibroblast Growth Factor Receptor 1 Tyrosine Kinase (FGFRIK) – This cell surface receptor has protein tyrosine kinase activity and is important in cell growth, differentiation, metabolism and oncogensis. When growth factor binds the receptor dimerises which results in autophosphorylation of specific tyrosine residues in the cytoplasmic domain. The phosphorylated tyrosines can stimulate tyrosine kinase activity or function as binding sites for Src-homolgy domains or phosphotyrosine domains. The structure of FGFRIK[82] is similar in overall topology to the insulin receptor kinase except that a loop carrying two tyrosine residues as shown in Figure 11 is pointing away from the active site suggesting a

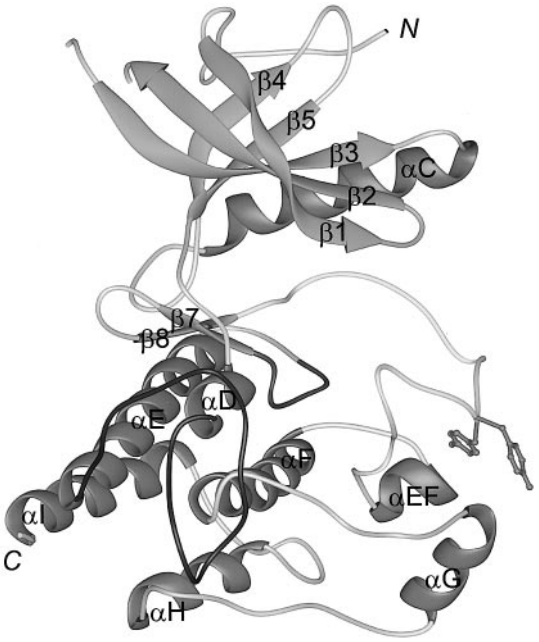

Figure 11. *Ribbon diagram showing the overall fold of the FGFRIK structure, and the side chains of the two tyrosines of the activation loop. Reproduced with permission from reference 82. © Cell Press.*

different mechanism of autoinhibition to the insulin receptor kinase. Other receptors are thought to dimerise as a result of hormone or a cyclic peptide agonist.[83]

5.13 α-Hemolysin – This protein from staphyloccal strains of bacteria is able to bind to cells such as human platelets by forming an organised structure that is capable to form transmembrane pores that disrupt the cell membrane causing leakage of low molecular weight molecules, destroying the osmotic balance and resulting in cell lysis. The protein organises a pore made of β sheets (antiparallel β-barrels). The structure of this heptameric membrane pore was described by Song *et al*.[84] The whole molecule is described as being mushroom shaped. The cap of the mushroom is made up of seven β-sandwiches, and so called amino latches from each monomer. The transmembrane stem is a 14 strand right handed β-barrel. The overall structure is remarkably organised to form a central pore of up to 26 Å as shown in Figure 12.

5.14 HIV Capsid Protein, N-Terminal Core Domain – The capsid protein of HIV-CA is studied in great detail since it is required for the viral morphogenesis and replication. The structure of the N-terminal core domain has been reported

