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Amino Acids, Peptides and Proteins

VOLUME 27

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A Specialist Periodical Report

Amino Acids, Peptides and Proteins

Volume 27

A Review of the Literature Published during 1994

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Preface

The task of compiling five of the six chapters has been carried out by authors who have been loyal to this series of Reports over many years. But this year one chapter (Chapter 3) has been written by two newcomers (Michael North and Stef Biagini) who have injected 'new blood' into our reporting of biologically active analogues and peptidomimetics. We welcome them, hoping that they can contribute further in future. This volume also sees the return of the biennial coverage on Metal Complexes of Amino Acids and Peptides (Chapter 5). For the convenience of editing, the order of the chapters has been changed so that Jennifer Littlechild's chapter on proteins appears as Chapter 6. The coverage in each of the chapters has not been drastically changed although the changing patterns in the peptide field demand annual self-assessment by our reporters. Generally reporters confirm buoyant activity in 1994, and somewhat surprisingly in this age of computer-aided information retrieval a number of specialist reviews have been reported. We thought that our team here were the only willing horses on Earth to undertake such tasks!!

There is a continuing shift of emphasis away from fundamental chemistry associated with making peptides into the more technical fine tuning of solid phase techniques. The expansion of combinatorial libraries has also made it more difficult to define the boundaries of our peptide synthesis coverage since choosing an efficient assay is as important as the synthetic methodology involved. Nevertheless, developments in HPLC and in mass spectometry (e.g. electrospray techniques) have added greatly to the armoury available, and assessing mixtures can now be done more confidently than ever before. NMR and X-ray techniques applied to pure proteins have augmented the Brookhaven database to over a thousand structures – a remarkable achievement.

Since the early days, peptide chemists have always cast a watchful eye for 'racemisation' in their products. A short note appeared in 1994 (*Int. J. Pept. Protein Res* 1994, **44**, 399) that the use of the term itself is inappropriate! The process should be termed 'enantiomerisation'. It will be interesting to see what terminology will survive into the next millennium. The reporting period coincided with a number of international symposia which provide excellent contact environments and the rapidly published reports from the symposia provide food for thought for future ideas. However the policy taken by the Reporters in this series is that the contents of these conference proceedings become 'reportable' when the works appear in refereed jourrnals.

Word-processing and printing technology has revolutionised the production of these Reports over the years, and printing is now made direct from floppy disks. However, the work still reflects very many hours of human toil from the Reporters, and the co-operation of the editorial team at the RSC, in particular Janet Freshwater. I am most grateful to everyone for all the hard work to get this volume into print.

John S. Davies
University of Wales, Swansea

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.

In 1995 the Joint Commission issued the following corrections to the above Recommendations:

Section 3AA–13.4 For Ala-Thr-Gly-Asp-Gly, read Ala-Thr-Gly-Asp-Gly

Section 3AA–13.5 The correct name is (7*E*,9*E*,11*Z*,14*Z*)-(5*S*,6*R*)-6-[(cysteinyl-glycin)-*S*-yl]-5- hydroxyicosa-7,9,11,14-tetraenoic acid.

A complete listing of the single-letter code 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 and unusual abbreviations are periodically compiled, and the latest examples were published in the abbreviations section of last year's volume (Volume 26).

List of Abbreviations

The following is a list of the abbreviations for the less well-known non-proteinogenic amino acids used in the text. Some of these compounds, for example 2,4-diaminobutyric acid, have more than one abbreviated form.

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-cyclopentamethylene propionic 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 G.C. BARRETT

1 Introduction

The 1994 literature covering the amino acids, from the point of view of their chemistry and biochemistry, is dealt with in this Chapter. The approach adopted is identical to that used in all previous Volumes of this Specialist Periodical Report. The Chapter concentrates on the literature covering the natural occurrence, chemistry, and analysis methodology for amino acids. Routine literature covering the natural distribution of well-known amino acids is excluded. Patent literature deals with material that also finds its way into the conventional literature, and is therefore excluded from this Chapter. It is easily reached through the appropriate sections of *Chemical Abstracts* (Section 34 in particular).

The flow of Journal papers and secondary literature continues to accelerate, as far as the amino acids are concerned, and papers have been collected for this Chapter from major Journals and from *Chemical Abstracts* [to Volume 122 (1995), issue 9]. Where it is helpful to refer to earlier Volumes of this Specialist Periodical Report, the formula '(see Vol. 23, p. 3)' is used.

Most of the papers cited are only briefly described, so that adequate commentary can be offered for particular papers presenting significant advances in synthetic and analytical methodology relating to the amino acids, with mechanistically-interesting chemistry being given prominence.

The coverage adopts the usual meaning of the term 'amino acids', i.e. aminoalkanoic acids $H_3N^+(R^1R^2C)_nCO_2^-$. Many conceivable structural types (for example, benzene derivatives carrying amino and carboxy groups) are excluded. Representative citations are offered, of analogues in which the carboxy group is replaced by a phosphorus oxyacid equivalent, H_3N^+-$(R^1R^2C)_n$-$P(O)(OH)(O^-)$,[1-4] e.g. (1S,2S)-phosphothreonine,[4] have important research applications; even the boron analogue $R_3N{\cdot}(BHR^1)_n$-CO_2R may hold some similar promise.[5]

2 Textbooks and Reviews

A substantial source of information on instrumental and analytical protocols[6] includes material on the amino acids. A similarly thorough coverage of topics in the synthesis of amino acids has been published.[7]

Several reviews will be found in appropriate sections of this Chapter, though others of a more general nature are collected here; these cover α-aminoisobutyric acid,[8] carboranylalanine in neutron capture therapy,[9] cyclopropane-based amino acids,[10] 1-aminocyclopropanecarboxylic acid synthesis,[11] cyclobutane-based amino acids,[12] synthesis of heterocyclic amino acids,[13] uses of amino acid esters as chiral auxiliaries in organic synthesis,[14] uses of α-amino acids in aminosugar synthesis,[15] and stereochemical details of metabolic reactions of amino acids.[16]

3 Naturally Occurring Amino Acids

3.1 Isolation of Amino Acids from Natural Sources – All fermentative processes for the production of amino acids require routine isolation of the product, and, like the production of amino acids through protein hydrolysis, the separation of mixtures is a common concluding stage to the process. This section is intended to select some less routine aspects of the isolation of amino acids, particularly those unexpected outcomes of otherwise straightforward procedures.

Protein hydrolysis is most commonly accomplished using hydrochloric or methanesulfonic acids, but several alternative protocols have been suggested; mercaptoethanesulfonic acid (160-180°) has been found to be effective.[17]

Continuous concentration of amino acids using a liquid emulsion membrane with a cation extractant, di-2-ethylhexylphosphoric acid, has been described.[18] Preparative chromatographic isolation (gel filtration and partition) of pyridinoline[19] and of hydroxylysyl- and lysyl-pyridinolines[20] from biological fluids, and preparative chromatography of benzyl esters of basic amino acids[21] and N-benzyloxycarbonyl amino acids (Z-amino acids)[22] illustrate standard methods.

3.2 Occurrence of Known Amino Acids – Where common amino acids are found in meteorites, and in ancient fossils, an obvious first question, but one only recently addressed in a rational scientific manner, is: is the amino acid indigenous or has it been introduced subsequently? Further studies (see Vol.25, p.3) based on sensitive GC-MS isotope-analytical techniques confirm the indigeneity of amino acids through identical $\delta^{13}C$ and $\delta^{15}N$ values for D- and L-enantiomers of a particular amino acid in thoroughly-cleaned 7000–10^5 y samples, and in Pleistocene fossils.[23] Quaternary land snails given more detailed study[24] confirm this diagnostic test as far as neutral amino acids are concerned, but differing $\delta^{13}C$ values for D- and L-enantiomers of aspartic and glutamic acids introduce an element of doubt; presumably these amino acids as constituents of shell protein are subject to more complex diagenesis.

Contemporary natural sources that have been shown to contain unusually interesting, though known, amino acids include D-aspartic acid (supplied by intestinal bacteria) in appreciable quantities in *Octopus vulgaris*,[25] the antimicrobial and antioxidant N-(p-coumaryl)pipecolic acid in rhizomes of *Cirsium brevicaule*.[26] Both D- and L-tert-leucine appear, together with D-kynurenine, as constituents of discodermin E, from the marine sponge *Discodermia kiiensis*.[27] α-Methylcysteine appears in condensed form in the cryoprotective agent

thiazohalostatin (1) from *Actinomadura*.[28] Another peptide from *Verticillium coccosporum* has been discovered to contain 2-amino-8-oxo-9-hydroxydecanoic acid.[29]

The presence of β-alanine in *Clitocybe acromelalga*, as its L-glutamide derivative[30] adds another natural location to those already established for this β-amino acid. γ-Hydroxy-L-glutamic acid occurs in bulbs of *Hemerocallis longituba* in the form of the amide, longitubanine (2; R = OH).[31]

Synthetic cis-4-methylproline is physically different from the compound located over the years in various natural sources, calling for some reconsideration of the structural assignments.[32]

The assessment of crosslinks that develop *in vivo* in proteins of higher species, as a result of ageing or disease, has become an important diagnostic criterion, and pyridinium crosslinks[33] and dityrosine crosslinks have been identified in bovine thyroglobulin.[34]

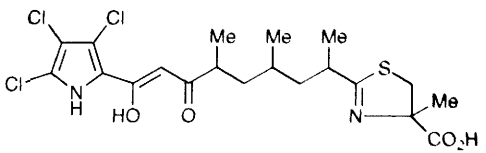
3.3 New Naturally Occurring Amino Acids – Mycestericins from *Mycelia sterilia* are potent immunosuppressants that have been shown to be hydroxylated α-hydroxymethyl-α-aminoalkanoic acids (3–5) of extraordinary types.[35] Another new acyclic aliphatic α-amino acid also owes its fascination to the functional group that it contains, the first natural azoxy-containing antifungal agent, L-azoxybacilin (6), from *Bacillus cereus* NR2991.[36] Sphingofungins (7) are a new family of antifungal metabolites from *Aspergillus fumigatus* ATCC 20857.[37] New opines (8)[38] and piperidine 2,4,5-tricarboxylic acid (9), are further metabolites from *Clitocybe acromelalga*[39] (see also Ref.30). Five new compounds (e.g., 10 and stereoisomers) related to domoic acid have been isolated from mussels.[40]

3.4 New Amino Acids from Hydrolysates – γ-Hydroxy-tert-leucine is a constituent of polytheonamides A-C from the marine sponge *Theonella swinhoei*,[41] and Zwittermicin A from *Bacillus cereus* is (11).[42] The other new amino acids are mostly lactams condensed into more complex structures; the 2,5-dihydrofuryl-γ-lactams, fulvanines D and E (12), (13) from *Hemerocallis fulva*,[43] anchinopeptolides B–D (14) from the sponge *Anchinoe tenacior*,[44] the antibiotic magnesidin A (15) from *Vibrio gazogenes*,[45] and the novel siderophore vibrioferrin (16) that develops in *Vibrio parahaemolyticus* in response to limitation of Fe in the culture fluid.[46]

4 Chemical Synthesis and Resolution of Amino Acids

4.1 General Methods for the Synthesis of α-Amino Acids – The term 'general methods' has been attached to a group of reactions that have become familiar through use for many years; these are covered in this Section as far as the α-amino acids are concerned.

Relatively few novel ideas have been introduced under this heading in recent years, and those that have, have been concerned with the burgeoning area of 'Asymmetric Synthesis'. Although given a Section of their own in this Chapter



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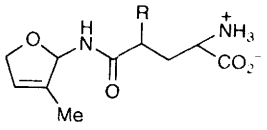

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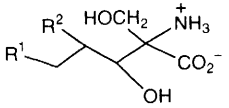
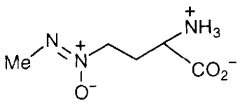


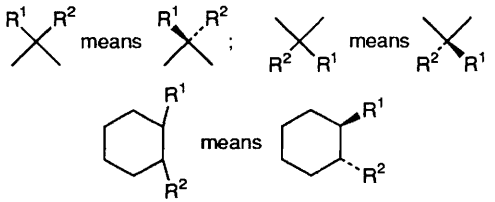
(3) R^1 = (*E*,*E*, *R*)-CH=CH$(CH_2)_4$CH=CHCH(OH)$(CH_2)_5$Me, R^2 = OH
(4) R^1 = (*E*)-CH=CH$(CH_2)_6$CH(OH)$(CH_2)_5$Me; R^2 = OH
(5) R^1 = $(CH_2)_8CO(CH_2)_5$Me; R^2 = OH; or R^1 = (*E*)- CH=CH
or R^1 = (*E*)-CH=CH$(CH_2)_6CO(CH_2)_5$Me; R^2 = H

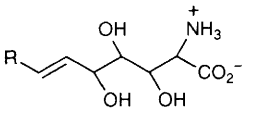


(6)

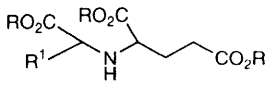
Three-dimensional features at chiral centres of structures depicted in this chapter follow the convention:–

(a) horizontally-ranged atoms, and their bonds, and atoms in rings, are understood to be in the plane of the paper;
(b) atoms and groups attached to these atoms in (a) are ABOVE the page if ranged LEFTWARDS and BELOW the page if ranged RIGHTWARDS:

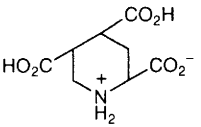




(7) R = $(CH_2)_6CH(OH)(CH_2)_5Me$ and
N-acetylated, for Sphingofungin D;
N-non-acetylated, for Sphingofungin B;
NH_3^+ replaced by $NHC(=NH)NH_2$ for Sphingofungin A



(8) R^1 = Pr^i, Bu^i, or Bu^s



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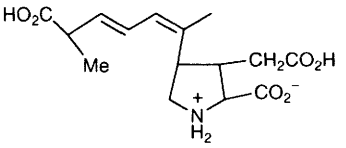

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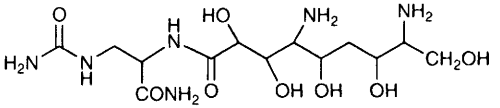

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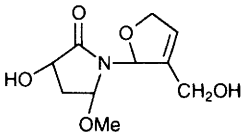

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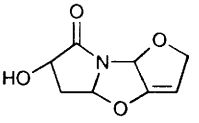

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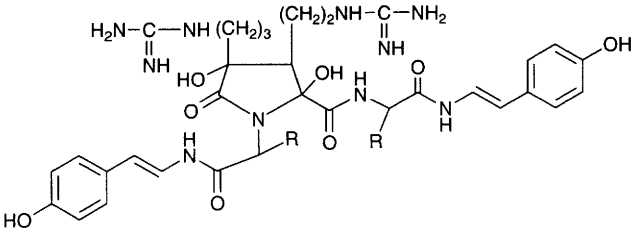
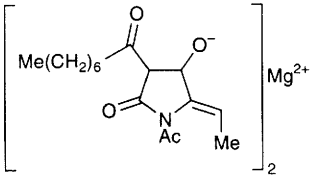


(14) various R



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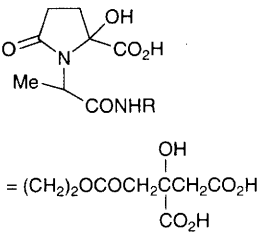
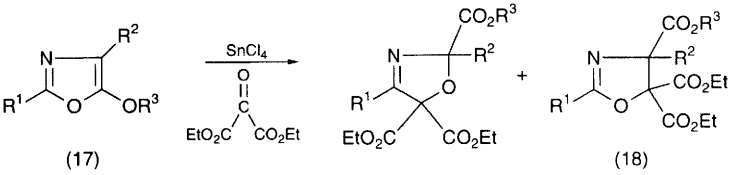


(16) R = $(CH_2)_2OCOCH_2\overset{OH}{\overset{|}{C}}(CH_2CO_2H)CO_2H$



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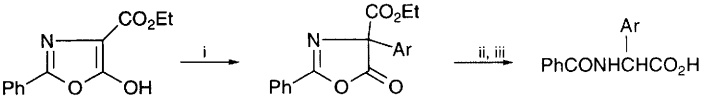


Reagents: i, $ArPb(OAc)_3$; ii, NaOH in EtOH–H_2O; iii, H_3O^+

Scheme 1

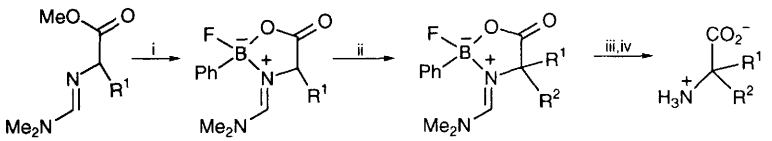
(Section 4.2), asymmetric synthesis methods are nearly always 'general methods of synthesis' too. Other general reactions by which one amino acid is used as starting material for the synthesis of another, are mostly covered in the later Section 6.3 (Specific Reactions of Amino Acids).

Long-established methods continue to be revisited as reliable routes, and many of these are used in syntheses of labelled amino acids (see Section 4.15). The alkylation of diethyl phthalimidomalonate (see Refs.166,256) and diethyl acetamidomalonate (see also Refs.254,258), e.g. for a synthesis of 2-amino-7,7-dimethyloctanoic and 2-amino-8,8-dimethylnonanoic acids,[47] and the alkylation of oxazolones, e.g. in an aspartic acid synthesis (17 → 18),[48] in a synthesis of α-amino-β-phosphonopropionic acid,[49] and in an arylglycine synthesis (Scheme 1)[50] and corresponding vinylglycine synthesis,[51] are typical long-established methods. Rearranged dimers that are well-known (usually unwanted) side-products from oxazolone alkylation, can be hydrolysed to give α-alkyl-α-amino acids.[52] Addition of a thiol to 4-benzylidene-2-methyloxazolone, followed by routine work-up, gives a threo/erythro-mixture of N-acetyl S-(p-methylbenzyl)-β-phenylcysteine methyl ester.[53] A novel variant of the oxazolone procedure is represented in the conversion of a 4,4-bis(isopropylthio)oxazolone into amides or peptides, and its chlorinolysis (SO_2Cl_2) to give halogenoglycine derivatives that are easily converted into other amino acids through halogen substitution.[54]

These two general methods are essentially glycine alkylation procedures; other routes in this category include alkylation of glycine Schiff bases (phase-transfer catalysed alkylation of $PhCH=NCH_2CO_2Me$ → phenylalanine, mediated by microwave energy),[55] and corresponding syntheses of leucine, serine and aspartic acid,[56] Michael additions, of $(R^1O)_2P(O)CH=CH_2$[57] and a two-step alkylation (by $R^1CH=CRCH_2Br$ then γ-elimination of Br) to give α-cyclopropylglycines.[58] Similar approaches employing N-phenacyl-N-benzylglycine[59] and N-(ω-chloroalkyl)-N-Boc-glycine[60] as starting materials lead to azetidinecarboxylic acids and higher homologues. The corresponding use of N-oxides of glycine Schiff bases to prepare α-(N-hydroxyamino) acids,[61] and of α-amidinoalkanoates (Scheme 2)[62] have a good deal in common, mechanistically.

The [3,3]-rearrangement of N-protected glycine allyl esters (19 → 20)[63] exemplifies an alternative glycine alkylation process that has been well studied from the 1960's.

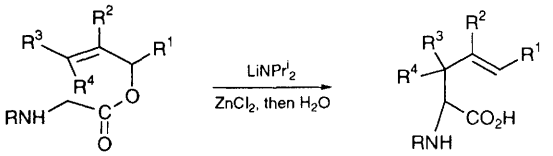
Alkylation of α-halogenoglycine synthons (Scheme 3) is significantly facilitated by $ZnCl_2$, indicating a radical mechanism where the catalyst is both a radical initiator and chelates the substrate.[64] Copper(I)-catalysed Cl-transfer radical cyclization of N-(alk-3-enyl)-α-chloroglycines gives prolines *via* 2-aza-5-alken-1-yl radicals.[65] A similar study of the generation of the glycine α-radical formed by stannanes from α-bromo-, -benzyloxycarbonyloxy-, and -methoxy-glycine derivatives, and its alkanesulfenylation with disulfides, has been described.[66] Xanthates $MeO_2CNHCH(S_2COEt)CO_2Me$ similarly yield radicals that add to alkenes to offer a valuable new general amino acid synthesis.[67] N-Protected α-hydroxyglycine esters are readily substituted, illustrated this year in a preparation of (p-vinylphenyl)glycine.[68] α-Acetoxy analogues have been employed in syntheses of vinylglycine[69] and propargyl homologues.[70]



(+ epimer at B)

Reagents: i, $K^+ PhBF_3^-/Me_3SiCl$; ii, $KOBu^t$, R^2hal; iii, refluxing MeOH; iv, ethylenediamine / MeOH

Scheme 2



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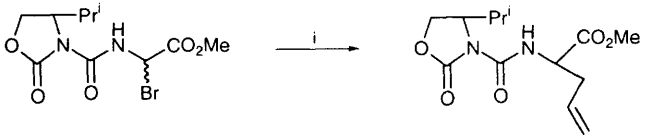


Reagents: i, $CH_2CH{=}CHSnBu_3$, $ZnCl_2.OEt_2$

Scheme 3

Isocyanoacetates $CNCH_2CO_2R$ (see also Ref.255) perform well in aldol additions that show high diastereoselectivity to provide β-hydroxy-α-amino acids.[71] α-Nitroacetates are readily alkylated, Michael addition of allyl acrylate followed by reductive cyclization giving N-hydroxy-pyroglutamate derivatives.[72]

Amination processes leading to amino acids constitute an established group of general methods that have been exemplified this year by some of the oldest variants: reductive amination of α-ketocarboxylic acids using NH_3/Raney nickel,[73] and of methyl (1S,2R,3R)-3-hydroxy-2-methoxycyclohexanecarboxylate;[74] ammonolysis or methylaminolysis of t-butyl bromoacetate,[75] and the corresponding process with diethyl bis(2-methylthioethyl)malonate.[76] Addition of ammonia, primary amines, or hydroxylamine to substituted fumaric acids leads to corresponding aspartic acid analogues,[77] and corresponding Michael addition of N-acylisoureas (formed from a carbodi-imide and a carboxylic acid) to methyl hydrogen maleate to give N-carbamylaspartic acids.[78] Further examples (see Vol. 26) of the formation of cyclic hydrazino-acids through cycloaddition of dienes to azodicarboxylates, have been published[79] (see also Ref.199). Condensation of a primary amine ($TiCl_4$) with a γ-chloro-α-ketoester to give a γ-chloro-α-iminoester is followed by cyclization to give a 1-amino-2,2-dialkylcyclopropanecarboxylic acid.[80]

Several examples of azidation, of enolates[81] and of α-methoxyacrylonitriles (giving α-azidonitrates),[82] have been described as stages in α-amino acid syntheses. Diazonium salts are electrophilic α-aminating agents towards esters in the form of their ketene silyl ketals, yielding α-azo- or -hydrazono-esters which on hydrogenation yield α-amino acid esters.[83] Use of an alkyl sulfenimine $R_2S{=}NH$ as aminating agent towards a latent nucleophilic carboxy group equivalent has been given a preliminary assessment.[84]

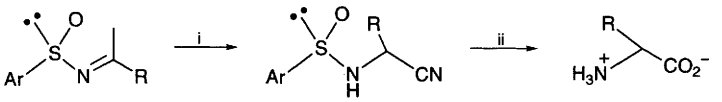
Amination through Stevens rearrangement of transient ammonium ylides formed between amines and diazoketones or diazoesters gives α-aminoketones or α-amino esters, respectively, in one step.[85]

Amidocarbonylation – the introduction of both amino and carboxy groups in a one-pot process – has been illustrated in an N-acetylglycine synthesis (paraformaldehyde, CO, and H_2, with a cobalt-phosphine catalyst),[86] and the distantly-related equivalent process from aldehydes and $CHCl_3$ continues to be studied.[87]

Introduction of the carboxy function into a protected amine, to lead to the corresponding α-amino acid, can be accomplished in certain cases, e.g. by the oxidation of a phenyl group (C_6H_5- → -CO_2H) using RuO_4.[88]

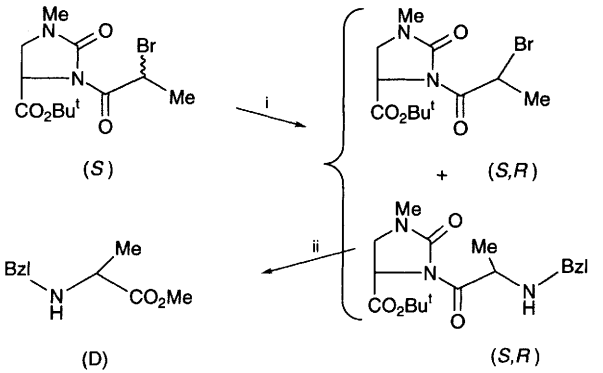
'Modifications to an amino acid side-chain' could be described as a general method of amino acid synthesis, although examples of this approach constitute a somewhat miscellaneous collection and are mostly located later in this Chapter (Section 6.3). However, an interesting set of procedures for the alkylation of the dehydro-alanine derivative methyl 2-acetamidoacrylate)tricarbonyliron(0), has been described,[89] leading to βββ-tri-alkyl amino acids through successive treatment with 2 eq MeLi and an alkyl halide (see also Vol.25, p.9).

Contraction of a β-amino acid backbone could also be described as a general synthesis method for α-amino acids, and further examples (see Vol.24, p.8) of the conversion of α-keto-β-lactams into α-amino acid N-carboxylic acid anhydrides



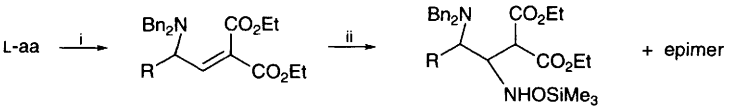
Reagents: i, Et_2AlCN; ii, 6M HCl, reflux

Scheme 4



Reagents: i, $PhCH_2NH_2$, K_2CO_3, CH_2Cl_2, r.t. 40 h; ii, NaOMe/ MeOH

Scheme 5



Reagents: i, established methods; ii, $Me_3SiNHOSiMe_3/CH_2Cl_2$, 22 °C, 18h

Scheme 6

have been accomplished by Baeyer-Villiger oxidation,[90] a process that is applicable to homochiral substrates, leading to β-alkylserine N-carboxylic anhydrides.[91] The method has been also been illustrated in a synthesis of (R)-α,β-di-amino-γ-hydroxyacid N-carboxylic anhydrides from β-lactams.[92]

4.2 Asymmetric Synthesis of α-Amino Acids – Activity in this area continues to increase, both in the provision of new methodology and in the development of established methods, including well-known standard general methods of synthesis, some of which are described in the preceding section, and revisited here in 'asymmetric versions'.

Two thorough reviews cover the overall topic[93,94] and another review deals with asymmetric synthesis of '2,3-methano'-amino acids (i.e., 1-aminocyclopropanecarboxylic acids).[95]

Modifications of standard general methods of α-amino acid synthesis are represented in a Strecker procedure employing a chiral ketone as catalyst for the equilibration of aminonitriles $R^1R^2C(CN)NHCHRCN$,[96] and in an equivalent process using homochiral sulfinimines (illustrated for the (S_S)-configuration in Scheme 4).[97]

Amination reactions incorporating kinetic resolution (Scheme 5),[98] and related hydroxylamination (Scheme 6),[99] illustrate further standard methods.

Several examples of the alkylation of glycine derivatives can be grouped together: Michael addition of $MeCH(CN)CO_2Me$ to vinyl ketones or acrolein catalysed by a Rh(I)-chiral phosphine complex, giving (R)-$RCOCH_2CH_2CHMe(CN)CO_2Me$ in 83–93% e.e. and thence to (R)-α-methyl-α-amino acids through routine elaboration;[100] stereoselective alkylation of glycine Schiff bases $Ph_2C{=}NCH_2CO_2Bu^t$ using active methylene compounds and a (-)-cinchonidine-derived chiral catalyst;[101] aldolization of glycine with PhCHO, catalysed by supramolecular bilayer assemblies containing L-alanine–lipid peptides with pyridoxal and Cu(II) salts, to give (S)-β-phenylserine in modest enantiomeric excess;[102] alkylation of (-)-menthyl N-acetyl α-bromoglycine by allyltrimethylsilane catalysed by $ZnCl_2$ to give (S)-(+)-norvaline after hydrogenation (see also Scheme 3);[103] for a synthesis of (-)-menthyl N-acetyl α-hydroxyglycine by this research group using (-)-menthyl glyoxalate + $MeCONH_2$, see Ref. 104.

Addition of a glycine enolate to the carbonyl group of α-D-ribohexofuranos-3-ulose gives the corresponding (S)-α-(glycos-2-yl)glycine.[105] The asymmetric benzylation of the carbanion from (-)-menthyl hippurate and similar glycine derivatives carrying chiral auxiliary groups, although thoroughly researched over many years, was recently found to be unsuccessful when only one equivalent of base is used.[106] In a broad study, the diastereoselectivity of this process was shown to be dependent upon the amount of additives and the nature of the N-acyl group and of the chiral ester. Alkylation of homochiral glycinamides gives generally good diastereoselectivity.[107] N-Boc-2-(tert-Butyldiethylsilyloxy)pyrrole is a glycine equivalent that underges aldol addition to homochiral aldehydes (Scheme 7) to give α-(polyhydroxyalkyl)-α-amino acids.[108]

Further conventional approaches are described for phase transfer-catalysed Gabriel synthesis employing (-)-bornyl α-bromoalkanoates (optical purities from

1.7–47%);[109] rather worse results are obtained in this reaction with various heterogeneous phase-transfer catalysts.[110] The conversion of lithium (1S,2R,4R)-10-dicyclohexylsulfamylisobornyl-2-cyano-3,3-diphenyl propanoate into ββ-diphenyl-α-methylalanine (21 → 22) involves a Curtius rearrangement.[111] A related approach for a synthesis of (S)-3,4-dichlorophenylalanine[112] is completed by a subtilisin resolution (see Section 4.17) in view of the disappointing optical purity of the product of the synthesis.

Photochemical amination of chiral silyl enol esters (23 in Scheme 8) has been illustrated in a synthesis of erythro-β-methyl-L-phenylalanine.[113]

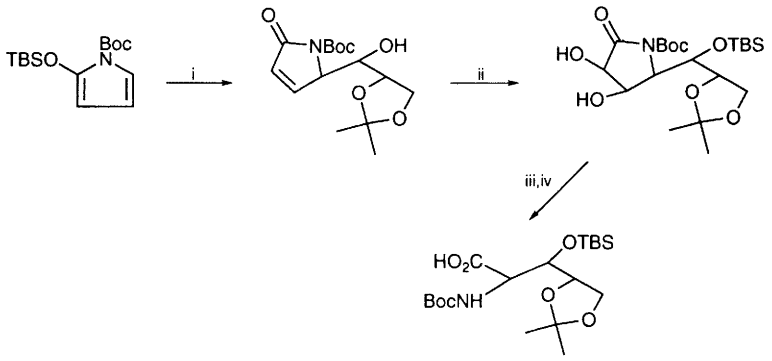
The Schollkopf bis-lactim ether synthesis continues in use (see also Ref.260) for the asymmetric synthesis of 2-amino-3-methyl-4-phosphonobutanoic acids,[114] 3-fluoro-4-nitro-L-phenylalanine,[115] bicyclic lactams,[116] and (2S,3R)-3-methylglutamic acid.[117] Alkylation of L-valine-derived piperazine-2,5-diones (24) followed by hydrolysis leads predominantly to (S)-α-amino acids.[118]

The S-configuration is induced by the chiral grouping in the substrate, during the hydrogenation of N-formyl-(Z)-dehydro-βββ-trifluorobutyrine (-)-menthyl ester (25 → 26).[119] The other main interest in the asymmetric hydrogenation of dehydro-amino acid derivatives over the years has concentrated on achiral substrates, and on the development of improved asymmetric hydrogenation catalysts. A clear enhancement of enantioselectivity (e.e.'s of L-amino acids up to 99%) accompanies the incorporation of electron-donating aromatic substituents in vic-diarylphosphonites derived from carbohydrates, as the chiral moiety in a Rh(I)-chiral phosphine catalyst.[120] The role of the protecting groups in cinnamates subjected to this procedure has been assessed.[121]

The main focus of development of synthetic methodology continues to be the use of heterocyclic chiral auxiliaries, most of which have been favoured for several years now. Predominantly (2R)-syn-β-substituted serines are formed (d.e. 84–100%) when the Ni(II) complex of the Schiff base of N-benzyl-L-proline o-benzoylanilide is aldolized by m-fluorobenzaldehyde or by a fluorine-substituted alkanal.[122] Alkylation of the Schiff base using alkyl halides[123] illustrates the approach employed by this research group for many years, though they have now established aldolization of the chiral Schiff base (27) by aldehydes to be an efficient (better than 90% e.e.) alternative route to substituted serines.[124] Use of this synthon in a synthesis of the photoactivatable 4′-(1-azi-2,2,2-trifluoroethyl)-L-phenylalanine has been described as extremely efficient.[125]

N-Acryloyl and -crotonoyl-camphorsultams can be used for the synthesis of stereodefined aziridine-2-carboxylic acids through bromination, dehydrobromination, and aminolysis stages.[126] Construction of a cyclopropane ring on the well-known aminoalkenyl synthon R*-CH = C(NHZ)CO_2Me [R*-CH is the (S)-glyceraldehyde moiety] and subsequent processing gives (1S,2R)-1-amino-2-vinylcyclopropanecarboxylic acid (*alias* γδ-dehydro-allocaoronamic acid).[127]

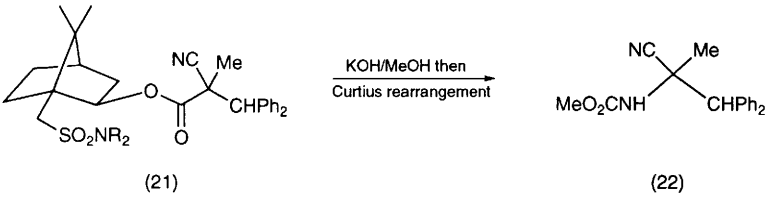
The use of chiral N-acyloxazolidinones in various contexts (see also Ref.64) continues to give excellent results, illustrated in the synthesis of (-)-pyrimidoblamic acid (Scheme 9) the early stages being based on [4 + 2]-cycloaddition of 2,4,6-tris(ethoxycarbonyl)-1,3,5-triazine to 1-(dibenzylamino)-1-propyne);[128] β-branched phenylalanines;[129] tryptophans;[130] and αβ-dimethyl-1,2,3,4-tetrahy-



protected 4-*epi*-polyoxamic acid

Reagents: i, *0,0*-isopropylidene-(*R*)-2,3-dihydroxypropanal, $SnCl_4$, Et_2O;
ii, $KMnO_4$, CH_2Cl_2; iii, LiOH, THF, then $NaIO_4$; iv, $NaIO_4$, RuO_2

Scheme 7



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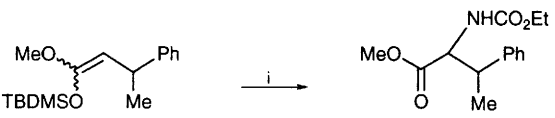

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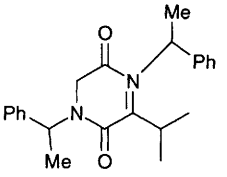
Reagents: i, ethyl azidoformate, *hv*

Scheme 8



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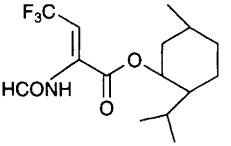

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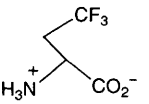

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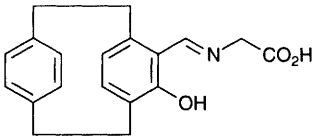

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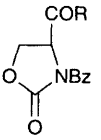

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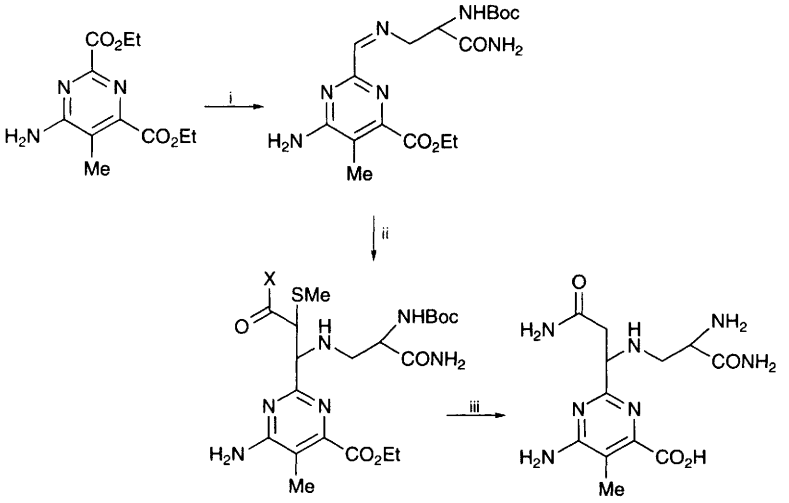


Reagents: i, $CO_2Et \longrightarrow CHO$ + Boc-Asp derivative; ii, Evans Sn^{II} enolate; iii, Bu_3SnH then HCl.EtOAc

Scheme 9

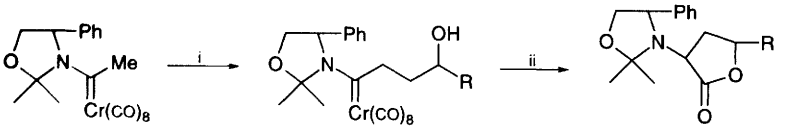
droisoquinoline-3-carboxylic acids;[131] homoserine analogues (using the alternative Karady/Seebach oxazolidinone approach);[132] and (2S,3S)-threoninol and related compounds using the (R)-glycidol-derived oxazolidinone (28).[133] Homologous chiral heterocycles used in similar ways include oxaziridines [reaction with copper(I) salts to give products of N-centred radicals leading to cis-5-benzyl-D-proline].[134] cis-4,5-Disubstituted oxazolidin-2-ones are epimerized at C-5 *via* a N/C-5 di-anion.[135] Piperazinones carrying a homochiral N-substituent[136] provide a new variant of the well-established imidazolidin-5-one.[137] An alternative to the oxazolidinones is the lactam (28; $-CH_2-$ in place of ring -O-), shown to be a useful chiral auxiliary.[138] N-Acylated 5-substituted 3,3-dimethyl-2-pyrrolidinones created in this study have been used in illustrative asymmetric syntheses.[139]

Routes based on recently-developed variants include the synthesis of γ-hydroxy-α-amino acids, illustrated for (+)-bulgecinine, based on aldolization of the Cr(0)-modified acyloxazolidinone (Scheme 10; see Vol.26, p.16),[140] a highly-diastereoselective aldol reaction of a Cr(0)-complexed benzaldehyde derivative starting a synthesis of (29), an analogue of the N-terminal amino acid of nikkomycin B,[141] synthesis of (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine from the serine-derived oxazolidine (e.g. compound 107),[142] and a synthesis of (2S)-N-benzoyl 2-t-butyl-4-methylene-oxazolidin-5-one (Vol,26, p.16) for use in asymmetric synthesis, by bromination (Br_2/hv) of the L-alanine-derived heterocycle, then dehydrobromination (NaI),[143] and synthesis of (2R,3S)- and (2S,3R)-precursors (30 and epimer, respectively; Scheme 11) illustrated for syntheses of β-methyl analogues of protein amino acids (Phe, Tyr, and His analogues; see also Vol.26, p.13).[144]

Alkylation of new heterocyclic 'chiral glycine derivatives' prepared from glycinamide (Scheme 12) follows the oxazolidinone philosophy,[145] and hydrogenation of homochiral 3-ethyl-5-phenyl-3,4-didehydro morpholinones (see Vol.26, p.16) has led to (R)- and (S)-2-aminobutanoic acids from 2-oxobutanoic acid.[146]

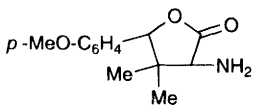
Particular examples, illustrating new synthons that are capable of being used in asymmetric syntheses more generally, have been reported; thus, the furan-2-one synthon (31), obtained from D-glucosamine, has been used in a synthesis of 4-hydroxy-L-pipecolic acid,[147] and D-glucosamine has been used in a preparation of Boc-L-serinal through periodate cleavage.[148] D-Mannosamine is a starting material for a synthesis of sphingofungin D (7; Scheme 13).[149] Kinetic resolution induced by L-(+)-di-isopropyl tartrate accompanies $Bu^tOOH/Ti(O^iPr)_4$ oxidation of α-furanylamines (32 → 33 + 34) and ozonolysis of the residual L-α-furanylamine leads to the N-toluene-p-sulfonyl L-amino acid.[150] Unconventional asymmetric synthesis of amino acids features a homochiral α-amino acid as chiral auxiliary to generate an enantiomer of γ-hydroglutamic acid,[151] 'ring-contraction' of 5-isonitroso-2,2-dimethyl-1,3-dioxan-4,6-dione through refluxing with ketones *via* a nitrosoketene (Scheme 14), cycloaddition to alkenes and routine elaboration yielding L-α-amino acids,[152] while ring-expansion is involved in a 4-oxoproline synthesis from a homochiral azetidinonecarboxylic acid (Scheme 15).[153]

4.3 Synthesis of Protein Amino Acids and Other Naturally Occurring α-Amino Acids – This section continues the coverage of α-amino acid synthesis, showing



Reagents: i, BuLi, RCHO; ii, *hν*, CO[–Cr(CO)$_6$]

Scheme 10



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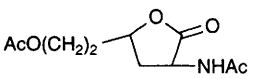

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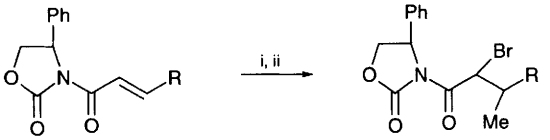
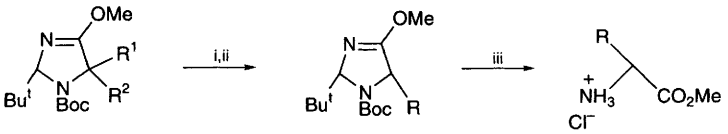


(30)

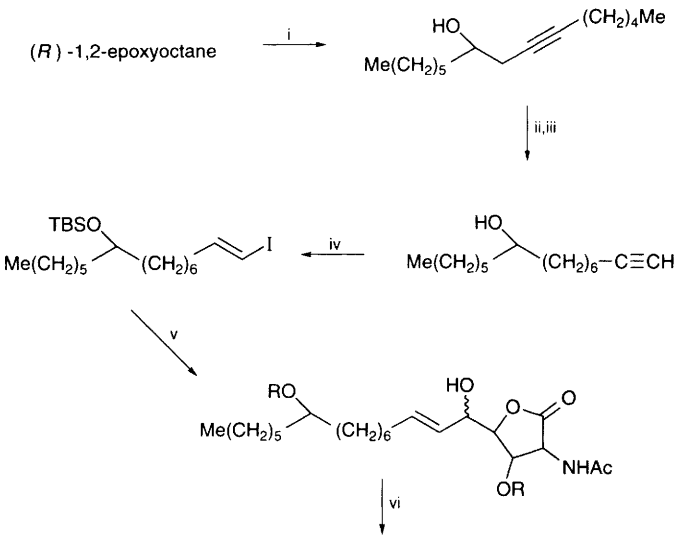
Reagents: i, MeMgBr–CuBr.SMe$_2$; ii, NBS

Scheme 11



Reagents: i, LDA/THF,–78 °C; ii, RX; iii, TFA/CH_2Cl_2 then 0.2M HCl

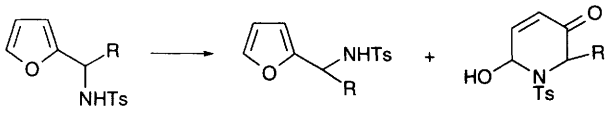
Scheme 12



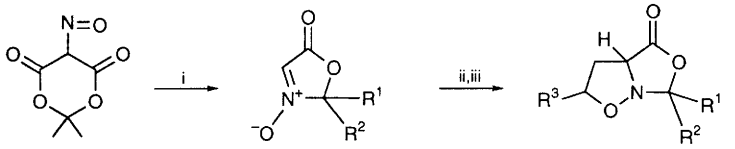
sphingofungin D (7)

Reagents: i, 1-heptyne, BuLi, $BF_3.OEt_2$; ii, Li, Bu^tOK, $H_2N(CH_2)_3NH_2$;
iii, $EtO_2C–N=N–CO_2Et$, Ph_3P, $PhCO_2H$ then TBSCl;
iv, Bu_3SnH, AIBN then I_2; v, synthon from *N*-acetyl-D-mannosamine, $CrCl_2/NiCl_2$/DMSO; vi, routine steps

Scheme 13

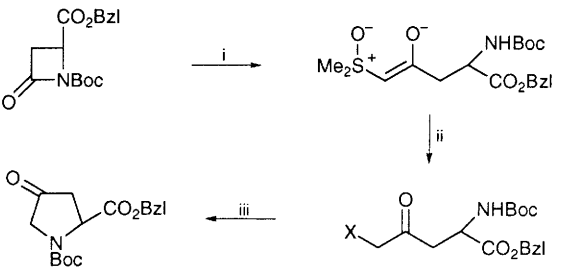


(32) (33) (34)



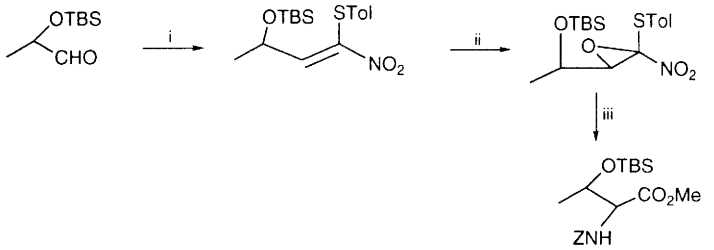
Reagents: i, R^1COR^2, reflux; ii, $R^3CH=CH_2$;
iii, ⟶ α-amino acid by aq. $NaHCO_3$, then H_2/5% Pd–C

Scheme 14



Reagents: i, Me_2SOCH_2, DMSO; ii, HX; iii, ML_n(cat.)

Scheme 15



Reagents: i, $TolSCH_2NO_2$, then $MeSO_2Cl$, ii, Bu^tCOOK; iii, NH_3 then ZCl

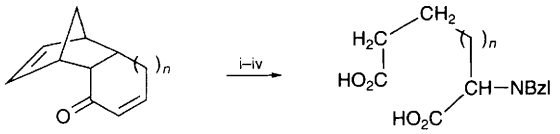
Scheme 16

applications of current methodology for the synthesis of natural products. Choice of route is usually tailored to the particular synthesis target, though alternative approaches are occasionally compared.

The usual crop from the primary literature dealing with aspects of fermentative production of the common amino acids [L-lysine using *Corynebacterium glutamicum*;[154] L-aspartic acid using intact coryneform *Brevibacterium flavum* MJ-233;[155] L-threonine using *Brevibacterium lactofermentum*;[156] L-phenylalanine methyl ester from methyl trans-cinnamate using phenylalanine ammonia lyase;[157] L-DOPA using a cell suspension culture of *Mucuna pruriens*;[158] and D-amino acids from enzymic hydrolysis of hydantoins by whole cells of *Agrobacterium radiobacter*[159]] is supplemented by reviews [production of amino acids using genetically-engineered *Serratia marcescens*;[160] using aminopeptidases and aminoamidases;[161] using hydantoinases;[162] L-aspartic acid using immobilized microorganisms;[163] and L-DOPA using tyrosine phenol lyase[164]]. Details of routine studies of the biosynthesis of amino acids in plants have not been collected in this Chapter over the years, though there has been the custom to mention unusual studies, e.g. the enzymic synthesis of β-substituted alanines in plants,[165] and of 5-hydroxy-4-oxo-L-norvaline in *Streptomyces akiyoshiensis*.[166]

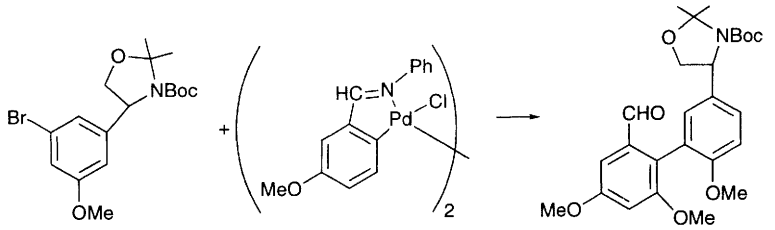
A new synthesis of D-alloisoleucine from (S)-2-methylbutanol[167] compares favourably with two standard procedures, *viz.* inversion at C-2 of L-isoleucine and racemization of N-acetyl-L-isoleucine and resolution with hog acylase. New syntheses of protected D-threonine and L-allo-threonine[168] employ a little-used general method of α-amino acid synthesis (Scheme 16). Extraordinary syntheses of L-glutamic acid and L-α-aminoadipic acid[169] use chiral equivalents of cyclopentadienone and cyclohexadienone respectively (Scheme 17).

The laboratory synthesis of 'MeBmt', the threonine derivative present in cyclosporin, has been reviewed.[170] A new synthesis in four steps[171] from (2Z,4R)-4-methyloct-6-yn-2-en-1-ol (Sharpless epoxidation and ring-opening with $MeNH_2$ are the essential steps), offers an improvement on the heroic multistage first synthesis of MeBmt (see Vol.17, p.8). A synthesis of 2-amino-4-hydroxy-3,3-dimethylbutyric acid (alias pantonine) from 3-chloro-2,2-dimethylpropanol,[172] β-hydroxyhomoserine (an intermediate in mugeneic acid syntheses) in 12 steps[173] from cis-2-butene-1,4-diol, (2S,3R)-2-amino-4-hydroxyadipic acid (a constituent of theonellamide F) by asymmetric reduction of the corresponding β-ketoester obtained from L-aspartic acid,[174] quisqualic acid from L-serine *via* the Garner aldehyde (107, R = H; -CHO → -CH=NOH → $-CH_2N(OH)CONHCO_2Et$, etc),[175] stizolobic acid through a biomimetic route starting with a catechol aldehyde,[176] and the alicyclic relatives anticapsin and bacilysin starting with a Diels-Alder adduct of dehydroalanine with O-TMS-cyclohexadienol,[177] In the last-mentioned study, independent confirmation is provided that the previously announced stereochemistry of anticapsin requires revision [it has the (S)-configuration at C-4; see Vol.26, p.4]. Construction of the side-chain of arogenic acid, (γS)-β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)-L-alanine, is conveniently achieved through Michael addition of the anion of methyl 1,4-dihydrobenzoate to methyl N-acetyldehydroalaninate.[178] Further exploration of routes to vancomycinic acid precursors (35)[179,180] and polyoxamic acid (36; a synthesis involving

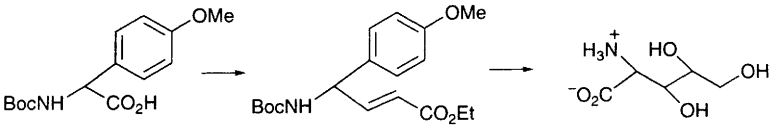


Reagents: i, $-CH{=}CH \longrightarrow -CH_2-CH_2-$; ii, $BzlNH_2$; iii, $NaBH_4$; iv, $NaIO_4$, $RuCl_4$

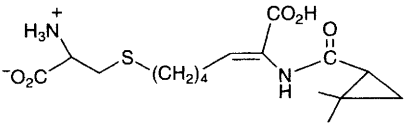
Scheme 17



(35)



(36)



(37)

$-C_6H_5 \rightarrow -CO_2H$)[181] has been reported. The same method of generating a carboxy group appears in a synthesis from α-aryl-β-alanines, of 2′-deoxymugineic acid and nicotianamine.[182] Complex synthetic targets are also represented in cilastatin (37), constructed from L-cysteine, 7-bromo-2-oxoheptanoic acid, and (+)-(S)-2,2-dimethylcyclopropanecarboxylic acid.[183] The synthesis of several other cyclopropanes [trans-α-2-(carboxymethyl)cyclopropyl]glycine from *Blighia unijugata*, synthesized by dibromocyclopropanation of (38) prepared from D-serine;[184] all four stereoisomers of carnosadine (39; a set of constrained arginines, see Vol.26, p.19);[185] (1R,2S)- and (1S,2S)-dehydrocoronamic acids from dimethyl 2-vinylcyclopropanedicarboxylic acid (selectively hydrolysed by successive treatment with two esterases with different regioselectivities) then routine introduction of the amino group through the Curtius rearrangement[186]] have been described. Full details (see Vol.25, p.21) of a synthesis of (2S,4R)- and (2S,4S)-diastereoisomers of hypoglycin A have been described, incorporating a Sharpless epoxidation stage in an asymmetric methylenecyclopropane synthesis.[187]

(2R,3S)-3-Hydroxylysine has been synthesized through Hayashi's chiral ferroceno-gold catalysed oxazoline formation from 4-phthalimidobutanal and methyl isocyanoacetate, and the (2S,3R)-enantiomer through a route incorporating a Sharpless cis-hydroxylation.[188]

Total synthesis of the azetidinecarboxylic acid derivative, mugineic acid (40, X = H; equally correctly described either as a β-hydroxyornithine derivative or an α-hydroxy-GABA derivative) (see Vol.25, p.23)[189] and its 3-epi-hydroxy-derivative (40; X = OH)[190] involve a common intermediate (41). The latter report also describes the synthesis of distichonic acid A, and 2′-hydroxynicotinamine.

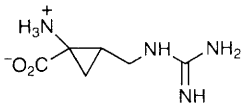
Naturally-occurring proline derivatives of continuing interest, the kainoids and bulgecinine, have been synthesized by new routes. Condensation of glyoxal with the (R)-phenylglycinol derivative (42) proceeds *via* a cyclic iminium ion that undergoes tandem aza-Cope–Michael reactions *en route* to (-)-α-allokainic acid.[191] A six-step route to DL-kainic acid (43 in Scheme 18) uses a similar reaction sequence to set up the ring system with the correct relative stereochemistry.[192] Further details have been published[193] of a synthesis of (-)-α-kainic acid employing a ring construction step based on the Pauson-Khand reaction (see Vol.26, p.24) and the same research group has reported an effective synthesis of α-allokainic acid (Scheme 19).[194] A short, efficient route to 4-arylkainoids starts with trans-4-hydroxy-L-proline.[195] Radical cyclization of a protected L-serinal (44) is at the heart of an elegant synthesis of (+)-bulgecinine,[196] also reached through reduction of appropriately substituted 2-amino-4-oxoalkanoic acids (45; somewhat capricious stereoselectivity is involved in the reduction).[197] Clavalanine and erythro-4-hydroxyornithine were also prepared from the same starting material in this study.

A route to (S)-(-)-pipecolic acid (46 → 47) employing the chiral oxazolidine approach is also capable of extension to the synthesis of 2- and 6-alkyl analogues.[198]

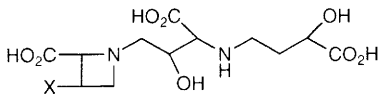
'PCA', an unusual hydrazino acid that is a constituent of the Luzopeptins, has been synthesized by the condensation of di-t-butyl azodicarboxylate with the di-anion of $(MeO)_2CHCH_2CH(OH)CH_2CO_2Et$ (see also Ref.79).[199]


Boc
N
O
O
O
H_3N^+
^-O_2C
CO_2H

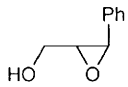
(38)


H_3N^+
^-O_2C
NH
NH_2
NH

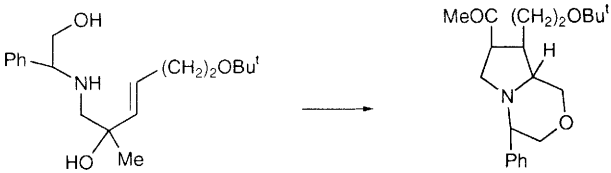
(39)


HO_2C
OH
HO_2C
N
N
H
CO_2H
X
OH

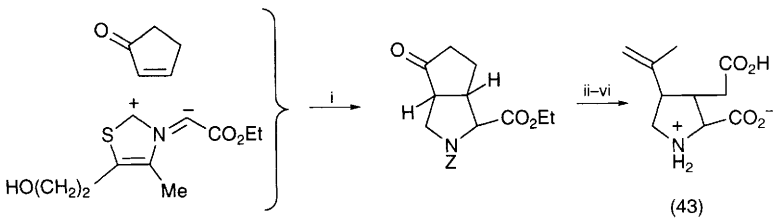
(40)


Ph
HO
O

(41)


OH
Ph
NH
$(CH_2)_2OBu^t$
HO
Me
MeOC
$(CH_2)_2OBu^t$
H
N
O
Ph

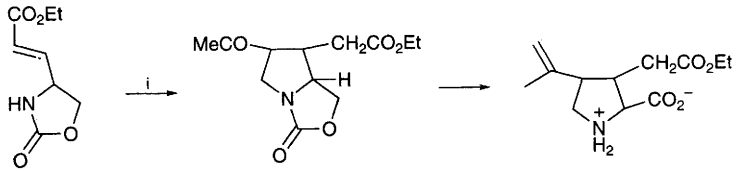
(42)



(43)

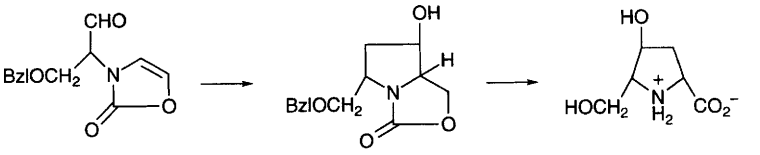
Reagents; i, Et_3N, MeCN, r.t., then $SnBu_3H$/AIBN, ZCl; ii, MeLi, $TiCl_4$; iii, $BF_3.Et_2O$; iv, oxidative ring-opening (RuO_2/$NaIO_4$); v, $CH_2{=}PPh_3$ or CH_2I_2/Zn; vi, separate diastereoisomers then OH^-, ion exchange

Scheme 18



Reagents: i, $MeCOCH{=}CH_2$; ii, $CH_2{=}PPh_2$

Scheme 19



(44)

(+)-bulgecinine

4.4 Synthesis of α-Alkyl Analogues of Protein Amino Acids – The synthesis of homochiral examples of known absolute configuration of this class of substituted α-amino acid has been considered to be a difficult enterprise, but the extraordinary fact that a configurationally-stable anion can be generated from N-Boc-N-methyl-L-phenylalanine using Li 2,2,6,6-tetramethylpiperidide opens the door to α-alkyl analogues.[200]

The conventional approach, α-methylation of a Schiff base of a protein amino acid, is illustrated in a synthesis of α-methylhomocysteine (Scheme 20).[201] α-Allylation of pipecolic acid and ensuing steps yield the α-(2-alkoxycarbonyl)ethyl analogue.[202] The hydantoin route is often inappropriate, because of the drastic conditions needed to release the amino acid, but 3-(toluene-p-sulfonyl)hydantoins carrying adenine and thymidine side-chains are easily hydrolysed to give the corresponding amino acids, in dilute alkali at slightly elevated temperatures.[203]

Grignard addition to fluoroacetonitrile followed by routine stages gives α-fluoromethylglutamic acid, whose cyclization leading to the glutamate racemase inhibitor, aziridinoglutamate, has been worked out.[204]

The need for resolution is avoided in the chiral oxazolidinone approach, used for a synthesis of (S)-2-methylproline,[205] although the oxazolidinone hydrolysis step that completes the procedure can be simplified.[206] (S)-α-2-Aminoethylmethionine has been obtained in 18% yield from the 5-(methylthioethyl)oxazolidinone, through enolate alkylation with $BrCH_2CN$ and routine elaboration.[207]

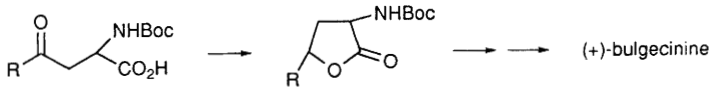
Stereoselective alkylation of isobornyl 2-cyanopropanoate is exemplified with syntheses of α-methyl-L- and D-tryptophan[208] and α-methyl-D- and -L-phenylalanine.[209]

αβ-Di-alkylaspartic acids are readily obtained *via* β-lactams prepared from Schiff bases $ArN=CRCO_2Me$ and ketenes.[210]

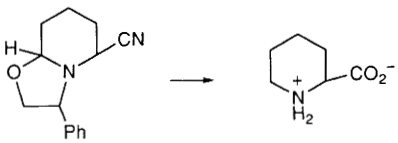
4.5 Synthesis of α-Amino Acids Carrying Alkyl Side-Chains, and Cyclic Analogues – With the proviso that 'use of one α-amino acid for the synthesis of another' is covered in the later Section 6.3 (Specific Reactions of Amino Acids) papers collected here deal with approaches to aliphatic synthetic targets from other starting points.

(S)-2-Aminosuberic acid has been prepared from $(E)\text{-}CH_2=CH(CH_2)_5CH=CHCH_2OH$ through asymmetric epoxidation, ring-opening with benzhydrylamine, and oxidative cleavage.[211] Other amination processes include Curtius rearrangement (diphenyl phosphoroazidate) of selectively-hydrolysed 2,2-dialkylcyclopropane-1,1-dicarboxylic acid esters,[212] and ring-closure of 2,5-dibromoadipic acid (R)-pantolactone esters using benzylamine to give trans-pyrrolidine-2,5-dicarboxylic acids.[213] 1,2-trans-Placing of NH_2 and OH in a 1,2-trans relationship on cyclopentadiene (AcOOH then NH_3/MeOH) and *Candida antarctica* resolution starts a route to (2S,3R)-3-hydroxyproline.[214] An unusual synthesis of 3-phenyl-3-hydroxyproline from an N-benzoylethyl-N-toluene-p-sulfonylglycinamide involves photochemical cyclization.[215]

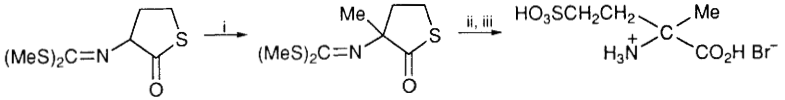
Carboxylation of 4-(hydroxymethyl)pyridine through reaction of its O-trimethylsilyl-N-oxide with Me_3SiCN and saturation of the ring leads to the 4-substituted piperidine-2-carboxylic acid.[216] Hydrogenation of dimethyl



(45)

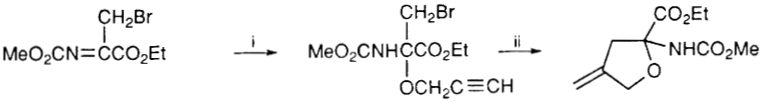


(46) (47)



Reagents: i, base, MeI; ii, aq. HCl; iii, Br_2, H_2O

Scheme 20



Reagents: i, $HOCH_2C{\equiv}CH$, $(PPh_3)_4Pd/CuI$; ii, $Bu_3SnH/AIBN$

Scheme 21

3,5-pyridinedicarboxylate provides a 1:1 cis-trans mixture of piperidine dicarboxylic acids.[217]

4.6 Models for Prebiotic Synthesis of Amino Acids – Conventional studies under this heading continue much as they have done for many years, represented by $CO_2/CO/N_2/H_2O$ mixtures subjected to electric discharge (giving 6 protein amino acids, glycine predominating, and 2 non-protein amino acids when CO is abundant),[218] and by ^{60}Co γ-irradiated aqueous glycine (Asp, Ser, Thr, and Glu, and $MeNH_2$ + $EtNH_2$ formed from reaction of a glycine radical with glycine breakdown products).[219]

Current speculation, that deep oceans were the sites of the origin of life, is helped by confirmation (hydrothermal synthesis; Vol. 25, p.34) of the generation of amino acids from $C_2H_2/H_2O/O_2/H_2/(NH_4)_2CO_3$ mixtures at 200-275°.[220]

4.7 Synthesis of α-Alkoxy α-Amino Acids, and Analogous α-Heteroatom-substituted α-Amino Acids – Asymmetric synthesis of (R)-α-sulfenylglycine has been achieved by the reaction of MeSSMe with 2-hydroxypinan-3-one Schiff bases.[221]

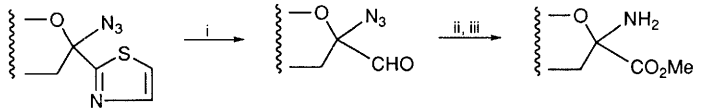
Fmoc-α-Methoxyglycine has been prepared by addition of Fmoc-carbamate to glyoxylic acid, then O-methylation by $MeOH/H^+$.[222] Anodic α-methoxylation of α-amino acids has become a routine step in many synthetic applications (notably, substitution by alkyl groups), and illustrated with asparagine and serine derivatives[223] and proline.[224]

Independent studies were aimed at the provision of anomeric tetrahydrofuranosyl amino acids and pyranosyl analogues (Scheme 21)[225] (Scheme 22)[226] within the context of analogues of the herbicide (+)-hydantocidin, whose short synthesis (see Vol.26, p.24) from β-D-ribofuranosyl amide results from fortuitous α-bromo β-amide formation and treatment with silver cyanate.[227]

Several examples of the uses in synthesis, of glycine derivatives conforming to the title of this section, are cited elsewhere in this Chapter.

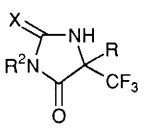
4.8 Synthesis of α-(ω-Halogeno-alkyl) α-Amino Acids – A review has appeared describing Ukrainian work on the synthesis of fluorine-containing amino acids.[228] A well-established route to ββ-difluoroalanine from $ZNHCH(CHF_2)CH=CH_2$ through oxidation to generate the carboxy group, has been rendered a practical proposition through an efficient synthesis[229] of $H_2NCH(CHF_2)SEt.HBr$. α-(Fluoromethyl)-β-fluoroalanine, an important intracell pH indicator, can be prepared in 44% overall yield from 1,3-difluoropropan-2-ol, through application of standard methods [1,3-difluoroacetone → $(FCH_2)C=NCHPhCH_2OH$ → $(FCH_2)CH(CN)NH_2$ using TMSCN].[230] α-Trifluoromethyl-α-amino acids are readily obtainable from imidazolidin-2,5-diones (48).[231] β-Difluoromethyl-m-tyrosine has been prepared through an uneventful Evans oxazolidinone synthesis.[232]

(2S,4S)-5-Fluoroleucine has been synthesized from L-pyroglutamic acid through diastereoselective methylation, followed by less stereochemically-demanding steps.[233]

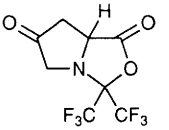


Reagents: i, TfOMe, then $NaBH_4$ and $HgCl_2$; ii, Ag_2O then CH_2N_2; iii, H_2–Pd/C

Scheme 22



(48) X = O or S



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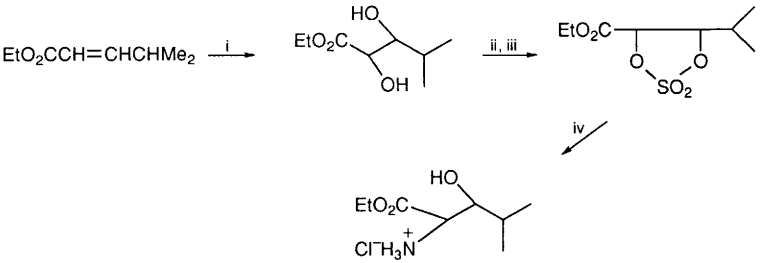


Reagents: i, Sharpless dihydroxylation; ii, $SOCl_2$; iii, $NaIO_4/RuCl_3$; iv, NaN_3 and reduction

Scheme 23

trans-4-Fluoro-L-pipecolic acid and the 4,4-difluoro-analogue have been prepared from di(ethylamino)sulfur tetrafluoride and the oxazolidinone (49), available from L-aspartic acid;[234] and (2R,5R)-5-chloropipecolic acid has been obtained by elaboration of the readily-available N-methoxycarbonyl (S)-5-TBDMSoxy-2-oxo-piperidine.[235]

Examples of side-chain halogenation of amino acid derivatives are to be found in the later Section 6.3, though it could be noted here, that 4-alkyl-5-ethoxyoxazoles (easily prepared from N-acylamino acids) are useful substrates for perfluorination.[236]

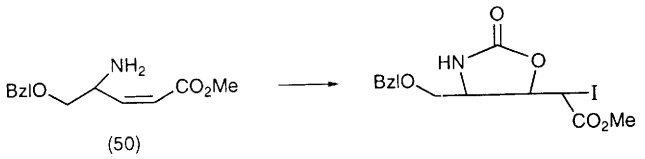
4.9 Synthesis of α-(ω-Hydroxyalkyl) α-Amino Acids – Hydroxylation of alkenes leading to hydroxyalkyl side-chains is represented in a number of different strategies. Where the α-amino acid moiety is in place, as with L-vinylglycine [$CH_2=CHCH(NH_2)CO_2H$], then 1,2-dihydroxylation using OsO_4 yields hydroxythreonine stereospecifically.[237] In a similar approach, 1-aminocyclohexene-1-carboxylic acids, formed from 4-arylideneoxazolin-5-ones by Diels-Alder addition to dienes, give iodohydrins that yield γ-hydroxy-α-amino acids through reductive dehalogenation and hydrolysis.[238] Protected aspartic acid enolates provide (3R)- and (3S)-hydroxy-L-aspartates through treatment with electrophilic hydroxylating agents.[239]

Grafting the α-amino acid moiety on to a hydroxylated structure is illustrated in a new synthesis of C-α-D-glucosyl-α-amino acids starting with a protected 1-allyl-1-deoxyglucose, then Sharpless epoxidation, selective mesylation, tritylation, and azidolysis and routine elaboration.[240] A similar approach leading to (2S,3S)- and (2R,3R)-3-hydroxyleucine (Scheme 23) also succeeds because of favourable regioselectivity.[241] Mercury(II) oxide oxidation of D-glucosamine gives D-glucosaminic acid, and straightforward replacement of the 3-hydroxy group by H, giving (2S,4S,5R)-4,5,6-trihydroxynorleucine.[242] Monosaccharide-derived azidolactones continue to serve (see Vol. 26, p.33) as starting materials for tetrahydroxy-1-aminocyclopentane- and cyclohexanecarboxylic acids with unambiguous control of stereochemistry.[243] threo-3-Hydroxy-L-glutamic acid[244] and (2S,3R)-3-hydroxyornithine[245] have been prepared through a highly stereoselective iodocyclocarbamation of the chiral alkene (50) obtained from O-benzyl-L-serine.

4.10 Synthesis of α-(ω-Amino-alkyl) α-Amino Acids – The synthesis of isoxazolidin-5-ones, and their use in the synthesis of β-amino- and β-(N-hydroxylamino)-alanines, has been described.[246] Substitution of aminating agent for hydroxylating agent in a process described in the preceding section[239] has been successful in leading to (3R)- and (3S)-amino-L-aspartates.

4.11 Synthesis of α-Amino Acids Carrying Unsaturated Aliphatic Side-Chains – As usual, there is interest in each of the categories covered by the heading of this Section: the αβ-unsaturated-α-amino acids, *alias* 'dehydro-amino acids', are constituents of natural peptides, while they and their homologues (vinyl- and allyl-glycine) are increasingly valuable in synthesis.

Protected dehydroalanine is easily formed from serine derivatives, treatment of



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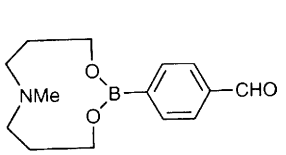

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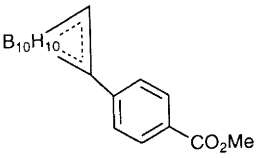

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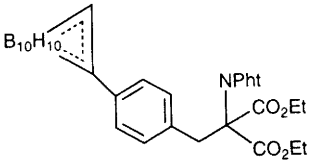

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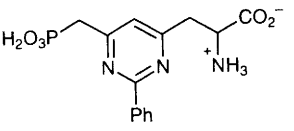

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Z-Ser(OTs)OEt with $Bu_4NI/NaOH$ being currently recommended.[247] The potassium salt of N-acetyl α-(diethylphosphonyl)glycine condenses readily with aldehydes, exemplified in the synthesis of dehydro-amino acids carrying long side chains, (E)- or (Z)-$EtO_2CC(NHAc)=CHCH_2(CH_2)_nCO_2Me$.[248] N-Acyl-2,3-dehydro-2-amino acid esters result from perrhenate-catalysed decomposition of α-azido acid esters in solution in organic solvents containing an acyl chloride.[249]

A general synthesis of αβ-dehydroamino acids from a glycine derivative is exemplified with the preparation and hydrolysis of 4-alkylaminomethylenethiazol-5-ones.[250] The reaction sequence from the aldehyde (51) formed from D-arabinose and L-serine, to the highly functionalized dehydroamino acid (52) postulated to be a constituent of azinomycins, includes a Wittig condensation with a glycylphosphonate.[251]

An αβ-dehydroamino acid with an extended side-chain, methyl (-)-(Z)-2-(Z-amino)-4,5-cyclopropane-hex-2-enoate has been prepared through manipulation of functional groups on 5-(t-butyldimethylsilyloxy)furan-2(5H)-one.[252] A more routine approach to such systems is Michael addition to a protected dehydroalanine, which offers convenient access to (Z)-dehydrotryptophans through $PdCl_2$/NaOAc-AcOH catalysed reaction with indoles (see also Refs.177,178).[253]

4.12 Synthesis of α-Amino Acids with Aromatic or Heteroaromatic Groupings in Side Chains – Increasing interest in the therapeutic use of common amino acids capable of neutron capture when carrying appropriate substituents has encouraged studies in the synthesis of organic boron derivatives (see also Refs.9,868). Standard methods have been applied for the synthesis of o- and m-borono-L-phenylalanines. Grignard reaction of o- and m-bromotoluene with $B(OMe)_3$ and functionalization so that alkylation of diethyl acetamidomalonate can be carried out, was followed by α-chymotrypsin resolution.[254] p-Borono-DL-phenylalanine and -DL-phenylserine were prepared by aldolization of methylisocyanoacetate using the aldehyde (53).[255] A series of papers has appeared, describing the synthesis of phenylalanines substituted in the phenyl moiety by carboranyl groupings. One of these starts with methyl p-bromobenzoate *via* p-$(HC\equiv C)$-$C_6H_4CO_2Me$ + decaborane → (54), and reaction of the derived benzyl bromide with diethyl phthalimidomalonate → (55).[256] Other standard methods are represented, e.g.[257] the elaboration of an allyl chain carrying a carboranylphenyl grouping, into the acyl group of a chiral N-acyloxazolidinone (*cf.* Section 4.2).

5-Fluoro-D- and L-DOPAs and ^{18}F-analogues have been prepared starting from 5-nitrovanillin, through the acetamidomalonate route and Balz-Schiemann substitution of a diazonium group by fluorine, followed by chromatographic resolution.[258] 6-Fluoro-L-DOPA and its 3-O-methyl derivative have also been obtained by standard synthetic methods.[259]

Homochiral bis-amino acid diaryl ethers exist in natural products, and O-arylation of tyrosine derivatives employing fluoroarenes has been established using suitably mild reaction conditions.[260]

Simple heteroaromatic side-chains are represented in β-(2-pyridyl)-L-alanine, prepared from 3-(2-pyridyl)acrylic acid through catalysis by L-phenylalanine

ammonialyase (present in *Rhodotorula rubra* mycelium);[261] and in the pyrimidine isostere (56) of the potent NMDA antagonist, SDZ EAB 515,[262] synthesized by alkylation of $Ph_2C=NCH_2CO_2Me$. β-Isoxazol-4-yl-L-alanines are potent NMDA agonists, and further synthetic studies (see Vol.25, p.38) have been described of homologues (57 and 58),[263] AMPA and 4-methylhomoibotenic acid have been prepared through cycloaddition of suitably substituted bromonitrile oxides to alkynes.[264] Resolution using (-)-phenylethylamine and absolute configurational assignment by X-ray crystal analysis is included in one of these preparations.[263]

A related alanine derivative that carries a β-heterocyclic moiety is the neurally-active quisqualic acid. Conformationally-constrained analogues have been synthesized, using standard methods.[265] The homochiral α-amino acid (59) carrying a thiazoline side-chain was obtained by a novel manipulation of the penicillin nucleus.[266]

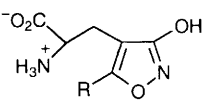
β-Substituted tryptophans (see also Refs.129-131, 144, in Section 4.2) have been obtained by Lewis-acid catalysed ring-opening by indoles, of the aziridine (60). Opposing regioselectivities are observed; the lactone (61, from 60, RR^1 = O) leads to β-amino acids, and the acetal (60, R = H, R^1 = OTBDMS) yields (62) from which β-substituted tryptophans were obtained.[267] erythro-β-Alkylated tryptophans can be obtained by conjugate addition to (63) formed from the tryptophan derivative (64) that Crich's research group has been establishing as a valuable synthon in recent years.[268] 3,4-Bridged tryptophans (65) are, likewise, obtained starting from tryptophan itself, *via* 4-bromodehydrotryptophan and cyclization of the derived 4-bromo-α-propenyl-DL-tryptophan.[269] Other 3,4-cyclized tryptophan analogues formed with the α-amino group through an isoleucyl bridge[270] are members of a family of conformationally constrained amino acids that includes potential protein kinase C modulators.

These analogues support a wide range of pharmacological studies that reflect the importance of derivatives of the parent amino acid, and the fact that N-ethyl-L-tryptophan benzyl ester is a weak antagonist at the Substance P(NK_1) receptor has stimulated the synthesis of indole-substituted analogues [N-acetyl-L-(3′,5′-di-trifluoromethyl)tryptophan was found to be particularly effective] by standard methods.[271] An efficient synthesis of 5-azidotryptophan[272] employs a standard route, starting from 5-nitroindole.

4.13 Synthesis of α-Amino Acids Carrying Sulfur- or Selenium-containing Side Chains – This class of amino acid is gaining further importance, since certain sulfur functional groups react with nitric oxide. L-Thiocitrulline (readily prepared from L-ornithine) and its S-methyl derivative, and L-homothiocitrulline, are potent inhibitors of nitric oxide synthase, and presumably owe this property to their structural similarity with arginine analogues.[273]

Conventional synthetic strategies are involved in the synthesis of S-alkyl-L-cysteines[274] (see also Ref.53) and of the conformationally-constrained L-methionine (66) obtained by elaboration of the Diels-Alder adduct of 5-norbornen-2-ol with a protected dehydroalanine.[275]

L-(+)-Selenomethionine has been prepared from L-homoserine lactone and MeSeLi.[276]



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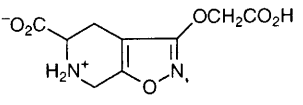

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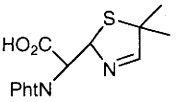

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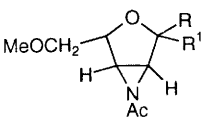

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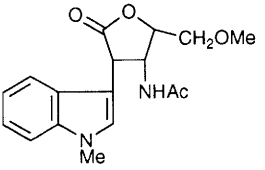

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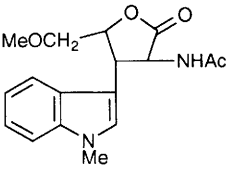

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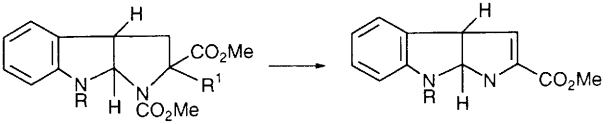

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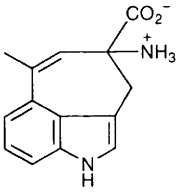

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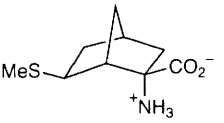

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4.14 Synthesis of α-Amino Acids Carrying Phosphorus Functional Groups in Side Chains – Main areas of interest in compounds of this class, as distant isosteres of some common amino acids, are being sustained by current research (see also Refs.99,114), e.g. into the synthesis of (R)-4-oxo-5-phosphononorvaline, $H_2O_3PCH_2COCH_2CH(N^+H_3)CO_2^-$ (a selective NMDA glutamate site antagonist) in four steps from D-aspartic acid.[277] Stereoselective synthesis of L-phosphinothricin [$MeP(O)(OH)CH_2CH_2CH(NH_2)CO_2H$], present in the herbicide 'glufosinate-ammonium', has been reviewed.[278] The (αα-difluoroalkyl)phosphonate analogue of phosphoserine has been synthesized starting from O-benzyl-N-Boc-L-serine.[279] 1-(Z-Amino)-5-(Boc-amino)-pentylphosphinic acid, an isostere of lysine, has been synthesized from 3,4-dihydro-(2H)-pyran.[280]

Phosphonate-bridged phenylalanine derivatives (67) that are capable of selective de-protection so that they can be incorporated into peptides have been synthesized from the corresponding p-iodo-L-phenylalanines (an improved synthesis of this amino acid has been worked out), through Pd-catalysed coupling.[281] The phosphonotyrosine isostere N-Fmoc-4-phosphono(difluoromethyl)-L-phenylalanine, has been prepared from the organozinc reagent from β-iodoalanine together with the requisite iodoarene,[282] and independently from the methyl ester of its diethylphosphonate.[283] N-Boc-(4′-dimethylphosphonomethyl)-L-phenylalanine (68) has been obtained through a route that employs the iodarene-organozinc reagent C-C-bond-forming methodology.[284]

4.15 Synthesis of Isotopically Labelled Amino Acids – All the familiar isotopes featured under this heading over the years are represented in the current literature. ^{2}H-Labelled protein amino acids, intended to assist biosynthetic studies, often call for the most ingenious synthetic strategies, as illustrated by (2S,4S)- and (2S,4R)-[5,5,5-$^2H_3$]leucine. The route starts from (R)-pulegone → [2-^{2}H]citronellal → [5,5,5-$^2H_3$]isovaleric acid (through de-carbonylation using Wilkinson's catalyst followed by oxidation), then follows standard steps.[285] Labelled aziridines (69; prepared from labelled malates, starting with fumarates/fumarase or by enzymic amination) serve as starting materials for syntheses of stereospecifically-[^{2}H]-labelled D-serine, D-cystine, and β-chloro-D-alanine, through nucleophilic ring-opening.[286] Synthesis of labelled D-(prop-2-ynyl)glycine (70) follows an identical strategy using a carbanion as nucleophile.[287]

Regiospecific labelling of the aromatic ring in phenylalanine is achieved by treating a protected tyrosine tetrazolyl ether or its o- or m-isomer, with ^2H_2O.[288]

The development of established methods (see Vol. 26, p.38) allowing the preparation of ^{2}H-L-glutamic acid, and ^{2}H-, ^{13}C- and ^{15}N-L-glutamic acids, on a gram scale from 2-oxoglutaric acid, includes several points of interest; e.g. the involvement of glutamate dehydrogenase with ^2H_2O as solvent, for C-3- and C-4-labelling, and exchange at C-4 by equilibration in 20% ^{2}HCl-^2H_2O.[289] A related approach to ^{13}C- and ^{15}N-L-alanine using alanine dehydrogenase, and to other ^{15}N-L-α-amino acids, includes full experimental detail.[290]

^{11}C-Labelled amino acids are accessible only through super-rapid methods of synthesis (and equipment ensuring the safety of operators), due to the short

half-life of the isotope, but this requirement has not only been met satisfactorily over recent years, but with sufficient leeway to permit purification to be included in the cycle. Thus, a 45-minute process (including HPLC purification) serves for the preparation of [β-^{11}C]-p-chloro-L-phenylalanine from 4-Cl-C_6H_4-$^{11}CH_2Br$ and the Li enolate of a chiral imidazolinone (see Scheme 5, Section 4.2) followed by hydrolysis.[291] The special reactivity of the L-tryptophan-derived bis(N-methoxycarbonyl)hexahydropyrrolo[2,3-*b*]indole (64, *cf.* Ref.137) allows rapid alkylation by $^{11}CH_3I$ *en route* to [α-$^{11}CH_3$]-L-tryptophan and its N-carboxylic anhydride.[292] The use of the Anatech RB-86 robotic synthesizer permits rapid synthesis of [1-^{11}C]-L-tyrosine from the analogous isocyanide[293] (see Vol.19, p.20) and of [CH_3-^{11}C]-L-methionine through robot-controlled methylation of homocysteine lactone.[294]

α-[^{14}C]Methyl-L-tryptophan has been prepared by methylation of the Li enolate of a Schiff base of an L-tryptophan, followed by enzymic resolution.[295] and by similar processing of the L-tryptophan-derived bis(N-methoxycarbonyl)-hexahydropyrrolo[2,3-*b*]indole (64).[296] The α-[^{3}H]methyl-analogue was also prepared in the latter study.

The simplest amino acid syntheses leading to labelled glycines, amination of [^{13}C]bromoacetic acid esters with $Boc_2{}^{15}N^-K^+$[297] and carboxylation of BocN-$MeCH_2SnBu_3$ with $^{14}CO_2$ after lithiation with MeLi,[298] are typical of numerous syntheses of labelled protein amino acids over the years, a further example being ^{15}N-[$^2H_3$]acetyl-L-aspartic acid as a standard for isotope dilution GC-MS analysis of N-acetyl-L-aspartic acid in urine.[299] The glycine isotopomers were used for spectroscopic assignments.[297]

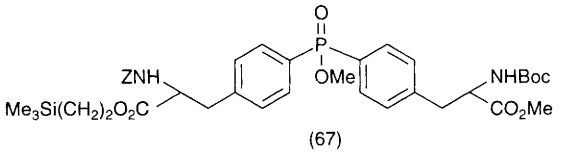
[^{18}F]-Labelled amino acids, e.g. β-[^{18}F]fluoro-alanine[300] provide useful substrates for *in vivo* drug delivery and similar diagnostic studies, a popular substrate being 6-[^{18}F]fluoro-L-DOPA, whose 3-O-methyl derivative has been prepared by fluorodestannylation of the corresponding stannylated DOPA.[301]

Saccharomyces cerevisiae mediates the synthesis of L-[^{35}S]cysteine and -methionine from $Na_2{}^{35}SO_4$.[302]

Aromatic iodination of tyrosine, by either the Chloramine-T/I_2 or analogous Iodogen methods, is a standard preparation of 2,5-di-iodotyrosine, and has been applied for [^{125}I]-labelling of α-methyl-L-tyrosine.[303]

4.16 Synthesis of β-Amino Acids and Higher Homologous Amino Acids – Extraordinary growth of interest in this topic is evident in the current literature. The driving force, apart from the usual mechanistic interest in novel bond-forming processes, must be the importance of the isolated examples of natural amino acids, amides and peptides within this category; perhaps also the intuitive expectation that many more physiologically-active natural β-amino acids are waiting to be discovered.

A major proportion of these studies now concerns stereoselective synthesis, and recent work with β-amino acids has been reviewed from this point of view.[304] Some general methods are extensions of those used for the stereoselective synthesis of α-amino acids, such as the C-5-aldolization[305] and C-5-alkylation[306] of six-membered chiral perhydropyrimidino-4-ones (Scheme 24) and alkylation at



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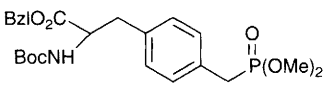

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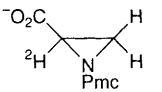

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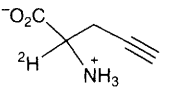

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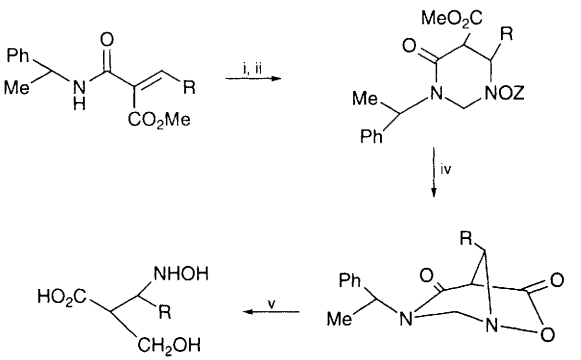
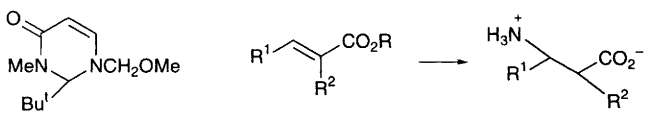


Reagents: i, NH_2OH, then ZCl/NaOH; ii, $(CH_2O)_n$, TsOH; iii, remove Z, then LiHDMS; iv, Amberlyst H-15; v, $LiAlH_4$, then H_3O^+

Scheme 24



(71) (72) (73)

C-6 of unsaturated analogues (71),[307] addition of chiral imines [(S)-PhCHMeN=CHR to (E)- or (Z)-α-silyloxyketene acetals mediated by chiral boron reagents,[308] or $^{-}CH_2CO_2Me$ to (+)-(S)-p-tolylS(O)N=CHPh to give (R)-3-amino-3-phenylpropanoic acid,[309] addition of the highly syn-stereoselective nitrogen nucleophile (R)-PhCHMeNLiCH_2Ph to alkyl cinnamates (72 → 73)[310] and anti-addition to crotonates;[311] the reasons for high stereoselectivity in the latter approach have been discussed.[312] The adducts can be further alkylated with excellent stereoselectivity, and an example of this is included in the novel establishment of an asymmetric Michael addition of a homochiral magnesium amide (R)-PhCHMeN(MgBr)CH_2Ph.[313] A further example of a synthetic target that has been achieved through asymmetric Michael reactions is (2S,3R)-2-methyl-3-aminopentanoic acid [(R)-PhCHMeNLiCH_2Ph + t-butyl (E)-2-methyl-penten-2-oate].[314]

Amination of ethyl 2-methyl-4,4,4-trifluoroacetoacetate using benzylamine and appropriate further steps (including penicillin acylase resolution – the (R)-enantiomer is most readily hydrolysed) forms the basis of a synthesis of all four stereoisomers of α-methyl-β-trifluoromethyl-β-alanine.[315] The [1,3]-proton shift at the heart of this process can be biased by (-)-cinchonidine to give enantiomerically-enriched (R)-β-fluoroalkyl-β-amino acid derivatives (up to 36% e.e.).[316] A similar approach is seen in the addition of benzylamine to ethyl (R)-trans-4,5-O-isopropylidene-4,5-dihydroxy-2-pentenoate (Scheme 25),[317] and in the addition of phthalimide salts to imides of chiral imidazolidinones.[318] An interesting alkylation process $NO_2CH_2CH_2CO_2Bu^t$ → $NO_2CH[CH(OH)CH_2F]CH_2CO_2Bu^t$ uses 2-fluoroethanol/$COCl_2$.[317] α-(o-Hydroxyphenyl)-β-alanines are available through the addition of $(Me_3Si)_2N^-Li^+$ to coumarins.[319] Homochiral N-diphenylamino-3-amino-1,2-diols formed by aminolysis of epoxyalkanols can be converted into β-amino acids *via* allylamines.[320] Reductive dimethylamination of αβ-unsaturated acids has been described.[321] β-Amination of 3-hydroxycyclobutanecarboxylate esters through treatment with carbonyldi-imidazole and sodium azide involves an acylnitrene insertion step.[322]

Nucleophilic addition to imines ($PhCH=NSO_2R$ + $BrZnCH_2CO_2Bu^t$ → $H_2NCHPhCH_2CO_2H$)[323] and the related process with N-acyl-α-methoxyamines[324] illustrate one general approach to β-amino acid synthesis, while 1,2-cyanohydroxylation of alkenes by nitrile-imines [$EtO_2CC\equiv N^+N^-CH_2Ph$ → 3-carboxypyrazolines → $RCH(CH_2CN)NHCH_2Ph$][325] provides an alternative amination pathway. Hydrogenation of homochiral aziridine-2-carboxylates over 3 days gives β-amino acid esters.[326]

Further syntheses of N-benzoyl-(2R,3S)-3-phenylisoserine methyl ester, the derivatized side-chain moiety of taxol, have been described, one[327] employing conventional synthesis and resolution, while the other incorporates yeast-catalysed reduction to introduce a second chiral centre into (S)-phenylglycine-derived acyl cyanides $PhCH(NH_2)COCN$.[328] Diastereoselective reduction of N-protected β-amino-α-ketoacids has been achieved, by H_2/RuCl/(R)-BINAP for the preparation of L-isoserine,[329] and employing microbial reduction for the preparation of (2R,3S)-(-)-phenylisoserine.[330] Although oxazolones offer standard routes to α-amino acids, exploitation of their reactivity at C-2 in a β-amino acid synthesis has

also been realized (Scheme 26).[331] This amounts to one-carbon homologation of an aldehyde, also achieved using nitromethane; the ensuing conversion ($-CH_2NO_2 \rightarrow -CO_2H$) involves drastic conditions (12M HCl,100°,46 h) but is nevertheless appropriate for an erythro-phenylnorstatine synthesis.[332]

Examples of the extension of standard practice in the α-amino acid field are: alkylation of β-alanine carrying two chiral auxiliary groups, *viz.* N-(hydroxypinanylidene)-β-alanine (-)-menthyl ester;[333] hydrogenation (H_2/Pd) of 3-aryl-2-aminomethacrylic esters $BocN^iPrCH_2C(=CHAr)CO_2Me$ gives racemic β-amino acid esters.[334] An asymmetric Diels-Alder approach gives the fused-ring didemnin analogues (74).[335] Ireland enolate-Claisen rearrangement of β-alanine allyl esters, (E)- and (Z)-$RCH=CHCH_2O_2CCH_2CH_2NR^1R^2$, leads to α-alkyl-β-amino acids (75 and 76).[336] Alkylation α- to the carboxy group of a β-amino acid derivative through the aldol route allows versatile chain extension.[337]

Amidiniomycin (77) has been synthesized from norbornylene via meso cis-dicarbomethoxycyclopentane; the route depends on enzymic discrimination between enantiotopic ester groups for its success.[338] A synthesis of (2S,3R)-3-amino-2-hydroxyalkanoic acids by amination of (78) may require a broader study if, as claimed, it is to be accepted as a 'general' synthetic route.[339]

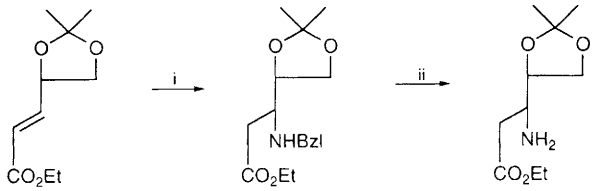
Syntheses starting with an α-amino acid include Wolff rearrangement of the diazomethylketone derived from an N-protected α-amino acid[340] followed by diazo-transfer and oxidation (dimethyl dioxirane) to give N-protected β-amino α-keto-esters without racemization. Homologation, through the Wolff procedure, of protected L-arginine gives dipeptides when irradiation is performed in the presence of an amino acid ester.[341] One-carbon homologation of α-amino acids, by their conversion into 2-(2-aminoalkyl)thiazoles followed by hydrolytic thiazole cleavage and further elaboration (*cf* Scheme 22, Ref. 226) has been demonstrated to give α-hydroxy-β-amino aldehydes and acids.[342]

Schmidt rearrangement of cis-1-carboxy-2-carbomethoxy-bicyclo[2.2.1]heptene gives the corresponding β-amino acid ester.[343]

Unprecedented syntheses of mechanistic interest have been described, by which unique β-amino acid targets have been attained (e.g. 79), by ring-contraction of tetrahydroisoquinoline alkaloids after lithiation,[344] and (80) from the 2-(β-naphthyl)oxazoline (81) by direct amination and alkylation followed by hydrolysis.[345]

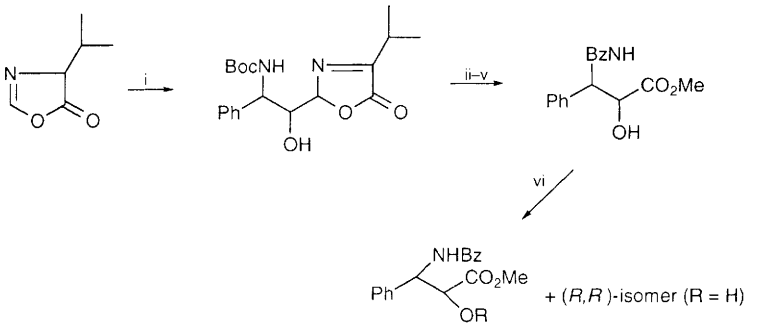
Ozonolysis of N-ethoxycarbonyl-2,3-dihydropyrroles in methanol gives the corresponding N-formyl-N-ethoxycarbonyl-β-amino acid methyl esters; the well-known oxidative ring-opening of 1,2,3,4-tetrahydropyridines to give 5-aminoalkanals is also explored further in this study.[346]

An extension of the amination approach leading to β-amino acids has been established. A tandem conjugate addition–hydroxylation protocol[347] using (S)-$PhCHMeNLiCH_2Ph$ and [(+)-camphorsulfonyl]oxaziridine leads to homochiral 3-amino-2-hydroxyalkanoic acids (82; R = Ph, *alias* allophenylnorstatine, a component of the HIV-protease inhibitor kynostatin);[348] and to (83; R = hexyl, *alias* microginin).[349] Both (2R,3R)- and (2S,3R)-diastereoisomers of the last-mentioned example were prepared, establishing the latter to be the absolute configuration of the natural ACE inhibitor. (3S,4S)-Statine and its isomers are



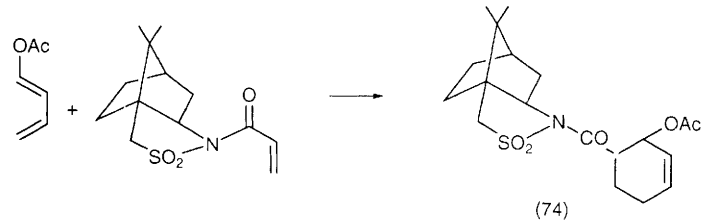
Reagents: i, $BzlNH_2$; ii, H_2, Pd/C, EtOH

Scheme 25



Reagents: i, Boc-phenylglycinal; ii, Et_3N; iii, 6M-HCl, 110 °C, 12 h;
iv. $SOCl_2$; v, BzCl; vi, *Pseudomonas fluorescens*, vinyl acetate

Scheme 26



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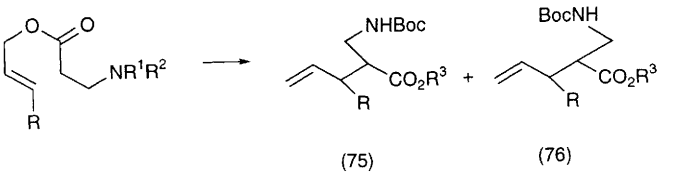

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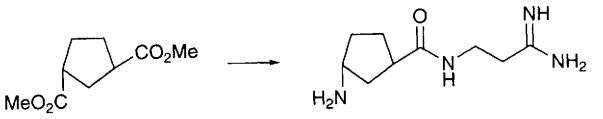

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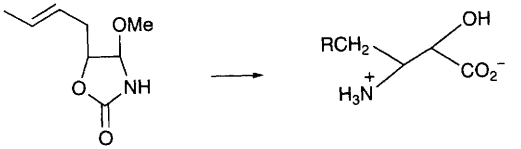

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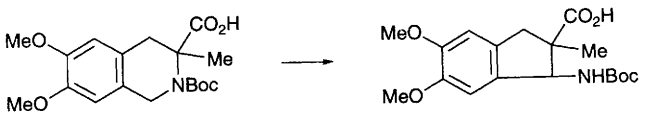

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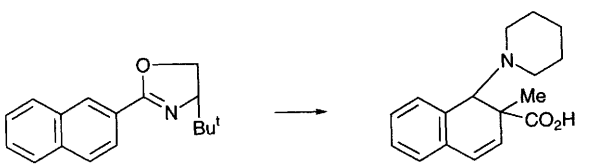

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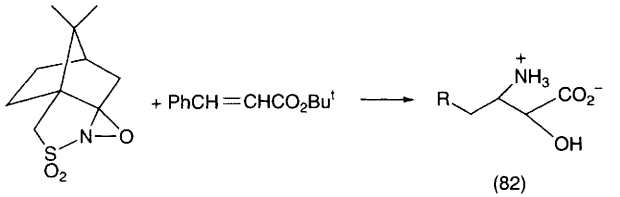
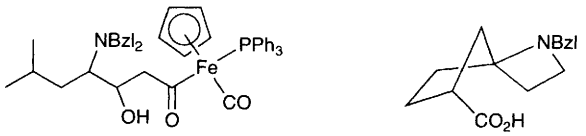
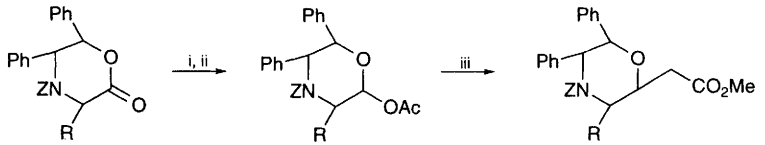






Reagents: i, DIBALH; ii, Ac_2O; iii, $CH_2=C(OMe)OTBDMS$, $ZnBr_2$

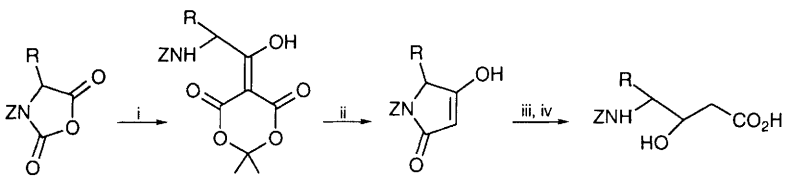
Scheme 27

easily prepared by aldol reactions of a protected L-leucinal, and a highly diastereoselective route has been established employing diethylaluminium enolates derived from $[\eta\text{-}C_5H_5]Fe(CO)(PPh_3)(COMe)$, to give the intermediate (84).[350] Simple alternative routes, addition of lithiated methoxyallene to an N-Boc-aminoalkanal followed by ozonolysis [$BocN(CH_2Ph)CH^iPrCHO \rightarrow BocN(CH_2Ph)CH^iPrCH(OH)C(OMe)=C=CH_2 \rightarrow$ (2S,3S)-norstatine],[351] amination of aldols [N-Boc-aminoalkanal/$PhCH_2CO_2H$ dianion] with $Ph_3P(O)N_3$ and ring-opening of the resulting diastereoisomeric 4,5-disubstituted oxazolidin-2-ones,[352] and azide ring-opening of the appropriate homochiral 2,3-epoxyalkanol, giving statine and its 3-epimer,[353] are typical of routes established over recent years.

