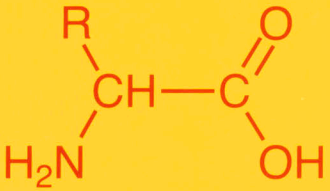

SPECIALIST PERIODICAL REPORTS

AMINO ACIDS, PEPTIDES AND PROTEINS

VOLUME 28

SENIOR REPORTER J.S. DAVIES

THE ROYAL SOCIETY OF CHEMISTRY

A Specialist Periodical Report

Amino Acids, Peptides and Proteins

Volume 28

A Review of the Literature Published during 1995

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Preface

We have reached an age when genetic engineering can generate orphan receptors awaiting suitable ligands to be discovered, bringing us to the era of inverse pharmacology. Prion proteins and their conformational changes are on their way to being acclaimed as the source of BSE and CJD and other diseases of the ageing process. Yet researchers in these fields complain that the lack of physical techniques to identify the modified prions is hampering developments. So as all our Reporters in this series once more report expansion and consolidation within their areas, we can only hope that amongst the hard work put into the original research, reported in 1995, there are developments which will assist in catching up with the increased demands at the frontiers. The strides taken in applying physical techniques to protein folding seem to be well advanced in this direction.

To bring together another compilation of published papers has again depended on long hours of dedication from my fellow reporters, listed on the title page of this book. The core of stalwart reporters from previous volumes remain a core source of Chapter reviews for this volume as well, and we welcome back Dr. Chris Schofield and his Oxford colleagues for their biennial look at the β-lactam scene, which is under continuous evolution in trying to win the battles against the microbes' wish to survive. Topics in Chapter 3 recently have been ably reviewed for us by Michael North and colleagues at Bangor. Due to other calls on his 'reviewing' time, Michael is withdrawing from the team for the next volume, but does so with much appreciation for his support for the series.

Two Peptide Symposia on the American (Columbus, Ohio) and European (Edinburgh) sides of the Atlantic have taken place since the appearance of Volume 27 in this series, and the books of Proceedings from them will serve as hors-d'oeuvres to await the appearance of full papers from which our Reports are compiled.

Once again, my sincere thanks go to my co-authors, and book editors at the RSC at Cambridge under the watchful eye of Janet Freshwater. It is their effort that has been instrumental in ensuring the continuation of this series into yet another volume – Diolch yn fawr.

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.

Recently the Joint Commission have 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.

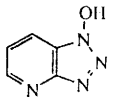
Chapter authors have been encouraged annually to include new abbreviations in their texts. With the ever increasing diversification in structures, lists of unusual abbreviations are periodically compiled. Some examples are listed below.

Abo	2-azabicyclo[2.2.2]octane-3-carboxylic acid
Abu	α-aminobutyric acid
A_2bu	2,4-diaminobutyric acid
ACCA	4-aminocyclohexanecarboxylic acid
εAhx	6-aminohexanoic acid
Aib	α-aminoisobutyric acid
Aic	2-aminoindan-2-carboxylic acid
A_2pr	2,3-diaminopropionic acid
Atc	2-aminotetralin-2-carboxylic acid
Ava	5-aminopentanoic acid
Aze	azetidine-2-carboxylic acid
Cha	3-cyclohexylalanine
Cpg	α-cyclopentylglycine
Cpp	1-mercaptocyclohexaneacetic acid, *or* β-mercapto-β,β-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

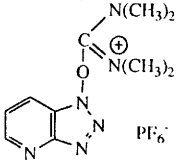
In the Abbreviations section of Volume 24 (1993) of this series, it was deemed timely to give structures to clarify the acronyms for the plethora of coupling agents currently in use. Since then the popularity of HATU and HOAt justifies structural clarification of these acronyms as follows:

HOAt



1-hydroxy-7-azabenzotriazole

HATU



O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate

1
Amino Acids

BY GRAHAM C. BARRETT

1 Introduction

The literature of the amino acids for 1995 is reviewed in this Chapter, aiming particularly at thorough coverage of developments in chemical and analytical areas. Although the literature covering routine biological studies of common amino acids is excluded, the more innovative biological and pharmaceutical work is covered. Scrutiny of the hard copy literature (the major journals, and *Chemical Abstracts* from issue 11 of Vol 122 to issue 9 of Vol 124 inclusive) has provided the citations that make up the Chapter.

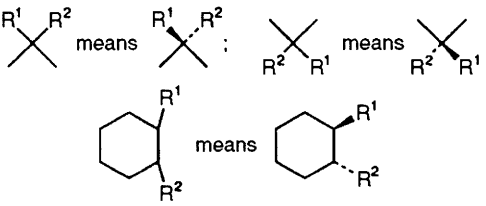
Continuity with preceding Volumes of this Specialist Periodical Report has been a prime consideration, so the Chapter has been sub-divided in the style used in all previous Volumes in this series. The device '(see p. XX, Vol YY)' that is used in this Chapter provides reference back to preceding Volumes, and helps the reader to keep track of important amino acid topics that have been developing over the years.