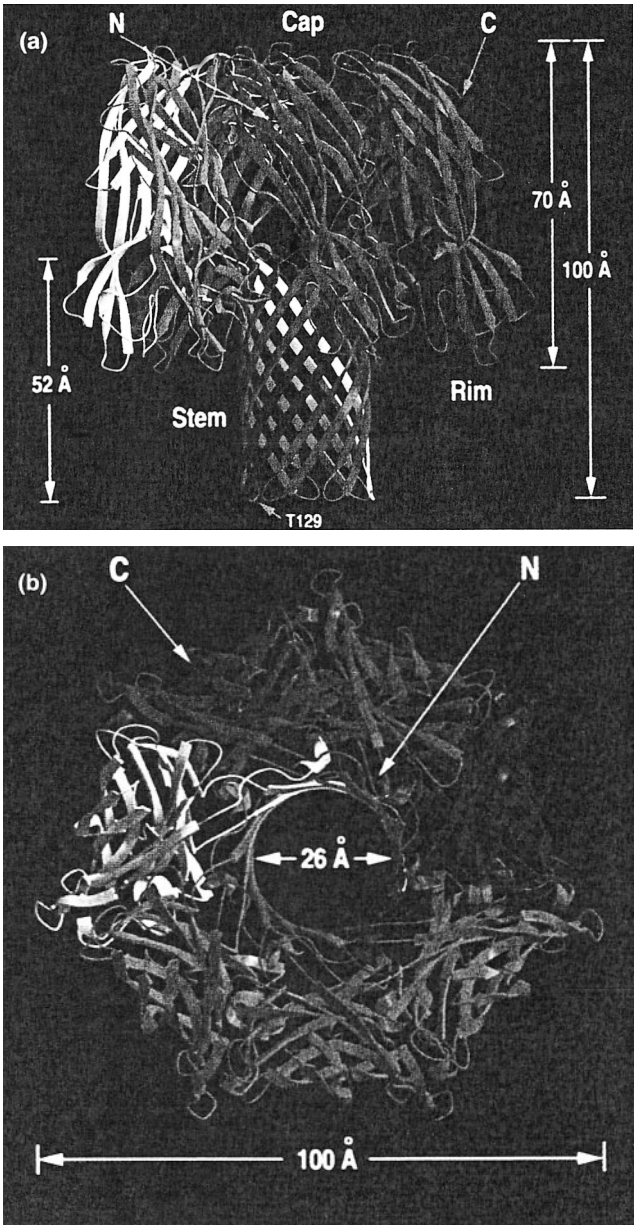

Figure 12. *Ribbon representation of the α-himolysin heptamer, viewed (a) perpendicular to the sevenfold axis and (b) from the top of the structure parallel to the sevenfold axis. The cap, stem and ring domains are labelled. Reproduced with permission from reference 84.*

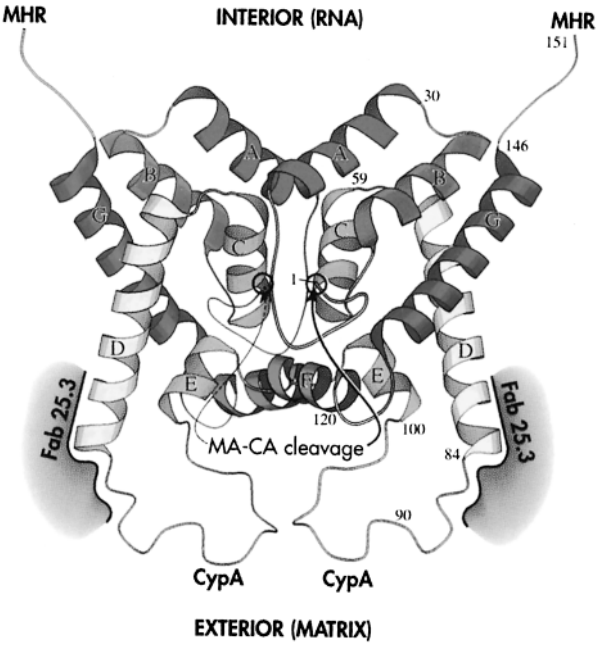

Figure 13. *Ribbon diagram of HIV capsid protein N-terminal domain dimer showing the HIV-1 protease cleavage site between the matrix (MA) and the CA, the site of interaction with Fab 25.3 and CypA binding side. The major homology region (MHR) which is the beginning of the disordered C-terminal domain is shown. Reproduced with permission from reference 86.*

by NMR[85] and by X-ray in complex with a Fab antibody.[86] The N-terminal domain is shown in Figure 13 and consists of seven α helices. The structure is stabilised by three conserved hydrophobic leucine residues on the inside of these helices. The whole of the protein was crystallised but the C-terminal region is too disordered to see in the crystal structure. The N-terminal domain is thought to form a dimer at the surface of the viral core. The five helix coiled coil is thought to be essential for retroviral maturation and is therefore a target for therapeutic agents.

5.15 HIV Matrix Protein – This protein along with the previously described capsid protein is a product of the viral gag gene. The matrix protein is responsible for membrane targeting and binding of the viral envelope protein whereas the capsid and nucleocapsid proteins condense and surround the RNA genome, the matrix protein remains in a structural shell associated with the inner face of the viral membrane. A crystal structure of the trimeric type I matrix protein has been described.[87] The protein is composed of an N-terminal globular domain with a

novel fold of five α helices and a three-stranded β sheet. This domain is responsible for protein assembly, membrane binding, viral envelope protein binding and nuclear localisation. The C-terminal α helix is essential for viral entry and projects away from the N-terminal domain towards the centre of the virus. The structure is similar to an earlier NMR structure published in 1994. A model for binding to the matrix membrane is proposed based on the trimeric structure where N-terminal myristoyl groups and basic residues are involved with the interaction.

5.16 Nef: Regulatory Factor – The *nef* gene of HIV virus encodes a protein that is important for the development of AIDS. Some HIV-1 positive individuals that show no sign of progression to AIDS consistently have deletions in the DNA that codes for this gene. The protein Nef is known to interact with the SH3 domain of the Src family tyrosine kinases. This interaction occurs at a specific proline rich amino acid motif (PXXP). The structure of a mutant C206A of Nef has been solved by NMR methods[88] and a complex of Nef, core domain THR mutant/Fyn SH3 domain, R961 mutant complex has been solved by X-ray methods.[89] The latter paper shows the detailed interaction between the two proteins at the conserved proline motif and at an additional area outside this motif which shows the reason for the increased affinity and specificity of binding. Nef provides a potential target for a rationally designed anti-HIV drug.