A further statine synthesis involves the use of a homochiral oxazinone (Scheme 27) that is alkylated in an unusual way.[354] Similar alkylation of N-protected N-α-amino acid carboxylic anhydrides (NCAs) uses Meldrum's acid (Scheme 28); reduction of the resulting tetramic acids gives statine analogues.[355]

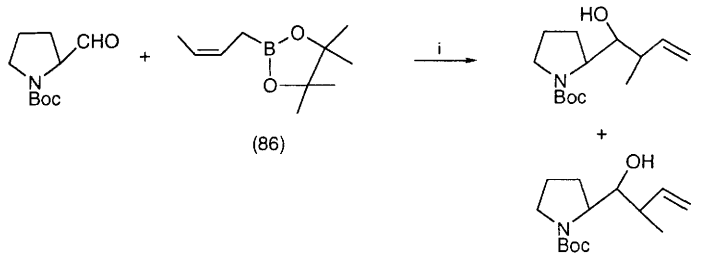
Synthesis of γ- and higher homologous amino acids is studied for much the same reasons that motivate efforts in the α- and β-amino acid area: the provision of authentic samples of biologically-important amino acids and their analogues, and also the growing interest in dipeptide and oligopeptide isosteres that δ-amino acids and higher homologues represent. 'Conformationally-constrained γ-aminobutyric acid' (GABA) is one way of describing the spirobicyclic condensation product (85) of 2-(N-benzylimino)cyclopentanecarboxylic acid with dibromoethane.[356] L-erythro-αβ-dihydroxyGABA and γ-erythronine have been prepared through oxidative degradation of 4-aminopent-1-ene-2,3-diols formed from penta-1,4-dien-3-ol by Sharpless epoxidation followed by amination.[357] Since dolaisoleucine t-butyl ester formed from the aldol adduct from 2-N-methyl-L-isoleucinal and t-butyl glyoxylate is identical with the natural γ-amino acid, the (3R,4S,5S)-stereochemistry can be assigned.[358] Dolaproine, a γ-amino acid from (-)-dolastatin 10, has been assigned the (2S,2'R,3'R)-stereochemistry through examination of the product from a corresponding route from the Boc-L-prolinal/(S)-$HOCPh_2CHPhO_2CEt$ aldol adduct, verified by X-ray crystal analysis.[359] An alternative route to natural dolaproine relies on preferential anti-addition of (Z)-crotylboron reagents (86) to homochiral N-Boc-aminoalkanals (Scheme 29).[360] A simplified route to E/Z-γ-amino acid esters $MeO_2CNHCHRCH=CHCO_2Et$, through a one-pot partial reduction (DIBALH) to the aldehyde and homologation with $(EtO)_2P(O)CHLiCO_2Et$, has been described.[361] Both enantiomers of 4-aminohex-5-enoic acid (vigabatrin) have been prepared in this way, starting with N-Z-L- or -D-methionine methyl ester and concluding with reduction of the double bond and introduction of the terminal alkene group through oxidative elimination of methanesulfinic acid.[362] Wittig reactions of $MeC(=PPh_3)CO_2Me$ (or the corresponding phosphonate) with α-Boc-alaninal gives a 2:1 anti/syn-mixture of 2-methyl-4-aminopentanoic acid derivatives after catalytic hydrogenation.[363]

A variety of routes exist, to lactams of various ring sizes, although their cleavage to give ω-amino acids can be problematical. A study of N-acylated lactams from this point of view has established the use of toluene-p-sulfonic acid



Reagents: i, Meldrum's acid, NEt_3; ii, Δ, AcOEt; iii, $NaBH_4$; iv, NaOH, aq. acetone

Scheme 28



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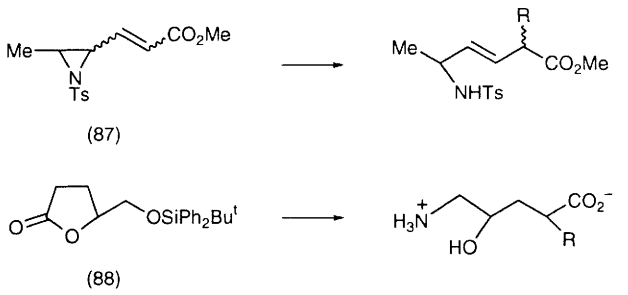
Reagents: i, THF, r.t.

Scheme 29



(87)

(88)

in methanol for the purpose.[364] Oxidation of N-Troc-piperidines with $RuCl_3$/$NaIO_4$ and hydrolysis under unspecified conditions gives 5-aminoalkanoic acids.[365] Pyrrolidin-2-one ring opening is the final stage in a synthesis of 4-amino-2,2-dimethylbutanoic acid.[366] Diastereoselective alkylation of δ-lactams carrying a chiral N-substituent has been established.[367]

A route to δ-amino acids has been established, exemplified by regioselective addition of alkylcopper reagents to each of the four stereoisomers of the N-toluene-p-sulfonylaziridine (87).[368] Analogues [(2R,4S)-5-amino-4-hydroxypentanoic acids] of a particular natural example of this family of amino acid have been synthesized from L-glutamic acid *via* the silylated α-hydroxymethyl-lactone (88).[369] α-N-Boc-Aminoaldehydes yield δ-amino γ-hydroxyacid derivatives through condensation with allylic bromides,[370] as do corresponding methyl ketones through reaction with α-bromoalkanoates.[371] Mitsunobu amination of an allylic alcohol with phthalimide is a step in a route to a δ-aminoalkenoic acid.[372]

4.17 Resolution of DL-Amino Acids – Active development of existing methodology, especially in topic areas under the heading of chromatographic resolution, would be an accurate description of current research. Analytical aspects (determination of enantiomer ratios by chromatographic and related means) are mostly covered in Section 7, and configurational assignments achieved through synthesis strategies are covered in earlier Sections.

Classical laboratory procedures for the resolution of amino acids are represented in diastereoisomeric salt formation with homochiral sulfonic acids.[373] The procedure is usefully complemented by salicylaldehyde-mediated racemization, illustrated by a preparation of D-p-hydroxyphenylglycine.[374] N-Salicylidene- and pyridoxylidene-DL-amino acids have been resolved analogously, through chromatography of derived diastereoisomeric copper chelates.[375] Marfey's reagent can be used both to generate diastereoisomeric derivatives and to assign absolute configuration to individual enantiomers, for example to micricystins.[376] Use has been made of the different rates of condensation of (+)-2-hydroxypinan-3-one with Schiff bases of DL-amino acids, to enrich samples with a particular enantiomer, to the extent of complete resolution in certain cases.[377] Examples of the resolution of higher homologous amino acids in this way are relatively rare, but vigabatrin enantiomers (see preceding Section, Ref.362) have been secured through acylation of 5-vinylpyrrolidin-2-one with (R)-$PhCHMeCO_2H$ and subsequent crystallization.[378]

Resolution of DL-amino acids through preferential crystallization of one enantiomer is underpinned by idiosyncratic physical solid state behaviour; thus, L-phenylalanine crystals added to a DL-glutamic acid sample favour the crystallization of the L-enantiomer, but all attempts failed to achieve the corresponding result using D-phenylalanine.[379] 4-Hydroxyproline assisted the resolution of DL-allothreonine in analogous fashion.[380] Spontaneous resolution under racemizing conditions in prebiotic times was probably not of major importance, either for the origin of enantiomeric imbalance or for the amplification of any microscopic imbalance.[381]

A widening range of both enzymes and non-protein DL-amino acids is being represented in resolution studies. The possibilities have been explored for the use of pronase with non-protein amino acid methyl esters,[382] *Aspergillus niger* lipase for kinetic resolution of pipecolic acid methyl ester,[383] *Candida cylindracea* lipase with aziridinecarboxylates,[384] lipases for α-vinylglycine after reduction,[385] thiol proteases with Z-amino acid methyl esters,[386] subtilisin Carlsberg,[51,112] an amino acid amidase from *Mycobacterium neoaurum* with α,α-disubstituted α-amino acid amides,[387] decanoyl-α-chymotrypsin with N-dodecanoylamino acid p-nitrophenyl esters[388] immobilized *Aspergillus oryzae* aminoacylase with N-acetyl-p-chlorophenylalanine[389] and for continuous column resolution of N-acetyl-DL-methionine,[390] and an aminoacylase from *Streptovercillium olivoreticuli* or penicillin acylase from *E.coli* for preparative-scale resolution of o- and p-fluorophenylglycines.[391] Novel procedures have been studied; one featuring a D-amino acid oxidase/aminotransferase/L-glutamic acid system that converts racemic mixtures of common and non-protein amino acids into the L-enantiomers;[392] another using immobilized enzymes in a tea-bag method, with N-acetylamino acid methyl esters in reverse micellar media;[393] and another establishing the resolution of N-acylamino acids using alcalase in supercritical CO_2.[394] Aldolase from *Streptomyces amakusaensis* catalyses the reverse aldol reaction with (2S,3R)-β-hydroxy-α-amino acids, and therefore provides enantiomers when applied to the racemates.[395]

Most of the current research studies of resolution are based on physical principles, particularly involving chromatographic separation over chiral stationary phases (CSP's). The technique continues to develop rapidly, and reviews have appeared of Pirkle CSP's[396] and preparative-scale resolution by this approach.[397] Long-established examples of CSP'S are represented in recent papers, including cellulose thin-layer chromatography with a cyclodextrin-containing mobile phase, showing additive contributions to discrimination leading to large separation factors for tryptophans.[398] Bovine serum albumin (BSA) achieves the resolution of N-alkanoyl DL-[^{3}H]leucines (the D-enantiomer is more strongly retained and separation factors are strongly dependent upon the solutes in the stationary phase.[399] For the free amino acids in solutions at pH 7.0, the L-enantiomer is more strongly bound to BSA when it is immobilized in the form of a membrane.[400] Cyclodextrins can accomplish the resolution of DL-alanine β-naphthylamide.[401] New examples include β-cyclodextrins to which D- or L-phenylalanine cyanomethyl esters are linked,[402] 6'-(3-aminopropylamino)-6'-deoxycyclomaltaheptaose (which binds L-tryptophan more strongly than β-cyclodextrin),[403] or chiral alcohols immobilized within a poly(ethylene) film (enantioselective transport of amino acids)[404] and 4-vinylpyridine–1-vinylimidazole copolymers imprinted with N-Z-L-aspartic acid (see Vol.26, p.49).[405] Crystals of imprinted D-phenylglycyl-D-phenylglycine formed from solutions containing N-acetyl-L- or D-leucine ethyl ester are capable of enantioselective binding of these solutes.[406] Capillary GLC of N-trifluoroacetyl-DL-amino acid n-butyl esters over L-Phe-tetra-amide CSPs has proved effective in enantiomer ratio determinations.[407] Chromatographic resolution on the ligand-exchange principle is represented in the use of copper(II)-6-deoxy-6-(N-histamino)-β-cyclodextrin

(binding of D-amino acids is favoured),[408] the use of copper(II)- or nickel(II)-N-substituted-L-proline-modified silica gel,[409] and a similar use of (R)-2-amino-alkanol for chiral modification of stationary phases.[410] Results obtained in ligand-exchange resolutions using a range of metal salts have been compared,[411] and the routine method employing a chiral mobile phase additive has been studied for the resolution of fluorine-substituted phenylalanines and phenylglycines.[412]

Permeable membranes have been mentioned in the preceding paragraph, in their role as enantioselective barriers that can form the basis of preparative resolution technology. Membranes to which L-phenylglycine is bonded are more permeable to the D-enantiomer of this amino acid,[413] and hollow-fibre membranes carrying N-(1-naphthyl)-L-leucine have shown promising enantioselectivity.[414] Liquid membranes are particularly promising in this respect, 5-cholesteryl-L-glutamate forming the basis of mixed micelles that preferentially bind the D-enantiomer from solutions of DL-phenylalanine in the presence of copper(II) ions.[415] 1,2,4-Triazole-containing cholesteryl esters offering preferential transport to D-phenylalanine primary alkylammonium salts.[416] Chiral discrimination is also revealed for Langmuir-Blodgett monolayers containing N-palmitoyl-DL-valine and DL-alanine and N-stearoyl-DL-valine, compared with L-analogues,[417] by (cholesteryloxycarbonyl)benzo-18-crown-6 monolayers,[418] and by a variety of emulsion liquid membranes;[419] and several other papers in this Symposium Volume, Ref.419). A promising development is the demonstration of enantiomeric enrichment of derivatized and free amino acids by foam-forming 'chiral collectors'.[420]

A 44-mer RNA that binds L-citrulline and D- or L-arginine has been reported.[421]

Speculation surrounding the methods by which DL-amino acid mixtures, generated in prebiotic times, were supplanted by L-enantiomers has shifted towards the consequences of parity-violating phase transition phenomena (Bose-Einstein condensation activation),[422] a theory advanced by Salam (see Vol. 24, p.40) that he has recently reviewed.[423] Many of the main protagonists in this field have presented their ideas in this publication; favouring either parity-violating energy differences between L- and D-enantiomers of an amino acid,[424,425] through chiral interactions at the ocean-air interface (see Vol.25, p.55),[426] or through spontaneous amplification.[427,428] The emergence of L-amino acids, as a consequence of parity-violating energy differences leading to electroweak neutral currents, has been reviewed more recently[429] by an advocate of an alternative theory (see Vol.26, p.51).

The origin of chirality in amino acids and carbohydrates is suggested to lie in sonically-induced phase transitions; a D- or L-amino acid will eventually accumulate through sonication of a racemic amino acid.[430] A much earlier idea, encapsulated in the Vester-Ulbricht theory, asserts that enantioselective destruction of the D-amino acids occurs more rapidly, relative to their L-enantiomers, under the influence of inherently asymmetric radiation. This has been re-investigated to show that DL-leucine bathed in radiation from a ^{22}Na weak positron source suffers more rapid destruction of its D-enantiomer.[431,432] ESR

study of D- and L-alanine[433] and of D- and L-leucine[434] degraded with ^{90}Sr-^{90}Y β-radiation shows that irradiation generates more radicals in the D-enantiomer, and the Vester-Ulbricht theory is therefore claimed to be given further support. Reviews of origins of chirality and life, giving explanations that make lesser demands on the reader, have appeared.[435,436]

5 Physico-Chemical Studies of Amino Acids

5.1 X-Ray Crystal Structure Analysis of Amino Acids and Their Derivatives – A pattern that has emerged in recent years, the revision of earlier data and determination of structures of new compounds, continues to be represented in the current literature. The fact that X-ray crystallographic instrumentation has become more sophisticated, so that results are more reliable, but also less tedious to obtain, is mainly responsible for the increased activity in this area. Crystal structures for free amino acids have been reported for DL-alanine,[437] DL-arginine (as its dihydrate, formate, and formate dihydrate),[438] L-arginine fluoroborate,[439] L-arginine maleate dihydrate,[440] L-histidine monoacetate,[441] L-histidinium phosphite and a re-investigation of monoclinic L-histidine,[442] and N-methylglycine monohydrogen phosphite.[443]

Crystal structures of amino acids have been surveyed with particular reference to their propensity towards polymorphism.[444]

Derivatives that have been subjected to study are benzyl 2(S)-Boc-amino-4-oxo-6-phenyl-5(E)-hexenoate,[445] DL-valine N-carboxyanhydride,[446] N-monochloracetyl-D-α-methyl-leucine,[447] N-acetyl-L-proline methyl ester, -(4S)-hydroxy-L-proline methyl ester, and -(4S)-fluoro-L-proline methyl ester,[448] N-chlorosulfonyl-L-proline benzyl ester,[449] Z-[(E)-5-(p-nitrobenzyloxycarbonyl-methinyl)]-L-proline t-butyl ester,[450] N-[N-benzyloxycarbonyl-L-1,2,3,4-tetrahydroisoquinolin-3-ylcarbonyl]-L-phenylalanine methyl ester,[451] and Boc-L-tryptophan (2-thymin-1-yl)ethyl ester.[452] While crystalline N-acetyl L-proline methyl ester is characterized by a cis-peptide bond, the ring-substituted analogues adopt the trans-configuration.[448]

5.2 Nuclear Magnetic Resonance Spectrometry – An NMR study qualifies for inclusion in this section only if the object of the study involves more than routine support of synthetic studies. Conformational studies are included within this policy, and a familiar example, interpretation of ^{1}H-NMR (as well as IR data) of C^2HCl_3 solutions of N-acetyl-L-proline N-methylamide, provides more reliable conformer ratios based on improved understanding of spectral parameters.[453] Related studies involving chloro-substituted tryptophans[454] and bacoflen analogues [3-(thien-2-yl)- and 3-(furan-2-yl)-γ-aminobutyric acids][455] adopt the common approach of calling for data from both ^{1}H- and ^{13}C-NMR measurements.

Establishment of absolute configuration of α-amino acid enantiomers, and of other α-chiral amines, through interpretation of ^{1}H-NMR data of amides formed with (S)-O-methylmandelic acid,[456] and enantiomeric purity determination of

deuteriated esters of amino acids through ^{2}H-NMR measurements using poly(γ-benzyl-L-glutamate) in CH_2Cl_2 as a chiral lyotropic liquid crystal solvent,[457] are studies that illustrate familiar stereochemical applications of ^{1}H- and ^{2}H-NMR Another analytical application is represented in the estimation of carbamate formation in aqueous solutions of amino acids,[458] while the extraordinary sensitivity of current NMR instrumentation is further illustrated (see Vol.23, p.46) in the detection of glutamic acid, glutamine, and N-acetylaspartic acid in brain tissue.[459] NMR parameters for glycine in frozen aqueous solutions have been collected;[460] values of ^{1}H-NMR spin-lattice and spin-spin relaxation times increase for aqueous solutions of glycine and proline with increases in temperature and concentration.[461] A 'magic angle spinning' study by ^{1}H-NMR of L-alanine has been directed at the assessment of molecular dynamics in the solid state.[462]

^{13}C-NMR studies with similar objectives have been reported; one broad study relates chemical shifts for each carbon atom to torsion angles for N-formyl-L-alanine- and -valine-amides[463] and L-threonine and L-tyrosine,[464] while another study illustrates the usefulness of ^{13}C-NMR measurements for assessing protonation equilibria for ornithine, lysine, and hydroxylysine in aqueous dimethyl sulfoxide.[465] Spectra of N′-alkyl- or aryl-N-carbamyl and their N-nitroso derivatives, have been correlated with structure.[466]

For ^{17}O-NMR, the frontiers continue to be pushed back but gaining results with little general applicability to revealing subtle details of amino acid structures in solutions; for example, further cross-polarized dynamic angle spinning data on ^{17}O-labelled amino acids have been collected.[467] Specialized applications continue, however, for ^{31}P-NMR, with accurate enantiomeric analysis using amides formed from amino acids and the chiral phosphorinane HP(O)(OCHMeCH$_2$NEt$_2$)$_2$,[468] ^{77}Se-NMR data can be used for absolute configurational assignments to amino acids converted into imidazol-2-selenones.[469]

5.3 Optical Rotatory Dispersion and Circular Dichroism – Reports over the years have described the use of these techniques to determine subtle structural details for amino acids, though empirical rules sometimes fail. Thus, the CD spectrum of N$^{\alpha}$-acetyl-L-ornithine and homologues is found to be what the simple rules predict for the D-configuration,[470] and anomalous optical rotation data have been found for N$^{\varepsilon}$-acetyl-L-lysine and N$^{\alpha}$-acetyl-L-lysine.[471] and N$^{\alpha}$-acetyl-L-arginine.[472] Reliable correlation, of CD with absolute configuration, has been established (L-derivatives show positive CD near 310 nm and negative CD at 280–290 nm) for Eu(fod)$_3$ complexes of amino acids[473] and of amino acid esters.[474] The CD features of trinuclear complexes between amino acids and $[M_3O(O_2CCH_3)_6L_3]^{n+}$ (L = water or pyridine, n = 0 or 1) are suitable for the assignment of absolute configuration through the application of a semi-empirical helicity rule.[475]

CD in the Soret wavelength region for solutions of homochiral amino acid derivatives and achiral porphyrins has been ascribed to hydrogen bonding association.[476]

Raman optical activity of amino acids (usually measured as CD) has been

reviewed.[477] This is a topic area that has developed slowly, and its fundamental basis is still being worked out, assisted by studies such as comparisons of measured and calculated Raman CD for L-alanine and its isotopomers.[478]

5.4 Mass Spectrometry – Increased activity associated with the newer instrumental techniques would be an appropriate inference based on the current literature covering mass spectrometric studies of amino acids and derivatives. There is also an awareness that some long-known variants of MS techniques have been under-used, such as negative ion measurement, which gives 'cleaner' spectra for N-(2,4-dinitrophenyl)amino acids,[479] intense $[M-1]^-$ peaks for N-phospho-amino acids,[480] and can allow discrimination between the four γ-hydroxyornithine diastereoisomers after bis(N-benzyloxycarbonylation).[481] Both positive- and negative-ion modes have been applied to generate spectra from free amino acids (glycine, methionine, histidine, and cysteine), the spectra showing prominent $[M-1]^-$ and $[M_2-1]^-$ peaks.[482]

New techniques providing spectra of amino acids themselves include time-of-flight MS of phenylalanine bombarded with 2.5 MeV carbon ions,[483] and pulsed laser-initiated ionization–desorption of tryptophan embedded in rhodamine B and glycerol[484] and of crosslinking amino acids pyridinoline and its deoxy- and glucosylgalactosyl-derivatives.[485] Cluster ion formation from a mixture of two different amino acids in a Na^+-containing matrix has been studied, leading to an Na^+ ion affinity scale for amino acids (a Li^+ affinity scale was constructed similarly).[486] ^1H-^2H-Exchange involving amino acids and CH_3O^2H,[487] and gas-phase proton affinity studies for amino acids, have been the subject of study over several years, applied to glycine and its isotopomers.[488] Corresponding FT-ion cyclotron resonance studies of phenylalanine and its N-methyl- and NN-dimethyl-analogues have been described.[489] Theoretical aspects have been presented, of kinetics of protonation leading to cluster ions $[(\text{amino acid})_2\text{–H}]^+$ in the gas phase.[490] Gas-phase basicities, a subtly-different parameter, have been determined for amino acids[491] with particular attention to lysine and histidine, by the kinetic method.

5.5 Other Spectroscopic Studies of Amino Acids – Several projects cited in preceding sections have relied on more than one spectroscopic technique, commonly including IR and other vibrational spectroscopic methods. A remarkably simple positive answer to the question: do diastereoisomeric interactions exist between enantiomers in the solid state? has emerged from IR spectra of L-proline, DL-proline, and of an equimolar mixture of the two, which is not simply the weighted average of the preceding two spectra.[492] The result does not, of course, clarify the nature of these interactions, but much more detail is available through IR studies of glycine in neon, argon, and krypton matrices (three different conformers are established for the first time),[493] and a similar result for proline and ^2H-labelled proline.[494] IR-Raman spectroscopy of solid L-aspartic acid and $^2H_4$- and ^{15}N-isotopomers[495] and of solid L-glutamic acid and $^2H_4$- and ^{15}N-isotopomers[496] yield fundamental vibration modes for these amino acids.

Another familiar interest is revealed in the establishment of intramolecular hydrogen-bonding patterns for derivatives of β- and γ-amino acids through IR study of CH_2Cl_2 solutions.[497]

The rotational spectrum of β-alanine determined in a free-expansion jet spectrometer provides evidence for the existence of the same types of intramolecular interactions within two conformers, as already found in gaseous glycine and alanine.[498]

Photoelectron spectra have been determined for phenylalanine and its N-methyl- and NN-dimethyl-analogues.[489]

5.6 Physico-Chemical Studies of Amino Acids – This section covers useful interpretations, in terms of the behaviour of amino acids, of some simple laboratory measurements. Thus, the solubility behaviour of fourteen amino acids in water as a function of pH and temperature has been considered on the basis of fundamental structural and thermodynamic parameters;[499] solubilities of L-isoleucine, L-leucine, and L-valine in aqueous NaOH increase as the NaOH concentration is increased, then decrease sharply after the 1:1-ratio has been passed.[500] The solubility of the dipeptide derivative Z-L-Asp-L-Phe-OMe (i.e., Z-aspartame) in water containing L-phenylalanine methyl ester shows complex dependence upon concentration, pH and on other parameters, showing that the solutes interact in more ways than simply through ionic attractions and repulsions.[501] Concentrated proline solutions show non-ideal behaviour (freezing point depression and isopiestic data), and this explains the protective effect of proline on enzyme activity (due to the fact that it exerts a role as inert space-filling solute to help maintain a native polypeptide conformation).[502]

The various lipophilicity scales for amino acids have been reviewed,[503] and a new hydrophilicity scale has been proposed based on calculations of solvation parameters.[504] A multi-channel sensor system has been trained to correlate the 'taste' characteristics of amino acids.[505]

Guest–host studies continue (see also Section 4.17), with water-soluble calix-[n]arenes (89; n = 4, 6, 8) that strongly bind amino acid methyl esters into their cavity.[506] The related cyclic tetramer (90),[507] forms 1:1-complexes with aromatic amino acids, and the chiral porphyrin (91) shows enantiodiscrimination towards amino acid esters.[508] The C_2-symmetric chiral porphyrin analogue [92; R = (R)-$CH_2CHMeOH$] is an effective transporter of lithium salts of amino acids through CH_2Cl_2 membranes,[509] and a phenanthroline–copper(I) template supports a bis(2-aminoacylpyridine) receptor that binds Z-L-glutamic acid and other dicarboxylic acids.[510] A similar mechanism accounts for the binding of Z-aspartic acid to an 2-acylaminopyridine-substituted heterocyclic template, to which a broader range of Z-amino acids shows modest binding.[511] Cyclo-oligomers of cylindrical shapes have been synthesized that present amide groups to guest molecules, and show high selectivity towards N-acetylamino acid N′-methylamides.[512] The crown ether (93) has been shown to use its carboxy-group as well as the macrocycle atoms to complex with amino acids.[513]

The enantiomeric discrimination factors that are sought in such studies are being put on a firmer numerical basis, as illustrated for standard molar enthalpies

of binding by cyclodextrins of L- or D-phenylalanine and L-phenylalaninamide. Values have been determined by microcalorimetry, and are independent of configuration.[514] Microcalorimetric data also show that the chiral discrimination of L-ascorbic acid–Fe^{3+} towards enantiomeric α-helical peptides is not exerted towards cysteine enantiomers.[515]

Heterogeneous liquid systems are of growing interest, from the point of view of amino acid transport; systems studied recently (see also Refs.413-419) are basic amino acids/water/$CHCl_3$ + sodium di(2-ethylhexyl)sulfosuccinate[516] organic solvent/water/arylboronic acids + crown ethers,[517] and organic solvent/water/18-crown-6 + picric acid.[518] Reverse micellar extraction of amino acids from aqueous media by di-octyldimethylammonium chloride is sensitive to co-solutes and physical parameters.[519] In the glycine or L-lysine/octyl β-D-glucoside/water systems, from 3 to 7 amino acid molecules can be bound by each molecule of the glucose derivative, resulting in a lowering of its critical micellar concentration.[520]

Equilibria involving transfer of arginine, from solutions containing HCl and NaCl to a cation exchange membrane, have been evaluated.[521] Sorption of amino acids on to ion exchange membranes has been studied.[522]

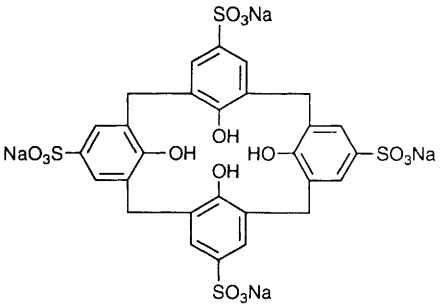
Dipole moment data have been collected for L-cysteine and L-cystine.[523]

A review has appeared covering solute–solute and solute–solvent interactions that occur in solutions of amino acids.[524] The viscosity of a solution of an amino acid has been related to the effects of the solute on water structure,[525] and data have been collected for viscosities of aliphatic α-amino acids in 0.5 and 2M urea.[526] Apparent molar volumes of aliphatic α-amino acids in 0.5 and 2M urea,[527] and of aqueous L-valine, L-isoleucine, and L-leucine[528] have been calculated from densities and volumetric heat capacity data.

Thermodynamic parameters, enthalpies of dilution of L-threonine and L-asparagine,[529] enthalpies of interaction of amino acids and peptides with crown ethers in water,[530] apparent molal heat capacities and volumes of aliphatic α-amino acids at 288–328K,[531] and similar thermal properties of aminopolycarboxylic acid solutions[532] have been collected. Microcalorimetric studies provide enthalpy of dilution data for ternary aqueous solutions that contain glycine, an alkanol, and another α-amino acid.[533]

Electrical measurements for the effects of weak static and alternating low-frequency magnetic fields on current flow through aqueous amino acids[534] and potentiometric titrations leading to protonation constants for glycine in aqueous NaCl[535] and the proton-binding isotherm for glycine[536] have been presented, as have corresponding dissociation constants for L-proline, L-histidine and L-tryptophan.[537] The protonation rates for L-tryptophan in acidic media decrease with increasing pH.[538]

5.7 Molecular Orbital Calculations for Amino Acids – Development of familiar themes under this heading is continuing with calculations of solvation energies of zwitterionic forms of glycine, alanine and serine in different conformations in water,[539] hydration parameters and conformations of N-acetylamino acid methyl esters,[540] and 2-(N-acetylamino)isobutyric acid N-methylamide[541] and of twenty common amino acids substituted in the same way.[542]



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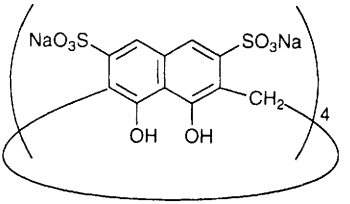

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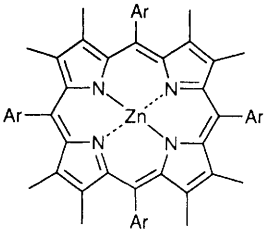

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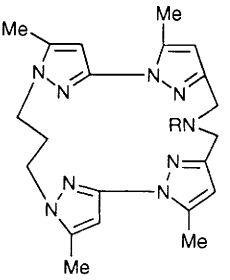

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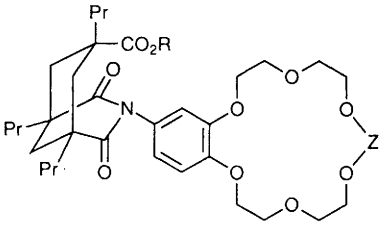

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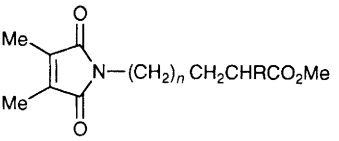


(94)

Quantum mechanical force fields generated by [glycine.nH_2O] supermolecules in basic glycine solutions,[543] electrostatic properties of amino acids modelled using atomic multiple moments,[544] and molecular connectivity models leading to structure–property relationships for amino acids,[545] illustrate another area of computational interest (see also Ref.546).

Spectroscopic data generated through molecular orbital calculations concern vibrational frequencies of three non-ionized conformations of cysteine and serine,[547] chemical shift changes related to dihedral angles for glycine and glycinamide,[548] and gas-phase proton transfer energy values for eight of the protein amino acids.[549] An erratum has been published[550] concerning Ref.461 in Chapter 1 of Vol.26 (p.55).

6 Chemical Studies of Amino Acids

6.1 Racemization – Preparative applications of racemization are covered elsewhere in this Chapter (Section 4; e.g., Ref.167), and the content of papers eligible for discussion in this section is usually limited to a narrow topic, e.g. that the rate of racemization of L-aspartic acid in water at 100° is increased when dimethyl sulfoxide is added.[551]

It has become clear that the dating of fossils based on the presumed constancy of racemization rates of their indigenous amino acids is liable to considerable error because of unspecifiable catalysis, though the basis of a claim, that the kinetics of amino acid racemization are non-linear, is obscure.[552] Application of the method to amber-entombed insects using samples ranging in age from 100 y to 130 × 10^6 y can only provide results matching those of other dating methods if it is assumed that the amber environment retards racemization rates by a factor of greater than 10^4.[553] This, indeed, represents slow racemization and is about the same rate as DNA degradation by de-purination.[554] The dating method applied to *Homo tirolensis* (i.e. the male corpse found in 1991 at a high altitude in the Austrian Tyrol) and also to a specimen of ginger from Egypt, both of the same age (5200 y), in fact gave considerably different age values, and not only that, but the racemization rate in the colder specimen was faster![555] Since o- and di-tyrosine were detected in *Homo tirolensis*, and these are markers for free radical attack on proteins, the authors suggest that the hydroxyl radical formed by sunlight at high altitudes may accelerate amino acid racemization.

6.2 General Reactions of Amino Acids – This Section covers reactions at the amino and carboxy groups (and reactions at both) as well as reactions at the α-carbon atom of α-amino acids $^+H_3NCHRCO_2^-$; the following Section covers reactions of amino acid side-chains R.

Thermolysis of butyrine, 3-amino- and 4-aminobutanoic acids gives many reaction products,[556] while decarboxylation of L-threonine and L-hydroxyproline occurs at 170° in cyclohex-2-enone, giving optically-active β-amino-alkanols.[557] Irradiation of DL-lysine with soft X-rays causes the change of zwitterion to free

base, and decarboxylation leading to 1,5-diaminopentane.[558] Radicals formed by γ-irradiation of DL-threonine[559] and by pyrolysis of DL-serine, DL-threonine and DL-tyrosine at 200–600° for 2-180 min[560] have been studied by ESR. γ-Radiolysis of aqueous phenylalanine leads to tyrosine.[561]

Studies of these types, that often provide essential warnings of sample breakdown to those preparing samples of amino acids for analysis, are few and far between. However, in-depth study of the N-halogenation of amino acids and decomposition of the reaction products, continues to expand (see Vol.26, p.56), with decomposition kinetics being determined for N-chloro-valine,[562] and compared with data for N-chloro-sarcosine, -N-methylalanine, -N-methylvaline, and -proline.[563] N-Bromo-amino acids have been studied.[564] The Grob fragmentation pathway that is followed by these derivatives in aqueous solutions[565] can be promoted by metal alkoxides.[566] N-Nitrosation (N_2O_4/CH_2Cl_2) of α-(acetylamino)acids gives more stable products than hitherto believed, but they fragment in alkaline media to give α-hydroxyacids.[567] Reaction within a Co(III) complex [formed with $K_3\{Co(CO_3)_3\}$] at a pyridoxylidene-amino acid ligand generates an α-hydroxy-α-amino acid.[568] A kinetic study of the nitrosation of imino acids has revealed intramolecular migration of the nitroso group from an intermediate nitrosylcarboxylate formed at high pH.[569]

Conversion of α-amino acids into α-nitro acids employing the powerful oxygen transfer agent HOF.MeCN (formed in aqueous MeCN with F_2) involves racemization.[570]

N-Acylation reactions include formylation of amino acid esters with tri-ethyl orthoformate[571] and conversion of N-formyl-α-trifluoromethyl-α-amino acids into isocyanides.[572] The analogous isocyanates $OCNCR(CF_3)CO_2Me$ and hydrazides are starting materials for the synthesis of azapeptides,[573] and N-acryloyl-L-prolinamides have been prepared for the first time, for use in copolymer preparations.[574] N-Acylation of amino acids using vinyl esters has been advocated[575] (the method is well-known in the literature). N-(L-Maleyl)ation of amino acids (with glycine and proline as exceptions) can be accomplished with the aid of aminopeptidase A.[576] Solid-state N-phthaloylation of amino acids[577] and 3-carboxybenzoylation of DL-alanine using isophthalic acid[578] has been assessed using differential scanning calorimetry. An analogous product (94), previously undetected, emerges from Maillard reactions involving γ-aminobutyric acid, 6-aminocaproic acid, N^{α}-acetyl-L-lysine, and pentoses.[579] Amadori compounds formed at an early stage of the Maillard process, have been generated from D-glucose and α-amino acids, and subjected to FAB-MS and NMR study.[580]

N-[2-(4-Nitrophenyl)sulfonylethoxy)carbonyl]ation of amino acids gives N-protection that can be reversed by bases in aprotic solvents.[581]

Novel N-alkylation of amino acid esters by tricarbonyl(cyclohexadienyl)iron cations,[582] and through high-pressure reaction with oxiranes,[583] together with more traditional reductive alkylation methods using aldehydes (preparation of N-allyl and -propargyl- derivatives,[584] use of PhCHO/NaTeH for preparation of N-benzylamino acids[585]) have been described. The preparation of pure N-methylamino acids by conversion of Z-amino acids into oxazolidinones using

formaldehyde followed by Et_3SiH/TFA reduction ($NaBH_4$ is somewhat less effective)[586] or by the corresponding reaction of N-benzylamino acids with the reduction step accomplished by hydrogenation),[587] follows established methodology. Direct methylation of N-Boc-O-TBDMS-D-tyrosine with MeI/BuLi/-78° seems, however, to proceed uneventfully.[588] Coupling (R)- or (S)-homophenylalanine ethyl ester with lactates gives (R,S)- and (S,R)-N-[(1-ethoxycarbonyl-3-phenyl)propyl]alanine.[589] Procedures have been described for the preparation of N-(9-phenylfluoren-9-yl)-L-alanine and -L-aspartic acid dimethyl ester.[590] Bis-N-alkylation through reductive aminocyclization of L-valine methyl ester with keto-aldehydes is accomplished with high diastereoselectivity (Scheme 30), but with little stereochemical bias for simpler ketones.[591] N-Alkylation of sodium salts of secondary amino acids uses 4-chloro-N-benzylpyridin-2(1H)-one (DMSO, 160–180°).[592]

Following recent results (see Vol.26, p.62) that have established the condensation of amino acids to form peptides in aqueous NaCl by copper salts,[593] another non-enzymic reaction with the same outcome under putative prebiotic conditions has been established. This is based on cyanamide driving the reaction: FeS + $H_2S \rightarrow FeS_2$ (Pyrite) which then brings about the condensation of thioglycollic acid $HSCH_2CO_2H$ so as to activate the amino acid carboxy group for peptide bond formation.[594] A quite different discovery, but connected in its prebiotic relevance, concerns the finding that mixtures of amino acids can exert the catalytic activities shown by β-galactosidase, carbonic anhydrase, and catalase.[595]

Carbamylation of L-aspartic acid[596] and N-(9-acridinylthiocarbamoyl)ation of amino acids[597] have been described, the first of these studies being aimed at establishing the finer details of the biogenesis of dihydro-orotic acid, and the second providing a derivatization protocol that is some 6–22 times faster than phenylthiocarbamoylation, and giving a fluorescent product.

γ-Irradiation of amino acid solutions in the presence of the spin-trap 2-methyl-2-nitrosopropane, gives isolatable t-butylaminoxyl acids $Bu^tN(O\cdot)CHRCO_2H$.[598] The solid-state reaction of p-benzoquinone with amino acids that is accomplished by grinding the mixture, gives a purple solid (λ_{max} 562 nm) that contains free radicals different from those generated when the reactants meet in aqueous solution.[599]

Selective removal of the t-butoxycarbonyl group from N-Boc-amino acid t-butyl esters occurs on treatment with dry HCl in EtOAc; t-butyl ethers also survive the process.[600]

Oxidation of amino acids by peroxomonosulfate in aqueous alkali starts with electrophilic attack at nitrogen to give an imino acid through involvement of the α-CH proton.[601] There are, as usual, numerous papers covering routine oxidation studies of amino acids in the current literature, representative examples dealing with kinetics of chromium(VI)/$HClO_4$ oxidation of alanine, valine, and phenylalanine,[602] and electrolytic oxidation of methionine to its sulfoxide[603] (this study employs carbon, platinum or gold electrodes modified with Langmuir-Blodgett films of stearic acid or N-stearoyl-L-valine, and in the latter case, faster oxidation of the D-enantiomer was observed).

Enhanced colour for the ninhydrin reaction has been reported for 5-arylninhy-

drins.[604] That the enolate ion of Ruhemann's Purple within two five-membered rings of partial anti-aromatic character (as seen in the cyclopentadienyl anion) is the chromophore responsible for the long-wavelength absorption feature is backed up by molecular orbital calculations.[605]

Esterification studies that carry special interest include benzyl ester formation accompanying N-benzyloxycarbonylation, resulting from reaction of excess Z-Cl with α-isopropyl- and α-vinyl-α-amino acids.[606] Also, the formation of aryl esters from N-phenylacetylglycine in the standard fashion (a phenol + dicyclohexylcarbodi-imide + py/TsOH) involves low yields,[607] which can be overcome through an indirect route *via* N-Boc-N-phenylacetylglycine, through esterification followed by Boc removal. N-Boc-Amino acid chloromethyl esters have been prepared using chlorosulfonylmethyl chloride $ClSO_2OCH_2Cl$.[608] The BOP reagent, although little used now in peptide synthesis after its replacement with safer alternatives, is advocated for mild esterification of N- and side-chain protected amino acids.[609] Dicyclohexylcarbodi-imide esterification (catalysed by DMAP) to couple syn-phenylisoserine to baccatin III to form taxol can be effected starting with the derivatized anti-compound.[610] Lipase-catalysed regiospecific esterification of the primary hydroxy group of butyl α-D-glucopyranoside to 2,2,2-trichloroethyl N-Boc-4-aminobutyrate has been reported;[611] lack of space precludes mention of further routine papers covering enzymic esterification of amino acids that are in the current literature.

Ester cleavage from N-acylated amino acid benzyl esters can be achieved using N-bromosuccinimide,[612] or lithium iodide in aprotic non-polar solvents.[613] Lithium iodide also cleaves methyl and tert-butyl esters. Studies of enantioselective hydrolysis of N-protected DL-amino acid esters show no signs of slackening. Remarkably large rate enhancements for the hydrolysis of L-isomers are achieved with careful optimization of salt concentration {[KCl] = 0.03M for the N-dodecanoyl-DL-phenylalanine p-nitrophenyl ester/Z-L-Phe-L-His-L-Leu-OH/di-tetradecyldimethylammonium bromide chiral micelle system}.[614] Similar studies exploring other chiral species have involved (2S)-N-benzyl-N-(long-chain alkyl)-β-aminoalkanols/Cu(II), Zn(II), or Co(II) salts[615] and N-dodecyl-NN-dimethyl 1-octadecylammonium bromide vesicles carrying chiral amine groupings and metal salts.[616] A more conventional approach is represented in subtilisin-mediated transesterification of Z-DL-Ala-ONP with 1-butanol.[617]

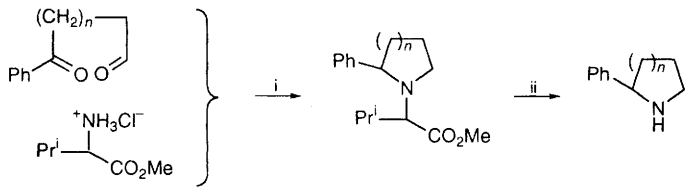
Reduction of the carboxy group of an N-protected α-amino acid to the primary alcohol function ($-CO_2H \rightarrow -CH_2OH$) is occasionally accomplished in a roundabout way, e.g. Boc-L-Val-OMe + RMgBr followed by 1%HF in MeCN,[618] but $BH_3.SMe_2$[619,620] or $LiAlH_4$ reduction[621–623] is straightforward. These last two papers describe the application of Swern oxidation to the alkanols, to give the corresponding aldehydes (see also Ref.624; for the use of $py.SO_3$, see Ref.619), while the conversion of the primary alkanol into iodomethyl (PPh_3/I_2) permits cyclization and further functional group transformations to be performed leading to 3-carboxycyclopentylamines;[625] the homochiral α-aminoalkanals are increasingly valuable in broad areas of organic synthesis, and may be secured in high yield by $LiAl(OBu^t)_3H$ reduction of either Boc-L-amino acid phenyl esters[626] (or the methyl ester of a protected arginine),[627] or Boc-L-amino acid

mixed anhydrides.[628] DIBALH Reduction of diethyl L-aspartic and glutamic acids to aldehydes is α-selective, and if conducted in the presence of a lithium trialkylphosphonoacetate, leads to N-protected γ-amino-αβ-unsaturated dicarboxylic acid esters.[629] Reduction of benzyl aspartates and elaboration of the alkanols to enantiomerically-pure 3-amino and 3,4-diaminoalkanols has been described;[630] conversion of α-(N,N-dibenzylamino)aldehydes into nitriles (-CHO → $-CH_2CN$) and ensuing alkylation and reduction gives 1,3-di-amines.[631] The reduction of the equivalent N-urethane-protected carboxyanhydrides by DIBALH or lithium tris[(3-ethyl-3-pentyl)oxy]aluminium hydride gives high yields without racemization[632] (sodium borohydride reduction gives the protected β-aminoalkanols[633]). Reduction of the Weinreb amide with lithium aluminium hydride gives the aldehyde [$-CO_2H$ → -CONMe(OMe) → -CHO], and ensuing reductive amination with an amino acid ester has been described (→ $-CH_2NHCH_2CO_2Me$),[634] though others have found this last step problematical.[635]

Dissolving metal reduction (Na/refluxing propan-1-ol) of α,α-disubstituted amino acid amides is a new method of obtaining β-aminoalkanols.[636] Conversion of the carboxy group into the acid fluoride is straightforward with NN-bis(alkoxycarbonyl)-L-α-amino acids, without racemization, using cyanuric fluoride; the same starting materials give Boc-N-carboxyanhydrides with the Vilsmeier reagent ($SOCl_2$/DMF)[637,638] Fmoc-amino acid chlorides are reduced [$Bu_3SnH/Pd(PPh_3)_4$] to corresponding aldehydes in rather low yields.[635] Conversion of the carboxy group of L-tryptophan into ketones *via* the Weinreb amide [→ -CONMe(OMe) → $-COCH_2P(O)(OMe)_2$ → -CH = CHAr etc],[639] or the one-step Weinreb-amide-to-vinyl-ketone route using allyl magnesium bromide,[640] conversion of a methyl ketone, formed from a protected phenylalanine in this way, into an isopropyl group,[641] and clean decarbonylation of an α-alkylpipecolic acid using diphenylphosphoroazidate,[642] illustrate further functional group manipulations.

Cyclization of β-aminoalkanols with Ph_2POCl gives N-diphenylphosphinoyl aziridines.[643] Reduction and cyclization of vinylglycine to the oxazolidinone, then N-allylation, can be accomplished in a one-pot process.[644]

The generation of heterocyclization products from N-acylated amino acids continues to attract mechanistic and synthetic interest, focussing on 2-alkoxyoxazol-5(4H)-ones formed from N-alkoxycarbonyl-L-amino acid mixed anhydrides and isopropenyl chloroformate[645] and from analogous symmetrical anhydrides on treatment with NN′-di-isopropylcarbodi-imide.[646] Ureas, e.g. Boc-L-Ala-L-N(Boc)CHMeCON(^{i}Pr)CONH^{i}Pr, are also formed in this process by rearrangement of the symmetric anhydride to the N(Boc)-dipeptide followed by addition to the carbodi-imide. The generation of a mixture of N-acylisourea, symmetrical anhydride and oxazolone through reaction of an N-protected amino acid with a carbodi-imide does not involve significant racemization, which becomes more pronounced when an amine is introduced (as in the normal course of peptide synthesis). As this process continues, it is aminolysis of the anhydride initially, and of the oxazolone at later stages, that introduces racemized products.[647] N-Methylamino acid esters react *via* an oxazolone and/or symmetrical anhydride

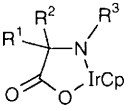


Reagents: i, $NaBH_3CN$ or $NaBH(OAc)_3$; ii, Bu^tOK, and Curtius rearrangement

Scheme 30

Reagents: i, TFAA, Py, 100 °C

Scheme 31



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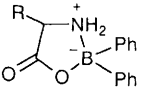

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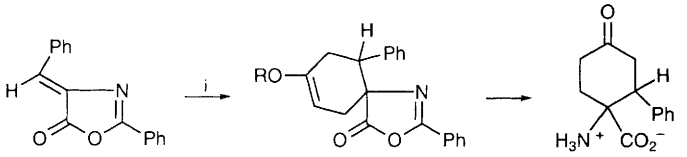


Reagents: i, $CH_2{=}C(OMe)C{=}CH_2$, etc.

Scheme 32

in giving acyloxyphosphonium salts with the currently popular phosphonium peptide-bond-forming reagents PyBrOP and PyClOP (PyBOP reacts sluggishly in this process).[648]

N-Acyl-N-benzylamino acids yield 5-trifluoromethyloxazoles when treated with TFAA/py in benzene (Scheme 31),[649] while N-2-hydroxybenzyl analogues yield tricyclic benzoxazolines.[650] Equilibrium mixtures of thiazolinones, phenylthiocarbamoylamino acids and N-phenylthiohydantoins (PTHs) formed in the Edman peptide sequencing process can be converted into thiazolinones that are amenable to ring-opening with a fluorescent amine.[651] Other interesting five-membered heterocycles include (95), formed from an amino acid and $[Cp^*IrCl(\mu\text{-}Cl)_2]$, and capable of highly diastereoselective ligand complexation[652] and (96) formed from amino acids and diphenylborinic acid (*cf.* Vol.26, p.62).[653]

1,3-Dipolar cycloadditions of amino acid-derived imines illustrated in previous Volumes of this series (see e.g., Vol.23, p.55) continue to provide highly-substituted five-membered heterocycles. Thus, decarboxylation of imines formed from amino acids and alloxan or 1-phenyl-3-methylpyrazolin-4,5-dione gives azomethine ylides that add to maleimides.[654] Highly-substituted homochiral pyrrolidines are formed between N-acryloyl-L-proline benzyl ester and $R^1CH{=}N^+(Li)R^2C^-CO_2Me$.[655] Formation of 3-(3,6-dioxopiperazin-2-yl)propanoic acid from γ-methyl glutamate and glycine ethyl ester[656] exemplifies a familiar six-membered heterocyclic amino acid condensation product, while a more unusual ring-enlargement process (see Vol.25, p.69) accompanies photolysis of N-phthaloyl-L-DOPA methyl ester protected in its side-chain by 3′,4′-methylenation (corresponding treatment of N-phthaloylthreonine and serine methyl esters gives phthaloylglycine through β-fragmentation).[657]

6.3 Specific Reactions of Amino Acids – The conventional use of this section over the years continues in this Volume, covering papers that concentrate mainly on reactions at the side-chain of α-amino acids. By their nature, these processes often amount to the use of one amino acid to synthesize another, and some papers that could have been located here can be found in the earlier synthesis Sections 4.1–4.15.

Side-chain halogenation of aliphatic L-amino acid esters (as their N-phthaloyl derivatives) is stereoselective (particularly so with t-butyl esters), giving (2S,3R)-β-hydroxyphenylalanine after substitution of the bromo-substituent that was introduced using NBS,[658] and 4-bromo-L-glutamates (stereoselectivity not investigated, but the diastereoisomer mixture was easily separated).[659] Fluorination of α-fluoromethyltyrosine with acetyl hypofluorite gives the β-fluoro-analogue.[660] ω-Iodoalkenylglycines $CH_2{=}ClCH_2CH(NHAc)CO_2Et$ undergo Michael-type chain extension with $CH_2{=}CHCO_2Et/Pd(OAc)_2$ to give $EtO_2CCH{=}CHCH{=}CHCH_2CH(NHAc)CO_2Et$.[661] The corresponding reaction occurs with electrophiles, e.g. $(E)\text{-}ICH{=}CHCO_2Et$ with $CH_2{=}C(SnBu_3)CH_2CH(NHAc)CO_2Et$.[662] The organozinc synthon formed from L-glutamic acid (side-chain $-CO_2H \rightarrow -ZnI$) undergoes Pd-catalysed condensation with an aryl iodide to give enantiomerically-pure homophenylalanines,[663]

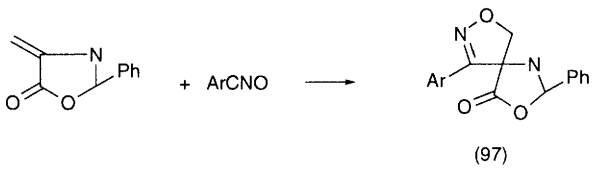
and the equivalent serine-derived synthon[664] reacts with acryloyl chloride, followed either by cyclization to give 4-oxopipecolic acid, or by addition of benzylamine to give 4-oxolysine.[665] Photo-activatable moieties have been added to the isopropenyl and carboxy side-chains of (-)-kainic acid.[666]

Diels-Alder additions of buta-1,3-diene[667] and of Danishefsky's diene [or (E)-PhCH = C(CN)CO_2Me or 2-methoxybuta-1,3-diene][668] to the 2-phenyloxazolone derived from αβ-dehydrophenylalanine (Scheme 32) gives 1-aminocyclohexene-carboxylic acids which can be categorized as conformationally-constrained analogues of common α-amino acids. A heterocyclic version (97) arises by 1,3-dipolar addition of 2,6-dichlorobenzonitrile oxide to 4-methyleneoxazolidin-5-one.[669] Methylenation of the D-glyceraldehyde-derived Z-oxazolone gives a mixture of five cyclopropanes, with the (1S,2R)-derivative as predominant product.[670]

Conventional modifications to the alicyclic moiety of alicyclic α-amino acids include ethylene biosynthesis from 1-aminocyclopropanecarboxylic acid[671] and hydrogenolytic ring-opening of aziridinecarboxylates (98).[672] Studies of saturated heterocyclic imino acids include stereoselective additions of alkylcopper reagents to cyclic acyliminium ions formed from pipecolic acid,[673] and a remarkable ring-expansion (Scheme 33) of 5- and 6-membered members of this class to 8- and 9-membered homologues through the reaction with acetylenic dipolarophiles of azomethine ylides formed with formaldehyde.[674]

Oxidation of urethane-protected L-proline methyl ester to L-pyroglutamic acid with iodosylbenzene/trimethylsilylazide/CH_2Cl_2 also causes 5-substitution (insertion of N_3, Cl, or OH; other substituents and reaction conditions can alter the pattern of reactions).[675] Electrochemical oxidation of L-proline followed by methylcopper addition to give trans-5-methyl-L-proline.[676] 3,4-Dehydro-L-proline (from hydroxy-L-proline) serves as starting material in a route to (2S,3R,4S)-epoxyproline (m-chloroperbenzoic acid) accompanied by its diastereoisomer.[677] The epoxides give an 8.3:1-mixture of 3-methyl-4-hydroxy- and 4-methyl-3-hydroxyprolines through $LiCuMe_2$ ring-opening.[678] Regio- and stereoselective hydroxylation of the enolate of 4-oxoproline is a stage in a route to swainsonine [(2R,3S,4R)-3,4-dihydroxy-2-hydroxymethylpyrrolidine].[679] Hydroxy-L-proline is also the starting point (conversion into its cis-isomer) in a synthesis of 'de(hydroxymethyl)desulfo-analogues' of the O-sulfonated glycopeptides, bulgecins A, B, and C.[680]

Aromatic side-chain modifications that have been accomplished include the preparation of Boc-L-(4-carboxy)phenylalanine from the corresponding tyrosine O-triflate,[681] preparation of O-glycosylated Fmoc-L-tyrosine pentafluorophenyl esters,[682] anodic oxidation of 3,5-dibromotyrosine methyl ester to generate caremicolin models (99),[683] anodic oxidation or thallium(III) nitrate oxidation and zinc reduction to give isodityrosine and dityrosine derivatives.[684] Electro-oxidation[685] and cyclic voltammetric monitoring of the oxidation[686] of L-DOPA, elaboration of the phenolic side-chain and transesterification of tyrosine, giving L-DOPA esters by combined tyrosinase and α-chymotrypsin treatment,[687] are also reported. 5-S-CysteinylDOPA undergoes oxidation under physiological conditions to give pheomelanins *via* 1,4-benzothiazines.[688] Azide anion radical



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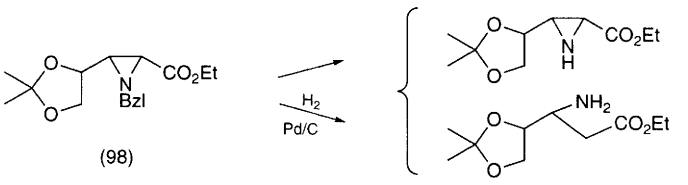

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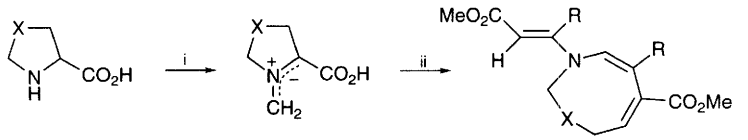
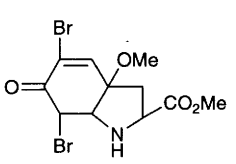


Reagents: i, HCHO; ii, $RC{\equiv}CCO_2Me$

Scheme 33



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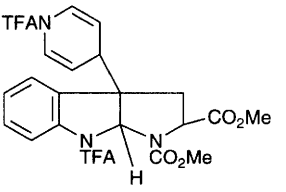

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attack on tyrosine generates the oxygen-centred phenoxide radical, and the eventual reaction product is di-m-tyrosine.[689]

Generation of a fluorescent species (λ_{excit} 320 nm, λ_{em} 392nm) by treatment of tryptophan with nitrous acid has been reported, without speculation or evidence for its structure.[690] X-Ray analysis[691] supports the structure (100) assigned to an N^{im}-trifluoroacetylated reaction product from the reaction of N-methoxycarbonyl-L-tryptophan methyl ester with TFA and pyridine; the minor reaction product (6%)[692] is the all-cis isomer (101; R = H) accompanying 84% of the isomer with inverted ring junction protons, which is the product expected on the basis of existing knowledge of tryptophan cyclic tautomers. Thus, the well-established N^{im}-toluene-p-sulfonyl analogue, which undergoes a highly diastereoselective aldol addition to benzaldehyde after deprotonation with LDA,[693] has appeared in several papers recently (see also Ref.268). The diastereoselectivity of Pictet-Spengler processing of tryptophan esters (RCHO + H_2N- → imine → carbolines) depends on the ester alkyl group.[694] (2-Hydroxyethylthio)-substitution at positions 2 and 7 of the indole moiety of tryptophan by treatment with $Hg(OAc)_2$ followed by mercaptoethanol explains a side-reaction observed during peptide synthesis.[695]

Histidine chemistry described in the current literature covers a 1:1:1-adduct involving the imidazole moiety of the N^{α}-acetylamino acid, with malondialdehyde and an alkanal (but no reaction in the absence of the alkanal),[696] formation of a Michael-type adduct N-Z-1(3)-(1′-formylmethyl)hexyl-L-histidine as a model for attack on proteins by lipid breakdown products,[697] and β-attack by hydroquinone on a protected histidine to give the β-(2,3-dihydroxyphenyl) homologue.[698] S-[2-Carboxy-1-(1H-imidazol-4-yl)ethyl]-3-thiolactic acid is a new histidine metabolite isolated from urine; it has been synthesized from the cysteine adduct through HNO_2 de-amination.[699]

Arndt-Eistert homologation of (2S,3S)-3-methylaspartic acid giving (2S,3R)-3-methylglutamic acid competes favourably with a bis(lactim ether) synthesis.[117] Rapoport-type alkylation of aspartic acid-derived enolates gives syn- or anti-2,3-pyrrolidinedicarboxylic acids,[700] also independently prepared in essentially the same way;[701] the same conformationally-constrained aspartic acid analogues have been synthesized from the glutamic acid-derived synthon (102).[702] α-tert-Butyl-γ-methyl N-Z-glutamate gives the γ-anion with lithium hexamethyldisilazide, which is a convenient source of γ-substituted glutamic acids through reaction with electrophiles.[703] The protected N-hydroxyornithine analogue $FmocNHCH(CO_2H)CH_2CH_2CHRNHOCH_2CH_2SiMe_3$ emerges from a synthesis starting with L-glutamic acid (side-chain $-CO_2H$ → -CH=NOTBDMS) and cyclized *via* the N-hydroxysuccinimide ester to give $N^{\alpha}N^{\delta}$-protected N^{δ}-hydroxycyclo-ornithine and its homologue.[704] 2-Indolylmethyl ketone formation through the side-chain carboxy group of aspartic acid provides the novel sweet compound monatin after stereospecific conversion of the side-chain carbonyl group into $-C(OH)(CO_2H)-$.[705]

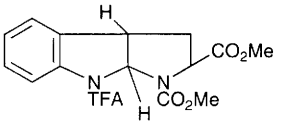
Protected L-pyroglutamic acid continues to be one of the most frequently-used chiral synthons in general organic chemistry, and no less so as starting material for the preparation of other amino acids. 4,4-Di-substitution can be accomplished

after LiHDMA de-protonation,[706] 4-Methylenation and -cyclopropanation have been described, either *via* the 4-(dimethylaminomethyl)pyroglutamate formed from the lithium enolate of a pyroglutamate and Eschenmoser's salt, or through an imidazolidinone synthesis (see Section 4.2; introduction of a $BuO_2CC(=CH_2)CH_2$- group).[707] Independently, the same route has been followed by other workers, but with some different minor details.[708] Reduction of the lactam carbonyl group of pyroglutamates to $-CH_2-$, leaving other reducible functions intact, proceeds *via* the hemiaminal (successively, $LiEt_3BH$ and $SiEt_3H/BF_3.Et_2O$).[709] The hemiaminal was reacted with stabilized phosphonates to give products of Wittig synthesis that were isolated as 5-substituted prolines (103 → 104; trans:cis = 5:1) through cyclization of the intermediate alkene.[710] L-Pyroglutaminol methoxymethyl ether has been used to synthesize (+)-1,8-di-epi- and (-)-1-episwainsonine through construction of a piperidine ring on NH and ether functions, and manipulation of the pyrrolidine functional groups.[711]

Pyroglutamic acid-derived synthons used in large-scale synthetic enterprises include (105; manzamine A);[712] and (106 and its epoxide; natural polyhydroxylated pyrrolidines).[713] The L-pyroglutamate-derived synthon (106) can be subjected to stereoselective epoxidation to give (2S,3S)-3-hydroxyproline from which ($-CO_2H$ → $-CH_2OH$) castanodiol can be obtained,[714] and (2S,3S)-3-methylproline and (2S,3R)-3-phenylproline were prepared similarly.[715] Pyroglutaminyl chloride (from oxalyl chloride and TMS-pyroGlu)[716] and its N-Fmoc derivative (from Fmoc-L-glutamic acid *via* the dichloride formed using $SOCl_2$) have been obtained.[717]

N^{β}-Glycosylated asparagines may be prepared by reaction of Fmoc-L-α-aspartic esters with a glycosyl azide under the influence of Et_3P/CH_2Cl_2.[718] N^{β}-Aralkyl-protected asparagines and glutamines can be cleaved by boron tris(trifluoroacetate) in TFA/AcOH.[719] A route to aspartic acid β-semi-aldehyde, based on ozonolysis of a protected allylglycine, avoids less satisfactory steps in routes from aspartic acid itself.[720] Low yields when reducing the acid chloride (30%) were encountered, however, in a route ($-CO_2H$ → $-COCl$ → $-CHO$) from α-methyl Z-L-aspartate, but could be improved when the reduction was performed in the presence of palladium.[721] Development of the N-acryloyl compound into the conformationally-constrained α-amino acid, 6-oxodecahydroisoquinoline-3-carboxylic acid, is described in this study.[722] The aldehyde was isolated as the dimethyl acetal, a device also used in a related study using glutamic acid γ-semialdehyde,[723] which also features in a synthesis of differentially protected meso-2,6-diaminopimelic acid.[724] Appropriate protection was involved in all these studies, and in the latter route, manipulation of the side chain carboxy group (Wittig homologation etc) of the oxazolidinone was used.

Formation of methyl ethers from protected serines and threonines[725] and benzyl ethers,[726] including benzylation providing precursors to photoactivatable side-chains,[727] follow standard phase transfer alkylation procedures. Routes to 2-acetamido-2-deoxy-β-D-glycosides,[728] corresponding 2-acetamido-galactosides,[729] galactosides,[730] and glycotetraoses[731] have been explored. A cumbersome route from ε-hydroxy-L-norleucine gives homochiral 3-amino-7-substituted azepinones.[732] (2R,3S,4R)-3,4-dihydroxy-2-hydroxymethylpyrrolidine and



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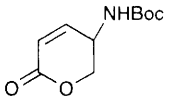

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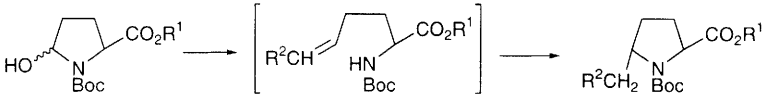

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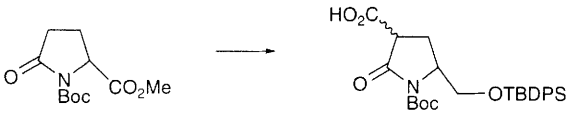

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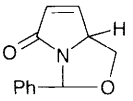

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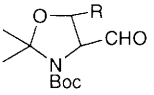

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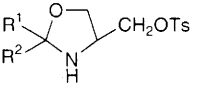
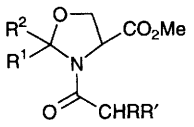


(108)

(2S,3R,4S,5S)-3,4-dihydroxy-2,5-dihydroxymethylpyrrolidine have been prepared from D-serine and D-ribonolactone, respectively.[733]

A number of synthetic opportunities follow from the juxtaposition of amino, carboxy and primary alcohol functions in serine, e.g. a lengthy synthesis of Fmoc-γγ-di-t-butyl-γ-carboxy-L-glutamic acid from D-serine.[734] Here, the carboxy group of the starting material becomes the extended side-chain of the product *via* an aldehyde, and the $-CH_2OH$ side-chain becomes the eventual carboxy group, accounting for the 'L-from-D' nature of the process. The synthetic uses of L-serinal derivatives are proliferating, and two papers report improved syntheses of a protected form, the Garner aldehyde (107; R = H), one route[735] avoiding the need for methyl iodide and benzene and the other, also explored for the threonine homologue (107; R = Me) using an $LiBH_4$ and $COCl_2$/DMSO sequence for the initial stages.[736] D-Threonine gives the corresponding synthon through standard steps.[737] β-Branched α-amino acids have been obtained through the sequence $-CHO \rightarrow -CH(OH)C{\equiv}CH \rightarrow$ bromoallenes.[738] A new electrophilic L-alaninol synthon (108), prepared from L-serine, undergoes nucleophilic substitution by Gilman cuprates or Grignard reagent/CuX complexes.[739] The related N-acylated synthon derived from L-serine (109; R^1 = Bu^t, R^2 = H) gives bicyclic Dieckmann products leading to useful homochiral tetramic acids.[740] N-Z-L-Serine β-lactone reacts with trimethylsilylamines Me_3SiNR_2 primarily by alkyl oxygen cleavage, to give optically-pure β-aminoalanines (certain reaction conditions cause acyl-oxygen cleavage leading to serinamides).[741] The chiral oxazolidinone (110), formed from serine, threonine or cysteine (S in place of ring O) using bis(trichloroethyl) carbonate[742] gives N-allyl derivatives that undergo intramolecular oxime–alkene cycloaddition, to give pyrrolidines.[743] Other ring-closure reactions involving serine include samarium(II) iodide-mediated cyclization of N-allyl-[744] and propargyl-serinals[745] to give 2,3,4-trisubstituted pyrrolidines (111). Access to homochiral 5-alkylpiperazine-2-carboxylic acids (112) is initiated by condensation of L-serine with an α-amino acid.[746]

(ω-Aminoalkyl)-α-amino acids, notably lysine, are also represented in broader organic synthesis, and the side-chain function has been developed into diazoacetamides $L\text{-}N_2CHCONH(CH_2)_4CH(NH_2)CO_2Et$ that have been added to C_{60}-fullerenes to give a [60]fullerene-fused cyclopropane carrying an amino acid structure.[747] Simpler side-chain modifications are illustrated by the preparation of N^{ε}-(carboxymethyl)-L-lysine and N^{δ}-(carboxymethyl)-L-ornithine through N^{δ}-(carboxyethyl)-L-ornithine synthase-mediated reductive condensation with glyoxylate,[748] and conventional preparations of N^{ε}-Fmoc-L-lysine and N^{δ}-Fmoc-L-ornithine from copper(II) complexes of the amino acids.[749] A more involved sequence (successively, sodium nitroprusside, CBr_4/PPh_3, p-bromoaniline) leads to N^{α}-Z-N^{ε}-(p-bromophenyl)-L-lysine [similarly, to N^{τ}-(p-bromophenyl)-L-histidine from Z-histidine).[750] The ε-amino group of N^{α}-Z-L-lysine methyl ester has been converted (dimethyldioxirane in acetone) into the nitrone [$-NH_2 \rightarrow -N^+(O^-){=}CMe_2$] from which ($H^+$ then Ac_2O) N^{α}-Z-N^{ε}-acetoxy-L-lysine methyl ester was secured.[751] There may be useful analytical applications associated with the finding that the ninhydrin–Fe(III) system reacts specifically



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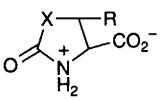

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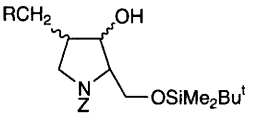

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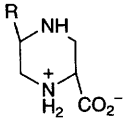

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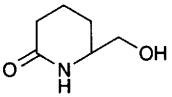

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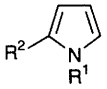

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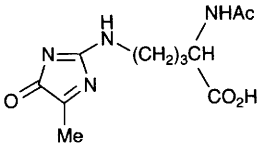

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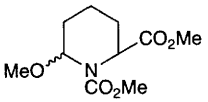

(116)

with lysine at pH 1 (but not with ornithine, arginine, histidine, proline or glycine.[752]

Reactions of the side-chain amino group of lysine, some of them modelling *in vivo* protein behaviour, have been studied. A useful synthon (113) for the synthesis of substituted piperidines, has been prepared in five steps from L-lysine using conventional precedents.[753] With 4,5-epoxy-Z(E)-heptenal (a lipid peroxidation product), lysine gives the pyrroles [114; R^1 = $CH(CO_2H)(CH_2)_4NH_2$ or $(CH_2)_4CH(NH_2)CO_2H$; R^2 = H, CH(OH)Et)] together with the isomeric compounds formed through the α-amino group.[754] With methylglyoxal, reversible glycosylation occurs through conversion of the initially-formed imine into unknown oligomers.[755] The process is irreversible with arginine, giving 4,5-dihydroxy-5-methylimidazolines (115), the imidazolin-4(5)-ones derived from these showing fluorescence (λ_{excit} 320 nm, λ_{em} 398 nm). L-Lysine has been converted into a protected L-α-amino-ε-mercaptohexanoic acid *via* a pyridinium analogue.[756] Anodic oxidation of di-N-methoxycarbonyl-L-lysine methyl ester in methanol gave the α-methoxyoxazolidinone (116) whose stereoselective azidolysis and reduction have provided the first example of an optically-pure α-aminoamine.[757]

Efficient access has been worked out, to N^G-methyl-D- and -L-arginine from the ornithines and $MeNHC(SMe)=NH_2^+$ I^-,[758] and further study of preparations of side-chain protected arginine, either the long way round [synthesis of ω,ω'-bis(urethane)s by N^δ-guanylation of ornithine with bis(urethane)protected 1-guanylpyrazoles],[759] or directly by ω-arenesulfonylation.[760] In the latter study, no improvement on a currently-used protecting group of this family, the Mtr-group, was achieved by the introduction of electron-donating alkyl groups into the aryl moiety. Electro-oxidation of arginine at Pt electrodes liberates nitrate ion through an electron transfer mechanism.[761] This is an interesting result, because it shows that inorganic nitrogen oxides can be released from arginine without the presence of nitric oxide synthase; the bustling activity in the nitric oxide area is illustrated by a synthesis of the NO synthase inhibitor N^ω-hydroxy-N^ω-methyl-L-arginine in 8 steps from N^G-Z-L-arginine.[762]

Tetrahydropyrimidines have been formed from reaction of 2,4-diaminobutyric acid with other amino acids,[763] and related tetrahydropyrimidin-4(1H)-ones have been identified as self-degradation products of bellenamine (R)-$H_2N(CH_2)_3CH(NH_2)CH_2CONHCH_2NH_2$, a β-lysinamide from *Streptococcus nashvillensis* that shows useful immuno-enhancing HIV protease inhibitory action. The formaldehyde needed to generate the reaction products is supplied by hydrolytic cleavage of the $-NHCH_2NH_2$ moiety of the natural product.[764] Methyl 4,5-diaminopentanoate cyclizes to 5-aminomethylpyrrolidinone and 5-aminopiperidinone in dilute aqueous alkali.[765]

The thiol function of cysteine shows important oxidation reactions, illustrated with a kinetic study of photo-oxidation (H_2O_2) of N-acetylcysteine[766] sensitized by N-(9-methylpurin-6-yl)pyridinium salts,[767] and one-electron oxidation by the azide radical anion at pH 10.5 leading to intramolecular proton abstraction and subsequent processes.[768] S-Arenesulfenylation of cysteine derivatives, with the objective of placing a photoactivatable group at sulfur, has been achieved using

the appropriate pyrid-2-yl disulfide.[769] Thiolysis of S-(SCM)cysteine hydrochloride in water gives S-(alkanesulfenyl)cysteines.[770] S-Trifluoromethylation of N,C-protected cysteines has been accomplished,[771] and other S-protection strategies are featured in the current literature: (novel) S-(allyloxycarbonylaminomethyl)ation,[772] and (traditional) benzylation with improved methods for introduction through benzyl cations or $ArCHO/Et_3SiH$.[773] Cyclic ketimine formation from (2-aminoethyl)-L-cysteine mediated by snake venom L-amino acid oxidase[774] parallels the process observed with the cystathionine de-amination product, S-(2-oxo-2-carboxyethyl)-L-homocysteine.[775] The ketimine readily undergoes autoxidation to the sulfoxide.[776] Nitric oxide is released from S-nitrosocysteine under physiological conditions, and there have been several recent studies of the consequences of this, one being the destruction of zinc-sulfur clusters in proteins.[777]

Studies of methionine and other S-alkyl cysteines reflect similar themes. Slow oxidation of S-(2-propenyl)cysteine and its sulfoxide by aqueous nitric oxide,[778] one- and two-electron oxidation of methionine by peroxynitrite HOONO *via* the radical HOONO· (leading first to the sulfoxide and ultimately to the liberation of ethylene),[779] and 2,2'-bipyridinium chlorochromate oxidation of methionine, established by a kinetic study to involve a sulfurane transition state.[780] N-Phthaloyl-L-methionine ethyl ester undergoes the expected reaction with SO_2Cl_2 to give a α-chlorosulfide mixture that yields the aldehyde-containing side-chain on hydrolysis (-CHClSMe → -CHO).[781] S-Phenyl-L-cysteine is best oxidized to the sulfone with magnesium monoperoxyphthalate, giving a versatile synthon for preparations of (S)- or (R)-cycloalkylglycines and prolines and analogues.[782] Facile alkylation α to the sulfone function with stereocontrol is also the basis of the use of the same synthon for syntheses of (2S,3S)- and (2R,3R)-pyrrolidine-2,3-dicarboxylic acids.[783]

6.4 Effects of Electromagnetic Radiation on Amino Acids – The topics covered in this section over the years continue to surface in the literature, which has provided acounts of γ-radiolysis of aqueous tyrosine[784] and 3,5-diiodotyrosine.[785] Photo-oxidation of 5-S-cysteinylDOPA at wavelengths longer than 320 nm gives benzothiazines, whereas cleavage of the aliphatic moiety to yield DOPA is the consequence of irradiation at 280–320 nm.[786] Time-resolved fluorescence of the protein cross-linking amino acid, dityrosine, has been evaluated.[787]

As usual, most of the papers concern tryptophan and its analogues, with investigations of products of photolysis of tryptophan in aqueous solutions,[788] and of quenching of singlet oxygen by aqueous tryptophan (comparisons with tyrosine, histidine, methionine and cysteine were also included in this study).[789] Fluorescence decay studies focus on constrained tryptophan analogues, e.g. 3-amino-1,2,3,4-tetrahydrocarbazole-3-carboxylic acid,[790] and on a new reference compound with an ultra-short fluorescence lifetime.[791] Supersonic gas expansion studies of tryptophan and substituted analogues permit the allocation of fluorescence lifetimes to individual conformers.[792] Phosphorescence decay of tryptophan involves energy transfer between individual molecules in the triplet excited

state.[793] Low-temperature UV photolysis of tryptophan yields stable products *via* anion and cation radicals, the products surviving warming to room temperature; they show themselves to be efficient luminescence quenchers.[794]

7 Analytical Methods

7.1 Introduction – Several useful reviews of methods of amino acid analysis have appeared, e.g. analysis of dansylamino acids.[795]

7.2 Gas-Liquid Chromatography – The importance of sample clean-up prior to derivatization and GLC analysis of amino acids has been stressed.[796] Derivatization incorporating an extractive alkylation of amino acids with pentafluorobenzyl bromide followed by N-heptafluorobutyroylation illustrates the greater attention being paid to sample authenticity, prior to GLC analysis incorporating negative ion CI-MS.[797] Similar derivatization approaches employ N(S)-isopropoxycarbonyl methyl esters (sulfur-containing amino acids),[798] and N(O)-isobutoxycarbonyl t-butyldimethylsilyl esters.[799] Alkyl chloroformates can provide a one-step derivatization procedure for amino acids in aqueous solution, to give N-alkoxycarbonylamino acid alkyl esters (the alkyl ester group derives from the breakdown of the chloroformate, but an alcohol is usually added in the reagent cocktail, to ensure complete reaction).[800] Use of a different alcohol, of course, gives more flexibility but also more complications, since ethyl esters are also formed when the $EtOCOCl/CF_3CH_2OH$/pyridine reagent is used.[801]

The stable isotope dilution technique has been employed in the otherwise standard GC-MS analysis of cysteic and homocysteic acids, and cysteinesulfinic and homocysteinesulfinic acids.[802]

Enantiomer separation by GLC has been reviewed.[803] Chiral GLC analysis of N-trifluoroacetylamino acid methyl esters on a 2,6-di-O-butyl-3-O-trifluoroacetyl-γ-cyclodextrin capillary column,[804] and of N-trifluoroacetylamino acid isopropyl esters with Chirasil-L-Val as stationary phase[805] has been accomplished.

7.3 Thin-Layer Chromatography – Routine they may be, but several projects have been reported recently that are useful; comparisons of cellulose with silica gel for their performance in quantitative TLC,[806] a study of resolution of DL-amino acids on borate-gelled guaran-impregnated silica gel[807] and on copper(II)-L-proline-impregnated silica gel[808] from the point of view of the analytical resolution of DL-amino acids. Careful attention to detail is rewarded by reproducible quantitative TLC of lysine, threonine and homoserine.[809]

The preceding studies base their results on conventional ninhydrin colour-formation, though other spray reagents (p-dichlorodicyanobenzoquinone, applicable down to 0.1 μg,[810] and 4-dimethylaminobenzaldehyde (for quantitative TLC of tryptophan)[811] continue to be advocated.

New solvent systems $py\text{-}C_6H_6$ = 2:5:20, $MeOH\text{-}CCl_4$ = 1:20, and $acetone\text{-}CH_2Cl_2$ = 0.3:8, have been suggested for the TLC of PTHs.[812]

7.4 High Performance Liquid Chromatography – Not all the papers cited here deal simply with analytical studies; the chemistry of stationary phases and of derivatization protocols is frequently involved in the reports.

Reviews have appeared of amino acid derivatization,[813] fluorogenic labelling,[814] and derivatization for enantiomeric analysis.[815]

Nearly all HPLC analysis protocols for amino acids call for pre-column derivatization, usually the formation of a N-substituted amino acid mixture from the sample, prior to chromatographic separation and quantitation of the individual components. One commonly-used derivatization protocol is N-phenylthiocarbamoylation (PTC; for a review, see Ref.816), for amino acids in general[817] and arogenic acid (the biogenetic precursor in plants of phenylalanine and tyrosine) in particular,[818] for aspartic and glutamic acids and asparagine,[819] and hydrolysates of proteinaceous material in pollen[820] and in old paintings.[821] Use of the analogous N-butylthiocarbamoylamino acids seems to have no extra justification, though they show excellent chemical stability.[822] 4-(3-Pyridinylmethylaminocarboxypropyl)phenylthiohydantoins, the cyclization-rearrangement products of the correspondingly-substituted PTC-amino acids, have been studied by HPLC-electrospray MS.[823] Conditions for HPLC analysis of PTH mixtures, avoiding reagent-related background peaks, have been established.[824]

N-(Fluoren-9-ylmethoxycarbonyl)amino acids (Fmoc-amino acids) are gradually gaining acceptance for HPLC analysis,[825] reactions following derivatization being avoided by the use of heptylamine to remove excess reagent.[826] A specialized interest is represented in HPLC of Fmoc-(S)-alk(en)yl-L-cysteine sulfoxides in garlic.[827] Analysis of imino acids as Fmoc derivatives after removal of primary amines from samples by conversion into isoindoles (OPA-amino acids) using o-phthaldialdehyde and mercaptoethanol has been represented in analysis of glyphosate (N-phosphonomethylglycine),[828] and in mainstream studies of protein amino acids,[829] including special reference to automated analysis.[830] The OPA-amino acid pre-treatment procedure also allows the estimation of 4-hydroxyproline in biological fluids as its PTC-derivative.[831]

OPA-Derivatization and quantitation of the derivatives continues to be confidently used, and a total time of 17 minutes has been claimed for analysis of an amino acid mixture.[832] It has been used for L-lysine in wheat gliadin proteins,[833] for primary amino acids in rat plasma,[834] and in a rare example of post-column OPA-derivatization of amino acids separated by ion-exchange chromatography.[835] As has been mentioned frequently in the recent literature (see Vol.25, p.75) there is some uncertainty about the reliability of OPA-derivatization because of limited stability of the derivatives, and a study has shown that OPA-amino acid derivatives decompose to the extent of about 6% over 15 h (though their methyl esters reach this point of decay after 8 h).[836] The structurally-related, highly fluorescent, derivatives formed using naphthalene-2,3-dialdehyde continue to be explored.[837]

Comparison of HPLC analyses of D- and L-threo-β-methylphenylalanine by the o-phthaldialdehyde-mercaptopropionic acid method, use of Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide), and alkaline acetic anhydride procedures, as well as the use of GLC (N-trifluoroacetyl isobutyl ester) leads to no

particular recommendation.[838] N-Acetylamino acids in urine have been estimated by HPLC with MS detection,[839] whereas derivatization at the carboxy group with 9-anthryldiazomethane has been advocated for the determination of N-acetyl-amino acids released from proteins using proteases.[840] Benzoyl chloride has also been revisited for derivatization, and converts amino acids into 2-phenyl-5-benzoyloxyoxazoles, permitting analysis at the 1 pmol level with the assistance of electrospray MS monitoring.[841] Analysis of kainic acid as either N-(4-azidoben-zoyl)- or N-(4-azidoPTC)-derivatives, has been described.[842]

Dansylation[843] and dabsylation[844] provide stable derivatives and good HPLC separation can be achieved. One of these studies[843] was aimed at verifying the stability of common amino acids in 6M HCl. Analysis by electrospray MS of underivatized basic amino acids and N-hydroxylamino acids is moderately successful, but dansylation gives improved reliability.[845] Dansyl-L-phenylalanine can be detected down to approximately 5×10^{-14} mol levels.[846] 4-Hydroxyproline assay through dabsylation followed by OPA-derivatization provides reproducible results for low levels of analyte.[847]

In a comparison with PTC-derivatization, 6-aminoquinolinyl-N-hydroxysucci-nimide treatment of amino acids (giving AQCamino acids; λ_{max} 248 nm)[848] is clearly superior unless there is a minimal time delay between derivatization and HPLC.[849] Highly fluorescent asymmetric ureas are formed between amino acids and the carbamate of the AQC reagent, and HPLC analysis with their help gives results that compare well with classical ion-exchange analysis.[850] Use of a polymeric reagent carrying this carbamate has been explored.[851]

Post-column derivatization with 1,2-naphthoquinone-4-sulfonate (λ_{max} 305 nm) has been applied to samples separated by ion-pair liquid chromatography.[852]

Particular structural characteristics that allow specific analytical targetting are shown by some common amino acids. This is illustrated for cysteine and homocysteine analysis, conversion into the N-acetyl S-pyridinium derivative,[853] or into derivatives formed with monobromobinane,[854] and derivatization using 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate.[855] Non-derivatized samples (electro-chemical detection of cysteine, using a glassy carbon electrode),[856] and of S-adenosyl-L-homocysteine and S-adenosyl-L-methionine by UV detection,[857] have proved to be suitable for analysis. An interesting development for the HPLC ion-exchange analysis of underivatized amino acids exploits the chemiluminescence formed between amino acids and *in situ*-generated $Ru(bipy)_3$.[858] Protocols for HPLC of related amino acids (excitatory amino acids carrying sulfonic and sulfinic acid side-chains),[859] and selenium analogues of sulfur-containing amino acids,[860] have been worked out.