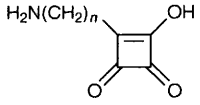
This Report has acknowledged in recent Volumes, that the term 'amino acids' has numerous meanings, but that the almost exclusive emphasis of this Chapter is on the α-aminoalkanoic acids. The reason for acknowledging this is the rising interest in the study of amino acids incorporating other oxyacid functions, particularly phosphorus analogues of the common aminoalkanoic acids. Short sections are included in this Chapter, on this topic, even though coverage must be

---

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:





(1)

selective in order that the literature of aminoalkanoic acids may receive thorough treatment. The unusually acidic 3-hydroxy-3-cyclobuten-1,2-dione grouping, established to be a bio-isostere of the carboxy group, has stimulated the synthesis of the corresponding amino acid analogues (1).[1]

2 Textbooks and Reviews

Several recent textbooks give either partial[2,3] or exclusive[4] coverage of amino acid topics. A symposium report[5] covers a wide range of recent studies on amino acids in higher plants.

Reviews have appeared covering derivatives of natural amino acids as radioprotectants,[6] and industrial aspects of the uses of amino acids.[7] The unusual amino acids hypusine [N^{ε}-(4-amino-2-hydroxybutyl)-L-lysine],[8] ovothiols (mercaptohistidines),[9] and the pyridinolines[10] have been reviewed from the point of view of their occurrence; members of the last-mentioned family, particularly pyridinoline itself and deoxypyridinoline, that derive from collagen breakdown, are present in urine at levels related to bone resorption activity, and these levels may be used as an osteoporosis index for individual patients (see also Ref. 849). The occurrence in proteins of the fluorescent crosslinking amino acid, pentosidine, has been reviewed;[11] its formation accompanies glycoxidation *in vivo*, and it accumulates at a greatly accelerated rate in uraemic patients; thus it is linked with the ageing process, and this suggests that its accumulation can be used as a diagnostic indicator. A review[12] covers the extensive literature on the non-natural amino acid threo-dihydroxyphenylserine, whose importance lies with the fact that it undergoes L-aromatic amino acid decarboxylase-catalyzed decarboxylation to give neurally-active norepinephrine.

Other recent reviews are more appropriately located in later Sections of this Chapter.

3 Naturally Occurring Amino Acids

The work described in this Section concentrates mainly on new amino acids, and on the more unusual of the known amino acids discovered in previously-unknown natural compounds.

3.1 Isolation of Amino Acids from Natural Sources – If reliable results are to emerge from such endeavours, then reliable methods of isolation of individual amino acids from complex mixtures are needed. This section has been introduced

into this Chapter in recent years as a collection point for citations on apparently routine current work that often includes salutary warnings of sources of error and contamination. Analytical and preparative scale purification of amino acids is covered in later sections of this Chapter.

A commercial cation exchange resin has been found[13] to be contaminated with several common amino acids (e.g. leucine: 260 pmol ml^{-1}resin). It is a mixed blessing, that there is no satisfactory method of clearing this background, which could have serious consequences for the reliable quantitation of amino acids in at trace levels if released by the resin into analytical samples. Concentration of solutions of amino acids can be achieved using ion exchange membranes,[14] and ion exchange chromatography has been used for large-scale purification of phenylalanine.[15] Other purification procedures with a similar context, for phenylalanine (continuous emulsion liquid membrane separation),[16] and for the separation of isoleucine from valine (kerosene – D_2EHPA partition),[17] rely on different physical principles. The extraction of phenylalanine and tyrosine from aqueous solutions, using hydrophilic solvents, has been precisely formulated,[18] a resource that may help in the work-up of complex amino acid mixtures. Milligram scale preparative HPLC allows the isolation of pure (96-99%) individual amino acids from mixtures, as their N-benzyloxycarbonyl derivatives.[19] The recovery (68-89%) of amino acids from mixtures in this way should surely be capable of improvement.

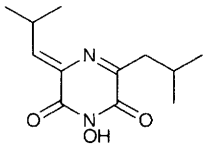
3.2 Occurrence of Known Amino Acids – Selection for this section is limited by excluding routine work with common amino acids; but the identification of αα-dialkyl α-amino acids in geological samples (they are minor components),[20] of common amino acids in resinous and other non-protein materials used by artists,[21] and in amber-entombed insects,[22] seem to be eminently worthy of mention. Samples for the last-mentioned study were aged from <100 y to 130×10^6y; the interpretation of amino acid racemization data determined for these samples would have given much younger ages; racemization rates for common amino acids must suffer retardation by the amber environment by a factor greater than 10^4.