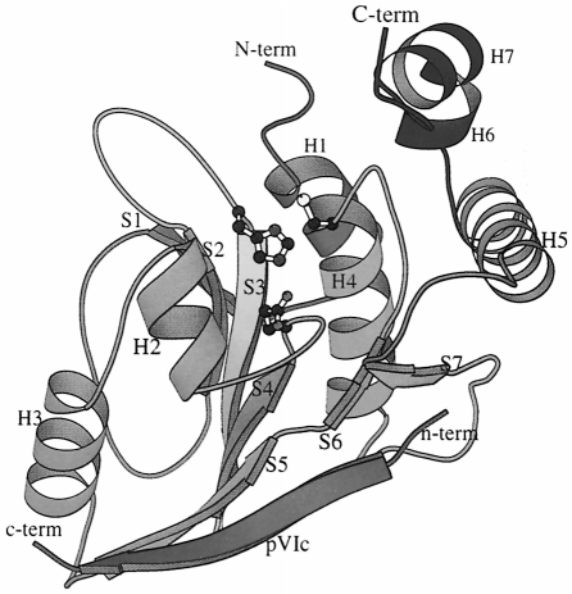

Figure 14. *Diagram of the structure of a denovirus proteinase with the active site Cys, His and Glu residues highlighted. the peptide cofactor pVlc is shown at the bottom of the structure forming an additional β sheet. Reproduced with permission from reference 90.*

5.17 Human Adenovirus-2 Endoproteinase/Cofactor Complex – This enzyme is required for the production of an infectious virus that is a leading cause of infant death in the third world. The recombinant adenovirus proteinase is simulated in activity by a peptide cofactor pVlc. It is a cysteine protease with catalytic triad residues in similar position to those in papain; however, the fold is different. A disulfide bond links the endoproteinase to the peptide cofactor which becomes the sixth strand of a five stranded β sheet. There are seven α helices also in the structure shown in Figure 14.[90]

5.18 Human Cytomegalovirus Protease – This is a herpes virus that is widespread in humans. It can cause death in people with suppressed immune systems. The protease catalyses its own cleavage and cleavage of the viral assembly protein precursor. It is a serine protease where the nucleophilic serine and histidine have been identified. The structure of this protease has been described by four groups.[91-94] It possesses a unique fold consisting of a seven stranded mainly antiparallel β barrel surrounded by seven α-helices. The third member of the catalytic triad is proposed to be a second histidine residue (Figure 15). A possible

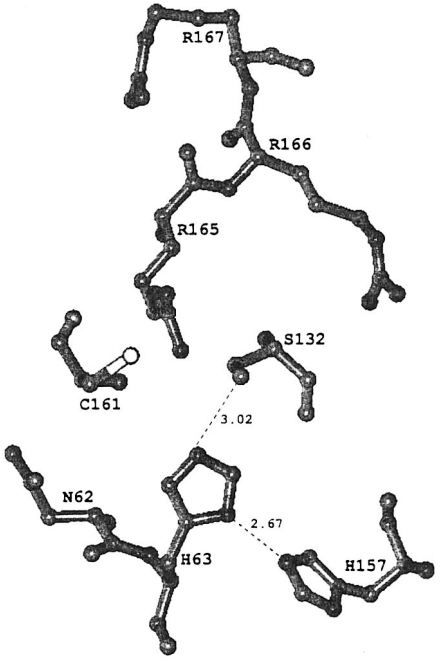

Figure 15. *The catalytic triad of human cytomegalovirus protease and its surroundings. Hydrogen bonds between the imidazole ring of His 63 and the side chains of Ser 132 and His 157 are shown. Reproduced with permission from reference 94.*

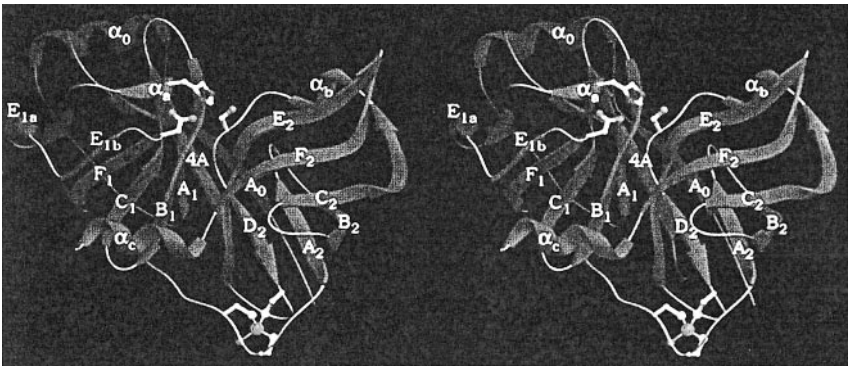

Figure 16. *Stereo view of the NS3 protease-NS4A complex. Residues of the catalytic triad are shown in ball-and-stick representation as are the cysteine residues and the water molecule bound zing. Reproduced with permission from reference 95.*

aspartic acid is also proposed. The inhibitory activity of zinc ions is explained by its binding to the active site. Regions of sequence conserved between LCMV protease and other herpes virus proteases correspond to the β barrel core which is considered to be the topology of the prototype of proteases from this virus family. The herpes proteases cleave a peptide bond between an alanine and a serine amino acid residue.