Tryptophan and its 5-hydroxy-derivative in cerebral fluid have been estimated by HPLC with electrochemical detection,[861] and through fluoresence detection (λ_{excit} 302 nm, λ_{em} 340 nm).[862]

Increasing numbers of HPLC studies of crosslinking amino acids reflect the importance of some of them as markers for metabolic disorders. Pyridinoline and its deoxy-analogue have dominated these reports,[863] which describe minor differences in protocol, one of which (use of acetylated pyridinoline as internal standard)[864] needs care so as to minimize its decomposition.[865] Estimation of

aldosine as its oxidative decarboxylation product (Fe^{3+}), 6-(3-pyridyl)piperidine-2-carboxylic acid,[866] and of desmosine and isodesmosine as dansyl derivatives.[867] Analysis of p-boronophenylalanine in tissue is an essential adjunct to studies of the uses of this amino acid in neutron capture therapy.[868]

Estimation of enantiomer ratios for amino acids is most commonly achieved now by modifications of the derivatization methods outlined above. Thus, the use of a homochiral thiol (N-acetyl-L-cysteine[869,870] or N-isobutyroyl-L- or D-cysteine[871,805]) in the OPA procedure permits the complete separation of 18 DL-amino acids. One of these papers[870] describes a dating study concentrating on aspartic acid extracted from dentin, and applicable down to 1 pmol levels. (+)-1-(9-Fluorenyl)ethyl chloroformate similarly yields diastereoisomer mixtures allowing the estimation of D/L-ratios of amino acids in crustacean nerve tissue.[872] A new fluorophore, N-[4-(6-methoxy-2-benzoxazolyl)benzoyl]-L-proline, has been introduced for the same purpose.[873] Protected amino acids for use in peptide synthesis have been derivatized with Marfey's reagent for enantiomeric purity estimations.[874]

The other main approach to HPLC determinations of enantiomer ratios involves the incorporation of chiral species into a silica gel stationary phase (a crown ether, applied to tyrosine and DOPA and analogues),[875] 3-aminopropylated silica gel acylated by 2,4-dinitrobenzoyl-(R)-(1-naphthyl)glycine for the analysis of tryptophan and aspartic acid,[876] tetra-esters of calix[4]arenes bonded to silica gel for the estimation of DL-amino acid esters[877]) and bovine serum albumin bonded to silica gel for the resolution of DL-tryptophan.[878] Pirkle-type columns have been used for enantiomeric analysis of amino acids derivatized with 4-fluoro-7-nitro- or 4-dansyl-7-fluoro-2,1,3-benzoxadiazole, the former reagent giving superior results.[879] 'Chiralcel-OD' [cellulose tris(3,5-dimethylphenyl carbamate)] gives good results for a series of 17 Fmoc-amino acids.[880]

7.5 Fluorimetric Analysis – This section collects miscellaneous exploratory studies that have not been covered elsewhere in this Chapter, such as separation of derivatized amino acids using the fast centrifugal analyser[881] and enhancement of the fluorescence of naphthalene-1,2-dialdehyde-derivatized amino acids by cyclodextrins as mobile phase constituents in HPLC.[882]

7.6 Other Analytical Methods – Clearly, the applications of capillary zone electrophoresis (CZE) and related methods are developing rapidly, as demonstrated by the dedication of a CRC volume to it.[883] There are several applications in the amino acids field.[884–886] One of these studies[885] concentrates on CZE of cysteine and cystine employing electrochemical detection, as does another[886] that also covers a broader range of analytes. Separation of a mixture of 24 dansylamino acids is sharpened by Tween micelles in the liquid phase,[887] and separation of PTHs with good distinction of artefactual peaks has been accomplished.[888] These studies use sample preparation protocols that are familiar from the HPLC field, and this is also seen in use of Fmoc-derivatization for estimation of hydroxyproline in serum after OPA-treatment,[889] enantiomeric

separations of PTHs using N-dodecanoyl-L-serine, -glutamic acid, or -valine micelles,[890] and of dansylamino acids[891] using cyclodextrins as buffer additives,[892] also used for dansyl- and OPA-amino acids.[893] The power of modern methodology is revealed in the estimation, at 140 ppm levels, of the enantiomeric purity of phenylalanine derivatized by 4-fluoro-7-nitrobenz-2,1,3-oxadiazole and separated by CZE with cyclodextrin-containing buffers and using laser-induced (488 nm) fluorescence detection.[894]

Chiral stationary phases are also compatible with CZE enantiomeric purity determination, and mechanistic aspects have been explored.[895] The practice of CZE resolution has been reviewed.[896]

Immunoassay techniques have not been surveyed thoroughly in this Chapter over the years, though attention is drawn to unusual relevant studies such as the estimation of desmosine in biological fluids.[897]

7.7 Assays for Specific Amino Acids – Most of the papers under this heading revolve around enzymatic methods, both in the biosensor category and in flow injection analysis techniques. However, the latter approach can also accommodate standard chemical methods of analysis, such as Chloramine-T oxidation of hydroxyproline to generate a stable colour with Ehrlich's reagent.[898] Photometric estimation of tryptophan depends on the formation of a coloured complex by Fe^{3+}/AcOH/glycollic acid oxidation.[899]

Simultaneous estimation of L-lysine and L-tyrosine based on enzyme-supported flow injection analysis uses two enzyme reactors and a single oxygen electrode.[900] A similar approach accommodates L-alanine and L-phenylalanine, with a flow injection fibre optic biosensor providing the means of quantitation,[901] also for chemiluminescence generated specifically when L-lysine is present, associated with a lysine oxidase/microbial peroxidase membrane.[902] Measurement of oxygen generated in the latter system defines the L-lysine content of a sample.[903]

More conventional biosensor studies also appear in the current literature, often leapfrogging existing technology, but usually adding further illustration of established methods. A 'micro-enzyme sensor', based on immobilized L-amino acid oxidase, has been proposed for the quantitation of L-amino acids in urine,[904] and a combined L- and D-amino acid oxidase version assays total D- and L-amino acids.[905] The immobilization of a peroxidase with a D-amino acid oxidase on an electrode measures D-amino acid concentrations in proportion to H_2O_2 liberated.[906] A process for the electrodeposition of poly(tyramine) on to electrodes provides amino groups through which an L-amino acid oxidase can be immobilized to give satisfactory sensors.[907] An estimation of D-amino acids is based on the formation of D-norleucine by bacterial D-amino acid transaminase coupled with 2-oxohexanoic acid.[908]

The majority of current amino acid–biosensor reports concern L-glutamic acid and its relatives, with immobilized glutamic acid oxidase[909] linked through glutaraldehyde to an aminopropyl-platinized platinum wire, for amperometric measurements.[910] A similar glutarate-immobilized glutamic acid oxidase + glutaminase sensor permits amperometry of glutamic acid and glutamine, which

together with lactic acid can be estimated as a total entity using rhodinized carbon electrodes.[911] The list is completed by an L-glutamic acid + NADH sensor,[912] a glutamic acid decarboxylase-coated electrode,[913] and an L-glutamine sensor comprising kidney slices and an ammonia-sensing electrode.[914]

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2
Peptide Synthesis

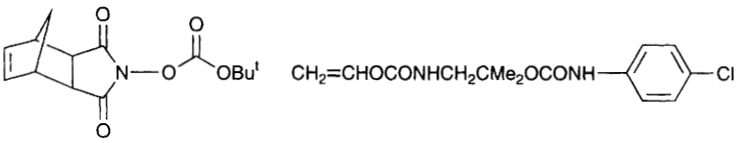
By D.T. ELMORE

1 Introduction

There are two significant changes in this report compared with its predecessor[1]. The total number of papers cited has markedly increased, although the number of papers devoted to the methodology section has decreased. As a result, the once familiar sections on general deprotection and selected examples of peptide synthesis have been omitted. The resulting compaction has assisted in controlling the length of the report. It has been felt necessary, however, to introduce a new section on nucleopeptides in the appendix. Some of the increase in the number of citations stems from the publication of new or revised books[2–10] and numerous reviews[11–64]. Most of the latter[11–43] cover similar methodological areas to those in this report. The remainder are more specialized and more akin to the appendix to this report.

2 Methods

2.1 α-Amino-group Protection – Reaction of α-substituted amino acids with ZCl in a variety of polar solvents in the presence of NEt_3 and 4-$Me_2NC_6H_4N$ (DMAP) at 50 °C gave Z-amino acid benzyl esters, probably via the unsymmetrical anhydride followed by its fragmentation[65]. Z groups can be removed from peptide derivatives containing an aliphatic αβ-dehydroamino acid by three standard methods, but treatment with CF_3CO_2H is preferred[66]. *N*-(t-Butoxycarbonyl)-5-norbornene-*endo*-2,3-dicarboximide (**1**), which has previously been used to synthesize Boc amino acids, has been prepared by reaction of the Tl(I) salt of *N*-hydroxy-5-norbornene-*endo*-2,3-dicarboximide with $(t\text{-}BuOCO)_2O$ instead of via the chloroformate for which $COCl_2$ is required[67]. It is a matter of personal choice whether one prefers to work with $COCl_2$, which announces its presence by an acute attack on the respiratory system or with Tl(I) derivatives which operate more slowly and insidiously on the brain. Convenient syntheses of *N*-Boc-*N*-alkylglycines, which are convenient intermediates for the preparation of peptoids, have been described[68]. It has long been known that *N*-Boc groups can be removed by a 1M solution of dry HCl in ethyl acetate, possibly because the resultant amine hydrochloride is precipitated, but it has now been shown that this process is selective since Bu^t esters and the Bu^t ether of serine survive during the time required to remove *N*-Boc groups[69]. The Bu^t ether of Tyr and *S*-Boc derivatives are rapidly cleaved by this process. The observation that *N*-Boc-*O*-tosylphenylalaninol in the presence of base loses both Boc and tosyl groups



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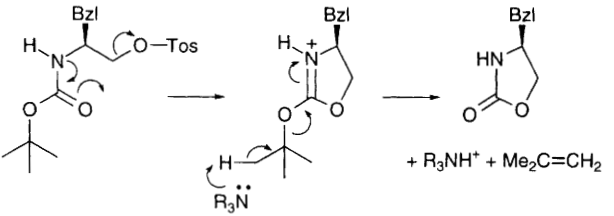
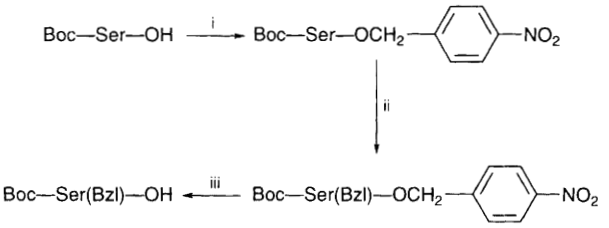
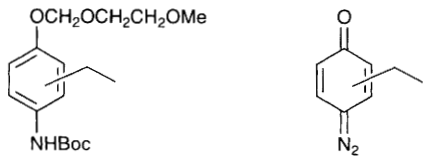


Scheme 1



Reagents: i, 4-$NO_2C_6H_4CH_2Br$; ii, $BzlBr/Ag_2O/CHONMe_2$;
iii, 1M-$Bu_4NF{\bullet}3H_2O$/tetrahydrofuran

Scheme 2



(3) (4)

probably proceeds according to Scheme 1[70]. This mechanism may explain why activation of Boc amino acids with DCCI can lead to loss of Boc group and formation of ninhydrin-positive products. A novel protecting group related to Boc has been generated from the reaction of an aryl isocyanate with the appropriate alcohol to give (**2**)[71]. Such compounds are of interest because of their high solubility and their sensitivity towards HBr/CH_3CO_2H, although application to the peptide field has not yet been reported.

Fmoc-L-4-azidophenylalanine has been synthesized by diazotization of 4-aminophenylalanine followed by successive treatment with NaN_3 and FmocONSu without isolation of the intermediates[72]. *N*-Fmoc groups can be selectively removed in the presence of various *O*-esters by treatment with KF and 18-crown-6 in $CHONMe_2$ at room temperature for 6-10 h with very good yields and no detectable enantiomerization[73]. A new protecting group, 2-adamantyloxycarbonyl, has been studied[74]. It is stable to CF_3CO_2H and 25% HBr, but is readily cleaved by CF_3SO_3H or HF. H-Lys(2-Adoc)-OH, which is available conventionally from the Cu complex of Lys, can be used in solid-phase synthesis with Fmoc as the orthogonal α-protecting group. Alternatively, the ε-2-Adoc group can be used in solution-phase synthesis in conjunction with the α-*N*-Boc group. α-*N*-2-(4-Nitrophenyl)sulfonylethoxy-carbamoyl (Nsc) derivatives of amino acids can be prepared by acylation of the Me_3Si derivatives in nonaqueous solution[75]. The *N*-protecting group can be removed by 1,8-diazabicyclo[5,4,0] undec-7-ene (DBU), 1,1,3,3-tetramethylguanidine or piperidine in aprotic solvents. One cleavage product is 4-nitrophenyl vinyl sulfone which can be trapped with piperidine to give $4\text{-}NO_2C_6H_4SO_2CH_2CH_2NC_5H_{10}$. Although the α-*N*-formyl group has declined in popularity, it may be of interest that formylation of α-amino acid ester hydrochlorides can be effected by reaction with $CH(CO_2R)_3$ under reflux[76]. Finally, the 4-nitrophthalimido group has been proposed as a protecting group[77] because it is easily removed with $MeNHNH_2$ in $CHONMe_2$ at room temperature, but application to peptide synthesis has not been reported.

2.2 Carboxyl-group Protection – There is very little to report on this front. 3-Methylbut-2-enyl esters can be prepared from the alcohol by either transesterification of Me esters or by direct esterification of the acid using DCCI in presence of DMAP[78]. Deprotection is effected by I_2 in cyclohexane at room temperature. The group has not been applied to peptide synthesis. In contrast, 2-cyanoethyl esters have been previously used and cleaved by K_2CO_3 in aqueous MeOH. An alternative method of deprotection is now available using Bu_4NF in a nonaqueous solvent[79]. In contrast to the foregoing, (±)-1-(4-Methoxyphenyl)ethyl esters, which are prepared from the alcohol with DCCI and DMAP, are easily deprotected under very mild acidic conditions such as 1% CF_3CO_2H or 10% Cl_2CHCO_2H in CH_2Cl_2 at room temperature[80]. Although alkylation of the indole ring of Trp by benzylic cations can occur, addition of 20% skatole as scavenger suppresses this reaction. These esters are only slowly cleaved by hydrogenolysis and this may inhibit their wide use; high sensitivity or complete stability are usually preferable to sluggish reaction in peptide synthesis. The removal of the 2-bromoethyl group by alkali is complicated by competing

hydrolysis of the alkyl halide[81]. Removal of the 2-bromoethyl group can be cleanly achieved by conversion into the 2-iodoethyl analogue followed by reaction with SmI_2 under anhydrous conditions in absence of oxygen. Although this is slightly long winded, no enantiomerization has been detected. Methyl esters can be prepared from *N*-protected amino acids with 2M NH_3 in MeOH in the presence of BOP at $-20\,°C$[82]. The authors overcame side reactions with Z-Glu-OH by carefully controlling conditions and they report an excellent yield, but alternative routes appear to be more attractive.

2.3 Side-chain Protection – A convenient synthesis of Boc-Ser(Bzl)-OH has been described (Scheme 2)[83], but a very impure product was obtained from Thr. During removal of side chain Bu^t groups with CF_3CO_2H, some *N*-trifluoroacetylation can occur and the yield of byproduct increases with reaction time[84]. A new protecting group for Ser (**3**) has been designed[85] which can be converted into (**4**). This is stable in the dark but is rapidly photolysed at 350nm. The t-butyldimethylsilyl group is a new protecting group for phenolic hydroxyl groups[86]; it is introduced by reaction of the silyl chloride in the presence of DBU in MeCN. It can be removed acidolytically by a variety of fairly mild protocols and it has been applied to the synthesis of some dipeptides of DOPA.

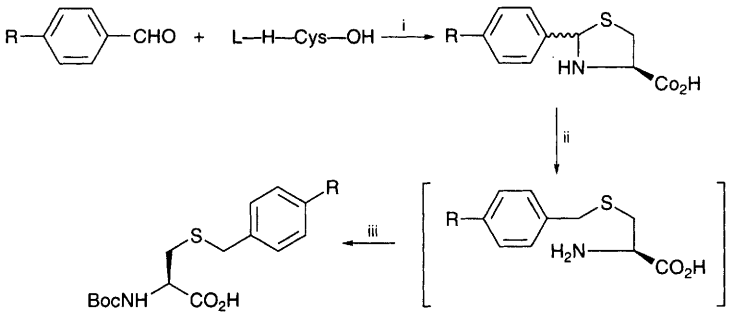
The β-carboxyl group of Asp can be protected easily as the cyclododecyl ester[87]. These derivatives tend to crystallize fairly readily but require HF or CF_3SO_3H for removal. Improved syntheses of the ω-*N*-Fmoc derivatives of Lys and Orn have been described[88]. In view of the long search for the most suitable protecting group for the guanidino group of Arg, it is intriguing that there is another report after a gap of many years that no protection is necessary[89]. Moreover, with Arg as the *C*-terminal residue, a partially protected tripeptide was coupled to an amino acid ester using either DCCI/HOBt or Bu^iOCOCl/*N*-methylmorpholine to give very high yields of tetrapeptide derivative with no detectable enantiomerization. Substantial enantiomerization, however, occurred with several other coupling techniques. In a more orthodox approach[90], the lability of four groups to acidolytic deprotection of Fmoc-Arg(R)-OH has been shown to be: 4-MeO-2,3,6-$Me_3C_6HSO_2$- > 2,4,6-$Pr^i_3C_6H_2SO_2$- > 4-MeO-3,5-$Bu^t_2C_6H_2SO_2$- > phenanthrene-3-SO_2-. The methylsulfonylethyl-oxycarbonyl group (Msc), which was proposed 20 years ago to protect α-amino groups, has been recommended for protecting guanidino groups[91]. It can be removed with 4M NaOH in dioxan. ωω′-Bisurethane arginine derivatives can be prepared by reaction of the appropriate derivative of 1-guanylpyrazole with the Cu complex of ornithine[92]. The α-amino group can subsequently be protected with Fmoc or Boc. The known modification of the indole nucleus of Trp during acidolytic deprotection of Arg residues bearing the Pmc group has been further studied[93]. The extent of Pmc transfer is dependent on the spatial separation of the Arg and Trp residues and is maximal when separated by one other amino acid, especially when this is not Gly or Pro. Group transfer cannot be completely prevented by scavengers. Protection of the imidazole group of His can be effected with the 2-Adoc group[94] (see ref. 74); the τ-*N*-atom is the site of reaction. The 2-Adoc group is stable to e.g. 7.6M HCl in dioxan, but is removed by HF and by mild treatment

with bases. Amide side chains of Asn and Gln, which have been protected by one of the following groups, 2,5-dimethyl-4-methoxybenzyl, 1-(3,4-dimethylphenyl)-ethyl, 2-methoxy-1-naphthalenemethyl, 1-(4-methoxyphenyl)ethyl, 2,4,6-trimethylbenzyl, diphenylmethyl and 4-methoxy-1-naphthalenemethyl, can be liberated with boron tris(trifluoroacetate) in CF_3CO_2H/CH_3CO_2H[95].

Thiol groups can be protected by the $CH_2=CHCH_2OCONHCH_2$- (allocam) group[96] and easily liberated by Pd-catalysed hydrostannolysis. The allocam group is stable during Fmoc removal, but is only marginally stable to the acidic conditions used to remove Bu^t and Boc groups. A useful route[97] to *N*-protected-*S*-aralkylcysteine derivatives is outlined in Scheme 3. Thiol groups can be protected by the phthalimidomethyl group (Pim) by reaction with *N*-chloro- (or *N*-bromo-) methylphthalimide/Et_3N in $CHONMe_2$ at room temperature[98]. The Pim group is not satisfactorily removed by Hg(II) salts, but is best achieved with $N_2H_4.H_2O$ in MeOH at 0 °C followed by reaction with $Hg(OAc)_2$ or $Cu(OAc)_2$ then with $HSCH_2CH_2OH$. If the treatment with $N_2H_4.H_2O$ is followed by reaction with I_2, disulfides are formed. If cysteine hydrochloride is treated with methoxycarbonylsulfenyl chloride (SmcCl), the NH_3^+ group is unaffected and H-Cys(Scm)-OH.HCl is formed[99]. Finally, *S*-oxides in side chains of peptides can be reduced with Me_3SiBr and $HSCH_2CH_2SH$ in CF_3CO_2H[100]. The method is applicable to peptide libraries.

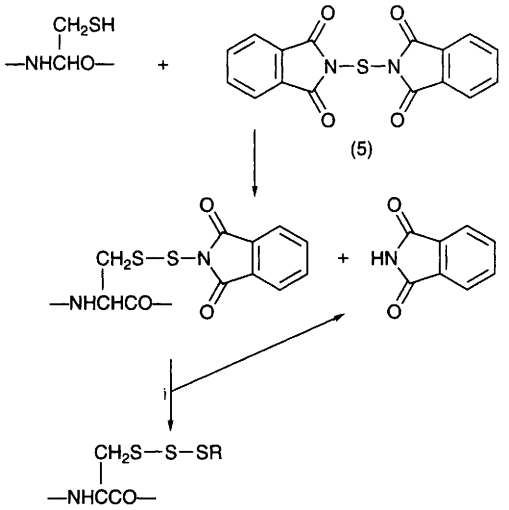
2.4 Disulfide Bond Formation – Methods used to form disulfide bonds during the period under review have not invoked any new chemistry apart from the use of the Pim group[98]. There are examples of the use of aerial oxidation[101] and of the use of pyridinesulfenyl groups for protection and activation[102,103]. A more complex synthesis of the antigenic region of the malaria merozoite surface protein (MSP-1) involved the formation of three disulfide bonds[104]. This was effected by protecting four thiol groups with Trt and two with Acm groups. The Trt groups were removed and the peptide was oxidized with MeSOMe. Fortunately, the correct disulfide bonds were formed. Finally, the two Acm groups were removed and treatment with I_2 afforded the required product. A similar synthesis of the *C*-terminal sequence of human factor IX has been accomplished by the same team[105]. Attachment of a protected cysteine residue at the thiol group to an insoluble support permits the assembly of a peptide chain on to the deprotected amino group. If this portion of the peptide chain contains another cysteine residue but bearing an Acm group on the side chain, treatment in $CHONMe_2$ with I_2 affords a cyclic disulfide in high yield and purity[106]. In less polar solvents, some parallel symmetrical dimer retaining Cys(Acm) residues is formed. Finally, a method has been reported for the synthesis of peptide trisulfides[107]. The method (Scheme 4) uses *NN*-thiobisphthalimide (**5**).

2.5 Peptide Bond Formation – The coupling of Fmoc amino acid chlorides can be mediated by the K salt of HOBt[108]. Reaction is fast and is claimed to be free from enantiomerization. Further examples of successful syntheses with Fmoc amino acid fluorides, especially Fmoc-Aib-F, have been reported[109]. The same



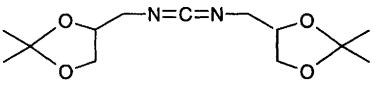
Reagents: i, 50% CF_3CO_2H/CH_2Cl_2; ii, CF_3CO_2H; iii, Et_3SiH then Boc_2O at pH 8

Scheme 3



Reagent: i, Cysteine derivative with free —SH group

Scheme 4



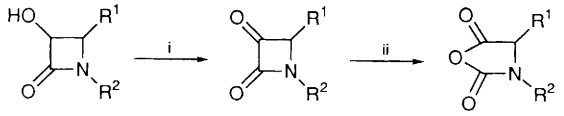
(6)

team identified cases where the use of *N*-carboxyanhydride failed because attack on the latter by the amino component occurred at the wrong carbonyl group.

Not everybody is seeking to assemble the most difficult sequences and so work on acylating agents less potent than acyl fluorides continues to flourish. Polyhalogenated aryl esters are still very much in vogue[110–113]. An interesting mainly theoretical paper discusses the possibility of designing superactive esters[114]. It was argued that the rate-determining step is commonly the collapse of the tetrahedral intermediate or a proton transfer therein and this renders the reaction more sensitive to the basicity of the nucleophile, its solvation and possibly steric effects leading to difficult couplings. It was suggested that most of the difficulties would disappear if the initial attack by nucleophile leading to the formation of the tetrahedral intermediate could be the rate-determing step. Several considerations, including some experimental data, led to the conclusion that esters of 2-hydroxy-1,3,5-triazine possess the desired properties. This paper should evoke a thorough examination of these concepts in the most adverse situations. In another example of the safety-catch principle, 4-methylthiophenyl esters were electrochemically activated to the corresponding 4-methylsulfonylphenyl esters for peptide synthesis[115]. 1-β-Naphthalenesulfonyloxybenzotriazole, which was previously used in solution-phase peptide synthesis, has now been used in solid-phase work[116]. The speed of coupling rivals that when using BOP and enantiomerization is minimal.

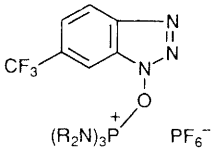
Carbodiimides, like reactive esters, retain their popularity especially when used with additives that effect good acceleration and minimize enantiomerization. An efficient synthesis of aryl phenaceturates using DCCI has been reported[117]. It is interesting mechanistically that rearrangement of *O*-acylisourea to *N*-acylurea proceeds with preservation of chiral integrity[118]. If an unsymmetrical carbodiimide is used in peptide synthesis, two isomeric *N*-acylureas can be formed[119]. When this occurs, the acyl group migrates to the less hindered nitrogen atom. Other byproducts are possible in the reaction of *N*-alkoxycarbonylamino acids and symmetrical carbodiimides[120]. A new carbodiimide (**6**) has been synthesized from 1,2-isopropylideneglycerol[121]. It offers several potential applications including the synthesis of alkyl esters of *N*-protected amino acids and the simple purification of peptides synthesized by its use. Small peptides were obtained in good yield and without significant enantiomerization. *N*-Cyclohexyl-*N'*-isopropylcarbodiimide is claimed to be better than either of the symmetrical carbodiimides in giving a better yield of product that was easily purified because of the advantageous solubility of the urea formed[122]. The successful synthesis of ACP(65-74) indicates its potential.

N-Protected amino acids including Arg, treated with $(Bu^tOCO)_2O$, pyridine and an arylamine in a one-pot procedure yielded the corresponding arylamide[123]. *N*-Methylmorpholine or Et_3N can replace pyridine. The pyridinium salt of a *NN*-bisBoc amino acid, treated with cyanuric fluoride in CH_2Cl_2, gives the corresponding acyl fluoride, but if Vilsmeier reagent in MeCN replaces cyanuric fluoride, the product is the *N*-Boc derivative of the *N*-carboxyanhydride[124,125]. *N*-Carboxyanhydrides are also available[126] by oxidizing α-hydroxy-β-lactams to the



Reagents: i, P_2O_5/MeSOMe; ii, 3-chloroperbenzoic acid/CH_2Cl_2 at –40 °C

Scheme 5



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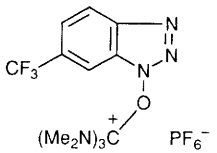

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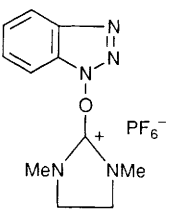

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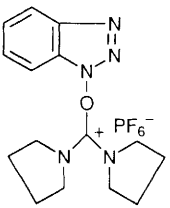

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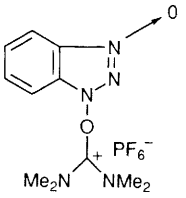

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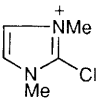

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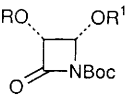

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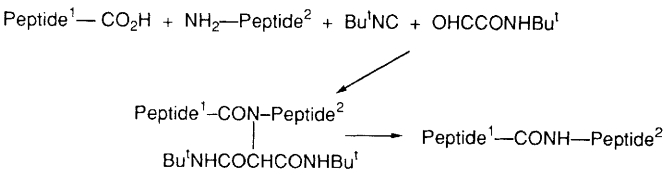


Scheme 6

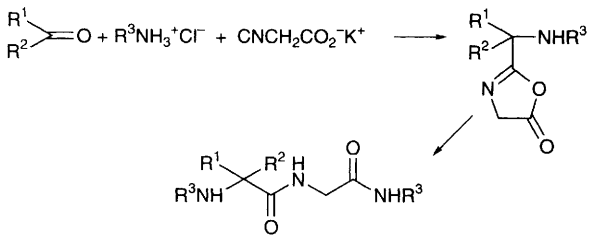
α-keto derivatives and then subjecting these to a Baeyer-Villiger rearrangement (Scheme 5).

Comparative studies with the phosphonium and uronium types of coupling agents continues to receive much attention. When BOP and $Pr^iN=C=NPr^i$ were used in multiple peptide syntheses, results were similar except when Asn was being incorporated. In this case, BOP gave inferior results that were not improved by addition of HOBt[127]. New coupling agents, CF_3-BOP (**7**; R = Me), CF_3-PyBOP (**7**; R_2N = pyrrolidino) and CF_3-HBTU (**8**), all of which are based on 6-trifluoromethyl-1-hydroxybenzotriazole, were found to be efficient for preparing peptides of Aib[128]. Carpino and coworkers have been very active in this field. Two new coupling agents, *O*-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleneuronium hexafluorophosphate (HAMDU) (**9**) and -1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate (HAPyU) (**10**), have been designed[129]. These rival HATU in achieving high coupling velocities. Of tertiary bases examined, the strongest bases such as Pr^i_2EtN effected fastest coupling but with unacceptable enantiomerization. The crystal stucture of HBTU and HATU have been determined and shown to be guanidinium *N*-oxide isomers (e.g. **11**) of the expected structure[130]. One of the most important advances of the year is the introduction of 1-hydroxy-7-azabenzotriazole (HOAt) as a better additive than HOBt in peptide coupling[131,132]. Yields in so-called difficult couplings were considerably improved and the extent of enantiomerization was significantly decreased. Moreover, as indicated above, the uronium and phosphonium salts of HAOt are much better coupling agents than those derived from HOBt. These results have been confirmed and extended[133,134], for example, in the synthesis of peptides containing *N*-MeLeu and *N*-MeVal. Finally yet another coupling reagent (**12**) has been developed and it effects the assembly of Aib peptides in presence of HOAt[135]. With chiral amino acids, there was no detectable loss of chiral integrity.

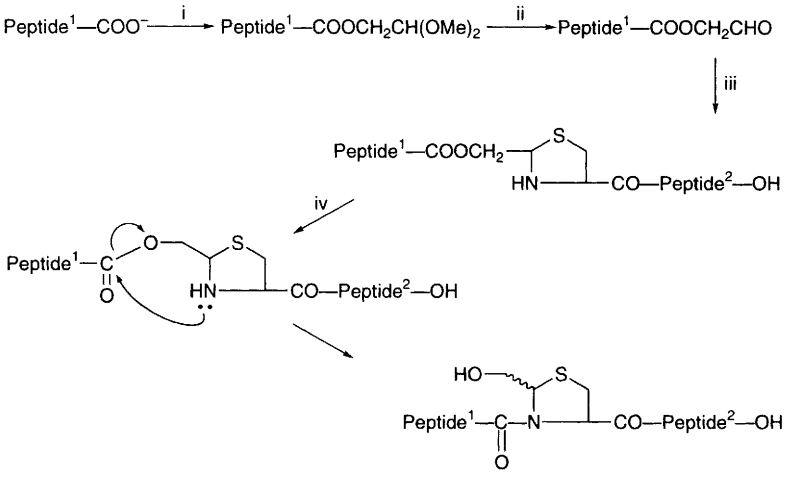
N-t-Butylglyoxylamide is a new reagent for coupling segments in an Ugi synthesis[136] (Scheme 6). A multi-component condensation using potassium isocyanoacetate permits the synthesis of *NN'*-diarylpeptide amides[137] (Scheme 7).

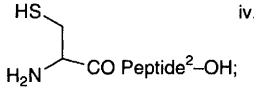
Reaction of 2,4-dimethyl-5(4H)-oxazolone with Z-Asp-OH followed by addition of H-Phe-OMe.HCl and *N*-methylmorpholine gave a 7:3 mixture of α- and β-isomers of Z-Asp-Phe-OMe[138]. Z-Asp-OH or Z-Glu-OH alone with the oxazolone gave the corresponding anhydride. The same school has described preparations of Fmoc pyroglutamic acid, the acyl chloride and the succinimidyl ester[139]. Ring opening of the β-lactam (**13**) with amino esters gives norstatine peptides[140]. An ingenious method[141,142] of coupling two unprotected peptide fragments involves (1) formation of an *O*-peptidylglycolylaldehyde, (2) condensation of the product with the α-amino group of a peptide bearing an *N*-terminal cysteinyl or threonyl residue to give a peptidyl-thiazolidine or -oxazolidine, (3) rearrangement at a slightly more alkaline pH to give a peptide containing a pseudoproline residue at the site of peptide union (Scheme 8).

Two simple tests for enantiomerization during assessment of a method for forming a new peptide bond have been described[143,144]. Finally, Benoiton has registered a justified plea for more precise nomenclature in describing loss or change of chirality in peptide synthesis[145]. With a feeling of guilt and remorse, the

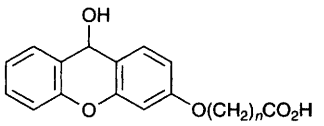


Scheme 7



Reagents: i, $BrCH_2CH(OMe)_2$, DMF, 60 °C 24 h; ii, 30% TFA in CH_2Cl_2 (2–5% H_2O); iii,  iv, pH 4–9

Scheme 8



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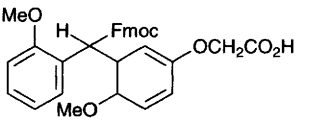


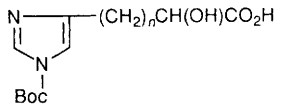
(15)

Reporter has taken this lesson to heart. It is appropriate, however, to point out another example of incorrect nomenclature widely used by peptide chemists. Peptide couplings of the type:

$$R^1CO_2H + ClCOOR^2 \rightarrow R^1COOCOOR^2$$
$$R^1COOCOOR^2 + R^3NH_2 \rightarrow R^1CONHR^3$$

usually involve the formation of an unsymmetrical anhydride, *not* a mixed anhydride. The latter term is best reserved to describe the mixture resulting from disproportionation when two anhydrides are admixed and the system is allowed to come to equilibrium.

2.6 Solid-phase Peptide Synthesis – A few new supports have been developed. Heterobifunctional polyethylene glycol (PEG) derivatives, $BocNH(CH_2CH_2O)_nCONHCH_2CO_2H$, have been attached to aminomethyl copoly(styrene – 1% divinylbenzene)[146]. These supports had good swelling properties in a wide range of solvents and behaved well when Boc, Fmoc or Dts protecting groups were used. A copolymer of tetraethyleneglycol diacrylate and polystyrene[147] behaved similarly. An improved synthesis of Rink's polymer has been described[148]; somewhat surprisingly, the potassium salt of 2,4-dimethoxy-4′-hydroxybenzhydrol gave better results than the caesium salt in the coupling process to the polystyrene support. A new resin consisting of beaded PEG-polyacrylamide copolymer with 4-(α-amino-2′,4′-dimethoxybenzyl)phenoxyacetic acid as linker is useful for the enzymic solid-phase synthesis of glycopeptides[149]. Aminopropyl cellulose in beaded form in conjunction with the 4-oxymethylphenoxyacetyl (HMPA) linker has been used to synthesize matrix-bound peptides for affinity chromatography and antibody generation[150]. A 3,9-substituted xanthene linker (**14**) has been designed and shown to be satisfactory in SPPS[151]. Its performance was satisfactory in the synthesis of three test peptides. Two trityl-type linkers permit easy peptide detachment under mild acidic conditions[152]. The linker (**15**) required the insertion of an ε-aminohexanoic acid residue between it and the resin in order to get satisfactory yields during coupling[153]. An analogue of gramicidin A was synthesized in a satisfactory test and detachment from the polyacrylamide support by CF_3CO_2H/CH_2Cl_2 was facilitated by ultrasonication. An imidazole derivative (**16**) is a linker with a safety catch[154]. It is coupled to Wang resin and peptides are assembled on the hydroxyl group with Fmoc protection of α-amino group. When the Boc group is removed from the imidazole ring, the latter, when unprotonated, provides anchimeric assistance for the hydrolysis of the peptidyl ester group. In a test synthesis, peptide detachment was complete in 5–7 min at 50 °C and pH 7.5. A nitrated polystyrene resin has been developed and the assembled peptide is detached photolytically[155]. Unfortunately, this process required exposure of the peptide to ultraviolet radiation for 18-24 h. A previously described allylic linker (**17**) has been further developed[156] since the assembled peptide can be detached by Bu_3SnH in the presence of $(Ph_3P)_2PdCl_2$. Another safety-catch linker involves the generation of an *N*-peptidyl benzimidazol-2-one (Scheme 9) which is very easily hydrolysed[157], a result to be expected from Kamiński's concept[114]. A large-



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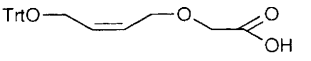
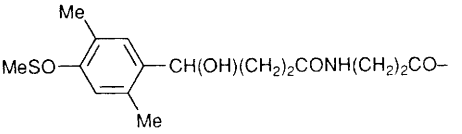


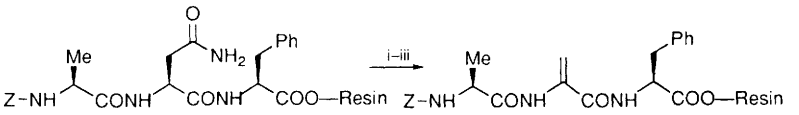
(17)

R = Ph or CCl_3CH_2

Scheme 9

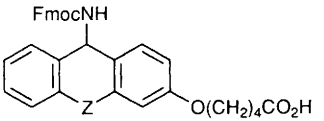


(18)

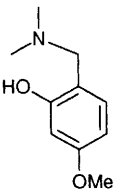


Reagents: i, 1.5 equiv. $PhI(O_2CCF_3)_2$, DMF/THF/H_2O + 1.5 equiv. pyridine
ii, MeI (10 equiv.), $KHCO_3$ (10 equiv.), MeOH, room temp;
iii, 10% NEt_3 in MeOH, room temp.

Scheme 10



(19) Z = CH_2CH_2 or CH=CH



(20)

scale preparation has been described [158] of (2′,4′-dichlorophenyl)-4-bromomethyl-phenoxyacetate for coupling to an amine-functionalized resin and attachment of the first amino acid. The linker (**18**) is intended to be used with a 2-dimensional protection scheme using acid-labile temporary protecting groups and acid-stable groups cleavable by reductive acidolysis[159]. Two tricyclic linkers (**19**; Z = $-CH_2CH_2-$, -CH=CH-) permit product detachment at low acid concentrations[160]. Indeed, peptides can be detached from one of these (Z = -CH=CH-) at such low acidity that acid-labile side chains remain intact. High and reproducible yields are obtained when a glycolamidic ester linkage is used for SPPS[161].

The problems caused by association of peptide chains under assembly appear to have been solved by selective reversible protection of peptide bonds. In an important series of papers, Johnson and Sheppard[162–166] have greatly extended the work reported last year. Val and Ile residues with high P_β values favour association but side-chain protecting groups in general have little effect. Aprotic polar solvents with strong hydrogen bond acceptor behaviour tend to prevent association. The most powerful technique so far found for combating association uses the 2-hydroxy-4-methoxybenzyl group (Hmb) on selected peptide N atoms (**20**). The 2-hydroxy group renders the group labile to acidolysis so that it can be easily removed when peptide assembly is complete. It is of course orthogonal to the Fmoc group. In order to stabilize the Hmb group during peptide synthesis, it is convenient to acetylate the 2-hydroxy group. The acetyl group can be removed by 20% pyridine at the end of the assembly. The Hmb group possesses the additional advantage that it increases the solubility of peptides carrying it. As expected, the method was tested by synthesizing ACP(65-74). It seems that this bête noire is now only a white mouse. By using the Hmb group on 5 residues, the β-amyloid fragment(1–43) was successfully synthesized[164]. Use of the Hmb group offers another additional bonus. The tendency for Asp(OBu^t) groups to form aspartimides or piperidides[166,167] during removal of Fmoc groups from the *N*-terminus is overcome by protection of the aspartyl peptide bond with Hmb[166]. It should be pointed out that an alternative analysis[168] of difficult couplings does not entirely agree with the views of Johnson and Sheppard. Perhaps it would be more appropriate to conduct a wider practical survey of the Hmb technique than to carry out further theoretical examination of coupling difficulties.

The *N*-carboxyanhydrides derived from Fmoc amino acids are recommended for attachment of the *C*-terminal amino acid to the Wang and Rink resins[169]. The same approach is proposed for Fmoc-His(Trt)[170]. Little or no enantiomerization is reported. In contrast, when Cys is to be the *C*-terminal residue, the use of piperidine causes enantiomerization and it is recommended that Fmoc-Cys(Acm) is attached to the 2-chlorotrityl linker[171]. Me_2SiCl_2 has been used to detach peptides from Wang resin[172]. $SiCl_3I$, generated from $SiCl_4$ and NaI, removed Boc and Z groups from protected peptides under assembly[173,174]. A mixture of $CF_3CHOHCF_3$ and CH_2Cl_2 (1:4 v/v) rapidly detached peptides from a 2-chlorotrityl resin[175]. A new protocol has been developed for detachment and partial deprotection of peptide fragments before purification, reprotection and fragment coupling[176]. The method uses the new 2-Adoc protecting group[74]. It has been shown that a mixture of Me_3SiN_3 and Bu_4NF (8:3) facilitated the removal

of Alloc groups by $Pd(PPh_3)_4$ in CH_2Cl_2 during SPPS[177]. The authors maintain that concentrations of Me_3SiN_3 are too low to generate formation of the highly explosive $CH_2(N_3)_2$. During treatment of a resin-linked peptide containing an -Asp(OFm)- with HF and PhOMe, the peptide was detached and Bzl groups were removed, but a substantial amount of impurity was detected in which the Bzl group was attached to the Fm group[178]. The problem is obviated by choosing an ester group that is not affected by benzylic cations. Another complication attended the acidolytic detachment from resin of peptides with *C*-terminal Met[179]. The latter was extensively converted into homoserine and it is recommended that all Bu^t groups are removed before detachment from resin. A final deprotection problem concerns incomplete removal of Fmoc groups during SPPS which is attributed to β-sheet formation[180]. Judicious use of the Hmb group could probably avoid this problem.

This section ends with a summary of some variations of the SPPS technique including multiple syntheses and the production of peptide libraries. Technical improvements include the use of a spectrofluorometric method of monitoring SPPS[181] and its acceleration by employing elevated temperatures[182]. Peptide amides have been made by assembling an amino-acid sequence on a Kaiser oxime resin then cleaving with an aliphatic primary or secondary amine[183]. Peptides containing α-hydroxyglycine, the biochemical precursors of peptide amides, have been synthesized starting from Fmoc α-methoxyglycine[184]. The methoxy group is removed when the peptide is detached from the support with CF_3CO_2H containing 5% of water. Treatment of the product with peptidyl amidoglycolate lyase (EC 1.4.17.3) should give the amide. An Asn residue in a peptide supported on a resin can be converted into a ΔAla residue (Scheme 10)[185]. Conversely, dehydrotripeptides made by SPPS can be asymmetrically hydrogenated using Rh complexes of chiral diphosphine ligands as catalysts[186]. Although cyclic peptides are not a main subject for this Report, a few references[187-190] will give an insight into the kinds of structure and methods available. In order to increase the solubility of hydrophobic peptides, polyethylene glycol monocarboxylic acid can be incorporated into the assembled structure[191,192], although the molecular size of the polymer may have a considerable influence on the rate of acylation steps.

Multiple concurrent peptide syntheses are increasing in importance although the multipin approach has perhaps passed its zenith[193-197]. The use of radiation-grafted crowns on polyethylene pins appears to be the most popular technique. If a DKP-forming handle is incorporated in the structure to be assembled, detachment can be achieved under very mild conditions using sonication to accelerate the process[197].

The use of combinatorial synthesis is revolutionizing organic chemistry and nowhere more than in the peptide field. The refinement of SPPS methodology with its associated hardware has contributed greatly to the progress reported this year[198–211]. It is possible, however, to assemble peptide libraries without having recourse to expensive hardware[198]. Peptide libraries can be constructed by two main methods:- (*a*) iterative division, coupling and recombination, (*b*) competitive coupling. The first method requires the researcher to steer between the Scylla of using an inordinate amount of resin for SPPS and the Charybdis of ending

with some peptides being present in amounts inadequate for screening. A statistical treatment of method (*a*) has been presented[199]. For method (*b*), the relative rates of coupling with immobilized amino acids has been determined[200]. A technique has been described for generating libraries in which all possible sequences are synthesized with certainty[201]. Another investigation focused on the assembly of a hexapeptide library which aimed to minimize both synthesis and screening[202]. A different hexapeptide library required to identify potential ligands for double stranded DNA has been produced[203]. A cellulose membrane served as support. All 8000 tripeptide amides consisting of coded amino acids were synthesized and one of these, H-Tyr-Pro-Gly-NH_2, was shown to inhibit HIV-1 Tat function and to block replication of the virus[204]. Although the concept of one support bead – one peptide is popular, it is by no means universal. A method has been described for the generation and screening of peptide libraries by assembling many peptides on each bead[205]. In contrast, a library of 10^6-10^8 peptides has been assembled on the one bead – one peptide concept[206]. The whole library was screened for binding to a specific acceptor. The bead that bound the peptide best was isolated and the structure of the peptide was determined. A secondary library was developed to obtain a peptide with further enhanced activity. In another development, a library of peptides can be subjected to chemical modification (e.g. methylation) while still attached to the support[207]. Each original peptide might give rise to a population of products. Location of particular peptides in a library can be more tiresome than the original multiple synthesis and increasingly chemical tagging of beads bearing peptides is being used[208]. A general method for tagging peptides[209] uses halogenated phenols which can be identified by electron capture gas chromatography at levels of < 10 pmol. The tag is attached to the solid support via a linker which is a derivative of vanillic acid. The -CO_2H of the latter is converted into a diazomethyl ketone and thence to carbene which reacts covalently with the support. The tags are read after oxidative detachment with ceric ammonium nitrate under sonication. A peptide library has been assembled[210] on a steroid polystyrene conjugate and screened for ability to bind Leu-enkephalin. The latter was tagged with a red dye to facilitate recognition of opioid binding. The peptide receptor library was tagged as indicated above so that its subsequent structural elucidation identified the peptides that bound the opioid peptide. Finally, a cyclic peptide template comprising 3 Lys and 1 Glu residues has been used to assemble a library for screening for chymotryptic inhibitory activity[211].

2.7 Enzyme-mediated Synthesis and Semi-synthesis – The use of charged, solubilizing *N*-α-protecting groups is recommended for the acyl donor when various Ping Pong proteinases are used[212]. Much higher substrate concentrations can be used and high yields are reported. In contrast, the use of 5(4H)-oxazolinones has been proposed[213], but it is debatable if much is to be gained since reaction of alkyl esters as acyl donors is sufficiently rapid that deacylation of acyl-enzyme is rate determining. Picolyl or *N*-methylated picolyl esters of hydrophobic amino acids have been recommended for couplings mediated by

trypsin[214]. The use of unprotected taurine as the nucleophilic substrate is possible using subtilisin or proteinase K[215].

A considerable amount of research has been devoted to the choice of solvent and to the immobilization of enzyme on a variety of supports. Immobilized thermolysin has been used for the synthesis of 'Aspartame' precursors[216–218]. In one case[218], a computer-optimizing technique was used to diminish the number of experiments required. A similar synthesis using papain in H_2O/EtOAc has also been described[219]. Other studies, often involving kinetic measurements rather than simply determining yields, have been carried out on several enzymes[220–228]. In general, the best yields were obtained with organic solvents of low polarity. If water was present, optimum yields were obtained with quite low concentrations of water and this bears out many earlier published results. In some cases, it was shown that V_{max} increased and K_m decreased as solvent polarity decreased. In one case[229], it was shown that α-chymotrypsin can be extracted from aqueous solution into organic solvents with low concentrations of surfactants such as sodium bis(2-ethylhexyl) sulfosuccinate (Aerosol 07) as a result of ion-pairing of surfactant and enzyme. Reverse micelles are not formed. An important contribution to proteinase-catalysed synthesis of peptides has arisen from the demonstration by X-ray diffraction studies that α-chymotrypsin in hexane retains its active conformation despite some changes in protein solvation[230]. Enhanced yields of peptides were obtained with trypsin or α-chymotrypsin that had been palmitoylated (presumably on ε-amino groups)[231]. When chymotrypsin was coupled to polyallylamine that had previously been partially lauroylated, the enzyme had enhanced thermostability[232]. A kinetic study of peptide synthesis by this modified enzyme would be very interesting. Some evidence has appeared suggesting that addition of cations to proteinases in organic solvents can enhance activity. Thus Ca^{2+} ions accelerate transesterification reactions catalysed by α-chymotrypsin and this is attributed to an increase in k_{cat}[233]. Similar results were obtained with KCl and both α-chymotrypsin and subtilisin[234]. It was proposed that the salt has a protective effect against deactivation by organic solvent. Somewhat surprisingly, peptide synthesis proceeds satisfactorily with heterogeneous mixtures of substrates provided that a liquid or semiliquid eutectic is formed[235,236]. The process is profoundly assisted by the addition of an adjuvant such as a hydrophobic solvent. Probably as a result of the increasing success using proteinases in hydrophobic solvents, research on reverse micelles has diminished. There are two publications[237,238] on the enhancement of reactions catalysed by α-chymotrypsin by reverse micelles. The effect of temperature on enzyme-catalysed peptide synthesis has received further attention. Although the transesterification of Moz-Leu-Bzl by EtOH was decelerated by lowering the temperature from 25-30 °C to 5 °C, the yield was improved because the competing hydrolysis was depressed even more[239]. There have been further studies of enzyme-catalysed peptide synthesis in frozen solution[240,241]. For example[241], the yield of product obtained in the coupling of Mal-Phe-OMe and H-Leu-NH_2 catalysed by α-chymotrypsin was optimal between 248 and 263 K. There have been several studies using proteinases coupled to PEG[242–246]. Depending on the M_r of PEG and the degree of substitution, the enzyme-PEG conjugate can become soluble in

benzene at the expense of a decrease in V_{max} for peptide synthesis. Conjugation also increases the thermal stability of the enzyme. The properties of proteinases immobilized on a variety of commercially available supports have been further studied[247,248].

There are several publications detailing work with less familiar enzymes. Small peptides have been produced using thrombin[249] and a proteinase from the extremely thermophilic organism *Thermus* Rt41A[250]. A dipeptidylpeptidase (EC 3.4.14.5) from *Lactococcus lactis* PepX has been used for the first time to generate peptide bonds involving Pro[251]. A new Glu/Asp-specific endopeptidase from *B. licheniformis* has been used to catalyse the syntheses of 'Aspartame' analogues[252]. Aminopeptidase A from *Staph. chromogenes* can be used to effect reversible protection of the α-amino group of a peptide with malic acid except when the *N*-terminal residue is Gly or Pro[253]. Genetic engineering has produced some useful mutants of better known enzymes. Four mutants of subtilisin 8397 are more stable than wild-type enzyme in organic solvents[254]. Enzyme preparations which are freeze-dried from inorganic buffers such as sodium phosphate (pH 8.4) have enhanced stability. Mutants of subtilisin BPN were made to accommodate large hydrophobic side chains at P_4 in substrates of the type Suc-X-Ala-Pro-Phe-pNA (X = Ala,Val,Ile,Leu,Phe)[255]. The Y104A mutant particularly favoured binding of the substrate where X = Leu. Mutants of carboxypeptidase Y have been generated to study the binding of substrates[256]. Asp^{51} and Glu^{145} function as a binding site for the terminal $-CO_2H$ of the substrate. Replacement of these by Ala or Gly has a beneficial effect on substrate binding and product yield.

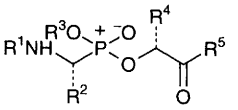
Some work, but less than might have been expected, has been carried out on the catalysis of peptide bond formation by monoclonal antibodies raised against transition-state analogues. A hapten of the general formula (**21**) is coupled to a carrier protein and used to immunize mice to generate monoclonal antibodies which should bind the transition state for peptide synthesis in view of the tetrahedral configuration of the phosphorus atom[257–259]. Although a good rate acceleration factor was obtained[257], the stereoselectivity was less impressive[259].

Finally, enzyme-catalysed semisynthesis continues to attract attention. Model experiments on transpeptidation reactions catalysed by carboxypeptidase Y have been reported[260]. The nature of the side chain of the attacking nucleophile is not crucially important, but amino acid amides react much more quickly than the parent amino acid. Use of the Glu/Asp-specific endopeptidase[252] from *B. licheniformis* has improved the semisynthesis of a potent analogue of hGHRF[261]. $4\text{-}HOC_6H_4CH_2CO$-D-Ala-Asp-OR and $[Ala^{15}]$GHRF(4-29)-NH_2 were coupled to give $[desNH_2Tyr^1,D\text{-}Ala^2,Ala^{15}]$GHRF(1-29)-$NH_2$ and alanine. A non-enzymic semisynthesis of a deuterated analogue of porcine insulin has been described[262]. The α-amino group of the A chain of insulin was selectively protected with the Mse group then Phe^{B1} and Val^{B2} were detached by two Edman cycles. The B1 and B2 residues were replaced by coupling sequentially with protected octadeuterated amino acid succinimidyl esters and protecting groups were removed. A chimeric molecule composed of EGF and insulin fragments has been prepared by enzymic coupling[263]. Three peptides representing

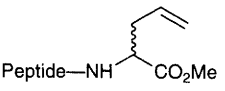
the complete sequence of triose phosphate isomerase have been reassembled using subtilisin in the presence of $MeCN/H_2O$ (3:2) or glycerol/H_2O (9:1)[264]. Perhaps the most enterprising example of semisynthesis concerns the assembly of RNase A from six fragments of 12–30 residues using esterified forms for acylation. A double mutant (P221C, P225A) of subtilisin, subtiligase, was used and produced about 70% yield per peptide bond formed[265]. The same technique was used to synthesize two mutants of RNase A in which 4-fluoro-histidine replaced either His^{12} or His^{119}. Both residues are involved in the catalytic process. Interestingly, although large changes in the pH-rate profiles resulted, there was little change in k_{cat}.

2.8 Miscellaneous Reactions Related to Peptide Synthesis – Aspartimide and its piperidine adducts were formed in the Fmoc-based synthesis of a domain resembling EGF in the blood coagulation factor VIII[266]. The sequences -Asp(OBu^t)-Asn(Trt)- and -Asp(OBu^t)-Gly- are especially prone to this side reaction. A fragment of thymopoietin, H-Arg-Lys-Asp-Val-OH, gives a complex mixture of products on digestion in aqueous solution resulting from an initial formation of aspartimide followed by formation of the isoAsp peptide, fragmentation to H-Arg-Lys-Asp-OH and valine and conjugation of two of the former to isomeric heptapeptides[267]. It is a warning that enzyme-catalysed synthesis might go astray if digestion times are prolonged for any reason. An Asp residue also caused problems in the solution synthesis of the octapeptide, thymic humoral factor γ2 (THF)[268]. The *N*-terminal tripeptide, H-Leu-Glu-Asp-OH and [$isoAsp^3$]THF were identified as impurities in the product. The apparently simple task of synthesizing protected derivatives of H-Glp-Glu-Asp-OH was complicated by the surprising ease with which the latter formed diketopiperazine derivatives[269].

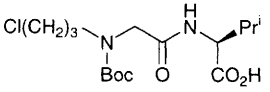
An interesting synthetic possibility exists with *N*-protected peptides terminating with -Gly-O-Allyl. These undergo an ester enolate Claisen rearrangemt on treatment with $LiNPr^i_2$, a Pd(0) catalyst such as $Pd(PPh_3)_4$ and a metal chloride such as $ZnCl_2$, $SnCl_2$, $CoCl_2$ or $AlCl_3$ to give (**22**) after esterification with CH_2N_2[270]. If an excess of $Hg(OAc)_2$ in the presence of $HSCH_2CH_2OH$ is used to remove Acm groups from a peptide containing a Trp residue, mercuration of the indole ring can occur at the 2 and/or 7 positions. Subsequent reaction with $SHCH_2CH_2OH$ then gives two mono- and one di-substituted derivatives bearing $HOCH_2CH_2S$-groups[271]. This side reaction can be prevented by using 50% CH_3CO_2H rather than a more dilute solution. Simple peptides containing a residue of aminomalonic monoalkyl ester have been synthesized[272]. These can be subjected to *C*-alkylation in the usual way. Although a benzyl ester group in the side chain could be removed by hydrogenolysis, methyl esters could not be saponified. It is possible that deprotection might be achieved using lipase N from *Rhizopus niseus*, especially if the ester group used is $MeO(CH_2)_2O(CH_2)_2O$-[273]. *N*-Boc-*N*-$Cl(CH_2)_n$-Gly-OH can be synthesized readily and can be elaborated into peptides (**23**)[274]. These undergo intramolecular *C*-alkylation with $LiNPr^i_2$ in a stereoselective fashion.



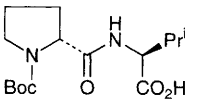
(21)



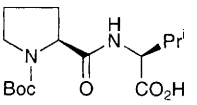
(22)



(23)



(LL, 8.5)



(DL, 1)

Scheme 11

3 Appendix: A List of Syntheses Reported Mainly in 1994

The syntheses are listed under the name of the peptide or protein to which they relate, but no arrangement is attempted under the subheading. In some cases, closely related peptides are listed together.

Peptide/protein *Ref.*

3.1 Natural Peptides, Proteins and Partial Sequences

ACTH
Fragments (1–10), (11–24) and (1–24) 275

β-Amyloid
Fragment (25–35) 276,277

Angiotensin
Analogues 278–280

Anthopleurin-A
Sea anemone anthopleurin 281

Antibiotic peptides
Analogues of antibiotic K-582A 282
Modelins, antiviral peptides 283
Chimeric peptide based on amoebapore and melittin 284
Hypelcin A-III from *Hypocrea peltata* 285

3.4 Conformation of Synthetic Peptides

3.5 Glycopeptides

3.9 Miscellaneous Peptides

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3

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

By S.C.G. BIAGINI and M. NORTH

1 Introduction

Readers may notice a slight change in the content and emphasis of this chapter this year, mainly due to the change in authorship. Also this year, the method of obtaining relevant references has been changed; thus references were initially obtained from the on-line science citation index for 1994 using a series of keywords. These were then whittled down to a more reasonable number by hand, after which the original articles were consulted when they were available in either the UWB or RSC libraries, otherwise an abstract of the paper was obtained. Two results of this change have been a significant increase in the number of journals abstracted in this chapter, and a limitation on references to those in the 1994 database which covers a few articles from the end of 1993 and most papers published in 1994. However, no 1995 papers have been included, and some papers published in December 1994 will be included in the next annual review. No work published in patents or in unrefereed form (such as the proceedings of the 23rd European peptide symposium) has been included.

The structure of this chapter means that many references could have been included in more than one place. However, in order to keep the chapter to a reasonable length, preference has been given to grouping references according to their biological activity rather than by the particular conformational constraint/isostere that they contain. An exception has been made however, when the above policy would have placed the referenced work in a miscellaneous section. Throughout this chapter, amino acids are referred to by their one or three letter codes following standard nomenclature. If no stereochemistry is specified, then the amino acid is of the natural L-configuration.

Whilst the general format of the review has been retained, a number of changes have been made to specific sections. The titles of some subsections have been broadened to permit more information to be placed within them, and reduce the length of the miscellaneous sections. A few sections have been deleted due to lack of material this year, and replaced by other sections. In particular, three totally new sections have been introduced, the first on amino acids with modified side-chains covering the substantial amount of work reported on the synthesis and applications of unusual amino acids with conformationally constrained side-

chains, or with transition state analogues within their side-chains. The second new section brings together the large amount of work done on RGD containing peptides and analogues. Finally, a section on helical peptides has been introduced to complement the existing section on peptide turns. The authors hope that the readers will find these changes helpful.

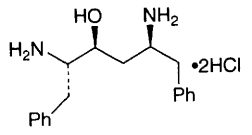
2 Peptide- backbone Modifications

A short review has been published, outlining the progress of peptides to drugs. Five examples describing the application of a peptidomimetic design cycle in medicinal chemistry were described, illustrating the progression from native peptides to therapeutically useful drugs.[1] Most of the many papers published on hydroxyethylene peptide bond isosteres are included in the various subsections of section 5 of this chapter, as they were designed for use within specific enzyme inhibitors. However, a practical synthesis of the hydroxyethylene dipeptide isostere **1**, has been reported. The synthesis started from phenylalanine, and involved the formation and stereospecific reduction of an enaminone.[2] Another protocol, for the preparation of peptides containing an hydroxyethylene isostere, has also been described. This method, which allows variation of the amino acid sequence, is based upon the $CrCl_2$ mediated allylation of *N*-protected α-amino aldehydes.[3]

2.1 ψ[NHCO]-Retro-inverso Analogues – The minimum-energy conformations of *N,N'*-dimethyl-2-methylmalondiamide and *N,N'*-dimethyl-2-dimethyl-malondiamide (ψ[NHCO]-retroamide analogues of Ala and Aib, respectively) have been computed using the AM1 semi-empirical method. The molecules were found to adopt a conformation possessing an intramolecular six-membered ring hydrogen-bond structure as shown in **Figure 1**, and the same trend was observed in malonamide derivatives substituted at the central carbon atom. The results were also compared with previous molecular-mechanics calculations.[4]

The optically pure fluorinated β-lactam **2** was used as a synthetic building block for the synthesis of the fluorinated retroamido peptide bond isostere **3**. This peptide isostere can be considered to be an analogue of the potent HIV-1 protease inhibitor **4**, though as yet no biological activity data has been reported.[5]

2.2 ψ[CH_2NH]-Amino Methylene and ψ[CH_2O]-Ether Analogues – The pseudo-pentapeptide *cyclo*-(Arg-Gly-Asp-D-Phe-ψ[CH_2NH]Val) has been synthesised by a combination of solution and solid-phase methods. The peptide showed a βII' conformation, and the ψ[CH_2NH] bond was found to be a strong hydrogen-bond donor which thus rigidified the peptide backbone.[6] Reductive amination of Fmoc-amino aldehydes by sodium cyanoborohydride, has been described and utilised as an approach to the synthesis of ψ[CH_2NH]-containing peptide analogues. Thus for example, a mixture of *N*-Fmoc-*O*-*t*-butyl-tyrosinal and glycine yielded mainly Fmoc-Tyr(O^{t}Bu)-ψ[CH_2NH]-Gly-OH when treated with



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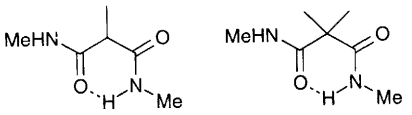
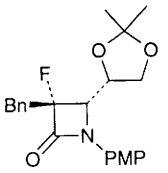


Figure 1



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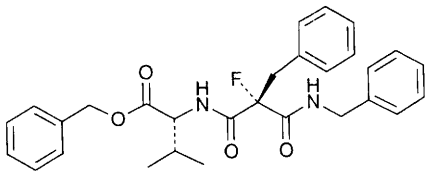

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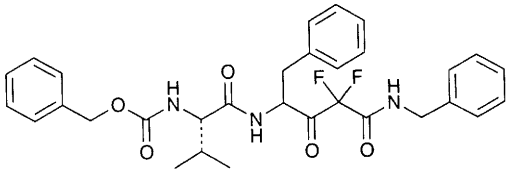

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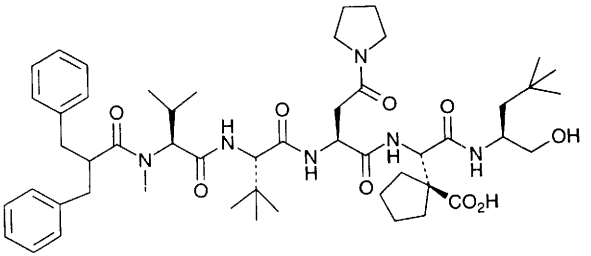


(5)

sodium cyanoborohydride.[7] For an example of the use of a $\psi[CH_2N(CH_3)]$ peptide bond isostere, see section 2.5.

YIGSR-NH_2, A synthetic peptide from the B1 chain of laminin, which reportedly has potential as an antimetastatic agent, has been studied, in an effort to analyse the structure-activity relationships and improve its antimetastatic potency. A series of $\psi[CH_2NH]$ peptide analogues of YIGSR-NH_2 and a number of peptides in which the Tyr residue was replaced with D-Tyr, Phe, *p*-FPhe, and *p*-NH_2Phe, were prepared. All new peptides were assayed *in vitro* for their ability to promote cell attachment in both B16F10 mouse melanoma cells and HT-1080 human fibrosarcoma cells. On the basis of the *in vitro* assay results, the most active peptides were tested *in vivo* for their ability to inhibit tumour metastasis to the lungs in mice that were coinjected with B16F10 melanoma cells and 1 mg of peptide. Of the nine new peptides, only the *p*-NH_2Phe derivative showed consistent *in vitro* cell attachment activity, but with only low *in vivo* antimetastatic activity.[8] The molecular mechanisms involved in the chain assembly of laminin have also been investigated using synthetic peptides. The research also revealed the importance of the isoleucine residues, in stabilising the heteromeric triple-stranded coiled-coil structure.[9] Laminin-related peptides, Tyr-Ile-Gly-Ser-Arg analogues, have also been prepared, showing that the L-Arg residue was very important for the production of inhibitory effects on experimental metastasis. Poly(ethylene glycol) hybrids of Tyr-Ile-Gly-Ser-Arg-Gly were also prepared, and found to have greater inhibitory potency than the simple peptide.[10]

p21^{ras} Farnesyl transferase (FTase), an enzyme responsible for post-translational modification of proteins, has been reported to be inhibited by simple CA_1A_2X peptidomimetics (A = aliphatic amino acid, and X = serine or methionine). The structural requirements of these peptide analogues was investigated. The central A_1A_2 dipeptide, was replaced by 3- or 4-aminomethylbenzoic acid (AMBA) and 3- or 4- aminobenzoic acid (ABA). The results indicated that interaction between FTase and the peptidomimetics, requires precise structural and conformational characteristics (Cys-4-ABA-Met, is 128 times more potent than Cys-3-ABA-Met). Computer modelling of the Cys-4-ABA-Met inhibitor showed that a folded conformation, where the thiol and carboxylate groups are close, was not possible. Therefore a β-turn conformation, that allowed simultaneous coordination of the Cys-thiol and Met-carboxylate to the zinc ion, is not important for inhibition of p21^{ras}FTase, as previously suggested.[11] A systematic study of CVFM, a CAAX-type farnesyl transferase inhibitor, has been undertaken to determine the structural elements important for intrinsic activity as well as substrate character. The results indicated a narrow profile for nonsubstrate farnesyl transferase inhibition.[12] Also, CAAX motif peptides have been derivatised with the light sensitive benzophenone group (Bz), in order to permit their use as catalytic site-directed covalent photocross-linking ligands for farnesyl transferase. Results, with the Bz_2-GYPCVVM peptide in particular, implied that the catalytic domain on farnesyl transferase is comprised of an interfacial cleft at the contact region between α- and β-subunits, a domain that is shared by both CAAX and farnesyl substrate moieties.[13] Pseudopeptides related

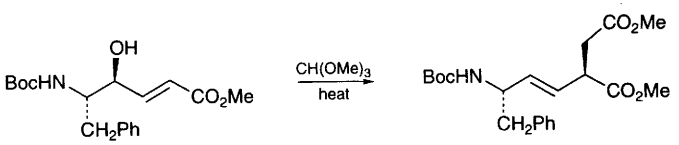
to the *C*-terminal tetrapeptide of the Ras protein that signals farnesylation, have been reported as inhibitors of Ras farnesyl transferase. The most potent *in vitro* enzyme inhibitor described was Cysψ[CH_2NH]Ileψ[CH_2NH]Phe-Met (IC_{50} = 5nM). Structure-activity studies also led to (Cysψ[CH_2NH]Val-Ile-Leu), a potent and selective inhibitor of a related enzyme, the type-I geranylgeranyl protein transferase.[14] Another report described the synthesis of several hydrophobic inhibitors of $p21^{ras}$ farnesyl transferase. These peptidomimetic structures, containing cysteine and methionine residues separated by an aromatic spacer, were functionalised as their methyl esters and Z-derivatives, and were shown to penetrate NM 3T3 cells to disrupt $p21^{ras}$ plasma membrane association.[15]

The conformation of the 29-residue rat galanin neuropeptide has been studied using Monte Carlo methods and the ECEPP/3 force field. The results were found to be in qualitative agreement with the available NMR and CD data of galanin.[16] Structure-activity relationship studies have revealed that the first three residues of galanin (Gly^1-Trp^2-Thr^3) are of critical importance for high-affinity binding to the galanin receptor. To obtain galanin receptor ligands with long-lasting biological activity the amino-terminus of galanin was protected. Analogues of rat galanin(1-16) carrying modifications at the three amino-termini of galanin, were prepared. All modifications of the peptide backbone flanking Trp^2 as in the analogues [*N*-Me-Trp^2]-galanin(1-16), [Tcc^2]-galanin(1-16), (Trp^2-ψ[CH_2NH]-Thr^3)-galanin(1-16) produced a dramatic loss of affinity toward the galanin receptor. [*N*-Me-Thr^3]-galanin(1-16) was the most active of the peptide backbone modified analogues (K_D = 997nM). Modifications to the indole ring in Trp^2 {[For-Trp^2]-galanin(1-16), [Tcc^2]-galanin(1-16)} yielded analogues which, at concentrations up to 10mM, did not displace [^{125}I]galanin binding. *N*-Methylation of Gly^1 by the introduction of sarcosine {[Sar^1]-galanin(1-16)} did not significantly affect the ligand-binding properties of galanin(1-16) (K_D = 8.7nM).[17]

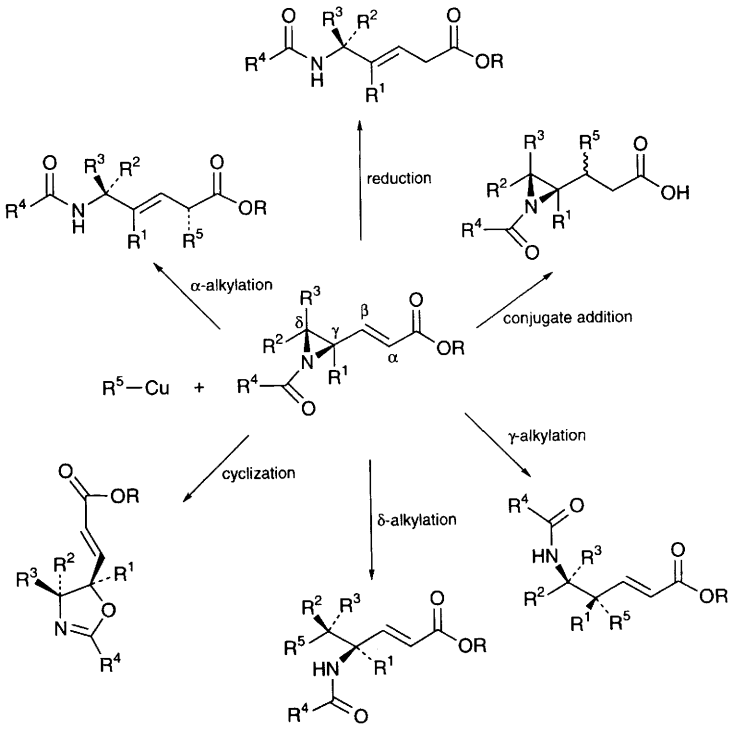
The solution conformations and molecular modelling studies of Boc-Val-ψ[CH_2O]-Leu-OH and the corresponding unmodified dipeptide Boc-Val-Leu-OH have been described by Villeneuve *et al.* The study also included the determination of the solid state conformations by X-ray crystallography. The results obtained showed a similar structural geometry for the two compounds, although they differed in the degree of conformational constraint. The dipeptide sequence was studied as a model for the Val^2-Sta^3 unit of pepstatin A.[18] An ether based peptide analogue containing an ether linkage in place of the Ser-Leu-Gly unit of the PKA substrate kemptide, has been prepared and found to be a competitive inhibitor of kemptide phosphorylation catalysed by PKA.[19]

The peptidomimetic BILD-1263 **5**, has been reported to be an inhibitor of HSV ribonucleotide reductase with *in vivo* antiviral activity. In cell cultures, compound **5** suppressed the replication of HSV-1, HSV-2 and acyclovir-resistant HSV strains.[20]

2.3 ψ[CH = CH] Isosteres and Related Analogues. – A stereospecific approach to ψ[*E*-CH = CH] isosteres in which the *C*-terminal amino acid is a replacement for



Scheme 1



Scheme 2

aspartic acid has been reported. The key step in the synthesis involved an orthoester Claisen rearrangement of an allylic alcohol derived from amino acids as shown in **Scheme 1**.[21] A diastereo- and enantioselective synthesis of the (*E*)-alkene dipeptide isostere of Ala-Ala from alanine has been developed. The synthesis proceeded in seven steps from alanine methyl ester, and involved stereocontrolled addition of a (*Z*)-vinyllithium reagent, followed by a [2,3]-Wittig rearrangement.[22] ψ[*E*-CH=CH] peptide isosteres have also been prepared in good diastereomeric excess by a stereoselective alkylation of peptide aziridines with organocopper/BF_3 complexes (**Scheme 2**).[23]

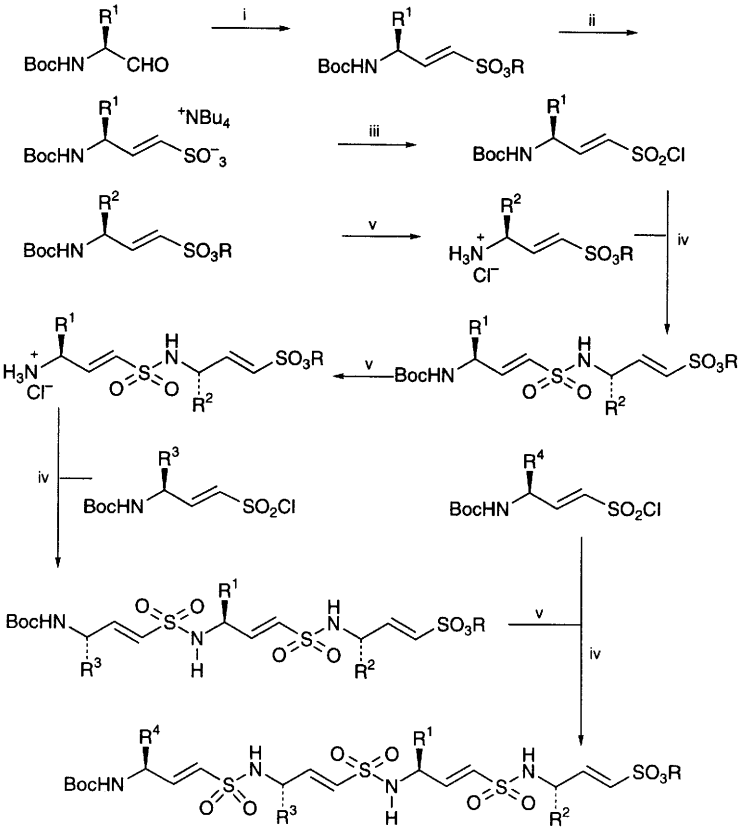
A new synthesis of fluoro-olefin peptide isosteres has been described, in which the Peterson olefination reaction was utilised. Four tetrapeptides incorporating this isostere [(*E*)- and (*Z*)-Ala-Gly-ψ[CF=C]-Pro-Phe, and (*E*)- and (*Z*)-Ala-Gly-ψ[CF=C]-(*R*)-Pro-Phe] were prepared, and three of these were found to be inhibitors of peptidyl-prolyl isomerase.[24]

2.4 Phosphorus Containing Peptide Bond Isosteres – Mechanistic studies and the insights into the biological pathways of peptide hydrolysis which can be obtained have maintained the interest in the synthesis of phosphorus based transition state analogues of amide hydrolysis during the period of this review, though most of the material will be found in the section on specific biologically active peptides. A stereospecific synthesis of phosphonate analogues of diaminopimelic acid has been reported. The analogues were generally found to be weak competitive inhibitors of diaminopimelic acid enzymes, and showed negligible antibacterial activity.[25]

The phosphinic isostere containing tetrapeptide, Z-Phe-ψ[PO_2CH_2]-Gly-Pro-Nle, was found to be a potent inhibitor of collagenase (from *Corynebacterium rathayii*) with a K_1 value of 8nM. Increasing the length of the phosphinic unit containing inhibitors from tetra- to hepta-peptides further improved the potency of these compounds. The heptapeptide analogue, Z-Phe-Gly-Pro-Phe-ψ[PO_2CH_2]-Gly-Pro-Nle-OMe, with a K_1 value of 0.6nM, is the most potent inhibitor reported to date for bacterial collagenases.[26]

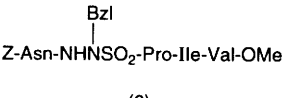
2.5 Sulfur Containing Peptide Bond Isosteres – An iterative process for the synthesis of sulfonamido-pseudopeptides, based on chiral vinylogous aminosulfonic acids (γ-amino-α,β-unsaturated) has been described as shown in **Scheme 3**.[27] The functionalisation of the double bond and the incorporation into solid-phase synthesis of these analogues has however yet to be reported. Azasulfonamidopeptide derivatives (-CONHNRSO_2NH-) have been suggested as a novel class of peptide bond hydrolysis transition state mimics. Synthetic approaches to these compounds, and the X-ray structure of two examples have been reported by Jones and co-workers.[28] This isostere was then incorporated into peptide **6**, which was designed as a HIV-1 proteinase inhibitor, but which showed only low activity.[29]

Bombesin (Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) and the related peptide litorin (pGlu-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) have been modified at positions 13-14 and 8-9 respectively, with ψ[CH_2S]



Reagents: i, $(EtO)_2PO\text{-}CH_2SO_3R$, Bu^nLi, THF, –78 °C; ii, Bu_4NI, acetone, reflux; iii, SO_2Cl_2, Ph_3P, CH_2Cl_2, 3 Å molecular sieves; iv, DBU, cat. DMAP, 25 °C; v, 3M HCl in MeOH

Scheme 3



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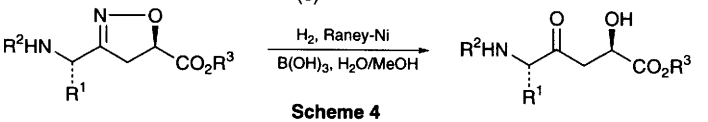


Scheme 4

and ψ[$CH_2N(CH_3)$] peptide bond isosteres. The physicochemical properties and biological activities were also examined. [Phe^{13}ψ[CH_2S]Leu^{14}]bombesin and [Phe^8ψ[CH_2S]-Leu^9]litorin bound to the murine pancreatic bombesin/gastrin releasing peptide receptor with similar dissociation constants ($K_d = 3.9$ and 3.4nM, respectively). Oxidation of the thiomethylene ether to two diastereomeric sulfoxides was found to increase potency, whereas further oxidation decreased potency. The analogues were determined to be receptor antagonists. ψ[$CH_2N(CH_3)$] substitution at the 8-9 amide position resulted instead in the formation of an agonist. All the peptides were determined to possess ordered conformations by CD, though different conformations corresponding to agonist and antagonist structures were suggested. Based on the pH dependence of the fluorescence spectra of the peptides in a zwitterionic detergent, two titratable groups were identified ($pK_a = 6.3$ and 8.5). The lower pK_a was found in the agonist analogues but not in the ψ[CH_2S]-containing antagonist.[30]

2.6 Ketone Containing Isosteres – A high yielding synthetic route to α-hydroxy ketomethylene dipeptide isosteres from the corresponding 2-isooxazoline dipeptide isosteres by hydrogenolysis and hydrolysis has been reported as shown in **Scheme 4.**[31]

α-Ketoamide units (-NH-CO-CO-) in intact peptides have been generated from Ser/Thr residues *via* Ru(VIII)-catalysed C^{α}-C side-chain scission. The reaction was found to be selective, with few unwanted side reactions; Cys and Met residues however were found to oxidise to sulfones under the reaction conditions.[32] The structures of a range of peptides having the oxalamide (-NH-CO-CO-NH-) unit located at their centre, have been determined. The oxalamide group was found to possess the *trans* conformation in two retropeptides, and an approximately orthogonal conformation in the peptide containing Pro residues. Torsional angles about the CO-CO bond were 180° in MeO-Aib-CO-CO-Aib-OMe, 175° in MeO-Leu-CO-CO-Leu-OMe, and -108 and -106° for the two independent molecules in the crystal of MeO-Pro-CO-CO-Pro-OMe, owing to steric hindrance between CO and the pyrrolidine ring. Other crystal data for these peptides were also reported.[33]

2.7 Hydrazine, Hydrazone, and Related Isosteres – The crystal structures of four hydrazino peptides Piv-Pro-h(N^{α}Bn)Gly-NH^iPr, Piv-Pro-hAla-NH^iPr, Moc-hPro-NH^iPr and Boc-hPro-Gly-N(OH)Me, derived from the hydrazino analogues of glycine (hGly), alanine (hAla) or proline (hPro) have been determined. A common folded structure of the α-hydrazino acid residue characterised by a bifurcated hydrogen bond closing an eight-membered ring, was revealed as illustrated in **Figure 2.** This folded structure is topologically similar to the βII′-turn in peptides, and the CO-NH-N hydrazine link can be considered as a good turn-inducer in peptide analogues.[34]

The semicarbazone moiety (C-CH=N-NR-CO-NH-C) has been proposed as a stable peptidomimetic. The semicarbazone moiety may be obtained either by the coupling of a peptide aldehyde with a semicarbazide, or by the action of an alkylisocyanate on a peptide hydrazone. This peptidomimetic has been used to prepare four analogues of the Pro-Gly, Pro-Ala and Pro-Phe dipeptides, which were

studied by ^{1}H-NMR and IR spectroscopy, and in the solid state by X-ray diffraction.[35] The semicarbazone unit has also been used in the synthesis of other peptide analogues. Thus H-Asp(O^{t}Bu)-Sc (Sc = semicarbazide) has been used to synthesise a series of mono-, di- and tripeptide aldehydes, as well as multigram quantities of Ac-Tyr-Val-Ala-Asp-H, a potent reversible inhibitor of interleukin-1b converting enzyme. Biological evaluation of these peptide aldehydes suggested that the tripeptide scaffold, Z-Val-Ala-Asp, retains good potency and selectivity for the enzyme.[36]

2.8 α,α-Dialkylated Glycine Analogues – The synthesis and crystal structures of fully protected tripeptides containing $C^{\alpha,\alpha}$-diphenylglycine (Dph), namely Z-Aib-Dph-Gly-OMe and Bz-Dph-Gly-OMe has been reported. The X-ray data suggested the formation of mixed local conformations in the crystals.[37] The fully blocked pentapeptide Tfa-(Deg)$_2$-Abu-(Deg)$_2$-O^{t}Bu (Deg = $C^{\alpha,\alpha}$diethylglycine) was found to adopt a 3_{10}-helical structure in the solid state. In solution, however, C_5 extended local conformations were observed. The conformation of the solid state structure was attributed to the perturbation caused by the Abu residue.[38] The X-ray diffraction crystal structures of the (αMe)Leu derivative Ac-D-(αMe)Leu-OH and the terminally protected tripeptide Z-D-(aMe)Leu-Ala$_2$-OMe showed the onset of the fully extended (C_5) conformation for the (αMe)Leu residue in both independent molecules in the asymmetric unit of the former compound and in two out of the four independent molecules in the asymmetric unit of the latter compound. The results represent an indication that this peptide secondary structure, uncommon for protein amino acids and other *C*-α-methylated chiral residues, is not a rare observation in (αMe)Leu derivatives and short peptides.[39] The conformational transition between the α- and the 3_{10}-helical states of α-methylalanine (Aib) have been studied by molecular mechanics. The activation free energies for the forward and reverse transitions were found to be significantly lowered, when entropic stabilisation effects were included in estimates based solely on potential energy.[40]

A diastereoselective cyclopropanation of a chiral α,β-dehydroamino acid **7**, obtained from D-mannitol, resulted in the formation of a single stereoisomer of compound **8** as shown in **Scheme 5**. This chemistry provides a new route to enantiopure 1-aminocyclopropane-1-carboxylic acid derivatives.[41] Conformationally strained tryptophan derivatives **9**, possessing a ring that bridges the α-carbon and the 4-position of the indole ring, have been prepared. The key step involved palladium-catalysed cyclisation of an α-propenyl tryptophan derivative under Heck conditions.[42]

A membrane-modifying peptide antibiotic, trichosporin B-VIa, has been synthesised by solution-phase methodology. The Aib^{14} analogue, in which Pro^{14} was replaced by Aib, was also synthesised, to modify the secondary structure of trichosporin B-VIa.[43] The effect of C^{α}-methyl groups on the conformational ensemble of gonadotrophin releasing hormone (GnRH) analogue peptides, has been examined using NMR methods. Two analogues were prepared: Ac-D-Nal-D-C^{α}Me-4-ClPhe-D-Pal-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2 and Ac-D-Nal-D-C^{α}Me-4-ClPhe-D-Pal-Ser-C^{α}Me-Tyr-D-Arg-Leu-C^{α}Me-Arg-Pro-D-Ala-NH_2. The results indicated that the C^{α}-methyl groups in residues 5 and 8 of the latter

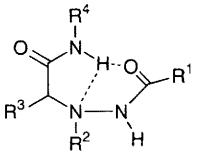
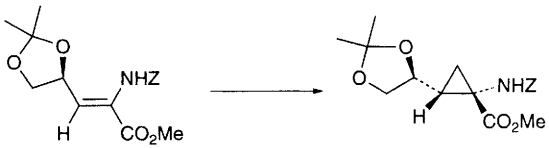
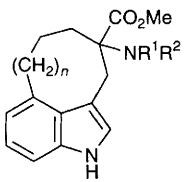


Figure 2



(7) (8)

Scheme 5



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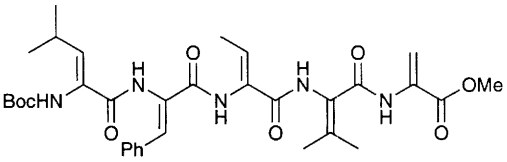

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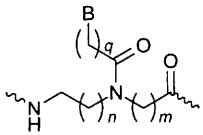
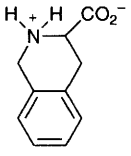


Figure 3



Tic

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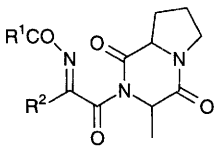


(12)

peptide do not influence the backbone or the global conformation of the peptide.[44]

2.9 Dehydroamino Acid Analogues – Ni^{II}, Zn^{II} and Co^{II} complexes of α,β-dehydro-dipeptides (also containing Gly, Leu, Ala, Val or Phe residues) have been studied by potentiometric and spectroscopic methods. Deprotonation and co-ordination of amide nitrogens occurred in all cases around the physiological pH range. The dipeptides with composition of Xaa-ΔAla formed octahedral species, whilst Gly-ΔXaa (Xaa= Leu or Phe) formed square planar *bis*-complexes with Ni^{II}.[45]

A synthesis of dehydropeptides (e.g. **10**), containing only α,β-dehydroamino acid residues has been described. The preparations, involved fragment condensation, followed by β-elimination of water from β-hydroxy amino acids in modest overall yields.[46]

The conformations of a series of model peptides Ac-Pro-ΔXaa-NHMe, where Xaa= Ala, (*Z*)-Abu, (*E*)-Abu, Val, (*Z*)-Leu or (*Z*)-Dhe, were investigated using, NMR, IR, CD and X-ray crystallographic methods. Generally, the peptides were found to adopt a βII-turn conformation in inert solvents, unfolded conformations in polar media, and a βI-like turn within crystals. However, the ΔAla containing peptides, did not form any β-turn containing structures.[47] The structure of a peptide containing a *C*-terminal dehydrophenylalanine, Z-Gly-Δ^{Z}Phe was determined from X-ray diffraction data. The peptide adopted a conformation folded at the glycine residue. Two intermolecular hydrogen bonds join the folded molecules into columns and link columns to each other respectively. FTIR spectroscopy, showed the presence of a third hydrogen bond, which was interpreted to be of the N-H...N type.[48] The synthesis and crystal structure determined molecular conformation of *N*-Ac-ΔPhe-Leu-OCH_3 and N-Ac-ΔPhe-NVa-OCH_3 have also been reported.[49]

Two model peptides containing dehydrophenylalanine, namely a tetrapeptide (Ac-Δ^{Z}Phe-Pro-Δ^{Z}Phe-Ala-OMe) and a heptapeptide (Boc-Gly-Δ^{Z}Phe-Val-Δ^{Z}Phe-Ala-Δ^{Z}Phe-Leu-OMe), have been synthesised, and their solution conformation investigated by NMR and CD techniques. The results indicated that the conformational restraints imposed by the dehydrophenylalanine residues in alternate positions favours the formation of 3_{10}-helices within the peptides.[50] However, the same workers also synthesised the pentapeptide Boc-Pro-ΔPhe-Ala-ΔPhe-Ala-OMe; the crystal structure determined conformation of which showed that this peptide adopted a non-helical β-bend ribbon structure.[51]

A quantum mechanical study to compare the ability of Aib, ΔAla and Ala to stabilise helical conformations has been effected. The results indicated that the Aib and ΔAla residues are better helical formers than Ala.[52] The conformations of poly(dehydroalanine) have been studied using molecular mechanics methodology. An exhaustive search of the available conformational space was carried out on a ΔAla octapeptide model. Two major groups of low-energy conformers were found, one corresponding to a regular 3_{10}-helix or type III turn, the other to an irregular conformation, possessing ψ= -157 to -170° and ϕ= -1 to 15° values which can however also be found in the $i + 2$ position of γ-turns. The data

confirm that ΔAla may induce turn-like structures in peptides and also indicate that it may confer flexibility to the peptide chain.[53]

2.10 Miscellaneous Modifications – The formyl tridepsipeptides HCO-Met-ψ[COO]Leu-Phe-OMe, HCO-Met-ψ[COO]Aib-Phe-OMe, and HCO-Met-Leu-ψ[COO]-Phe-NHBz have been synthesised, in an effort to investigate the effect of the introduction of an ester bond at various positions in the chemotactic tripeptides. Only the third of these compounds retained biological activity.[54] The benzodiazepine containing peptidomimetic BZA-5B has been reported to inhibit the MAP kinase activation pathway in H-Ras-transformed Rat-1 cells, but not in untransformed Rat-1 cells.[55]

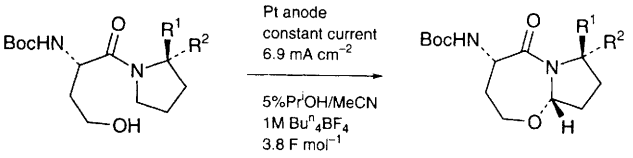
Peptide nucleic acids (PNA's) are currently attracting much interest as novel and highly specific DNA binding agents which have the added advantage of being relatively easily prepared. PNA oligomers where one of the repeating backbone units is extended with a methylene group to either *N*-(2-aminoethyl)-β-alanine or *N*-(3-aminopropyl)glycine have been prepared. Alternatively, the linker to the nucleobases could be extended from methylenecarbonyl to ethylenecarbonyl as shown in **Figure 3**. The PNA oligomers bound modestly to complementary DNA oligonucleotides in an antiparallel direction.[56]

The constrained dipeptide, Z-Tic-Phe-OMe, (Tic= **11**) has been studied by X-ray diffraction. The conformation of the peptide linkage was found to be *trans*, and in the crystal the molecules are held together by an intermolecular hydrogen bond and by van der Waals forces between the hydrophobic phenyl groups.[57]

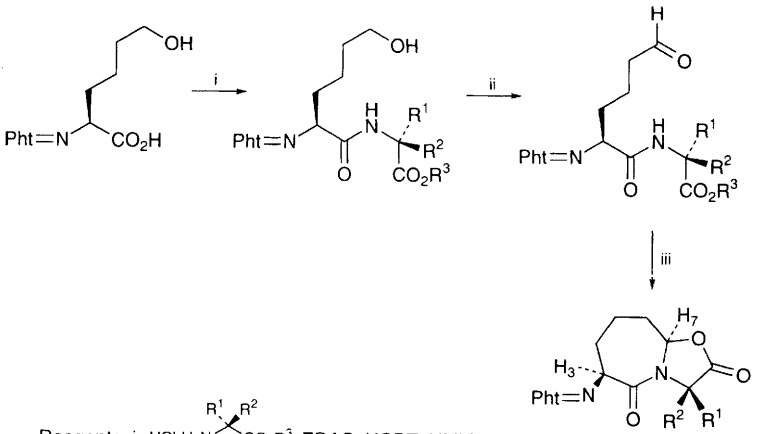
As models for the synthesis of tetrahedral intermediates related to ergopeptines, *N*-(α-acylimino)acyldioxopiperazines **12** such as *N*-(Ac-D-Phg)-*cyclo*-(Val-D-Pro) have been prepared.[58] The synthesis of a common main skeleton of thiostrepton peptides, A10255G and J, containing thiazole and oxazole rings has been described.[59]

3 Conformationally Restricted Cyclic and Bridged Analogues

3.1 Rings and Bridges Formed *via* Amide Bonds – A variety of methodologies have been reported in recent years for the head-tail cyclisation of peptides. Recently, McMurray and Lewis have reported the synthesis of three such cyclic decapeptides using Fmoc-solid phase peptide synthesis on a 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMBA) linked polystyrene resin, followed by cleavage of the protected peptides from the solid support, and cyclisation in solution. The authors report high yields and purity of the peptides along with rapid coupling times using this methodology.[60] A synthetic improvement to standard solid-phase synthesis followed by cyclisation methods, for the synthesis of cyclic penta- and hexapeptides, has been suggested by Sowemimo *et al.*, based on the employment of the DAS-resin for solid support. Three cyclic peptides were synthesised in good yields and purity.[61] Starting from the α-(2,4-dimethoxybenzyl) ester of *N*-Fmoc-aspartic acid, side-chain-protected resin-bound Fmoc-peptides containing an *N*-ε-1-(4,4-dimethyl-2,6-



Scheme 6



Scheme 7

Figure 4

dioxocyclohexylidene)ethyl lysyl residue have been prepared. Cyclic peptides comprising 7-14 amino acid residues were subsequently obtained employing this procedure. As a model conjugation, *cyclo*-[Thr-Asn-Asn-Asn-Leu-Lys(SAMA)-Thr-Lys-Asp] was coupled with bromoacetamide. The same peptide was also coupled with a bromoacetylpeptide to give a well defined peptide/peptide conjugate. All of the peptides prepared in this study were conjugated to bromoacetylated tetanus toroid for immunisation purposes.[62]

Bicyclic peptide mimetics, based on lactams and aminals have been synthesised in reasonable yield by the anodic oxidation (NCH_2 to NCH(OMe)) of a *C*-terminal proline moiety in a dipeptide containing unsaturated amino acids or homoserine derivatives, followed by a diastereoselective cyclisation as shown in **Scheme 6**. The anodic oxidation however did not work for unsaturated substrates containing an oxygen substituent α to the amide bond.[63] Bicyclic lactams, were also prepared by workers in another laboratory, whereby the key step involved an intramolecular *N*-acyliminium ion cyclisation to generate 7,6- and 7,5-fused rings,[64] however the incoroporation of these bicyclic lactams into a peptide backbone has yet to be reported.