5.19 NS3 Protein, Protease Domain, Hepatitis C Virus – This protease is essential for hepatitis C virus replication. It is estimated that 1% of the human population are infected with the virus and 20% of these develop an acute infection. The protease is interesting since it contains zinc but is a serine protease with a fold similar to chymotrypsin.[95,96] The structurally important zinc is found in the C-terminal domain and is tetrahedrally coordinated by three cysteines and a histidine (indirectly via a water molecule). The X-ray structure described by Kim *et al.*[95] is in complex with a peptide NS4A and it appears that the NS3 protein requires the peptide as a cofactor for efficient catalysis. The peptide contributes one strand of an eight stranded β barrel in the N-terminal domain of the protein. The active site is found between the N and C terminal domains and contains the catalytic triad, histidine, aspartic acid and serine. The structure of this interesting protease is shown in Figure 16.

5.20 Vaccinia H1-related Phosphatase – The vaccinia virus is a member of the poxvirus family, which includes large DNA viruses that replicate exclusively in the cytoplasm of eukaryotic cells. This protein is a dual specificity phosphatase that hydrolyses both phosphotyrosine and phospothreonine residues. This group of phosphates share a common active site motif HCXXGXXR (S or T) which contains an essential catalytic cysteine residue. The structure described by Yuvaniyama *et al.*[97] shows the enzyme to be related to the Yersinia and human

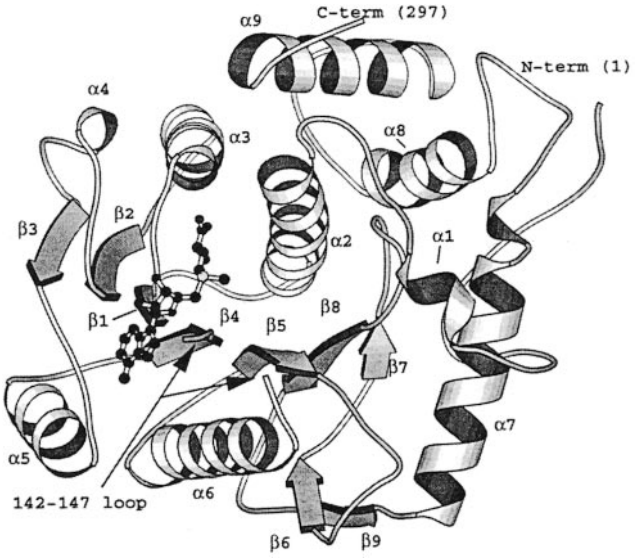

Figure 17. *Ribbon diagram showing the secondary structure elements of VP39. Ball and stik representation is used for the AdoMet molecule. The position of the missing loop near the AdoMet binding site is indicated. Reproduced with permission from reference 98.* © *Cell Press.*

protein tyrosine phosphatases but is smaller and is thought to represent the core structure required for phosphatase action.

5.21 Vaccinia Virus VP39 – These viruses, since they are found only in the cytoplasm, cannot use the transcriptional machinery of the host protein. VP39 is a protein encoded by the viral genome that is involved in the modification of both ends of the viral mRNA. Despite a lack of sequence homology, the catalytic core of VP39 is very similar to that of other AdoMet-dependent methyl transferases. The structure of VP39, determined by Hodel *et al.*, is shown in Figure 17.[98] The VP39 appears to be different from the other related structures since that RNA recognition and binding are incorporated into a single domain where loops and helices present the side chains which contact RNA.

5.22 Nuclear Transport Factor – This protein is interesting since it is involved in the transport of proteins from the cytoplasm to the nucleus of the cell. The proteins that must pass into the nucleus contain signals (NLS's) which consist of short stretches of basic residues. These proteins interact with cytosolic importins α and β, to form a macromolecular complex which binds to a nuclear pore which itself is very complex and comprises 5-100 different proteins. Transport across the pore requires energy in the form of GTP provided by a GTPase, Ran and the

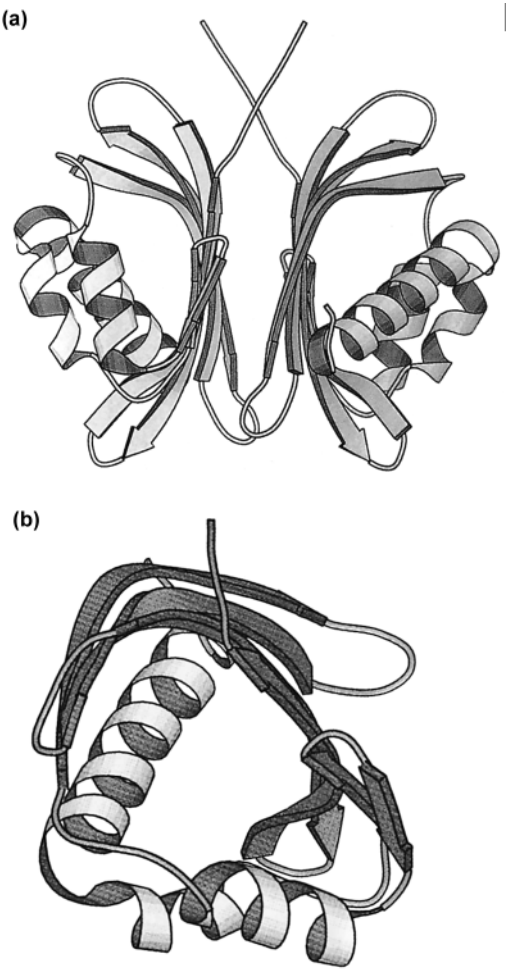

Figure 18. *(a) Ribbon representation of the NTF-2 dimer viewed perpendicular to the non-crystallographic diad. The C-terminus is situated at the top of each monomer. (b) Ribbon representation of the NTF-2 dimer looking down into the hydrophobic cavity. Reproduced from* J. Mol. Biol.*, 1996,* **260***, 422, by permission of the publisher Academic Press.*

nuclear transport factor. This protein is a dimer of identical subunits that form a $\alpha+\beta$ barrel which is closed at one end and has a deep hydrophobic cavity accessible from the other end as shown in Figure 18.[99] This protein interacts with nucleoporin p62 and this is thought to be via a repeated X-F-X-F-G motif found near the N-terminal domain.

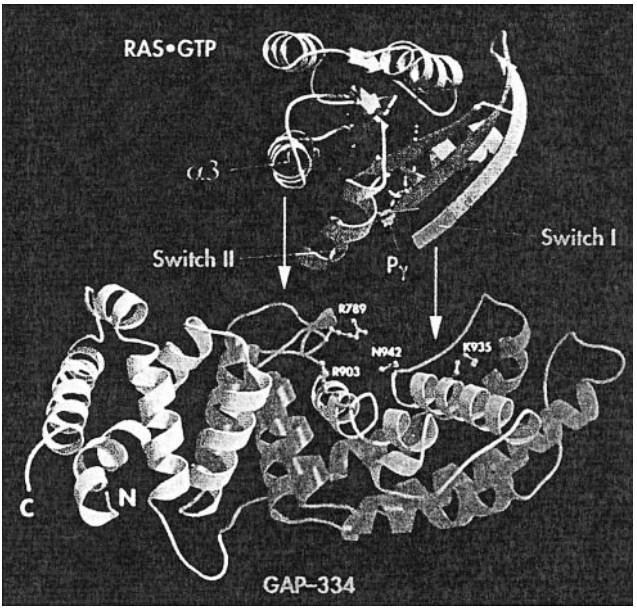

Figure 19. *Docking approach between Ras and GAP modelled manually on the basis of genetic and biochemical studies. Ras is approaching GAP such that its regions implicated to be involved in GAP-accelerated GTP hydrolysis contact the catalytic domain of GAP-334 and the γ-phosphate of GTP is close to residues suggested to participate in the enzymatic reaction. Reproduced with permission from reference 100.*

5.23 Human p120GAP, GTPase-Activating Domain – This protein is involved in activating GTPase proteins such as Ras which is similar to Ran described above. Mutations in these regulatory proteins and Ras are associated with several human diseases. The structure of the GTPase-activating domain from human p120GAP has been determined by Scheffzek *et al*., and a docking model of how it interacts with Ras has been presented.[100] The structure is α helical and adopts a novel fold. Two arginine residues in GAP are thought to participate directly in catalysis of Ras-mediated GTP hydrolysis as shown in Figure 19.

5.24 p53 Tumour Suppressor, Core Domain (Human) S3BP2 Protein, C-Terminal Domain (*E. coli* Complex) – The p53 tumour suppressor pathway is important in suppressing neoplasmic transformation. The structure of the p53 tumour suppressor bound to the ankyrin and SH3 domains of S3BP2 protein has been determined by X-ray methods and suggests that this complex forms *in vivo*.[101] The p53-S3BP2 interface is highly conserved across species and is frequently mutated in cancer.

5.25 6-Phosphofructo-2-kinase/Fructose 2,6-Bisphosphatase – This enzyme is involved with the regulation of the cytosolic levels of fructose 2,6-bisphosphate, which is a key regulatory molecule in glycolysis. It is one of the enzymes that regulates the glucose level in the blood. It is a bifunctional enzyme with both kinase and phosphatase activities. The monomer is divided into two domains, one carrying each function. The kinase domain is related to adenylate kinase and the phosphatase domain with cofactor dependent phosphoglycerate mutases and the acid phosphatases. The structure of the rat enzyme determined by X-ray shows the active enzyme to be a dimer.[102]

5.26 Inosine 5′-Monophosphate Dehydrogenase (IMPDH) – Increased levels of this enzyme have been observed in rapidly proliferating human leukemic cell lines and solid tumour tissues which makes it a target for cancer therapy. The structure of this enzyme has been described in complex with inosine 5′-monophosphate and mycophenolic acid (MPA).[103] This is a potent uncompetitive inhibitor of the enzyme. The enzyme is a tetramer in which each monomer consists of two domains. The larger domain is a αβ barrel and this domain makes up most of the subunit contacts of the tetramer. The MPA is thought to inhibit the enzyme by mimicking the nicotinamide portion of the NAD cofactor and a catalytic water molecule.