γ-Lactams, formed by the regioselective aza-annulation of enamines with acryloyl chloride, followed by hydrogenation, have been prepared as β-amino acid analogues which possessed structural features similar to those of established peptide isosteres.[65] A new method has been described for the preparation of substituted seven-membered ring lactams as shown in **Scheme 7**.[66]

The cyclic dipeptides *cyclo*-[(*S*)-His-(*S*)-Leu] and cyclo-[(*S*)-His-(*S*)-Phe] are catalysts for the asymmetric preparation of cyanohydrins from aldehydes and HCN. The solution state conformations of these diketopiperazines has been studied extensively by NMR techniques as well as by molecular modelling calculations. The results showed that for *cyclo*-[(*S*)-His-(*S*)-Leu], the imidazole ring is folded over the diketopiperazine ring as shown in **Figure 4**. This is the opposite conformation to that determined for cyclo-[(*S*)-His-(*S*)-Phe], and may explain the opposite sense of asymmetric induction between the two catalysts.[67]

Three cyclic hexapeptides, *cyclo*-(Gly-Pro-Phe-Val-Xaa-Phe) where Xaa = Phe, D-Phe or D-Pro, have been prepared and investigated by NMR methods. Each peptide exhibited two conformations, due to *cis-trans* isomerisation about the Gly-Pro peptide bond. The *cis*-Gly-Pro segment in the minor isomer was found not to be involved in a βVI-turn, but formed a turn structure with *cis*-Gly-Pro in the i and $i+1$ positions. The turn possessed no hydrogen bond but closely resembled a βI-turn and was thus labelled a pseudo-βI-turn. The major *trans*-isomers were found to adopt a standard βI-turn, with the proline in the $i+1$ position.[68] The preparation *via* solution state methods of *cyclo*-(Pro-Phe-β-Ala-Phe-Phe-β-Ala), has also been described. The conformation of the peptide was analysed in both the solution and solid state, and the results confirmed the authors expectation of the low propensity of β-alanyl residues to be positioned at the corners of a turned structure.[69] The same group also prepared and analysed the cyclic hexapeptide *cyclo*-[(Pro-Phe-β-Ala)$_2$], and coined the term 'pseudo type II β-turn' to describe the conformation of the *cis* β-Ala-Pro peptide bond found in this peptide.[70]

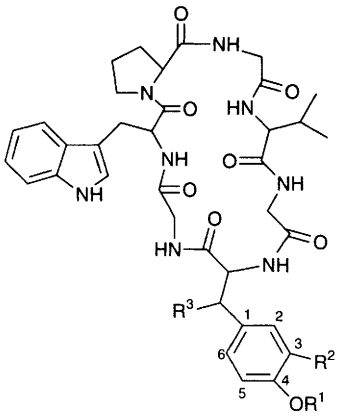
A theoretical analysis of the conformational constraints imposed by *N,N'*-ethylene and *N,N'*-propylene-bridges has been reported. The size and/or pucker type of the ring constraint was found to have little effect on the peptide backbone, which always adopted either one of two distinct semi-extended forms.[71] 24-Membered ring pseudopeptides, *cyclo*-[Gly-eXX-Gly]$_2$ {eXX; *N,N'*-ethylene-bridged dipeptide, X = (*S*)-alanine, leucine, isoleucine, or phenylalanine} and *cyclo*-[Sar-eXX-Gly]$_2$ {X = alanine or phenylalanine} have been prepared, and their conformations investigated by NMR spectroscopy in DMSO. It was found from NMR measurements that the major conformers of the six peptides studied had C_2-symmetric structures, though they differed in the number of *cis*-amide bonds. Thus for *cyclo*-[Gly-eXX-Gly]$_2$ (X = A, L, or I), and for *cyclo*-[Sar-eAA-Gly]$_2$, all of the amide bonds were *trans*, whilst the two Gly-eFF peptide bonds of *cyclo*-[Gly-eFF-Gly]$_2$ and the two Sar-eFF bonds of *cyclo*-[Sar-eFF-Gly]$_2$ were found to be *cis*. The structures of *cyclo*-[Gly-eAA-Gly]$_2$ and *cyclo*-[Sar-eAA-Gly]$_2$ were optimised by molecular mechanics on the basis of the NMR data.[72]

The solution-phase synthesis and conformational analysis of the *S*-glycosylated cyclic hexapeptide *cyclo*-(D-Pro-Phe-Cys(tetra-*O*-acetyl-β-D-galactopyranosyl)-Trp-Lys(*Z*)-Phe) has been effected, in order to investigate the influence of a saccharide residue in position *i* of a standard β-turn on the formation of reverse turns and on the biological activity. The peptide was investigated by NMR techniques and refined by molecular dynamics calculations. Comparison of the *S*-glycosylated-Cys^3 peptide with the analogous Thr^3 peptide showed that they exhibited a similar overall conformation of the hexapeptide [βII' D-Pro-Phe and another β-turn about Trp^4-Lys^5(*Z*)]. The glycopeptide showed a dynamic flip between βI, and βII type turns, whereas the Thr analogue only populated the βI type turn, owing to the presence of a hydrogen bond bridge lacking in the glycopeptide.[73]

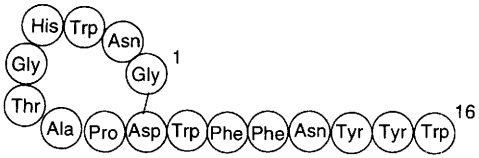
Cyclo-(Phe-Pro)$_4$ and DL-noradrenalin hydrochloride have been shown to form a 1:1 complex, when examined by ^{13}C-NMR and titration techniques. The complex was found to be linked through hydrogen bonds between the carbonyl groups of the Phe^1 and Pro^2 residues and the hydrogens on the ammonium moiety of DL-noradrenalin hydrochloride.[74]

The structure in solution of the vasoconstrictor hormone urotensin II *cyclo-S,S*-(Ala-Gly-Thr-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val), using NMR spectroscopy, has been determined. A combination of distance geometry and dynamical simulated annealing techniques was used to calculate the structure. Nine resultant structures with few distance constraint violations were selected. The conformation of the molecule in the cyclic hexapeptide segment (core region) was well-defined whereas the *N*-terminal segment was disordered. This result correlated very well with the earlier predictions about the biologically active and inactive roles played by the core and the *N*-terminal segment respectively.[75]

The conformation of two cyclic hexapeptides, *cyclo*-[Ala-D-Ala-Ser-Phe-Gly-Ser] and *cyclo*-[Ala-Gly-Ser-Phe-Gly-Ser], both derived from CD4, have been characterised by high resolution NMR spectroscopy and molecular dynamics, in an effort to design peptide mimetics suitable for competitive binding.[76] The solution conformation of the linear CD4 fragment 81-92 TYICEVEDQKEE and



(13) R^1 = H, R^2 = Prenyl, R^3 = OH
(14) R^1 = H, R^2 = H, R^3 = OH



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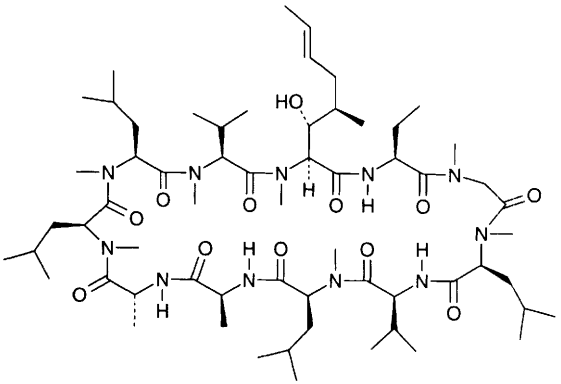


(16)

two of its benzylated analogues have also been determined by NMR spectroscopy, distance geometry and simulated annealing techniques. The structures of both benzylated derivatives were similar but distinct from that of the wild-type dodecapeptide. It was concluded from structural analysis that bulky side chains of amino acids at an appropriate position can have a marked effect on the conformation and thus the functions of a peptide.[77]

The structures of two novel cyclic heptapeptides **13** and **14**, isolated from an *Aspergillus flavipes* culture, have been elucidated by chemical and spectroscopic studies. Peptide **13** is a potent substance P antagonist at the human NK1 receptor (K_i = 8 mM), and contains the new amino acid 3-prenyl-β-hydroxytyrosine. Several analogues of **13** were prepared, and structure-activity results indicated that the 3-prenyl-β-hydroxytyrosine moiety is critical for biological potency.[78]

Cyclinopeptide A [*cyclo*-(Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe)], its tyrosine analogues with one or both phenylalanines substituted by tyrosine, and their linear counterparts with the starting sequence Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe, have been studied by NMR, CD and fluorescence methods. The results indicated that the cyclic analogues have conformations most similar to cyclinopeptide A, and that aromatic rings in the cyclic peptides are situated perpendicularly to one another, manifesting edge-to-face pairing.[79] In the same work, cyclinopeptide A and analogues thereof have also been investigated as suitable models for studying the spectroscopic properties of aromatic side-chains. The NMR studies, showed a distinct differentiation in the chemical shifts of aromatic protons, which were possibly due to edge-to-face interaction of the rings. The optical activity of the aromatic side-chains was found to depend upon their position in the peptide chain, and corresponded to differences in the side-chain conformation of both aromatic residues.

The cyclic peptide RES-701-1 **15**, was found to be a selective endothelin type B receptor antagonist. The characterisation of the producing strain of *Streptomyces sp.* RE-701, as well as the structure, physico-chemical and biological properties of RES-701-1 have been reported.[80] The solution conformations in methanol and chloroform of endothelin receptor antagonists *cyclo*-(dV-dW-dD-P) and *cyclo*-(dV-N$^{\alpha}$-MeL-dW-dD-P) have been studied by NMR methods. Both peptides were found to have a well defined peptide backbone conformation composed of a type II β-turn at the Leu-D-Trp and a γ′-turn at Pro.[81]

Cyclosporin A **16** has been used as the starting material for the semisynthetic preparation of a variety of cyclosporins.[82]

Leualacin, an L-type specific Ca^{2+} channel blocker has previously been established as a cyclic pentadepsipeptide containing leucine, *N*-methylphenylalanine, β-alanine, and *S*- and *R*-leucic acids, based upon its MS and NMR spectra.[83] A conformational analysis of leualacin in $CDCl_3$ has now been conducted, in which distance constraints were derived from ^1H-^1H and ^1H-^{13}C nOe's, and dihedral angle constraints calculated from $^3J_{H,H}$ and $^3J_{C,H}$, supplemented with ring current chemical shift information. Conformers satisfying the NMR data were obtained by a conformational grid search followed by energy minimisation. The functionally essential motifs of leualacin were thus speculated

to be the carbonyl and alkyl groups on the leucic acid residues and the phenyl ring of the *N*-methylphenylalanine.[84]

A series of 20-amino-acid containing peptides derived from the primary structure of an antigen from *Echinococcus granulosus*, have been studied using NMR and CD techniques. The linear peptide corresponding to the sequence 93-112 in the antigen was found to adopt in large proportion the α-helix structure. Derivatives containing lactam bridges were found to destabilise the α-helicity of the peptides.[85]

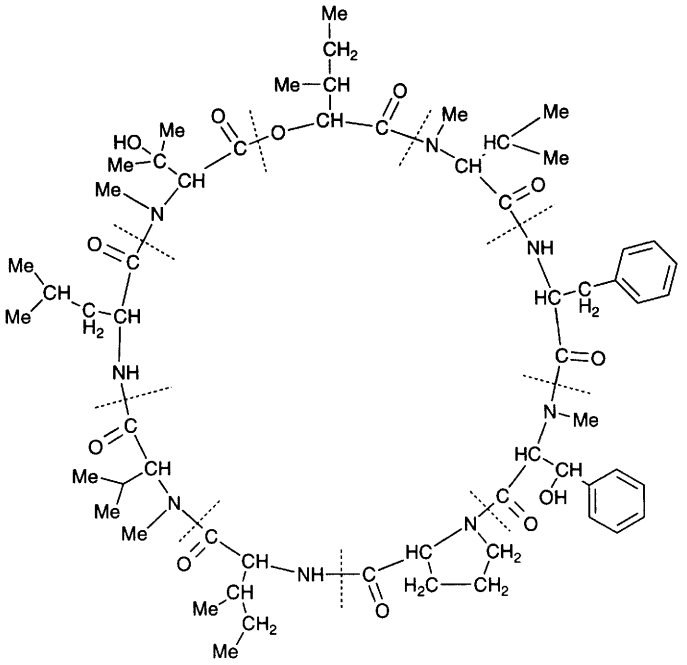
The solid-state and solution conformations of aureobasidin E **17**, a new type of cyclic depsipeptide antibiotic, have been analysed by X-ray diffraction and NMR spectroscopy. NMR spectroscopy showed that the solution conformation was similar, though more flexible, to the solid state conformation. The structure activity relationship of certain functional groups of aureobasidin E was also discussed.[86]

(-)-Bistramide C **18,** a macrocyclic hexapeptide, which contains thiazole and oxazole rings, has been synthesised using a modified Hantzsch reaction, to prepare the required enantiomerically pure thiazole amino acid derivatives.[87] In a related field, the stereostructure of the highly modified thiazole containing cyclic peptide lissoclinamide-5 **19** has been revised by Pattenden and Boden, who synthesised both lissoclinamide-5 and its stereoisomer **20**, thus showing that the natural product has structure **19** and not **20** as previously reported.[88] The structure of crystals of patellamide A **21**, a cytotoxic cyclic peptide having a non-C_2-symmetric methyl group, displayed a C_2-symmetric and saddle-shaped rectangular conformation in which the methyl group is disordered into two C_2-symmetric positions.[89]

3.2 Bridges Formed by Disulfide Bonds – The chemical synthesis of disulfide bonds, in the context of scale-up preparations, has been discussed in a mini-review. The authors paid special attention to their own methodologies, involving the addition of peptides *via* syringe pumps to standard oxidising agents.[90]

The effect of a disulfide crosslink between two peptide chains on the stability of β-ribbon secondary structures formed between the peptide chains has been investigated. Two sets of 9-residue peptides incorporating cysteine in one peptide and (*S*)-α-amino-ε-mercaptohexanoic acid in the other, were synthesised. Comparison of the CD data showed that the dimer containing a disulfide bond between the longer sidechains of (*S*)-α-amino-ε-mercaptohexanoic acid possessed higher β-ribbon character as compared to the dimer with cystine disulfide bond.[91]

The conformational characteristics of elcatonin, an analogue of eel calcitonin in which an ethylene linkage mimics the Cys^1-Cys^7 disulfide bridge, have been reported. Some degree of α-helicity was observed for both alcatonin and eel calcitonin (**Figure 5**) in solutions of trifluoroethanol/water provided the trifluoroethanol concentration was at least 15%.[92] New analogues of the calcitonin gene-related peptide (CGRP), designed from the results of molecular modelling studies of active fragments, have been synthesised and tested. The analogues were found to have affinities for the receptor comparable to those seen for native CGRP.



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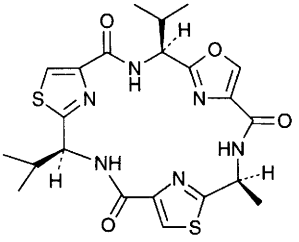

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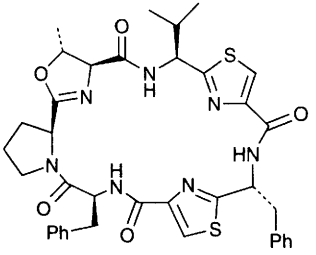


(19)

Two analogues constrained by disulfide bridges were found to be agonists, and so gave an insight into the bioactive conformation of CGRP.[93]

The structure activity relationships of human interleukin-8 have been probed using chemically synthesised analogues with single or double amino acid substitutions, as well as CXC chemokine hybrids. The results described, indicated that the significant requirements for activity are the disulfide bridges, and a 30-35 turn, which provide a structural scaffold for the NH_2-terminal region which includes the primary receptor binding site.[94] The conformational analysis of peptide **22**, related to cyclinopeptide A, has been effected using solid-state, solution and MD techniques. The studies showed that the solid-state structure is an energy minimum, and is reproduced *in vacuo* and in solution simulations.[95]

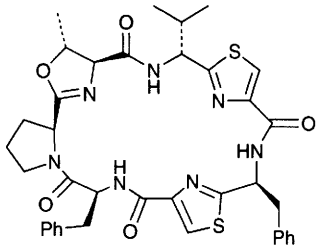
The 32 amino-acid natriuretic peptide urodilatin was chemically synthesised and subjected to 2D NMR spectroscopy studies in aqueous solution. No long range nOe's could be detected, except between residues close to the single cystine bond. This led to the conclusion that urodilatin in aqueous solution is a random coil peptide with the exception of the region around the cystine bond.[96] CD and FTIR studies have been effected on an immunogenic disulfide cyclised octadecapeptide, a fragment of a snake curaremimetic toxin. These studies indicate that the immunogenic disulfide cyclised peptide (23-40) can adopt in solution an ordered structure.[97]

Ω-Agatoxin-TK (Ω-Aga-TK), a 48-amino-acid containing peptide isolated from the venom of the funnel web spider (*Agelenopsis aperta*), is a selective and potent inhibitor of P-type calcium channels in the nervous system. A peptide that has the amino acid sequence identified for native Ω-Aga-TK, has been synthesised. The results showed that Ω-agatoxin containing a D-serine at position-46, but not synthetic Ω-[L-Ser]-agatoxin, exerted blockade of P-type calcium channels in cerebellar purkinje neurones. Molecular dynamics calculations showed that the carboxyl-terminal, six-amino-acid peptide of Ω-Aga-TK containing D-Ser(46) assumes a different conformation than does the peptide containing L-Ser(46). The results suggested that the specific conformation of the carboxyl-terminal region of Ω-Aga-TK, particularly the configuration of Ser(46), together with a β-sheet structure formed by four disulfide bonds, might be essential for blockade of P-type calcium channels.[98]

Much additional work has been published on cyclic disulfide containing peptides; this is referred to in the appropriate section for the particular peptide studied.

3.3 Helices and Helix Inducers – In recent years, Kemp and co-workers have been developing a number of α-helix initiators. In the latest development of this work, the authors report the synthesis of peptide **23**, in which an amidinium ion is used to constrain one of the amide bonds within a tripeptide. It remains to be seen whether or not compound **23** is an effective α-helix initiator.[99]

p-Bromobenzoyl, linked to the *N*-terminus of a peptide, has been reported as a useful CD probe for determining the solution conformation of peptides. The *p*-bromobenzamido chromophore was used to determine the screw sense of a series of 3_{10}-helical peptides.[100] Systematic studies of peptides by circular



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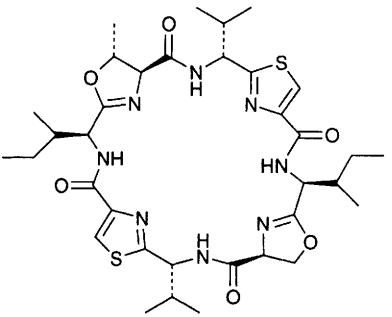

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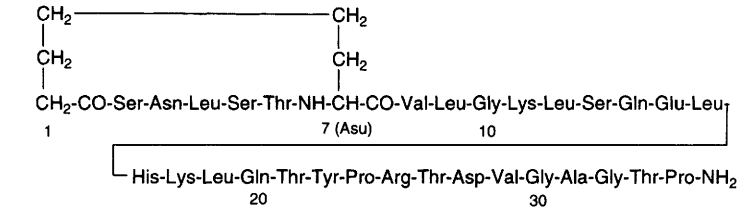

elcatonin

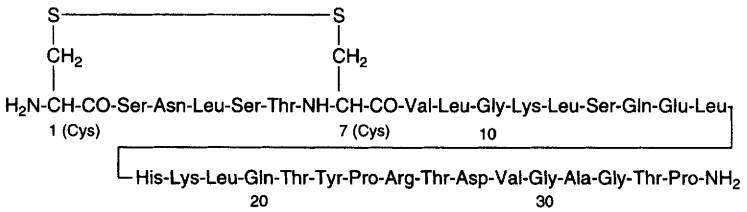

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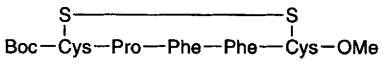
Figure 5



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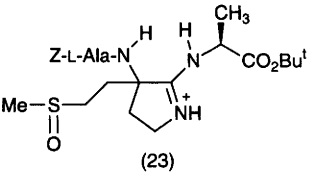


(23)

dichroism models spectroscopy in various micellar/vesicular media have been effected, to study the ranking order of helical propensity for uncharged amino acids in the membrane environment. In contrast to their conformational preferences in water, the helical proclivity of amino acids in membranes was shown to be governed by their side chain hydrophobicity, and by the hydropathy of the local peptide segments in which the residues reside.[101] A synthetic peptide, known to form L-type calcium channels, when templated, has been studied in its non-templated form by CD spectroscopy. In aqueous fluoroalcohol media, the peptide was mainly helical, whilst in dodecylphosphocholine micelles a β-sheet motif appeared to predominate.[102]

The relationship between α-helical secondary structure and the fluorescence properties of an intrinsic tryptophan residue have been investigated. A monomeric α-helix forming peptide and a dimeric coiled-coil forming peptide containing a central tryptophan residue were synthesised. The fluorescence parameters of the tryptophan residue were determined for these model systems at a range of fractional α-helical contents.[103] A series of peptides with the sequence acetyl$(AAQAA)_3$amide have been synthesised, each containing a different residue having its carbonyl carbon enriched in ^{13}C. This allowed the evaluation of the helicity distribution within the molecule, which was found to be more helical at the *N*-terminal region of the peptide, than had been previously estimated by molecular dynamics studies.[104]

α-Helical hydrophobic polyamino acids have been reported to produce proton-conducting defects in lipid bilayers that may be used to model functional proton channels in biological membranes.[105]

A simple thermodynamic formalism has been presented to model the conformational transition between a random-coil monomeric peptide and a coiled-coil helical dimer. The model, which is appropriate particularly for short peptides, is an alternative to the theory developed by Skolnick and Holtzer. The theory of the helix-coil transition in denaturation experiments was also discussed.[106] Helix folding simulations with various initial conformations, and using solvent-referenced energy calculations, have been presented. In particular, a 16-residue peptide with alanine side-chains, folded into a predominantly α-helical conformation, during constant temperature simulations.[107]

The control of secondary structure in a designed peptide has been achieved by chemical oxidation of methionine to the sulfoxide form. The peptide Ac-YLKAMLEAMAKLMAKLMA-NH_2, which can adopt the α-helical structure, was oxidised to the sulfoxide form, which displayed the polar/non-polar alternation required for amphipatic β-strand formation.[108] In an approach to the *de novo* design of α,α-motifs, the 16 residue peptides Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-Xxx-Pro-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (Xxx = D-Phe; Phe) have been spectroscopically studied in solution. NMR and CD spectra established helical conformations in both the peptides. Despite the presence of a potentially helix breaking, central, D-Phe-Pro segment, the first peptide was forced into a continuous helical fold presumably as a consequence of the overriding stereochemical dominance of the Aib residues.[109]

Lys(+) to Arg(+) substitutions which are known to increase the helix content

of designed helical peptides, have been effected on the helical peptide Ac-$(AAAAK)_3A.NH_2$. The unsubstituted sequence contains a significant amount of 3_{10} helix, however, single Lys(+) to Arg(+) substitutions shifted the peptide conformation toward α-helix in a position-dependent fashion. The single substitution closest to the carboxy terminus induced the largest conformational change at the helix amino terminus, which suggested that a single strategically-placed arginine can exert long range control on helix structure.[110] The tetrapeptide Boc-D-Glu-Ala-Gly-Lys-NHMe and its L-Glu analogue have been prepared and their solution conformation examined by NMR methods. A weak type II reverse-turn conformation was attributed to the L-Glu-peptide, whereas the D-Glu analogue showed a stable tandem type II′ turn-3_{10}-helix conformation in apolar solvents. The latter conformation is postulated to be of use as an *N*-terminal helix cap in the formation of longer 3_{10}-helices.[111] Three peptides, H-$(Ala-Arg-Leu)_8$-OH, H-$(Val-Arg-Leu)_8$-OH and H-$(Leu-Arg-Leu)_8$-OH, have also been synthesised, and found to adopt 3_{10}-helical structures in the presence of phospholipid bilayers. The peptides were able to form cation-selective ion channels, but showed no antimicrobial activity when tested.[112] The solid-state structure of dodecapeptide Boc-$(Ala-Leu-Aib)_4$-OMe has been determined using X-ray diffraction, and assigned as possessing two independent 3_{10}-helical molecules in a triclinic unit cell.[113]

The conformation of the CN1 peptide, derived from the nervous system P2 protein, has been studied in D_2O solution using Fourier transform infrared (FTIR) spectroscopy. The peptide was found to be mainly random, though with some α-helix and β-sheet structure present. The study produced different quantitative estimations of secondary structures to those previously reported in CD studies, but the FTIR results were presented as more reliable.[114] The structure of a chemically synthesised 25-residue-long functional signal peptide of *Escherichia coli* ribose binding protein was compared with that of a non-functional mutant-signal peptide using circular dichroism and two-dimensional ^1H NMR in solvents mimicking the amphiphilic environments. Because both of these peptides have stable helices within the hydrophobic stretch, it was speculated that the additional 2 turns of the α-helix in the NH_2-terminal region of the wild-type signal peptide are important for its function.[115]

The binding of apolipoprotein A-I model peptides to lipid bilayers has been reported. Two 18 amino acid peptides (DWLKAFYDKVAEKLKEAF and KWLDAFYKDVAKELEKAF), possessing ampiphilic helices, were employed in the measurement of binding isotherms and peptide-lipid headgroup interactions.[116] Cell recognition of the α1(IV)531-543 region of type IV collagen has been reported as being independent of substrate conformation. The study involved the use of single stranded and triple-helical peptide models of the α1(IV)531-543 region.[117] The 3D structure in water of endothelin-1 **24** has been determined using ^1H-NMR and molecular dynamics studies, and the peptide was shown to possess a compact stable conformation with an α-helical segment between residues 9-16, and a hydrophobic cavity formed by the aromatic and alkyl groups on residues 13, 14, 17, 19, 20 and 21.[118] The influence of proline on bending of the α-helix was investigated by replacement of the proline-61 residue

of the Fis protein with alanine, serine or leucine. The study showed that the kink of the α-helix does not require the presence of proline.[119]

A synthetic peptide, belonging to a predicted α-helical domain, corresponding to residues 106-126 of human prion protein, was observed to undergo polymorphism depending upon environment conditions. In particular, stable β-sheet structures were observed at acidic pH. This may help explain the shift from α-helix to β-sheet associated with the conversion of cellular prion protein to post-translationally modified prion protein.[120] The antibiotic [Arg^2]K-582 A (Arg-Arg-D-Orn-Thr-D-Orn-Lys-D-Tyr) has been subjected to a detailed theoretical conformational analysis. The results indicated that the linear peptide has a high propensity to form a quasi-cyclic conformation in equilibrium with π(L-D) helices. The findings were corroborated by the synthesis of two inactive analogues with an L-Lys in place of D-Orn^3 or D-Orn^5, confirming the importance of the folding pattern for the antimicrobial activity of K-582.[121]

A model 16 amino acid containing-peptide of endothelin-1 (**25**), which possessed the minimised sequence homology to the corresponding part of endothelin-1, has been synthesised in order to study the requirements of the cystine bridge in stabilising the α-helix motif. The results indeed showed the important role for the stabilisation of peptide conformation by the disulfide bonds.[122] The solution structure of a monocyclic analogue **26** of endothelin, [^{1,15}Aba]endothelin, has been determined by NMR. The implications for the pharmacological activity and selectivity of the analogue was also discussed. The *N*-terminus was proposed to be a key structural region for differentiation of binding activity at the ET_A and ET_B receptor sites.[123] The neuropeptide Y analogue *N*-acetyl[Leu^{28},Leu^{31}]NPY(24-36)-amide binds specifically to prejunctional or Y-2 receptors acting to inhibit neurotransmitter release. The structure of this biologically active mutant has been studied by NMR spectroscopy. Structure analysis of the peptide was performed using distance geometry and dynamic simulated annealing, revealing the presence of a helical structure exhibiting an amphiphilic character and slight constriction in the segment 24-29.[124]

The helical sense of gramicidin A channels was studied by residue substitutions, to evaluate individual amino acid contributions. The study reported that the helix handedness is most probably a complex function of the arrangement of differing segments of the peptide.[125] A comparative monolayer and infrared study of analogues of gramicidin A containing either tyrosines or naphthylalanines instead of tryptophans indicated that the nature of the aromatic residues influences the favoured conformation of the peptides. Polar residues favour formation of a single stranded helix, whilst non polar residues favour a double stranded helix.[126]

Gramicidin A analogues, labelled with ^{13}C in the backbone carbonyl groups and the C-2 indole carbons of the tryptophan-11 and tryptophan-13 residues, have been synthesised using Boc-protected amino acids, and the purified analogues were incorporated into phosphatidylcholine bilayers with a 1:15 molar ratio. The orientations of the labelled groups within the channel were investigated using solid-state NMR, to determine the effect of a monovalent ion (Na^+) on the orientation of these groups. However, the presence of sodium ions did not

perturb the ^{13}C spectra of the tryptophan carbonyl groups. These results contrast with earlier results in which the $Leu^{10,12,14}$ carbonyl groups were found to be affected by the presence of sodium ions and imply that the tryptophan carbonyl groups are not directly involved in ion binding. The channel form of gramicidin A has been demonstrated to be the right-handed form of the β(6.3) helix, consequently the tryptophan carbonyls would be directed away from the entrance to the channel and take part in internal hydrogen bonding, so that the presence of cations in the channel would have less effect than on the outer leucine residues. Sodium ions also had no effect on the C-2 indole resonance of the tryptophan side chains. However, some sensitivity of the gramicidin side chains to the surrounding lipid, was observed.[127] High resolution dynamic and structural information has been obtained from ^{2}H solid-state NMR spectra of the Val^{1} side-chain of the gramicidin channel in a lipid bilayer. Both powder pattern lineshapes and spectra from uniformly aligned samples of gramicidin in lipid bilayers have been analysed to achieve a consistent interpretation of the data.[128] Solid state deuterium NMR inversion-recovery and Jeener-Broekaert relaxation experiments have also been performed on oriented multilamellar dispersions consisting of 1,2-dilauroyl-sn-glycero-3-phosphatidylcholine and ^{2}H exchange-labelled gramicidin D, at a lipid to protein molar ratio of 15:1, in order to study the dynamics of the channel conformation of the peptide in a liquid crystalline phase.[129,130]

Corticotropin releasing factor (CRF) is a 41-amino acid containing peptide amide which has previously been postulated to assume an α-helical conformation upon binding to its pituitary receptor. A series of cyclic analogues have been prepared, with the rationale that side chains necessary for binding could also be replaced by side-chain bridges. Computer modelling was used to predict likely side chain bridging opportunities and evaluated the effects of such replacements by correlating biological results with those derived from CD spectroscopy. Thirty-eight monocyclic peptide amides, competitive antagonists of human/rat CRF, were synthesised. CRF antagonists were tested for their ability to interfere with CRF-induced release of ACTH by rat anterior pituitary cells. In most cases, one of the bridge heads was located at a position where substitution by a D-residue was tolerated (*i.e.*, positions 12 and 20). Careful optimisation of bridge length and chirality was found to be critical. In fact, out of the 38 analogues that were synthesised and tested, only two, {*cyclo*-(20-23)[D-Phe^{12},Glu^{20},Lys^{23},-$Nle^{21,38}$]CRF(12-41) and *cyclo*-(20-23) [D-Phe^{12},Glu^{20},Orn^{23},$Nle^{21,38}$]CRF(12-41)}, were found to be more potent (3 and 2 times, respectively) than [D-Phe^{12},$Nle^{21,38}$]CRF(12-41), the parent compound. Six other analogues belonging to two different families were found to be half as potent as the standard, and 18 further compounds had 2-20% of the potency of the standard. All other analogues were significantly less potent. CD results of all of the analogues in 50% TFE {a concentration of TFE that induced nearly maximum helicity of [D-Phe^{12},$Nle^{21,38}$]CRF(12-41)} suggested that whilst helicity may be an important factor for CRF analogue recognition, there was little correlation between percent helicity as determined by spectral deconvolution and biological activity *in vitro*.[131]

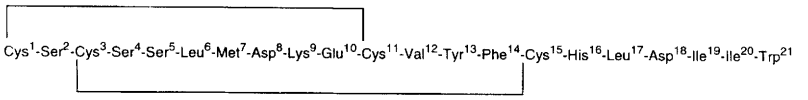
A series of apolipoproteins related to ApoE, containing the residues involved

in mediating the binding between lipoproteins and the low density lipoprotein receptor (LDL), have been synthesised. Their lipophilicity was increased by acylation with non-polar groups. The results indicated that the receptor binding properties of apoE depend on its association with phospholipid; transfer of peptide fragments of the receptor-binding domain of apoE, from the aqueous phase to a lipid surface converts them from a random coil to an α-helical conformation that is recognised by the LDL receptor.[132]

The secondary structure of amino terminal fragments of human parathyroid hormone related protein (PTHrP) in aqueous solution, in trifluoroethanol solution and in the presence of model membrane systems, has been studied by circular dichroism spectroscopy. The results indicated that the conformational properties of the functionally significant amino terminal 1-34 region parallel those reported for the corresponding, but largely nonhomologous region of parathyroid hormone. The conformational similarities were speculated to account for the ability of PTHrP to mimic the functional properties of parathyroid hormone.[133] The structure of the biologically active mutant PTHrP[Ala^{26}](1-34)amide in 10% trifluoroethanol has been studied by proton NMR spectroscopy. Complete assignments of all backbone and side chain hydrogens were made. The NMR data were further refined using computational techniques. The major structural features include two segments of α-helix extending from Glu^{4} to Lys^{13} and from Phe^{21}/Phe^{22} to Ala^{34}, with a turn from Gln^{16} to Arg^{19} and a hinge around Ser^{14}/Ile^{15}. A close resemblance to the structure of PTH(1-34)amide in water was noted. A comparison of the structural features common to PTH and PTHrP in different solvents was made which enabled the key structural features likely to be involved in PTH receptor binding to be identified.[134]

The PTH antagonists Tyr^{34}bovine-βPTH-(7-34)NH_2, D-Trp^{12},Tyr^{34}-βPTH(7-34)NH_2, and PTHrP(7-34)NH_2, have been administered to hypercalcemic athymic nude mice bearing a human squamous cell carcinoma of the lung in 60- to 500-fold molar excess of a dose of PTHrP(1-34) known to produce hypercalcemia. The antagonists had no significant effect on serum calcium levels. In an adenylyl cyclase assay using the ROS 17/2.8 cells, a potent PTH antagonist Leu^{11},D-Trp^{12}PTHrP(7-34)NH_2 was rapidly inactivated in the presence of rat or human plasma. This inactivation by plasma was not blocked by common inhibitors of proteolysis (aprotinin, soybean trypsin inhibitor, and leupeptin). Preliminary studies demonstrated that inactivation of the PTHrP antagonist was caused by a plasma component with an apparent molecular weight of 230,000 Daltons. The knowledge of the structure of the PTH/PTHrP receptor combined with the identification of a hormone-inactivating plasma factor should facilitate the design of PTH-antagonists that are effective *in vivo*.[135]

3.4 β-Turn Mimetics and Miscellaneous Bridges – Cyclopeptides containing a pseudo-amino acid with a biphenyl moiety have been prepared (**Figure 6**). NMR techniques and molecular modelling calculations, inferred that these cyclopeptides, adopt an antiparallel β-sheet conformation.[136] The synthesis of pyrrolinone-based β-strand peptidomimetics **27**, involving the cyclisation of



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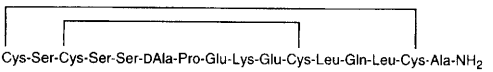

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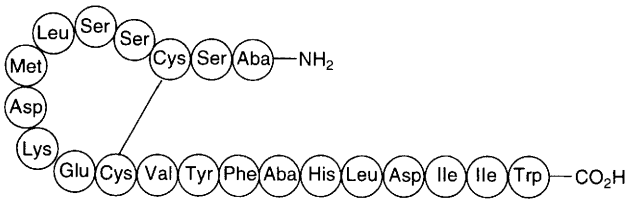

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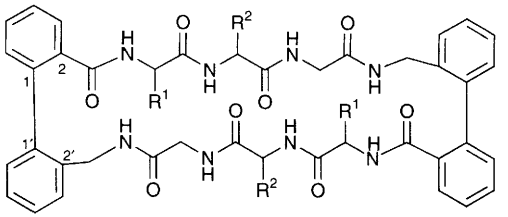
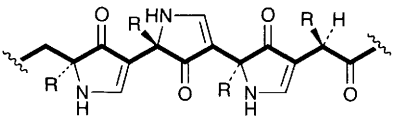


R^1 = Me, R^2 = $PhCH_2$
R^1 = Me, R^2 = Pr^i

Figure 6



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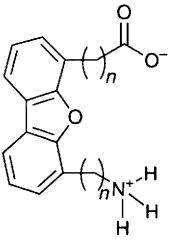


(28) $n = 2$

metalloimines, with α-substituted α-amino ester building blocks, has also been described. The enaminone NH protons form hydrogen bonds, which stabilise the β-strand conformation, and the presence or absence of nitrogen protecting groups controlled antiparallel versus parallel sheet formation.[137] Three dibenzofuran-based amino acid residues, namely 4-(2-aminoethyl)-6-dibenzofuranpropanoic acid **28**, (4-aminoethyl)-6-dibenzofuranethanoic and 4-amino-6-dibenzofuranmethanoic acid, have been prepared, in order to investigate the ability of **28**, to nucleate antiparallel β-sheet formation. The results indicated that **28** forms a 15-membered ring intermolecularly hydrogen-bonded hydrophobic cluster, which appears to act as a partial β-sheet template.[138]

The intramolecular amidoalkylation reaction of phenylalanine or homophenylalanine and α-methoxyglycine containing dipeptides, was used to prepare derivatives of 4-amino-2-benzazepin-3-one-1-carboxylic acid **29**. Compounds **29** are conformationally restricted peptides containing a bridged Phe-Gly moiety (**Scheme 8**).[139]

CD Spectra are widely used to detect the presence of β-turn/ sheet structures in peptides. However, the use of the dichromophoric CD assay for β-turn formation in peptide sequences has been investigated by Baldwin *et al.* The investigations were paralleled by NMR studies, which revealed the presence of a previously unreported hydrogen bond in the β-turn conformers, which appeared to play a role in the generation of observed Cotton effects in the CD spectra. The authors therefore suggested caution in the use of the CD technique alone as an assay for β-turn conformers in peptides.[140]

The folded conformation of the peptide H_2^+-Ser-Tyr-Pro-Phe-Asp-Val-O^-, in water has been evaluated, using NMR techniques and molecular dynamics calculations. The peptide was found to adopt a type VI β-turn and the major source of stabilisation appeared to be the stacking of the aromatic and proline rings.[141] The solution conformation of a biologically active *C*-terminal hexapeptide analogue of the pheromone biosynthesis activating neuropeptide Tyr-D-Phe-Ser-Pro-Arg-Leu-NH_2 has also been studied by NMR spectroscopy. In a mixed solvent of water and DMSO, a β-turn conformation was identified, suggesting therefore the biologically active conformation of the peptide.[142] Conformational studies on analogues of the invertebrate peptide pGlu-Asp-Pro-Phe-Leu-Arg-Phe-NH_2, using NMR techniques, suggested that this peptide can adopt a β-bend conformation. This could explain the selectivity of this and related peptides towards receptors governing neurological functions.[143]

X-ray diffraction studies have been effected on the peptide (Z)-Aib-Gly-Ile-Leu-OMe.H_2O, which is a protected analogue of the *C*-terminal sequence of the membrane-active peptabiol antibiotic trichogin A IV. The peptide backbone was found to be folded, and the urethane carbonyl *O*-atom acts as the acceptor of two intramolecular hydrogen bonds, giving rise to both a β-bend and to an α-bend. The geometry of the latter is significantly distorted from that observed in α-helices. This structure represents the first observation of such an α-bend in a protected tetrapeptide sequence.[144]

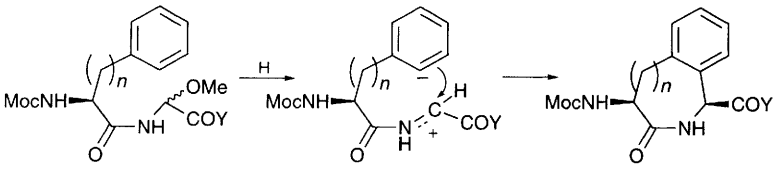
The type II and type III collagen α-1 chain *C*-telopeptides are a 27mer with the sequence *N*Ac-GPGIDMSAFAGLGPREKGPDPLQYMRA, and a 22mer,

*N*Ac-GGGVASLGAGEKGPVGYGYEYR respectively. Their conformations have been studied in CD_3OH/H_2O (80/20) solution by means of two-dimensional proton NMR and CD spectroscopy. The conformation of the type II *C*-telopeptide was found to be essentially extended. The conformation of the type III C-telopeptide was also mostly extended, except for a β-turn ranging from Gly^8 to Glu^{11}, which is stabilised by a hydrogen-bond between NH of Glu^{11} and the carbonyl group of Gly^8. The low temperature coefficient of NH(E^{11}) and, in particular, the observation of a medium range nOe between H-α(A^9) and NH(E^{11}) corroborated the existence of a β-turn in this region. Although spectral overlap prevented a precise conclusion with regard to the type of β-turn present, there was some evidence that it might be type II.[145]

A T-cell stimulating peptide H-Val-Gln-Gly-Glu-Glu-Ser-Asn-Asp-Lys-OH, the 163-171 fragment epitope of interleukin-1 β (IL-1 β), has been synthesised using solution phase techniques. The backbone conformation of the synthetic fragment, investigated in aqueous solution by circular dichroism spectroscopy, was found to be a mixture of β-turns and random coil structures. This β-turn containing structure has also been found to be a theoretically preferred conformation using Chou-Fasman proclivity data and is in accordance with the presence of an all-β-globular conformation for its parent molecule IL-1 β. Thus, the β-turn conformation was concluded to probably be involved in retention of T-cell stimulation activity in this synthetic epitope.[146]

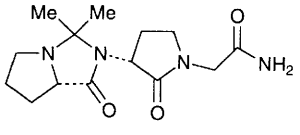
The 60 amino acid proline-rich neutralisation (PRN60) domain of the external surface unit glycoprotein of feline leukaemia virus was chemically synthesised in total, and in fragments. The ability of these retroviral peptides to form ordered conformations was examined using ^{1}H-NMR, circular dichroism spectroscopy, and intrinsic viscosity measurements. The conclusions researched were that PRN60 forms a β-turn helix, and that this region of FeLV-gp70 is a separate folding domain of the retroviral surface unit glycoprotein. The unique conformational properties of PRN60 and its critical role as the predominant target for neutralising antibody responses suggested that this peptide is a reasonable candidate for producing a synthetic peptide vaccine for FeLV.[147]

An improved algorithm for packing polypeptide chains with fixed geometry, which converges to a local energy minimum rapidly and efficiently, has been described. The speed of convergence of the new algorithm is comparable to that of existing algorithms for minimising the energies of single polypeptide chains, and it is several times greater than the speed of convergence of previous algorithms for minimising the energy of structures consisting of several polypeptide chains. The algorithm has been used to minimise the energy of three-stranded Ala_8 β-sheets, three-stranded Val_6 β-sheets, and five-stranded Ile_6 β-sheets, starting from regular structures found previously; of the three-stranded regular and truncated $(Gly\text{-}Pro\text{-}Pro)_4$ structures used in earlier work to model collagen; and of the stacked β-sheet$(Ala\text{-}Gly)_6$ structures used to model silk. The antiparallel Ala β-sheet, and Gly-Pro-Pro triple helices, and the silk II structure remained nearly regular after energy minimisation, but by contrast with results from earlier computations the other structures became significantly irregular.[148]



n = 1 or 2 (29)

Scheme 8



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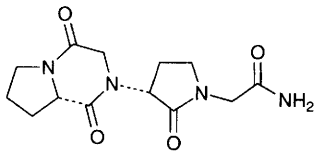

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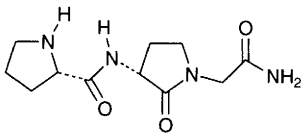

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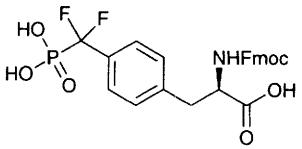

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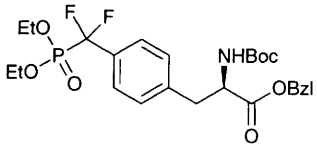

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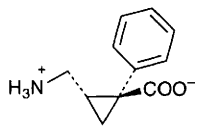

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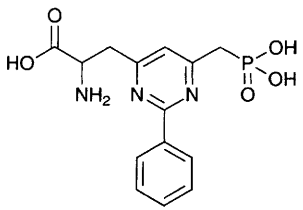


(36)

A series of linear and cyclic thymopentin (TP5, Arg-Lys-Asp-Val-Tyr) analogues, has been investigated by CD, NMR and MD methods. The stability of the γ-turn conformation on the residue located in position 3 of the peptide, was found to vary with the immunostimulating cavity of the peptides.[149] The same research group, also investigated a series of six proline-containing thymopentin (TP5) analogues (Pro^1-TP5, Pro^2-TP5, Pro^3-TP5, D-Pro^3-TP5, Pro^4-TP5, and Pro^5-TP5). The importance of the stability of the conformation with a γ-turn centred on residue 3 was again discussed.[150]

A 3D model has been proposed for peptide T, an HIV reproduction inhibitor with amino acid sequence corresponding to a fragment of the T4 receptor-binding site of the viral protein gp120. The structure was modelled using a method designed previously and based on combining molecular mechanics algorithms with NMR data. Six types of low-energy structures were obtained which differed in the spatial folding of the peptide backbone. All types were shown to lack strict determination of the side group conformations, which could assume several states providing (within the given type) about the same stabilisation of the backbone. Despite certain differences, all the structures selected were marked by two consecutive reverse turns of the polypeptide chain within the *C*-terminal pentapeptide segment. This region is supposed to be responsible for peptide binding with the T4 receptor and for the antiviral effect.[151]

The X-ray structure of the dipeptide $H\frac{+}{2}$-Lys-Leu-OH·OAc has been resolved. The two molecules in the unit cell showed almost identical conformations, and the dipeptide backbone was folded.[152] The crystal structure of *cyclo*-(Thr-D-Val-Pro-Sar-*N*-MeVal-*O*Thr), has been described. This cyclic penta-depsipeptide represents one of the two peptide units of actinomycin, a 16-membered depsipeptide. The cyclic pentapeptide backbone adopted a flat conformation similar to that found for an analogous *N*-protected pentapeptide lactone, previously reported in the literature.[153]

The nucleocapsid protein of Moloney murine leukaemia virus (NCp10) is a 56-amino acid protein which contains one zinc finger of the $CysX_2CysX_4HisX_4Cys$ form, a highly conserved motif present in most retroviruses and retroelements. The three-dimensional structure of NCp10 has been determined in aqueous solution by 600MHz ^1H NMR spectroscopy, followed by computational studies. The solution structure was characterised by a well-defined central zinc finger, surrounded by flexible *N*- and *C*-terminal domains. The Tyr^{28}, Trp^{35}, Lys^{37}, Lys^{41} and Lys^{42} residues, which are essential for activity, lie on the same face of the zinc finger, forming a bulge structure probably involved in viral RNA binding. The significance of these structural characteristics for the various biological functions of the protein was also discussed, taking into account the results obtained with various mutants.[154]

The tripeptide amide Pro-Leu-Pro-NH_2, a dopamine-receptor modulating agent, has been studied by X-ray diffraction, and found to be folded at the *N*-terminal Pro residue, and semi-extended at the central Leu and *C*-terminal Pro residues. The Leu side chain was in the common g^- (t,g^-) disposition.[155] The pseudopeptides **30** and **31** were synthesised as mimics of the 'C5' hydrogen bond structure found in the compound **32**, itself a mimic of Pro-Leu-Gly-NH_2. These

analogues were tested for their ability to enhance the dopamine D2 receptor agonist *N*-propylnorapomorphin, in the absence and in the presence of 5′-guanylylimidodiphosphate. Both compounds enhanced binding by increasing both the binding affinity of the agonist and the number of high-affinity sites available for binding.[156]

4 Amino Acids with Modified Side-chains

A method for the large scale synthesis of all four stereoisomers of β-methyltyrosine has been described by Hruby *et al.*, based upon methodology previously used for the preparation of other β-methyl amino acids. The key reactions were an asymmetric Michael-like addition of an organocuprate to a chiral α,β-unsaturated acycloazolidinone (establishing the stereochemistry at the β-centre), and subsequent stereoselective electrophilic bromination of the resulting product (fixing the stereochemistry at the α-centre). Conversion of the bromide to the azide, catalysed hydrolysis to the azido acid with simultaneous recovery of the chiral auxiliary, reduction of the azide, and final deprotection of the phenol group afforded the desired amino acids.[157]

The Fmoc-4-phosphono(difluoromethyl)phenylalanine derivative **33**, has been prepared from the previously reported Boc-derivative **34**, and used in the synthesis of two model peptides to show its potential applicability to peptide studies.[158] A similar set of derivatives was also prepared by Smyth and Burke.[159] A related non-fluorinated series of Boc-4-dimethylphosphonomethyl-phenylalanine derivatives has also been reported.[160] Cyclic hexameric peptides based on the amino acid sequence, Gly-Xxx-Val-Pro-Met-Leu, where Xxx was either a phosphotyrosyl (pTyr) residue, or a hydrolytically stable pTyr mimetic, have been examined for their ability to bind to the *C*-terminal SH2 domain of the p85 phosphoinositol 3-kinase (PI 3-kinase). The cyclic peptides retained significant binding affinity relative to their linear counterparts. Potency varied depending on Xxx in the order: phosphonomethyl phenylalanine (Pmp, ID_{50} = 5.2 mM) < phosphonodifluoromethyl phenylalanine (F_2Pmp, ID_{50} = 2.2mM) < pTyr (ID_{50} = 1.0mM), with Xxx = Tyr being inactive (ID_{50} > 500 M). Greatly reduced potency was observed when Xxx was of the unnatural D-configuration. The cyclic peptides represent conformationally constrained ligands which should be useful in the development of p85 SH2 domain-directed inhibitors.[161] A tris-sulfotyrosyl dodecapeptide (TRDIY(S)ETDY(S)RK-amide), whose primary sequence is identical to the 1142-1153 sequence of the insulin proreceptor, has been reported to inhibit tyrosine dephosphorylation of the insulin receptor *in situ*.[162]

Phosphonomethyl phenylalanine (Pmp) has been utilised as a non-hydrolysable phosphotyrosyl (pTyr) mimetic, and has been incorporated into peptides that have previously been reported to competitively inhibit the protein-tyrosine phosphatases PTP1 and PTP 1B. The hexameric peptide sequence Ac-D-A-D-E-X-L-amide, where X = (D/L)-Pmp or L-F_2Pmp, resulted in half maximal inhibition values of these two peptides against PTP 1B-mediated dephosphorylation of autophosphorylated insulin receptor of 200mM and 100nM, respectively. These

data indicated that F_2Pmp induces a three orders of magnitude enhancement in affinity relative to Pmp, resulting in a potent peptide-based PTP inhibitor. F_2Pmp may therefore be useful in the preparation of selective, high affinity PTP inhibitors.[163]

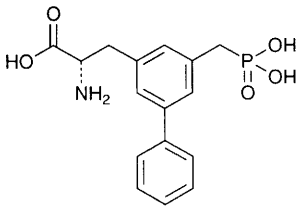
Vascular endothelial growth factor is a potent angiogenic factor which binds to two structurally similar receptor tyrosine kinases, KDR and FLT1. An enzymatically active form of the cytosolic domain of the KDR receptor has been expressed in bacteria. The expressed protein underwent autophosphorylation in both bacterial cells and in its purified form. Using peptide mapping and sequencing techniques, four tyrosine residues that are phosphorylated were identified, and corresponded to residues 951, 996, 1054 and 1059 of the KDR protein. The location of the phosphorylated residues in the bacterially expressed protein, and/or the consensus sequences around these sites, suggested that they may be identical to the phosphorylated sites of KDR in mammalian cells.[164]

The synthesis of all the isomers of α,β-dimethylphenylalanine and α,β-dimethyl-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, in good yield and high optical purity, has afforded new amino acids, potentially suitable for the topographical control of peptide conformation.[165] High-affinity analogues of neuropeptide Y containing conformationally restricted non-proteinogenic amino acids (*e.g.* **11** and **35**) have been reported. The work also aimed at reducing the size of neuropeptide Y analogues, whilst maintaining Y^1 receptor affinity.[166]

The synthesis of a pyrimidine isostere **36** of the *N*-methyl-D-aspartate (NMDA) receptor antagonist SDZ EAB 515 **37** has been described. The isostere **36** was found to be more than two orders of magnitude less active than **37**, even though the structural differences are apparently minimal.[167] Two diastereomeric non-hydrolysable phosphonopeptide dead end inhibitors of the catalytic subunit of cAMP-dependent protein kinase, have been synthesised. The diastereomers of Leu-Arg-Arg-Ala-DL-(2-amino-4-diethylphosphonobutyric acid)-Leu-Gly were prepared as a mixture starting from DL-2-amino-4-phosphonobutyric acid, and were separated on a C-18 reverse phase HPLC column. One of the diastereomers was found to inhibit the enzyme by a ten fold higher factor than the other diastereomer, and was therefore assigned as possessing the L-configuration.[168]

A haemoregulatory peptide analogue derived from the cystine-dimerised pentapeptide Glp-Glu-Asp-Cys-Lys-OH, in which the cystine residue had been replaced by an all carbon isosteric *L,L*-2,7-diaminosuberic acid moiety, was prepared by segment condensation methodology.[169]

All four stereoisomers of carnosadine **38**, a conformationally constrained arginine analogue, were prepared from either the lactone **39** or the diester **40** (or their enantiomers). Products from both series were manipulated into protected forms for peptide synthesis using the Boc or Fmoc approach.[170] The side chain conformations shown by arginine and lysine in amino acid and peptide crystal structures and bound to oxyanions in proteins have been analysed in an attempt to understand the behaviour of these long-chain amino acids in an ionic environment. Except for χ_1 ($N\text{-}C_\alpha\text{-}C_\beta\text{-}C_\gamma$), torsional angles were found to have a preference for the *trans* conformation. Factors that could prevent a χ-angle from being *trans* were the simultaneous binding of the anion by the main- and side-



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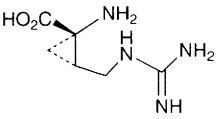

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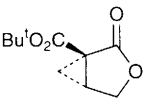

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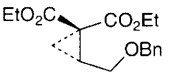

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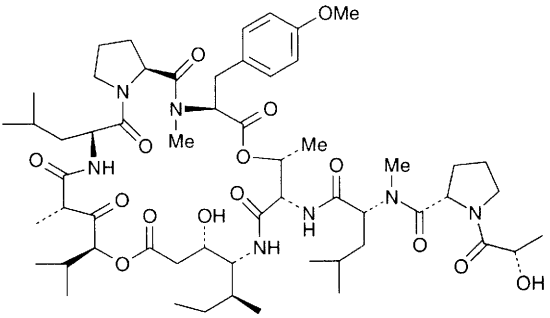

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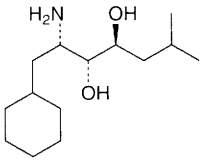

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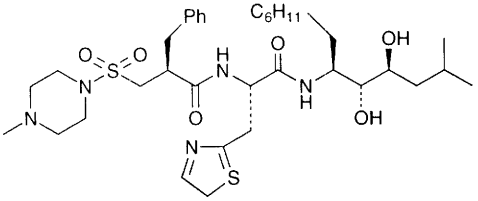

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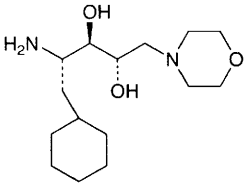


(44)

chain nitrogen atoms, or the sharing of the anion between two different molecules in the crystal structure. Small molecules containing arginine showed a tendency to crystallise with two molecules in the asymmetric unit. This was speculated as being a general phenomenon for all extended molecules which have hydrogen-bond donors (or acceptors) embedded in a rigid set-up.[171]

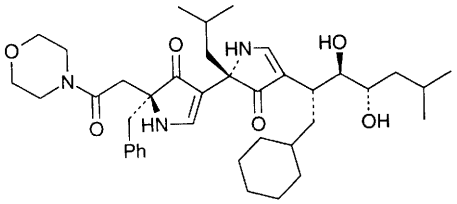
The surface topology-probing of lysine residues in bovine ribonuclease A, lysozyme and horse heart myoglobin has been effected by amino-acetylation or -succinylation, followed by mass spectrometry. Electrospray and ^{252}Cf-plasma desorption mass spectrometry of multiply protonated molecular ions and deuterium exchange experiments provided a relative conformational characterisation of the proteins. Further characterisation of HPLC-separated modified peptides, provided exact identification of acylation sites.[172] Modifications were introduced in the side chain of didemnin B **41**, to afford several analogues for biological testing in order to identify the features responsible for the bioactivity of the natural product.[173]

5 Enzyme Inhibitors

5.1 Renin Inhibitors – A ten step stereocontrolled synthesis of the dihydroxyethylene dipeptide isostere **42**, *via* an *erythro*-selective addition of a Grignard reagent to a *cis* α,β-epoxy aldehyde, has been reported. The isostere was required as part of the synthesis of the orally active renin inhibitor A-72517 (**43**).[174] The synthesis and structure-activity relationships of transition-state mimicking inhibitors containing the dihydroxyethylene isostere at the scissile site has also been described. The compounds with (2*S*,3*R*,4*S*)-4-amino-5-cyclohexyl-1-morpholino-2,3-pentanediol **44** at the P_1-$P_{1'}$ site were found to be potent and specific renin inhibitors.[175] The same research group have also reported a stereospecific synthesis of the (2*S*,3*R*,4*S*)-4-amino-5-cyclohexyl-1-morpholino-2,3-pentanediol isostere unit required for the above transition state mimics starting from 3-cyclohexyl-alanine, and utilising iodo-cyclocarbamation as the key reaction.[176]

A radioactive form of the peptidomimetic inhibitor ditekiren has been synthesised, with tritium labels in the *N*-methyl-histidine moiety and in the Leu-Val alcohol transition state mimic. The label was introduced into the histidine residue by iodination of the parent drug, followed by hydrodehalogenation with tritium gas. Biological tests however, showed prolonged retention of radioactivity in laboratory animals. In order to overcome this problem, the preparation of ditekiren, labelled in the 'unnatural' Leu-Val portion of the molecule, was undertaken. In this case the tritium was introduced by reduction of a keto-analogue with sodium borotritride. The peptide thus prepared, was found to be metabolically suitable for drug disposition studies.[177]

The Smith and Hirschmann development of pyrrolidinone based peptide mimetics continued with the preparation of selective renin inhibitors **45** and **46** (IC_{50} = 18mM and 600nM, respectively), both of which were designed to dock into the renin active-site model. Similarly, pyrrolidinone-based inhibitors for



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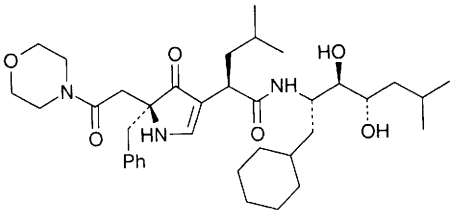

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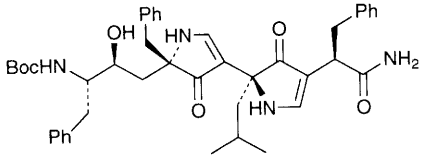

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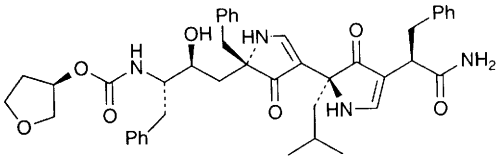

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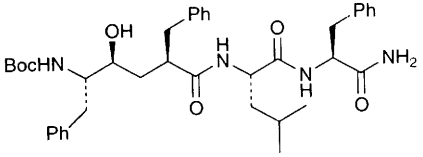

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HIV-1 protease were also designed (**47** and **48**), based upon the known peptidal inhibitor **49**, and shown to have improved cellular transport properties.[178]

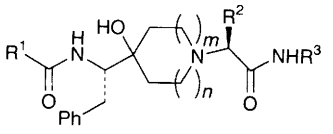
5.2 HIV-1 Protease Inhibitors – Some of the work reported in section 5.1 is also relevant to work on HIV-1 inhibitors. Conformationally restricted HIV-1 protease inhibitors containing the transition state hydroxyl group incorporated into piperidine or pyrrolidine ring systems **50** have been synthesised stereoselectively *via* a convergent double reductive amination strategy. The inherent stereochemistry of an amino acid derivative was utilised to control the stereochemistry of the products. Some of these compounds exhibited submicromolar inhibitory activity towards HIV-1 protease.[179]

The cyclohexylalanine hydroxyethylene dipeptide isostere **51** (Cha-ψ[H.E.]-Ala) has been employed for structure-activity relationship studies on HIV-1 protease inhibitors by systematically replacing the positions corresponding to scissile sites of the P_4-P_2 subsites of substrate-based HIV-1 protease inhibitors. In particular, compounds (Boc-Orn-Val-Cha-ψ[H.E.]-Ala-NH^{n}Bu, $K_i = 11$nm) and (Z-Orn-Val-Cha-ψ[H.E.]-Ala-NH^{n}Bu, $K_i = 8$nm) exhibited good enzyme selectivity, when tested on other proteases.[180] Conformationally constrained macrocyclic peptides (17-19 membered rings) containing hydroxyethylamine isosteres have also been prepared as HIV protease inhibitors, with good IC_{50} values.[181]

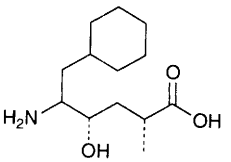
The hydroxyethylene-based inhibitor, SB 206343, was based on a model derived from the structure of the MVT-1O1/HIV-1 protease complex and contains a 4(5)-acylimidazole ring as an isosteric replacement for the P-1′-P-2′ amide bond. It is a competitive inhibitor of HIV-1 protease with an apparent inhibition constant of 0.6 nM at pH 6.0. The three-dimensional structure of SB 206343 bound in the active site of HIV-1 protease has been determined at 2.3Å resolution by X-ray diffraction. All of the interactions were in qualitative agreement with those predicted by the model.[182]

A series of analogues of LY289612, containing a β-hydroxy sulfide isostere, have been prepared, and evaluated *in vitro* for inhibition of HIV-1 protease.[183] Similar compounds containing the Phe[CH(OH)CH_2N(NH)]Phe dipeptide isostere have also been prepared, and have been shown to be potent inhibitors of the HIV-1 protease.[184] A series of HIV protease inhibitors possessing a hydroxylaminepentanamide transition state isostere have been developed. Molecular modelling studies and solid-state conformational data of the inhibited enzyme complex led to the design and synthesis of L-735,524 **52**, a potent and competitive inhibitor of HIV-1 and HIV-2 proteases, with K_i values of 0.52 and 3.3nM, respectively. Inhibitor **52** is orally bioavailable in three animal models, using clinically acceptable formulations, and is currently in phase II human clinical trials.[185]

A series of peptidic HIV-1 protease inhibitors, which also contained the structural segment of the vitamin *biotin*, have been prepared (e.g. **53**). The strategy was aimed at improving the oral availability of peptide based inhibitors, by absorption and distribution *via* biotin transporters and receptors. Cell-internalisation was shown to occur, though the weak antiviral capacity of these



(50) m = 0, 1; n = 0, 1



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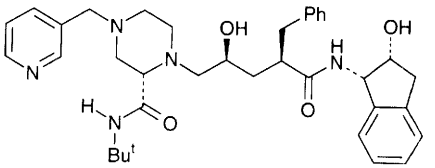

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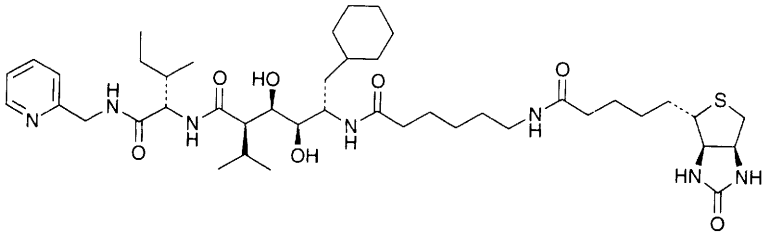

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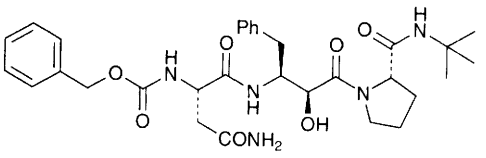

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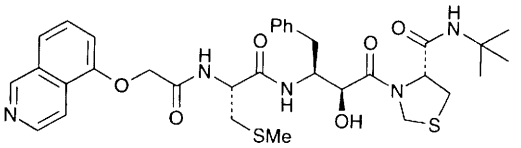


(55)

biotinylated inhibitors, precludes as yet, their consideration as practical anti-HIV therapeutics.[186]

Molecular dynamics calculations have been employed to elucidate the solution structure of two HIV-1-inhibitor complexes, namely **54** and **55**.[187] A thermodynamic cycle perturbation approach has been combined with molecular mechanics and crystallographic data to design novel inhibitors for the HIV-1 protease. The results led to the preparation of two modest inhibitors, and illustrated the potential of this methodology for screening proposed derivatives of lead compounds.[188]

Structural and conformational studies have been effected on both [Ile^7]surfactin **56a** an anti-HIV agent, and [Leu^7]surfactin **56b**. The results showed that the two surfactins exist in different conformational states in both polar and apolar solvents.[189]

NewPred is a semiautomated procedure to evaluate alternate binding modes and assist with three dimensional quantitative structure-activity relationship (SD-QSAR) studies. The predictive power of this package has been applied to a series of 30 HIV-1 protease inhibitors. Five comparative molecular field analysis models based on 59 HIV-1 inhibitors were tested. The test set included 18 compounds having a hydroxyethylurea transition state isostere to investigate the binding mode in $P_{1'}$ and $P_{2'}$. Twelve dihyroxyethylene peptide bond isostere containing compounds were also used to investigate binding in P_2 and P_3 as well as in $P_{2'}$ and $P_{3'}$. Six other compounds with known or inferred binding structure were part of the test set, but not investigated with NewPred. Energy comparisons allowed the selection of the lowest energy structures to be included in the test set. The models predicted the poor inhibitor activity of 1-(*S*)-amino-2-(*R*)-hydroxyindan-containing peptides, which was explained and interpreted from a 3D-QSAR perspective. The use of a new, flexibility-based, semiautomated method to explore alternate binding modes for 3D-QSAR models was demonstrated.[190]

Comparative molecular field analysis (CoMFA), a three-dimensional, quantitative structure-activity relationship (QSAR) paradigm, has been used to examine the correlations between the calculated physicochemical properties and the in vitro activities of a series of human immunodeficiency virus (HIV-1) protease inhibitors. The training set consisted of 59 molecules from five structurally-diverse transition-state isostere classes: hydroxyethylamine, statine, norstatine, keto amide, and dihydroxyethylene. X-ray data was used to provide further conformational information. Additional alignment rules were derived from minimisations of the ligands in the active site of the semirigid protease. The predictive ability of each resultant model was evaluated using a test set comprised of molecules containing a novel transition-state isostere: hydroxyethylurea. Crystallographic studies previously reported indicated an unexpected binding mode for this series of compounds which precluded the use of the field-fit minimisation alignment technique. The test set molecules were, therefore, subjected to a limited systematic search in conjunction with active-site minimisation. The conformer of each molecule expressing the lowest interaction energy with the active site was included in the test set. Field-fit minimisation of neutral molecules to crystal ligands and active-site minimisations of protonated ligands yielded

predictive correlations for HIV-1 protease inhibitors. The use of crystallographic data in the determination of alignment rules and field-fit minimisation as a molecular alignment tool in the absence of direct experimental data regarding binding modes was strongly supported by the results.[191]

5.3 Inhibitors of Other Protease Enzymes.

5.3.1 Serine Protease Inhibitors – Structural variants of basic pancreatic trypsin inhibitor (BPTI) have been synthesised, employing a chemo-enzymatic procedure. The P_1-P_2 amide bond of the inhibitor molecule, was replaced by either a ketomethylene function or an ester bond, yielding molecules with inhibitory activity. The two backbone-mutated BPTI derivatives showed increased dissociation constants of their respective trypsin complexes, due to the lack of a single hydrogen-bond interaction in the enzyme-inhibitor complex.[192]

Three cucurbita-maxiam trypsin-inhibitor-III (CMTI-III) analogues containing a Val residue in the reactive site (position 5) were synthesised by the solid-phase method. The analogues displayed an elastase inhibitory activity. Removal of the *N*-terminal Arg residue and introduction of the Gly-Pro-Gln tripeptide in the region 23-25 decreased the antielastase activity by two orders of magnitude. Removal of the disulfide bridge in positions 16-28 and the substitution of Ala for Cys^{16} and Gly for Cys^{28} decreased the activity by five orders of magnitude as compared with [Val^5]CMTI-III.[193] The same research laboratories also prepared four further analogues by the solid-phase method. The introduction of glycine in position 9, Gly-Pro-Gly in the region 17-19, or Gly-Pro-Asn in the region 23-25, respectively, did not change the antitrypsin activity of any of the modified peptides. All of these substitutions were presumed to be outside the trypsin-binding loop as judged from the X-ray structure of the complex between β-trypsin and the related inhibitor CMTI-I. Finally, a fourth analogue which possessed all three of the above substitutions exhibited full activity.[194]

A conformational study of peptides *cyclo-S,S*-[H-Cys-Ala-Leu-Ser-Tyr-Pro-Ala-Gln-Cys-OH] and *cyclo-S,S*-[Ac-Cys-Thr-Leu-Ser-Asn-Pro-Pro-Gln-Cys-OH], derived from the Soybean Bowman-Birk inhibitor of chymotrypsin, has been reported. The relationship between structure and activity of these two peptides, with regard to their protease inhibitory power, was also discussed.[195]

The potential of hydrazinopeptides as protease inhibitors has been investigated. The peptide Z-Ala_2-Pro-Val-hIle-Leu-OMe, where hIle represents hydrazinoisoleucine, was prepared together with Z-Ala_2-Pro-Val-Ile-Leu-OMe. The interactions of the peptides with human leukocyte elastase and porcine pancreatic elastase were analysed comparatively. The hydrazinopeptide behaved as a substrate for both enzymes, and was cleaved at the same site (-Val-//-NH-) as the parent peptide, albeit with a slight delay in hydrolysis (k_{cat}/K_M decreased by a factor of 2.7 for the h-leukocyte elastase catalysed hydrolysis). The presence of the hydrazinopeptidic bond reduced by a factor of 4.7 the apparent enzyme activity without abolishing it. These findings suggest that hydrazinopeptides, if suitably designed, could represent targets in the search for protease resisting pseudopeptides.[196]

Investigations into the mode of heparin binding to protein-C, a plasma serine proteinase inhibitor, concluded that a major binding site is *via* the H-helix.[197] The first defined sequential epitope of the tissue plasminogen activator (t-PA) was determined using a monoclonal antibody raised against a synthetic peptide segment corresponding to the amino acid sequence 341-354 of t-PA. This segment was selected by computer assisted epitope prediction. The sequential epitope was detected by Pepscan method using overlapping octa- and nonapeptides. By fine epitope mapping with tetra-, penta-, hexa- and heptapeptides the epitope was minimised to the pentapeptide EEEQK (347-351). Replacement set analysis confirmed the importance of this amino acid sequence, especially of the amino acid E(348), for antibody binding. Functional assays of rt-PA were not affected by this antibody indicating that the epitope has no influence on the enzymatic centre or the binding site of the inhibitor. The analysis demonstrated that the predicted recognition site of the monoclonal antibody 17-134/11 is exposed on the surface of the native rt-PA molecule.[198]

The synthesis by solid-phase methods of a series of inhibitors of prohormone convertase-1 (PC1) and furin, which are peptidases involved in the biosynthesis of peptide hormones, has been described. The core sequence of the inhibitors corresponded to D-Tyr-Arg-Ser-Lys-Arg-Xaa-Val-Gln-Lys-Asp, where D-Tyr replaced the natural Glu residue and Xaa (the $P_{1'}$ position) corresponded to Ser, Leu, or the unnatural amino acids, D-Ser, β-Ala, γ-Abu, β-Cha or γ-Hyp. Except for the γ-Hyp, L/D-Ser and Leu analogues, the others were found to be competitive inhibitors of human PC1 with K_i values from 1 to 8.6mM. The three analogues containing β-Cha, γ-Abu or β-Ala also proved to be potent inhibitors of the human furin activity, with K_i ranging from 0.8 to 2.2mM. Two more peptides, modified at P1 by addition of a semicarbazone moiety, were also found to be competitive inhibitors of human PC1.[199]

A mode of internal motion of single tryptophan, Trp^{86}, of *Streptomyces subtilisin* inhibitor, has been analysed from its time-resolved fluorescence. The intensity and anisotropy decays of Trp^{86} were measured in the picosecond range. The obtained results revealed that the internal motion of the indole ring became faster, the quenching rate of the fluorescence of Trp^{86} was enhanced and the height of potential energy became lower at higher temperatures, and suggested that Trp^{86} was wobbling around the long axis of the indole ring in the protein.[200]

The proteolytic activity of the multicatalytic proteinase complex, which preferentially cleaves amide bonds after branched chain amino acids, has been found to be inhibited by substrate-related peptidyl aldehydes. The most potent of the inhibitors, Z-Gly-Pro-Phe-leucinal, inhibits the enzyme complex competitively with a K_i of 1.5mM.[201]

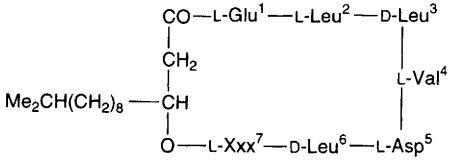
A number of peptides inhibiting prolyl endopeptidase have been screened and isolated from bovine brain by monitoring the inhibition of prolyl endopeptidase produced by *Flavobacterium meningosepticum*. The amino acid sequence of the peptide having the highest inhibitory activity was determined as H-Met-Pro-Pro-Pro-Leu-Pro-Ala-Arg-Val-Asp-Ala-Leu-Asn-OH. This peptide showed IC_{50} and K_i values of 38.4 and 8.6mM, respectively, for prolylendopeptidase isolated from bovine brain.[202] The synthesis of Eurystatin A **57**, a prolyl

endopeptidase inhibitor, employing the Passerati reaction in a key step, has been reported.[203]

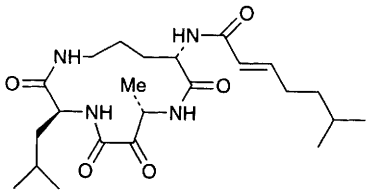
A broad series of *N*-(3-mercaptoacyl) amino acid derivatives have been evaluated for their ability to inhibit atriopeptidase (neutral endopeptidase, EC3.4.24.11) *in vitro* and *in vivo*. Structural parameters studied were (i) the substituent on the 2-position of the 3-mercaptopropionyl moiety, (ii) the amino acid component, (iii) the *S*-terminal derivative, and (iv) the C-terminal derivative. Optimum activity was observed for derivatives of methionine and *S*-alkylcysteines. *N*-[3-Mercapto-2(*S*)-[(2-methylphenyl)methyl]-1-oxopropyl]-methionine **58** was identified as a highly effective inhibitor of atriopeptidase meriting further evaluation as a potential cardiovascular therapeutic agent.[204]

Tripeptidyl peptidase II is an intracellular exopeptidase, which has been purified from rat liver and human erythrocytes. An efficient inhibitor of this enzyme has been prepared through the β-elimination of phosphate from the phosphonopeptide H-Arg-Ala-pSer-Val-Ala-OH. The dehydroalanine-containing peptide thus formed, was a competitive inhibitor with a K_i of 0.02mM. This value, was 45 times lower than for the corresponding Ser-analogue, illustrating the potential value of dehydroalanine containing peptides for peptidase inhibitors.[205]

5.3.2 Cysteine Protease Inhibitors – A number of epoxysuccinyl amino acid benzyl esters (HO-Eps-AA-OBzl) **59** in which the amino acid (AA) had been systematically varied, have been tested as inhibitors of cathepsins L, and S. These analogues of known inhibitor E-64 were designed to investigate whether selectivity for cathepsin L, or cathepsin S could be attained by varying the amino acid bound to the essential epoxide ring which induces inhibition by alkylating the active site thiol of the cysteine proteases. The results indicate that the specificity of these analogues does not parallel that observed for substrates. The greatest selectivity was obtained with HO-Eps-Arg-OBzl which exhibited an 89-fold preference for cathepsin L, over cathepsin S. A change from the L- to the D-stereochemistry for the phenylalanine analogues resulted in a 19-fold drop in k_2/K_i for cathepsin L and a 14-fold drop for cathepsin S. Both E-64 and Z-Phe-Ala-CH_2Cl form two hydrogen bonds with Gly 66 in the active site of papain. With the benzyl esters (HO-Eps-AA-OBzl) one of these hydrogen bonds is necessarily absent. In order to evaluate the importance of this hydrogen bond, three benzyl amide derivatives (HO-Eps-AA-NHBzl) were synthesised. In all cases the potency of the inhibitor was increased and indeed the HO-Eps-Phe-NHBz analogue was 64-fold more potent than the corresponding benzyl ester. For cathepsin L, there is also a 237-fold preference for L-Phe over D-Phe in the benzyl amide analogue. Although the information available from S-2-P-2 interactions with substrates could not be used to enhance the selectivity of the E-64 I analogues in a rational manner, the hydrogen-bonding interaction between the amide proton of the benzyl amide group in HO-Eps-AA-NHBzl and the S-2 subsite for both cathepsins L and S contributed in increasing the potency of these types of inhibitors.[206]



(56)a Xxx = Ile
b Xxx = Leu



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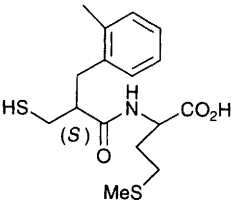

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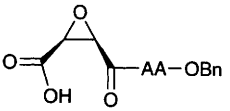

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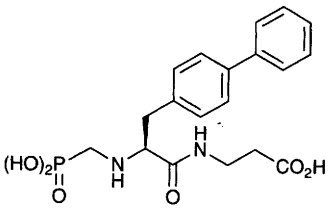

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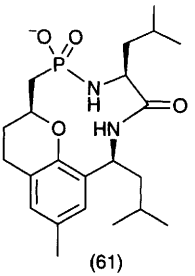

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5.3.3 Metalloprotease Inhibitors – Inactivation of the *Streptomyces griseus* metallo-endopeptidase II by $ClCH_2CO$-DL-(N-OH)Leu-OMe and by $ClCH_2CO$-DL-(N-OH)Leu-Ala-Gly-NH_2 has been the subject of a kinetics study. The results suggested that these reagents bind reversibly and react irreversibly at the activation site of the enzyme.[207] *N*-Phosphonomethyl dipeptides bearing a central (4-phenyl)phenylalanine residue have been reported as inhibitors of the zinc protease neutral endopeptidase. In particular the acid **60** displayed a high inhibitory potency *in vitro*, but lacked oral bioavailabilty. However, several diaryl phosphonate derivatives of **60**, performed as effective prodrugs with suitable bioavailability.[208] The macrocyclic phosphonamidate **61**, an inhibitor of the zinc peptidase thermolysin (K_i = 4nm), has been synthesised. The design of the inhibitor was based upon the structure of the complex formed between thermolysin and Z-$NHCH_2PO_2$-Leu-Leu (K_i = 9nm).[209]

A series of cyclic peptides containing a phosphinic bond have been synthesised and evaluated as inhibitors of a zinc bacterial collagenase from *Corynebacterium rathaii*. Among this series of pseudopeptides of different ring sizes, only two molecules *cyclo*-(Gly-Pro-Phe-ψ[PO_2CH_2]-Gly-Pro-Ahx) and *cyclo*-(β-Ala-Pro-Phe-ψ[PO_2CH_2]Gly-Pro-Ahx) were found to be potent inhibitors of this protease, with K_i values of 120 and 90nM, respectively. Besides determining the influence of the peptide ring size on activity, this study suggested that both the stereochemical and the conformational properties of the pseudophenylalanine residue in these cyclic peptides may determine their potency. Interestingly, the kinetic analysis for the binding of the cyclic peptide inhibitors to collagenase, as compared to a linear parent compound, revealed that the lower potency of the cyclic peptides is mostly the consequence of a lower rate constant for association to the enzyme. This is the first report on cyclic phosphinic peptides and their activities as inhibitors of a zinc protease.[210]

A bacterial collagenase peptide inhibitor (HS-CH_2-CH_2-CO-Pro-NMe-Ala-OH) has been investigated by molecular dynamics and energy minimisation studies, in the presence of solvent molecules. In particular the *cis-trans* conformational isomerism about the amide bonds was investigated. The number of interactions between water molecules and the oxygen atoms of the inhibitor was found to be larger in the *trans* form than in the *cis*. The organisation of water molecules around the inhibitor was thus determined to be crucial in determining the relative population of the *cis* and *trans* conformers.[211]

Joint inactivation of endothelial angiotensin converting enzyme (ACE) and epithelial neutral endopeptidase (NEP), both belonging to the same zinc metallopeptidase family, by a single compound would be of therapeutic interest in the treatment of cardiovascular diseases. Aimed towards this target, the cyclic ACE inhibitor 3-(mercaptomethyl)-3,4,5,6-tetrahydro-2-oxo-1H-1-benzazocine-1-acetic acid was selected as a template. Various aliphatic constraints were introduced on the benzyl moiety of the potent NEP inhibitor N-2-(mercaptomethyl)-3-phenylpropanoyl-tyrosine (IC_{50} NEP = 2nM, IC_{50} ACE = 25nM) to improve the fit between the computed most stable conformers of these molecules and the ACE template. New dual inhibitors, of general formula, *N*-2(*R,S*)-(mercaptomethyl)-3(*R,S*)-phenylbutanoyl-L-amino acid with IC_{50} values in the

nanomolar range for both enzymes were generated by this approach. The separation of the four stereoisomers using chiral amines, and the stereoselective synthesis of the 2-(mercaptomethyl)-3-phenylbutanoyl moiety showed that inhibitors with the 2*S*,3*R* configuration are the most potent against both NEP and ACE. The *in vivo* potency of various prodrugs of these inhibitors was also examined. These results indicated that an efficient and orally active dual inhibitor of NEP and ACE produced beneficial changes in hemodynamics and could represent a therapeutic progress in the treatment of cardiovascular diseases.[212] A structure-activity study of thiazepines and thiazines, acting as dual NEP/ACE inhibitors, has also been undertaken, in an effort to determine the critical parameters for activity against ACE and NEP *in vitro*.[213]

The ability of human collagenase to bind to the tissue inhibitor of metalloproteinases (TIMP) and to TIMP-2 resides mainly in the active site area of the 22000 M(r) *N*-terminal domain of the molecule, but the 27000 M(r) *C*-terminal domain also has a role in stabilising these interactions. The 22000 M(r) fragment was shown to form a complex with TIMP and TIMP-2 which was stable to gel filtration in a similar manner to the whole molecule, but no such complexes were formed by the 27000 M(r) fragment. Complex formation with the whole molecule was prevented by EDTA and by 1,10-phenanthroline demonstrating the importance of the active site. Additionally, TIMP and TIMP-2 competed with a reversibly bound peptide hydroxamic acid inhibitor for the active site. The inhibition of enzyme activity by TIMP and TIMP-2 was less pronounced in the 22000 M(r) fragment when compared to the whole molecule and a similar effect was seen with the peptide hydroxamic acid inhibitor and also with α(2)-macroglobulin, suggesting a role for the *C*-terminal domain in interacting with these inhibitors. Whole molecule collagenase and the 27000 M(r) fragment bound to type 1 collagen-Sepharose while the 22000 M(r) fragment exhibited no such binding, suggesting that the *C*-terminal domain has an important role in the binding of enzyme to substrate.[214]

5.4 RGD Containing Peptides and Analogues – There continues to be much interest in the synthesis of conformationally constrained cyclic peptides containing the RGD-sequence. The chondrolitic activities of integrin-binding and non-binding Fn-fragments (Fn-f) have been shown to be blocked by synthetic peptide analogues of the Arg-Gly-Asp-Ser sequence. The results also suggested that these peptides may be useful for blocking other activities of Fn-f.[215]

Structural features of RGD-related sequences have been investigated by NMR methods. Two linear peptides which inhibit platelet aggregation have been studied: D-Arg-Gly-Asp-Trp and L-Arg-Gly-Asp-Trp. The findings suggested that these fragments adopt a type II′ β-turn structure in solution. Folding features of a non-active cyclic peptide based on the same sequence (*cyclo*-[Arg-Gly-Asp-Trp]$_2$), have also been investigated. The biological relevance of these structures was also discussed.[216] The selective recognition of a number of cyclic RGD peptides of defined conformations, has been investigated in binding assays with $\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.[217]

The cyclic, RGD sequence containing peptide *cyclo*-[Asp-Ser-Lys-Arg-Gly] has

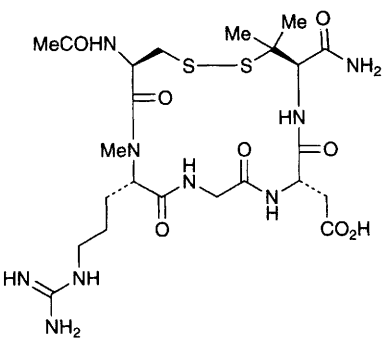
previously been shown to exist in solution in a β- and γ-turn containing conformation. Continuing their investigations, the Davies group have described an optimised synthesis of *cyclo*-[Asp-Ser-Lys-Arg-Gly], and have also reported specific differences in its effect on adhesion and cell spreading for fibroblast 3T3 and macrophage Bac1 cells.[218] A class of potent orally active cyclic peptide antagonist of the glycoprotein IIb/IIIa adhesion molecule have been prepared by linking a tetrapeptide, RGD-containing sequence between the two ends of a semirigid linker. The general structure of these analogues is *cyclo*-(Xxx-Arg-Gly-Asp-*m*-(aminomethyl)benzoic acid), and the conformational constraints imposed on several such compounds by the cyclisation were investigated to determine their effects on receptor binding.[219,220]

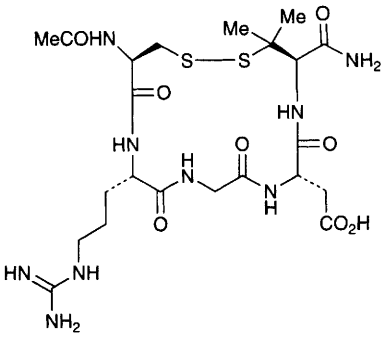
1,3-Dipolar cycloaddition methodology has been used to join together two amino acid derivatives one containing a backbone nitrone, and the other a backbone alkene. The cyclic peptides thus derived were found to be rigid β-turn mimics of RGD; however they did not inhibit activity in the ADP-induced human platelet aggregation (GP IIb/IIIa receptor). This corroborated previous evidence which suggested that the bio-active conformation of this receptor has a turn at Arg, extended ϕ, and ψ angles at Gly, and a γ-turn at Asp.[221]

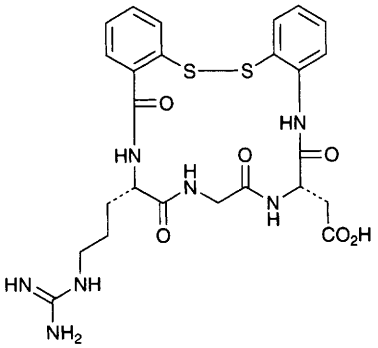
Another report described the cellular binding and internalisation by a filamentous phage, of the cyclic integrin-binding peptide sequence GGCRGDMFGC, in which the conformation of the RGD motif is restricted within a hairpin loop formed by a disulfide bridge between the two cysteine residues.[222] Evidence has been presented that shows that the conformationally restricted RGD containing peptide, *cyclo-S,S*-[1-adamantaneacetyl-Cys-Gly-Arg-Gly-Asp-Ser-Pro-Cys], is a potent inhibitor of cell adhesion mediated by $\alpha_4\beta_1$.[223] Analogues of *cyclo-S,S*-CRRGDSPASSC were synthesised to investigate the structure activity relationship of the peptide.[224,225] Structure-activity studies, have also been effected on *cyclo-S,S*-[Ac-Cys(Nα-Me)Arg-Gly-Asp-Pen]NH_2, in order to improve potency and affinity for the GPIIb/IIIa receptor. The lead compounds are shown in **Figure 7**; in particular compounds **62** and **63** displayed substantial affinity and potency.[226]

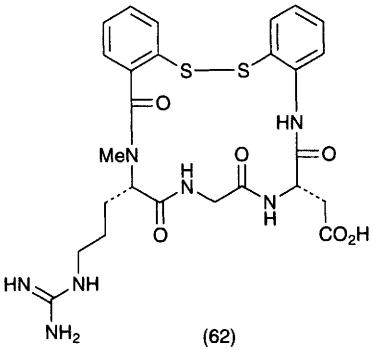
The aqueous conformation of the RGD peptide *cyclo-S,S*-[Ac-Cys-Arg-Gly-Asp-Phe-Pen-NH_2], was determined by NMR and molecular dynamics methods. The study compared the findings with results previously obtained for the conformation of the peptide *cyclo-S,S*-[Ac-Pen-Arg-Gly-Asp-Cys-NH_2], in an effort to understand the flanking residue's effect in RGD peptides.[227] The solution conformation of a cyclic RGD peptide analogue, *cyclo-S,S*-[2-mercaptobenzoate-arginine-glycine-aspartate-2-mercaptoanilide], has also been determined using separately, a binary genetic algorithm, and a molecular dynamics simulation. The two independent approaches were consistent with NMR and CD spectroscopic data; and the resulting backbone conformations of the structures from the two methods were very similar.[228] An ensemble molecular dynamics method has been used to map consensus conformations of the RGD sequence which are accessible to a potent set of structurally diverse inhibitors of fibrinogen-glycoprotein association. A low-energy RGD conformation was identified, which is consistent with known solution structures of potent RGD containing peptides.[229]

The synthetic peptide IASRYDQL has been prepared in an effort to determine









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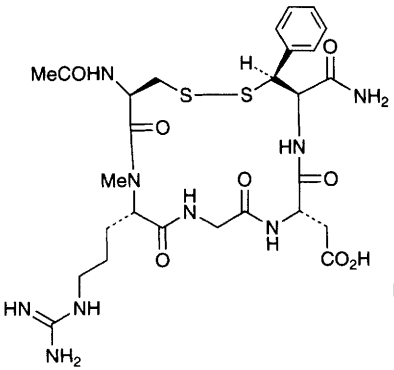
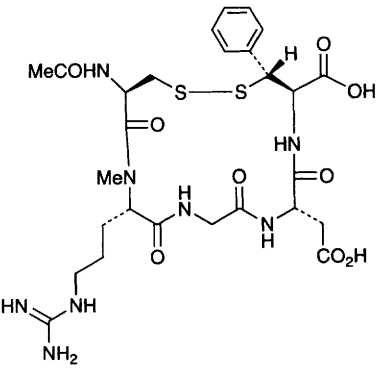




(63)

Figure 7

whether or not the SRYD sequence which is conserved in all gp63 proteins was involved in RGD binding. This peptide was shown by NMR studies in DMSO, to adopt different conformations, depending upon the amounts of residual water present in solution.[230]

5.5 Miscellaneous Enzyme Inhibitors – The crystal structure of an influenza virus peptide complexed with the human class MHC protein HLA-DR1 has been obtained. Thirty-five per cent of the peptide was found to be accessible to solvent and potentially available for interaction with the antigen receptor on T-cells.[231] The rational design of ligands for the substrate-binding site of trypanothione reductase (TR) has been reported. Peptides were designed to be selective for TR over human glutathione reductase, and Bz-Leu-Arg-Arg-β-naphthylamide was found to meet the criteria.[232]

A number of inhibitors of human carbonic anhydrase II (HCAII, EC4.2.1.1) that bind with nanomolar dissociation constants have been reported. These inhibitors were developed by exploiting interactions with hydrophobic 'patches' in the lip of the active site of this enzyme. The patches are molecular surfaces presented by a phenylalanine on one face of the active-site cleft (Phe-131) and three adjacent hydrophobic residues on the opposite face (Leu-198 and Pro-201/202). Attempts to design inhibitors capable of binding simultaneously to Phe-131 and Leu-198/Pro-201/202 did not lead to molecules that-bound more tightly than those binding to these hydrophobic sites individually.[233]

6 Side Chain Interactions Studied by Residue Substitution or Deletion and Similar Modifications

6.1 Peptides with 'Opioid Characteristics' – The conformational features of the δ-selective, cyclic peptide *cyclo-S,S*-[Tyr-D-Cys-Phe-D-Pen] were investigated using a combination of solution, solid-state and theoretical techniques. Two distinct conformers were revealed for the more rigid cyclic backbone of the molecule, but the flexible elements of the molecule were thought not to adopt any fixed structure in aqueous solution.[234] The Mosberg group continued their investigation, first by preparing a series of analogues in which the conformationally labile Tyr residue was replaced with several less flexible analogues (*e.g.* 1,2,3,4-tetrahydroquinoline-3-carboxylic acid). This determined that the side chain of residue 1 adopts a *trans* conformation when bound to the δ-receptor. This work also identified an extended conformation of the exocyclic peptide, which when combined with the conformation of the cyclic tripeptide, gave the conformation required for δ-receptor binding.[235] The same group also investigated the *in vitro* pharmacological and conformational properties of a series of analogues of peptide *cyclo-S,S*-[Tyr-D-Cys-Phe-D-Pen], in which the Phe^3 residue was replaced by each of the four stereoisomers of β-methylphenylalanine. The modest conformational constraints imposed by the β-methyl group, precluded definite assignment of low-energy conformers, for each of the analogues individually. However, it was possible to superimpose the

structures and arrive at complete model regarding the conformational features of the pharmacore in the δ-receptor bound state.[236]

The properties of di- and tri-peptides containing 1,2,3,4-tetrahydroquinoline-3-carboxylic acid (Tic **11**) in the second position showed that the message domain of opioid peptides can be composed of only two residues.[237] The authors continued their study by converting enkephalin and dermorphin into δ-selective opioid antagonist by insertion of the Tyr-Tic message domain into these peptides. NMR conformational studies, suggested that the orthogonal arrangement of the two aromatic rings found in the *cis*-Tyr-Tic moiety, typical of *N*-methyl naltrindole and other δ-selective opiate antagonists, is responsible for the antagonistic activity of all these peptides.[238] A theoretical conformational analysis of the δ-antagonist H-Tyr-Tic-Phe-OH and the μ-agonist H-Tyr-D-Tic-Phe-NH_2 showed that whilst the Tic residue imposed conformational restriction on the peptide, a certain amount of flexibility was maintained. The generated structures of these tripeptides were also correlated with naltrindole, and showed good spatial overlap of minimum energy structures within the pharmacophoric groups.[239]

The crystal structure, thermal vibrations and electron density distribution of Leu-enkephalin, (Tyr-Gly-Gly-Phe-Leu), have been analysed using X-ray diffraction data and SCF calculations.[240] [D-Pen^2,D-Pen^5]enkephalin, a highly potent δ-opioid receptor-selective compound, has been examined by X-ray diffraction and found to adopt a solid state conformation similar to that determined previously by NMR techniques.[241] Deltakephalin is a synthetic opioid peptide which differs from enkephalin in that a D-Thr has substituted for Gly^2, and a sixth residue, Thr has been added. The crystal structure of deltakephalin revealed that the peptide possesses a pseudo type I' β-bend, which is stabilised by an intramolecular side-chain to backbone hydrogen bond. This is the first reported observation of a pseudo β-bend conformation in a solid-state structure of an enkephalin analogue.[242]

Twelve analogues of the peptide H-Tyr-Gly-Gly-Phe-Met-Lys-Arg-Tyr-Gly-Gly-Phe-Met-OH, which corresponds to the peptide segment encompassing residues 100-111 of proenkephalin, have been synthesised using solid-phase methods. Opioid activities of the peptides were determined in the guinea pig ileum (GPI) assay and in the mouse vas deferens (MVD) assay. Some analogues showed higher potency than Leu-enkephalin in the GPI assay, whereas all compounds were less potent than Leu-enkephalin in the MVD assay.[243]

Cyclic analogues of deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) and delorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) have been synthesised. These peptides have the general structure [D-Xaa^2,Yaa^5]deltorphin I or II, where Xaa^2 represents D-cysteine or D-penicillamine, and Yaa^5 is an L- or D-penicillamine residue. In biological assays, the analogues proved to be highly δ-selective. This study also confirmed the authors' suggestion that an increase in the lipophilicity of the surface of the *C*-terminus of the peptides, would increase the receptor selectivity of the analogues.[244] Another series of substitutions on deltorphin I, in which the Phe^3 residue was replaced with specific aromatic and non-aromatic amino acids, has also been effected. The analogues displayed

slightly lower binding constants than deltorphin I, except when the residue was shorter than Phe, or non-aromatic, in which case the binding constants were markedly decreased. These results are compared with a similar study effected in Tyr-*cyclo*-*S,S*-[D-Cys-Phe-D-Pen].[245]

In an effort to correlate the topography of the bioactive structures of *cyclo*-[D-Pen^2, D-Pen^5]enkephalin (DPDPE) and the deltorphins, a number of chimeric peptides have been synthesised in which the *C*-terminal dipeptide δ-address of the deltorphins have been linked to the highly δ-opioid selective DPDPE and DPLPE peptides. A major structural feature determining high potency of hybrid analogues was found to be the chirality of residue 5. The importance of the hydrophilicity of amino acids in positions 2 and 5 for δ-selectivity was consistent with previous findings. The results also suggested that the δ-receptor interacts with hybridised enkephalins and deltorphins, differently than with DPDPE.[246]

A highly constrained tyrosine derivative, (2*S*,3*S*)-β-methyl-2′,6′-dimethyltyrosine has been prepared by asymmetric synthesis and incorporated into [D-Pen^2, D-Pen^5]enkephalin and deltorphin I. The results of binding assays and bioassays showed that the two analogues acted very differently at δ-opioid receptors. Further pharmacological evaluations suggested that they actually interact primarily with the δ(1) and δ(2) receptor subtypes, respectively. These results, and conformational studies using NMR and computer-assisted modelling, provided insights into the different stereochemical requirements for these two δ-opioid ligands to recognise the δ-opioid receptor and its subtypes.[247]

An *N*-substituted 2-benzazepine, previously reported to possess morphine-like analgesic activity *in vivo*, has been adapted for use as a constrained mimic of the *N*-terminal residues of leucine enkephalin. Molecular modelling was used to evaluate the suitability of the 2-benzazepine nucleus for this purpose. The principal peptide constraint structure, 2-(7-methoxy-2,3,4,5-tetrahydro-1H-2-benzazepin-2-yl)-ethanoic acid, and some structurally related benzazepine analogues were synthesised and incorporated into peptides using solid-phase protocols. Radioligand receptor binding studies were then used to evaluate the general opioid receptor affinity of the target compounds, and affinities comparable to the corresponding leucine enkephalin analogue were obtained.[248]

Leucine enkephalin has been reacted with mushroom tyrosinase under reductive conditions to produce an analogue hydroxylated at the Tyr^1 moiety of the peptide. The affinity of [HO-Tyr^1]leucine enkephalin to receptors in rat brain homogenate was compared to that of leucine enkephalin itself. Hydroxylation of leucine enkephalin was found to decrease receptor affinity to both μ- and δ-opioid receptor sites by a factor of about 20.[249]

A comparative conformational analysis of dermorphin and deltorphin II has been effected in aqueous solution by NMR techniques, in an effort to examine the conformational characteristics that relate to the respective selectivities towards μ- and δ-opioid receptors. The conformers which fulfilled the required geometry constraints were then subjected to molecular dynamics studies. Although dermorphin and deltorphin II were found to exist in an equilibrium among many flexible conformers, some differences could be observed. The conformers of dermorphin showed a structure rounded at the *N*-terminal Tyr-D-Ala-Phe-Gly-Tyr and

C-terminal Gly-Tyr-Pro-Ser-NH_2 moieties, which were found to be almost at right angles to each other, while those of deltorphin II were characterised by a 'hook'-shaped backbone structure in which the nearly extended conformation of the Val-Val-Gly-NH_2 sequence was located under the folded conformation of the *N*-terminal Tyr-D-Ala-Phe-Glu sequence. The possible relationship between these conformational characteristics and the μ/δ-opioid receptor selectivities was also discussed.[250]

The opioid receptor antagonist properties of the μ-opioid receptor selective peptide, Boc-Tyr-Lys-Lys-Trp-Trp-NH_2 and its systematically modified analogues were determined in guinea pig ileum, mouse vas deferens and rabbit vas deferens bioassays to locate the necessary structural features to develop κ-receptor selective antagonist(s) of substantial affinity. Replacing the tyrosine residue by phenylalanine as well as increasing the lipophilicity of the *C*-terminal by isoamylamide substitution yielded enhanced κ-receptor affinity. The presence of the *C*-terminal lipophilic Trp-Trp-NH_2 region was found to be necessary as revealed from the equilibrium dissociation constant values. Recognition that only one lysine residue is required for the antagonist activity led to the synthesis of the tetrapeptide Boc-Tyr-Lys-Trp-Trp-NH_2 having a κ/μ selectivity of 22 and a K_e of 5.4mM.[251]

Analogues of the potent and moderately μ-opioid-receptor-selective cyclic β-casomorphin-5 derivative *cyclo*-[H-Tyr-D-Orn-Phe-D-Pro-Gly] (**64**) have been prepared by conventional solution synthesis. Replacement of the Phe^3 residue by 2-naphthylalanine led to a peptide **65** with high affinity for both μ- and δ-opioid receptors. This compound turned out to be an agonist in the μ-receptor-representative guinea pig ileum (GPI) assay, but a moderately potent antagonist against various δ-agonists in the 6-receptor-representative mouse vas deferens (MVD) assay. It thus represents the first known cyclic opioid peptide analogue with mixed μ-agonist/δ-antagonist properties. Interestingly, replacement of 2-naphthylalanine in compound **65** with 1-naphthylalanine resulted in an analogue **66** showing high affinity for μ-receptors and a full agonist effect in the MVD assay that was mediated by both μ- and δ-receptors. Further reduction of the peptide ring size, as achieved by deletion of the Gly^5 residue, produced a compound which was a full agonist in both bioassays. Conformational analysis of the analogues, using NMR spectroscopy and molecular mechanics studies, suggested that the antagonist properties of analogue **65** may not be due to a difference in its overall conformation as compared to the agonist **64** but may be a direct effect of the 2-naphthyl moiety *per se*, preventing proper alignment of the peptide for receptor activation.[252]

A systematic series of dynorphin A (H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH) analogues, in which the derivatives incorporated the sulfhydryl amino acids L- and D-Cys and L- and D-Pen in positions 5 and 11, have been synthesised. The study was aimed at investigating the potency and selectivity of dynorphin A analogues towards κ-opioid receptors, and suggested that the requirements for binding are not the same for the κ, μ and δ-central receptors.[253]

The low energy conformations of Met-enkephalin (Tyr-Gly-Gly-Phe-Met) on

the AMBER potential energy surface have been located by an efficient search procedure based on the simulated annealing method. The observed conformations were classified into three distinct conformational groups; those with a type II' β-turn at Gly^3-Phe^4; those possessing a type II β-turn at Gly^2-Gly^3; and those containing three consecutive γ-turns along the backbone. The lowest energy conformation was classified within the first group and the β-turn at Gly^3-Phe^4 further extended into an antiparallel β-sheet including Gly^2 and Met^5.[254]

trans-5-Methylproline has been prepared from proline, by a route involving electrochemical oxidation followed by methylcopper substitution. Incorporation of this proline derivative into the 2- and 4-positions of β-casomorphin-5 (H-Tyr-Pro-Phe-Pro-Gly-OH) was shown by NMR investigations to have little effect on the *cis*/*trans* ratio of the peptide bond. The opioid receptor affinities did not allow the confirmation of the requirement for a *cis*-Tyr-Pro peptide bond for biological activity.[255] A dipeptide phenylalanyl-glycine has been crystallised as a 1:1 complex with trichloroacetic acid. The *trans*-peptide unit showed significant deviation from planarity, and the peptide backbone was folded with torsion angles at glycine adopting a typical D-residue conformation. The relevance of this study to bioactive conformations of enkephalins was also discussed.[256]

6.2 Cholecystokinin Analogues – Conformational properties of all four stereoisomers of a synthetic amino acid, β-methylphenylalanine (β-MePhe), when incorporated into the bioactive octapeptide sequence of cholestocystokinin, *i.e.* H-Asp-Tyr-β-MePhe-Gly-Trp-Nle-Asp-Phe-NH_2, have been studied by using ^1H and ^{13}C-2D NMR spectroscopy. β-Methylphenylalanine residues were found to introduce significant perturbations to the side-chain conformations.[257]

Cionin, a protochordate-derived octapeptide amide related to the gastrin/cholecystokinin family of peptides, contains two consecutive tyrosine sulfate residues. In order to gain insight into the role of each of these tyrosine sulfate residues in the biological activity, cionin and its derivatives in which one of the two tyrosine sulfate residues was replaced by tyrosine have been prepared by two Fmoc-based solid-phase approaches. Cionin and mono-Tyr(SO_3H)-containing derivatives were assayed on exocrine pancreas in dogs.[258]

6.3 Angiotensin Analogues – The role of the NH_2-terminal domain of angiotensin II (ANGII) and [Sar^1]ANGII has been investigated by NMR techniques, and compared with the biologically inactive COOH-terminal pentapeptide [des,1,2,3]ANGII. The findings indicated that the NH_2-terminal domain of ANGII appears to have an essential role in generating the biologically active charge relay conformation of the hormone.[259]

A series of analogues of ANGII, with a combination of methylations at positions 1 and 7, have been prepared by solid-phase methods. Biological assays indicated that all modifications resulted in reduced affinity. Simultaneous modifications induced affinity loss in a synergic manner. Agonistic (Phe^8) analogues containing Aib in position 7 all showed reduced intrinsic activity, indicating an influence of this position on the activation mechanism of the angiotensin receptor of the type AT_1.[260]

A series of cyclic amide-linked ANGII analogues have been synthesised involving various cyclisations between terminal amine and acid groups, and side-chain amine and acid groups. All of the analogues were biologically inactive, except for cyclic [Sar^1, Asp^3, Lys^5]ANGII which had high contractile activity in the rat uterus assay (30% of ANGII), and [Lys^1,$Tyr(Me)^4$,Glu^8]ANGII which had weak antagonist activity. Precyclic linear peptides synthesised using 2-chlorotrityl chloride resin and *N*-α-Fmoc-amino acids with suitable side chain protection were obtained in high yield and purity and were readily cyclised with benzotriazol-1-yloxy-tris (dimethylamino)-phosphonium hexafluorophosphate as coupling reagent. Molecular modelling suggested that the ring structure of the potent analogue can be accommodated in the charge relay conformation proposed for ANGII.[261]

The effect of substitutions at the imidazole 5-position has been studied on esters and amides of DMP 811. Good binding constants were recorded for some analogues.[262]

The effect of structural changes in the *N*-terminal amino acid of angiotensin-IV, with respect to AT receptor binding, has been examined by competition with [^{125}I]angiotensin-IV in bovine adrenal membranes. Replacement of the 1-2 peptide bond of angiotensin-IV with the methylene bond isostere (CH_2-NH) increased the K_i approximately five fold, indicating that the peptide bond may be replaced whilst maintaining relatively high-affinity receptor binding.[263]

6.4 Oxytocin and Vasopressin Analogues – Oxytocin, [Trp^9]oxytocin and [Val^2,*p*-$FPhe^9$]oxytocin have been prepared for the purpose of determining the average distance between the chromophores (Tyr^2 and Trp^9, in the first analogue; and *p*-$FPhe^9$ and the disulfide bridge, in the second). The inter-chromophore distances determined by fluorescence spectroscopy were similar to those obtained from molecular mechanics calculations.[264]

A series of new linear photoactivatable and iodinatable antagonists of the neuropeptidic hormone vasopressin have been synthesised. These were based on modifications of a previously reported potent and selective antagonist (phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2) of the vasopressor response (V-1a receptor) to [arginine]vasopressin. (Azidophenyl)alkyl substitutions, of the general structure N_3-$C_6H_4(CH_2)_nCO$ (n = 0, 1, 2, or 3), were employed in position 1. The seven new analogues were:

4-N_3-C_6H_4CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**67**),
3-N_3-C_6H_4CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**68**),
4-N_3-$C_6H_4CH_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**69**),
3-N_3-$C_6H_4CH_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**70**),
4-N_3-$C_6H_4(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**71**),
3-N_3-$C_6H_4(CH_2)_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**72**),
4-N_3-$C_6H_4(CH_2)_3$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2 (**73**).

All the analogues were tested for their affinity to the rat hepatic V-1a receptor. Analogues **67** and **68** had a low affinity (K_i approximate to 20nM), and analogues **69-73** showed a high affinity (K_i between 0.04 and 0.3nM). Analogues **69-73** were

iodinated on the Tyr-9 residue, giving a further series of compounds. All five iodinated derivatives exhibited K_i values of 0.2-1nM for rat liver membranes. Their affinities for oxytocin and renal V-2 vasopressin receptors were however much lower. Moreover, all analogues completely antagonised the vasopressin-stimulated inositol phosphates production in WRK(1) cells and were devoid of any agonistic potency. They are promising candidates as potential high-affinity, highly selective, photosensitive ligands for the V-1a receptor site.[265]

Conformational energy calculations, have been effected on an oxytocin antagonist, *cyclo*-[prolyl-D-phenylalanyl-isoleucyl-D-dehydropiperazyl-dehydropiperazyl-D-(*N*-methyl)phenylalaninyl] (L-365209). The favourable conformations, were compared with selected low-energy conformations of the potent antagonist [Dmp[1], *cyclo*-(Glu[4],Lys[8])]oxytocin, and a spatial match was found. This implied a common pharmacore responsible for binding of the two peptides to the uteronic receptor. The antagonist [Dmp[1], *cyclo*-(Glu[4],Lys[8])]oxytocin, was in fact found by molecular dynamics calculations to adopt three distinct conformations, two of which matched the spatial requirements of the common pharmacore, with one of these conformations also being identified by NMR methods.[266,267]

6.5 Thrombin Binding Peptides – The conformation of the cyclic hirudin *C*-terminal peptide **74** bound to the exosite of thrombin, has been studied by theoretical and experimental NMR techniques. The exosite of thrombin was found to be very specific to both the backbone and the side-chain conformations of the hirudin peptide, especially those of residues Phe[56] and Ile[59].[268] The solid phase synthesis of a new bivalent hirudin analogue **Figure 8**, which includes the hydrolytically stable 4′-phosphono-phenylalanine mimic of tyrosine-*O*-sulfate, has been reported. The peptide inhibits α-thrombin, acting therefore as an anticoagulant agent.[269] The structural essentials of Ser[1] in receptor activation by SFLLRNP, a ligand peptide tethered to the thrombin receptor, were investigated by the synthesis of a series of analogues in which Ser[1] had been replaced.[270]

X-ray data for hirudin, and the thrombin active-site inhibitor D-Phe-Pro-Arg-CH_2Cl, have been employed in the design of a new thrombin inhibitor. In the new inhibitor, the *C*-terminal amino acid residues 53-65 of hirudin are linked by a spacer peptide of four glycines to the active-site inhibitor NAPAP (*N*-α-(2-naphthylsulfonyl-glycyl)-DL-*p*-aminophenylalanyl-piperidide). The conformational flexibility of the linker peptide in the new inhibitors was investigated with molecular dynamics techniques. A correlation between the $P_{1'}$ position and the interactions of the linker peptide with the protein was suggested. Modifications of the linker peptide were proposed based on the distribution of its main-chain torsion angles in order to enhance its binding to thrombin.[271]

The *N*-terminal peptide from a new type of thrombin receptor has been shown to exhibit thrombin receptor agonist activity. The effects of this synthetic thrombin receptor against the peptide (SFLLRNPNDKYEPF, TRAP) on human umbilical vein endothelial cells (HUVECs), was examined. TRAP induced rapid morphological changes in HUVECs, with marked increase in the release of prostacycline, endothelin, platelet activating factor, tissue type plasminogen

activator, and plasminogen activator inhibitor-1. Incubation of cells with TRAP also induced a rapid decrease in cell-surface thrombomodulin. Activation of the described thrombin receptor may therefore alter their role in the hemostatic pathway.[272] A synthetic heptapeptide H-Ser-Phe-Phe-Leu-Arg-Asn-Pro-NH_2, which corresponds to the ligand peptide latent in rodent thrombin receptors, has been shown to activate the thrombin receptor in the absence of thrombin. In order to evaluate the structural requisites of two consecutive phenylalanines, three sets of analogues with substitutions at position either 2 or 3 were synthesised and examined for their stimulatory activity in phosphoinositide turnover in SH-EP epithelial-like cells. The replacement of Phe^2 by Ala completely eliminated the activity, while that of Phe^3 retained about 50% activity with a full stimulation. The Phe/Leu substitution resulted in a large increase (37-fold) in EC_{50} value for Phe^2, but in insignificant change for Phe^3. Substitution of *para*-fluorophenylalanine ((*p*-F)Phe) for Phe^2 enhanced strongly (4-fold) the activity, in contrast to a reduction by the Phe^3/(*p*-F)Phe substitution. Elimination of either Phe^2 or Phe^3 resulted in a complete loss of activity. The results indicated that Phe^2 and Phe^3 play different roles in the receptor activation. A highly specific aromatic π-π interaction was suggested between Phe^2-phenyl and thrombin receptor binding site, while Phe^3 appeared to be important for retaining a bioactive conformation.[273]

Simulated annealing techniques have been used to explore the conformational space of the potent antithrombotic peptide Lys-Arg-Asp-Ser (KRDS) and of two analogues: D-Lys-Arg-Asp-Ser (K(D)RDS) which is inactive, and Lys-Arg-Glu-Glu (KREE), which exhibits a strong biological activity. A reduced number of conformational classes were obtained and conformations corresponding to predominant classes were found to be in qualitative agreement with structural parameters deduced from ^1H NMR spectra. A comparison between the conformational classes of active and non active peptides showed that some conformations were found to be specific for active peptides.[274]

6.6 Tachykinin Analogues – The solution structure of a hexapeptide, *cyclo*-(Gln-Trp-Phe-Gly-Leu-Met), which is a selective neurokinin-2 antagonist, has been studied by NMR and molecular dynamics techniques. Comparison was made with other neurokinin-2 antagonists, and the activity of the cyclic antagonist was suggested to be inversely related to the conformational rigidity of the cyclic peptides.[275]

Analogues of the *C*-terminal fragment, residues 4-11, of substance P (SP_{4-11}), have been synthesised in which the methionyl residue was replaced successively by the Glu(OEt), Glu(OBn), Hse(Me) and Glu(CONHMe) residues. Analogues of neurokinin A, residues 4-10 (NKA_{4-10}), and of neurokinin B, residues 4-10 (NKB_{4-10}), were also prepared, in which the methionyl residue was replaced by the Hse(Bn) and Hse(Me) residues respectively. The SP_{4-11} analogues were tested with *in vitro* preparations representative of neurokinin-1, 2 and 3 receptor types. Only against the neurokinin-2 preparations were the SP_{4-11} peptides found to be more powerful than the parent octapeptide. The selectivity of all the analogues was found to be reduced when compared with the corresponding hexapeptide

(Suc-Phe-Glu-Glu-Ile-Pro-Lys-Glu)

(74)

Asn-Gly-Asp-Phe-Glu-Abo-Ile-Pro-Glu-Glu-Xaa-Leu-D-Glu-OH

$(Gly)_4$

Pro-Arg-Pro-D-Phe-H

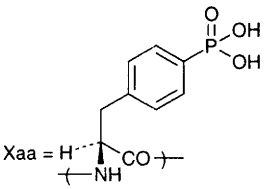
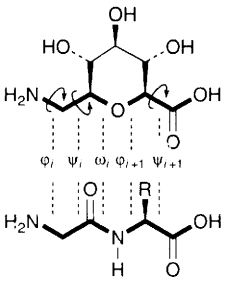
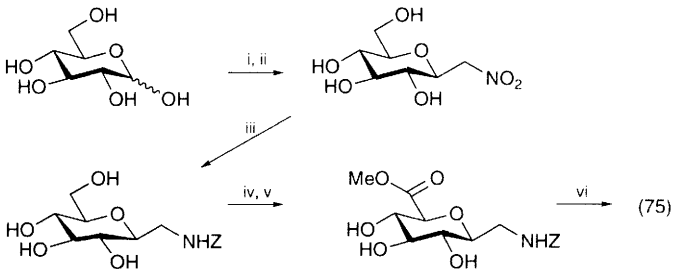


Figure 8



(75)



Reagents: i, $MeNO_2$, NaOMe; ii, H^+/H_2O, Δ; iii, H_2, Pd/C, Z-Cl; iv, O_2, Pt/C; v, MeOH, DCC/DMAP; vi, NaOH, H_2Pd/C,

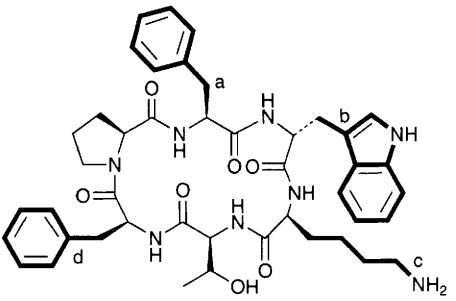
Scheme 9

analogues. The SP_{4-11} analogues showed reduced affinity for neurokinin-1 receptors, whilst the NKA_{4-10} and NKB_{4-10} peptides showed similar affinities to neurokinin A and neurokinin B for neurokinin-2 and neurokinin-3 receptors, respectively. The effect of the lipophilicity of the Met^{11} side chain, especially when a phenyl group is present in the side chain, at the neurokinin-2 receptor was also discussed.[276]

Constrained analogues of phenylalanine have been designed for analysing the binding pockets of Phe^7 (S-7) and Phe^8 (S-8), two aromatic residues important for the pharmacological properties of substance P (SP). The amino acid derivatives used were tetrahydroisoquinoleic acid, diphenylalanine, 9-fluorenylglycine (Flg), 2-indanylglycine, the diastereomers of 1-indanylglycine (Ing) and 1-benz-[f]indanylglycine (Bfi), and the *Z*- and *E*-isomers of dehydrophenylalanine (Δ^ZPhe, Δ^EPhe). According to the binding data, the S-7 subsite was interpreted to be small, as only the gauche (-) probe [(2*S*,3*S*)-Ing^7]SP presented a high affinity for specific NK-1 binding sites. The [$\Delta^E Phe^7$]SP analogue, which projects the aromatic ring toward the trans orientation, was over 40-fold more potent than the corresponding *Z*-isomer. These conflicting results could be due either to the binding protein quenching the minor trans rotamer of [(2*S*,3*S*)-Ing^7]SP, or to the constrained amino acid side chain rotating when inserted in the protein. In position 8, the high binding affinities of [Flg^8]SP and [(2*S*,3*S*)-Bfi^8]SP suggested that the S-8 subsite is large enough to accept two aromatic rings in the gauche (-) and one aromatic ring in the trans orientation. Peptides bearing two conformational probes in positions 7, 8, or 9 led the authors to postulate that the S-7, S-8, and S-9 subsites are independent of one another.[277]

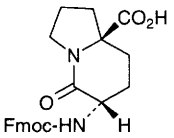
6.7 Somatostatin Analogues – The sugar amino-acid (Gum) **75** has been proposed as a novel peptidomimetic. The analogue was prepared in four steps from glucose (**Scheme 9**), and presumed to act as a conformationally constrained dipeptide isostere. Two cyclic somatostatin analogues, *cyclo*-(Gum-Tyr-D-Trp-Lys-Val) and *cyclo*-(Gum-Phe-D-Trp-Lys-Thr), were prepared, and found to be active in the submicromolar range for the inhibition of the release of growth hormone (IC_{50}, 0.47 and 0.15mM respectively).[278] β-D-Glucose, has also been used as scaffolding in the synthesis of a non-peptide peptidomimetic of somatostatin, shown in **Figure 9**, along with the structure of the cyclic peptide L-363,301 which was being mimicked. The critical amino acid side chains were retained for analysis of structure-activity relationships.[279]

A group of new peptide ligands displaying high selectivity for binding to somatostatin receptor subtypes (SSTR) 2, 3 or 5 have been used to characterise somatostatin receptor involvement in the inhibition of glucagon secretion in rats. It was found that NC-8-12 and DC-25-100, which have high affinity for SSTR2 and much less affinity for the type 5 receptor, were by far the most potent inhibitors of glucagon secretion with EC_{50}s of 48 and 18nmole, respectively, relative to somatostatin itself (EC_{50} 131nmole). These two analogues were however much less potent than somatostatin in inhibiting glucose-stimulated insulin release. In contrast, DC-23-99 (a type 5 receptor selective analogue), which was previously found to be a more potent inhibitor of insulin secretion

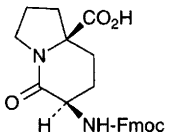


L-363,301

Figure 9



(76)



(77

than somatostatin, had considerably less potent (EC_{50} 410nmole) effects on glucagon release. The SSTR3-specific ligands, DC-25-12 and DC-25-20, were not effective at the doses tested. The differing spectra of activities of these analogues suggest that inhibition of insulin and glucagon secretion in rats is mediated by entirely different somatostatin receptor populations.[280]

Structure-activity studies of glucagon-like peptide-1 (GLP-1), were effected by replacing each amino acid with alanine to identify side-chain functional groups required for interaction with the GLP-1 receptor. Side chains in positions 7, 10, 12, 13 and 15 were reported to be directly involved in the receptor interaction, whilst positions 28 and 29 were important for the conformation recognised by the receptor.[281]

6.8 Bradykinin Analogues – Two Fmoc-protected bicyclic β-turn mimics **76** and **77** were synthesised in nine steps from (*R*)-2-allylproline for use as *cis*-Gly-Pro peptide mimetics and incorporated into analogues of bradykinin.[282] However, the resulting peptide analogues showed little activity at the bradykinin B_2 receptor. This corroborated evidence that the active conformation of bradykinin comprises a *trans*-Ser^6-Pro^7 geometry, as opposed to a *cis*-Ser^6-Pro^7 amide bond.

NMR and CD studies have been carried out on solutions of bradykinin and a bradykinin antagonist in solutions of aqueous trifluoroethanol. The results indicated that bradykinin may be a mixture of at least two conformers, and that one predominates at concentrations of trifluoroethanol of 80% or higher. The conformation of bradykinin at lower trifluoroethanol concentrations appeared similar to that of the bradykinin antagonist.[283]

The preparation by solid phase methods of linear and cyclic kinin analogues has been described. Amongst the peptides prepared were *cyclo*-bradykinin, *cyclo*-kallidin (*cyclo*-Lys-bradykinin) and a *cyclo*-kallidin-vespulakinin 1 hybrid [*cyclo*-(Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly)]. Preliminary pharmacological experiments showed that the cyclic kinin analogues are much less potent than bradykinin but still show specific bradykinin-like action, results which support the hypothesis of the presence of a pharmacore in the centre of the (brady)kinin molecule.[284]

6.9 Miscellaneous Examples – Inhibition of NADPH oxidase activity by synthetic peptides mapping within the carboxyl-terminal domain of small GTP-binding proteins has been reported. The inhibition however was found not to be sequence specific but related to the presence of a polybasic motif.[285] The binding properties of human Factor IX were investigated by the solid phase synthesis of two peptide sequences, Factor IX-(1-47) and Factor IX-(1-42). When monitored by fluorescence quenching, calcium ions induced the prototypical conformational transition that is observed in Factor IX in Factor IX-(1-47) but not in Factor IX-(1-42).[286] Analogues of mast cell degranulating peptide have been prepared, by removing residues 16-18 (Arg-Lys-Ile), 1-2 (Lys), 1-2 and 16-18 and by acetylation of the amino end (Ile). The analogues were tested on mast cells for histamine-releasing activity. The findings suggested that the *C*-terminus is more important than the *N*-terminus in determining bioactivity of MCD peptide.[287]

New semisynthetic analogues of human insulin, modified in the *C*-terminal region of the B-chain, have been prepared in order to study the role of particular amino acid residues in the expression of hormone biological properties. Biological *in vitro* potencies (specific binding to cultured human lymphocytes IM-9 and lipogenic potency in isolated rat adipocytes) of the semisynthetic analogues were estimated, ranging from 0.2 to 100% relative to porcine insulin.[288]

A nona-peptide GAAVLEDSQ corresponding to the *N*-terminus of a novel cytokine, monocyte cytotoxicity inducing factor, (MCF), has been prepared. Also synthesised were two truncated peptides, GAAVL and LEDSQ, and the substituted peptide, GAAVLENSQ. The authentic *N*-terminal peptide was biologically active in the nanomolar range, while substitution of asparagine for aspartic acid at position 7 diminished biological activity. Biological activity was observed from the *C*-terminal fragment LEDSQ, but the *N*-terminal pentapeptide (GAAVL) was devoid of biological activity. The *N*-terminus of MCF therefore appears important in interacting with the binding site on monocytes.[289] The free and Ca^{2+}-bound conformations of the synthetic peptide Boc-Leu-Pro-Tyr-Ala-$NHCH_3$, a substrate for a protein tyrosine kinase, have been examined, using CD, NMR and molecular modelling methods. Besides its relevance in terms of the possible involvement of divalent cations in substrate-tyrosine kinase interaction, the conformational characterisation of 'Boc-Leu-Pro-Tyr-Ala-$NHCH_3$ and its Ca^{2+} complex should help understand the conformational determinants for Ca^{2+}-binding by linear peptides.[290]

Six insect neuropeptide proctolin analogues modified in position 4 of the pentapeptide skeleton, such as Arg-Tyr-Leu-X-Thr, where X = Hyp, Hyp(4-OMe), Thz, homo-Pro, Ach (1-aminocyclohexane-1-carboxylic acid) or Sar were synthesised by solution-phase methods. Their cardiotropic effects were examined on two insect species (*Tenebrio molitor L.* and *Periplaneta americana L.*), and the importance of the pyrrolidine ring in Pro residue for the entire biological activity of proctolin was inferred.[291] The same laboratories, continued their investigations of proctolin, with the preparation of (Arg-Tyr-Leu-Pro-Thr) and 42 analogues modified in positions 1-4. The activities of proctolin and its analogues were examined in various biological preparations, such as: myotropic effects in selected insect species *in vitro* and behaviour of rats *in vivo*. The structure/activity relationship in these varied preparations was discussed.[292]

Molecular dynamics simulations of the conformational behaviour of the conformers of PIDOTIMOD, an interleukin-2 lymphocyte T receptor agonist dipeptide, have been carried out. Molecular dynamics simulations at constant temperatures of 300 and 400K and constant total energy runs at high temperatures were performed, and trajectories and statistical properties of motions investigated. At 300K two *trans* and one *cis* conformer were found to be dynamically stable. At 400K both *trans* and *cis* conformers were thermally mixed. At high temperatures large fluctuations of the peptide bond between the oxoprolyl and thiazolidine rings were observed. Nevertheless in the short time scale *trans cis* interconversion did not occur. Suggestions on the nature of the structure activity relationship were also made.[293] An X-PLOR scheme for imitating the action of ribosomes in aiding synthesising peptides to find their ultimate

conformations has been introduced. The scheme was tested with an example from the Delta-Sleep-Inducing-Peptide mutant family.[294]

A general method for estimating the statistical barrier height distribution in a disordered system has been presented. The method is based on the interpretation of the temperature dependence of the instantaneous normal mode density of states. An integral equation is derived which relates the fraction of unstable instantaneous normal modes at a particular temperature to the intrinsic distribution of one-dimensional energy barriers for the 3N-6 internal degrees of freedom in the system. A technique for solving the integral equation was presented and applied to derive the energy barrier distribution for the isobutyryl-Val-Ala_2-methylamide tetrapeptide, the S-peptide of ribonuclease A, and the bovine pancreatic trypsin inhibitor. The results were compared with the random energy model and a suggestion was offered as to how parameters for the statistical Hamiltonian used in that theory might be derived using computer simulation.[295]

The solution conformation of griseoviridin, a broad spectrum antibiotic, has been determined by ^{1}H-NMR in deuterated dimethylsulfoxide. Five structures were obtained from restrained molecular dynamics calculations. These structures satisfied well the experimental restraints, with small values of nOe violation and total energies. On comparison with its crystal structure, a good agreement was noted. However, a small variation between the structures was observed at the aminodecanoic acid part of the molecule.[296]

Synthetic peptides corresponding to various regions of G_s were evaluated for their ability to mimic G_s effects on the agonistic affinity of the β-adrenergic receptor, and to disrupt the β-adrenergic activation of G_s. The findings showed that at least two regions on the α-subunit of G_s participate in high affinity G_s binding to the β-adrenergic receptor, and in fact contain much of the information for specific interaction.[297]

The development of Leuplin, a highly potent LHRH derivative for prostrate cancer/endometriosis, has been described.[298] The synthesis and *in vitro* cytotoxicity of diastereomerically modified dolastatin-15 analogues has been reported.[299]

A *C*-terminal hexapeptide analogue [N$^{\alpha}$Me-Arg-Lys-Pro-Trp-Tle-Leu, Tle=*tert*-leucine] of neurotensin (NT), has recently been reported to have a high affinity for the NT receptor, and appears to have central activity after systemic administration. The Pro-Trp motif was substituted by a series of natural and unnatural amino acids, in an effort to probe the structure-activity relationship. In the case of D-amino acid substitutions, the peptides had negligible binding affinity. In general, the Pro residue appeared more tolerant of substitution by amino acids that favour a reverse turn, than those that favour an extended conformation. The Trp position accepted steric bulk more readily than conformational constraints.[300] The rational design of novel neurotensin mimetics through use of the Multiple Template Approach, has been reported. This approach culminated in the discovery of a pharmacologically unprecedented agent, which behaved as a neurotensin antagonist at low concentration and as a full neurotensin agonist at high concentration.[301]

The gas-phase and solution energy minima of the tetrapeptide Arg-Cys-Gly-Val have been searched in its dihedral angle conformational space. A combina-

tion of random search and continuum dielectric methods yielded the energy minima and also their probable conformational distribution. No preferred conformation was predicted for the RCGV peptide in solution. This was due to a fine balance between the electrostatic free energy, which favoured compact conformations with opposite charges close together, and the solvation free energy, which favoured extended conformations with well separated charged groups that can be optimally solvated. In general, solvation reduced the tendency for opposite charges to come close together and increased the number of favourable conformations.[302]

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4
Cyclic, Modified and Conjugated Peptides

By J.S. DAVIES

1 Introduction

In an era when combinational peptide libraries[1] are being actively assessed for lead compounds in drug discovery, this Chapter covers Nature's multi-millennial lead in providing a rich harvest of bioactive molecules, which have become the focus of pharmacophoric exploration over many years. Naturally occurring cyclic and modified peptides are again the pillars of the subject coverage, but phosphorylated and glycosylated peptides have become a significant part. Nature's templates have often become the core for peptidomimetic design. Part of the tailoring often involves introducing cyclic constraints, but these analogues are not covered in this Chapter, but fit better into the coverage in Chapter 3. The various approaches to peptidomimetic design from a lead natural pharmacophore have been recently reviewed[2,3].

The core source of the 1994 papers reviewed here was again CA Selects[4] on Amino Acids, Peptides and Proteins (up to Issue 10, 1995 has been surveyed), with many mainstream Journals being scanned manually to pick up the 15-20% or so titles that seem to miss key-word abstracting. Patents have not been scanned, and although two symposia Proceedings[5,6] appeared during the gestation period of the Chapter, neither have been used for source material, although very relevant topics are covered in these very useful publications. But as one of the Editors[6] of the Proceedings states, their strength is the rapidity of the dissemination of information rather than its maturity. Most of the Symposia proceedings reappear as full papers and these therefore become the mainstay of this report.

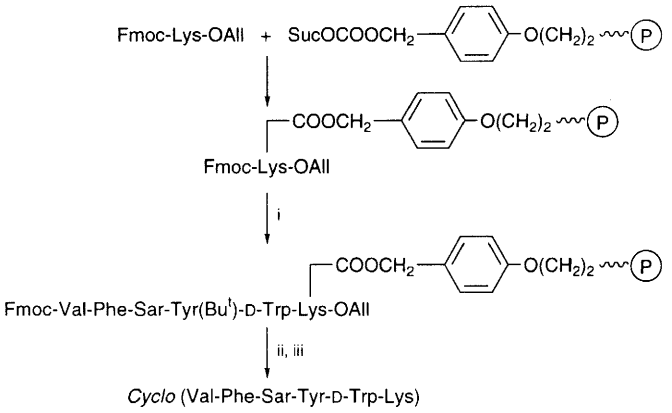
2 Cyclic Peptides

2.1 General Considerations – The last decade has seen a revolution in the powerful techniques available for structural elucidation and conformational investigation. Papers dealing with structural elucidation are now very often all-encompassing, in that high-field NMR techniques, X-ray studies and other physical methods tend to be included in the one publication. Hence productivity in structural elucidation is obviously on the increase and some structures especially from the marine environment continue to amaze. In this report, reference to a structure elucidated by high-field 2D-NMR techniques reflects that techniques such as nOe, COSY, ROESY, TOCSY, *etc.* have been used. One cautionary note,[7] albeit derived from linear peptides, has appeared on the use of

circular dichroism studies alone in an assay of β-turns. Other non-β-turn H-bonds can interfere with the observed Cotton effects, although examples using cyclic peptides do not seem to be affected.

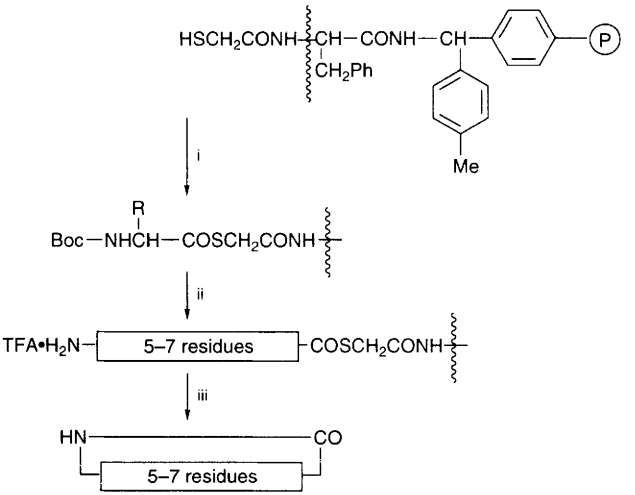
The traditional approach of cyclisation of linear peptide precursors in the solution phase has now found an efficient competitor in total synthesis in the solid phase mode. Over a hundred references to this technique have now been reviewed.[8] One approach requires the anchoring of the first amino acid to the resin *via* its side chain. While Asp or Glu side-chains have been used in the past, it is now reported[9] that using N,N′-disuccinimidyl carbonate, a linkage involving the side chain of lysine can be created as summarised in Scheme 1. Head to tail cyclisation can also be carried out *via* a suitable 'activated' linker group as *e.g.* using Kaiser's oxime resin. As a development of this approach a thioester function in the linker position has now been shown[10] to be more efficient as summarised in Scheme 2. To secure a racemisation-free cyclisation it would be best to leave a glycine residue at the C-terminal position. Yields of about 30% were achieved for a series of representative cyclohexapeptides. Anchoring the first residue *via* the side chain of Fmoc-Asp-ODmb (Dmb = 2,4-dimethoxybenzyl) was the starting point[11] used for the simultaneous multiple synthesis of cyclised peptides derived from a surface loop of a meningococcal class 1 outer membrane protein. As shown in Scheme 3 the use of the Dde group (1[-4,4′-dimethyl-2,6-dioxocyclohexylidene]ethyl) to mask the ε-NH_2 of lysine, provided a selective means of producing peptides for further conjugation for immunological experiments.

The above methods have been primarily designed to enable the cyclised product to be cleaved from the support, but immobilised cyclic peptide libraries are feasible.[12] In a check on the methodology 50–65% of monomeric cyclic peptide, *e.g. cyclo*-(Phe-Gly-Gly-Phe-Ala-Gly-Glu) was present on cleavage from the support. To be able to carry out solution phase cyclisations after growing suitably protected linear precursor on a resin, requires mild conditions for release of the protected peptide from resins. Cyclic penta-and hexa-peptides have been prepared[13] by this approach using a new DAS resin, cleaved under weak acid conditions, with HBTU and PyBOP being reagents of choice for the cyclisation step. Side-chain-side-chain cyclisations are often chosen as options in conformationally restraining peptides. Such cyclisations are now feasible both in the solution and solid phase. In a series of cyclic amide-linked analogues of angiotensins II and III, the approach taken[14] was to construct the sequences on a 2-chlorotritylchloride resin using the Fmoc strategy and suitable protecting groups for a head to tail cyclisation, or by using benzyloxycarbonyl/benzyl protection at the termini for side-chain cyclisation using the BOP reagent. Backbone to backbone linkages not involving amide bonds (*e.g.* disulfide links) have been very useful means of constraining peptides. An example of the process being carried out completely on a solid phase has recently been reported[15] for the synthesis of the analogue (1). Attempts[16] to carry out a 10-membered ring lactam formation on solid support by cyclisation of adjacent α,γ-diaminobutyric acid (Dab) and D-Glu residues, *e.g.* [Dab^2,D-Glu^3,Leu^5]-enkephalinamide, failed using HBTU, the main product resulting from a transfer of the tetramethyluronium



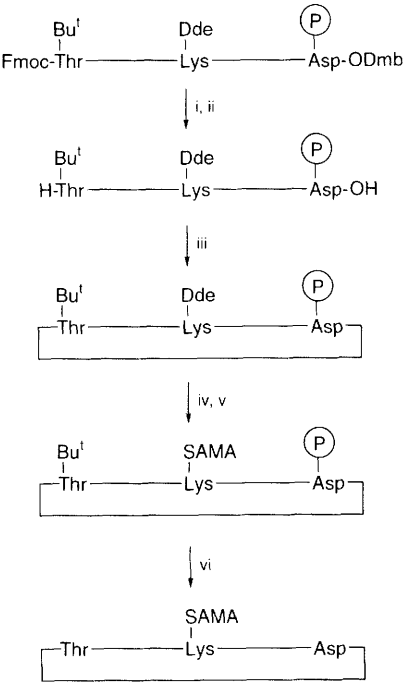
Reagents: i, Stepwise addition/deprotection; ii, Fmoc/allyl groups deprotection; iii, BOP/HOAt

Scheme 1



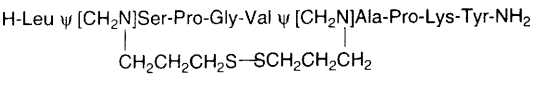
Reagents: i, Diisopropylcarbodiimide coupling of 1st residue; ii, Assembly of units as Boc-amino acids; iii, 0.1 equiv. DMAP/DIEA

Scheme 2

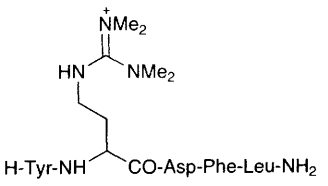


Reagents: i, TFA/anisole; ii, piperidine/DMA; iii, PyBOP; iv, N_2H_4/H_2O/DMA; v, *S*-acetylmercaptoacetate pentafluorophenyl ester (SAMA-OPfp); vi, 95% TFA

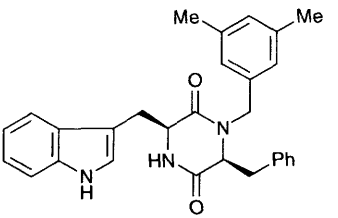
Scheme 3



(1)



(2)



(3)

moiety from HBTU to the amino acid side chain of Dab to give (2). Compared with other coupling agents it has been reported[17] that benzotriazolyloxy-bis(pyrrolidino)carbonium hexafluorophosphate gives a higher yield and shorter cyclisation times.

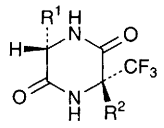
Many of the synthetic studies on specific examples discussed throughout this Chapter tend to be variations on the general ideas discussed above.

2.2 Dioxopiperazines (Cyclic Dipeptides) – There were no reports of novel naturally occurring cyclic dipeptides during this review period, but this small ring structure continues to generate great interest. A conformational study[18] has highlighted fundamental differences in the solution conformations of cyclo-[(*S*)-His-(*S*)Phe] which catalyses the formation of (*R*)-cyanohydrins and cyclo-[(*S*)-His-(*S*)-Leu] which leads to (*S*)-cyanohydrins. In the former dioxopiperazine the phenyl ring shields the top face of the imidazole ring, whilst in the latter it is the bottom face that is shielded by the dioxopiperazine ring. X-ray crystallographic studies[19] have revealed that in cyclo-(Gly-L-Pgl) and cyclo-(Ala-D-Pgl) where Pgl = phenylglycine, the phenyl rings are almost perpendicular to the mean planes of the dioxopiperazine rings which assume flattened twist-boat conformations. Comparisons of NMR data[20] with structures derived from molecular modelling of (3) showed that the aromatic rings are not stacked face to face over the dioxopiperazine ring. The phenyl ring is over the dioxopiperazine but the indole ring points away in the [G_o-] rotamer form. Molecular dynamics simulations[21] of the conformation of cyclo(L-Tyr-L-Tyr) have been used to predict its CD spectrum. There was good agreement with experimental for the strong negative band at 200 nm, but less of a magnitude correlation with the lower energy bands near 230 and 280 nm.

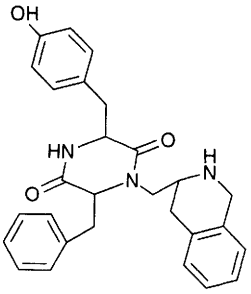
The 'gas-solid-phase' approach to dioxopiperazine synthesis reported in 1992 for amino acids, has been applied[22] to various dipeptides in the presence of silica. Yields of between 40-80% have been quoted with little loss of chirality. Replacement[23] of bromides at the α-positions of N-benzylpiperazin-2,5-diones by heterocyclic amines such as piperidine in the presence of sodium hydride has yielded symmetrically substituted α-*N*-functionalised piperazine-2,5-diones. Asymmetric incorporation of the R^2 group into (4), where R^2 = Me, benzyl or allyl has been carried out[24] using Grignard reagents or cadmium compounds with *in situ* generated cyclic acyl imines.

Dioxopiperazine formation is often an unwanted complication in peptide synthesis and there have been two examples reported. During the preparation[25] of protected H-Glp-Glu-Asp-OH, cyclo-[Glu(OBu^t)-Asp(OBu^t)] readily formed and accounted for some racemisation of the Asp residue. However more surprising was the spontaneous formation[26] in DMSO and MeOH of (5) from H-Tyr-Ticψ[CH_2NH]-Phe-OH, where Tic = tetrahydroisoquinoline-3-carboxylic acid, presumable *via* the intermediate (6).

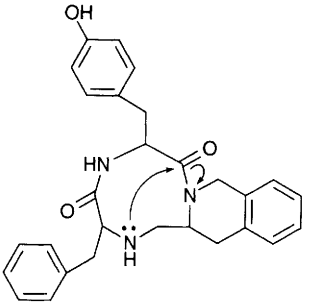
Fluorescence decay measurements on tryptophan-containing dioxopiperazines have shown[27] that fluorescence lifetimes correlate with protonation of titratable sites on the partner amino acid residue. Dioxopiperazines have been explored[28] as potential gelling agents to harden organic fluids. Cyclodipeptides consisting of



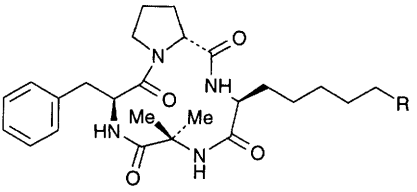
(4) R^1 = Me, Bu^i or benzyl



(5)

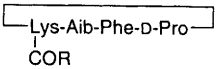


(6)

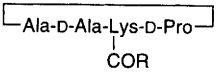


(7) R = 

(8) R = 

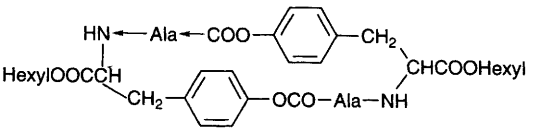


(9)



(10)

R = $ClCH_2CH(OH)$, oxiranyl, $ClCH_2CH_2N(NO)$ or 4-$(ClCH_2CH_2)_2NC_6H_4$



(11)

different amino acids proved to be superior to those containing similar residues. In a host-guest investigation[29] using C_2 symmetric macrolactam hosts with cyclo-(Gly-Leu) and cyclo-(Leu-Leu) as guest molecules moderately high enantioselective and diastereoselective recognition could be recorded with FT-IR. The solubilities[30] of five simple dioxopiperazines have been used to determine the molar entropies of dissolution, while free energies of adsorption of 2,5-piperazinediones on silica have been estimated[31] from HPLC retention data. The ability of tetrapeptides bearing a dioxopiperazine ring in the middle, to self-assemble has been investigated[32] using scanning electron micrographs.

2.3 Cyclotetrapeptides – The structure of a phytotoxic cyclotetrapeptide from *Verticillium coccosporum* has been found[33] to be (7), an analogue of chlamydocin (8). The cyclopeptide analogues (9) and (10) of chlamydocin have also been synthesised[34] and show inhibitory potencies in L1210 cell lines. Urethane bonds derived from the phenolic group of tyrosine have been used[35] as amide surrogates for the construction of the pseudotetrapeptide structure (11). X-ray and NMR studies confirmed an oblong shaped structure with tight type II β-turns with the urethane groups adopting a *trans-trans* conformation.

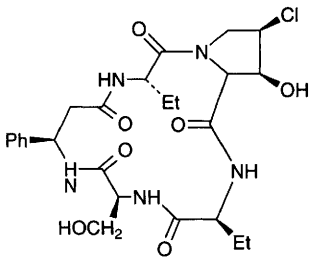
2.4 Cyclopentapeptides – Pharmaceutical interest in endothelin, the vasoconstrictor associated with impaired cardiovascular and renal function, has spurred on equal interest in the naturally occurring endothelin receptor antagonist, cyclo-(D-Trp-D-Asp-Pro-D-Val-Leu) BQ123. This explains the interest in two large scale synthetic strategies to make BQ123. In one approach[36] a sodium salt of BQ123 was prepared on a 100g scale using a methyl ester to protect the D-Asp side chain allowing the benzyl group to be used for the C-terminal leucine. HBTU was found[37] to be efficient at head to tail cyclisation in another multigram preparation of BQ123. Another NMR and molecular modelling study[38] on this cyclopentapeptide, this time in DMSO and water, has revealed no difference in the backbone conformation from previous studies but the different solvents can significantly affect the preferred side-chain conformations. Conformational flexibility of the Leu-Trp hydrophobic cluster was not observed in either DMSO or water. BQ123 and a N-methylated analogue, cyclo-(D-Val-MeLeu-D-Trp-D-Asp-Pro) have also been studied[39] in methanol and chloroform solutions at low temperatures. The peptide backbone conformations were again well defined as type II β-turns at Leu-D-Trp and a γ'-turn at proline, with evidence of a close approach of the Leu and D-Trp side chains. N-Methylation did not perturb either the backbone or side-chain interactions.

Studies on higher plants have recently revealed cyclopeptide structures with interesting tyrosinase inhibitory activities, with some, such as pseudostellarin A, cyclo-(Gly-Pro-Tyr-Leu-Ala) showing β- and γ-turn conformations[40] typical of cyclopentapeptide structures. Astin B, *cyclo*-[Pro(Cl)$_2$-aThr-Ser-βPhe-Abu] from *Aster tataricus* has been studied[41] by 2D-NMR and restrained molecular dynamics calculations and compared with an X-ray determination. Little difference was found between the conformations in the solid and solution forms except that

intramolecular H-bonds, especially the one between the a Thr NH and β-PheCO, might be stronger in the solid state. The *Aster* species has also yielded[42] a novel β-hydroxy-γ-chloroproline containing cyclopentapeptide having structure (12).

Other sections in this book (*e.g.* Chapter 3) have highlighted the current interest in the RGDS motif as a pharmacophore for antithrombotic agents and as a model in cell adhesion studies. A number of active cyclopentapeptide analogues have been assessed and at least one (13), although not strictly a homodetic cyclopentapeptide, is of sufficient interest for a large scale preparation to be attempted using solution phase techniques.[43] The cyclisation was carried out using HBTU between the C-terminus of glycine and the aspartyl residue, with a final detosylation step giving a product which precipitated out pure, from the reaction mixture. A combination of solid phase synthesis of protected linear precursors on an acid sensitive resin, followed by solution phase cyclisation with soluble carbodiimide has been used[44] to make cyclo-(Arg-Gly-Asp-Ser-Lys), cyclo-(Arg-Gly-(DL)Gla-Ser-Lys] and cyclo-(Arg-Gly-Asp-Phlac-Phlac). The biological activity of the Gla analogue (Gla = γ-carboxyglutamic acid) is interesting as in previous examples any changes to the Asp position of the basic motif has not been fruitful. A 2-chlorotrityl resin was used[45] in the solid phase synthesis of protected linear precursor, Boc-D-Pheψ[CH_2NH]Val-Arg(NO_2)-Gly-Asp(OBzl)-OH, prior to cyclisation by diphenylphosphoryl azide (DPPA) to yield the pseudocyclopentapeptide cyclo(Arg-Gly-Asp-D-Phe-ψ[CH_2NH)Val). The formation of the [CH_2NH] link was carried out *via* a nickel boride reduction of the corresponding [CSNH] analogue at the dipeptide stage. NMR studies confirmed a difference between the reduced analogue and its parent cyclopentapeptide with the H-bonding characteristics of the former tending to rigidify the structure under physiological conditions. Conformational averaging on the NMR time scale has been examined[46] by computer simulations of multiple copies of the molecule cyclo(D-Pro-Ala-Ala-Ala-Ala). Ensembles generated using only nOe's or coupling constants do not account for all the NMR restraints, but the type II′ β-turn is reproduced very well with the simulations. Only the portion which is undergoing averaging shows a wide range of conformations.

2.5 Cyclohexapeptides – The roots of *Stellaria yunnanensis* have yielded[47] two new cyclopeptides, stellarin B and C which have been given the structures cyclo-(Gly-Ser-HOIle-Phe-Phe-Ala) and cyclo-(Gly-Ser-HOIle-Phe-Phe-Ser) respectively, on the basis of spectral data. Seeds of *Vaccaria segetalis* have given rise[48] to segetalin A which has estrogen-like activity and a structure elucidated as cyclo-(Trp-Ala-Gly-Val-Pro-Val). In a series of linear and cyclic peptide T derivatives, cyclo-(D-isoAsp-Thr-Thr-Asn-Tyr-Thr) seemed[49] the most suitable for CD_4 receptor binding. Cyclic hexapeptides isolated from the roots of the Rubiaceae family have become of interest as anti-tumour reagents, with one of their members RA-VII (14) in clinical trials in Japan. N-Alkylation[50] of the Ala^2 residue to give (15) retains the cytotoxic properties of the parent with the N-prenyl derivative showing the most prominent *in vitro* activity. Increased activities have also been reported[51] for the thioamide analogues, the surrogate thioamide bonds being introduced between Tyr^3 and Ala^4, and Tyr^6 and $D\text{-}Ala^1$



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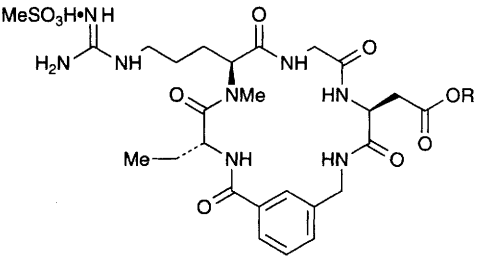

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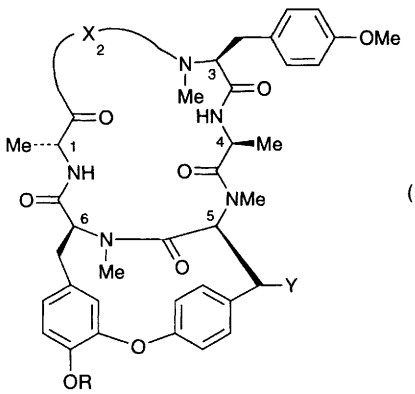
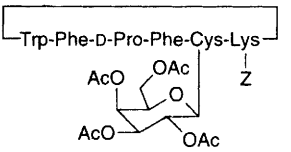


(14) X = Ala, Y = H, R = Me
(15) X = *N*-alkyl, alkenyl, aralkenyl-Ala, Y = H, R = Me
(16) X = Ser, Y = H, R = Me
(17) X = (*S*)-$OCH_2CH_2CH(NH_2)CO$; R = Me, Y = H
(17a) X = Ala, Y = OH, R = H



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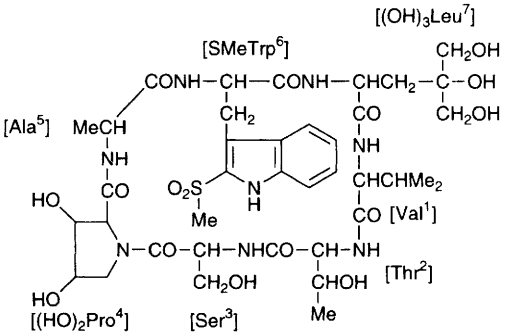


(19)

using Lawesson's reagent. By using the serine analogue RAIII (16), the potential for ring expansion *via* a N → O acyl shift has been explored.[52] The resulting macrocyclic lactone (17) which does not show much conformational change does show promising antitumour activity. However potentiation of cytotoxic activities does not seem to be very feasible[53] if the changes are restricted to the R group on the cycloisodityrosine ring. A concise total synthesis[53a] of bouvardin (17a) and an O-methylated analogue has been reported. A particularly successful Ullman macrocyclisation yielded the key diphenyl ether unit which was then coupled with Boc-D-Ala-Ala-MeTyr(OMe)-Ala-OC_6H_5 followed by macrocyclisation.

Cyclic hexapeptides based on the template molecule, cyclo-(Xxx-Trp-Phe-Gly-Leu-D-Leu), have been synthesised[54] as part of a study which included glycoconjugates being introduced into the Xxx position. Assembly of the peptides was carried out on a solid phase resin with Gly as the C-terminal residue with cleavage from the resin being undertaken by Pd(II) acetate/ammonium formate catalytic transfer conditions to prevent loss of protection on Lys and Glu residues introduced into Xxx. Glycosylamine residues were introduced with TBTU, and soluble carbodiimide/DMAP conditions were used for cyclisation in the solution phase. All the analogues proved to be selective NK-2 antagonists, showing that differently charged residues can be accommodated by the receptor. In order to preserve a high antagonist activity at the NK-2 receptor in a series of analogues based on[55] cyclo-(Leuψ[CH_2NH]Xaa-Gln-Trp-Phe-β-Ala) the pseudopeptide bond [CH_2NH] has proven advantageous, and the antagonist activity reflected the degree of lipophilicity of the residue introduced at position Xaa. For the synthesis of the peptides, solid phase techniques were used to produce the linear precursors followed by cyclisation between the C-terminal β-Ala and the N-terminal of Leu under high dilution conditions using the BOP reagent. Cyclic hexapeptides have also been designed[56] as structural mimics of the distorted type I β-turn based on -Trp^{18}-Arg^{19}-Tyr^{20}-found in the 74-residue tendamistat protein which inhibits α-amylase. The optimised 'designer' cyclohexapeptide came to be cyclo-(D-Pro-Phe-Ala-Trp-Arg-Tyr) which inhibited α-amylase with a K_i value of 14-32 μM, significantly better than linear analogues, and better than cyclic analogues containing the Nagai-Sato type II β-turn mimic on the other side of the cyclic hexapeptide ring. The solution conformation of the selective NK-2 antagonist cyclo(Gln-Trp-Phe-Gly-Leu-Met) has been studied[57] by a combination of 2D-NMR and molecular dynamics techniques. The resulting conformation derived contains a variation type I′ β-turn in the Gly-Leu-Met-Gln segment and when this has been compared with other NK-2 antagonists, their activity appears to be inversely related to the conformational rigidity of the cyclic peptides.

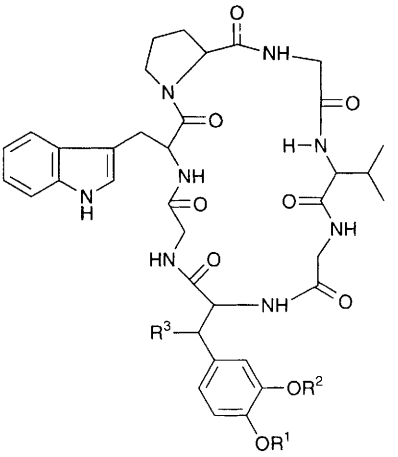
For the last two decades cyclohexapeptides have often been considered prototypes for the application of many physical techniques, and this interest is still continuing. Thus coupling constants and H-bonds, as experimental restraints for cyclo-(Gly-Pro-Pheψ[CSNH]Val-D-Phe-Pheψ[CSNH]) in a distance geometry refinement protocol, have been explored[58] and compared with the oxo-analogue. The *trans-cis*-isomer ratio for the Gly^1-Pro^2 bond was 58:42 in the thio-analogue and 68:32 in its oxo-counterpart, but overall the conformations were very similar. Two cyclic hexapeptides cyclo-(Ala-D-Ala-Ser-Phe-Gly-Ser) and cyclo-(Ala-Gly-

Ser-Phe-Gly-Ser) derived from the loop portion of the C'C'' ridge of T-cell surface receptor CD-4 have been studied.[59] Both display, in DMSO solution, a single conformer with two intramolecular H-bond stabilised β-turns, but in aqueous solution the β-turn conformation is no longer the predominant structural form. The influence of an S-glycosylated residue on the conformation of the cyclohexapeptide ring in (18) has been checked[60] by an array of high-field NMR techniques and molecular dynamics simulations. Comparison of (18) with its Thr^3 parent system shows that it exhibits a similar overall conformation (βII' D-Pro-Phe and another β-turn about Trp^4-Lys^5(Z)). However the glycopeptide analogue shows a distinct dynamic βI, βII flip, whereas the Thr-analogue only populates βI. Diphenylphosphorylazide DPPA/$NaHCO_3$ without using high dilution was the cyclisation method chosen to make the analogues. Three cyclic hexapeptides cyclo-(Gly-Pro-Phe-Val-Xaa-Phe) with Xaa = L-Phe, D-Phe or D-Pro have been examined[61] as mimetics of the hydrophobic part of the antibiotic cinnamycin. Each peptide exhibited the two rotamers about the Gly-Pro peptide bond. The *cis*-Gly-Pro segment in the minor isomers is not involved in a βVI-turn but forms a turn structure with the *cis*-Gly-Pro in the i and i + 1 positions defined as a pseudo βI-turn. An azide-based cyclisation at the Gly residue was used to make the cyclic peptides.

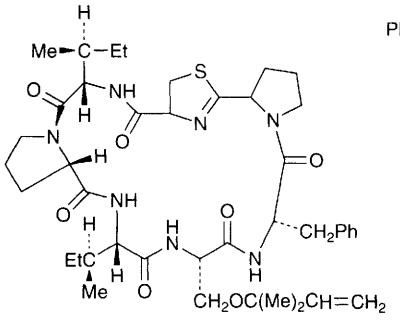
An X-ray crystal structure[62] on the Li complex of cyclo-$(Pro\text{-}Gly)_3$ has revealed two conformations in the crystal: one conformer has three CO's on one side and three on the other side of the cyclic peptide plane, while the other has all six on the same side. Both bind independently to the Li ion, and maybe considered as prototype conformations for explaining the transport of ions across lipid membranes. Two slightly different almost hexagonal backbone conformations of the β-type have been detected[63] in an X-ray analysis of single crystals of cyclo-(D-Leu-L-MeLeu-D-Leu-L-MeLeu-D-Leu-L-MeLeu) from MeOH solution. There was also evidence for pairing of molecules forming dimeric units with six interannular H-bonds supporting the idea that stacks of β-rings can serve as molecular channels.

X-ray, NMR and constrained molecular dynamics have been used to study β-Ala containing cyclohexapeptides. In cyclo-(Pro-Phe-β-Ala-Phe-Phe-β-Ala), the solid state conformation is characterised[64] by a *cis*-β-Ala^6-Pro^1 bond and the Pro^1-Phe^2 is incorporated in a pseudo type I β-turn while Phe^4-Phe^5 is in a typical type I β-turn. In DMSO two slowly interconverting *cis-trans* isomers around the β-Ala^6-Pro^1 bond were detected, and confirmed expectations of a low propensity of β-Ala residues at the corners of the turns. A study[65] of cyclo-$(Pro\text{-}Phe\text{-}\beta\text{-}Ala)_2$ by similar approaches revealed that in the solid state two identical halves of the molecule adopt two different conformations, due to *cis* and *trans* rotamers about the β-Ala-Pro bond. All the Pro-Phe segments in both solid and solution display angles close to type II β-turns. In both these references to β-Ala peptides the cyclisation techniques involved DCCI in dichloromethane for the cyclisation stage.

Cyclo (ε-Aca-Pro-Xxx-ε-Aca-Pro-Xxx), where ε-Aca = ε-aminocaproyl and Xxx = Ala, Thr(OBzl) have been studied[66] both in acetonitrile and in D_2O solutions using IR techniques, while computer simulations[67] using the locally



	R^1	R^2	R^3
(20)	H	prenyl,	OH
(21)	H	H,	OH
(22)	Me	prenyl,	OH



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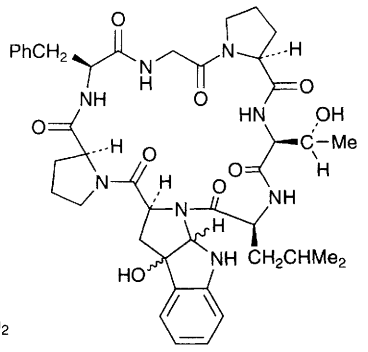


(24) α-H, α-OH
(25) β-H, β-OH

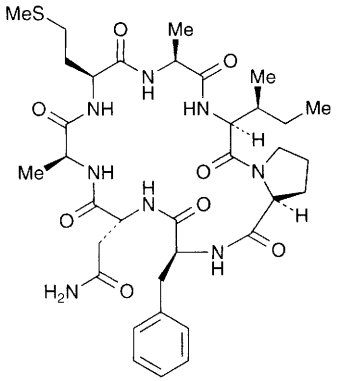
enhanced sampling method confirmed a unique conformation for Ac-Cys-His-Asp-Leu-Phe-Cys-NHMe, probably due to the hydrophobic interactions between Phe and Leu, which are not possible in AcCys-(Ala)$_4$-Cys-NHMe.

2.6 Cycloheptapeptides and Cyclooctapeptides – This section is again this year dominated by a wealth of naturally-occurring structures. Viroisin (19)[68] from the mushroom *Amanita phalloides* has the same biological function as phallotoxin although the structures are different. In solution the cycloheptapeptide seems to have a well-ordered conformation, with all the functional groups (Me of Ala^5, OH groups at CβSer^3 and Pro^4, and the side-chain of Leu^7) orientating themselves for binding to target proteins. During natural product screening for potential molecules with affinity for the NK-1 receptor, cultures of *Aspergillus flavipes* produced two novel cycloheptapeptides, WIN 66306 (20) and WIN 68577 (21). Methylation of (20) to form (22) gave an even better competitive antagonist to substance P at the human NK-1 receptor with a K_i of 0.12 ± 0.03 μM. The 3-prenyl-β-hydroxytyrosine moiety within the molecules appears to be critical for the biological potency. Marine taxa continue to give rise to cyclic peptides with high levels of cytotoxicity. Added to this tradition is mollamide (23), from the ascidian *Didemnum molle* whose structure has been elucidated[70] by NMR experiments and confirmed by an X-ray crystallographic structure. Tested in cell lines its cytotoxicity has been quoted as an IC_{50} value of 1 μg/ml against P388 and inhibition of RNA synthesis at an IC_{50} value of 1 μg/ml. The Western Indian ocean sponge *Phakellia carteri* has yielded[71] two isomeric cycloheptapeptides, phakellistatin 3 (24) and isophakellistatin 3 (25) which include in their structure a photooxidation product of the tryptophanyl residue. Again the full structures were confirmed by high-field NMR experiments and an X-ray structure for (24). The marine sponge *Phakellia costada* produces yet another variation on the cycloheptapeptide theme, phakestallin 5 (26), a new human cancer cell growth inhibitor.[72] All the residues except asparagine were shown to have the (S) configuration. In order to hold the Glu^{58} to Lys^{61} domain in a conformational form to simulate the binding of the C-terminal fragment of hirudin to the exosite of thrombin, the cyclic peptide, Suc-Phe-Glu-Ile-Pro-Lys-Glu-OH has been used[73] in a nOe/distance geometry study. The conformation came out to be very similar to those in a native 11-residue peptide in the thrombin bound state.

In last year's report we reviewed the structures of five cycloheptapeptides, hymenamides A-E from the Okinawan marine sponge *Hymeniacidon sp.* This year four new members of the family, hymenamides G, H, J and K have been shown[74] to be a family of cyclooctapeptides with the following structures:

Hymenamide G	cyclo(-Pro-Pro-Tyr-Val-Pro-Leu-Ile-Leu)
Hymenamide H	cyclo(-Pro-Leu-Thr-Pro-Leu-Pro-Trp-Val)
Hymenamide J	cyclo(-Pro-Tyr-Asp-Phe-Trp-Lys-Val-Tyr)
Hymenamide K	cyclo(-Pro-Tyr-Asp-Phe-Trp-Lys-Ala-Val)

Axinastin 5 is the name given[75] to the cancer growth inhibitor (GI_{50} 0.3-3.3 μg/ml) isolated from the marine *Axinella cf. carteri*, and has been shown[75] to be cyclo(-Pro-Pro-Tyr-Val-Pro-Leu-Ile-Leu), which appear to be identical with



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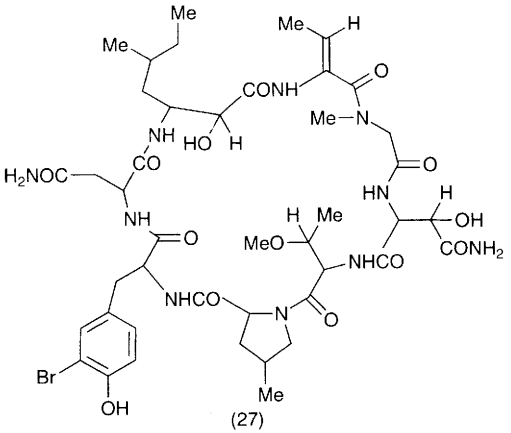

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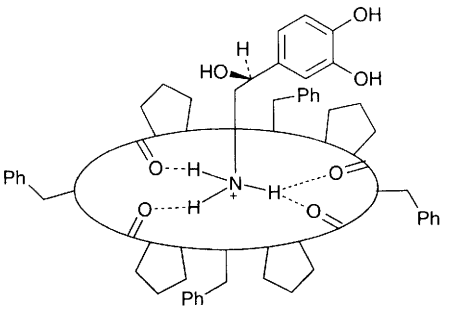


(28)

hymenamide G above. Perthamide B, from the sponge *Theonella sp.* has been shown[76] to have the structure (27), and is a weak inhibitor of the binding of [^{125}I]IL-1β to intact EL 46.1 with an IC_{50} of 27.6 μM.

The roots of *Pseudostellaria heterophylla* have been the source of a number of cyclic peptides with potent tyrosinase inhibition properties. Not all of them fit into the cyclooctapeptide classification but for convenience all the components elucidated so far, pseudostellarins A-C[77], D-F[78] and G[79] are listed below:

Pseudostellarins

A	cyclo(Gly-Pro-Tyr-Leu-Ala)
B	cyclo(Gly-Ile-Gly-Gly-Gly-Pro-Pro-Phe)
C	cyclo(Gly-Thr-Leu-Pro-Ser-Pro-Phe-Leu)
D	cyclo(Gly-Gly-Tyr-Pro-Leu-Ile-Leu)
E	cyclo(Gly-Pro-Pro-Leu-Gly-Pro-Val-Ile-Phe)
F	cyclo(Gly-Gly-Tyr-Leu-Pro-Pro-Leu-Ser)
G	cyclo(Pro-Phe-Ser-Phe-Gly-Pro-Leu-Ala)

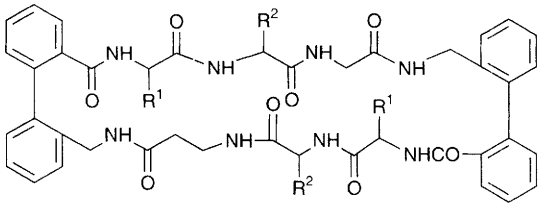
The ^{13}C NMR spectrum[80] of cyclo(L-Phe-L-Pro)$_4$ and DL-noradrenaline hydrochloride in a mixture of $CDCl_3/CD_3OD$ suggested the formation of a complex which retained a C_2-symmetric conformation as depicted in (28). In a NMR comparison[81] between the linear antiaggregatory sequences D-Arg-Gly-Asp-Trp and L-Arg-Gly-Asp-Trp and their non-active cyclooctapeptide analogue cyclo(Arg-Gly-Asp-Trp)$_2$, it is revealed that cyclisation leads to a conformational rearrangement away from the type II β-turn shown in the solution conformation of the linear fragments. A strong ROE value was observed between the Arg and Gly amide protons in the cyclic analogue, with the evidence pointing towards a β-turn involving the Trp/Arg residues at positions 2 and 3. With the incorporation[82] of two biphenyl residues as spacers in the structures (29) and (30), interconverting diastereoisomers due to the atropisomerism of the biphenyl units have been detected in the NMR data. However the presence of a β-sheet conformation has been confirmed for the major diastereoisomers detected for (29) and (30).

Octapeptide fragments of thioredoxin and thioredoxin reductase having the structures Ac-Ala-Cys-Ala-Thr-Cys-Gly-Phe-NH_2 and Ac-Trp-Cys-Gly-Pro-Cys-Lys-His-Ile-NH_2 have been synthesised[83] using both solution and solid phase technologies. For the solution phase S-t-butylthio and t-butyl protection in combination with N^{α}-Z and N^{α}-Nps protection was used for building up the sequence followed by a final cyclisation of the bisulfide link using azodicarboxylic acid di-t-butyl ester. For the solid phase, Fmoc UNCA's or TBTU/HOBt couplings with the same side-chain protection were used, but the protocol suffered from irreversible re-attachment of cleaved peptide *via* alkylation of the various side chain functions.

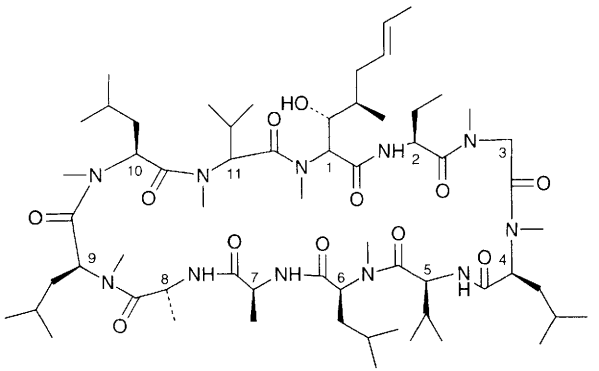
2.7 Higher Cyclic Peptides – Cyclosporin A (CsA) (31) continues to elicit great interest because of its significance in the immunosuppression field. From previous studies the OH group in the MeBmt residue is important to its activity, and in attempts[84] to O-alkylate the OH, only phase transfer catalysed conditions using

40% KOH/1 eq benzyltriethylammonium chloride and the halide proved successful. A detailed mechanistic study[85] on the aqueous acid catalysed degradation of cyclosporin A, has shown that the MeBmt OH group is involved in a N → O acyl migration *via* a hydroxyoxazolidine intermediate. The rearrangement is summarised in Scheme 4 and shows that the isocyclosporin A is the predominant degradation product in acid solution. In another study[86] of acidic hydrolytic cleavage, HPLC separation and FAB/Tandem mass spectrometry has shown evidence of cleavage at residue 11 and both residues 10 and 11 to give two open-chain nona- and decapeptides. Mechanism of action of cyclosporin A is believed to involve binding to cyclophilin (CyP), and the resulting complex interacts with calcineurin inhibiting phosphatase activity. While probing the reaction of the CsA/CyP complex with calcineurin it has been found[87] that the calcineurin has a tight binding pocket for N-MeLeu in position-4 of CsA. To explore this, Me[(*4S*)-Me]norLeu^4-CsA and Me[(4R)(4*S*)-Me]norLeu^4-CsA have been synthesised. The former had 4 × affinity for CyP compared with CsA, but the immunosuppressive activity was 2-3 times lower which is in line with the proposed tight binding involving MeLeu-4. In an attempt to manipulate one domain of CsA while preserving others, Lawesson's reagent has been shown[88] to be capable of regioselectively modifying secondary amide bonds at positions 4 and 7 to form thioamide analogues. Yields were low but appeared better if acetylated CsA was used. The importance of the MeBmt residue in CsA is reflected by the great interest[89] shown in its asymmetric synthesis.

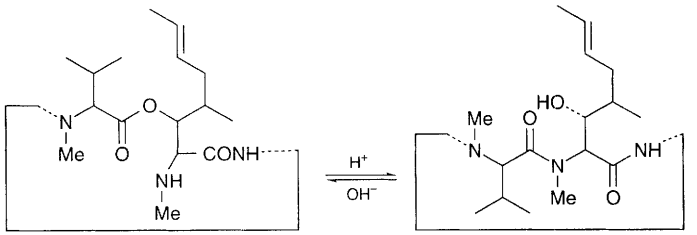
Past NMR measurements of CsA in various solvents have shown the molecules to possess different conformations if the CsA is free, bound to cyclophilin, or in a complex with alkali metal ions. However, using acetone as solvent,[90] which allows low temperature studies, the conformation turns out to be different from those previously reported. A *cis* peptide bond was detected between MeLeu^9-MeLeu^{10} and one of the amide protons gives an intramolecular H-bridge stabilising a β-turn around Sar^3-MeLeu^4. So the solvent did not induce the biologically active *trans*-conformation around MeLeu^9-MeLeu^{10}. The conformation of cyclosporin A in the CsA/CyP complex has been compared[91] with its conformation in a complex with an antibody fragment (Fab). The Fab-bound conformation of CsA as determined by X-ray crystallography is significantly different from the CsA/CyP complex, which can be explained by different side-chain contacts in the two complexes. It is suggested that in the Fab/CsA contacts there is mutual adaptation of both receptor and ligand during complex formation. Two molecular mechanics methods,[92] template forcing and dynamic forcing, have been applied to a simulation of the receptor binding of CsA. It was not easy to induce the receptor-bound forms so it seems the receptor environment has to provide the necessary energy transitions for conformational change in the CsA. FTIR Analysis[93] of CsA analogues in the presence of Mg^{2+}, Ca^{2+}, Na^+ or Li^+ ions has been carried out, confirming the formation of complexes with all the ions. The ion transport efficiencies[94] of cyclosporins A, C and H which for Li^+ and Ca^{2+} are in the order H > A >> C, contrasts with their immunosuppressant activity which has the order A > C >> H.



(29) R^1 = Me, R^2 = $PhCH_2$
(30) R^1 = Me, R^2 = Pr^i



(31)



Isocyclosporin A Cyclosporin A

Scheme 4

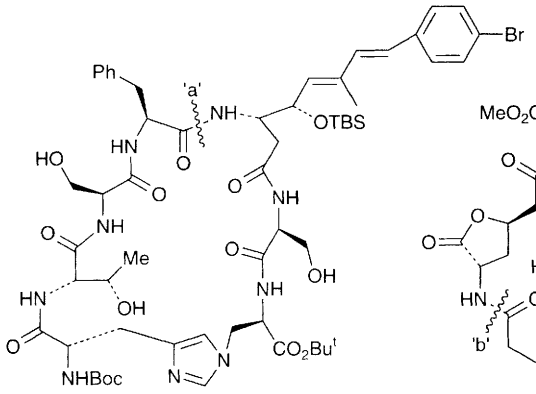
Theonellamide F from a Theonella sponge is a unique bicyclic dodecapeptide. Steps in the synthesis have now been reported[95] in preliminary form, and the approach taken involves separate syntheses of the protected two cyclic dodecapeptide rings (32) and (33). Cyclisation of the linear precursor to (32) was carried out at position 'a' using DPPA and at position 'b' in (33) using the same reagent.

The same coupling conditions (DPPA) were also used[96] for the cyclisation of linear kinin analogues which had been prepared by solid phase protocols. An example of the type of glycosylated cyclic analogues produced is compound (34). A [$Bpa^{2,2'}$] gramicidin S analogue has been prepared[97] where L-5-bipyridylalanines replace the ornithine residues. This analogue formed stable complexes with Co^{2+} Ni^{2+}, Cu^{2+} and Zn^{2+} ions without much change in the cyclodecapeptide conformation. In order to study the interactions between aromatic residues in the cyclolinopeptide A (CLA), cyclo(Leu-Ile-Ile-Leu-Val-Pro-Pro-Phe-Phe), the tyrosyl analogues, [Tyr^8]CLA,[Tyr^9]CLA,[$Tyr^{8,9}$]CLA have been synthesised[98] together with their linear analogues. BOP or DPPA were used for the cyclisation step, and it appears from 1H NMR, CD and spectrofluorimetry that edge to face pairing of aromatic rings (at the 8 and 9 positions) occurs in the cyclic compounds but not in the linear ones. Smaller sized cyclic cystinyl analogues of cyclolinopeptide, containing the full or part of the Pro-Pro-Phe-Phe sequence have been investigated[99] by X-ray and molecular dynamics simulations.

During screening assays for new anti-HIV agents, a secondary metabolite, RP-71955, was isolated[100] from Streptomyces and was found to have the structure (35). Anti-viral activity was also detected[101] in the crude extracts from the tropical tree *Chassalia parvifolia* (Rubiacceae) and on isolation of two novel macrocyclic peptides circulins A (36) and B (37), the structures found probably represent the largest naturally occurring homodetic cyclic peptides. Side-chain cyclisation[102] on the resin using Fmoc/Bu^t/Z and carboxyamidomethyl ester protection has enabled an analogue (38) of endothelin to be synthesised where the Cys^3-Cys^{11} and Cys^1-Cys^{15} disulfides have been replaced by two β-Ala residues and a Dpr (Dpr = 2,3-diamino propionic acid) residue linked to the side chain of aspartic acid.

A review has appeared[103] on peptidyl-prolyl *cis/trans* isomerases and their effectors, and a total synthesis has been reported[104] for an analogue of the immunosuppressant FK506, in which the functionalised cyclohexyl ring at C_{28} has been replaced by a phenyl group. The 'peptidic' content of the steroidal cyclopeptide (39) is barely at the tetrapeptide level, but the impressive overall structure justified its inclusion under this sub-heading. To achieve the synthesis[105] of (39), the starting materials included lithocholic acid and (*S*)-phenylalanine, and the strategy required acyclodimerisation of a linear steroidal peptide precursor. The conformation of (39) involves a lipophilic cavity.

2.8 Peptides Containing Thiazole/Oxazole Rings – The ascidian *Lissoclinium patella* has been a rich source of cyclic peptides, and a recent member studied[106] by X-ray crystallography has been given the structure (40) when a total synthesis[107] of lissoclinamide 5 from the same organism was initially carried out, synthetic material differed from the authentic natural product. On the basis of a



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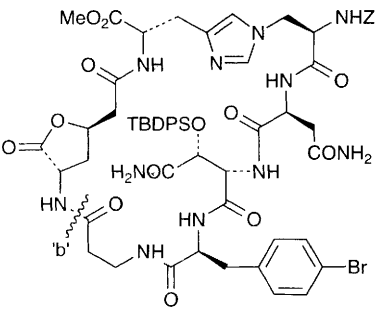

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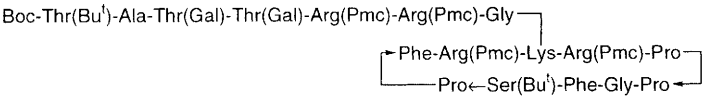

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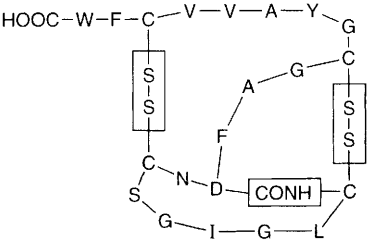

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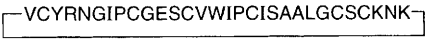

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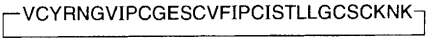

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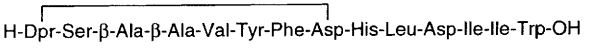

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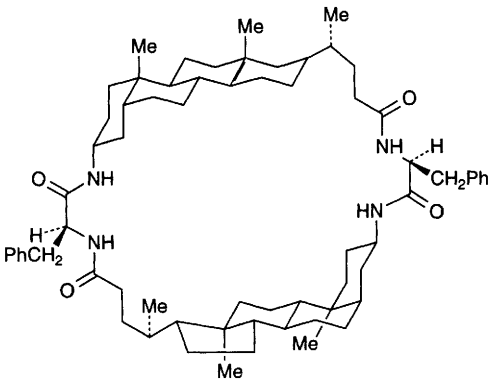

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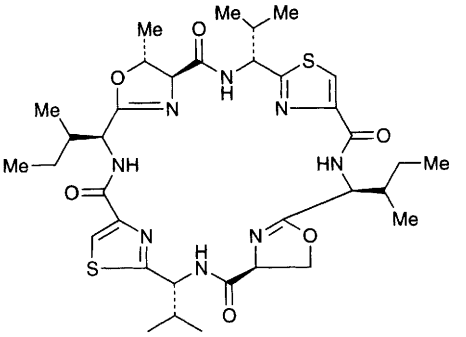

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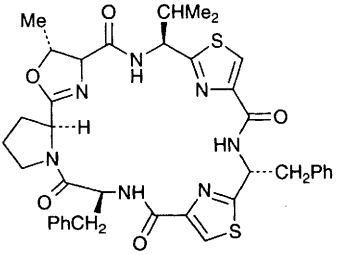

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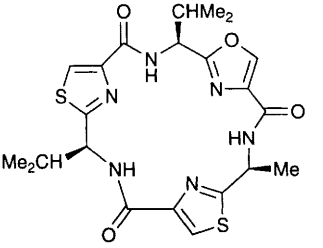


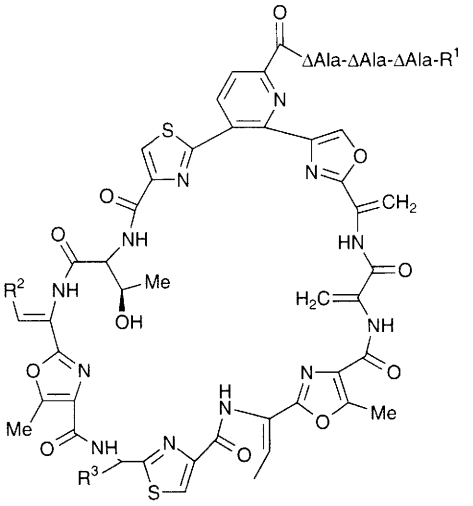
(42)

re-synthesis using L-valine and D-phenylalanine it is now confirmed that the original structure should be modified to the structure (41), with opposite configurations at two of the centres to what was suggested originally. The cyclisation step took place at the amide bond between the thiazole rings using DPPA. The same reagent proved[108] successful in the cyclisation step used in making (-)bistatramide C (42). Thiotipin, a novel thiopeptide from *Streptomyces sp.* DT31, which possesses a *tipA* promoter inducing activity (min. induction concentration 80ng/ml) has been found to have[109] the structure (43), very similar to the previously known sulfomycin I (44). An abstract entitled the structure elucidation of the novel antibiotic of amythiamicin D,[110] implies a polythiazole-containing cyclic peptide, but lack of access to the journal prevents a representative structure being submitted with this report.

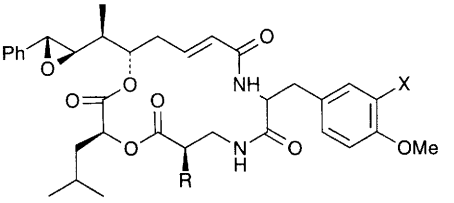
2.9 Cyclodepsipeptides – Strict adherence to nomenclature would restrict discussion under this subsection to depsides formed within the primary backbone of the molecules. However, as in recent years depside links formed by utilisation of the side-chains of serine or threonine, *e.g.* macrocyclic lactones, have been included in this category, which this year again is one of the biggest sub-sections in the Chapter.

The marine environment has retained its status as a rich source of depsipeptides. The Okinawan marine sponge *Dysidea arenaria* produces a potent (IC_{50} = 5 pg/ml) cytotoxic agent, arenastatin A having the structure (45)[111] which corresponds to a β-alanine analogue of cryptophycin B.[112] Another potent antitumour cyclodepsipeptide, and potent fungicide, from the blue-green alga *Nostoc. sp.* strain GSV 224 has been elucidated[112] as structure (46) and named cryptophycin A. The determination of the absolute stereostructure of arenastatin A (45) has been achieved[113] using high field NMR, and confirmed[114] by total synthesis. There have been previous reports on the five bioactive tridecapeptide lactones theonellapeptolides I_a-I_e from *Theonella swinhoei* . This year, theonellapeptolide IId has been characterised[115] as structure (47), a congener of the earlier structures. Theonellapeptolide IId prevents fertilisation of the sea urchin *Hemicentrotus pulcherrimus*. The mollusk Onchidium has not up to now been a rich source of cyclic peptides (apart from the dolastatins), but onchidin[116] (48) has a structure very reminiscent of valinomycin and represents the first report of a dimeric cyclodepsipeptide from a mollusk. Another member of the didemnin family from the tunicate *Tridemnum cyanoporum* has been designated[117] didemnin H with structure (49). In the light of a structural determination[118] on three new cytotoxic and antimicrobial peptides discodermins F (50), G (51) and H (52) from the sponge *Discodermia kiiensis*, the structures of discodermins A-D have had to be revised to accommodate a reversal of order in the sequence of the 12/13th residues Asn/Thr, respectively. Discodermin E has been shown[119] to be an analogue of discodermin A where D-Trp has been replaced by a D-kynurenine residue.

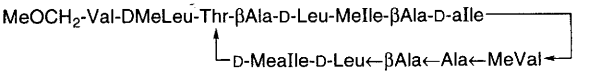
The power of the NMR technique in combination with X-ray diffraction is well illustrated[120] by the elucidation of the structures of salinamides A (53) and (B) (54) from a marine streptomycete. Both share a rigid bicyclic structure with



(43) R^1 = ΔAla, R^2 = Me, R^3 = OH
(44) R^1 = NH_2, R^2 = CH(OH)Me, R^3= OMe



(45) R = H, X = H
(46) R = Me, X = Cl



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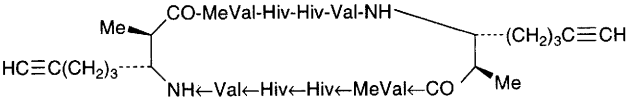

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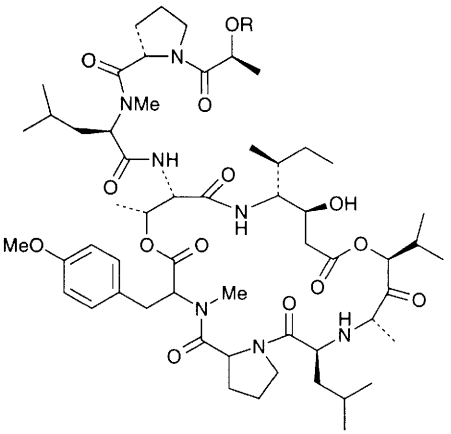
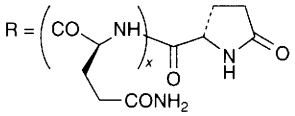
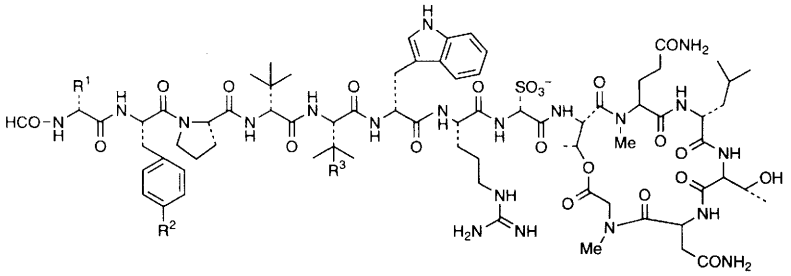


(49) R = H didemnin B



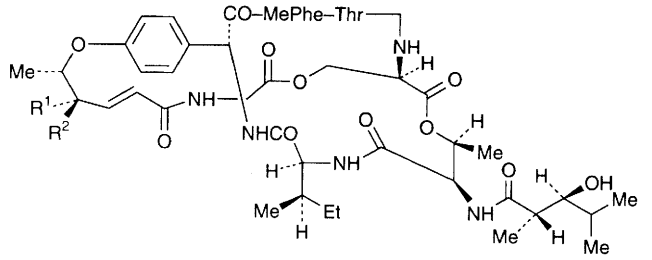
x = 1 didemnin H
x = 2 didemnin E
x = 3 didemnin D



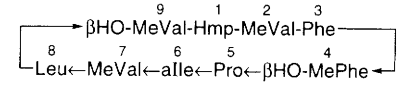
(50) $R^1 = R^2 = H$, $R^3 = Et$
(51) $R^1 = R^3 = Me$, $R^2 = H$
(52) $R^1 = H$, $R^2 = OH$, $R^3 = Me$

half the molecule dominated by hydrophobic interactions while the other half has an extensive network of H-bonds. A new type of antifungal cyclodepsipeptide, aureobasidin E (55) has also been elucidated[121] by advanced physical methods, and is novel on the basis that it lacks a fatty acid residue usually characteristic of related molecules. The structure of (56), one of the two peptide units in actinomycin has been elucidated[122] by X-ray crystallographic techniques, and adopts a flat conformation similar to that found in an analogous N-protected pentapeptide lactone by Mauger *et al.* (1985). The structure of FR90*1*228 produced by *Chromobacterium violaceum* No. 968 has similarly been deduced[123] to be (57). Hapalosin, from blue-green alga, has been given the structure (58) by a combination of spectroscopic techniques.[124] Hapolosin represents a new class of multidrug-resistance reversing (MDR) agents. Halobacillin (59) from a marine *Bacillus* has turned out[125] to be an analogue of surfactin with replacement of a Glu by a Gln residue. It inhibits growth of human colon tumour cells at an IC_{50} value of 0.98 μg/ml, but shows none of the antibacterial activity of surfactin. The 3D structure of protonated [Leu^7]-surfactin from *Bacillus subtilis* has been determined[126] by NMR techniques and two distance geometry protocols, refined by restrained and unrestrained molecular dynamics (GROMOS). The conformations are characterised by a 'horse saddle' topology for ring atoms on which are attached the two polar Glu and Asp side chains. To partner the structure for BZR-cotoxin II worked out in 1992, it has now been found[127] that BZR-cotoxin I from *Bipolaris zeicola* race 3, the cause of leaf spot disease in corn has the structure (60).

Confirmation of the structure of the calcium blocker leualacin (61) has come from its synthesis.[128] Final closure of the cyclopentadepside ring was achieved in 85% yield *via* the unhindered NH_2 group of Leu attacking a pentafluorophenyl ester using a 2-phase system ($CHCl_3$/$NaHCO_3$/solution) *(cf.* DPPA low yield, pentafluorophenyldiphenylphosphinate 60% yield and HATU 50% yield). A conformational analysis[129] of leualacin, using NMR and energy minimisation programmes, indicated a γ- and β-turn profile analogous to cyclic pentapeptides and stabilised by transannular H-bonds between L-Leu and β-Ala. L-MePhe and (*S*)-leucic acid were connected by a *cis* peptide bond. The cytotoxic doliculide from the Japanese sea hare *Dolabella auricularia* has been shown[130] to have the structure (62), obviously a metabolite of mixed peptide-polyketide origins. There have been two full reports[131] of the synthesis of (62) from the same laboratory, the key steps being the construction of the stereogenic centres of the fifteen carbon polyketide derivative by a combination of Evans Aldol reaction and a Barton deoxygenation reaction. For the final cyclisation step of constructing the N-methyl amide bond, BOP-Cl was the reagent chosen giving (62) in 74% yield. In order to improve upon low stereoefficiencies and low yields in the final lactone formation in past attempts, new syntheses have been reported[132] for geodiamolide A (63) and jaspamide (64). Use of an Evans asymmetric alkylation, a Mitsunobu esterification, and DPPA for carboxylic group activation in the macrolactamisation step has given improved efficiency. A cyclic cytotoxic peptide geodiamolide TA (65) from the marine sponge *Hemiasterella minor* (Kirkpatrick) has been shown[133] to be a higher homologue of geodiamolide D where the Ala

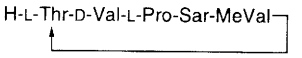


(53) R^1 = OH, R^2 = CH_2Cl
(54) R^1, R^2 = OCH_2



Hmp = 2(*R*)-hydroxy-3(*R*)-methylpentanoic acid

(55)



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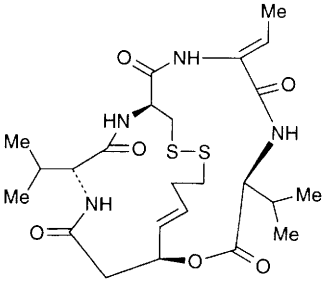

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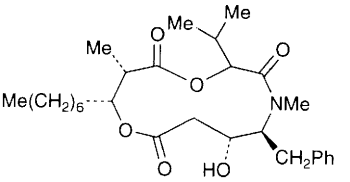

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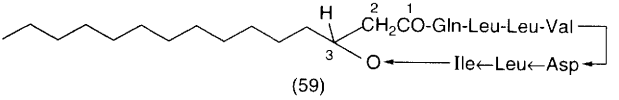
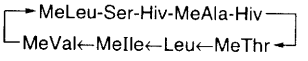


(59)

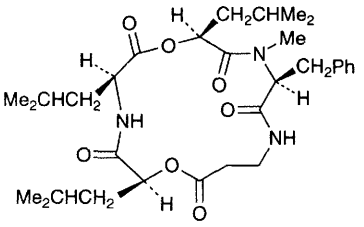


(60)

residue has been replaced by Val. Intensive structure/activity studies[134] on cyclopeptolide (66) from the fungus *Septoria sp.* NRRL15761 have revealed that two derivatives (67) and (68) gave superior activities against yeasts *in vitro* at pH 6.5. Methylation by MeI/KH/18-crown-6 gave permethylated compound (69). In these studies macrocyclisation by DCC/pentafluorophenol at high dilution was used. A derivative of cyclopeptolide which ranks as the most potent resistance modulating agent so far developed has been found[135] to be (70), where the (*R*)-lactic acid has been replaced by the (*S*)-isomer. The synthesis[136] of the macrocyclic ring of the antitumour antibiotic A83586C (71) is only a few steps away following the synthesis of a hexapeptide precursor (72). The potential clinical importance of didermin B (73) has spawned[137] structural/activity studies in its side-chain, which have included modification to the proline residue. As part of the study macrocyclisation yields were also improved by the use of the new phosphinate FDPP which gave a 68% cyclisation yield compared with DPPA (42%, with dimerisation as a major by-product).