5.27 Inosine-Uridine Nucleoside N-Ribohydrolase – This enzyme is found in parasites such as *Crithidia fasciculata* and is a target for a specific drug to treat such diseases. Since these parasites cannot make purines they obtain them from their hosts. Inosine-uridine nucleoside N-ribohydrolase is one of the enzymes involved in this salvage pathway. Its structure has been determined by Degano *et al.*[104] The enzyme is a tetramer where the core of each subunit is an eight stranded central β sheet surrounded by six α helices forming a dinucleotide finding fold. The active site is at the C-terminal end of the β sheet and the cavity has an overall negative charge which may interact and stabilize the positive charge of the ribooxycarbonium ion transition state.

5.28 Human Kinesin Motor Domain – Kinesin is a microtubule-based protein involved in intracellular organelle transport. The structure of this ATP utilising molecular motor has been described by Kull *et al.*[105] The structure shown in Figure 20 is very similar to myosin catalytic domain. The proteins do not exhibit any sequence homology and have different enzymatic and motile properties. Loop residues in the protein are proposed to interact with microtubules and the C-terminal region is thought to propagate and amplify conformational changes induced by the hydrolysis of ATP. The crystal structure of the motor domain of the kinesin-related motor ncd from *Drosophila* has been determined in complex with MgADP.[106] Both kinesin and ncd are involved in the formation and maintenance of mitotic and meiotic spindles. They move in opposite directions with kinesin being plus end-directed and ncd being minus end-directed. The structures have 40% sequence homology but are structurally superimposable except for several discrete differences at the top end of the molecule.

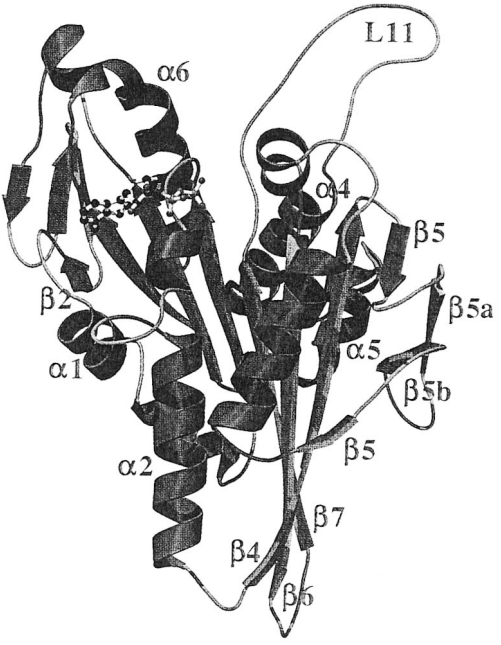

Figure 20. *The secondary structure elements of kinesin where the ADP molecule is shown in ball and stick representation. Reproduced with permission from reference 105.*

5.29 Yes Kinase Associated Protein, WW Domain (Human)/Peptide Complex – The WW domain is essential for control of epithelial sodium channels where disruption of the interaction leads to Liddle's syndrome. The structure of the domain has been solved by NMR methods.[107] It is found to be ~38 amino acids with a high proportion of hydrophobic, aromatic and proline residues. Two tryptophans, spaced by 20-22 residues are highly conserved. The structure is solved in complex with a proline rich peptide with a consensus sequence PPXY. The core of the WW domain is a triple-stranded anti parallel β sheet. The central prolines of the peptide make contact with one of the conserved tryptophans.

5.30 Human Transthyretin, FAP Amyloid Protofilament – Amyloid diseases, such at Alzheimer's, transmissible spongiform encephalopathies, type-II diabetes and FAP involve the deposition of protein that is normally soluble into insoluble abnormal fibrils that invade the extracellular space of essential tissues. Synchrotron X-ray studies have been carried out with the transthyretin amyloid fibril.[108] A novel protofilament structure is proposed for the fibrils from patients with FAP which is a condition caused by a mutation of a valine to a methionine aminoacid in transthyretin. The model comprises a 24 β strand repeating unit with β strands arranged perpendicular to the fibre axis, forming a complete