Two separate groups[138,139] have reported syntheses for the anthelmintic PF1022A which has the structure cyclo(MeLeu-Lac-MeLeu-PhLac)$_2$. A specific cleavage[140] of the 3-hydroxypicolinyl residue in pristamycin I_A using Zn reduction in aq. acidic conditions releases an amino group available for further derivatisation. For an efficient synthesis of the antitumour cyclodepsipeptides, the luzopeptins, methods for the synthesis of the pyridazine carboxylic acid (74) have to be developed. A five-step synthesis[141] has now been evolved, but doubts are raised as to whether the unit is stable enough to be incorporated into the peptide chain. Under negative ion fast atom bombardment conditions glutathionyl adducts of cyclodepsipeptides can be detected,[142] and 2D-NMR and modelling techniques have been used[143] to assign a molecular conformation to the antibiotic enopeptin A.

2.10 Cyclic Peptides Containing 'Other' Non-Protein Ring Components – Marine sponges produce some fascinating bioactive structures under this category also. Representative examples are, discobahamins A (75) and B (76) characterised[144] from the sponge Discodermia, and konbamide (77) and keramamide A (78) from Theonella, whose stereochemical details[145] have now been worked out. Sponges of the genus Microscleroderma produce cyclohexapeptides which have been elucidated[146] as microsclerodermins A (79) and B (80). A new structural elucidation protocol[147] has been found useful in assignment of structures to new nodularins. The protocol involves (i) FAB mass spectrometry data on the parent, (ii) ^{1}H NMR and chiral GC analysis for an acid hydrolysate, (iii) ozonolysis/borohydride reduction and (iv) FAB mass spectrometry/collision induced dissociation mass spectrometry on linear peptides. The nodularins, [DMAdda^3]-nodularin (81), [(6Z)-Adda^3]-nodularin (82) and [D-Asp^1]-nodularin (83) were characterised in this way. Cypemycin (84) appears to be a structurally unique[148] peptide antibiotic with a sulfide bridge and four α,β-unsaturated amino acids. Cyclisation[149] by FDPP between the Arg amino group and the prolyl carboxy group proved to be a key stage in carrying out the total synthesis of cyclotheonamide B (85) and derivatives (86) and (87). Further insights into the



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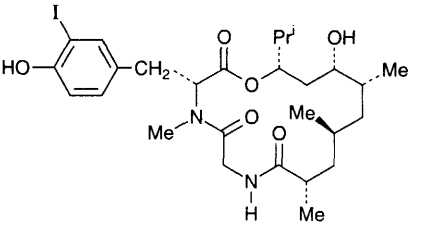

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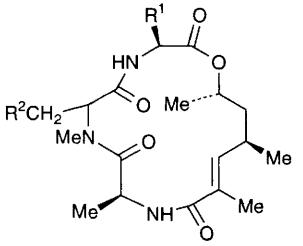
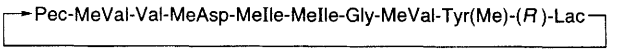
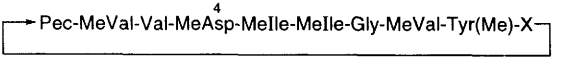


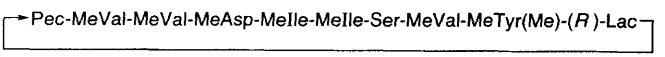
(63) R^1 = Me, R^2 = 3-iodo-4-hydroxyphenyl,
(64) R^1 = 4-OH-Ph, R^2 = 2-bromoindolyl,
(65) R^1 = Me_2CH, R^2 = 3-iodo-4-hydroxyphenyl



(66) Pec = L-pipecolic acid
Lac = lactic acid



(67) X = (*S*)-Lac (68) X = D-Ala
(70) X = (*S*)-Lac, $MeAsp^4$ as $MeAsp(OBu^t)$



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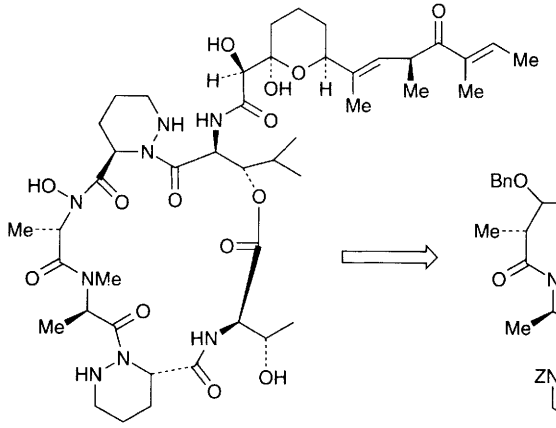

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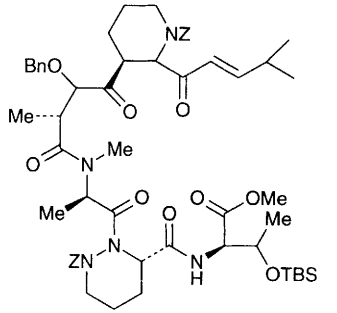

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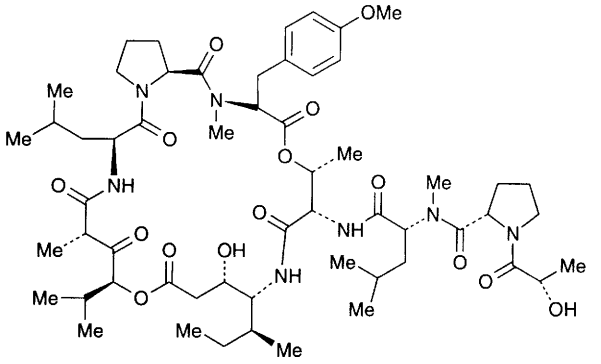

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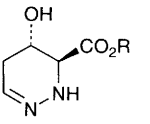


(74)

binding between immunophilin receptors and FK-506 have been obtained[150] from the synthetic availability of analogues such as (88). It is speculated that FK-506 uses a trisubstituted double bond to mimic a trisubstituted amide unit, and its side atoms are a mimic of the Ile side-chain.

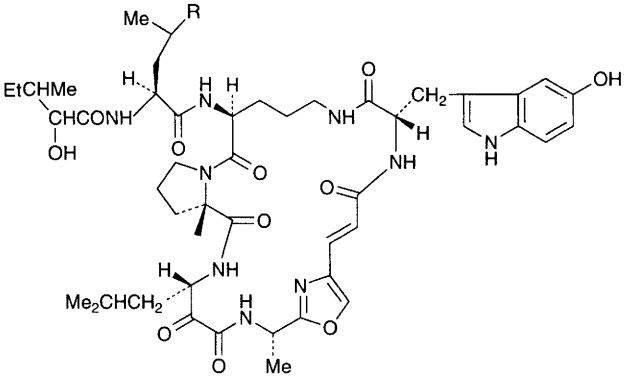
Many thrombin inhibitors, such as the cyclotheonamides, postatin, and the prolyl endopeptidase inhibitors eurystatin A (89) and B (90), contain a β-amino-α-oxocarboxylic acid moiety at the active centre. A synthesis[151] of this residue from (*S*)-Z-alaninal and methyl (*S*)-2-isocyano-4-methylpentanoate *via* the Passerini reaction, allowed a total synthesis of the eurystatins utilising the pentafluorophenyl ester, $CHCl_3/HCO_3^-$/water two-phase system for cyclisation without high dilution. The receptor molecule (91) has been synthesised[152] in 2 steps from suitably protected units and shows selective binding of tripeptides containing N^5-trityl-D-glutamine as an end group. Few steps only were needed for the synthesis[153] of enniatin analogues based on the dinicotinic acid containing structure (92). Two new cyclopeptide alkaloid structures (93) and (94) have been deduced[154] in extracts from the roots of *Ziziphus mucronata*.

3 Modified and Conjugated Peptides

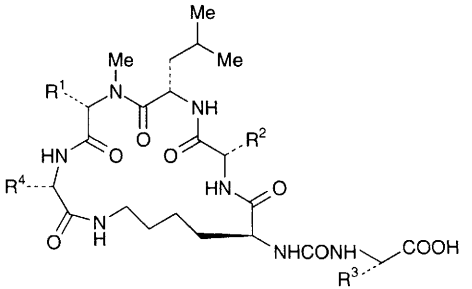
This section brings together reports on peptides which have non-peptidic conjugates attached to their side-chains. Again this year the number of examples in this category have increased.

3.1 Phosphopeptides – Two approaches to the introduction of phosphate groups onto the side chains of Ser, Thr or Tyr are still in vogue: (a) protected phosphate units are included in the building blocks of the sequence or (b) the phosphate group is introduced post-assembly. For the former approach use of Boc-Ser(PO_3Ph_2)-OH and Boc-Thr(PO_3Ph_2)-OH has been reviewed[155], but in the application[156] of Boc-Ser(PO_3Ph_2)-OH to the synthesis of phospholamban 11-19 sequence, extensive dephosphorylation of the phenyl phosphate groups occurred during HF cleavage from the resin. Problems have also been encountered[157] during the use of Boc-Tyr-(PO_3Me_2)-OH as a building block in making the phospho-tyrosine containing fragment of the regulatory domain of $pp60^{c\text{-}src}$. There was incomplete incorporation of the Tyr(PO_3Me_2) residue, partial demethylation and phosphorylation, and an N → O migration of the N-terminal Phe residue to the OH of the adjacent Thr residue. Better success seems to have been achieved[158] for the synthesis of H-Ser-Ser-Ser-Tyr(PO_3H)-Tyr(PO_3H)-OH, phosphorylated angiotensin II and neurotensin 8-13 by using Fmoc-Tyr[$PO(OBu^t)_2$]-OH as a building block.

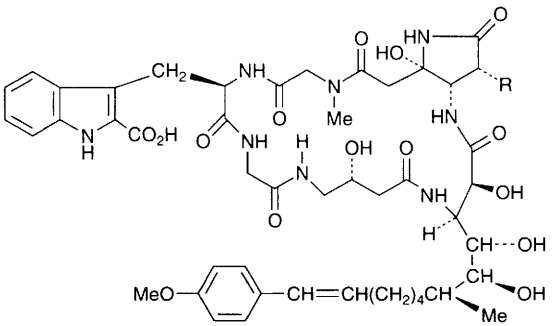
For the post-assembly approach (b), global phosphorylation of unprotected hydroxyl amino acids with $Et_2NP(OBu^t)_2$ and 1H-tetrazole followed by *in situ* oxidation with Me_3COOH has proved to be successful[159] in the phosphorylation of the EGF receptor sequence, H-Ala-Glu-Asn-Ala-Gly-Tyr-Leu-Arg-Val-Ala-Pro-Gln-Ser-NH_2 and its analogues. If oxidation was carried out with dibenzoyl tetrasulfide then thiophosphorylation took place. It was shown that the



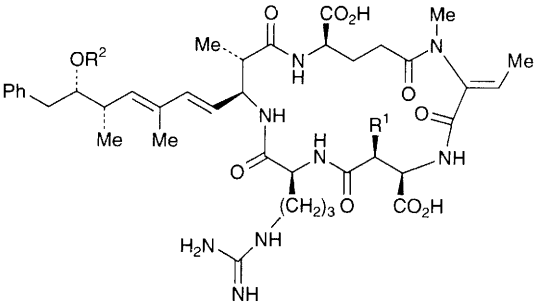
(75) R = H
(76) R = Me



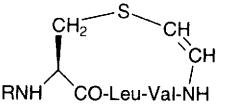
(77) $R^1 = CH_2CHMe_2$, R^2 = Me, $R^3 = CH_2CHMe_2$, R^4 = 2-bromo-5-hydroxytryptophan
(78) R^1 = 6-chloro-5-hydroxy-*N*-methyltryptophan, $R^2 = CH_2CHMe_2$, R^3 = benzyl, R^4 = benzyl



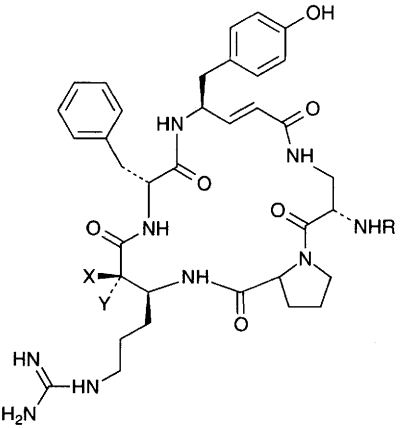
(79) R = OH
(80) R = H



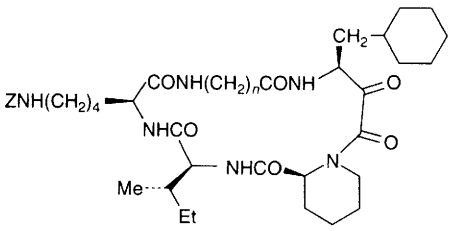
(81) R^1 = Me, R^2 = H
(82) R^1 = R^2 = Me
(83) R^1 = H, R^2 = Me



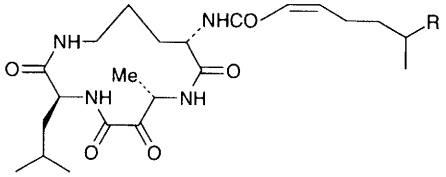
(84) R = Me_2Ala-Dhb-Pro-Ala-Dhb-Pro-Dhb-Val-Gln-Phe-Val-aIle-Gln-Gly-Ser-Dhb-aIle, where Dhb = (*E*)-2-aminobut-2-enoic acid



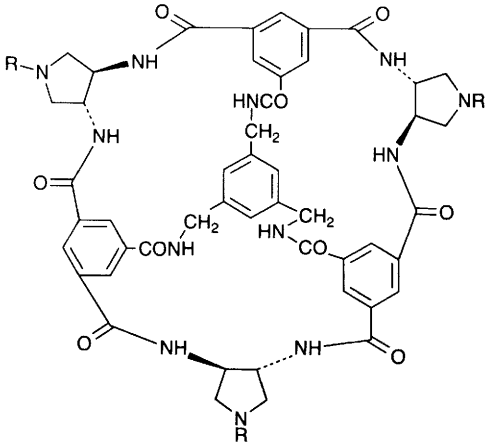
(85) R = Ac, X,Y = O
(86) R = Ac, X = H, Y = OH
(87) R = Ac, X = OH, Y = H



(88) n = 5–7



(89) R = Me
(90) R = Et

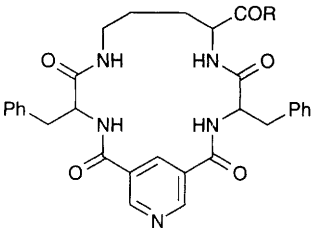


(91)

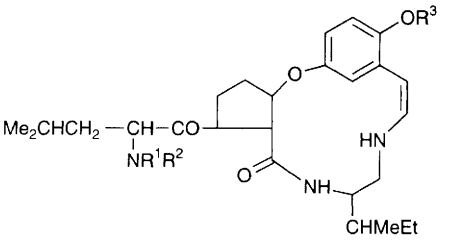
Tyr-thiophosphorylated molecule was unstable in acid conditions. The t-butyldimethylsilyl protecting group successfully protects the serine residue to be phosphorylated while the sequence is built-up on solid phase.[160] Deprotection with 2 eq $N^+Bu_4F^-$ on the resin yields a specific Ser residue for phosphorylation with $(Pr^i)_2NP(OBzl)_2$ followed by Me_3COOH. As illustrated in Scheme 5 it is also possible to use a H-phosphonate method[161] to introduce the phosphate group. Studies[162] using ^{31}P and ^1H NMR have shown that the pK value for Tyr(PO_3H)- is 5.9, for Thr(PO_3H) and Ser(PO_3H) it is 6.1. Phosphorylation also leads to significant shifts in the ^1H NMR resonances of the residues. FAB Mass Spectra of Boc-Ser(PO_3R_2)-OH and derived tripeptides show a strong β-elimination fragmentation.[163]

There has been an expansion in the demand for satisfactory isosteres for phosphate derivatised residues due to the mechanistic interest in protein phosphorylation and in the design of effective inhibitors. The most popular isostere for -Ser(PO_3H) appears to be -Abu(PO_3H)- having the structure (95). Fmoc-Abu(PO_3Me_2)-OH has been prepared[164] and successfully incorporated into H-Leu-Arg-Arg-Val-Abu(P)-Leu-Gly-OH and H-Ile-Val-Pro-Asn-Abu(P)-Val-Glu-Glu-OH. Convenient syntheses[165] of racemic Fmoc-Abu(PO_3Me_2)-OH and Fmoc-Abu(PO_3Et_2)-OH have been reported, and using the building block Fmoc-L-Abu(PO_3Allyl_2)-OH, analogues of the partial sequence of neuromodulin where Ser^{34} is replaced by -Abu(PO_3OH), have been synthesised.[166] The bislactim ether method of Schöllkopf, followed by a selective enzymatic ester hydrolysis have led to a successful synthesis[167] of (96) a protected isostere of phosphothreonine. When racemic Fmoc-Abu(PO_3Et_2)-OH was used in the solid phase synthesis[168] of H-Leu-Arg-Arg-Ala-DL-Abu(PO_3Et_2)-Leu-Gly-OH an inhibitor of the catalytic subunit of cAMP-dependent protein kinase separation of the diastereoisomers could be carried out on a C-18 reverse phase HPLC column.

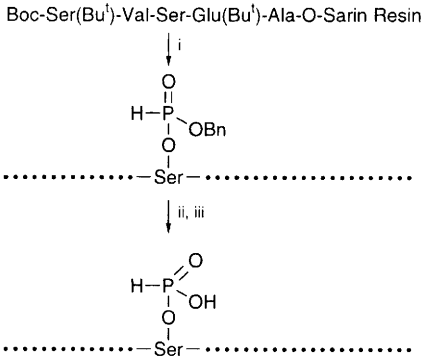
Protected isosteres (97) of phosphotyrosine have been used to incorporate[169] a replacement for Tyr in linear and cyclic analogues based on H-Gly-Tyr-Val-Pro-Met-Leu. A super acid sensitive Rink resin was used to build up the sequence and the protected diastereoisomeric cyclic peptides could be separated on HPLC. The quest for tyrosine kinase inhibitors has produced[170] an enantioselective synthesis of p-hydroxymethyl L-phenylalanine which has been further elaborated to make (98), a tyrosine kinase substrate. It has been speculated that the incorporation of fluorine at the α-methylene position of 4-phosphonomethyl-phenylalanine would produce an isostere with a pK_a closer to phosphotyrosine. Derivative (99) has been enantioselectively synthesised[171] for incorporation as a phosphotyrosine mimetic, and it has been shown[172] that an unprotected form (100) can be used directly to give the model peptides Ac-F_2Pmp-Ile-Asn-Gln-NH_2 and Ac-Glu-F_2Pmp-Ile-Asn-Gln-NH_2 where Pmp represents the 4-phosphonomethylphenylalanine residue. The synthon for O-thiophosphotyrosine, Fmoc-Tyr[PS($OBzl)_2$]-OH has been prepared[173] in 63% yield by phosphinylation of protected tyrosine with $(BzlO_2PNPr^i_2$/tetrazole, followed by oxidation of P(III) to P(V) with S_8 in CS_2. The latter stages can also be used in a global thiophosphorylation approach. Sequences H-Thr-Glu-Pro-Gln-Tyr(PS)-Gln-Pro-Gly-Glu-OH and H-Thr-Arg-Asp-Ile-Tyr(PS)-Glu-Thr-Asp-Phe-Phe-Arg-Lys-OH corresponding



(92) R = H or OMe

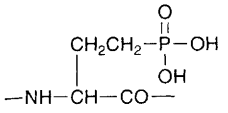


(93) $R^1 = R^2 = R^3 = Me$
(94) $R^1 = R^3 = H$, $R^2 = COCH(NMe_2)CH_2Ph$

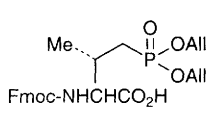


Reagents: i, $PhCH_2OPH(=O)O^-$/Bu^{t}COCl/pyridine/dichloroethane; ii, I_2/pyridine; iii, TFA

Scheme 5



(95)



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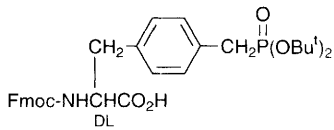

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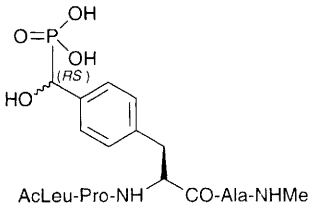

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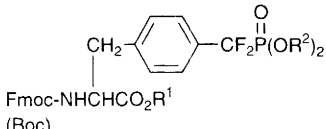
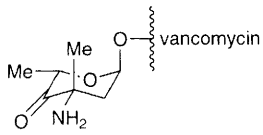


(99) R^2 = Et
(100) R^2 = H



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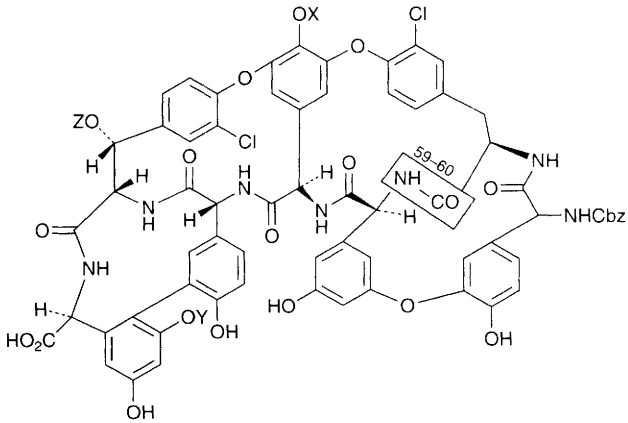


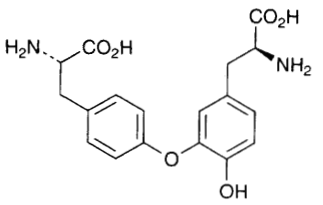
(102) X = *N*-acyl-β-D-glucosaminyl
Y = α-D-mannosyl
Z = *N*-acetyl-β-D-glucosaminyl

to positions 523-531 of $p60^{Src}$ protein and positions 1142-1153 in the insulin receptor respectively, have been synthesised.[173] However purification in 0.1% CF_3COOH/EtOH caused decomposition of the thiophosphopeptide in solution, but a pH of 7.5 proved more beneficial for stability.

3.2 Glycopeptide Antibiotics – Small steps towards a better understanding of the physiological action of antibiotics such as vancomycin continue to be made. It has been suggested[174] that homodimerisation enhances their biological activity, so that structural information on the dimeric antibiotic eremomycin becomes vitally important. Association constants determined[176] using H-D exchange have been used to calculate dimerisation constants K_{dim} for a number of strongly dimerising glycopeptide antibiotics. Bahhimycin, isolated from the broth of *Amycolatopsis sp.* Y-86,21022, has been shown[176] to have an aglycone core identical with vancomycin. The two sugar units are D-glucose and the unusual dehydrovancosamine residue (101). A semi-synthetic derivative (102) of teicoplanin has been used[177] to study the aggregation and rate of association between it and the model peptide H-Ala-Asp-Leu-Ala-Ala-OH. The advantages gained in chemoselective modification of the glycopeptide antibiotics have been explored[178] on teicoplanin through the cleavage of the 59-60 amide bond using ethanolic solution of a large excess of $NaBH_4$. Synthesis of segments of vancomycin continue, with most of the activity centred around non-peptidic parts of the structure. Hence biomimetic phenolic oxidation using an electrochemical method has led[179] to isodityrosine (103) and dityrosine, and the biaryl AB segment (104) of vancomycin can be constructed[180] using a triphenylphosphine-catalysed coupling of a lithio aryl compound, and displacement[181] of a fluorine *ortho* to the nitro group generated the D-O-E 16-membered ring system (105). The first synthesis[182] of vancomycinic acid (106) using nucleophilic displacements of the halides on 2,6-dibromobenzoquinone has been reported, together with an expeditious approach[183] to the β-hydroxyaryl α-amino acids in vancomycin. Overlapping with the fluorine displacements reported in ref. 182 is the similar S_NAr displacements[184] to give (107). Displacement of fluorine took place in 4 eq K_2CO_3 at room temperature for 6 hrs without high dilution. The mild conditions allow the inclusion of racemisation prone p-methoxyphenylglycine. When compound (107) was prepared, two atropisomers due to restricted rotation of the NO_2 containing ring were isolated and characterised.

The S_NAr displacement of fluorine discussed above has also been instrumental in achieving a novel synthesis[185] of K-13 (108). Routes for the synthesis[186] of a model (109) for the C-F-G ring system of ristocetin A have been described. The key aryl ether bond was constructed by reaction of phenoxides of hydroxyarylglycine derivatives with an arenemanganese tricarbonyl complex.

3.3 Glycopeptides – The expansion in this subject area is reflected this year in the number of reviews published in the area, with some having more than 200 references. Thus the classification, occurrence and discovery of glycopeptides have been reviewed.[187] Glycopeptide syntheses[188,189] have been authoritatively



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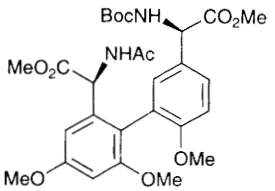
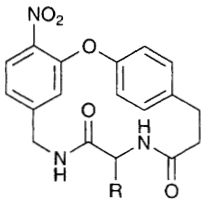
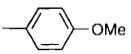
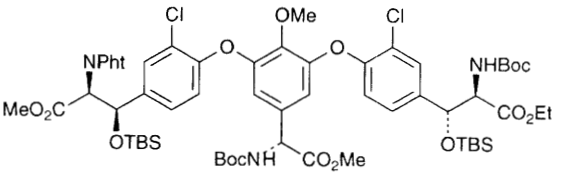


(104)



(105) R = H

(107) R = 



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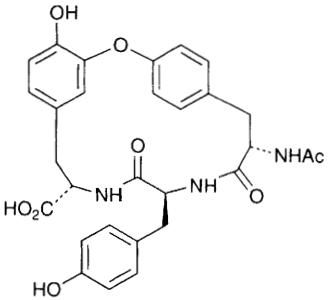

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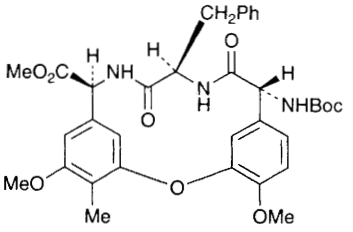
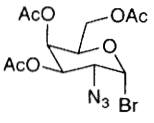
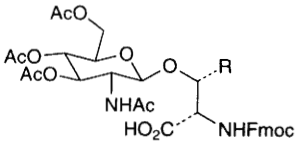


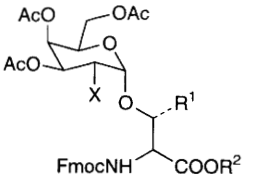
(109)



(110)



(111) R = H
(112) R = Me



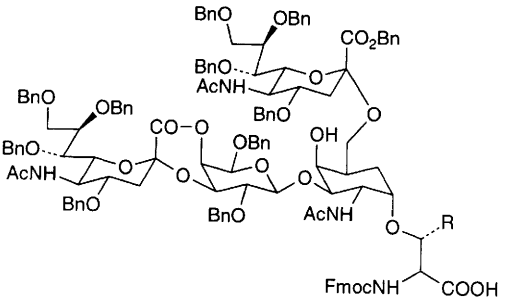
(113) X = AcNH, R^1 = H, Me, R^2 = H
(114) X = N_3, R^1 = H, R^2 = C_6F_5
(115) X = N_3, R^1 = Me, R^2 = C_6H_5

reviewed, together with new enzymatic protecting group techniques[190] and the remodelling[191] of glycopeptides for affinity labelling. In the synthesis of glycopeptides on solid supports it is important to have compatibility between the growing chain and the polymer support, so a polyethylene glycol polyacrylamide copolymer $PEGA_{1900}$ has been developed[192] for this purpose. A key component in many glycopeptide syntheses, azidodeoxygalactopyranosyl bromide (110), has been made by improved synthetic methodology.[193] It is still convenient this year again to subdivide the discussion to the O- and N-glycopeptide categories.

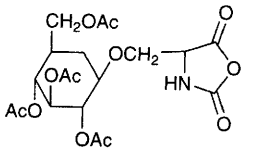
O-Glycopeptides – The sub-division of syntheses to the 'building block' approach, and the 'global glycosylation' approach is more diffuse this year, but there are sufficient examples of useful protected building units for incorporation into solid phase syntheses to review them together. Thus the building blocks (111) and (112) suitable for the synthesis of GLCNAc residues β-linked to Ser or Thr have been made.[194] The α-linked analogues (113) have been incorporated[195] as building blocks, as part of a protocol to make H-Val-Thr(R)-Ser(R)-Ala-Pro-Asp-Thr(R)-Arg-Pro-Ala-Pro-Gly-Ser(R)-Thr(R)-Ala-Pro-Pro-Ala-His-Gly-OH (R = α-D-GalNAc). 2-Azido-2-deoxy-D-galactosyl residues α-linked to Thr or Ser can be introduced to the solid phase protocol *via* derivatives[196] (114) and (115), with the azido group being a precursor of a 2-acetamido group. Thirty four O-glycopeptides based on the repeating unit of human intestinal mucin have been synthesised[197] using the derivative (115) in a multiple column synthesiser. The rather impressive tetrasaccharide derivative (116) has been used[198] as a unit in the solution phase total synthesis of the N-terminal glycoheptapeptide of glycophorin AM, while Fmoc-[$GalNAc(Ac)_3$-α]Thr-OH was the unit of choice to synthesise[199] the glycohexapeptide H-Val-(GalNAc-α)Thr-His-Pro-Gly-Tyr-OH characteristic of oncofetal fibronectin. Polymerisation[200] of the N-carboxyanhydride unit (117) gives a relatively high molecular weight poly[O-(β-D-glucopyranosyl)]-L-serine polymer.

To overcome the vulnerability of alkyl esters of glycosylate amino acid derivatives to alkali, polyethylene glycol esters have been used[201] successfully as in (118), because they can be deblocked by lipases. A stereocontrolled synthesis[202] of a triglycosyl-L-Ser-Pro-OMe (119) which has phytoalexin elicitor activity has been carried out by glycosylation of a disaccharide with a protected glycosylated serine derivative. The water soluble neoglycolipid (120) has been synthesised[203] *via* glycosylation of Fmoc-Ser-OC_6F_5 with perbenzoylated lactosyl bromide. α- and β-Acetylated D-glucopyranosyl esters of a number of enkephalins,[204] when tested in the gpi and mvd assays were less potent than [Leu^5]-enkephalin. It appears from work carried out[205] on synthetic O-glycosylated models, and a 23-residue glycopeptide from human intestinal mucin, that O-glycans are less likely to undergo β-elimination than had been previously supposed.

In a series of detailed papers[206] on the synthesis of bleomycin A_2 (121), the glycosyl residue is reported as having been introduced *via* the protected fragment (122). β-Galactosidase catalyses the synthesis[207] of galactosyl or lactosyl derivatives of serine and threonine, while α-mannosidase functions in the same way for introducing mannose. Glycosylation of amino acids and peptides with 3-amino glycals (L-acosaminal and L-ristosaminal) has been reported[208] to be successful



(116) R = H, Me



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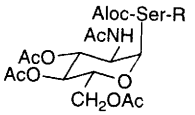
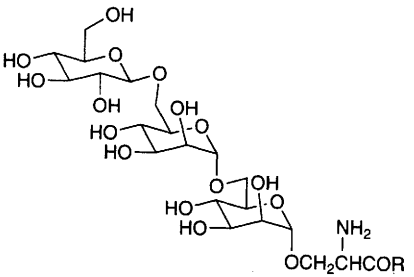
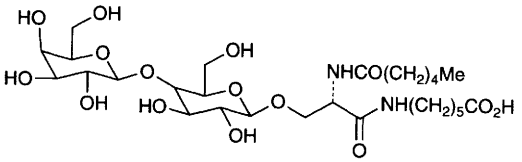


(118) R = $OCH_2CH_2OCH_2CH_2OMe$



(119) R = OH, Pro-OH



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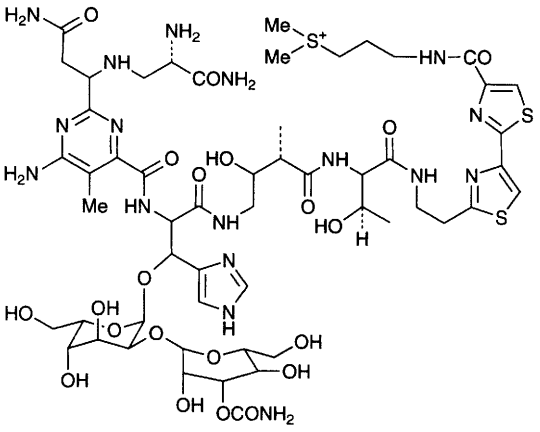
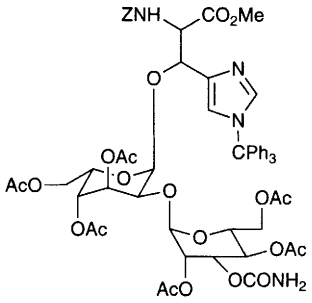
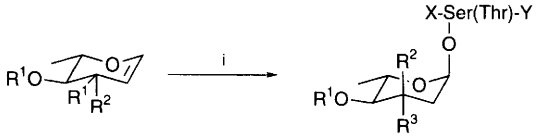


(121)



(122)



R^1 = H, R^2 = H, R^3 = NH_2 (L-acosaminal)
R^1 = H, R^2 = NH_2, R^3 = H (L-ristosaminal)

Reagent: TMS triflate/CH_2Cl_2

Scheme 6

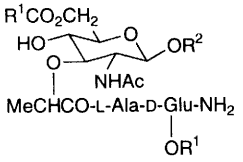
via the TMS triflate catalysed reaction summarised in Scheme 6. Multiple column peptide synthesis, incorporating the glycosyl component as suitably protected Fmoc-pentafluoro phenyl esters, has been successful in the synthesis[209] of 6′-O-phosphorylated mannose disaccharide-containing glycopeptides. Glycopeptides containing two disaccharide units were shown to be potent inhibitors of receptor binding. A series of N-acetyl-L-Ala-D-isoglutamine analogues (123) containing an acridine derivative have been synthesised[210] as potential anti-HIV-1 and anticancer agents. Reductive β-elimination of O-glycosylated peptides liberates peptides with a specially modified residue where the carbohydrate was attached. Mass spectrometric study[211] of the released peptide can pinpoint the position of carbohydrate link. The potential of using electrospray mass spectrometry to study the sequences of non-derivatised glycopeptides has been explored.[212]

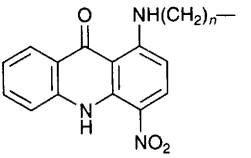
N-Glycopeptides – A protocol for solid phase synthesis involving specific allylic protection of Glu or Asp residues, and using Fmoc/Bu^t strategies to build up the sequence has been developed.[213] Removal of the allyl protection releases a specific carboxyl group which can then be condensed with glycosylamines using PyBOP/HOBt. Conversion of maltooligosaccharides with 2-7 sugar moieties into β-1-amino-1-deoxy derivatives allows them to be coupled[214] to Fmoc-Asp(OPfp)-OBu^t. After treatment with trifluoroacetic acid the resulting glycosylated asparagines were used for the solid phase synthesis of T-cell epitopic glycopeptide analogues. For the synthesis[215] of a fucosyl chitobiose hexapeptide related to the leukemia virus envelope glycoprotein, fucosylated chitobiosyl asparagine conjugates were synthesised using allyloxycarbonyl and t-butyl protecting groups. O-Acetyl protected fucose seemed to have the correct acid stability profile for the synthesis. An allylic linkage as an anchoring group has proved[216] to be a successful way of synthesising (124), a glycosylated peptide T derived from the sequence of gp 120 of HIV-1. For the synthesis[217] Ac-Asn(R)-Gly-Asn(R)-Ala-Ser-Ala-OH where R = (125), the key attachment between sugar and amino acid was carried out as summarised in Scheme 7. An amino-propyl silica support has proved[218] compatible with both the organic and aqueous environment required for a combination chemical-enzymatic synthesis of glycopeptides. Initially the peptide sequence is 'grown' *via* a hexaglycine and a α-chymotrypsin-sensitive phenylalanine ester bond, with the Asn-sugar residue incorporated as Boc-Asn(GlcNAcβ)-OH. Further extension of the carbohydrate sequence is then carried out on the solid support using appropriate glycosyltransferases. The same approach[219] has been used to synthesise the multivalent sialoglycopeptides, *e.g.* (126).

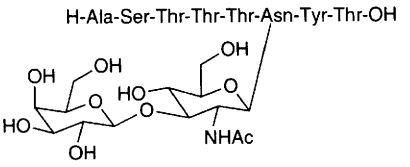
Comment has been made before on the diverse structures emanating from marine sponges. Structure (127) for theonegramide from *Theonella swinhoe*[220] could have been included elsewhere in this report, but as it contains an intriguing arabinose-peptide link it is worth emphasising under this category.

3.4 Lipopeptides – Effective immuno-modulating compounds for enhancement of the body's defence system have been a pharmaceutical goal for some years. Synthesis of lipopeptide (128) based on naturally occurring FK156 was reported in 1993, but it was rapidly metabolised after oral application. The methylated

(123) R^2 = H, CH_2Ph and *e.g.* R^1 can be







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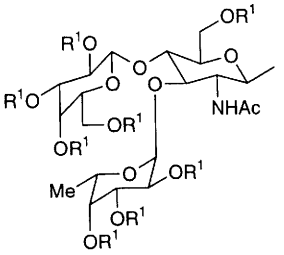
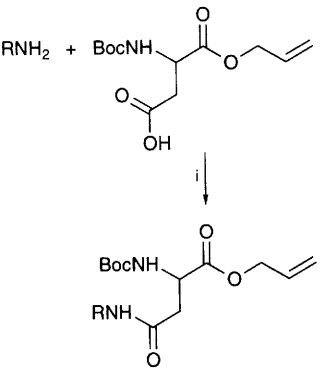


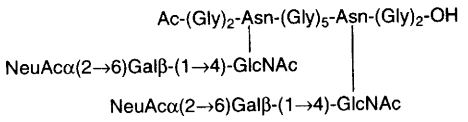
(125) R^1 = H



R = (125), R^1 = Ac

Reagents: I, isobutyl-2-isobutoxy-1,2-dihydroquinoline-1-carboxylate

Scheme 7

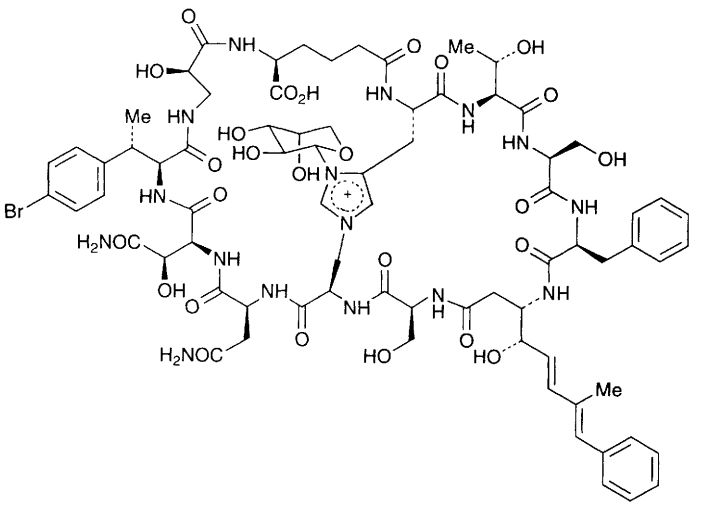


(126)

analogue (129) has now been synthesised[221] and found to be more stable. Lipopeptide WS1279 derivatives with (*R*)-glycerol moieties (130) have been synthesised[222] together with their (*S*)-analogues and the former (the natural configuration) were shown to have higher mitogenic activity. The structure and conformation of syringotoxin, a lipodepsipeptide from *Pseudomonas syringae pv. syringae* have been elucidated[223] by 2D NMR and molecular dynamics, and N-benzoyl (or N-pivaloyl)-*S*-farnesyl-L-cysteines have proved useful[224] in working out the putative role of methyltransferase in mediating human platelet aggregation.

3.5 Nucleoside-Oligonucleotide Conjugates – Nucleopeptides, defined as conjugates with a covalent phosphodiester bond between the terminal OH group of an oligonucleotide and a side-chain hydroxyl from the peptide, have been synthesised in the past using a convergent approach in which the two different components have been assembled separately and linked. In a new approach[225] for the synthesis of Phac-Phe-Val-Ser(P^{31}ACT)-Gly-OH, where Phac is phenylacetyl, the peptide was first assembled on an insoluble matrix, and oligonucleotide elongation carried out at the Ser hydroxyl on the resin. Oligonucleotide-peptide hybrids, having potential as antisense inhibitors of gene expression have been prepared[226] by coupling thiol-derivatised oligonucleotides with peptides derivatised at their N-terminus with a maleimido functionality. The synthesis and NMR assignments have been carried out[227] on the nucleoside dipeptide (131).

3.6 Miscellaneous Conjugates – General procedures have been evolved[228] for the site specific pegylation [(PEG = monomethoxypoly(ethylene glycol)] of peptides at the N-terminus, side-chain positions (of Lys, Asp and Glu) or at the C-terminus using Fmoc/Bu^t solid phase methodologies. A model tridecapeptide fragment of interleukin-2IL-2(44-56)NH_2 was chosen for study. The role of the tyrosine sulfate residues in cionin, H-Asn-Tyr(SO_3H)-Tyr(SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2 have been explored[229] using (i) Fmoc-Tyr(SO_3Na)-OH as a building block to assemble the peptide on the solid phase and (ii) Tyr(Msib) where Msib = p-(methylsulfinyl)benzyloxycarbonyl, was used in the assembly to achieve sulfation of a selective Tyr residue. After resin cleavage SO_3-DMF complex achieved the sulfation of the free Tyr residue. Reduction of the Msib group to TFA-labile derivatives followed by final treatment with 90% aq. TFA with scavengers gave the desired cionin derivative. Yields obtained *via* synthetic route (ii) appeared to be considerably higher than approach (i).



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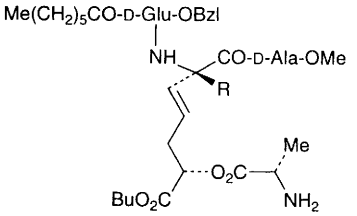
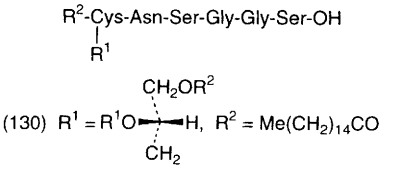
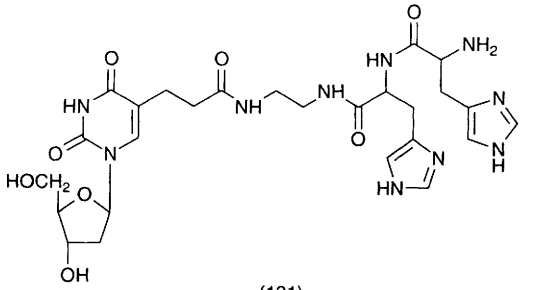


(128) R = H
(129) R = Me



(130) $R^1 = R^1O$, H, $R^2 = Me(CH_2)_{14}CO$



(131)

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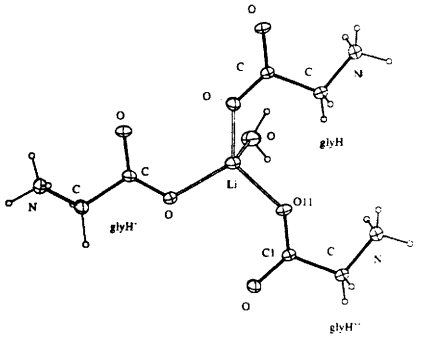
5
Metal Complexes of Amino Acids and Peptides

By K.B. NOLAN, A.A. SOUDI and R.W. HAY

1 Introduction

This chapter deals with the synthesis, structures and reactions of metal-amino acid and metal-peptide complexes and covers material published in 1993 and 1994. A new interdisciplinary journal entitled 'Metal-Based Drugs' in which metal-amino acid and metal peptide complexes feature prominently commenced publication in 1994.[1] In volume 1 there are three review articles which involve metal-amino acid or metal-peptide complexes. These describe chelating agents and the regulation of metal ions,[2] the chemistry of gold drugs including gold complexes of biomolecules,[3] and the chelating tendencies of a variety of bioactive aminophosphonates.[4]

A number of other reviews in this area have been published. Chelates of N-alkylated α-amino acids with metal ions particularly copper(II) are the subject of one review in which steric and enantioselective effects and distortion of the copper(II) polyhedron are discussed.[5] Redox decomposition reactions of copper(III)-peptide complexes are also reviewed.[6] Other reviews on copper include some physical properties,[7] and crystal structures,[8] of copper(II)-amino acid complexes. A comprehensive review containing 244 references on work carried out since 1975 on platinum(II) and platinum(IV) complexes of amino-acids and peptides has been published.[9] A critical survey of the stability constants of metal complexes of aliphatic amino acids has been carried out.[10] The various modes of binding of amino acids to metal ions have been described.[11] Metal binding peptides in plants is the subject of two reviews.[12,13] One of these[13] deals with the role of phytochelatins in the transportation of heavy metal ions to vacuoles and plant tissues, in the metabolism of trace elements and sulfur compounds in plants and in the development of tolerance to some heavy metals by some plants. Analysis of metalloproteins and metal-binding peptides by capillary electrophoresis[14] and the oxidation of amino acid residues in proteins by radiolysis and by metal (Cu^{2+}, Mn^{2+}, Fe^{2+}) catalysed reactions are also reviewed.[15] A new book entitled 'Bioinorganic Chemistry: Inorganic Elements in the Chemistry of Life' which contains many references to metal-amino acid and metal peptide complexes has been published.[16] Volume 2 of 'Perspectives in Bioinorganic Chemistry' has also appeared.[17]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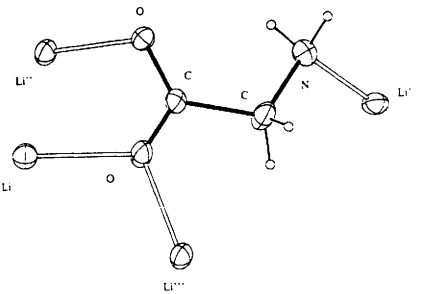
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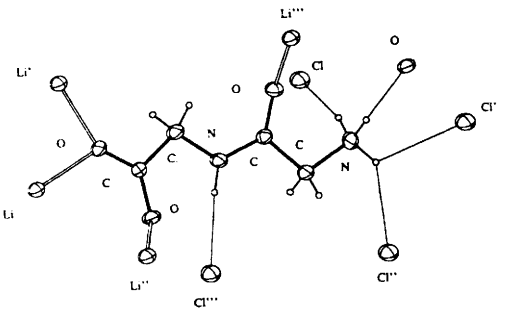
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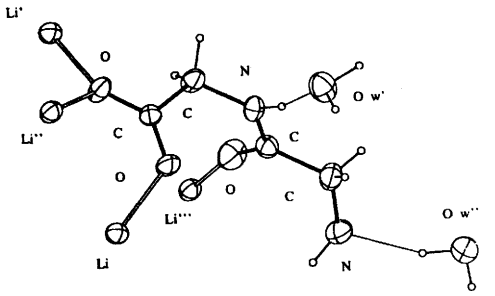
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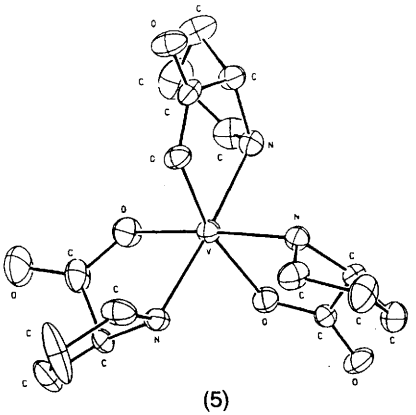
2 Amino Acid Complexes

2.1 Crystal and Molecular Structures – A number of lithium complexes of neutral and anionic glycine and glycylglycine have been prepared and crystallised from water or water/ethanol mixtures.[18] The crystal structures of [Li(GlyH)(H_2O)]Cl **(1)**, LiGly **(2)**, [Li(GlyGlyH)]Cl **(3)** and LiGlyGly·H_2O **(4)** have been determined. In all four complexes the Li^+ cation is tetrahedrally coordinated. Three of the coordination sites are occupied by carboxylate oxygen atoms from three different but crystallographically equivalent Gly or Gly-Gly ligands respectively. The fourth coordination site at Li^+ is different in each complex. In the case of complex **(1)** it is occupied by an aquo ligand, in **(2)** by the amino group of Gly and in **(3)** and **(4)** by the peptide oxygen atoms. The crystal structures of lithium L-pyroglutamate mono- and di-hydrates have also been reported.[19]

A series of vanadium(III)-amino acid complexes of formula VA_3 where AH = L-Pro, D-Pro, L-Phe, D-Phe, DL-Phe, L-Try and L-Val have been synthesised and the structures of V(L-Pro)$_3$·DMSO **(5)** and V(D-Pro)$_3$·DMSO **(6)** have been determined by X-ray diffraction.[20] Structure **(5)** corresponds to the mer, Δ diastereoisomer while structure **(6)** is the mer, Λ form, each of these being one of the four possible diastereoisomers formed. The addition of 2-hydroxynaphthalene-2-carbaldehyde to solutions of $VOSO_4·5H_2O$ and amino acids gave complexes VO(H_2O)L where H_2L is the Schiff base formed between the aldehyde and Gly or Phe.[21] Reaction of these with amines (imidazole, methylimidazole, pyrrole, pyridine, histidine and histidine derivatives) gave VO(OH)L(amine) complexes and with alcohols gave VO(OR)L(ROH). It was also found that His can replace Gly in the complex VO(H_2O)Gly forming a histidine-Schiff base species [VO_2{$O_2CCHCH_2C_3H_4N_2$}N=CHC_4H_6O] **(7)** the crystal structure of which was determined. In this complex the geometry around the metal is square pyramidal with the Schiff base coordinated through the carboxylate, imino and phenolate groups. The nitrogen atoms in the imidazole ring are involved in hydrogen bonding to the carboxylate and oxo groups.

The complex VO(Sal-Gly)H_2O was found to dissolve in pyridine to form brown coloured VO(Sal-Gly)(py)$_2$ **(8)**, SalGly = N-salicylideneglycinate.[22] The complex VO(Sal-L-Ala)(bipy) **(9)** was obtained by adding 2,2′-bipyridine to VO(Sal-L-Ala)H_2O in methanol. Complexes **(8)** and **(9)** are both octahedral with the tridentate Schiff base ligand in equatorial positions and the heterocyclic nitrogen atoms occupying the remaining equatorial and one axial position.

The coordination behaviour of N-substituted 4-toluenesulfonyl (Tos) and benzenesulfonyl (Bs) amino acids (Gly, DL-Ala, β-Ala, Asn, Gln) towards Mn(II) ion has been investigated.[23] The crystal structure of [Mn(Tos-Ala)$_2$(H_2O)]·2.78H_2O·0.92CH_3OH **(10)** shows that the amino acid monoanion bridges two Mn atoms through the carboxyl group giving rise to a one dimensional polymer, the coordination around the metal being completed by two water molecules. In [Mn(Tos-Gln)$_2$] **(11)** each amino acid monoanion bridges two Mn atoms through the carboxyl group and binds a third Mn atom through the carbonyl oxygen of the terminal amide group giving rise to a two dimensional polymer.



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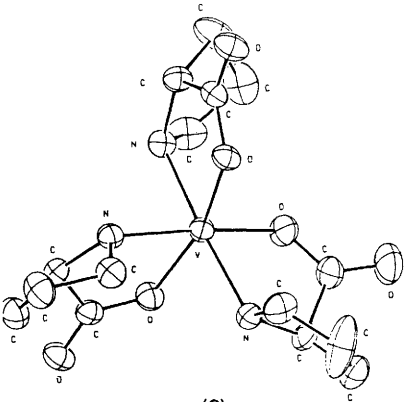

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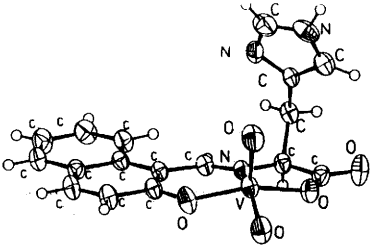

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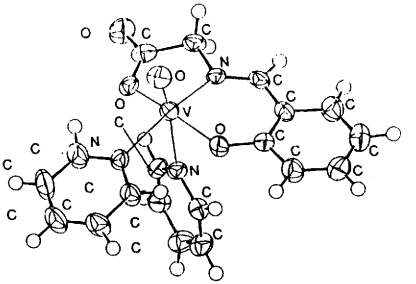

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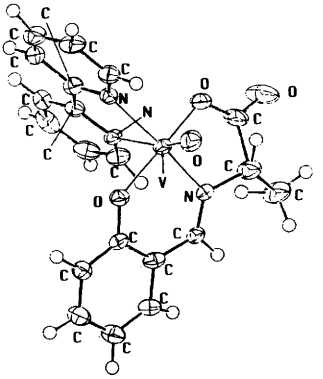

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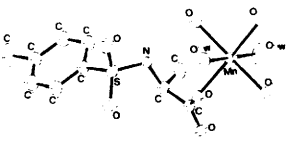

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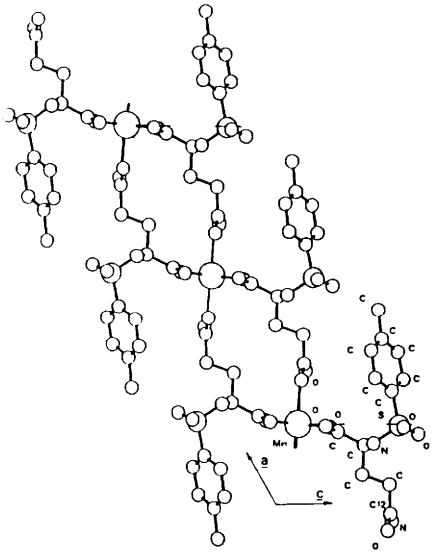

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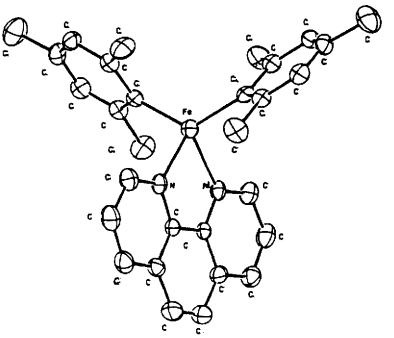

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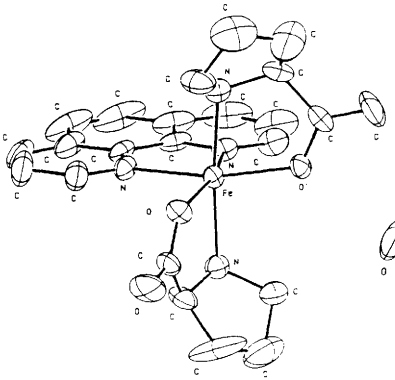

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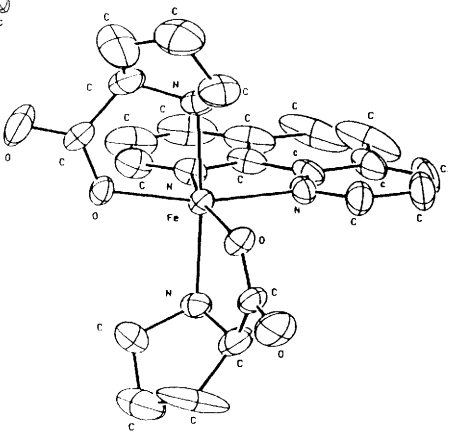


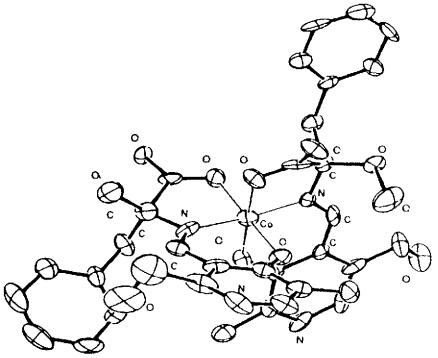
(14)

The arylation of $FeCl_2(THF)_{1.5}$ in THF with MesMgBr (Mes = 1,3,5-$Me_3C_6H_2$) in the presence of 1,10-phenanthroline (phen) gave the crystalline [Fe(phen)$(Mes)_2$] **(12)** which contains a tetrahedral iron(II), σ-bonded to two aryl groups.[24] These can be removed by a variety of protic ligands under aprotic conditions. The reaction of **(12)** with amino acids, AH, gave monomeric iron(II)-amino acid complexes [Fe(phen)$(A)_2$] where A = L-Pro, D-Pro, L-Phe, D-Phe, D,L-Phe, L-Trp, L-Val. These products show a diversity of solubility properties depending on the amino acid residue involved. All of them have been fully characterised and the crystal structures of [Fe(L-Pro)$_2$(phen)] **(13)** and [Fe(D-Pro)$_2$(phen)] **(14)** have been reported.

The structure of the ternary cobalt(III) complex [Co(pyridoxylidene-α-hydroxyphenylalaninato(pyridoxylidene-α-hydroperoxyphenylalaninato)]$^-$ **(15)** has been determined on the basis of ^{1}H NMR, positive-ion FAB mass spectrometry and X-ray diffraction studies.[25] This complex was obtained by reaction of pyridoxylidene-L-phenylalanine and cobalt(II) salts in the present of H_2O_2 and is an example of a novel type of fixation of active oxygen species onto a Schiff base ligand. The complex [Co(phen)$_2$(Gly)]$Cl_2 \cdot 4H_2O$ **(16)** was synthesised and characterised by IR, NMR and X-ray diffraction techniques.[26] A cobalt(III) complex with the $(N)(O)_3$-type tripodal, tetradentate ligand, bis-N,N-carboxylmethyl-L-phenylalanine (BCMPA) was found to site-specifically coordinate a bidentate amino acid in the trans-N,N rather than the cis-N,N configuration through non-covalent, weak interligand interactions.[27] The crystal and molecular structures of cis, **(17)** and trans, **(18)** isomers of the complex K[Co(BCMPA)L-Leu] have been determined.

The structures of the complexes trans-(O)-[Co(aeida)Cl(H_2O)] **(19)** and mer-(N)-[CoCl-(i-dtma)(H_2O)]ClO_4 **(20)** where H_2aeida is N-2-aminoethyl-N-(carboxymethyl)glycine and Hi-dtma is N,N-bis(2-aminoethyl)glycine, have been reported.[28] The potentially quinquedentate (SS,SS)-3-(2-aminocyclohexylamino)-2-(2-aminocyclohexylaminomethyl)-propionic acid (HL′) **(21)**, prepared via a metal-directed condensation reaction, readily formed a (carboxylato-tetraamine)-chlorocobalt(III) complex cation as well as the rhodium(III) and chromium(III) analogues.[29] The crystal structure of the complex SS,SS-[CoL′(Cl)]$ClO_4 \cdot 0.75HClO_4 \cdot 2H_2O$ **(22)** has been determined. In this complex the chloride and carboxylate ligands are trans and the two pairs of nitrogen donors from the cyclohexane-1,2-diamine residues are coplanar with the cobalt ion. The $NHCH_2CH(CO_2^-)CH_2NH$ core of the ligand necessarily occupies an octahedral face. The synthesis and characterisation of the complexes cis-β-[Co(L)Gly]$^+$ and cis-β-[Co(L′)AA]$^+$ as PF_6^- or mixed PF_6^-/Cl^- salts where LH is N-{2-[(2-aminoethyl)thio]ethyl}-2-aminoacetamide, L′H is N-(2-aminoethyl)-2-[(2-aminoethyl)thio]acetamide and AA = Gly, L-Ala, L-Leu, L-isoLeu, is described as well as the crystal and molecular structure of cis-β_1-[Co(L)Gly]$PF_6 \cdot H_2O$, in which the carboxylate group of Gly is trans to the amido group of L.[30]

In the complex [Ni(Lys)$_2$$(H_2O)_2$]$Cl_2 \cdot H_2O$ the Ni(II) ion has a distorted octahedral geometry.[31] The α-amino group and the carboxylate group of lysine coordinates to Ni(II) to form a square plane at distances of between 2.052(2) Å and 2.100(2) Å. Water ligands are located in apical positions at distances of



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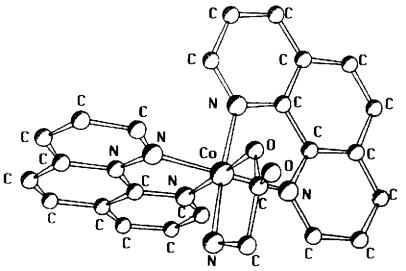

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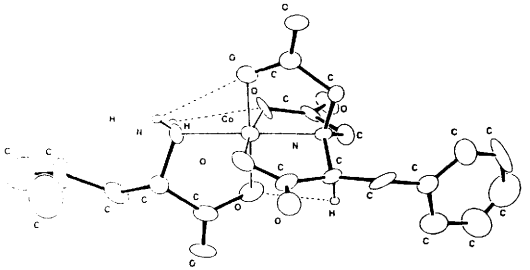

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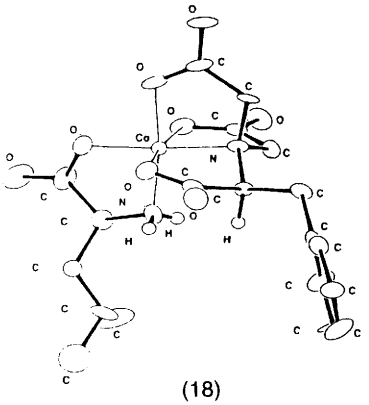

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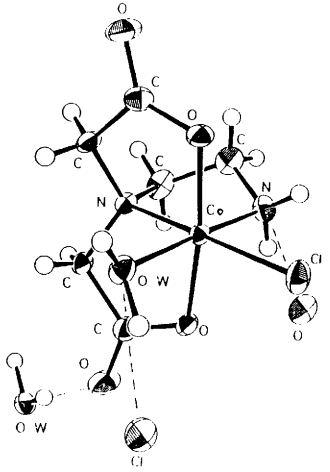
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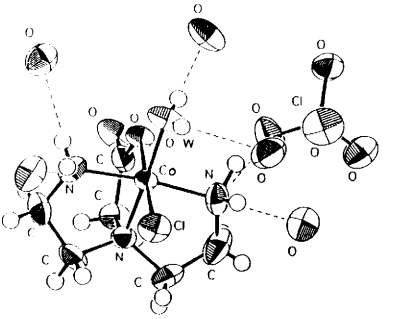
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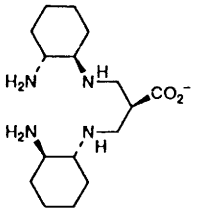
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CO2−
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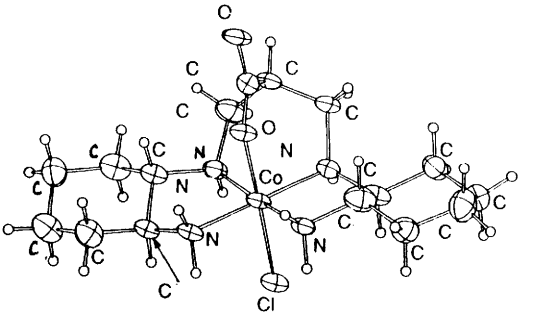
N
H

(21)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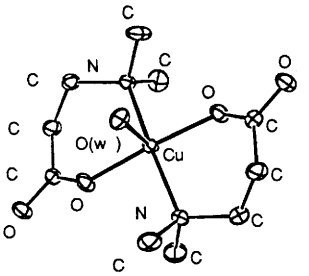
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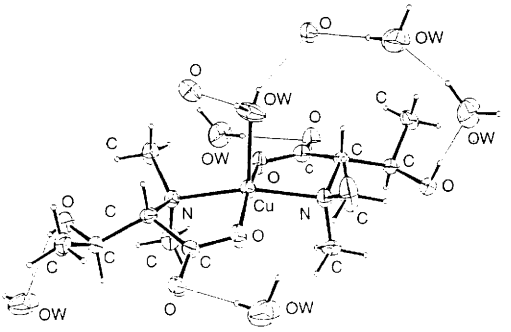
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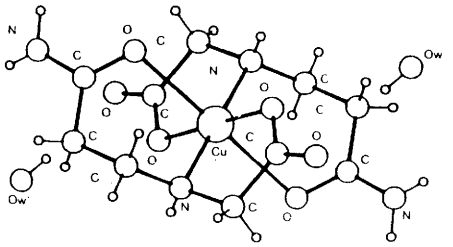
(24)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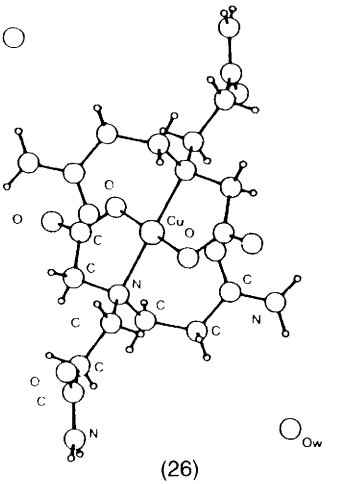
(25)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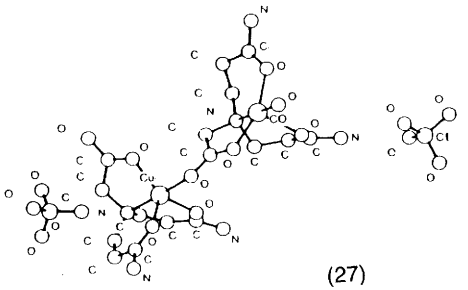
(26)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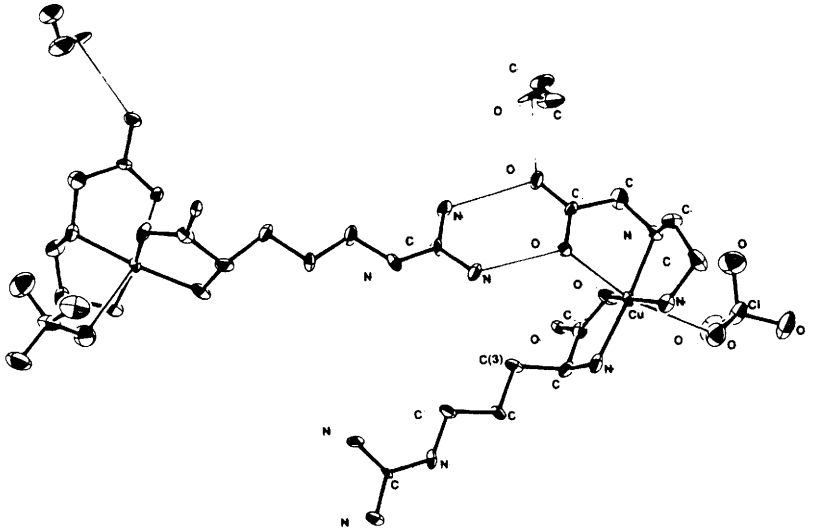
(27)

2.144(4) Å and 2.153(3) Å respectively. The structure involves an elaborate network of hydrogen bonds. The crystal structure of the nickel(II) complex of (2S, 3S)-2-(trifluoromethyl)threonine has also been reported.[32]

The crystal and molecular structures of several amino acid complexes of copper(II) have been determined. In the complex aquobis(N,N-dimethyl-β-alaninato)copper(II) hexahydrate **(23)**, obtained by the reaction of bis(β-alaninato)copper(II) with formaldehyde the copper ion is five coordinate and exists in a distorted square pyramidal geometry with an N_2O_2 donor set of two β-alanine ligands defining the basal plane and a coordinated water molecule in the axial position.[33] In the complex aquobis(L-N,N-dimethylthreoninato)copper(II) dihydrate **(24)** the geometry around the copper is also square pyramidal with threonine N and O atoms in the basal plane and the aquo ligand in the apical position.[34] The structure of the complex bis(N-tert-butyl-N-benzylglycinato)copper(II) consists of discrete molecules in which copper has square planar coordination.[35] Reaction of $Cu(L\text{-}Pro)_2$ with formaldehyde resulted in the formation of the N-hydroxymethylated amino acid complex $Cu(N\text{-}CH_2OH\text{-}L\text{-}Pro)_2(H_2O)$ the crystal structure of which has been reported.[36]

The crystal and molecular structures of the complexes bis(N-propionamidoglycinato)copper(II) hydrate **(25)**, bis(N,N-dipropionamidoglycinato)copper(II) dihydrate **(26)** and bis(N,N-dipropionamidoglycinato)-μ-carboxylato-dicopper(II) perchlorate hydrate **(27)** have been reported.[37] In all three complexes the amide group coordinates to copper through its carbonyl oxygen. In complex **(25)**, which is Jahn Teller distorted, the ligands are tridentate and coordinated to the metal through the amine nitrogen and the carboxylate and amide oxygen atoms. Complex **(26)** has a similar structure with the extra amide groups in the ligand being uncoordinated. Complex **(27)** is binuclear, the geometry around each copper being square pyramidal with an amide oxygen occupying the axial position in each case and an amine nitrogen, a carboxylate oxygen and an amide oxygen in the equatorial positions. The remaining equatorial position is occupied by a water ligand in the case of one copper and by a bridging carboxylate oxygen in the case of the other copper.

The crystal structures of orthorhombic $[Cu(EDMA)(L\text{-}Arg)ClO_4]\cdot{}^1/_2C_2H_5OH$ **(28)** and monoclinic $[Cu(L\text{-}Arg)_2](NO_3)_2\cdot 3H_2O$ **(29)** where EDMA is ethylenediamine-N-monoacetic acid have been determined.[38] In **(28)** the copper ion is in a slightly distorted square pyramidal geometry with the two nitrogen atoms of EDMA and the nitrogen and oxygen atoms of L-Arg coordinated at the equatorial positions while the carboxylate oxygen atom of EDMA is coordinated axially. A perchlorate oxygen atom is weakly coordinated at the other axial site. The coordinated carboxylate group of EDMA is hydrogen bonded to the positively charged guanidinium group of L-Arg in a neighbouring complex molecule. Complex **(29)** is square-planar with two nitrogen atoms and two oxygen atoms of two L-Arg ligands in a cis configuration with respect to the amino groups, which are hydrogen bonded to an uncoordinated nitrate ion. The guanidinium group is hydrogen bonded to two nitrate ions. The stability constants of these complexes were found to be enhanced by interligand interactions. The crystal and molecular structure of $[Cu(L\text{-}Ala)(H_2O)_2]SO_4$ **(30)** obtained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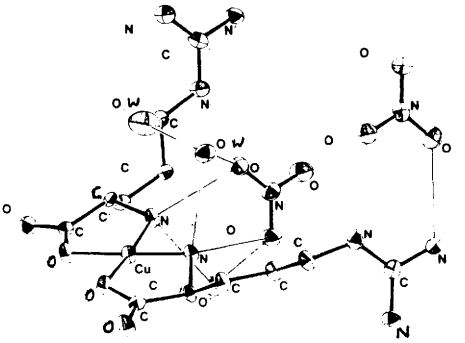
(28)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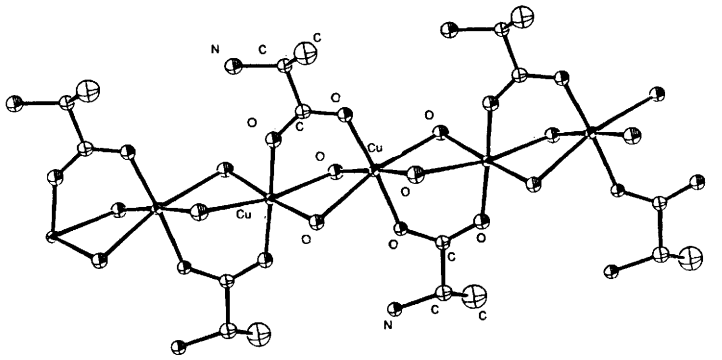
(29)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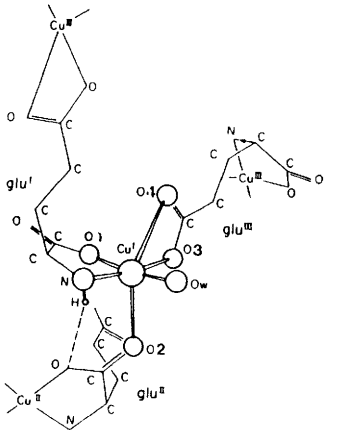
(30)

from equimolar aqueous solutions of $CuSO_4 \cdot 5H_2O$ and L-Ala (~ 0.5 mol dm^3) is also described.[39] The structure contains a polymeric chain of copper ions bridged by water molecules and the carboxylate groups of zwitterionic L-Ala ligands. Each copper ion has four equatorial water ligands and oxygen atoms from bridging carboxylates axially coordinated. The crystal and molecular structures of the histamine (Hm) complexes $Cu(Hm)_2(ClO_4)_2$ and Cu(Hm)enCl, where en = 1,2-diaminoethane, have been determined.[40]

The crystal structures of Cu(Glu) and Zn(Glu), both previously determined, have been described and EPR measurements have been carried out on single crystals of Cu(Glu) and Zn(Glu) doped with ^{63}Cu in order to determine exchange interactions.[41] In Cu(Glu) **(31)** the coordination around the metal is distorted octahedral with two oxygens, O1 and O3, from Glu1 and Glu3, the amino nitrogen from Glu1 and a water oxygen as equatorial ligands at distances of about 1.99 Å. An oxygen atom, O2, from Glu2 occupies an apical position at 2.299 Å while an oxygen, O4, from Glu3 completes the distorted octahedron. O4 is considerably displaced from the apical position due to the rigidity of the carboxylate group which also occupies an equatorial position. The coordination geometry of Zn(Glu) **(32)** is similar.

Mannich aminomethylation reactions involving $M(Gly)_2$ complexes, where M = Zn, Ni, Cu or Co, with formaldehyde and acetamide results in the formation of the respective metal(II) complexes of (N,N-di-N′-methylacetamido)-glycine, which were characterised by various physical means, including X-ray structural analysis of the zinc(II) complex **(33)**.[42] In this complex the ligand is tridentate; each of the amino nitrogen atoms of the chelated glycinates is linked by methylene bridges to two N′-acetamido groups, resulting in two methylacetamido pendants, one of which is coordinated to the central zinc atom via its carbonyl oxygen, while the other remains free. The zinc atom exists in an octahedral geometry, with the ZnN_2O_2 equatorial plane being defined by two glycinate moieties such that the oxygen donor atoms, as well as the nitrogen atoms, are trans. The results of a crystal structure determination of the complex $ZnCl_2$(L-His)·HCl revealed tetrahedral $ZnOCl_3$ coordination.[43] The complex Zn(His-OMe)$(BPh_4)_2 \cdot H_2O$ has ZnN_4 coordination with chelating histidine units. Other complexes prepared in this study include Zn(His-NH_2)$(ClO_4)_2$, Zn(Ac-His)X·H_2O (X = ClO_4, BF_4) which is a coordination polymer in the solid state, [Zn(Bz-His-OMe)(2,9-Me_2phen)]$(ClO_4)_2$ and [Zn(Bz-His-OMe)$_4$]$(ClO_4)_2$. The complex ethyl-L-cysteinatetechnetium(V) **(34)** has been prepared.[44] The structure of this is a distorted square pyramid in which the Tc atom is displaced from the mean plane defined by Cl, P, S and N(2) toward the N(1) atom by 0.594(1) Å.

Bis(2-diphenylphosphinoethyl)phenylphosphineruthenium(II) complexes of amino acids and dipeptides have been prepared.[45] These complexes are monomeric and of the type [RuCl(AA)(triphos)] where AA = L-Ala **(35)** and L-Val **(36)**, in which the amino acids display a κ^2N,O coordination. An X-ray analysis of complex **(36)** showed that the triphosphine ligand occupies a facial position. This is also the case for the triphosphine ligand in the dipeptide complexes in [Ru(Gly-GlyH$_{-1}$)(triphos)] **(37)** and Ru(Met-GlyH$_{-1}$)(triphos) **(38)**. Facile dehydrogenation of α-amino acids chelated to a bis(bipyridine)ruthenium(II) complex



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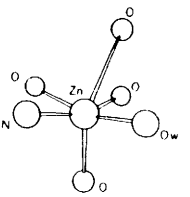

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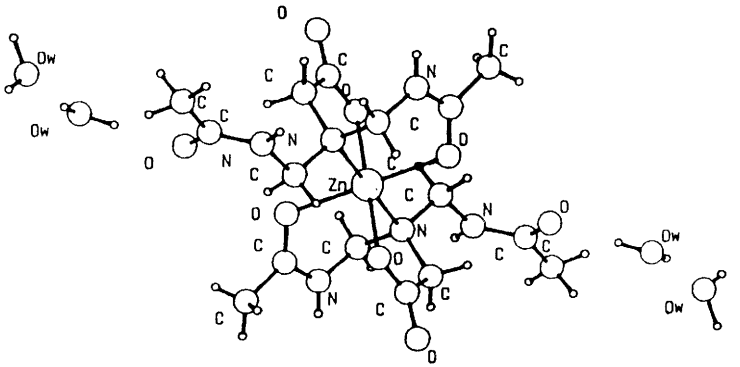

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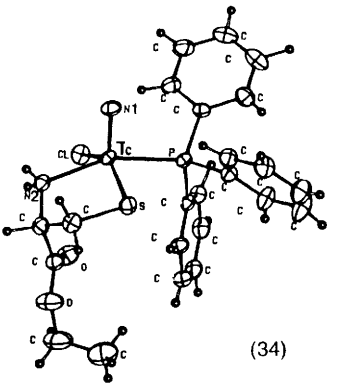

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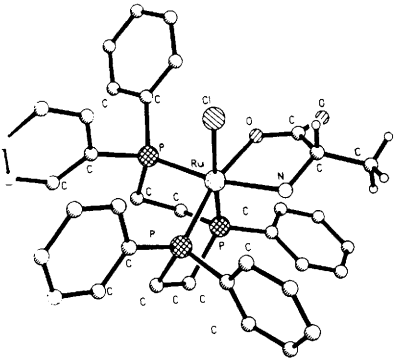

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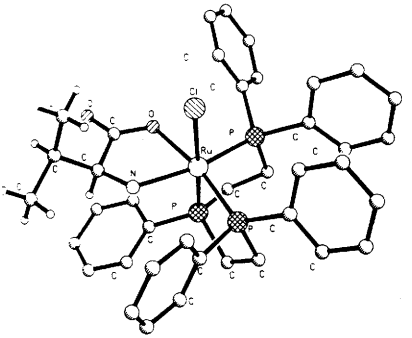

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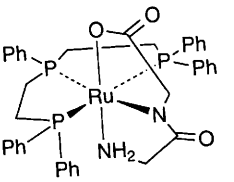

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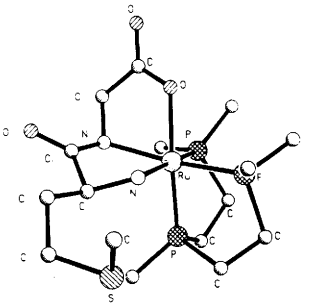

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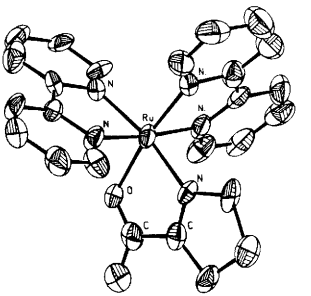


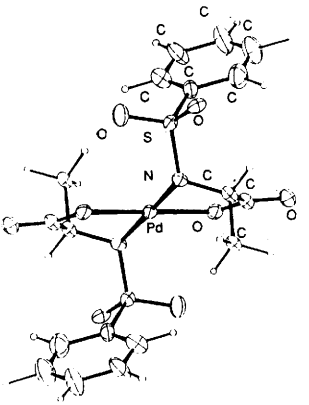
(39)

has been reported.[46] The dehydrogenation of $[Ru(Me\text{-}Ala)(bipy)_2]ClO_4$ (and the chloride salt) was achieved by anodic oxidation to give the α-iminoacidato complex $\{Ru^{II}[N(CH_3)=C(CH_3)COO](bipy)_2\}ClO_4 \cdot 2^1/_2H_2O$ in 78% yield, the first reported synthesis of an α-imino acidato ruthenium(II) complex. The complex $[Ru(Pro)(bipy)_2]ClO_4$ was oxidised similarly to give the α-imino acidato complex $[Ru(Pro\text{-}2H)(bipy)_2]ClO_4 \cdot 2H_2O$ **(39)** in 68% yield and the structure of which has been determined by X-ray diffraction. The α-imino acidato ring is almost planar and the Ru(II)-N(imino) bond distance, 2.04(1) Å, is shorter than Ru(II)-N(amino) bond distances, 2.07-2.14 Å, in amino acid complexes.

The crystal and molecular structures of the complexes $K_2[Pd(Bs\text{-}Ala)_2] \cdot 3^1/_2H_2O$ **(40)**, [Pd(Tos-Gly)(bipy)] **(41)** and $[Pd(Bs\text{-}\beta\text{-}Ala)(bipy)] \cdot H_2O$ **(42)** have been determined by X-ray diffraction.[47] In compound **(40)** the Pd atom is in a square planar environment, being trans coordinated by two centrosymmetrically related Tos-Ala dianions which act as bidentate ligands bonded to the metal through the carboxylate oxygen and the deprotonated sulfonamide nitrogen. Compounds **(41)** and **(42)** are also square planar and contain a Pd^{2+} ion coordinated to the N atom of the bipy ligand and to the deprotonated sulfonamide nitrogen and the carboxylate oxygen of the amino acid ligand. Several palladium(II), platinum(II), rhodium(III) and iridium(III) complexes with N-6-deoxygalactopyranosyl-α-amino acids have been prepared and characterised by spectroscopic methods and the structures of four of these have been determined by X-ray diffraction.[48]

A number of platinum complexes of amino acids and peptides have been prepared.[49] The structure of the complex $[Cl(Ph_3P)Pt(H\text{-}Aib)]$ **(43)**, where H-Aib is α-aminoiso-butyrate, has been determined. In this complex the Pt(II) ion displays square planar coordination with the amino acid acting as a bidentate ligand, the amino group being trans to the P atom of the phosphine ligand and the carboxylate being trans to the chloride. The reaction of cis-$[Pt(CH_3)_2(OD)_4]^{2-}$ with N-phosphonomethyl glycine, $NH_2(CH_2COOH)CH_2PO_3H$, H_3impa, in D_2O at pD 11 gave two isomers of $[Pt(CH_3)_2(OD)_2impa\text{-}N,O]^{3-}$ in which N and either carboxylate oxygen, O_c, or phosphonate oxygen O_p, are trans to CH_3.[50] The crystal structures of the complexes $[Pt(CH_3)_2(Himpa)H_2O] \cdot H_2O$ **(44)**, $Ag[PtBr(CH_3)_2Himpa]$ **(45)** and $Ag_3[PtBr(CH_3)_2Himpa]\text{-}[PtBr(CH_3)_2impa] \cdot 1^1/_2H_2O$ **(46)** have been determined. In complex **(44)** the nitrogen and phosphonate oxygen atoms are trans to the methyl groups. In complex **(45)** the silver ions are bonded tetrahedrally by bridging bromide and three oxygen atoms. In **(46)** the complex anions form an extended ribbon structure linked together through three independent silver ions by tripyl bridging bromide ions and oxygen atoms from both carboxylate and phosphonate groups. The crystal and molecular structure of a platinum(II) complex of the diacid diamide of EDTA, $[CH_2N(CH_2COOH)CH_2CONH_2]_2$, has been reported.[51]

The crystal structures of Gd(III) and Y(III) complexes of 10-(2-hydroxypropyl)-1,4,7,10-tetraazacylododecane-1,4,7-triacetic acid have been reported.[52] The 9-coordinate Gd(III) and Y(III) ions are 8-coordinated by the ligands, with the water molecule occupying the ninth (apical) positions. The structures of $[La_2(Aib)_4(H_2O)_8](ClO_4)_6$ **(47)** and $[Pr_2(Aib)_4(H_2O)_8]Cl_6 \cdot H_2O$ **(48)** (Aib = α-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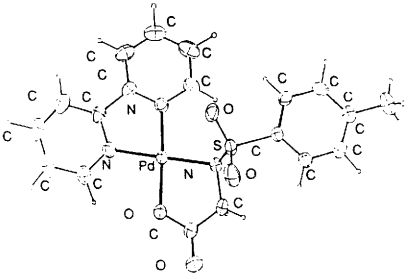
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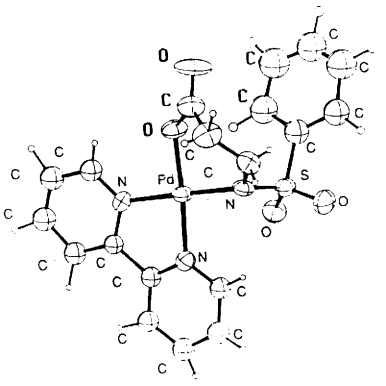
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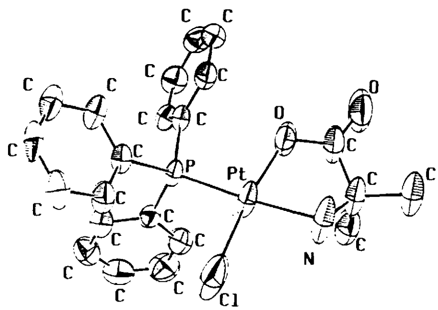
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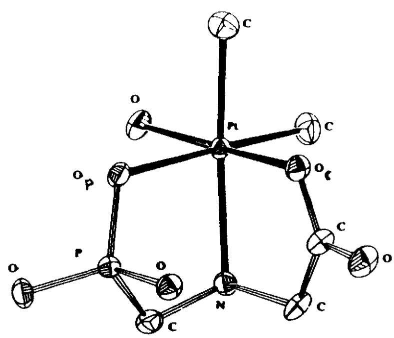
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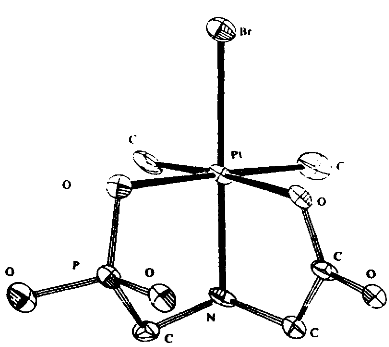
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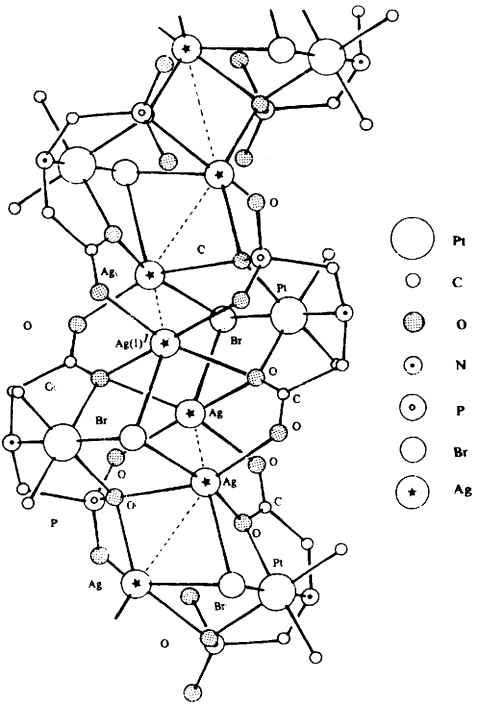

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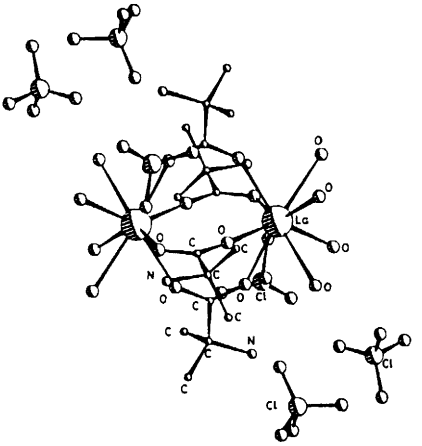

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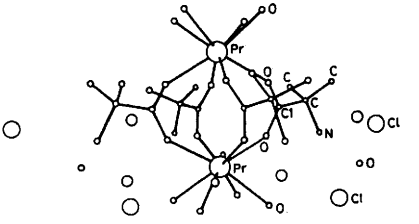

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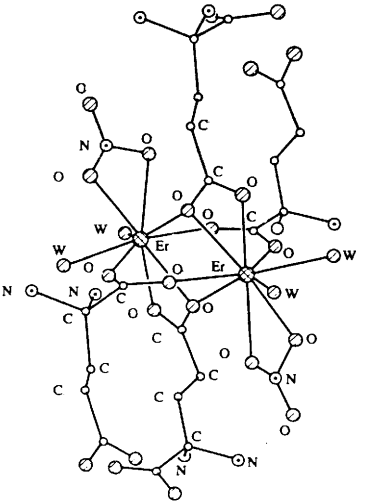
amino isobutyric acid) have been determined by X-ray crystallography which shows that the two lanthanide cations are bridged by carboxy groups of four Aib molecules and are surrounded by four oxygen atoms from water molecules thus making the coordination number 8.[53] The geometry around the lanthanum ion is a distorted bicapped trigonal prism whereas the praseodymium ion exhibits a square antiprism arrangement of ligands. The complex $\{[La(Gly)_3 \cdot 2H_2O] \cdot (ClO_4)_3\}_n$ was synthesised and structurally characterised.[54] In this complex the glycine ligands coordinate with lanthanum atoms through alternate 'two-four carboxy bridges' to form a one-dimentional chain compound of infinite length.

The crystal structure of the complex $[Er_2(Glu)_2(NO_3)_2(H_2O)_4](NO_3)_2 \cdot 5H_2O$ **(49)** has been determined.[55] The complex has an infinite chain structure built from dimeric units in which two erbium ions are connected by four bridging carboxylate groups from four glutamate ions. Each erbium is also coordinated by a bidentate nitrate anion and by two water molecules. The crystal structure of the complex $[UO_2(Van\text{-}L\text{-}His)bipy] \cdot MeOH \cdot H_2O$ containing N-o-vanillylidene-L-histidine has been reported.[56] Other similar complexes have also been synthesised and their chiroptical and electrochemical properties studied.

The complexes $Et_4N[W(CO)_4Gly]$ **(50)**, $Et_4N[W(CO)_4(Me\text{-}Gly)]$ **(51)** and $Et_4N[W(CO)_4(Me_2\text{-}Gly)]$ **(52)** have been synthesised from $W(CO)_5THF$ and the tetraethylammonium salts of the amino acids,[57] and their structures determined. The geometry of the anion in each case is that of a distorted octahedral arrangement of four CO groups and a puckered 5-membered glycinate ring. Despite the similarity in geometry the CO lability in complexes **(50)** and **(51)** is greater than in **(52)**. This is attributed to solvent assisted deprotonation of an amine ligand leading to a labile amide transient species.

The synthesis and structural characterisation of dimeric diorganotin(IV) complexes of formula $[R_2Sn(OC_{10}H_6CH{=}NCH_2CH_2COO)]_2$ have been carried out and the crystal structure of the dimethyltin complex, R = Me, **(53)** has been determined.[58] This centrosymmetric complex exhibits octahedral coordination for each tin atom. The tridentate ligand chelates meridionally via the phenolate oxygen, the imino nitrogen and one carboxylic oxygen atom. Two further trans positions in the tin coordination sphere are taken up by the two methyl groups while the apical (sixth) postion is filled by a shared carboxylate oxygen atom from the other organotin unit.

2.2 Synthesis – The penicillamine (H_2pen) complexes $K[Cr(D\text{-}pen)_2] \cdot 2H_2O$ and K[Cr(D-pen)L-pen] have been prepared and characterised by analysis of the splittings in the ligand field bands.[59] The D-pen complex has the trans-S, cis-N, cis-O configuration while the D-pen/L-pen complex is cis-S, cis-N, cis-O. Chromium(III) complexes of L-threonine, L-histidine, L-cysteine and D,L-alanine have been prepared by reacting $CrCl_3(pyridine)_3$ with the amino acid in 1:1 and 1:3 mole ratios.[60] Salts containing the octahedral ion $[Cr(dpt)L\text{-}Lys]^{2+}$, where dpt is bis(3-aminopropyl)amine, and $[Cr(Gly\text{-}Gly)L\text{-}Glu]^-$ in which the amino acid ligands are tridentate and occupy mer positions have been reported.[61] Mixed ligand complexes of chromium(III) and titanium(III) with succinimide



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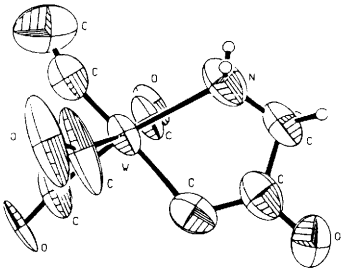

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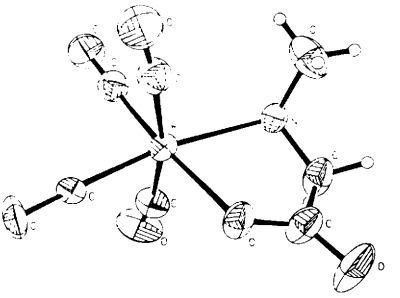

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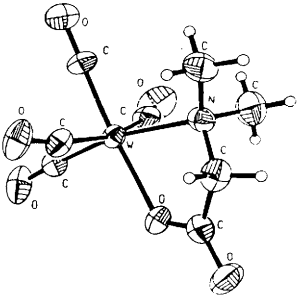

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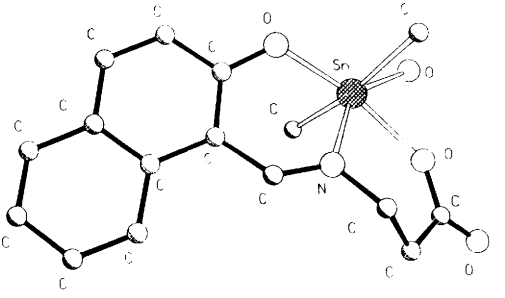


(53)

and amino acids (aminobenzoic acids, Gly, L-Pro, L-Leu, L-Cys, L-Tyr, N-Ac-D, L-Met) have been reported.[62]

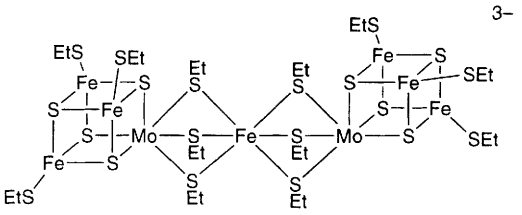
Tris(amino acid)cobalt(III) complexes containing L-alanine, L-2-aminobutanoic acid, L-valine and L-leucine, all having the fac configuration were synthesised and separated into their optical isomers.[63] The solubilities of the chelates were determined over the range 5-55°C and the thermodynamic parameters of solution analysed in terms of hydrobic effects. Several cobalt(III) complexes containing 1-amidino-O-alkylurea (AAUH) and the amino acids, AA, (Gly, Ala, Val and His) of formula $[Co(AA)(AAUH)_2]^{2+}$ have been prepared and characterised.[64] Octahedral cobalt(II) complexes with dithiocarbamates of α-amino acids (Gly, D,L-Ala, D,L-Val and L-Leu) have also been prepared.[65]

The reactions of fac-(S)-$Ir^{III}(aet)_3$ where aet = 2-aminoethanethiolate or Δ_{LLL}-fac-(S)-$H_3[Ir(L\text{-}Cys\text{-}N,S)_3]\cdot H_2O$ with Co^{2+} followed by air or H_2O_2 oxidation gave linear type S-bridged trinuclear complexes of formulae $[Co^{III}\{Ir^{III}(aet)_3\}_2]^{3+}$ and $\Delta_{LLL}\Delta_{LLL}$-$[Co^{III}\{Ir^{III}(L\text{-}Cys\text{-}N,S)_3\}_2]^{3-}$.[66] The aet trinuclear complex was optically resolved and the cysteinate complexes studied by absorption, CD and ^{13}C NMR spectroscopy.

Nickel(II) complexes of Schiff bases derived from 2-hydroxy-3-methoxybenzaldehyde or 2,4-dihydroxybenzaldehyde with Gly, D,L-Ala, D,L-Val, D,L-Met, L-Leu and D,L-Phe were synthesised and characterised by a range of techniques.[67] Copper(II) and zinc(II) complexes of Schiff bases derived from the same amino acids and o-vanillin have also been prepared and characterised.[68] Complexes of formula $CuL_2\cdot 2H_2O$, $ZnL_2\cdot H_2O$, $CoL_2\cdot 2H_2O$ and $NiL_2\cdot H_2O$ containing the Schiff base ligand L derived from 3-chlorobenzaldehyde and glycine have been prepared and characterised by microanalysis, conductivity measurements and spectroscopy (IR, ^1H NMR and electronic solution).[69] The antifungal properties of these complexes have also been investigated. Cobalt(II), nickel(II), copper(II) and zinc(II) complexes with Schiff bases derived from 2-hydroxy-1-naphthaldehyde and L-Ala or L-iso-Leu have been reported.[70] Cobalt(II), nickel(II) and copper(II) complexes with Schiff base ligands derived from 2-hydroxyacetophenone and various amino acids have also been synthesised and studied.[71]

The synthesis of copper(II)-amino (Gly, Ala, Leu) complexes by one step solid state reactions at room temperature is described.[72] The complex Cu(Val)Ser has been prepared and studied by electronic and ESR spectroscopy.[73] Mixed ligand complexes of general formula MLL′ where M = N(II) or Cu(II), L = Pro or Asp and L′ = imidazole or 2-methylimidazole were synthesised and characterised.[74] Complexes of zinc(II) and cadmium(II) with mannose α-amino acid derivatives have been prepared and characterised.[75]

The reactions of the double-cubane and monocubane clusters $(NEt_4)_3[Mo_2Fe_7S_8(\mu\text{-}SEt)_6(SEt)_6]$ **(54)** and $(NEt_4)_3[MoFe_3S_4(SEt)_3\{Fe(cat)_3\}]$ **(55)** where cat is catecholate, with ethyl L-cysteinate hydrochloride both gave the double cubane product $[Net_4]_4[Mo_2Fe_7S_8(\mu\text{-}SEt)_6(Cys\text{-}OEt\cdot HCl)_6]$ which was characterised by ^1H NMR, UV-vis, IR and Mossbauer spectroscopies.[76] The conversion of **(54)** into the product proceeds via the intermediate $[Mo_2Fe_9S_8(\mu\text{-}SEt)_6Cl_6]^{3-}$, **(56)**, Scheme 1. Reaction of **(54)** or **(55)** with methyl L-tyrosinate hydrochloride gave only the chloro containing cluster **(56)** or $(NEt_4)_3[MoFe_3S_4Cl_3\{Fe(cat)_3\}]$



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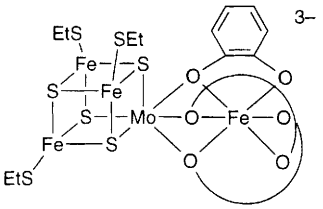

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$[Mo_2Fe_7S_8(\mu\text{-}SEt)_6(SEt)_6]^{3-}$

↓ 6 $HSCH_2CH(CO_2Et)NH_2 \cdot HCl$

$[Mo_2Fe_7S_8(\mu\text{-}SEt)_6Cl_6]^{3-}$ + 6 $HSCH_2CH(CO_2Et)NH_2$ + 6 HSEt

(56)

↓

$[Mo_2Fe_7S_8(\mu\text{-}SEt)_6\{SCH_2CH(CO_2Et)NH_2 \cdot HCl\}_6]^{4-}$

Scheme 1

respectively. Reaction of **(56)** with 6 equivalents of Et_4N (Tyr-OMe) gave $[Mo_2Fe_7S_8(\mu\text{-SEt})_6(\text{Tyr-OMe})_6]^{3-}$ for which spectroscopic parameters have also been reported.

The reaction of dithiocarbamate derivatives of several amino acids, dtcAA, (AA = Gly, Ala, 2-Abu, nor-Val, Val, nor-Leu, Leu and Ile) and sodium molybdate in acid medium resulted in the formation of the linear oxygen bridged dimolybdenum(V) complexes $Mo_2O_3(\text{dtcAA})_4{\cdot}2H_2O$, containing bidentate dtcAA ligands.[77] In organic solvents these are converted to molybdenum(IV) complexes $MoO(\text{dtcAA})_2$. When the reaction was carried out with molybdenum(V) chloride in an inert atmosphere molybdenum(V) complexes of formula $Mo_2O_4(\text{dtcAA})_2{\cdot}2H_2O$ were obtained.[78] Several new peroxo complexes of molybdenum(V), uranium(VI), zirconium(IV) and thorium(IV) containing bidentate amino acids have been synthesised.[79] These have formulae, $M(O)(O_2)(AA)_2$ where AA = Gly or Leu and M = Mo(VI) or U(VI), $Mo(O)(O_2)\text{Tyr}{\cdot}H_2O$, $M(O_2)\text{AA}{\cdot}H_2O$ where AA = Leu or Tyr and M = Zr(IV) or Th(IV) and $Th(O_2)AA$ where AA = Leu or Tyr. These complexes react with trans-stilbene, PPh_3 and $AsPh_3$ giving oxides. Molybdenum(VI) amino acid complexes of formula $[Mo_2O_5(OH)_2L]{\cdot}x\text{DMF}$ where L = His, x = 1 or L = Phe, Trp, Val, Ala, x = 0 have been synthesised and studied by IR and ^1H and ^{195}Mo NMR spectroscopy.[80]

Diamagnetic complexes of formula $[(Ru^{III}\text{edta})_2(\mu\text{-AA})_2(\mu\text{-O})]^{6-}$ **(57)** where AA = Phe, Val or Ala have been characterised in the solution and solid states.[81] These are stable in inert atmospheres, but in the presence of oxygen oxidative deamination of the bridging amino acid ligand occurs to give an α-keto acid. The reaction is catalytic.

A general synthetic procedure for the synthesis of palladium(II) and platinum(II) complexes of aminopolycarboxylate ligands is described.[82] Hence the complexes $M(H_4L)Cl_2$ and $H_6L[MCl_4]$ were obtained from acetic acid solutions of MCl_2 and H_4L (M = Pd or Pt, L = edta, pdta or cdta). Complexes $M(H_2L)$ have also been obtained from aqueous solutions of the chloro complexes. The $H_6L(MCl_4]$ salts in aqueous solution are slowly converted to complexes containing ligands L. The reaction of palladium halides with D,L-ethionine (EthH) in dichloromethane containing excess 2,6-dimethyl-4H-pyran-4-one (DMP) gave complexes $Pd(\text{EthH})X_2$, X = Cl, Br or I, which were isolated.[83] In benzene the adducts $Pd(\text{EthH})X_2{\cdot}\text{DMP}$ were obtained. Four palladium(II) and platinum(II) complexes of formula $[M(\text{dipy})AA]^+$ where dipy is 2,2′-dipyridylamine and AA is Gly or L-Ala have been prepared and their interactions with calf thymus DNA studied.[84]

There are several reports on synthesis of amino acids involving metal complexes as catalysts. These include the asymmetric synthesis of β-hydroxy-α-amino acids by condensation of aliphatic and aromatic aldehydes with zinc(II) and copper(II) complexes of (1R)-3-hydroxymethylene bornan-2-one glycine imines,[85] a study on the role of transition metal complexes in the asymmetric synthesis of β-hydroxy-α-amino acids,[86] the asymmetric synthesis of α-amino acids by copper catalysed conjugate addition of Grignard reagents to optically active carbamatoacrylates,[87] synthesis of β,γ-unsaturated amino acid derivatives by alkyne

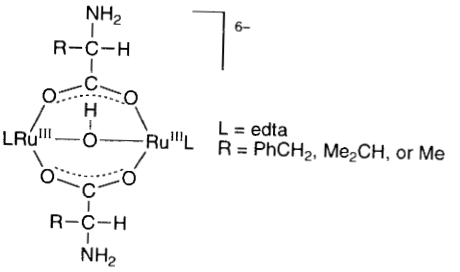
carbometallation,[88] elaboration of the side chain of α-amino acids containing a vinyl iodide by palladium catalysed coupling,[89] α-hydroxylation of pyridoxylidene-amino acids mediated on a cobalt(III) complex,[90] a novel synthetic route to cyclic α-amino acids using transition metal catalysed chlorine transfer cyclisations of carbon centred glycine radicals,[91] enantioselective formation of amino acids by isomerisation of mixed ligand copper(II)-Schiff base complexes,[92] a new route to 2,3-methano-amino acids by palladium(0)-catalysed azidation and amination of 1-alkenylcyclopropyl esters,[93] synthesis of α-amino acids from palladium(II) and rhodium(III) coordinated imines and oximes of α-oxycarboxylates,[94] asymmetric synthesis of α-alkyl-α-amino acids from chromium carbene complex derived β-lactams.[95] An attractive stereoselective 'one-pot' synthesis of β-lactams by the zinc-mediated condensation of an α-amino acid ester with an imine is described.[96] The copper(II) assisted synthesis of N^{α} protected N^{ω}-acridin-9-yl-α,ω-diamino acids has been described.[97]

2.3 Reactions/Structures in Solution – The reaction mechanism for the oxidation of L-methionine by chromium(VI) in $HClO_4$ solution has been reviewed.[98] The major oxidation product is methionine sulfoxide and this as well as 3-(methylthio)propionaldehyde are produced in parallel redox steps. NH_3 and CO_2 have also been identified. N-acetylation resulted in only one product, N-acetylmethionine sulfoxide. The kinetics and mechanism of the acid catalysed oxidation of the amino acids Ala, Val and Phe by chromium(VI) have also been reported.[99]

The reactions of mono- and di-peroxovanadate with several amino acids in aqueous solution as model systems for vanadium haloperoxidases have been studied by ^1H and ^{51}V NMR spectroscopy.[100] Monoperoxovanadate reacts with amino acids such as Gly or Pro giving two types of bis(amino acid) products, one having both amino acids bidentate, the other having one bidentate and the other monodentate, N-coordinated. No reaction with imidazole was observed. In contrast diperoxovanadates gave no products having bidentate coordination and complex strongly with imidazole, substituted imidazoles (e.g. His) and pyridine.

The reaction of the cluster $[FeS_4(SBu^t)_4]^{2-}$ with the α-benzylester of aspartic acid, Asp-OBzl, in DMSO resulted in the formation of $[Fe_4S_4(Asp\text{-}OBzl)_4]^{2-}$ which was characterised by ^1H NMR spectroscopy.[101] The mixed ligand clusters, $[Fe_4S_4(Cys\text{-}OEt)_x(Asp\text{-}OBzl)_{4-x}]^{2-}$, were similarly prepared and the temperature dependence of the isotropically shifted ^1H NMR signals of the α-CH and β-CH_2 protons was used to distinguish between the bound amino acids. The reaction of $[Fe_4S_4(SBu^t)_4]^{2-}$ with nonapeptide amides containing three Cys or Ser residues was also studied.

The formation kinetics and thermodynamic stability of iron(III) complexes of the new tetradentate ligand N^{α}-salicyl-L-alaninehydroxamic acid have been investigated by UV-vis. and stopped flow spectrophotometric methods.[102] The H^+-initiated dissociation of mono-, bis- and tris-complexes of iron(III) with N-methylacetohydroxamic acid as model systems for ferrioxamine were studied and rate constants and equilibrium constants have been reported.[103] An acid independent pathway was also observed for the dissociation of the mono complex. The



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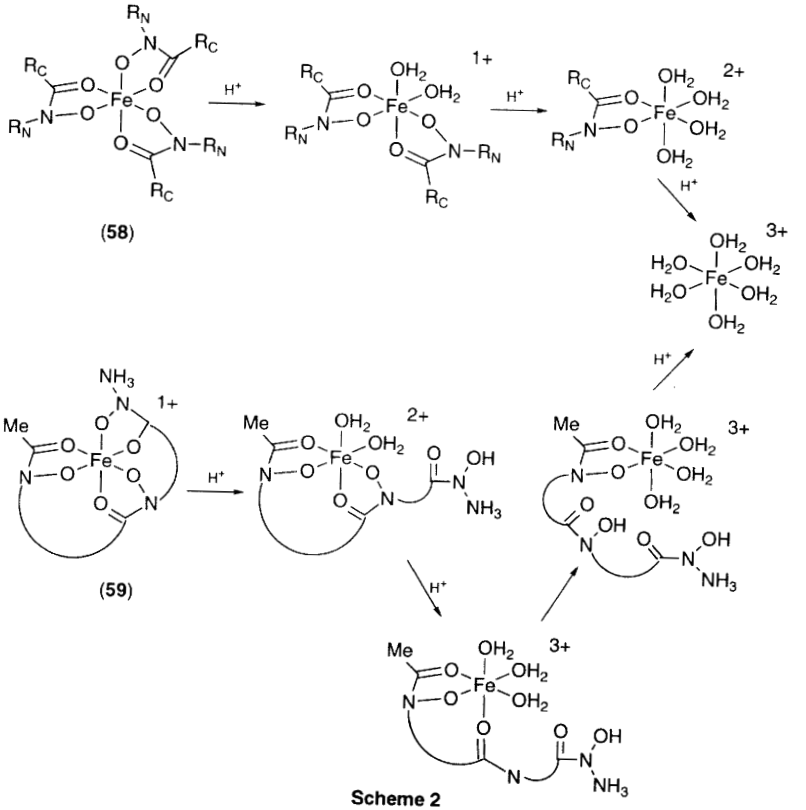

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(59)

Scheme 2

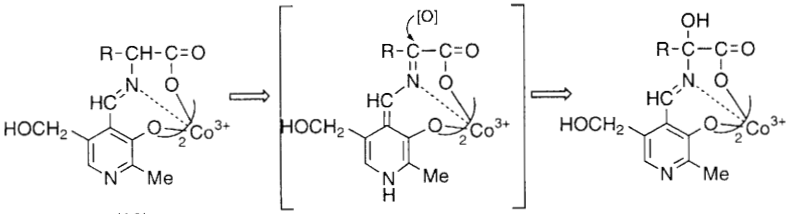
dissociation pathways for the tris complex **(58)** and the open chain trihydroxamate siderophore ferrioxamine B **(59)** are shown in Scheme 2. The kinetics of oxidation of cysteine and proline by $[Fe(CN)_6]^{3-}$ and $KMnO_4$ in basic solution,[104] and of L-methionine by polypyridyliron(III) complexes in aqueous solution,[105] have been studied.

The effect of methyl substitution in a tetraamine on the ligand field strength in $[Co(R\text{-}Ala)(tetramine)]^{2+}$ complexes has been examined by ^{59}Co NMR and electronic absorption spectroscopy.[106] It was established that methyl substitutions decrease the ligand field strength considerably and increase the lability of the normally inert Ala ligands. The oxidation of $[Co(phen)_3]^{2+}$ (phen = 1,10-phenanthroline) by $[Co(Gly)(ox)_2]^{2-}$ (ox = oxalate) has been investigated.[107] When Δ-$[Co(Gly)(ox)_2]^{2-}$ was used as oxidant chiral induction amounting to a 37% enantiomeric excess of the Δ isomer in the $[Co(phen)_3]^{3+}$ product was detected. Dioxygen complexes formed in basic solutions containing cobalt(II) and asparagine have been investigated by potentiometric, volumetric and spectroscopic methods.[108]

The reactions of the complexes [Co(en)Cl(His)]Cl and [Co(dien)His]Cl with diethylpyrocarbonate resulted in reversible carbethoxylation of histidine.[109] Conversion of an amino acid to an α-hydroxy amino acid has been observed in a cobalt(III) pyridoxylidene amino acid complex **(60)** and a mechanism for the hydroxylation is proposed, Scheme 3.[110] The ^{15}N chemical shifts in β-Ala and Gly ligands in the series of complexes $Co(Gly)_{3-n}(\beta\text{-}Ala)_n$ (n = 1-3) has been investigated.[111] It was found that for nitrogen trans to carboxylate, shifts are about 20 ppm greater than for nitrogen trans to amino groups. Shifts in β-Ala ligands are on average 10 ppm smaller than for those of Gly.

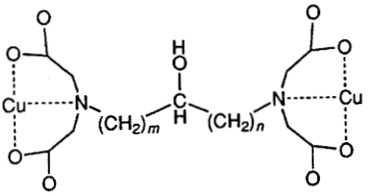
An extraordinarily high separation factor amounting to about 7 was obtained in the resolution of fac-$Co(\beta\text{-}Ala)_3$ with an optically active ob_3 isomer of $[Co(trans\text{-}dachxn)_3]^{3+}$, where dachxn is 1,2-diaminocyclohexane, adsorbed on a cation exchange resin.[112] The efficiency is due to the linearity of the three hydrogen bonds formed between the lone pairs of fac-$Co(\beta\text{-}Ala)_3$ and the three NH groups of Δ-ob_3-$[Co\{(+)\text{-}dachxn\}_3]^{3+}$. Optical resolutions of phenylalanine and mandelic acid were accomplished by complex formation with copper(II).[113] D- and L-mandelic acids were completely resolved by forming a ternary complex with L-phenylalanine while the maximum optical purity of D- and L-phenylalanine using D-mandelic acid was 65%. Accelerations in the rates of base hydrolysis of amino acid esters in the presence of cobalt(II), cobalt(III), platinum(II), palladium(II), copper(II) and nickel(II),[114] aquo-complexes containing ethylenediamine, diethylenetriamine and triethylenetetramine ligands, and in the presence of copper(II)-Schiff base complexes derived from amino acids,[115] have been reported. The kinetics of complexation of O-bonded glycinato cobalt(III) complexes, $[N_4Co(GlyH)Gly]^{2+}$, where N_4 represents two ethylenediamine or a triethylenetetramine ligand with $[Ni(H_2O)_6]^{2+}$ leading to the formation of $[N_4Co(GlyH)GlyNi]^{4+}$ have been investigated in the pH range 6.08-6.82 by stopped flow methods.[116]

The reduction steps of ternary complexes of copper(II) with Gly-Gly and the amino acids Ala and Tyr and with His and the amino acids Ala, Tyr, Phe and

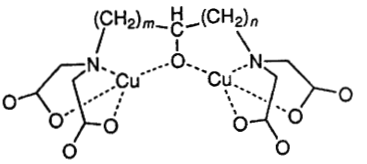


(60)

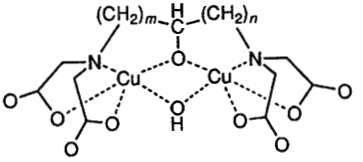
Scheme 3



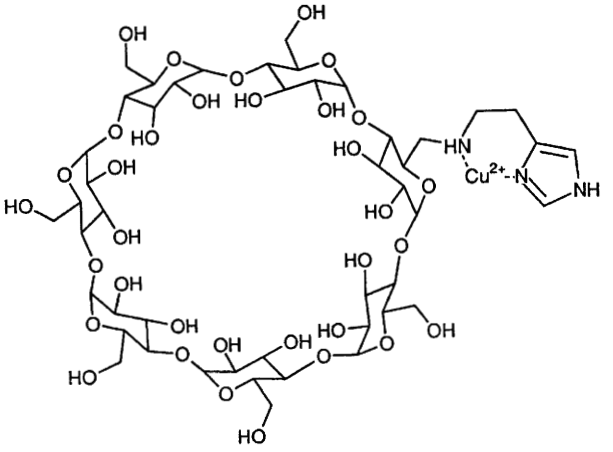
(61)



(62)



(63)



(64)

Trp have been studied by cyclic voltammetry in aqueous solution.[117] Reduction proceeds through a one electron process resulting in the formation of ternary copper(I) complexes. In the Gly-Gly ternary system these are unstable and disproportionate. In the histidine systems however they are stable due to imidazole group coordination to copper(I). The implications of these findings for the existence of copper(I) states in biological systems are described.

Binuclear copper(II) complexes of 2-hydroxy-1,3-diaminopropane-, 2-hydroxy-1,4-diaminobutane- and 3-hydroxy-1,5-diaminopentane-N,N,N′,N′-tetracetic acids have been investigated by potentiometric titration, ESR spectroscopy and magnetic susceptibility measurements.[118] At low pH the two copper ions are coordinated independently by the aminocarboxylate groups **(61)** and weak magnetic dipolar interactions exist. In all cases deprotonation of the alcohol OH groups occur at pH < 6 to give μ-alkoxo complexes **(62)**. For the propane and butane derivatives μ-alkoxo-μ-hydroxo double-bridged complexes **(63)** form as the pH is increased while for the pentane derivative the double-bridged complex is formed from the non-bridged complex at pH 5-8. The bridged complexes have strong spin exchange interactions between the paramagnetic centres through the bridges. The kinetics of the reaction of $[Cu(bigH)_2]^{2+}$ (bigH = biguanide) with Gly and Ala in the pH range 7.6-9.0 and temperature range 30-40 °C have been studied by stopped flow spectrophotometry.[119]

The hydrolysis of 4-nitrophenyl esters of picolinic acid and N-protected amino acids by metal (Cu^{2+}, Zn^{2+}) complexed vesicular assemblies is described.[120] The hydrolysis of 4-nitrophenylphosphate in the presence of amino acidato zinc(II) complexes has been studied at 40 °C over the pH range 5-10 and mechanisms involving the formation of ternary complexes are proposed.[121] Optical isomers of some α-hydroxy acids were separated by capillary zone electrophoresis using Cu(II)-amino acid complexes in the background electrolyte.[122] The kinetics and mechanism of the reaction of ninhydrin with $[Cu(Gly)]^+$ and $[Cu(Ala)]^+$ have been reported.[123]

A series of copper(II)-amino acid complexes (Gly, Ala, Val, iso-Leu, Ser) were shown to possess superoxide dismutase activity.[124] In a separate study it was shown that the high superoxide dismutase activity of some copper(II)-amino acid complexes does not correlate with their antitumour activity.[125]

The reactions of $[Pd(en)(H_2O)_2]^{2+}$ with betaine, $Me_3{}^+NCH_2COO^-$, Gly, β-Ala, and GABA have been studied by ^{15}N NMR spectroscopy using the ^{15}N-enriched complex $[Pd(en\text{-}{}^{15}N_2)(H_2O)_2]^{2+}$.[126] With betaine at 277 K the products are $[Pd(en)(bet\text{-}O)H_2O]^{2+}$ and $[Pd(en)(bet\text{-}O)_2]^{2+}$. Reaction of excess $[Pt(en)(H_2O)_2]^{2+}$ with Gly gave the complex $[Pd(en)(Gly\text{-}N,O)]^+$ as the dominant species in the pH range 4-10. At pH > 10, [Pd(en)(Gly-N)OH] formed. With Gly in excess at high pH, $Pd(en)(Gly\text{-}N)_2$ is the dominant species. Near pH 2, $[Pd(en)(HGly\text{-}O)H_2O]^{2+}$ is in equilibrium with $[Pd(en)Gly\text{-}N,O]^+$, free glycine and $[Pd(en)(H_2O)_2]^{2+}$. Reactions of $[Pd(en)(H_2O)_2]^{2+}$ with β-Ala were similar to those of Gly except that the N,O-chelate was less stable relative to $[Pd(en)(H\text{-}\beta\text{-}Ala\text{-}O)H_2O]^{2+}$ at low pH. Reactions with GABA over the pH range 4-8 gave a mixture of $[Pd(en)\gamma\text{-}Aba\text{-}N,O]^+$ and the isomers of $[\{Pd(en)(\mu\text{-}GABA)\}_2]^{2+}$. The reaction of cis-$[Pd(NH_3)_2(H_2O)_2]^{2+}$ with Gly gave initially $[Pd(NH_3)Gly\text{-}N,O]^+$

which on standing gave $[Pd(NH_3)(H_2O)(Gly\text{-}N,O)]^+$, in which NH_3 is trans to glycinate oxygen, as well as $[Pd(H_2O)_2Gly\text{-}N,O]^+$.

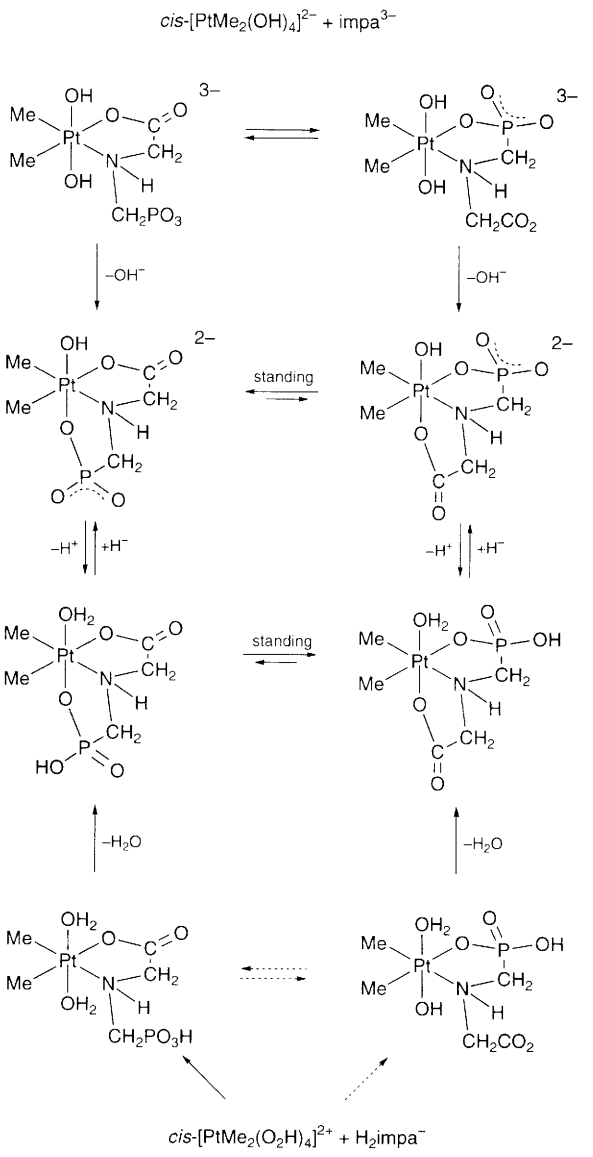
The reactions of N-(phenylsulfonyl)glycine, N-tosylglycine, N-(phenylsulfonyl)-D,L-alanine, N-tosyl-D,L-alanine, N-tosyl-β-alanine, N-benzoylglycine, N-acetylglycine and N-benzyloxcarbonylglycine with Pd^{2+} was investigated by d.c. polarography and by ^1H NMR and electronic spectroscopy.[127] Ternary complex between these amino acids, 2,2′-bipyridine and Pd^{2+} has also been investigated.[128] The formation of the ternary complexes involves initial binding of bipy to Pd^{2+} followed at higher pH by binding of an amino acid dianion. The reaction of cis-$[Pt(CH_3)_2(OH)_4]^{2-}$ with N-(phosphonomethyl)glycine, H_3 impa, in aqueous solution over a pH range has been investigated in detail.[50] These reactions are summarised in Scheme 4.

Ternary complexes of Pt^{2+} with the nucleotides 5′-GMP, 3′-GMP and 5′-dGMP where GMP is guanosinemonophosphate and with the amino acids N_α-Boc-2-His, N_α-Boc-Met and 1-methylimidazole have been studied as models for platinum mediated DNA-protein cross links.[129]

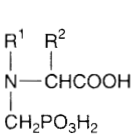
Positive and negative ion electrospray mass spectrometry was used to study solutions of amino acids (Gly, His, Met and Cys) and glutathione both in the absence of and in the presence of methylmercury(II) and alkali metal ions.[130] Interaction of alkali metal ions was weak but methylmercury interacted strongly. In addition to 1:1 complexes the species $[(MeHg)_2AA]^+$ was observed while glutathione formed a 3:1 species.

^{13}C and ^{119}Sn NMR spectra of thirteen tri-n-butyltin derivatives of amino acids have been reported.[131] The potential of lanthanide-amino carboxylic acid chelates as photoluminescent markers and cleaving agents of nucleic acids has been investigated.[132]

2.4 Formation Constants – Potentiometric studies of the ternary complexes formed by $[Cu(CDhm)]^{2+}$ **(64)** where CDhm is histamine modified cyclodextrin with aromatic amino acids (Trp, Phe, Tyr) show that stability constants of complexes containing the D-enantiomers are larger than those of the L.[133] In the case of aliphatic amino acids however there is no enantioselectivity. Calorimetric investigations confirmed that the enantioselectivity is due to preferential inclusion of the aromatic side chain of the D-amino acid in the cyclodextrin cavity.[134] The CD spectra of the complexes containing the aromatic D-amino acids are much more intense than those of the L, the difference increasing as the side chain is increased.[133] Enantioselectivity in fluorescence was also observed. These results were applied to the HPLC separation of the enantiomers of Phe, Trp and Tyr using the complex $[Cu(CDhm)]^{2+}$ added to the eluent and an achiral C_{18} column. Protonation and copper(II) complexation of 6-deoxy-6-[1-(2-amino)ethylamino]-β-cyclodextrin were also studied by NMR, ESR, pH-metric and calorimetric methods.[135] In ternary complexes with enantiomeric pairs of Ala, Phe and Trp no enantioselectivity was observed. Formation constants of ternary complexes of copper(II) containing the amides of S-Phe, S-Pro or S-Trp and R- and S-Val, Phe, Pro or Trp show that significant enantioselectivity exists for the (S,S)

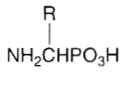

cis-[PtMe2(OH)4]2− + impa3−
3−
−OH−
standing
2−
−H+
+H−
−H2O
cis-[PtMe2(O2H)4]2+ + H2impa−

Scheme 4



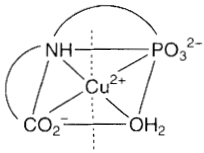
$R^1 = R^2 = H$
$R^1 = H, R^2 = CH_2CHMe_2$
$R^1 = H, R^2 = Ph$
$R^1 = H, R^2 = CH_2OH$
$R^1 = H, R^2 = CH(OH)Me$
$R^1R^2 = CH_2CH_2CH_2$
$R^1 = CH_2PO_3H_2, R^2 = H$

(**65**)

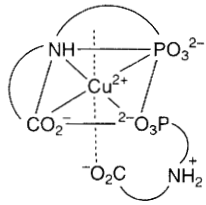


$R = H_2N(CH_2)_4$
$R = CH(OH)Me_3$

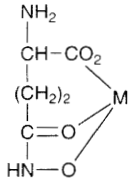
(**66**)



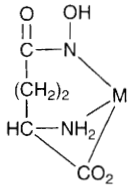
(**67**)



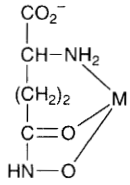
(**68**)



(**69**)



(**70**)



(**71**)

$TrpNH_2$/Pro, $ProNH_2$/Trp and $PheNH_2$/Pro pairs relative to the corresponding (S,R) pairs.[136]

Spectroscopic (vis., ESR) and pH-metric methods have been used to study complex formation between copper(II) and N-(phosphonomethyl)amino acids **(65)** or aminophosphonic acids **(66)** in aqueous solution.[137] Stability constants and spectroscopic data show that the first phosphonomethylamino acid ligand binds strongly in a tridentate manner **(67)** while the second binds less strongly to the remaining equatorial and to an axial position **(68)**. Equilibria in aqueous solutions containing nickel(II) and the plant growth regulators N-(phosphonomethyl)glycine, N,N'-[phosphinicobis(methylene)]bisglycine, N,N-bis(phosphonomethyl)glycine and N-(phosphonomethyl)iminodiacetic acid have been investigated using glass electrode potentiometry.[138] Complexes of the last of these ligands with manganese(II), iron(II), cobalt(II) and zinc(II) have also been investigated and with copper(II) in the presence of 4-nitrocatechol, a strong competing ligand.

The complexation of L-glutamic acid-γ-hydroxamic acid, H_2A, with Ni(II), Cu(II), Zn(II) and Fe(III) in aqueous solution has been studied by pH-metric, UV-vis., ESR, IR and ^1H NMR spectroscopies.[139] Mononuclear complexes containing bidentate HA^- and tridentate A^{2-} are formed. Coordination of the hydroxamate and carboxylate oxygens to iron(III) **(69)** is proposed while complexes with different bonding modes in equilibria exist in the case of nickel(II) and copper(II) **(70)**, **(71)**. Coordination via the hydroxamate oxygen atoms and the amino group **(71)** is preferred for zinc(II). Potentiometric and spectrophotometric studies have shown that the 2:1 complex of β-alaninehydroxamic acid wih nickel(II) has 4N, square planar coordination.[140] Formation constants of ternary complexes of nickel(II), zinc(II) and cadmium(II) with β-alanine hydroxamic acid and ethylenediamine have been reported.[141] Protonation and metal (Co^{2+}, Ni^{2+} and Cu^{2+}) complex formation constants are reported for L-lysinehydroxamic acid hydrochloride.[142] Formation constants are also reported for copper(II), nickel(II) and cobalt(II) complexes with L-isoleucinehydroxamic acid.[143]

Several studies refer to interligand interactions in ternary complexes and their influence on stability. Copper(II) complexes of formula Cu(A)B where A is ethylenediamine-N-monoacetic acid or D,L-2,3-diaminopropionic acid and B is L-Arg or L-Lys have been investigated by electronic absorption and CD spectra, equilibrium studies and X-ray diffraction.[38] Ligand-ligand hydrogen bonds or electrostatic interactions give rise to stability enhancement which has been quantified by comparing the stability constants with those of complexes in which the ligands have no interacting side chains. Formation constants are reported for ternary complexes of nickel(II) and copper(II) with 2,2',2''-terpyridine (terpy) and the amino acids, Ala, Phe, Tyr, Trp, Thr, Met and His.[144] The ternary complexes of copper(II) are less stable than those of nickel(II). In the latter the enhanced stability is stated to be due to Ni(II) → terpy π interactions in the non-aromatic amino acid complexes and due to these and metal-mediated stacking interactions in the aromatic amino acid complexes. Hydrophobic interactions between ligands in cobalt(II) and cadmium(II) complexes containing 2,2'-bipyridyl or 1,10-phenanthroline and Ala, Leu, Phe or Trp have been investigated.[145,146] Inter-

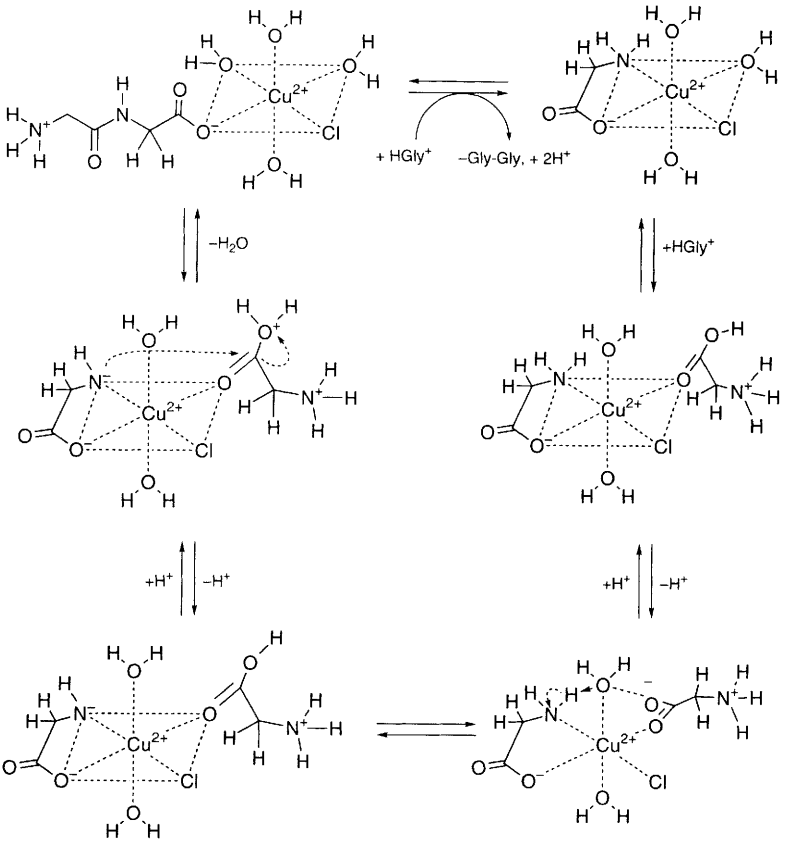
ligand interactions in zinc(II) and cadmium(II) complexes containing a phenolate ligand and amino acids or peptides,[147] and in zinc(II) and cadmium(II) complexes containing various amino acids ligands (Ala, Phe, Tyr, Trp, L-His) and peptides (Gly-Gly, Gly-L-Ala, Gly-L-Leu)[148] have been reported. Ternary complexes of cadmium with vitamin D_3 and Gly, Ala, His or Phe show interligand hydrophobic interactions.[149]

Stability constants are reported for binary 1:1 complexes between Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} or Cd^{2+} and imidazole and for ternary complexes containing in addition N,N-bis(2-hydroxyethyl)glycinate.[150] The stability of the ternary complex involving zinc(II) was found to be unexpectedly high and was explained in terms of reduction in coordination number upon binding imidazole, thus entropically favouring formation of the ternary complex. Association constants between a series of amino acid esters and trans-5-15-bis(2-hydroxy-1-naphthyl)-2,3,7,8,12,17,18-octaethylporphyrinatozinc(II) in chloroform follow the order Ala < Gly < Val < Leu.[151] The free energy charge for the binding of Leu-OMe was separated into two contributions i.e. metal coordination and hydrogen bonding. Equilibria between dimeric, trimeric and polymeric forms of molybdenum(VI) – amino acid complexes in solution have been investigated and stability constants reported.[80] Stability constants are also reported for cobalt(II), nickel(II), copper(II), zinc(II) and cadmium(II) complexes with a sugar-α-amino acid,[152] and for complexes of UO_2^{2+} and VO_2^{+} with glycine.[153]

The following ternary complexes have been investigated and formation constants have been reported; copper(II) – diamine – amino acid complexes containing 1,2- or 1,3-diaminopropane and 2-, 3- or 4-aminobutyric acid or 4-amino-3-hydroxybutyric acid;[154] copper(II) – dioxotetraamine macrocycles – amino acids;[155,156] cobalt(II), nickel(II), copper(II) and zinc(II) complexes with the tranquilizer promethazine and the amino acids Gly, His or Glu;[157] copper(II), nickel(II), zinc(II) and cadmium(II) complexes with 2,2'-bipyridylamine and a series of amino acids;[158] copper(II), nickel(II), cobalt(II), manganese(II) and zinc(II) complexes with phenylhydrazones and amino acids;[159] cobalt(II), nickel(II) and copper(II) complexes of amino acids and imidazoles;[160] iron(II), cobalt(II), nickel(II), copper(II), cerium(III), lanthanium(III) and UO_2^{2+} complexes of benzoic acids and amino acids;[161] complexes of copper(II) with some amino acids and tartrate;[162] complexes of palladium(II) with diethylenetriamine an amino acids.[163]

3 Peptide Complexes

3.1 Synthesis, Structure and Reactivity – The lanthanide ions are frequently used as isomorphic substitutes for the Ca(II) ion in biological systems. Lanthanide(III) complexes with the dipeptide glycylglycine, $Ln(Gly\text{-}Gly)_2(ClO_4)_3{\cdot}4H_2O$ (Ln = Nd, Eu), have recently been obtained as single crystals and their structures determined (the first for a lanthanide with a dipeptide).[164] Well resolved absorption and luminescence spectra at room and helium temperatures are reported and analysed. The bonding mode of the Ln^{3+} ions



Scheme 5

with the peptide are compared with the available X-ray data for the calcium complexes of diglycine and triglycine.

Metal salt-induced peptide bond formation has been studied by variation of the inorganic salt providing chloride ions which cause the dehydrating effect.[165] The dipeptide yields are mainly determined by two factors: the pH of the solution should be maintained below 3 to prevent metal catalysed peptide hydrolysis and to provide an optimum species distribution for peptide formation, and above 2 to prevent proton-catalysed peptide hydrolysis. The suggested mechanism for the reaction is outlined in Scheme 5.

Eighteen di- and tripeptides containing N-terminal cysteine residues have been prepared and reacted with basic zinc carbonate to give complexes ZnL_2 in which L is the peptide anion resulting from deprotonation of the SH group.[166] Tetrahedral ZnN_2S_2 coordination in these complexes is confirmed by a crystal structure determination of $Zn(Cys\text{-}GlyNH_2)_2$. The peptides Ac-X-Cys (X = Gly, Ala, Gly-Gly) react with basic zinc carbonate to give complexes $Zn(LH)_2$ which react with KOH to give complexes $K_2[ZnL_2]$.[167]

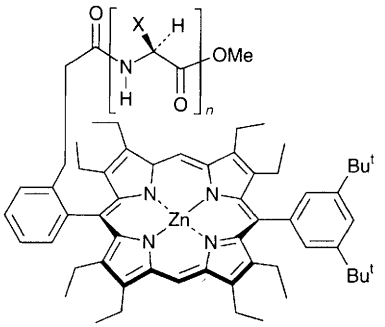
The structures of the complexes $Cu(Gly\text{-}L\text{-}Lys)\cdot H_2O$ and $Cu(Gly\text{-}L\text{-}Lys)\cdot 2H_2O$ in which the peptide is tridentate and coordinated through the NH_2, deprotonated amide and COO^- groups have been reported.[168] Copper(II) of formula $Cu(Gly\text{-}Gly)L\cdot nH_2O$ where L is imidazole, substituted imidazole, acetylhistamine or ammonia were prepared and characterised and the crystal structure of the acetylhistamine complex has been reported.[169]

Lithium complexes of neutral and anionic glycine (GlyH) and glycylglycine (Gly-GlyH) have been prepared.[18] The complexes $[Li(GlyH)(H_2O)]Cl$, LiGly, [Li(Gly-GlyH)]Cl and $LiGly\text{-}Gly\cdot H_2O$ have been characterised. The complexation of Li^+ by the amino acids and dipeptides is accompanied by characteristic low field shifts of their ^{13}C NMR resonances. The crystal structures of the complexes have been determined. In all four complexes the Li cation is tetrahedrally four coordinate. Three of the coordination sites are occupied by carboxylate oxygen donors from three different but crystallographically equivalent glycine or diglycine molecules. In the peptide complexes the fourth coordination site on lithium is occupied by a peptide oxygen atom.

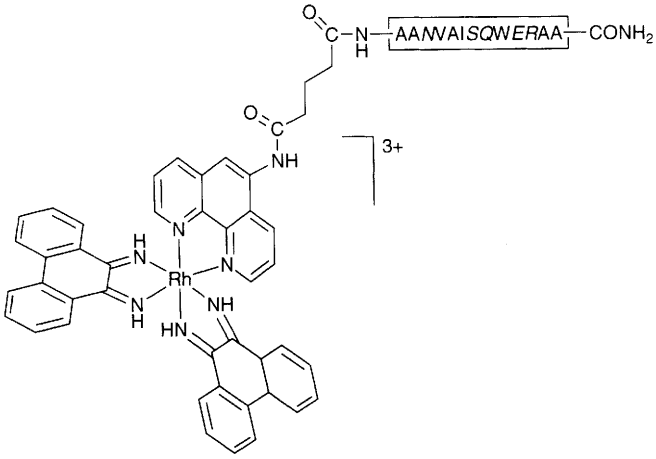
The introduction of allylic side chains into peptides by a Pd(0)- catalysed 'ester enolate Claisen Rearrangement' has been described.[170] Allylic esters of peptides undergo the rearrangement on treatment with LDA in the presence of various metal salts. The best results are obtained using zinc chloride. In the presence of a catalytic amount of palladium the rearrangement products are formed in high yield.

Oligopeptide-linked zinc porphyrins have been prepared (oligopeptide = $-Phe_m\text{-}Ala_n\text{-}OMe$ and porphyrin = 5,15-diaryl-2,3,7,9,12,13,17,18-octaethylporphyrin).[171] ^1H NMR, IR, visible and CD spectra obtained in $CDCl_3$ or CH_2Cl_2 established that the carbonyl oxygen binds to the zinc to form a pentacoordinated zinc porphyrin **(72)**.

The copper(III) complexes $Cu(Gly\text{-}Gly\text{-}His\text{-}H_{-2})$ and $Cu(Gly\text{-}Gly\text{-}His\text{-}H_{-3})$, prepared from the copper(II) complexes by azide oxidation, decompose rapidly


O
X
H
N
H
OMe
O
n
Zn
N
Bu^t
Bu^t

(72)


O
C
N
H
AANVAISQWERAA
CONH$_2$
O
C
NH
3+
N
H
N
Rh
N
N
H
NH
HN

(73)

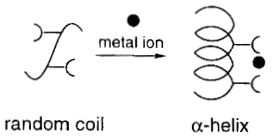
to give CO_2, Gly-Gly-NHCH(OH)CH_2 imidazole and Cu(I) in aqueous solution.[172] The mechanism of decomposition of the former complex is described.

The attachment of di-, tri- and tetrapeptide units to the ω-NH_2 end of each Lys in the complex $Cu(Lys)_2$ gave symmetric complexes with peptide units as recognition sites which bind to DNA in a sequence specific manner and effect scission at specific sites.[173] A family of metal-peptide complexes have been prepared by coupling short oligopeptides (13 residues) onto the metallointercalcating $[Rh(phi)_2(phen')]^{3+}$ (phi = 9,10-phenanthrenequinone diimine ; phen' = 5-(amidoglutaryl)-1,10-phenanthroline).[174] The complexes were prepared to see if side chain functionalities of small peptides may be used to augment metal complex recognition. The metal-peptide complexes bind with DNA and with photoactivation cleave it. The DNA site-specificity is found to depend on the peptide sidechain functional groups. A single glutamate at position 10 is found to be essential in directing DNA site-recognition to the sequence 5'-CCA-3'. Methylation of the glutamate side chain or direct substitution of glutamine for glutamate abolishes the 5'-CCA-3" selectivity. A representative example of one of the complexes used in the study is shown in **(73)**.

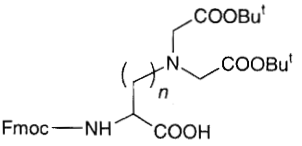
Non-eukaryotic amino acids which exert defined conformational restraints are important in the design of peptides and proteins. Chelating side arms such as 2,2'-bipyridyl and EDTA side chains bearing amino acids are capable of metal chelation, thus forcing the backbone of otherwise flexible peptides to attain an α-helical conformation **(74)**, Scheme 6. The synthesis of Fmoc/Bu^t protected amino acid chelators **(75)** have been described.[175] The peptide Ac-Ada(1)-Ala_3-Ada(1)-Ala_4-Glu-Lys-NH_2 was assembled by solid phase peptide synthesis in 75% yield.

The synthesis of the cyclic peptide **(76)** the first example of a new family of ion-binding peptides has been described.[176] The conformational and ion binding properties of **(76)** have been studied by NMR and CD techniques. The cyclic peptide displays solvent and temperature dependent conformations and forms complexes in acetonitrile with barium(II) (log K_{Ba} = 9.09). The formation constants with other alkaline earth cations are considerably lower (log K 3-5). The complexation properties of the tetrapeptide cyclo(L-His-Gly)$_4$ with Cu^{2+}, VO^{2+} and Mn^{2+} has been studied by EPR and NMR spectroscopy.[177]

The selective hydrolysis of peptides promoted by palladium aqua complexes has been studied.[178,179] Palladium(II) aqua complexes attached to the sulfur atom of methionine in peptides promote, under relatively mild conditions, regiospecific hydrolysis of the amide bond involving the carboxylic group of the methionine residue to which they are attached as shown in **(77)**, Scheme 7. The hydrolysis of the peptides Ac-Met-Gly, Ac-Met-Ala, Ac-Met-Ser, Ac-Met-Val, Ac-Met-Leu and Ac-Met-Ala-Ser in the presence of Pd(II) complexes containing aqua, hydroxo, ethylenediamine and 1,5-dithiacyclo-octane ligands has been studied. The half lives of the reactions are as short as 13 min at 40 °C and the rate constants depend on the steric bulk of the leaving fragment so that the reaction is somewhat sequence selective. Peptides devoid of side chains hydrolyse slowly in the presence of Pd(II)-aqua complexes. Di- and tripeptides were found to be

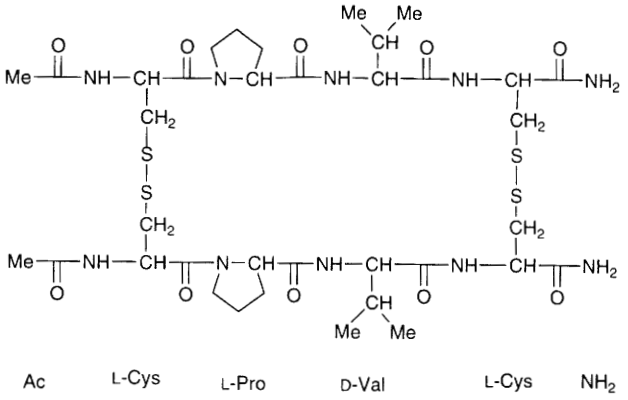


(74)

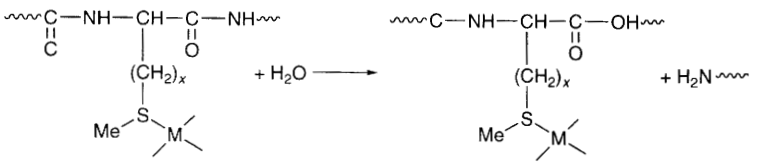


(75) $n = 1, 3$ or 4

Scheme 6



(76)



(77)

Scheme 7

efficiently hydrolysed by the cerium(IV)-γ-cyclodextrin complex in neutral and homogeneous solutions.[180]

The redesign of metal binding sites in peptides and proteins is attracting attention. The redesign of copper binding sites in copper-zinc superoxide dismutase and blue copper proteins such as azurin have given metal sites with altered spectroscopic and catalytic properties. The His_3-Zn site in carbonic anhydrase II has now been successfully engineered to the tetrahedral zinc polyhedron of composition His_2-Asp-Zn^{2+}.[181] The His_2-Asp-Zn^{2+} site resembles that found in alkaline phosphatase and is chemically comparable to the His_2-Glu-Zn^{2+} sites found in zinc proteases such as carboxypeptidase A and thermolysin. The interaction of some metal-ligated amino acids and dipeptides with chymotrypsin and trypsin has been studied.[182] Small structural analogues of substrates carrying the postively charged penta-amminecobalt(III) group at the carboxyl terminal [-$COOCo(NH_3)_5$] were prepared. These compounds do not undergo catalytic conversion and were found to inhibit their target enzyme reversibly. Nuclear magnetic resonance studies of the complex between consensus zinc finger peptide CP-1 and the paramagnetic cobalt(II) ion have allowed the orientation of the magnetic susceptibility tensor to be determined.[183] The knowledge of this tensor will allow refinement of the three-dimensional structure of the peptide and its complexes with DNA.

The structures of metal(II) complexes of tyrosine containing dipeptides L-Tyr-X (X = Gly, D/L-Ala, Tyr, Trp and Phe) and the diamines ethylenediamine (en), 2,2′-bipyridyl (bpy) and 1,10-phenanthroline (phen) have been studied by crystallographic, spectroscopic and potentiometric methods.[184] The complexes [Pd(bpy)(L-Tyr-Gly)]·$3H_2O$ and [Cu(phen)(L-Tyr-Gly)]·$3H_2O$ were crystallised and their X-ray structures determined. In the former complex the Pd(II) ion is in a square planar geometry with the two nitrogens of bipyridyl and two nitrogens of L-Tyr-Gly acting as donors. The phenol ring of L-Tyr-Gly is situated above the coordination plane and stacked with bipyridyl with an average spacing of 3.28 Å. In the copper complex the copper(II) ion has a five-coordinate square-pyramidal geometry; the two nitrogens and one oxygen of L-Tyr-Gly and one of the nitrogens of phen occupy the equatorial sites and the other nitrogen of phen is coordinated in the axial site. Intramolecular aromatic ring stacking occurs in the complex. The reactions of $[Pd(en)(H_2O)_2]^{2+}$ with N-acetylglycine, glycinamide and glycylglycine have been studied by multinuclear NMR (^{15}N, ^{13}C, ^{1}N) and detailed reaction schemes are presented.[185]

A range of bis(2-diphenylphosphinoethyl)phenylphosphineruthenium(II) complexes of amino acids and dipeptides have been prepared.[186] The dipeptides display N,N′,O coordination in these complexes. A variety of chiral half-sandwich complexes of cobalt(II), rhodium(III), iridium(III) and ruthenium(II) with amino acid amides and peptide ester ligands have been prepared and characterised, in some cases by X-ray crystallography.[187] The ^{19}F NMR signals of peptides such as Z-Cys-Pro-Leu-Cys-GlyX where X = m-NHC_6H_4F, p-NHC_6H_4F, p-$NHCH_2C_6H_4F$ or p-$NHCH_2CH_2C_6H_4F$ were found to be isotropically shifted both upfield and downfield by coordination to iron(II) in the complexes $(NEt_4)_2$[Fe(Z-Cys-Pro-Leu-Cys-GlyX)$_2$].[188] The results of this study

are consistent with formation of NH···S (Cys) hydrogen bonds and π-π interactions between the aromatic group and the S atom of Cys. The crystal and molecular structures of the complexes [Au(III)(Gly-Gly-L-His-H_{-2})]Cl·H_2O,[189] and Na_2[ReO(MA-Gly-Gly-AMS)]·$3H_2O$ where MA is mercaptoacetyl and AMS is aminomethanesulfonate,[190] have been reported. The binding of [Au(dien)Cl]Cl_2, where dien is diethylenetriamine with tripeptides over the pH range 2-10 has been investigated.[191] The Schiff bases 4-N-hydroxysalicylidene-glycylglycine and N-O-vanillal-glycylglycine and their complexes with manganese(II), cobalt(II), nickel(II) and copper(II) have been synthesised and characterised.[192]

3.2 Formation Constants, Species in Solution – α,β-Dehydropeptides (Δ peptides) have been receiving much attention because of their interesting biological and chemical properties. The nickel(II), zinc(II) and cobalt(II) complexes of α,β-dehydro-dipeptides containing Gly, Leu, Ala, Val or Phe residues have been studied by potentiometric and spectroscopic methods.[193] Deprotonation and coordination of amide nitrogens occurred in all cases in the physiological pH range. The dipeptides with the composition AA-Δ-Ala formed octahedral complexes while Gly-ΔAA (AA = Leu or Phe) formed square planar bis complexes with nickel(II).

The disulfide bridge is a common structural fragment in peptides and proteins e.g. oxytocin, vasopressin, insulin. There have been limited studies of the interaction of such compounds with metal ions. Coordination of copper(II) to cyclic peptides with a cysteinic disulfide bridge has recently been studied by potentiometric methods and by visible absorption spectra using the cyclic oligopeptide Cys-$(Gly)_n$-Gys ($0 \leqslant n \leqslant 4$).[194] The single peak in the visible spectrum shifts to lower wavelength (740 nm → 540 nm) with increasing deprotonation and hapticity, indicating changes in the coordination geometry of the copper(II) ion from tetragonal to distorted tetrahedral. Formation constants are also reported.

The equilibria in the systems oxovanadium(IV), VO^{2+}, with the peptides glycylglycine and glycylglycylglycine have been studied by a combination of potentiometry and spectroscopy (EPR and visible spectra).[195] If HL denotes the peptide, the equilibrium model includes the species MLH, ML_2H_2 and MLH_{-1} and several hydrolysis products. Possible structures for each stoichiometry are discussed.

Reaction of copper(II) with glycylglycine (H_2G) and zinc(II) in the ratios 1:2 and 1:1:2 respectively in the presence of excess H_2O_2 results in the formation of the novel peroxy complex [$Cu(O_2^{2-})(H_2G)_2$]·$2H_2O$ and a mixed peroxo carbonate complex [Cu, $Zn(O_2^{2-})(CO_3)(H_2O)_4$] respectively.[196] A notable feature of the reaction is the facile decomposition of the peptide bond at room temperature on addition of zinc to the system. Thermodynamic and spectroscopic (ESR, electronic and CD) investigations have been carried out on the copper(II) – Gly-L-His-Gly-L-His system in aqueous solution.[197] The species CuL^+, $CuLH_{-1}$ and $CuLH_{-2}$ have been identified, the first in the pH range 3-5.5 and the others between pH 5.5 and 7.8. The most active O_2^- scavenging species is [$CuLH_{-2}$]$^-$. The protonation constants of the tripeptide glyclyglycylhistidine (L^-) have been determined at 25 °C and I = 0.1 mol dm^{-3} as log K = 8.06, 6.82 and 2.80.[198]

Complexation with copper(II) gives the species $[CuLH]^{2+}$, $[CuL]^{+}$, $[CuLH_{-1}]$ and $[CuLH_{-2}]^{-}$, while in the case of nickel(II) only $[NiLH]^{2+}$, $[NiL]^{+}$ and $[NiLH_{-2}]$ are of importance. The displacement of the tripeptide from the nickel(II) complex by L-histidine has been studied kinetically over the range 7 – 8. There is a small solvolytic reaction and a reaction which is first-order in the hydrogen ion concentration. Under the experimental conditions employed, the reaction is independent of the L-His concentration and displacement occurs by a proton-assisted nucleophilic pathway with rate-determining cleavage of the first nickel(II)-N(peptide) bond.

Copper(II) complexes containing carboxylate and imidazole-N3 donors (α- and γ-Glu-Val, α and β-Asp-Gly, β-Asp-His, γ-Glu-His and homocarnosine) have been studied by potentiometric and spectroscopic methods in solution.[199] The presence of an α-carboxylate group in the N-terminal portion of the peptide molecule significantly enhances the metal-binding ability of the ligands due to bis complex formation involving amino acid like coordination. The interaction of copper(II) with β-Asp-His was characterised by the formation of an imidazole-bridged dimeric species, while formation of bis complexes was detected in the case of γ-Glu-His. Binding of the imidazole N3 and deprotonated amide nitrogen was presumed in the copper(II)-homocarnosine system.

Lead toxicity is generally considered to be initiated by coordination of lead(II) to appropriate functional groups on amino acids and proteins. However, there is currently little detailed information as to the nature of these interactions. Only one X-ray crystal structure has been reported for a lead amino acid complex, namely that of D-penicillamatolead(II). In this complex D-penicillamine acts as a terdentate ligand forming strong bonds to lead via amine, carboxyl and sulfide groups. High field proton and carbon-13 NMR spectroscopy has recently been used to study the interaction of lead(II) with both cysteine and the tripeptide glutathione in D_2O over a wide pD range.[200] No binding of lead(II) to either ligand was observed in acid solution. In basic solution PbL and/or PbL_2 complexes were formed with cysteine depending on the lead:cysteine ratio. Chemical shift measurements indicate a mixture of terdentate (NH_2, COO^-, S^-) and bidentate coordination with binding through the sulfur and carboxylate groups favoured in the bidentate case. With glutathione, both PbL and PbL_2 complexes are formed in basic solution. Proton chemical shift data are inconsistent with a previously proposed tetradentate binding mode for glutathione. Binding appears to occur only via the S^- group in both the mono and bis complexes.

The formation constants of complexes of the cyclic octapseudopeptide cyclo-(Gly-eLL-Gly)$_2$ **(78)** where eLL is N,N′-ethylene-bridged (S)-leucyl-(S)-leucine with eleven transition metal ions have been determined by CD spectra in acetonitrile at 25 °C.[201] For divalent metal ions the stability order is $Mn^{2+} > Fe^{2+} > Co^{2+} > Ni^{2+} < Cu^{2+} < Zn^{2+}$ while for the zinc group cations it is $Zn^{2+} < Cd^{2+} > Hg^{2+}$. The trends may be rationalised in terms of the cavity size of the ligand. ^{13}C NMR measurements establish that six amide oxygens in the ligand coordinate to Cu(II) and Fe(III) in CD_3CN.

Weak interactions between Ser, Phe and Met residues in peptides with metal

ions in aqueous solution are difficult to observe. Recent measurements on copper(II) complexes of oligopeptides containing these amino acid residues indicate that the Met side-chain (the sulfide sulfur) and the aromatic ring in the Phe residue stabilise CuL species by additonal interactions with copper(II).[202]

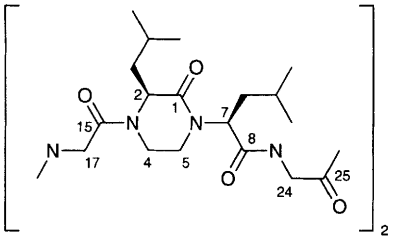
The Atrial Natiuretic Factor (ANF) is a 28-peptide released by mammalian atria and is a key regulator of blood pressure. Its structure is determined by a disulfide bridge with a C-terminal tail of 5 amino acid residues (-Asn-Ser-Phe-Arg-Tyr). This pentapeptide tail is necessary for the full natriuretic activity of the hormone. The pentapeptide fragment of ANF, Asn-Ser-Phe-Arg-Tyr-NH_2 has been shown to coordinate to copper(II) with the same four nitrogen donors as simple pentapeptides such as penta-alanine.[203] However, the complexes have a much higher stability due to the highly organised side-chain structure which is present in the complex but absent in the free ligand.

Potentiometric and spectroscopic studies on copper(II) and zinc(II) complexes of peptides containing bis(imidazolyl) ligands have been reported.[204] Stable mono- and bis(ligand) complexes were formed with all the ligands and the imidazole nitrogens were the main binding sites. The involvement of the side-chain imidazole residues of the peptides Bu^tOCO-Pro-His-Gly-$NHCHR_2$ and R_2CHCH_2CO-Ile-His-Gly-OEt (R = imidazol-2-yl) was shown to occur.

Patellamide D ($patH_4$) is a cyclic octapeptide from the ascidian Lissoclinium Patella. The peptide has the 24-azacrown-8 macrocyclic structure **(79)** with two oxazoline and two thiazole rings. The octapeptide forms mononuclear copper(II) complexes in which there are three nitrogen donors from a deprotonated amide and the oxazoline and thiazole rings.[205] A variety of mononuclear complexes are formed due to the presence of the four binding sites in the molecule. In addition the binding of different ligands such as Cl^- or water in the fourth equatorial site can occur. Patellamide D also forms three binuclear copper(II) complexes, $[Cu_2(patH_2)]^{+2}$, $[Cu_2(patH_2)(OH)]^+$ and $[Cu(patH_2)(CO_3)]$ in which each copper is coordinated by three nitrogen donors from a deprotonated amide and oxazoline and thiazole rings. The remaining coordination sites may be occupied by either Cl^- or H_2O in the first two complexes, while carbonate is assumed to bridge the two copper(II) ions in the third complex as shown in **(80)**.

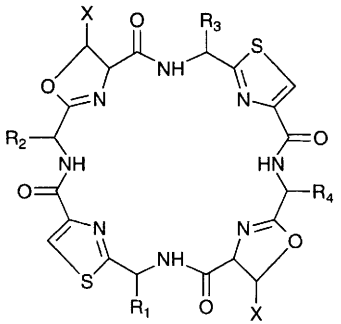
The extent of complex formation between copper(II) and many biologically active oligopeptides has been shown to change significantly in the presence of SDS micelles, a recognised model for cell lipid membranes.[206] Protonation constants of peptides can be increased by up to 2 log units, especially when they contain hydrophobic side chains. Metal complex formation is generally less extensive and the conformations of peptides can be dramatically altered when compared with those observed in simple aqueous solution.

Thymopoietin is a thymic hormone consisting of 49 amino acids. The synthetic pentapeptide fragment (TP5 = Arg-Lys-Asp-Val-Tyr) and other C-terminally shortened tri- or tetrapeptide derivatives are reported to correspond to the biologically active site of the hormone. Copper(II) complexes of tri- and tetrapeptides containing either carboxylate or amide groups in the side chain have been studied by potentiometric and spectroscopic techniques.[207] The ligands are tri- and tetrapeptide segments of the hormones thymopoietin and splenin. It was

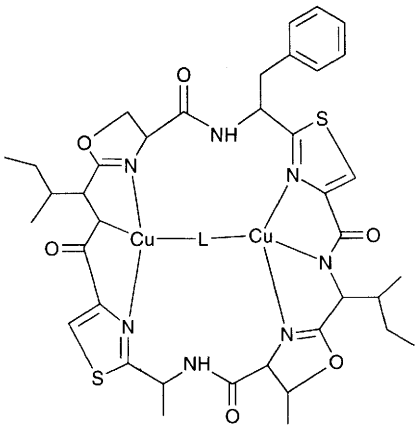


cyclo(Gly-eLL-Gly)$_2$

(**78**)



(**79**)



(**80**)

found that the presence of internal aspartyl residues significantly enhances the metal binding ability of the oligopeptides resulting in the cooperative deprotonation of the amide nitrogen atoms preceding the aspartyl residue. The subsequent amide groups do not take part in metal ion coordination. It was found that glutamyl residues have no significant effect on the complex formation processes of oligopeptides.

The acid-base properties and zinc(II) complexes of glycylhistamine, sarcosylhistamine, carcine and carnosine have been investigated by potentiometric, ^{13}C and ^{15}N NMR methods.[208] Macroscopic constants for the three states of protonation (LH_2^{2+}, LH^+, L) and the corresponding microscopic constants for the three protonation sites (terminal amino, N-1 and -3 imidazole nitrogens) have been quantitatively estimated for the ligands. Zinc(II) complexation is shown to reverse the tautomeric preference between 1- and 3-H tautomeric forms of the imidazole ring (in LH^+ and L), as compared to the free ligands where the 1-H tautomer is predominant. The acid-base properties and copper(II) complexes of glycyl- and sarcosyl-histamine (histamine = imidazole-4-ethanamine) have also been studied by potentiometric, spectrophotometric and EPR methods and compared with those of analogous histidine-containing dipeptides and carcinine (β-alanyl-histamine).[209] At pH 4-9 the predominant species is the 3N-coordinated complex $CuLH_{-1}$ together with minor amounts of CuLH and CuL. The pK values for the metal ion promoted deprotonation of the peptide nitrogen are exceptionally low (pK = 3.20 and 3.66 respectively). The bis complexes CuL_2 and CuL_2H_{-1} also form in the presence of excess ligand. In the pH range 9 – 11 the monomeric 3N-coordinated hydroxo-complex $CuLH_{-1}$ (OH) and a polymeric 4N-coordinated species are in equilibrium. The latter complex is assumed to be tetrameric $Cu_4L_4H_{-8}$, with the imidazole rings as bridging bidentate units coordinating through both N^3 and N^1-pyrrolic nitrogens. At high pH (ca. 11) a further deprotonation results in the formation of the monomeric 3-N coordinated hydroxo-complex $CuLH_{-2}(OH)$ with a pendant deprotonated N^1-pyrrolic nitrogen.

The dipeptide complex $[Pd(Gly\text{-}L\text{-}HisH_{-1})Cl]\cdot 1\cdot 5H_2O$ (Gly-L-$HisH_{-1}$ = the monoanion of Gly-His deprotonated at the amide nitrogen) has been prepared and structurally characterised.[210] Coordination of palladium occurs via the terminal amino group of glycine, N(π) of the imidazole ring of histidine and the deprotonated amide nitrogen. Reactions of the complex with the model nucleobases 1-methyluracil, 1-methylcytosine, 9-methyladenine, 9-ethyladenine and 6-methoxy-9-methylguanine have been studied in solution by ^1H NMR. The 1-methylcytosine complex $[Pd(Gly\text{-}L\text{-}His\text{-}N, O)(myct)]\cdot 3\cdot 5H_2O$ has been structurally characterised and nucleobase coordination shown to occur through N^3.

The interaction of zinc(II) with cyclo(L-histidyl-L-histidyl) has been studied by potentiometric and calorimetric methods in aqueous solution.[211] A comparison between the thermodynamic parameters of the complexes formed with those of the analogous species formed with cyclo(glycyl-L-histidyl) provide evidence for the formation of chelate rings of unusual size. The role of the different stereochemical requirements of copper(II) and zinc(II) in the formation of large chelate rings is discussed.

An X-band EPR study of the interaction of the paramagnetic metal ions Cu^{2+}, VO^{2+} and Mn^{2+} with the tetrapeptide H-(L-His-Gly)$_2$-OMe has been published.[212] The gas phase binding chemistry between Ca^{2+}, Co^{2+} and Ni^{2+} and 33 peptides (tri- to deca-) has been studied with respect to aqueous phase and theoretical aspects.[213] It is shown that metastable ion decomposition mass spectrometry has a future in the direct elucidation of important metal binding sites in peptides and proteins.

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6
Current Trends in Protein Research

By JENNIFER A. LITTLECHILD

1 Introduction

This chapter seeks to report some of the advances in protein research that were carried out in 1994. This area has been covered in volumes 25 and 26 of this series of Specialist Reports in Amino Acids, Peptides and Proteins. This field is very large and it would be impossible to cover all of the reported research in this area. Some highlights of this subject which include protein folding, motifs and some important new structures are included in this review.

2 Protein Folding

2.1 Theoretical Approaches – The field of protein folding has continued to receive much attention. It is clear that a number of distinct conformations exist for the polypeptide chain of a protein. The protein spends most of its time in the native conformation. The thermodynamic requirement is that the sequence must have a unique folded conformation under physiological conditions and the kinetic requirements that the denatured polypeptide chain can fold into this conformation with reasonable speed. Many theoretical studies have been carried out on protein folding and unfolding. A review by the group of Dobson published in 1994 considers how proteins fold in regard to a study with lysozyme.[1] The group of Levitt also published a review[2] considering protein folding/unfolding dynamics. A review of the molecular dynamics of protein unfolding with emphasis on barnase simulations was presented by the group of Karplus.[3] A diffusion-collision model is described by the same group and its qualitative and quantitative predictions compared with experimental data and alternative models.[4] Two recent papers have described methods for simulating the dynamics of proteins represented by secondary structure segments connected by loops.[5,6] A solvent induced organisation of proteins using a physical model of myoglobin has been described by Calloway.[7] A folding mechanism for a random 27-mer heteropolymer sequence on a cubic lattice has been described by Sali *et al.*, 1994.[8] Another similar study described the folding of a 36-mer sequence designed to have a pronounced global energy minimum, simulated by Monte Carlo dynamics on a cubic lattice. It was found that the formation of a specific nucleus leads to subsequent rapid folding to the native state.[9] Monte Carlo simulations of protein folding is also described with regard to lattice models and

an interaction scheme[10]. A new computational protocol has been developed for calculating globular and compact protein structures consistent with hydrophobic constraints.[11] Another paper by the group of Levitt has described exploring conformational space with a simple lattice model for protein structure.[12] Using simple structural and energetic criteria, based on a database of known protein structures, lattice structures can be selected that have significant simularities to the known native structures.

Sun has published a paper concerned with the reduced representation model of protein structure prediction-statistical potential and genetic algorithms.[13] In this work a reduced model in continuous space is described and applied to the binding of melittin and avian pancreatic polypeptide. Wallqvist and Ullner have described a simplified amino acid potential for use in structure predictions of proteins.[14] An article in a new journal, *Nature, Structural Biology* describes the barriers to protein folding.[15] A high-temperature all atom simulation of unfolding of chymotrypsin inhibitor 2 in low density solvent has been described by Li and Daggett.[16] A review article exploring the relationship of the results of the lattice simulations to thermodynamics and kinetics of the folding of proteins has been published called 'Matching speed and stability'.[17] A further paper by Karplus[18] addresses the question of the differences between the folding and non-folding sequences using a 27-mer random heteropolymer model on a cubic lattice and Monte Carlo dynamics. A pronounced global energy minimum is found to be a sufficient and necessary condition for a sequence to fold in this model. Vieth *et al.*,[19] have used a hierarchical approach consisting of Monte-Carlo and molecular dynamics simulations to predict folding pathways and the structure of the GCN4 leucine zipper. This is an example of a folding simulation that successfully combines coarse-grained lattice simulations and all-atom molecular dynamics. The cooperative creation of a hydrophobic core is observed that leads to side-chain fixation. A transition matrix method developed for heteropolymers has been used to look at transition states and folding dynamics of proteins.[20] A detailed *ab initio* prediction of lysozyme-antibody complex with 1.6 Å accuracy using Monte Carlo optimisation of a detailed energy function in dihedral space has been carried out.[21] Two other papers have considered folding kinetics of proteins. The first of these describes the free energy landscape for protein folding kinetics: intermediates, traps and multiple pathways in theory and lattice model simulations.[22] The second examines the folding kinetics of protein-like heteropolymers.[23] Covell has published a paper describing lattice model simulations of polypeptide chain folding.[24] Another paper by Shakhnovich shows that proteins with selected sequences fold into a unique conformation.[25] A phase diagram of a model protein derived by exhaustive enumeration of the conformations has also been described.[26] A comprehensive treatment of the statistical mechanics of designed protein sequences has shown that 'over-design' leads to slower folding.[27] Compact denatured states of proteins have also been modelled.[28] It is proposed that denatured states consist of broad ensembles of chain backbone conformations that involve common localised hydrophobic clustering and helical contacts, which depend on the amino acid sequence. The side chain entropy and packing in proteins has been examined by Bromberg and Dill.[29] They show that side chain

degrees of freedom oppose folding and it is proposed that packing in proteins is more like the packing of nuts and bolts in a jar than like pairwise matching of jigsaw puzzle pieces.

The properties and origins of protein secondary structure has been studied in several recent reports.[30,31] The latter paper asks whether compactness induces secondary structure in proteins. This uses poly-alanine chains computed by distance geometry. Monte Carlo simulations of protein folding have been applied to the specific examples of protein A, ROP and crambin.[32] The folding pathways of these three enzymes involve a collection of early intermediates that are followed by the rate-determining transition from compact intermediates resembling the molten globule state to the native-like state. The best predictions have an α carbon root mean square error of 2.25 Å. Monte Carlo simulation of first order transition for protein folding has been reported by Hao and Scheraga.[33] The method has been used to simulate folding of a 38-residue polypeptide with a hydrophobic potential that included both local and global interactions. It is determined that the first-order transition arises because the entropy increases more slowly than the energy when the protein initially unfolds from the native state.

2.2 Practical approaches – During folding or unfolding of proteins it has been suggested that the protein exists as a molten globule. There is a question whether this state has a native like tertiary fold or whether it is compact but structureless. Several papers have appeared in 1994 to address this problem. Peng and Kim have reported a protein dissection study of the molten globule.[34] The helical subdomain of α-lactalbumin, in solution from the β-subdomain, adopts a molten globular state with a native-like tertiary fold. A paper by Ptitsyn and Uversky has examined an important question as to whether the molten globule represents a specific thermodynamic state or whether it is a squeezed random coil. They have established that the molten globule is a third thermodynamic state of the protein.[35] They have found that the slopes of the native ↔ molten globule and molten globule ↔ unfolded equilibrium transition induced by urea and guanidinium chloride in one-domain proteins are proportional to their molecular weights. This is what would be expected for a phase transition of the first order in small systems. This is supported by previous studies and a study appearing in 1994 by Barrick *et al.*,[36] where they examined the molecular mechanism of acid denaturation and the role of histidine residues in the partial unfolding of apomyoglobin. Based on the substantially irreversible temperature-induced partial unfolding, a second order molten globule ↔ unfolded phase transition has been suggested for apomyoglobin.[37] It has been suggested by Ptitsyn[38] that the two equilibrium phase transitions accompanying the folding (or unfolding) of protein molecules represent the formation (or destruction) of the tertiary fold and the tertiary structure. Computer simulations of the folding of a model lattice protein suggests a natural protein can easily and rapidly fold into its unique native structure by a first order transition.[25]

The group of Ptitsyn[39] has described a new equilibrium state of protein molecules with properties intermediate between the molten globule and the

unfolded state. This 'partly folded' state was found by guanidinium chloride induced unfolding of β-lactamase at low temperature. Fink *et al.* have carried out a acid denaturation study of 20 proteins.[40] Some proteins unfold at intermediate pH and they fold to the molten globule or a more expanded state at low pH, while the others directly transform to the molten globule state. The acid denatured form of interleukin-4 is found to preserve the highly ordered non-polar core and all four α helices of the native state, but also to have some characteristics of the molten globule state.[41] A related study on the thermodynamics of staphylococcal nuclease denaturation by the group of Privalov[42] has found a tertiary structure substantially more pronounced than the classical molten globule. A highly ordered molten globule state has been observed for apocytochrome.[43] Apocytochrome b_{562} differs from the holoprotein by a shortening of one of the α helices. This leads to a loosening of the packing of the α helices which in turn leads to distortions of the molecule that result in the production of a large cavern which is capable of accommodating about 50 water molecules. A study by Lin *et al.*, on apomyoglobin suggests that this protein has a fluctuating structure. The addition of heme is thought of as funneling a diverse fluctuating population of substrates into the native state.[44] A trifluoroethanol-induced partially folded state of α-lactalburin has been studied.[45] Dobson has summarised the evidence for the existence of highly ordered molten globules.[46] The barriers involved in protein folding have been addressed in a recent study on cytochrome c. This demonstrated that the transformation of the molten globule to the native state is not necessarily the rate limiting step in protein folding. The energetic ups and downs of protein folding are discussed by Creighton.[47] *In vivo* protein folding is thought to be controlled by other proteins called chaperonins. The role of these very often large protein complexes in the folding of α lactalbumin has been studied by Okazaki, *et al.*[48] α-Lactalbumin in its molten globule state does not bind to GroEL chaperonin but the expanded state with fully reduced disulfide bonds does. Similar results are obtained by a different study using hydrogen exchange protection experiments.[49]

The group of Fersht have continued in their experimental approach to the folding problem and have published some important papers in 1994. The structure of the transition state for the folding/unfolding of the borley chymotrypsin inhibitor 2 and its implications for mechanisms of protein folding have been studied.[50] They have shown that by making a large number of mutations in the same elements of structure that the fractional values do not result from parallel pathways with a mixture of fully formed and fully denatured states but must represent genuinely weakened interactions.[51] One approach to simplify the analysis of unfolded states of proteins is to look at isolated fragments. The isolated fragments can bind to each other and reconstitute native-like structures. Two fragments of the barley C1-2 inhibitor associate to form a native-like structure.[52,53] The transition state for association has a structure that is remarkably like that for the refolding of intact protein.[54] NMR studies have continued to be an important way to look at the structure of unfolded states of proteins. The group of Fersht has reported the complete backbone ^{13}C, ^{15}N and ^{1}H NMR assignments of the pH denatured state of barnase.[55]

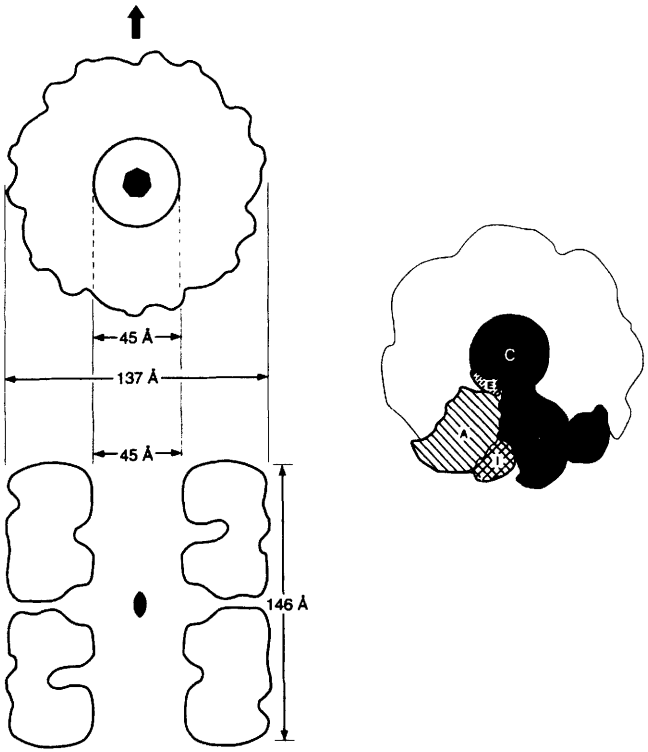


Figure 1 The structure of the large protein chaperonin, GroEL. The figure shows principal dimensions on a schematic diagram of the double ring cylinder, showing a view into the central channel (top) and a vertical cut through the cylinder made by a plane defined by a diameter and the cylindrical axis (bottom). The figure on the right shows the location of the E, I and A, equatorial intermediate and apical domains of the individual monomers. C is the central channel. Reproduced with permission from reference 57.

The role of molecular chaperones in protein folding was reviewed in 1994.[56] This year has also seen the X-ray structure determination of the large chaperonin GroEL.[57] This is the structure of the multisubunit protein at 2.8 Å resolution. The complex is a porous cylinder containing a large central channel. There are two rings of seven large subunits arranged back to back. Each subunit comprises three domains. The large channel through the structure of 47 Å provides enough space for a possible accommodation of a 'compressed' unfolded protein of about 50-60 kDa. Alternative access for other solution components to the central channel are provided by seven elliptical windows penetrating each ring and connecting it to the outside. The structure combined with mutational studies[58] indicates that a large portion of the functional surfaces are provided by the surface of the inner channel. The structure of this large protein complex is shown in Figure 1.

3 Protein Motifs

3.1 PH Domain – Proteins can fold in characteristic domains many of which have functional significance. One of the first to be recognised was the so called 'Rossmann fold' which is an αβαβ motif that is the binding site for mononucleotides.[59] Since then many other motifs have been described. One of these first described in 1993 was called a PH domain named after pleckstrin homology.[60,61] This domain has received considerable interest in 1994. It is approximately 100 amino acids long and was originally found in a number of signalling proteins and in the cytoskeletal protein spectrin. Since then the domain has been found in many other proteins including protein kinases, phospholipases, activators or inhibitors of small G proteins, including several oncogene products, cytoskeletal proteins and in proteins involved in budding of yeast cells.

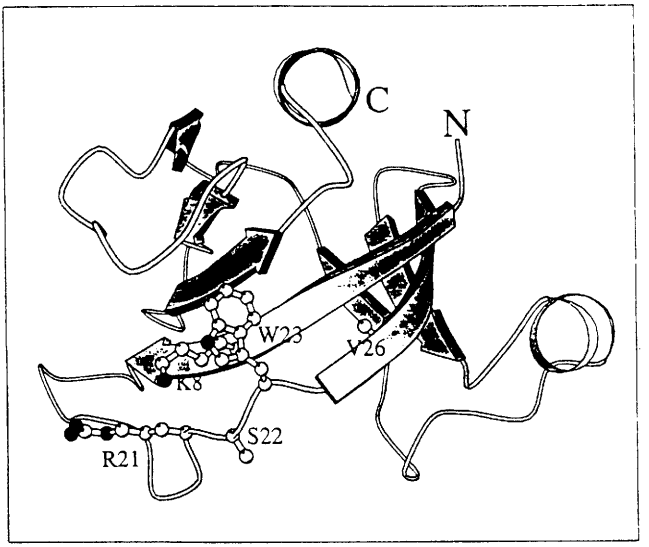


Figure 2 The PH domain of β-spectrin. Important amino acids involved in PIP_2 and IP_3 binding are indicated. Reproduced with permission from reference 68.

There have recently been five papers all published in 1994 on the three dimensional structure of this domain. All of these structures except two are obtained from NMR methods. The first was the β-spectrin domain.[62] Other NMR structures were the N-terminal domain of pleckstrin[63] and the PH domain in dynamin.[64] In addition two structures have been solved by X-ray crystallographic methods: these are the pleckstrin homology domain from human dynamin.[65,66] The basic structure of this PH domain is a seven stranded antiparallel β sheet that has a strong bend leading to a configuration called an orthogonal sandwich or an up-and-down β barrel. The domain has a characteristic C-terminal α helix that blocks one end of the bent sheet. There appears to be

a conserved sequence motif along the buried helical edge that contains a conserved tryptophan residue. The structure of the PH domain is shown in Figure 2. A general article discussing PH domains was published by Gibson *et al.*, in 1994.[67] The PH domains have a positively charged end that has been proposed to anchor this domain to membrane lipids[65,68] reported evidence to show that pleckstrin homology domains bind to phosphatidylinositol-4,5-bis-phosphate. NMR studies of the complex were able to locate the lipid binding site to the lip of the β-barrel.[69] Davies and Bennett have identified two regions of b_G spectrin that bind to distinct sites in brain membranes. PH domains are involved in signal transduction and cytoskeletal dynamics. They bind inositol phosphates which are key compounds of lipid metabolism involved with cellular signalling and are thought to interact with the $G_{\beta\gamma}$ complex which is part of the signalling complex associated with seven transmembrane helix receptors.[70] Clearly we will hear more about the role of the PH domain in the future.

3.2 Cysteine Knots – Another unusual feature which has been found in several proteins is involved with intramolecular disulfide bridges. This was first seen in 1992 in the protein transforming growth factor – β2 (TGF-β2)[71-74] and platlet derived growth factor.[75] Nerve growth factor has also been shown to contain this fold.[76] In these structures, six cysteines form three disulfide bridges that are arranged in a knot-like topology.

A similar 'knot' arrangement has been found in several enzyme inhibitors.[77] The inhibitor structure has been compared from ω-conotoxin GV1A a neurotoxin from the venom of *Conus geographus*, Kalata B1 a cyclic peptide from the tropical plant *Oldenlandia affinis* DC and *Curcurbita maxima* trypsin inhibitor-1 (CMTI-1). In addition Narasimhan *et al.*[78] have compared the structures of two polypeptides, ω-conotoxin CV1A and ω-agotoxin 1VA from the venom of the funnel web spider. All of these small polypeptides whose structures have been determined by NMR methods have a cystine-knot structure. The global fold is similar, starting with a peripheral β-strand followed by a connecting region containing turns or 3_{10} helix, then the other peripheral β strand which is connected by a turn to a central strand. The last two strands form a β hairpin structure. Another paper appearing in 1994 by Chang *et al.*[79] examines the disulfide folding pathway of the 'cystine knot' structure found in potato carboxypeptidase inhibitor. No simple sequence motif is associated with this knot.

The growth factor 'cystine knots' vary in that they have structures where cystine I-IV, II-V, III-VI disulfide linkages with Cys (I-IV) passing through the ring. All have two distorted β-hairpin loops on one side of the knot and a single loop on the other. There does seem to be a definite amino and sequence motif observed with growth factors. The sequence Cys-X-Gly-X-Cys is formed between cysteines III and IV and Cys-X-Cys between cysteines V and VI. An important publication in 1994 by the group of Issacs[80] shows that the crystal structure of human chorionic gonadotropin contains cystine-knot motifs in each subunit of the protein; see Figure 3. This structure was also reported by the group of Henrickson[81] at 2.6 Å resolution.

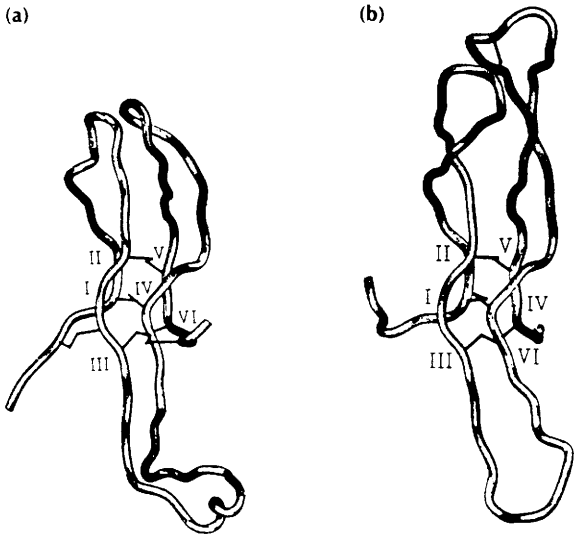


Figure 3 The crystal structure of human chorionic gonadotrophin. The structures of the α (a) and β (b) subunits illustrate the typical disulfide connections in the cysteine-knot growth factors. Reproduced with permission from reference 80.

Two groups have started to modify the cysteines involved in the 'knot' formation by site directed mutagenesis. This is with the protein activin A, β subunit[82] and with human gonadotropin α subunit.[83] Change of the cysteines altered the biological activity of the proteins.

The 'cystine knot' proteins can be divided into the two groups discussed above and in both cases the arrangement of the three disulfide links into a 'knot' like structure provides a peptide fold that provides a specific function. Clearly many other proteins will be found that have this structural motif.

3.3 Leucine-rich Sequences – Another motif that has received increasing interest in 1994 is the leucine-rich repeat (LRR). This motif has now been found in over sixty proteins. The LRR is defined by the consensus sequence XLXXLXLXLXXN±XaXX±a±±±±a±±X±±, where X is any amino acid, a is an aliphatic amino acid and ± denotes possible deletions.[84] The consensus leucines can often be replaced by other aliphatic amino acids and the consensus asparagine can sometimes be replaced by a cysteine or a threonine. LRR's can differ in length but many have 20-29 amino acid residues. Several LRR's are normally found joined to each other in one protein. The crystal structure of the ribonuclease inhibitor protein[85] contains 15 LRR's and its structure was discussed in RSC Specialist Reports, vol. 26. LRR proteins can have many functions for example in signal transduction, cell adhesion, development, DNA repair, recombination, transcription and RNA processing. This is a motif that is used throughout nature. A single leucine rich motif probably does not fold into a defined structure. Several LRRs appear to jointly

form a module. In contrast to most other modules the function of the LRR can be modulated by changing the number of repeats within a single domain. In 1994 many proteins have been identified that contain LRR's. These include the G proton coupled receptor GRL 101 from *Lymnaea stagnalis* ,[86] the Drosphilia 18 wheeler protein which functions in morphogenesis,[87] the Drosphilia Toll-like receptor (Tlr) protein which functions in development,[88] Ln 47 protein which is a peripheral membrane protein in Drosphilia,[89] a plant defense protein from *Arabidoppsis thaliana*, RPS2,[90,91] a tobacco N defense protein,[92] FIL2 signal transduction protein from the flower *Antirrhinum* ,[93] the wheat meiosis protein pAWJL3,[94] the mouse development protein Fig 1,[95] leucine-rich acidic nuclear protein (LANP) involved with the differentiation of cerebral neurons,[96] the human ST4 oncofetal trophoblast glucoprotein involved in cell adhesion,[97] and human Garp protein which is amplified in carcinomas.[98] There is still only one three dimensional structure of this group of proteins:- the ribonuclease inhibitor. With peptides of 20-29 residues containing the LRR consensus sequence the structure should be similar although short repeats of 20 or fewer amino acids could be related to other leucine rich proteins[84] such as pectate lyase.[99,100]

4 Metal Containing Proteins

4.1 Zinc Containing Proteins – Zinc has now been found to play important roles in enzyme active sites and as a structural role to hold the polypeptide chain into the correct conformation for interaction with other molecules:- for example in the case of the zinc finger. During 1994 a greater understanding of the role of zinc in proteins has been achieved.

4.1.1 Zinc Metalloproteinases – Zinc has been known for sometime to be important for the catalytic activity of a group of proteases that have gained increased importance. These enzymes can be grouped into four distinct families, the astacins, the adamalysins the serralysins and the matrix metalloproteinases (matrixins). Hooper has discussed these families in a recent article.[101] They all share the elongated zinc-binding motif HEXXHXXGXXH. The three dimensional structures of some of these enzymes have already been determined and have been reported in volumes 25 and 26 of this series. In 1994 the group of Bode[102] have described the refined 2.0 Å X-ray crystal structure of the snake venom zinc endopeptidase adamalysin II and have compared the structure with other members of the adamalysin/reprolysin family. Zhang *et al.*[103] have looked at the structural interaction of natural and synthetic inhibitors with the venom metalloproteinase, atrolysin C. The crystal structure of the 50kDa metalloprotease from *Serratia marcescens* has been reported.[104] Perhaps the most medically interesting papers appearing recently are those describing the structure of matrixins which are the collagenase from human neutrophils and the collagenase from human fibroblasts. Knowledge of the structure of these enzymes is important for the design of inhibitor molecules to be used in cancer therapy. An important paper by the group of Bode[105] describes the crystal structure of

human neutrophil collagenase inhibited by a substrate analog which reveals the essentials for catalysis and specificity of this enzyme. A hydroxamate inhibitor was bound to the enzyme which revealed that substrates bind along a cleft in an extended conformation which is different from the situation previously seen for the enzyme thermolysin. A further paper by the group of Bode[106] describes the structural implications for the role of the N-terminus in the 'superactivation' of collagenases. This is the first structure of a collagenase with a correctly 'native like' N-terminus. It is thought that this processing step might be involved in the 'superactivation' of this enzyme. The structure of the catalytic module of human neutrophil collagenase has also been studied by Stams and colleagues[107], complexed with a hydroxamate inhibitor. Several papers in 1994 have reported the structure of the human fibroblast collagenase. The structure of this collagenase at 1.56 Å resolution was reported[108] the protein complexed to itself[109] and in complex with inhibitors.[110,111] An NMR structure has been reported for the inhibited catalytic domain of human stromelysin 1.[112]

All of the protein families have enzymes that are divided into two domains by the substrate binding cleft, with the zinc at its bottom. The N-terminal domains of the catalytic modules are made up from a twisted β-sheet covering two long α helices. A difference between the enzymes is seen which is vital in substrate binding and determining the specificity of the proteinase. In collagenase a long S-shaped loop, encloses both a structural zinc ion and a calcium ion.[105] The central α helices in the active site of the enzymes are very similar. One α helix supplies two of the histidine residues which are required to coordinate the active site zinc. The third histidine zinc ligand of the metzincins is part of the C-terminal domain. An invariant methionine is important for a unique tight turn. The ε-methyl group of the methionine residue is directed towards the planar face of the imidazole of the first and second histidines. The structure of human neutrophil collagenase is shown in Figure 4. The catalytic zinc is coordinated by

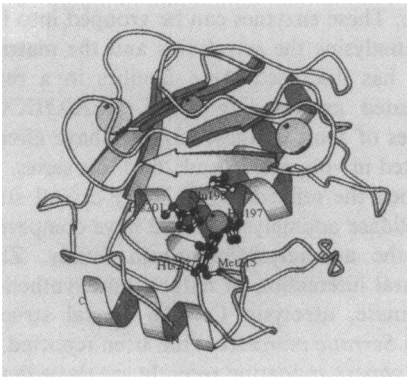


Figure 4 The structure of human neutrophil collagenase. The metal ions are shown as small (zinc) and large (calcium) spheres. The atomic structures of the ligands to the catalytic zinc, the methionine of the Met-turn and the catalytic glutamic acid are shown. Reproduced with permission from reference 105.