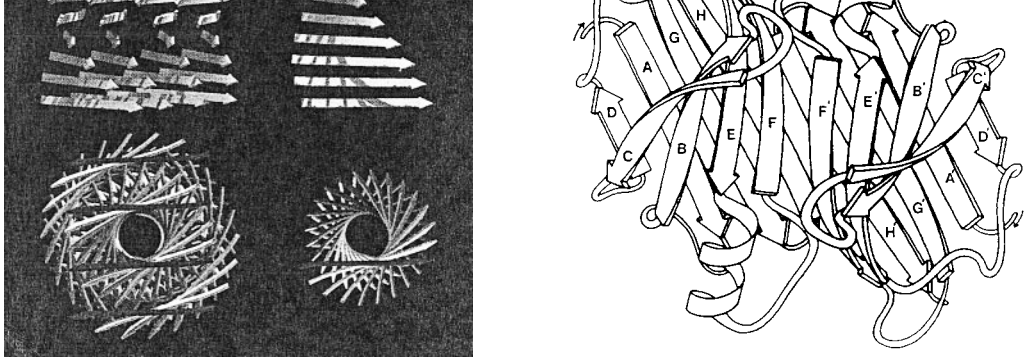

Figure 21. *(a) Model of the core structure of the FAP amyloid profibril and (b) a ribbon drawing of the transthyretin dimer. Reproduced with permission from reference 108.*

helical turn around an axis parallel to the fibre axis (as shown in Figure 21). The stability of the helix is thought to be due to hydrogen bonding between the sheets over a long distance. The proposed structure requires that the transthyretin building block be structurally different from the isolated homotetramer. It is proposed to be a monomer or dimer with reorganised or truncated β sheet. The amyloid formation would require significant structural changes in the precursor proteins.

6 Other Interesting Proteins

6. 1 D-Amino Acid Oxidase – Although proteins are composed of L amino acids the existence of the enzyme D-amino acid oxidase (DAAO) has been known for many years and is found in bacteria and in eukaryotes. In humans it is found in kidneys and the cerebellum where it is thought to have a role in the regulation of D-amino acids now known to be involved in neurotransmission as free amino acids or as part of neuroactive peptides. Two papers were published in 1996 which described the structure of the enzyme. These were of the pig kidney D-amino acid oxidase.[109,110] The crystal structures which are very similar although they were determined from different symmetry of crystals and under different conditions. They show that the active site of DAAO is formed by a cavity lined by hydrophobic residues and only a few polar side chains. Several mechanisms had previously been proposed for the mechanism of the enzyme, one of which was the carbo-anion mechanism. However, the structure has no residues in the active site that would act as the active-site base. The structure is consistent with the reaction occurring via the transfer of a hydride ion from the amino acid α carbon to the flavin as shown in Figure 22. An analysis of the active site

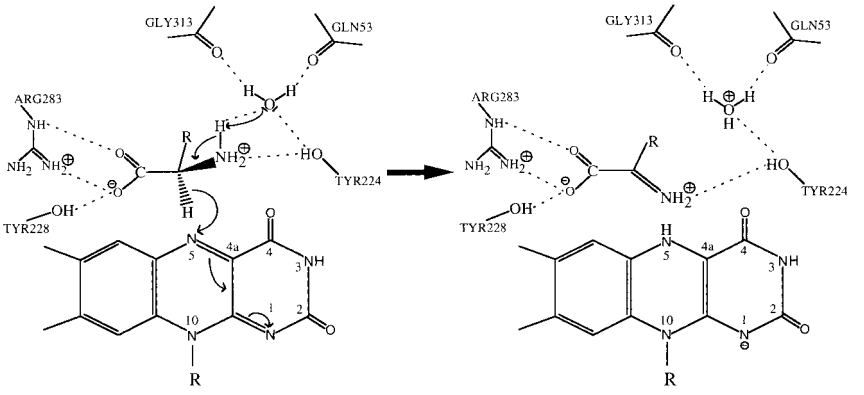

Figure 22. *Proposed mechanism for the reductive half cycle carried out by D-amino acid oxidase. The proton of the substrate α-amino group is accepted by the active site water molecule. Reproduced with permission from reference 109.*

architecture reveals the mirror image convergent evolution with flavocytochrome b_2FCB2. New insights into the catalytic mechanism of flavocytochrome b_2 were reported by Daff *et al.*[111] The catalytic centre of FCB2 has a catalytic histidine residue that could act as the active-site base, abstracting the substrate α hydrogen as a proton, as postulated by the carbo-anion hypothesis. However a hydride transfer could also be possible with the catalytic histidine acting as the base abstracting the proton from the substrate hydroxyl group. The major difference in the active site of these two enzymes is that the active centres are located on opposite faces of the flavin ring. They use similar active site architecture but solve the problem of different stereochemistry of their substrates using opposite sides of the flavin ring to place their active centres.