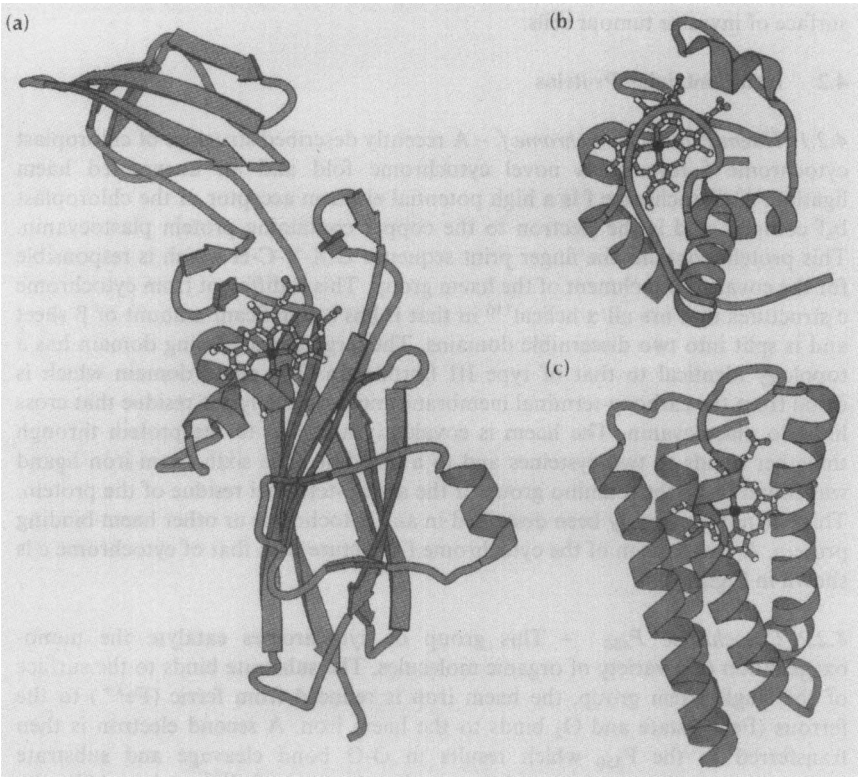


Figure 5 A comparison of the fold of cytochrome f with known folds of c type cytochromes a) cytochrome f, b) yeast cytochrome c, c) cytochrome c'. Reproduced with permission from reference 115

the three histidine residues with the distance between ε-nitrogen and zinc varying between 2.0 and 2.2 Å. In the absence of a substrate or an inhibitor, a water molecule forms an additional fourth ligand to zinc. The ligands surround the zinc in a trigonal pyramidal coordination sphere where the ε-nitrogen of the second histidine being at the tip of the pyramid and the zinc being almost coplanar with the other ligands. In the astacins and serralysins the hydroxyl oxygen of the conserved tyrosine is a fifth ligand to zinc which expands the coordination sphere to a trigonal-bipyramidal geometry.[104,113] The amino acid sequence homology between the families of zinc metalloproteases is relatively low with 13-27% identity and greater homology around the zinc binding active site. However the structural studies described above show that the enzymes are related and have evolved from a common ancestor. This is an area that is important in our understanding of the spread of cancer cells around the body. A recent paper[114]

has described a membrane bound metalloproteinase which is expressed on the surface of invasive tumour cells.

4.2 Iron Containing Proteins

4.2.1 Haem Protein Cytochrome f. – A recently described structure of chloroplast cytochrome f revealed a novel cytochrome fold and an unexpected haem ligation.[115] Cytochrome f is a high potential electron acceptor of the chloroplast b_6f complex and is the electron to the copper containing protein plastocyanin. This protein contains the finger print sequence C-X-Y-C-H which is responsible for the covalent attachment of the haem group. This is different from cytochrome c structures that are all α helical[116] in that it has a significant amount of β sheet and is split into two discernible domains. The large haem binding domain has a topology identical to that of type III fibronectin. The small domain which is distal from the carboxy-terminal membrane anchor has a lysine residue that cross links to plastocyanin. The haem is covalently attached to the protein through thioether bonds to two cysteines and to a histidine. The sixth haem iron ligand was found to be the α amino group of the amino-terminal residue of the protein. This has not previously been described in any cytochrome or other haem binding protein. A comparison of the cytochrome f structure with that of cytochrome c is shown in Figure 5.

4.2.2. Cytochrome P_{450} – This group of cytochromes catalyse the mono-oxygenation of a variety of organic molecules. The substrate binds to the surface of the single haem group, the haem iron is reduced from ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state and O_2 binds to the haem iron. A second electron is then transferred to the P_{450} which results in O-O bond cleavage and substrate hydroxylation. The structure of P_{450cam} has been studied[117] and in 1994 the structure of $P_{450terp}$ has been reported at 2.3 Å resolution.[118] The structures have a similar fold and the structure of $P_{450terp}$ is shown in Figure 6a. The different

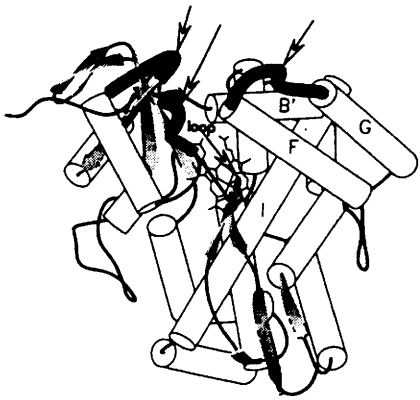


Figure 6a Structure of the P_{450} terp enzyme. Reproduced with permission from reference 118.

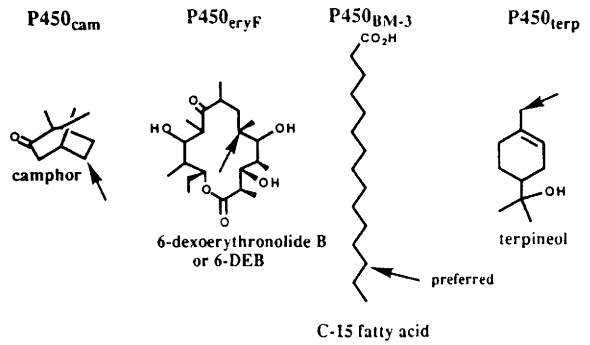


Figure 6b Structure of camphor, 6-deoxerythronolide B, C15 fatty acid and terpineol. This shows the variation of substrates for P_{450} structures already solved.

substrates of camphor and terpineol (see Figure 6b) show that the active site can vary in size and shape but the mechanism described above is similar. Several studies have used molecular modelling to understand the effects of specific mutations in P_{450} cytochrome structures. Mutation of key residues in rabbit cytochrome P_{450} 2c3v alters the regioselectivity of progesterone metabolism.[119] The application of three dimensional homology modelling of cytochrome P_{450} 2B1 has been described[120], cytchrome P_{450} 14a demethylase (*Candida albicans*) from P_{450cam}[121] and the active site of human P_{450}cl7.[122] A study to compare the 3D model of human thromboxane synthase using P_{450cam} and BM-3 as templates has given some information on the substrate binding pocket.[123] P_{450} BM-3 is shown to be a better template for thromboxane synthase than is P_{450cam} based on the higher sequence homology and identity between thromboxane synthase and P_{450} BM-3. An evaluation of molecular models of the cytochrome P_{450} *Streptomyces griseolus* enzymes P_{450}SUI and P_{450}SUZ has been carried out.[124] This paper provides a useful analysis of modelling methods. Specifically made mutants of cytochrome $P_{450's}$ have been used to evaluate the role of specific amino acid residues in the proteins. Those reported in 1994 are mutation of the homolog of Thr 252 (P_{450cam} numbering) in other eukaryotic $P_{450's}$[125] and a role for Asp251 in cytochrome P_{450cam} oxygen activation has been established.[126]

4.2.3. Haem Peroxidases – Several haem peroxidase structures have been reported in 1994. It is thought that the active iron-oxo intermediate formed in peroxidases is similar to that formed in P_{450} cytochromes. The crystal structure of a fungal peroxidase from *Anthromyces ramosus* at 1.9 Å resolution has been determined,[127] a recombinant peroxidase from *Coprinus cineerus* at 2.6 Å resolution,[128] a manganese peroxidase from *Phanerochaeta chrysosporium* at 2.06 Å resolution[129] and a recombinant pea cytosolic ascorbate peroxidase.[130] It is possible to trap and study intermediates in the reaction pathway with peroxidase enzymes and this has been achieved by Laue X-ray diffraction.[131] In this case the crystal structure of the Fe^{4+}=O centre has been determined. A recent paper

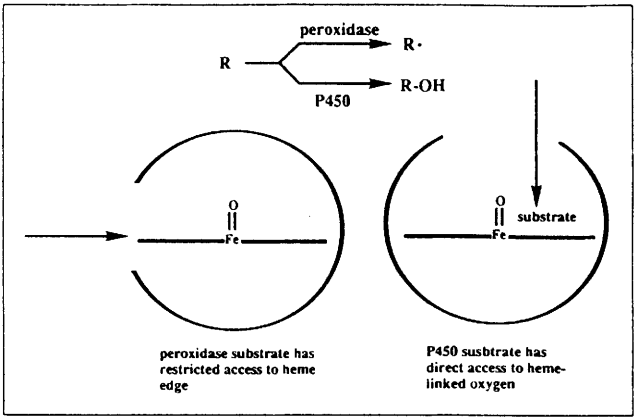


Figure 7 Difference in substrate access to the haem in peroxidase and P_{450} enzymes

appearing in 1994 has compared the structures of peroxidase and P_{450} cytochrome.[132] It appears that the peroxidase substrate interacts at the haem edge where electron transfer occurs whereas in the P_{450} cytochrome the haem edge is not accessible and instead a substrate pocket is situated directly adjacent to the $Fe^{4+}=O$ centre, allowing direct interaction between the substrate and $Fe^{4+}=O$ oxygen atom (see Figure 7).

4.2.4. Flavocytochrome c Sulfide Dehydrogenase – This enzyme is thought to be located in the periplasm of the anaerobic purple sulfur bacterium, *C. vinosum*. It catalyses the reversible conversion of sulfide to elementary sulfur *in vitro*. The structure of this enzyme was described in 1994 by Chen *et al.*[133] It is a heterodimer consisting of a 46kDa flavoprotein subunit in which flavin-adenine dinucleotide (FAD) is covalently bound and a 21kDa dihaem cytochrome subunit. The two domains of this cytochrome are equal in size and similar in structure despite there being low sequence identity. Each domain has four α helices and intervening loops wrapped around the haem group. The edges of the porphyrin rings are 11.4 Å apart and the two iron atoms are separated by 19.0 Å. The haem planes are inclined to each other by 30 and are orientated in a unique manner. The flavoprotein contains three domains. The site of covalent attachment of FAD is a cysteine residue. On one side of the flavin ring there is a disulfide bridge and there are hydrogen bonds between the N1-02 edge of the flavin ring and the backbone nitrogens on the N-terminus of an α helix. Kinetic studies have demonstrated an unusually fast interprosthetic group electron transfer between the haems and flavin in the complex. Figure 8 shows four potential pathways for electron flow from the flavin to the haem in this protein.

4.3 Dinuclear Iron Centre Proteins – An important class of iron containing proteins are the proteins that contain dinuclear iron centres that are coordinated by histidines and additional carboxylate ligands in the protein. These can be

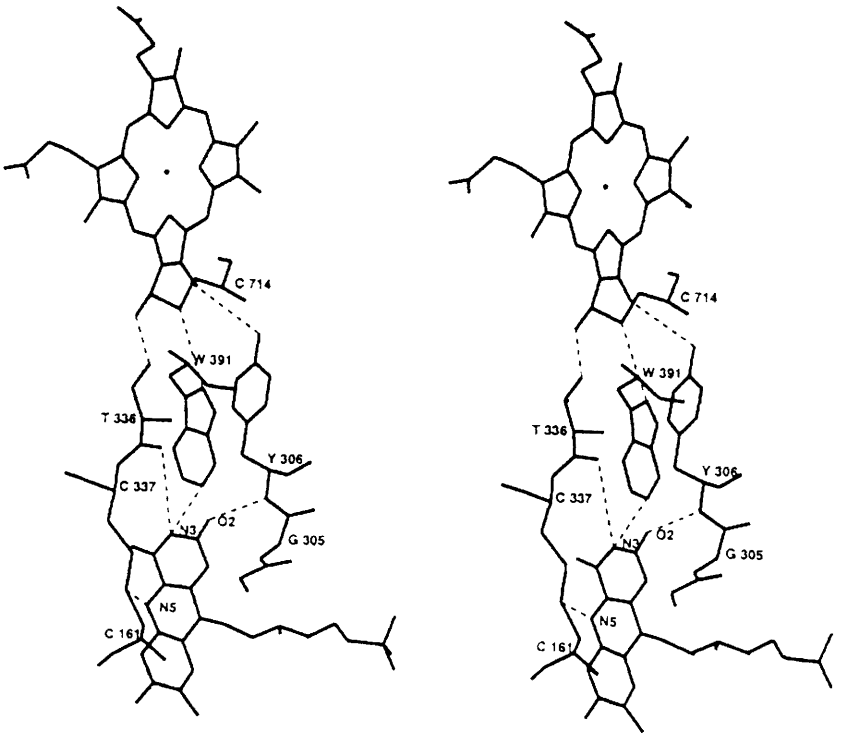


Figure 8 Stereo view of four potential pathways for electron flow from the flavin to the haem of flavocytochrome c sulfide dehydrogenase. Dashed lines indicate jumps through space or between atoms of the polypeptide chain and the flavin or haem groups. Reproduced with permission from reference 133.

divided into different classes the first of which includes ribonucleotide reductase, methane monooxygenase, stearoyl-acyl carrier protein Δ^9 desaturase, toluene hydroxylase, phenol hydroxylase, and alkene hydroxylase. A resonance Raman spectroscopy study for stearoyl-ACP desaturase and a primary sequence comparison with this and other di-iron-oxo proteins was reported in 1994.[134] The crystal structures of ribonucleotide reductase[135,136] and methane monooxygenase[137] have previously been reported. The mechanism of ribonucleotide reductase protein R1 has been examined by Uhlin and Eklund.[138] This enzyme is an $\alpha_2\beta_2$ tetramer in which the α subunits (R1) contain the substrate-binding site and the β subunits (R2) contain the di-iron centre. This centre catalyzes the oxidation of a neighbouring Tyr amino acid residue resulting in the generation of a catalytically essential tyrosyl radical which is involved in the activation of the ribose region of the ribonucleotide substrate and is not consumed in the process. A long range electron transfer chain communicates between the buried tyrosyl radical and the ribonucleotide substrate bound at the active site on the R1 subunit. Methane monoxygenase is made up of three components, the hydrolase,

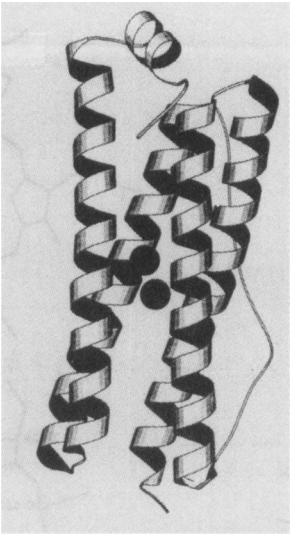


Figure 9 The structure of the iron binding subunits of bacterioferritin. The metals are drawn as black balls. Reproduced with permission from reference 143.

the coupling protein and the reductase. The reductase supplies electrons from the reduced nicotinamide adenine dinucleotide (NADPH) for reduction of the di-iron site. The di-iron cluster coordinated by the α subunit of this enzyme has a similar structure to that found in ribonucleotide reductase. The biochemistry of soluble methane monooxygenase has been reviewed in 1994 by Lipscomb.[139] The chemical reaction catalysed by this enzyme has been used for several commercial uses for example the processing of toxic waste, the conversion of methane to the more useful fuel methanol and the stereospecific hydroxylation of hydrocarbons. Spectroscopic studies on these two enzymes reported in 1994 include three papers on ribonucleotide reductase by the group of Stubbe. Firstly using Mössbauer spectroscopy to characterise the diferric radical precursor[140] secondly to study the kinetics of the excess of Fe^{2+} reaction by optical, EPR and Mössbauer spectroscopy[141] and thirdly to study the kinetics of limiting Fe^{2+} reaction by the same techniques.[141] Spectroscopic detection of intermediates in the reaction of dioxygen with reduced methane monooxygenase have been carried out by the group of Lippard.[142]

The second class of this type of protein are the bacterioferritin and rubrerythrin. The structure of bacterioferritin was published in 1994[143] and is shown in Figure 9. The *E. coli* bacterioferritin is a 24 subunit envelope protein. Both groups of protein described above catalyse dioxygen-dependent oxidation-hydroxylation reactions and have some structural similarities. They have a motif containing two consecutive helices. An iron coordinating glutamic or aspartic acid is located in the first helix and there is a EXXH amino acid motif in the second. The next type of protein containing a dinuclear iron centre is the well studied hemerythrin and myohemerythrin whose structures have been known for

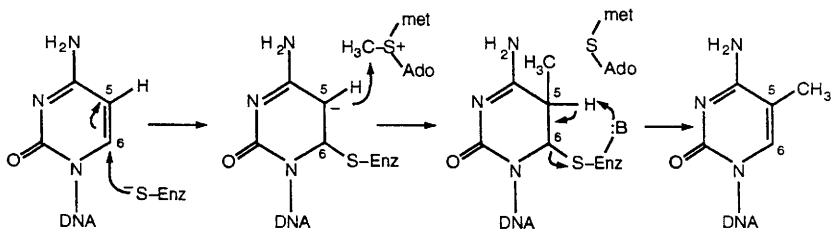


Figure 10a A schematic representation of the reaction pathway for the *Hha*I methylase enzyme.

some time.[144,145] In this case the iron ions are coordinated in a four-helix bundle by five terminal histidine residues and bridged by two carboxylate residues and an oxo group. These proteins are carriers of oxygen.

Another type of related protein which has been studied recently is a purple acid phosphatase which has been reported from a variety of sources including kidney beans[146] and human macrophage and asteodast.[147] The protein from kidney beans appears to have a dinuclear Fe(III)-Zn(II) active site, whereas the mammalian equivalent contains one ferric and one ferrous ion.

5 Protein–Nucleic Acid Interactions

5.1 Methyltransferases – One of the most interesting structures reported in 1994 was the description of the interaction between an enzyme responsible for DNA methylation and its complex with DNA. The enzyme was the *Hha*I methyltransferase[148] which is responsible for methylation of the DNA from a bacteria to protect the host DNA from the cells restriction enzymes. This structure showed that the base of the DNA to be modified, which in this case was cytosine, is flipped out of the DNA helix by the enzyme. The enzyme uses S-adenosyl methionine as the methyl donor. The catalytic mechanism of this enzyme had been proposed for some time[149] and has now been confirmed by the crystallography. The enzyme has a conserved cysteine residue which is involved in a covalent intermediate in the enzymic reaction. A key step is nucleophilic attack on carbon 6 of the target cytosine by a cysteine thiol as shown in Figure 10a. The monomeric enzyme is folded into two domains, a larger catalytic domain containing both catalytic and cofactor binding sites and a smaller DNA recognition domain which is involved in sequence-specific DNA recognition and in the penetration of the DNA helix to flip the target cytosine. The enzyme undergoes structural rearrangement on binding to DNA. In the ternary structure the cytosine flips completely out of the DNA and is positioned into the catalytic pocket of the enzyme, next to the cofactor. The structure implies that the target cytosine can flip out only through the minor groove and all of the sequence specific interactions with the target site occur in the major groove. The structure of the *Hha*I methyltransferase in complex with DNA is shown in Figure 10b.

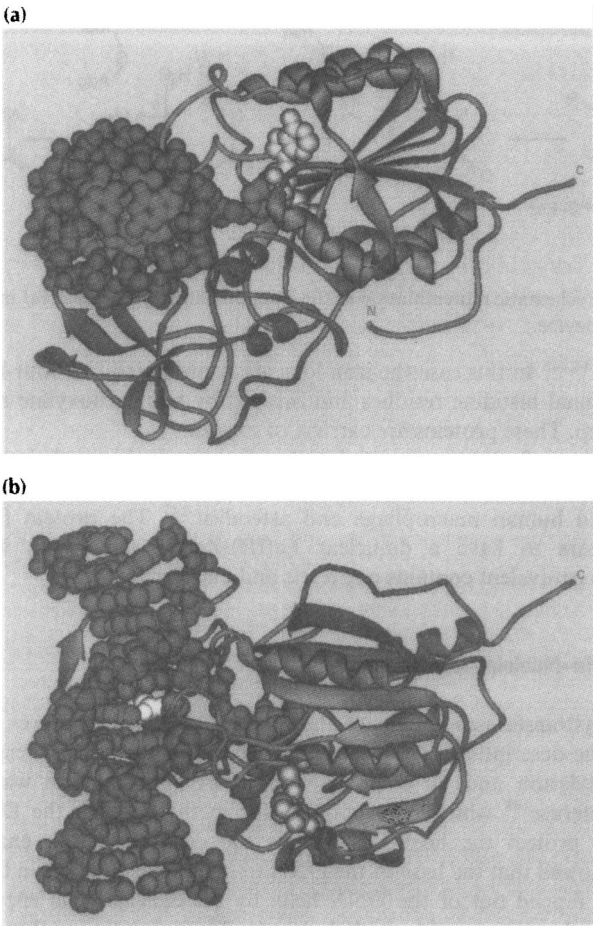


Figure 10b Graphic representation of the complex of *Hha*I methyltransferase covalently bound to a 13-mer DNA duplex containing the recognition sequence. The end product of the reaction is shown in space filling mode. Reproduced with permission from reference 148.

Also in 1994 the structure of a related enzyme has been published.[150] This is a catechol O-methyltransferase which catalyses the transfer of the methyl group from S-adenosyl methionine to one hydroxyl group of catechol. Catechol like cytosine contains a six membered ring and the single domain structure is similar to the catalytic domain of *Hha*I DNA methyltransferase discussed above.

Other C5-cytosine methyltransferases from eukaryotic sources including human DNA methyltransferase share a set of ten conserved blocks of amino acid residues. A comparison of these enzymes is made in a recent review published in 1994.[151] DNA methylation in eukaryotes has an important role to play in

gene regulation, genomic imprinting, DNA replication foci and embryonic development.

5.2 Restriction Endonucleases – The other partner to the DNA methyltransferases in bacterial systems are the restriction endonucleases. These two enzymes are thought to be the bacterial defense system to foreign DNA. In 1994 two new structures for these enzymes were reported. The structures of the *Eco*RI and *Eco*RV restriction endonucleases have been discussed in volumes 25 and 26 of the Specialist Reports on Amino Acids, Peptides and Proteins. One of the two new enzyme structures is for *Bam*HI[152,153] which is found to be similar to *Eco*RI despite the lack of amino acid sequence similarity between the two enzymes. The other new structure is for *Pvu*II with and without DNA.[154,155] The enzyme *Pvu*II is found to be similar to *Eco*RV. The two different pairs of enzymes have an overall similar structure at the point of DNA cleavage. All four enzymes have equivalent β-sheets which have a cluster of conserved acidic residues which can coordinate one or more Mg^{2+} ions which are essential for activity. A difference does exist between the two pairs of enzymes. *Bam*HI and *Eco*RI both cut DNA at a restriction site with a four base pair stagger, leaving 5′ phosphate overhangs or 'sticky ends'. They both approach DNA from the major groove for sequence recognition. In contrast *Eco*RV and *Pvu*II cleave DNA with 'blunt ends' and approach the DNA from the minor groove and wrap β strands or loops around the DNA and into the major groove for sequence recognition.

5.3 *E. coli* Ada Protein – DNA can become modified to form O^6-methylguanine by interaction with S-adenosyl methionine or by interaction with endogenous electrophiles. This modification can induce transition mutations during DNA replication. The Ada protein from *E. coli* can transfer the methyl group from O^6-methylguanine onto a cysteine residue in the C-terminus of the protein. The crystal structure of a 19KDa fragment of C-Ada has been reported in 1994.[156] In this structure the active cysteine residue is buried and it is proposed that the enzyme must undergo a conformational change to effect the methyl transfer reaction, which is proposed to involve a nucleophilic substitution mechanism of the S_N2 type, with the cysteine thiolate acting as the nucleophile. The Ada protein also responds to alkylation damage of DNA. The methyl groups of the Sp diastereoisomer of methyl-phosphotriesters in DNA are transferred to a cysteine in the N-terminus of the protein. In the cell the 39kDa Ada protein is cleaved into a 23kDa C terminal fragment. The N-terminal of the Ada protein is a zinc binding protein which binds specifically to DNA where the metal is tetrahedrally coordinated by four cysteine residues, including the active site cysteine. [^{13}C] filtered one-dimensional heteronuclear multiple-quantum spectroscopy (COSY) has been used to demonstrate direct interaction of the methylated active-site cysteine with zinc.[157,158] Other DNA repair enzymes have been studied in 1994. One of these is the *E.coli* Fpg protein. Grollman[159] and coworkers have investigated the substrate specificity of this protein and have defined oligonucleotides containing various 8-oxopurines as substrates. The C8

keto group of 8-oxoguanine appears to be important for binding of the protein in the major groove of DNA. In addition the C6 keto group of deoxyguanosine is critical for catalysis. The other enzyme is T4 endonuclease V which acts as a glycosylase on pyrimidine dimers. Iwai and colleagues[160] in 1994 have studied the interaction of this enzyme with cis-syn thymine and modified components of the phosphodiester backbone which include Rp-methyl phosphonate, an Sp-methylphosphonate and a phosphorothiolate containing 3′ sulfur. The two modifications which create a change in the minor groove of DNA, Rp-methylphosphonate and phosphorothioate had a significant effect on endonuclease V but the Sp-methylphosphorate group which is located in the major groove, did not have effect. The authors conclude that the endonuclease V interacts with pyrimidine dimers in the minor groove of DNA . Since these types of repair enzymes for DNA act to correct mutagenic and toxic damage to DNA that in turn leads to cancer, aging and death, their study will be of upmost importance in the future.

5.4 DNA and RNA Polymerases – A recent review appearing in 1994 discusses the structural relationships between the Klenow fragment of *E.coli* DNA polymerase and HIV-I reverse transcriptase enzymes.[161] The authors predict that the polymerase superfamily may share a universal polymerase active site architecture and mechanism. In the previous volume of RSC specialist reports on Amino Acids, Peptides and Proteins the structure of HIV-1 reverse transcriptase (RT) was discussed. This enzyme has become a major target for anti-AIDS therapy. In 1994 the group of Steitz reported the 2.9 Å resolution structure of this enzyme in complex with the non-nucleotide inhibitor nevirapine.[162] Many other papers have also appeared and these include a comparative study again by the group of Steitz with a new crystal form of the enzyme which shows that HIV-I RT has a specific flexibility that allows rotation of the polymerase active site located in the palm subdomain of p66 relative to the rest of the molecule. It is thought that this swivelling motion may permit the polymerase to accommodate movements required during DNA replication. The 2.2 Å resolution structure of the N-terminal region of HIV-I RT (which includes the fingers and palm domains) has been reported by Unge and collaborators.[163] A new crystal form of the intact enzyme in complex with non-nucleotide inhibitors has been described recently.[164] These crystals diffract to 2.2 Å resolution so a more detailed picture of the enzyme should emerge in the near future. An important area of research has involved the development of resistance to HIV-I RT inhibitors that are used in the treatment of AIDS. This has recently been reviewed in 1994 by Tantillo and collaborators.[165] Another recent paper suggests that the sensitivity of HIV-I RT to deoxynucleotides depends on template length whereas the sensitivity of drug resistant mutants does not.[166]

Three other polymerase structures have been reported in 1994. These are the ternary complexes of rat DNA polymerase β, a DNA template-primer and ddCTP,[167] the 2.3 Å crystal structure of the catalytic domain of DNA polymerase β[168] and the catalytic domain and the apoenzyme of the rat DNA polymerase β.[169] The overall structure of all the polymerases seems to be similar suggesting

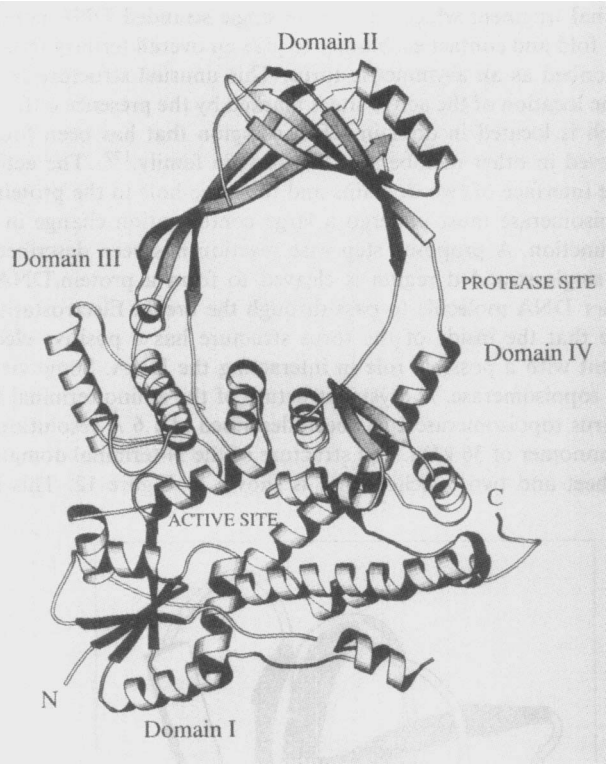


Figure 11 Structure of the 67kDa fragment of *E. coli* DNA topoisomerase I, depicting the domain structure and the large hole in the middle of the protein. The active-site region, the proteolytically sensitive strands and the N- and C-termini are labelled. Reproduced with permission from reference 171

they have a common mechanism for nucleotidyl transfer. It is suggested that this is similar to the two metal ion mechanism proposed for the phosphonyl reaction.[170]

5.5 DNA Topoisomerases – These enzymes are found in prokaryotic and eukaryotic cells and some viruses. They are involved in DNA replication, transcription and genetic recombination.

In 1994 the three dimensional structure of the 67 kDa N-terminal fragment of *E. coli* DNA topoisomerase was published.[171] This is the most well studied topoisomerase enzyme which is a 77 kDa monomeric zinc metalloprotein. The enzyme which only requires magnesium for activity has a strong preference for regions of single stranded DNA and is capable of cleaving single stranded DNA or short single stranded oligonucleotides. The *E. coli* topoisomerase I contains three Zn^{2+} ions which are bound in the C-terminal 30 kDa of the protein where

three putative tetracysteine domains are present. The crystal structure of the 67 kDa N-terminal fragment which can cleave single stranded DNA contains four domains that fold and contact each other to give an overall tertiary structure that has been described as an asymmetric torus. This unusual structure is shown in Figure 11. The location of the active site is marked by the presence of the catalytic tyrosine which is located in domain III in a region that has been found to be highly conserved in other members of this protein family.[172] The active site is located at the interface of two domains and the large hole in the protein suggest that the topoisomerase must undergo a large conformation change in order to perform its function. A proposed step wise reaction has been described. In this reaction the single stranded region is cleaved to form a protein/DNA gate to allow the other DNA molecule to pass through the break. Electrostatic calculations indicate that the inside of the torus structure has a positive electrostatic field, consistent with a possible role in interacting the DNA. Some viruses also have a DNA topoisomerase. A crystal structure of the amino terminal fragment of vaccinia virus topoisomerase I has been described at 1.6 Å resolution.[173] This protein is a monomer of 36 kDa. The structure of the N-terminal domain has a 5 stranded β sheet and two α helices and is shown in Figure 12. This structure

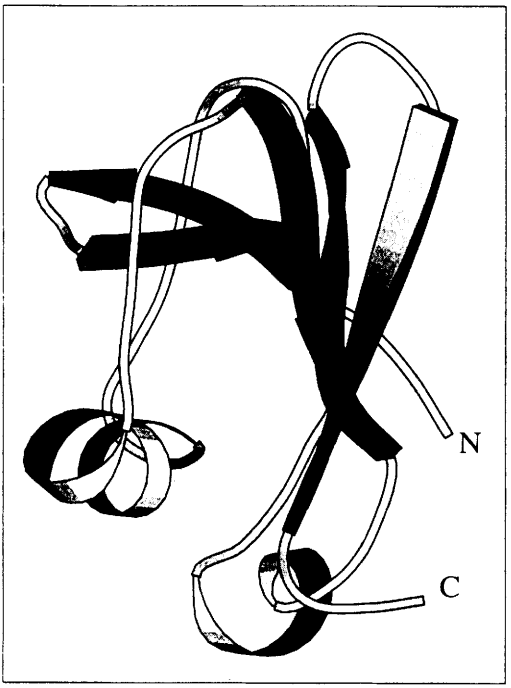


Figure 12 Structure of the 9kDa N-teminal domain of Vaccinia virus DNA topoisomerase I. The N- and C-termini are labelled. Reproduced with permission from reference 173.

shows that these poxviral proteins may not be structurally similar to eukaryotic topoisomerases which had been previously proposed. The viral topoisomerase has been the subject of single turnover and steady state kinetic analysis of the DNA strand cleavage and ligation reactions.[174] Poxviral topoisomerases are similar to the eukaryotic-like proteins since they form a phosphotyrosine bond with the 3'OH of the phosphodiester backbone of DNA, do not require magnesium and relax negatively and positively supercoiled DNA. Unlike all other topoisomerases, poxviral topoisomerases display a marked DNA-sequence preference for cleavage. In contrast to the eukaryotic proteins, poxviral topoisomerases are resistant to camptothecin, a potent alkaloid that has been used as a chemotherapeutic agent.

5.6 DNA Gyrase – A related enzyme is DNA gyrase. This enzyme is capable of introducing negative supercoils into a relaxed molecule in a reaction coupled to ATP hydrolysis. Gyrase is composed of a B subunit responsible for ATP binding and hydrolysis and an A subunit responsible for DNA binding. The intact protein with a A_2B_2 complex is the target for the antibiotics, coumarins which bind to the B subunit and quinolones which bind to the A subunit. The structure of a 43 kDa fragment of the B subunit has been known for some time.[175] This structure was solved in the presence of a non-hydrolysable analogue of ATP, ADPNP. This binds to the centre of domain I of this structure which is a dimer with a hole in the middle of the structure. Domain I has also been shown to be the binding site for antibiotics targeted to this subunit. Two recent papers reinforce this proposal. Gilbert and Maxwell[176] show that a 24 kDa N-terminal subdomain of DNA gyrase B protein binds coumarin drugs. Another interesting paper shows a three dimensional model of the *E. coli* gyrase B subunit crystallised in two-dimensions on novobiocin-linked phospholipid films.[177] This is a three dimensional electron microscopy reconstruction of the structure of the intact B-subunit of DNA gyrase. The reconstruction showed that the protein has a 'V' shape with the arms of the 'V' pointing away from the novobiocin-binding site. The 43 kDa fragment for which the crystal structure is known is located at the bottom of one of the arms of the 'V' suggesting that the antibiotic binding site may be close to the ATP-binding site. This would explain its mode of action and is leading to a more rational approach to antibiotic design.

6 Nucleic Acid Related Proteins

6.1 GTPases – Enzymes called GTPases are involved in many cellular processes which range from regulation of transmembrane signalling by hormones and light, to protein synthesis on ribosomes. One of the enzymes involved in signalling pathways leading to cell proliferation is called Ras. Study of this protein has been very active in 1994. The GTP bound form of Ras binds to the serine/threonine kinase c-Rafl locating it in the cell membrane where it can be activated. This has been reviewed in 1994 where Daum and colleagues discuss the 'ins and outs of Raf kinases'.[178] The topology of the Ras-binding

domain of human Rat-1 has been determined by heteronuclear three-dimensional NMR.[179] The critical binding and regulatory interactions between Ras and Raf occur through a small, stable N-terminal domain of Raf and specific Ras effector residues.[180] The solution structure and dynamics of Ras p21 – GDP has been determined by heteronuclear three and four dimensional spectroscopy.[181]

Human ADP-ribosylation factor (ARF1) is involved in vesicular transport and in activation of phospholipase D[182,183] and cholera toxin.[184] In 1994 the structure of human ADP-ribosylation factor 1 complexed with GDP was reported.[185] The structure reveals an amphipathic N-terminal helix, which is proposed to be responsible for GTP-dependent binding to the membrane. In the crystal ARF dimerises via formation of an antiparallel β sheet. It is proposed that the amphipathic helix lies in a groove on the surface of the protein in the structure of the water soluble GDP complex but becomes exposed along with a myristol moeity, upon GTP binding.

Another group of GTPases is the heterotrimeric G-proteins. In 1994 crystal structures were reported.[186,187] The 2.2 Å crystal structure of transducin α complexed with GTPγS had been reported by the group of Sigler.[188] Crystallisation and preliminary crystallographic studies of $Gi\alpha_1$ and mutants of $Gi\alpha_1$ in the GTP and GDP bound states have also been reported.[189]

The bacterial elongation factor EF-Tu which is involved in protein synthesis on the ribosome is also a well studied GTPase. Two crystal structures of this enzyme have been reported for the thermophilic bacteria, *Thermus thermophilus* [190] and *Thermus aquaticus*.[191] In 1994 the crystal structure of another bacterial GTPase, elongation factor G was published.[192] Interestingly the arrangement of the first two domains of EF-G-GDP resemble that of their counterparts in EF-Tu-GTP. The remaining three domains look like RNA-binding modules. The three-dimensional structure of elongation factor G in the nucleotide-free state has been solved from *Thermus thermophilus*.[193] This structure is very similar to that found in the GDP complex. EF-Tu is the binding site for the antibiotics kirromycin and pulvomycin. Three papers published in 1994 have used mutants of *E.coli* which show antibiotic resistance to map the position of binding of kirromycin[194,195] and pulvomycin.[196]

Several papers recently have focused on the mechanism of GTP hydrolysis based on the structural information available from the various GTPase enzymes. GTP hydrolysis in Ras and EF-Tu leads to inversion of configuration of the γ-phosphate as a consequence of a direct attack of a water molecule on the phosphorus atom, in line with the scissile γP-O bond. A catalytic water has been observed in the crystal structures of these enzymes. An experimental and theoretical study has been carried out by analysis of the role of a glutamine - 61 in the hydrolysis of GTP by $p21^{H\text{-}Ras}$.[197] It was suggested that Gln-61 in Ras might abstract a proton from water. This was supported by the observation that replacement of this glutamine by glutamate resulted in a 20 fold increase in GTPase rate.

A study has been made by mutagenesis of specific amino acid residues in G-protein α subunit, GTP hydrolysis.[198] A role for the conserved glutamine in

Figure 13 Structure of the large segmented protein ATPase. The ribbon diagram shows the arrangement of the α,β,γ subunits. The subscripts DP, TP and E refer to band ADP, AMP-PNP and no substrate respectively. Reproduced with permission from reference 201

these proteins being used to stabilise the transition state of the reaction rather than as a general base has received attention.[199] This suggestion has arisen from the crystal structure at 1.7 Å resolution of transducin α GDP. AlF_4^- (fluoroaluminate). It has now been suggested that the base might be the substrate - the γ-phosphate of GTP.[200]

6.2 ATPase – Adenosine triphosphate synthase (ATPase) is the central means of energy conversion in mitochondria, chloroplasts and bacteria. It uses a proton gradient to drive ATP synthesis from ADP and inorganic phosphate. It is a multisubunit assembly composed of a globular domain, an intrinsic membrane domain connected by a 45 Å stalk. The structure of the globular domain was determined in 1994.[201] This domain forms a flattened sphere in which three α and three β subunits are arranged like the segments of an orange as shown in Figure 13. They are arranged around a central α-helix formed by the C-terminal amino acids of the γ subunit. The nucleotide-binding domains have a similar topology to RecA protein with the nucleotide binding sites between the α and β subunits, and non-catalytic nucleotide-binding sites predominantly by residues

from the α-subunits. It is proposed in an elegant model that rotation of the α3/β3 assembly around the central helix leads to conformational changes in each catalytic site, thus driving the synthesis of ATP. This is supported by the fact that in the crystal structure the three catalytic sites have different conformations.

6.3 Nucleotide Synthesis Enzymes

6.3.1. Glutamine S-Phosphoribosyl 1-Pyrophosphate Amidotransferase – This enzyme catalyses the first committed step of purine biosynthesis and regulates flux through the entire 10 step pathway by feedback control. The structure of this allosteric enzyme was reported in 1994 by Smith *et al.* [202] The enzyme structure is from *Bacillus subtilus* and contains [4Fe-4S] clusters which have a regulatory function and allow control of purine biosynthesis in this bacterium by oxygen dependent activation of the enzyme. The enzyme is a doughnut shape with four subunits in a homotetramer which are related by three mutually perpendicular twofold axes. Each subunit consists of two domains. The N-terminal domain has a newfold consisting of a four-layer structure in which two antiparallel β sheets are sandwiched between layers of α helix. The C-terminal domain consists of five-stranded parallel β-sheet sandwiched between α helices. A loop and helix make contacts with the N-terminal domain of another subunit and appear to be involved in feedback regulation of the amidotransferase. The four [4Fe-4S] clusters are positioned between the N-terminal and C-terminal domains of each subunit. Ligation of the protein to the iron atoms is by four cysteine sulfur atoms. One AMP molecule is bound to each of the four subunits and is thought to protect the enzyme from oxygen dependent degradation. The allosteric enzyme controlling pyrimidine synthesis is aspartate transcarbamylase. The structure of this enzyme has been known for some time but an extensive review on this subject was published in 1994.[203]

6.3.2 Ribonucleotide Reductase – This enzyme converts ribonucleotides to 2'-deoxyribonucleotides. The structure of the R1 catalytic subunit of this enzyme has recently been reported.[204] An unusual new fold is observed for this enzyme. The R1 subunit domain is a ten-stranded α/β barrel of $(\beta\alpha)_4\beta$ halves. Each half comprises a curved, parallel sheet which is anti-parallel to the other half. Because the barrel has ten strands rather than the more common eight it has a finger like peptide loop which fills the barrel interior. One of the three catalytic cysteines is at the top of the loop (Figure 14). The R1 subunit binds the ribonucleotide substrate and uses the tyrosine free radical generated on the R2 subunit during oxidation of an Fe-O-Fe centre, as a catalyst to reduce ribose to deoxyribose while oxidising two cysteine thiols to a disulfide.

6.3.3. Thymidylate Synthase – This enzyme methylates deoxyuridine monophosphate to form thymine monophosphate. Two new thymidylate synthase structures were reported during 1994.[205,206] The first was from *Leishmania major* and the second the thymidylate synthase from T4 phage. The structures are similar to that reported for the enzyme from *E. coli* in 1991. The enzyme is a

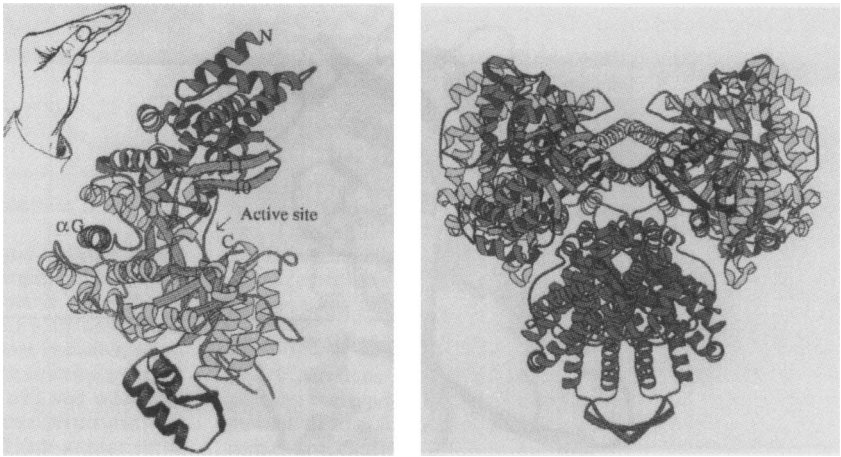


Figure 14 Structure of ribonucleotide reductase. The figure on the left is the monomer R1 showing the active site. The N-terminus would be at the tip of the longest finger as shown, and the thumb would illustrate the β hairpin (β10-β11) packed against the 4-helix bundle in the N-terminal domain. The longest helix in the barrel (αG, 28 residues long) is situated as the knuckles between the fingers and the palm. The figure on the right shows the postulated docking of the R2 dimer (bottom) onto the R1 dimer. Reproduced with permission from reference 204.

target for anti-cancer drugs. The suicide inhibitor 5-fluoro-dUMP forms covalent bonds to the active site cysteine residue and the cofactor N^5,N^{10}-methylenetetrahydrofolate. The T4 enzyme is part of a multi-enzyme dNTP-synthesising complex present in T4 infected *E. coli* and a specific interaction has been demonstrated with T4 dCMP hydroxymethylase.

6.3.4 Phosphoribosyltransferase Enzymes – These enzymes are involved in salvage pathways for nucleotides. The structure of human hypoxanthine-guanine phosphoribosyl-transferase GMP complex was described in 1994.[207] The enzyme catalyses the magnesium-dependent transfer of a ribosyl monophosphate group from α-D-5-phosphoribosyl pyrophosphate to the N9 nitrogen of either guanine or hypoxanthine. The lack or deficiency of this enzyme is associated with a number of pathological conditions, including Lesch-Nyhan syndrome, hyperuricemia, nephrolithiasis and gouty arthritus. The core structure of the enzyme resembles a nucleotide binding domain with a parallel five-stranded β-sheet surrounded by four α helices. This domain is formed by residues in the central region of the polypeptide chain. Flanking regions of the chain, together with the secondary sheet form a separate lobe at the C-terminal end of the central sheet. Similar structures have been observed for other phosphoribosyltransferase enzymes such as orotate phosphoribosyltransferase and glutamine-amido phosphoribostransferase. The GMP molecule binds in an *anti*-conformation in a solvent-exposed cleft located above the central β-sheet of the core region, with one edge of the lobe forming one of the sides of the cleft. The structure of the human enzyme is shown in Figure 15.

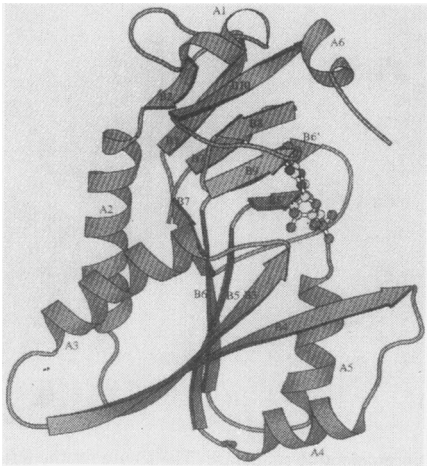
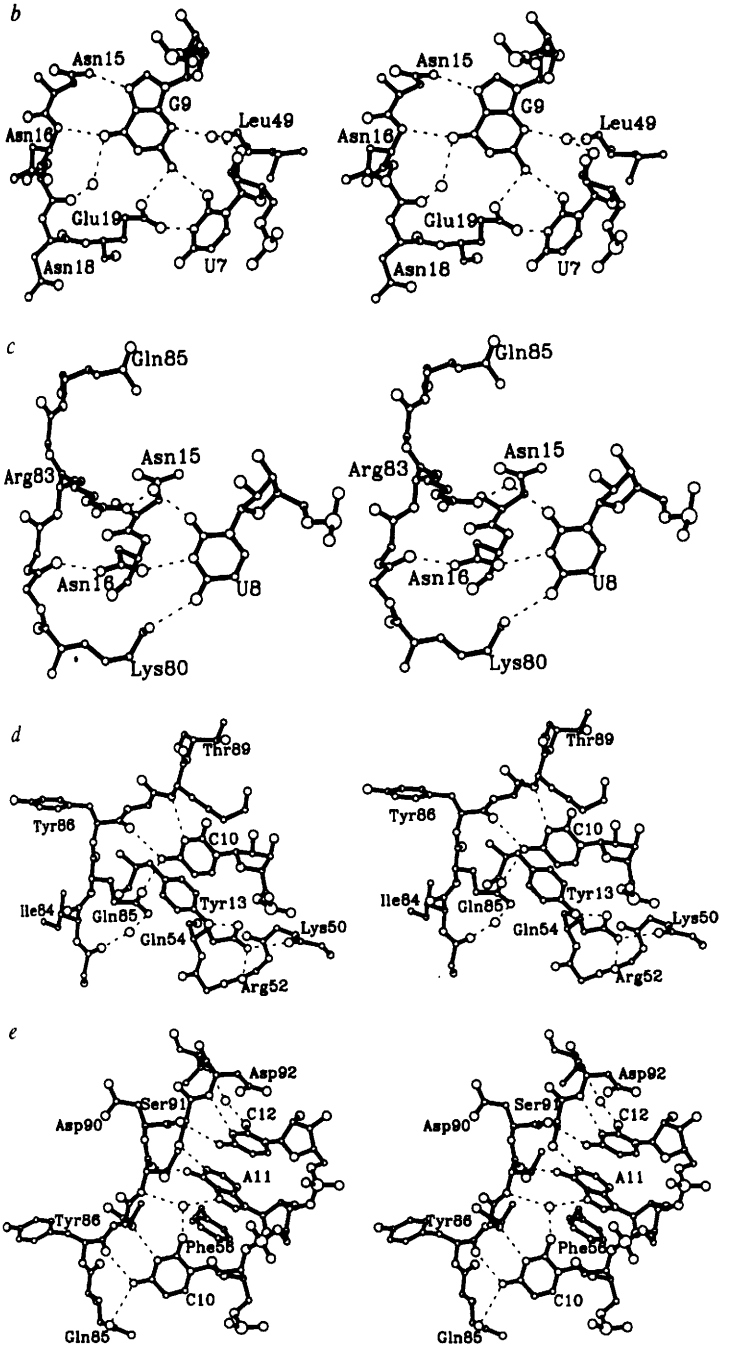


Figure 15 Structure of human phosphoribosyltransferase. Reproduced with permission from reference 207

6.4 Ribosomal Proteins – Current structural studies are focussing on the structure of ribosomal proteins from thermophilic bacteria. These proteins are easier to crystallise and show good homology with the well characterised *E. coli* proteins. Bacterial ribosomes contain more than 50 small ribosomal proteins and further structural characterisation of these proteins was reported in 1994. Many of the ribosomal proteins have a characteristic β-α-β motif structure. One protein that has this motif is ribosomal protein S6. This protein is located on the small ribosomal subunit facing the large subunit and although its precise function is

Figure 16 The interaction of the AUUGCAC heptanucleotide with the U1A protein. *a*, The fork where the stem of the hairpin ends and the loop begins, including two base pairs in the stem and nucleotides A6 and U7 which continue the stacking of the stem into the loop region. The critical residue Arg 52 is shown hydrogen-bonding to A6 and G16, and is buttressed by main-chain carbonyl oxygens. The amide group of Arg 47 also makes a hydrogen bond with the phosphate of G16. *b*, Contacts between nucleotides U7 and G9 and amino acids in the conserved RNP1 and RNP2 region. The stem stacking does not continue past U7, which makes a hydrogen-bond contact with G9. The carboxyl group of Glu 19 makes contacts with both U7 and G9 and Asn 15 makes a hydrogen bond to G9. G9 is also contacted by a main-chain amide and carbonyl from this region. *c*, Nucleotide U8 makes contacts with Asn 16 of RNP2 and with Lys 80 and Arg 83 (via H_2O) residue in the β4 stand. *d*, The pyrimidine ring of C10 stacks on Tyr 13 and is also hydrogen-bonded to main-chain groups of the C terminus and the side chain of Gln 85. The buttressing of Tyr 13 by a hydrogen bond between its phenolic oxygen and the Gln 54 side chain is a requirement for RNA binding. *e*, The A11 and C12 bases are stacked between the Phe 56 ring and the carboxyl group of Asp 92. The phosphate backbone follows the main chain of the C teminus, and main-chain carbonyls and amides make numerous interactions with C10, A11 and C12. Reproduced with permission from reference 210.


b
Asn15
G9
Leu49
Asn16
Glu19
Asn18
U7
Asn15
G9
Leu49
Asn16
Glu19
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U7
c
Gln85
Asn15
Arg83
Asn16
U8
Lys80
Gln85
Asn15
Arg83
Asn16
U8
Lys80
d
Thr89
Tyr86
C10
Tyr13
Ile64
Gln85
Gln54
Lys50
Arg52
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C10
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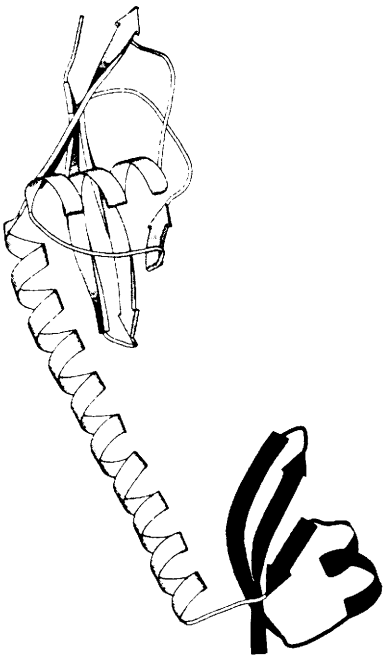

Figure 17 A diagram showing the extended structure of ribosomal protein L9. Reproduced with permission from reference 211.

not known it is thought to bind to 16 S rRNA as a complex with another protein, S18. The structure of this protein from the thermophile, *Thermus thermophilus* was determined in 1994.[208] The S6 protein forms a single domain comprising a four stranded β sheet with two α helices packed against one side. The folding pattern of S6 is similar to that reported for other ribosomal proteins L7/L12, L6 and L30. The conserved features appearing in these proteins are discussed by Burd and Dreyfuss in 1994.[209] Interestingly the structure of protein S6 is similar to the RNA-binding domain of the spliceosomal protein U1A for which the structure has also recently been reported.[210] This paper describes the crystal structure at 1.92 Å resolution of the RNA binding domain of the U1A spliceosomal protein complexed with an RNA hairpin. Conserved aromatic and basic residues in the middle of two β strands are thought to be involved in the interaction with rRNA. Some specific interactions that occur are shown in Figure 16. It is suggested that ribosomal proteins with this motif may interact with rRNA in a similar fashion. Another unusual ribosomal protein with a β-α-β motif is the protein L9 which is found in the large 50S ribosomal subunit where it is involved in rRNA binding. Two crystal forms of this protein have recently been solved.[211] Both show on unusual structure where in which two α/β domains are connected by a nine-turn α helix as shown in Figure 17 . The overall structure

is flat and extended. The length of the long connecting helix is invariant in other prokaryotic L9 sequences. It is thought that this helix has an architectural role in fixing the relative separation and orientation of the N- and C-terminal domains within the ribosome. It is the N-terminal domain that shows structural homology to the smaller ribosomal proteins L7/L12 and L30.

7 Lipases

The structures of several lipase enzymes have been solved in 1994. The study of these enzymes have given some insight into their mechanism and specificity of action. The catalytic activity of lipases is dramatically enhanced by lipid-water interfaces which is a phenomenon known as interfacial activation. In 1994 the structure of *Candida rugosa* lipase was solved by X-ray methods in a closed conformation where the active site serine amino acid residue, which is part of the catalytic triad Ser-His-Asp, is buried by the polypeptide chain.[212] The 'open' structure had previously been reported in 1993.[213] The two structures are thought to correspond to the active conformation adopted at interfaces and the inactive conformation that exists in aqueous solution. The structural changes between the two *Candida rugosa* lipase structures are confined to the flap residues and the oxyanion hole is a preformed feature in the closed form of the enzyme. The motion of the flap appears to take place about hinge points in the protein defined as Glu 66 and Pro 92. The proline residue undergoes a cis-trans isomerisation that is suggested to be the rate determining step in the interconversion of the two forms of the enzyme. The transition from open to closed form of the enzyme buries a large hydrophobic surface area, decreasing the total hydrophobic surface area by 9%. This area may be important for interaction with lipid substrates. N-linked carbohydrate residues may interact with the residues that form the flap and may have a role in activation of the enzyme.

A related structure to that of *Candida rugosa* lipase was also reported in 1994. This was from *Candida antarctica*.[214] This yeast has two different lipases A and B with different specificities and thermostabilities. Lipase B is less thermostable but very stereospecific in both hydrolysis and organic synthesis reactions such as esterification and transesterification. The B lipase has potentially important application in glucolipid synthesis. The lipase crystallises in several forms. The two forms that have been studied have a similar structure with an 'open' active site. Two helices in the structure and a loop form a narrow channel restricting access to the active site, the walls of which are very hydrophobic and lined with aliphatic residues. The channel is thought to account for the high specificity of the lipase. The enzyme seems to readily adopt an 'open' conformation and has low activity for triglyceride substrates. It is for this reason that lipase B is thought to be an intermediate between an esterase and a lipase hydrolysing water soluble substrates. These lipases from *Candida antartica* are being used increasingly in biotransformation reactions due to their specificity and thermal stability.

Another lipase enzyme structure has been solved recently and that is the *H. lanuginosa* enzyme.[215] This is another fungal lipase and shows 29% sequence

identity to the smaller *Rhizomucor miehei* lipase. Both enzymes show the common αβ hydrolase fold which was described in Vol 25 of Specialist Reports Amino Acids, Peptides and Proteins. The structure of the lid covering the active site appears disordered in this structure. The C-terminal subdomain contains a cluster of buried polar residues as well as a number of solvent molecules. It is postulated that this unusual polar core contributes to the stabilisation of the enzyme when it is partially embedded in the oil micelle during catalysis. Another fungal lipase which shares 41% sequence identity with the *H. lanuginosa* lipase and is part of the same family is that from *P. camembertii*.[215] This structure appears to be that of the closed or inactive form of the enzyme. A further related enzyme is that from *R. delemar* which shares 55% sequence identity with the *R. miehei* enzyme. The structure of this enzyme has also been described.[215] The crystal structure of the *R. delemar* enzyme shows the two molecules in the asymmetric unit in different conformations of the lid, one representing the molecule in its closed form and the other in the semi-open conformation stabilised by non-specifically bound detergent used in the crystallisation conditions.

A different mammalian lipase structure has also been reported in 1994. This belongs to the triacylglycerol lipases which are key enzymes in fat digestion. They convert insoluble triacylglycerols into more soluble products that can easily be assimilated by the organism. The structure of human pancreatic lipase was reported in 1990. This is the structure of horse pancreatic lipase.[216] The pancreatic lipases are made up of two domains in contrast to the fungal lipases which only have a single domain. The N-terminal domain is the catalytic part of the molecule and the C-terminal domain is responsible for binding another protein called colipase. The role of this smaller protein, colipase, is to help the lipase absorb into the bile salt coated lipid-water interface. The structure of this enzyme is shown in Figure 18 and is very similar to the human lipase with a flap covering the catalytic triad.

7.1 Lipid-Transfer Protein – Another protein which interacts with lipids is the wheat lipid transfer protein. The structure of this protein has been solved by multidimensional ^{1}H NMR.[217] These proteins are found in many plant tissues and are thought to facilitate *in vitro* exchange or transfer of different amphiphilic lipids such as phospholipids, fatty acids and glycolipids. They were initially thought to have a major role in intracellular traffic of lipids in plant cells although more recently since they are thought to occur on the cell wall, it has been suggested they could be involved in plant defence mechanisms against pathogens. The polypeptide backbone of the protein folds into a simple helical structure. The protein is stabilised by hydrophobic interactions and four disulfide bridges.

8 Receptor Structure

8.1 Receptor/Cytokine Structures – Small molecules called cytokines mediate intracellular signalling by binding with high affinity to the extracellular regions of cell-surface receptors. In 1994 several new receptor/cytokine structures have been

Figure 18 Stereo diagram showing the overall fold of horse pancreatic lipase showing the strands and helices. Residues of the catalytic triad (Ser, Asp, His) and the disulfide bridges are represented in ball-and-sticks. The common core of eight β strands is shown in dark shading. Reproduced with permission from reference 216.

reported which have served to further our understanding of these important interactions. The structure of the human growth hormone and the extracellular domain of its receptor had been reported in 1992[218] and the structure of the tumour necrosis factor/tumour necrosis factor receptor complex in 1993.[219] A recent study in 1994 has shown that growth hormone is also able to bind with the prolactin receptor despite the extracellular region of this receptor having only 28% sequence homology with the growth hormone receptor[220] (Figure 19). Differences are seen in the relative dispositions of the receptor domains such that binding of the prolactin receptor at the high affinity site on human growth hormone precludes the attachment of human growth hormone receptor at the low affinity site. The prolactin receptor structure provides an example of the WSXWS box which is a signature sequence for molecules in class 1 of the haematopoietic receptor superfamily. The crystal structure of tissue factor was also reported in 1994.[221] This is a class 2 haematopoietic receptor. The structure of this receptor is shown in Figure 20. The molecule consists of the two fibronectin type III domains. Another paper by Muller *et al.*[222] has information about the ligand binding site (factor V11a) of human tissue factor which have been inferred from functional data.

8.2 Cell Adhesion Molecules – Another important group of cell surface receptors are the cell-adhesion molecules that direct interactions between cells

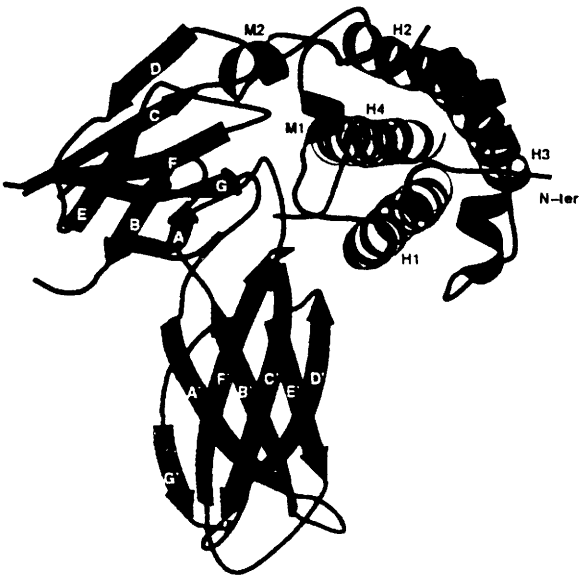

Figure 19 A ribbon representation of the growth hormone-prolactin receptor complex. The four helices making up the core of the growth hormone one labelled H1 through H4; small additional helical segments are denoted M1 and M2. The strands in the two receptor domains are labelled alphabetically; A-G for the N-terminal domain and A'-G' for the C-terminal domain: residues making up the WSXWS-box are circled. Reproduced with permission from reference 220.

or the interaction of cells with the extracellular matrix. The selectins are a family of C-type lectins which are calcium dependent carbohydrate binding proteins. In 1994 structural information became available for a ligand binding region of epithelial (E)-selectin.[223] This paper indicates a defined region and specific amino acid side chains that may be involved in ligand binding. Progress has also been made in the determination of the structure of soluble mannose binding proteins (MBP's). These are C-type lectins which have carbohydrate binding domains similar to those of selectins. These proteins can rapidly distinguish the pattern of sugars on the surface of potential pathogens from that on the surface of mammalian cells. Two papers in 1994 have determined structures of longer fragments of MBP which trimerise and can explain these effects.[224,225] The structure reported now includes a 'neck' by a short extended linker. The neck consists of a triple helix coiled-coil which stabilises the trimer. The carbohydrate recognition sites are arranged distant from each other. This arrangement describes why mammalian high-mannose carbohydrates cannot bind multivalently and the repetitive structures used by fungi and bacteria can bind.

Galectins or S-type lectins bind lactose and β-galactosidases. In 1994 the structure of S-lectin was reported by Liao.[226] The crystal structure is for a 14KDa

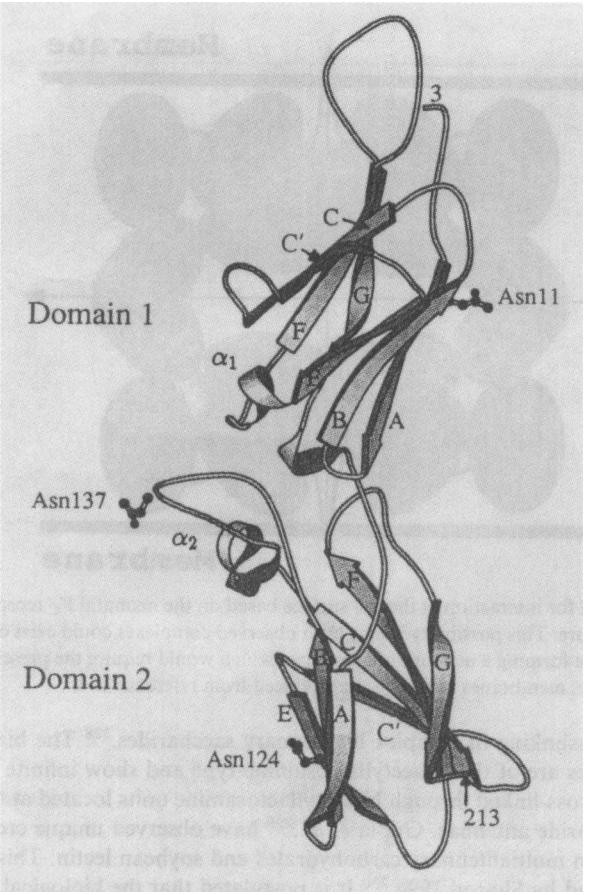

Figure 20 A diagrammatic representation of tissue factor. Strands of domain 1 and 2 are labelled A to G. The two α helices and three asparagines that provide the glycosylation site are also shown. The orientation of the molecule is such that the phospholipid membrane would be at the bottom. Reproduced with permission from reference 221.

galectin complexed with the disaccharide N-acetyllactosamine. The protein shows some structural similarity to legume lectins. Two monomers associate to form an extended β sandwich, each with the same jelly-roll topology. The carbohydrate-binding site possesses a network of salt bridges with specificity for β-linked galactose arising from interactions constraining the position of the O4 atom. Another example of a larger S-type lectin was also described in 1994. This is the structure of the baby hamster kidney carbohydrate-binding protein CBP30.[227] This is an example of an animal type lectin and in addition to the C-terminal carbohydrate binding domain has some repetitive sequences towards the N-terminus of the protein. Another study on mammalian lectin (galectin-1) has

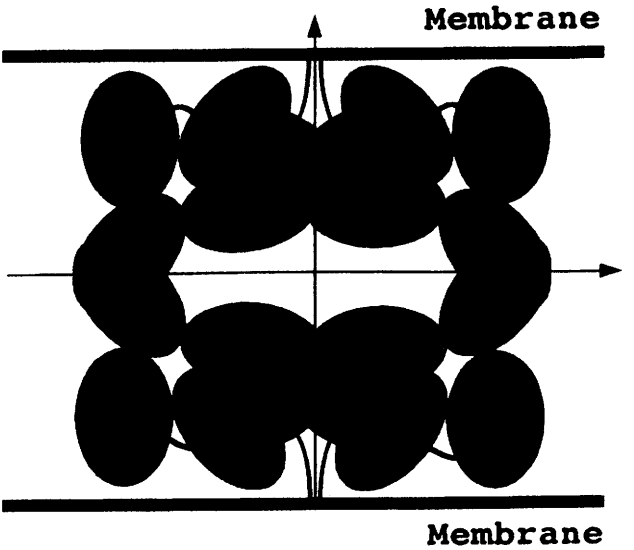

Figure 21 Model for interaction at the cell surface based on the neonatal F_C receptor structure. This possibility is that both observed complexes could exist on the cell surface forming a network of complexes which would require the presence of two parallel membranes as shown. Reproduced from reference 234.

shown the crosslinking of complex biantennary saccharides.[228] The biantennary oligosaccharides are of the N-acetyllactosamine-type and show infinite chains of lectin dimers cross-linked through N-acetyllactosamine units located at the end of the oligosaccharide antennae. Gupta *et al.*,[229] have observed unique cross-linked lattices between multiantennary carbohydrates and soybean lectin. This area has been overviewed by Sharon 1994.[230] It is postulated that the biological effects of lectins on cells depends on formation of specific cross-linked lattices on the target cell.

An interesting group from the immunoglobulin superfamily are also cell adhesion proteins. The first structure of a protein from this group was reported in 1992.[231] More recently the crystal structure of the extracellular region of the human cell adhesion molecule CD2 was reported at 2.5 Å resolution.[232] Analysis of this structure has allowed a model to be proposed for the adhesive interactions. The crystal structure of a MHC-related neonatal Fc receptor was reported in 1994.[233] The structure of this receptor in complex with Fc was also reported.[234] The Fc binds to the receptor at a site quite different from the site of T-cell receptor binding to the homologous MHC class I molecule. This has led to the suggested model shown in Figure 21 for interaction at the cell surface. The neonatal Fc receptor transfers IgG to the newborn.

8.3 Glucose/Galactose Receptor – One of the other receptor structures reported in 1994 was that of the periplasmic glucose/galactose receptor from *Salmonella*

typhimurium.[235] Receptors of this type can exist in an open and closed form. The protein has a 'hinge mechanism' to carry out its function and exists in the closed form when the ligand is bound. It is this form that is active in chemotaxis and transport.

8.4 Aspartate Receptor – Other receptors are involved in transmembrane signalling. Scott and Stoddard discuss transmembrane signalling and the aspartate receptor.[236] A spectroscopic study using ^{19}F-NMR has examined the conformational changes involved in the ligand binding domain of the aspartate receptor.[237] A review in 1994 by Kim discusses a 'frozen' dynamic dimer model for transmembrane signalling in bacterial chemotaxis receptors.[238] Negative cooperativity in the serine receptor and the aspartate receptor has been described.[239,240] Alterations in the structure of the receptor resulting from the binding of the first molecule of aspartate render the second binding site less receptive to aspartate.

9 Protein Phosphatases

The ability to reversibly phosphorylate and dephosphorylate proteins is the basis for the control of many cellular processes. In 1994 the structures of two such enzymes were determined. They were the catalytic domains of human protein tyrosine phosphatase 1B[241] and the structure of *Yersinia* protein tyrosine phosphatase.[242] The structures of these enzymes are closely related even though they share only 15% sequence identity. A domain called the PTP domains consists of a highly curved, central eight stranded mixed β sheet surrounded by two α helices on one side and by five α helices on the other side. This motif provides the majority of the functional groups for phosphate binding and catalysis and constitutes the fundamental component of the catalytic site. This type of protein phosphatase has an absolute specificity for pTyr-containing proteins. Protein tyrosine phosphatases exist as cytosolic forms as described above and receptor like transmembrane forms. They are characterised by homologous catalytic domains of 250 amino acid residues which contain the 11-residue signature motif (I/V) HCXAGXGR(S/T)G that is shared with the dual specificity phosphatases. The structural information now available together with previous kinetic and mutagenesis data allows the proposal that these enzymes catalyse dephosphorylation by forming a thiophosphate enzyme intermediate involving a cysteine residue in the PTP signature motif. At physiological pH the cysteine side chain exists as the more nucleophilic thiolate species, suited for S_N2 attack on the substrate. Other protein phosphatases can phosphorylate proteins at serine, threonine and histidine amino acid residues. Histidine phosphorylation was discussed in an article by Swanson *et al.* 1994.[243] The chromosomal location of human serine/threonine phosphatase genes is discussed by Cohen.[244] These different phosphatases can be split into four structurally distinct families. The structure of the tyrosine kinase domain of the human insulin receptor was described in 1994.[245] The insulin receptor is a disulfide linked α2β2 heterote-

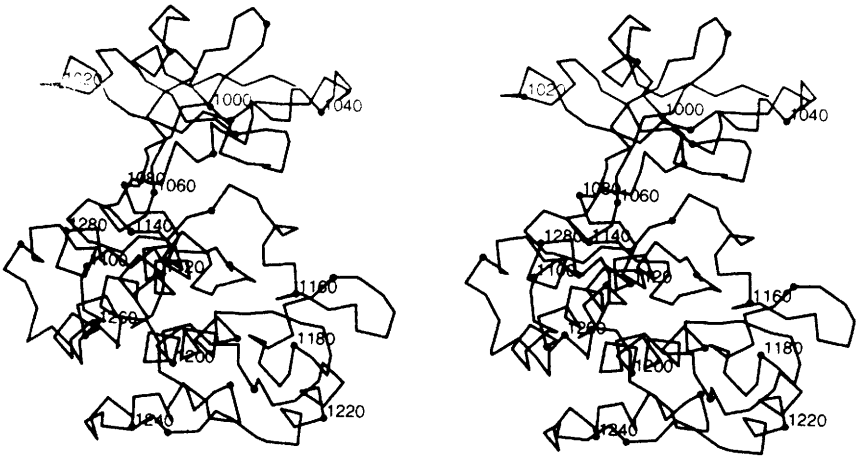

Figure 22 The insulin receptor kinase structure. Stereo view of the Cα trace of the protein. Every tenth residue is marked with a filled circle and every twentieth residue is labelled. Reproduced with permission from reference 245.

tramer. The binding of insulin to the extracellular α chain is thought to lead to a change in quaternary structure that results in autophosphorylation of specific tyrosines in the cytoplasmic portion of the β-chain. Three of the autophosphorylation sites are on tyrosine residues within the activation loop of the kinase domain, phosphorylation of which increases the activity of the kinase domain. This report is the first structure of a tyrosine kinase. The structure allows some mutations in the insulin receptors of patients with non-insulin dependent diabetes mellitus to be rationalised. The overall structure of the insulin receptor kinase is similar to the protein serine/threonine kinases.[246] The insulin receptor kinase is composed of two lobes (as shown in Figure 22). The smaller N-terminal lobe consists of five anti-parallel β strands and a single α helix and the larger C-terminal lobe comprises eight α helices and 4 short β strands. The activation loop, which includes three tyrosines that are not phosphorylated in the structure but are on activation, appears to act as an auto-inhibitory pseudosubstrate. It is also suggested that the structure observed in which one of the tyrosines is engaged in the active site and both substrate and ATP binding sites are inaccessible will be in equilibrium with another in which ATP is bound. It is suggested that the ATP-bound conformation will be stabilised by *trans*-phosphorylation of the three tyrosines in the activation loop only if the two kinase domains are correctly orientated.

10 Other Protein Structures

10.1 Acetyl-CoA Carboxylase – This enzyme catalyses the biotin-dependent carboxylation of acetyl CoA to form malonyl CoA which is the first committed

step in the synthesis of fatty acids. A study of the biotin-carboxylase subunit of this enzyme was described in 1994.[247] This subunit catalyses the first half reaction involving the ATP-dependent carboxylation of the 1′ nitrogen of biotin to form carboxybiotin. It is thought that the chemical mechanism of biotin carboxylase involves the reaction of bicarbonate and ATP to form a carboxyphosphate intermediate. This biotin carboxylase subunit folds into three domains. The N-terminal region folds as a dinucleotide binding motif. The second domain has two α helices and three β sheets and the third domain is an eight-stranded anti-parallel β sheet, a smaller three stranded antiparallel β sheet and seven α helices. Soaking experiments with a Ag^+ biotin complex have tentatively identified the active site in the third C domain.

10.2 D-Alanine: D-Alanine Ligase – D-alanine: D-alanine ligase catalyses the condensation of two D-alanines to yield D-Ala-D-Ala dipeptide which is one of the components of peptidoglycan of bacterial cell walls. The enzyme is a target for rational drug design. The enzyme structure has been solved in complex with ATP and an S,R-methylphosphinate inhibitor.[248] The fold of this enzyme is similar to *E.coli* glutathione synthetase and is divided into three domains. It is proposed that the catalytic mechanism is centred on a helix dipole and a catalytic triad of tyrosine, serine and glutamic acid able to assist binding and deprotonation steps as shown in Figure 23. The reaction proceeds by initial

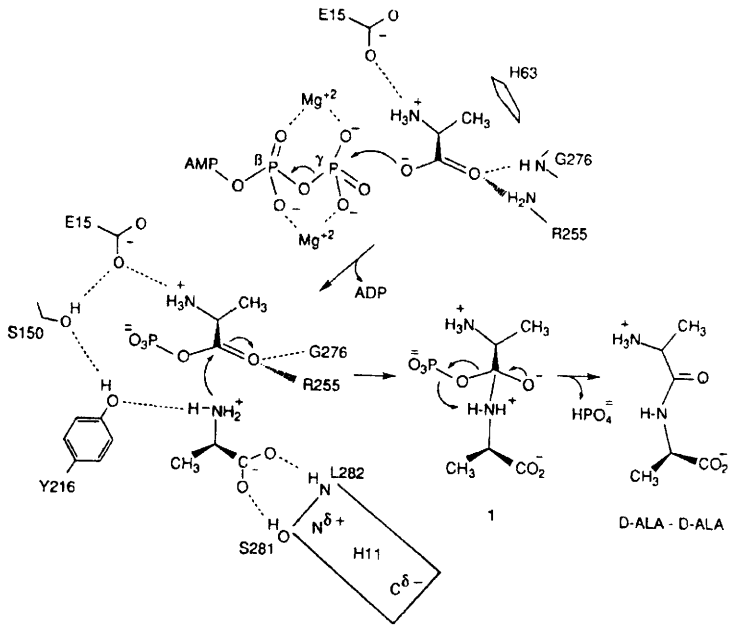

Figure 23 Diagram to show the catalytic mechanism of D-alanine : D-alanine ligase. Reproduced with permission from reference 248.

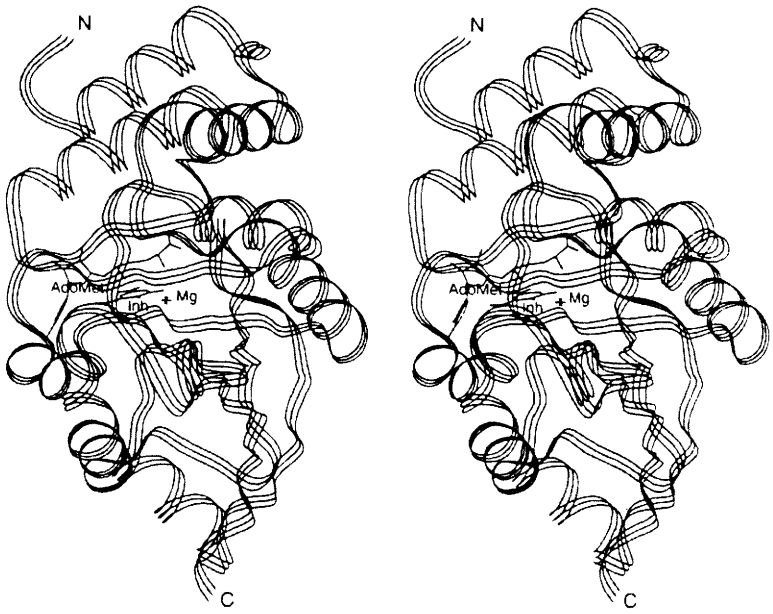

Figure 24 A stereo view of the enzyme catechol O-methyl transferase. The ligands AdoMet Mg^{2+} and inhibitor (Inh) are indicated. Reproduced with permission from reference 249.

attack on the γ-phosphate of ATP by the first D-alanine, whose N-terminal part is correctly positioned by Glu 15 to generate a D-alanyl-acylphosphate intermediate. The second D-alanine which is properly oriented by a hydrogen bond with Tyr216 and by an interaction with the macrodipole of helix H11, attacks this to form an intermediate that decomposes to D-Ala-D-Ala.

10.3 Catechol O-methyl Transferase – Catechol O-methyl transferase metabolises catecholamine neutrotransmitters such as dopamine, in the central nervous system. The enzyme catalyses the transfer of a methyl group from AdoMet to one hydroxyl group of catechols. Selective inhibitors of this enzyme are being investigated for the treatment of Parkinsons disease. The structure of this enzyme was described in 1994.[249] The structure is shown in Figure 24. The binding site for the inhibitor 3,5-dinitrocatechol is in a shallow groove on the enzymes surface. The catecholamine binding is thought to be very similar to the inhibitor binding. The methyl transfer from AdoMet to the catechol substrate is direct biomolecular transfer to the methyl group from the sulfur of AdoMet to the oxygen of the catechol substrate via an S_N2 like transition state.

10.4 Cellobiohydrolase I – This enzyme catalyses the hydrolysis of crystalline cellulose by cleaving chain ends. This enzyme structure has been solved from the

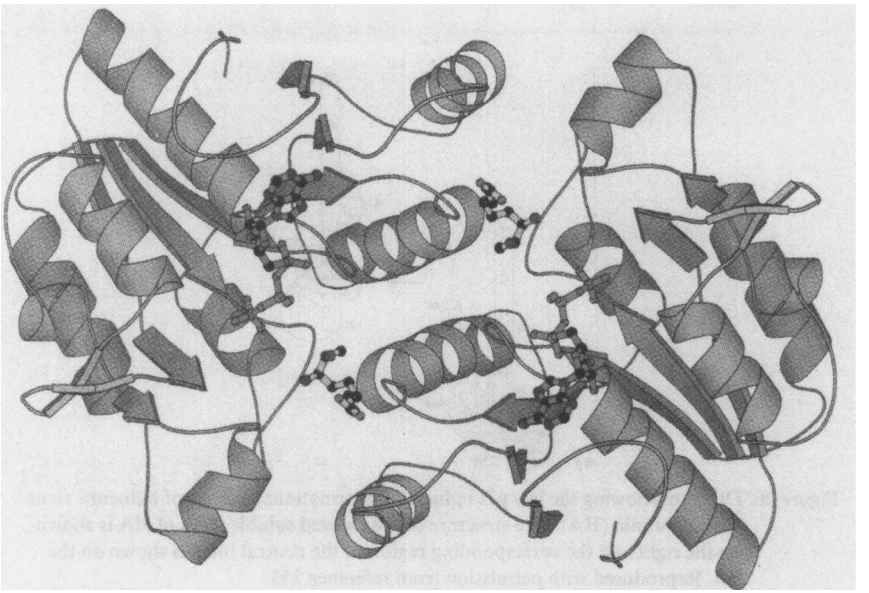

Figure 25 The structure of dethiobiotin synthetase dimer. The parallel β-sheet comprising β-strands β2, β5, β1, β6, β7 and β8 is wrapped around the helix α1. The ATP molecule has been modelled into the sulfate ion positions. The substrate modecule is also shown. Reproduced with permission from reference 252.

fungus *T. reesei*.[250] The enzyme structure is similar to a family of bacterial β-glycanases and has the main chain topology of legume lectins.

10.5 Dethiobiotin Synthetase – This enzyme catalyses the conversion of 7,8-diaminopelargonic acid to dethiobiotin which is the penultimate step in the synthesis of biotin. This enzyme uses carbon dioxide as a second substrate and requires Mg-ATP. The structure of the enzyme has been solved from *E.coli*.[251,252] The structure is a dimer and is shown in Figure 25. The substrate-binding site has been identified by soaking the non-hydrolysable ATP analogue adenyl-imidodiphosphate into crystals.[252] The enzyme works as a dimer and it is proposed that inter-monomer movements accompany the reaction to bring the substrate closer to ATP. This enzyme shows similar topology to the oncogene product p 21 and adenylosuccinate synthetase from *E.coli*.

10.6 Farnesyl Diphosphate Synthase – This enzyme is involved in the isoprenoid biosynthetic pathway. In humans it is necessary for the synthesis of farnesylated proteins, cholesterol and ubiquinones. The enzyme structure has been solved from chicken.[253] The protein represents a novel fold of 10 α helices. There are two asparate rich sequences necessary for enzyme activity. These are located on either side of a cleft near the surface of the protein. In the structure two

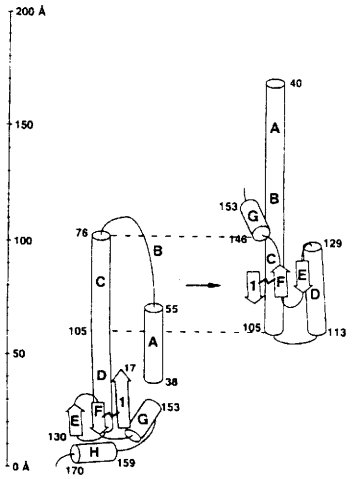

Figure 26 Diagram showing the law pH induced conformational change of influenza virus haemaglutinin (HA). The structure of the cleaved soluble form of HA is shown on the right and the corresponding region of the neutral form is shown on the left. Reproduced with permission from reference 255.

samarium atoms bind at these sites where it is thought that the substrate-magnesium complex binds during enzyme function. The structure of this enzyme is particularly important since it will enable the design of inhibitors to modulate cholesterol biosynthesis in humans.

10.7 β-Galactosidase – This enzyme structure has been solved from *E.coli*.[254] β-galactosidase catalyses the hydrolysis and transgalactosylis of β-galacto-pyranosides. In *E.coli* this enzyme is involved with the hydrolysis of the β1-4 linkage of lactose producing glucose and galactose and for the transgalactosylic formation of allolactose. It is proposed that the mechanism of this enzyme is a general acid-catalysed hydrolysis similar to that of hen white lysozyme. The β-galactosidase monomer has five domains made up from a 1023 residue polypeptide chain. The third domain which resembles a TIM-barrel forms the core of the monomer and contains the active site at one end. The active enzyme is a tetramer.

10.8 Haemoglutinin – This glyco-protein is necessary for infection of influenza virus. Its role in membrane fusion is induced at low pH where the protein undergoes a structural change. An interesting study has solved the structure of a soluble fragment of this protein at low pH which has given insight into the nature of this structural change.[255] Figure 26 shows a diagrammatic representation of this large conformational change that occurs in the protein structure.

10.9 Hevamine – Hevamine is a chitinase that is produced in plants after

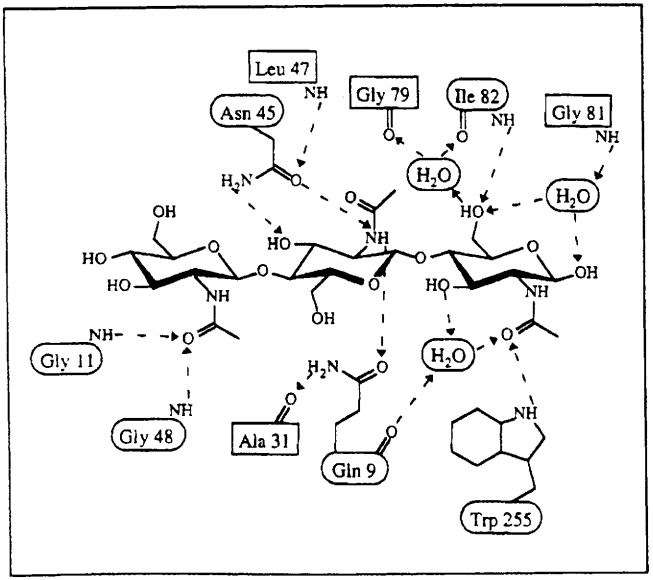

Figure 27 Diagram to show the interactions between the protein hevamine and the chitin fragment. Hydrogen-bonding contacts are shown between a trisaccharide and hevamine. The residues of the first and second hydrogen-bonding shell are depicted with ellipsoid and rectangular boxes respectively. Reproduced with permission from reference 256.

wounding. It can hydrolyse chitin which is a linear chain of β(1-4) linked N-acetylglucosamine residues and is a component of the cell wall of fungi and exoskeleton of anthropods. Hevamine from rubber tree is a $(\beta\alpha)_8$ barrel protein which shows lysozyme activity.[256] The protein has three disulfide bonds and the position of the active site has been confirmed by determining the structure with a bound chitin fragment. A 30 Å long binding cleft has been determined as shown in Figure 27. Hevamine is important for its potential to confer disease resistance in plants.

10.10 Inositol Polyphosphate 1-Phosphatase – This enzyme removes the 1-position phosphate from inositol 1,4-bisphosphate and inositol 1,3,4-trisphosphate yielding inositol 4-phosphate and inositol 3,4 bisphosphate. The inositol polyphosphates have important regulatory roles in the cell. This enzyme structure has been solved from cow.[257] It does not show significant sequence homology to other proteins but two short sequence motifs are also found in mammalian inositol monophosphatase and fructose 1,6-bisphosphatase. This similarity is also conserved at the structural level with a conserved core comprising 155 residues that form five α helices and 11 β strands.

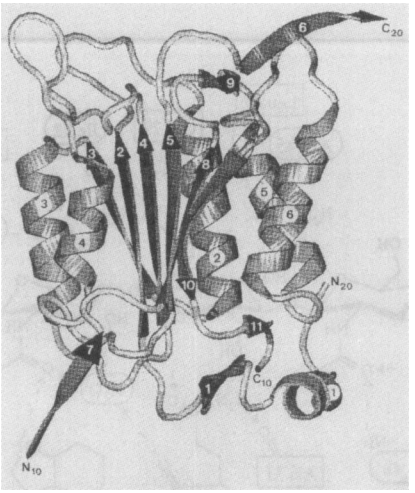

Figure 28 A ribbon diagram of the interleukin-1β converting enzyme. Reproduced with permission from reference 259.

10.11 Interleukin-1β Converting Enzyme – Two structures have been reported for this enzyme in 1994.[258,259] This is an important enzyme since it is a cytoplasmic cysteine protease that cleaves pro-interleukin 1β to give the active form of the protein. There is a sequence homology between this enzyme and the *Caenorhabditis elegans* cell death gene ced-3. This suggests that this family of cytoplasmic proteases may be involved in both the generation of cytokines and the mediation of apoptosis. The active form of the enzyme is composed of two subunits. The active cysteine is in one subunit although the other is required for activity. The minimal substrate for the enzyme is a tetrapeptide with an aspartate residue at the P1 site. Figure 28 shows a ribbon diagram of the interleukin converting enzyme heterodimer. A bound tetrapeptide inhibitor makes contact with both subunits and is covalently bound to the sulfur atom of the active site cysteine residue.[258] This appears to be a new class of cysteine proteases. It is suggested that the hydrolytic mechanism of this enzyme proceeds by activation of the cysteine residue by polarisation and/or deprotonation by a histidine residue resulting in a tetrahedral intermediate.

10.12 Glucose-6-phosphate Dehydrogenase – This is the first enzyme of the pentose phosphate pathway. The structure of this protein was solved to 2.0 Å resolution.[260] The enzyme is a dimer with each subunit consisting of a classic dinucleotide-binding domain and a large new fold β + α domain. This latter domain which has not previously been seen is predominantly antiparallel nine-stranded β sheet. The structure has allowed the determination of the active site of the enzyme.

The structure of glucose dehydrogenase has also been solved from the Archaeon *Thermoplasma acidophilum*.[261] The sequence of this enzyme shows no

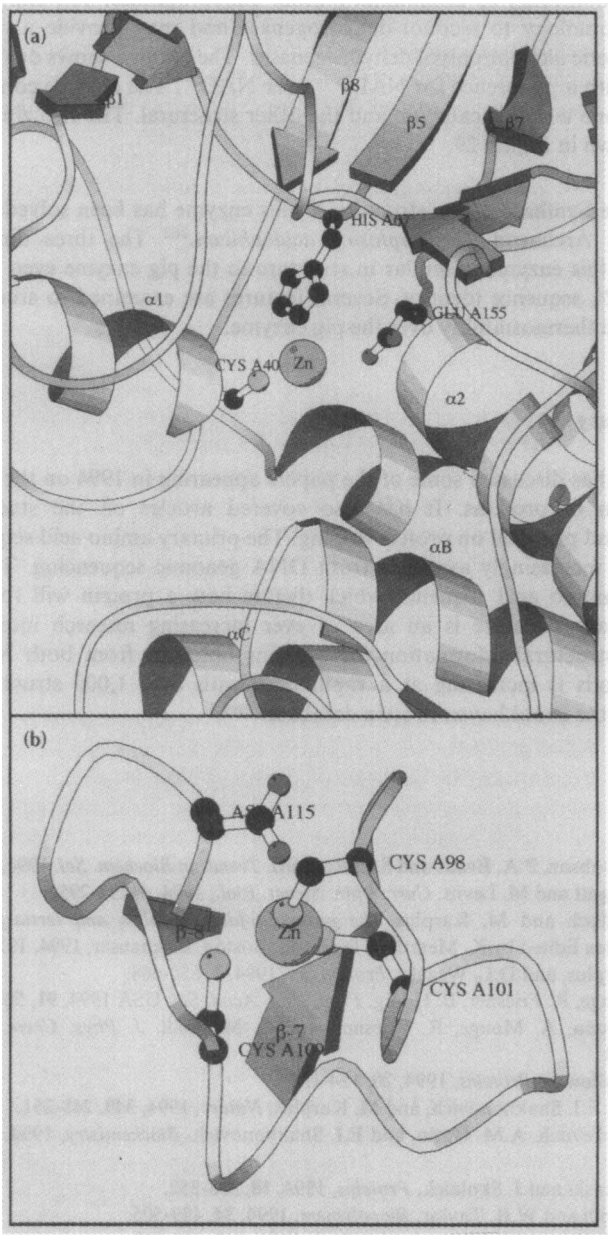

Figure 29 Schematic representation of the zinc binding sites of *Thermoplasma* glucose dehydrogenase a) the catalytic zinc, b) the structural zinc. Reproduced with permission from reference 261.

appreciable homology to eubacterial glucose dehydrogenase. The enzyme has a structural homology to alcohol dehydrogenase and may provide a model for other tetrameric alcohol/polyol dehydrogenases. The enzyme shows dual cofactor specificity with a preference for $NADP^+$ over NAD^+. The enzyme contains two zinc atoms one which is catalytic and the other structural. The two zinc binding sites are shown in Figure 29.

10.13 Citrate Synthase – The structure of this enzyme has been solved from the thermophilic Archaeon *Thermoplasma acidophilum*.[262] The three dimensional structure of this enzyme is similar in structure to the pig enzyme even though it has only 20% sequence identity. Several features are examined to study why it has increased thermostability over the pig enzyme.

11 Summary

The chapter has discussed some of the papers appearing in 1994 on the structure and function of proteins. It has also covered articles on the studies both theoretical and practical on protein folding. The primary amino acid sequences of proteins are increasingly available from DNA genomic sequencing. The subtle changes in amino acid sequence which dictate how a protein will fold into a specific tertiary structure is an area of ever increasing research interest. The amount of structural information for proteins obtained from both X-ray and NMR methods is increasing at a rapid pace with over 1,000 structures now deposited in the Brookhaven Protein database.

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