6.2 Green Fluorescent Protein – An interesting protein structure solved in 1996 was that of the green fluorescent protein.[112,113] This structure is shown in Figure 23a and consists of a β barrel with eleven strands on the outside of a cylinder of diameter ~30 Å and length ~40 Å. Inside the cylinder is a fluorescent centre composed of a modified tyrosine sidechain and cyclized protein backbone as part of an irregular α helical segment. This represents a new class of protein fold called the 'β can'. The fluorophore is thought to originate from an internal Ser-Tyr-Gly sequence that is post-translationally modified to a 4(p-hydroxybenzylidene)-imidazolidin-5-one structure. The fluorescence absorbance/excitation peak is at 395 nm with a minor peak at 475 nm. The normal emission peak is at 508 nm. Femtosecond time resolved spectroscopy studies by Lossau *et al.*[114] and Chattoraj *et al.*[115] have revealed that the two states corresponding to the two major absorption bands can interconvert quickly in the excited state and the presence of an isotope effect implicates proton movement in the interconversion. The environment of the fluorophore is crucial for its function. Model compounds identical to the hydroxyphenyl imidizolidinone core of the fluorophore have been synthesised and are shown not to be significantly fluorescent in solution.[116] The nearby basic amino acids histidine, glutamine and arginine appear to stabilise anionic oxygen atoms at the opposite ends of the fluorophore and the acidic group from glutamic acid forms a hydrogen bond with the hydroxyl group of a serine residue as shown in Figure 23b. Mutants of the protein have been made, to understand its mechanism of fluorescence.[117,118] Removal of more than seven amino acids from the C-terminus or more than the N-terminal methionine leads to loss of fluorescence.[119] In the crystal structure the C-terminus loops back outside of the cylinder. The N-terminal segment forms a cap on one end of the protein.

7 Summary

In this review I have tried to summarise some of the wealth of literature which has appeared on proteins in 1996. The research in this area appears to grow at an ever increasing rate and clearly only certain topics and proteins have been discussed.

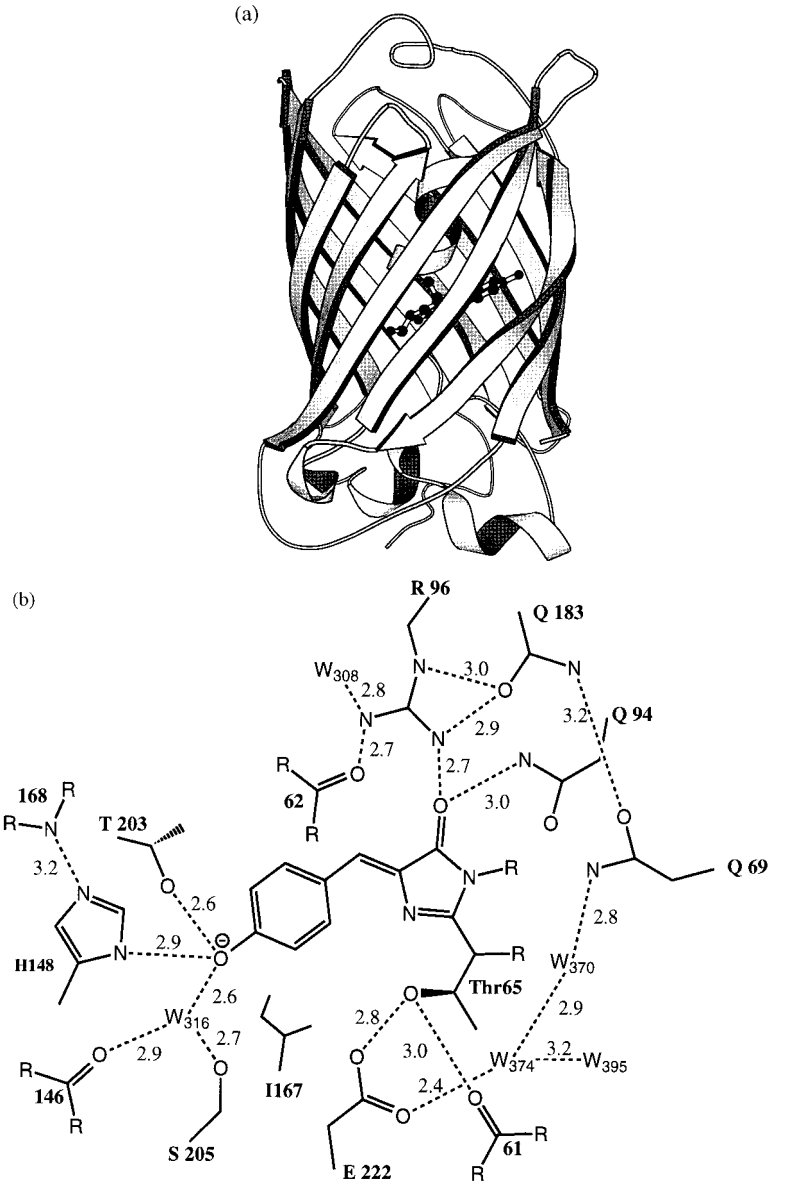

Figure 23. *(a) Schematic drawing of the backbone of green fluorescent protein with the chromophore shown as ball and stick representation. (b) Schematic diagram showing the first and second spheres of co-ordination of the chromophore. Hydrogen bonds are shown as dashed lines and have the indicated lengths in angstroms. Part (a) reproduced with permission from reference 113.*

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