Streptomyces akiyoshiensis cultures accumulate N-acetyl-L-DOPA,[23] which is shown in this study not to be involved in the biosynthesis of 5-hydroxy-4-oxo-L-norvaline (the major metabolite of this bacterium). Fungal and other plant sources shown to contain unusual amino acids include *Tricoloma muscarium* (ibotenic acid; the first report of this isoxazole derivative in a mushroom not belonging to the genus *Amanita*),[24] *Ateleia glazioviana Baillon*, a tree that is insect-repellent and toxic to cattle (1-aminocyclobutane-1,3-dicarboxylic acid and δ-acetylornithine),[25] and roots of *Glycyrrhiza yunnanensis* (NNN-trimethyl tryptophan betaine).[26]

Rhizocticins A, B, and D contain (Z)-L-2-amino-5-phosphonopent-3-enoic acid,[27] an α-amino acid already described to be a component of plumbemycins but thought to be of the D-configuration in these members of the latter family, whose structures are now in need of correction as far as absolute configuration is concerned. Structures have been established for new microcystins, which contain

2-aminobuten-2-oic acid.[28] Cyanobacteria (blue-green algae) are already known to produce bioactive cyclic peptides, and new examples are: anabaenopeptins A and B (*Anabaena flos-aquae* NRC 525-17) that contain, together with other common L-amino acids, D-lysine, N-methyl-L-alanine and homo-L-tyrosine;[29] and the cyclic depsipeptide oscillapeptin (*Oscillatoria agardhii* NIES-204)[30] together with the cyclic hexapeptide oscillamide Y,[31] which contain homotyrosine and N,O-dimethyl-L-tyrosine (in the former case) and N-methyl-L-alanine (in the latter case) amongst other constituents. The dioxopiperazine, flutimide (2) has been isolated from a new fungus *Delitschia confertaspora*.[32]

The rapidly accumulating literature covering the discovery of D-enantiomers



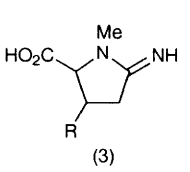
(2)

of the common amino acids in natural sources is illustrated for D-serine in rat brain and D-aspartic acid in peripheral organs[33] (see Ref. 897 for the identification of D-amino acids in serum samples). The occurrence of D-aspartic acid in proteins, and the broader picture concerning *in vivo* racemization of amino acids, have been reviewed.[34] D-Alanine occurs in free form in 15 out of 24 species of marine micro-algae.[35]

3.3 New Naturally Occurring Amino Acids – New aliphatic α-amino acids include (2S,3S,4R)-β-hydroxy-γ-methyl glutamic acid and its (2S,3R,4R)-epimer (together with pipecolic acid and 5-hydroxypipecolic acid) in seeds of *Gymnocladus* dioicus (see also Ref. 828),[36] (S)-cis-2-amino-5-chloropent-4-enoic acid in *Amanita vergineoides*,[37] (2S)-2-amino-5-chloro-4-hydroxyhex-5-enoic acid from *Amanita gymnopus* fruit bodies (together with (2S)-2-aminohex-5-enoic acid a first natural occurrence of an amino acid already known in the laboratory and (2S)-2-aminohexa-4,5-dienoic acid and (2S)-2-aminohex-5-ynoic acid).[38] The pyroglutamic acid relatives (3; R = OH, or H), found in *Streptomyces sp. SA-3501*, have been christened pyrostatins A and B, respectively; they have potential importance due to their role as inhibitors of N-acetyl β-glucosaminidase.[39]

New natural aromatic and heteroaromatic α-amino acids include purealidins J – R [nine new bromotyrosine derivatives, e.g. (4) and (5), from *Psammaplusilla purea*, an Okinawan marine sponge],[40] and the cysteine derivative (6) from *Streptomyces SB212305*.[41] A new mycosporin-like amino acid (7) from the reef-building corals *Pocillopora damicornis* and *Stylophora pistillata* contains methylamine in place of the glycine moiety usually seen in the mycosporins.[42]

The novel β-amino acid (S)-3-amino-5-mercatopropionic acid (8) occurs in caledonin (from the marine tunicate *Didemnun rodriguesi*),[43] while the higher



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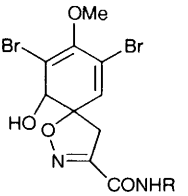

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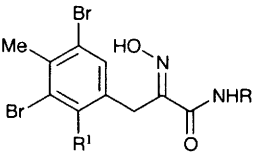

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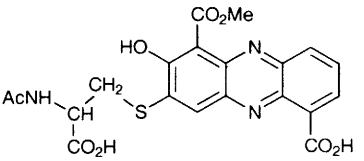

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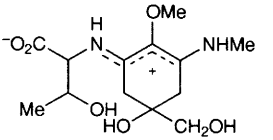

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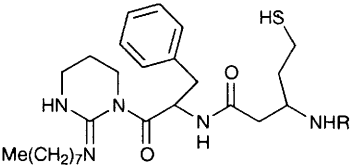

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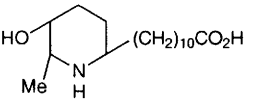
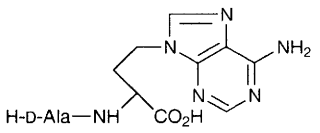


(9)

homologue (9) is one of four new piperidine alkaloids from leaf extracts of *Cassia leptophylla*.[44]

3.4 New Amino Acids from Hydrolysates – The title of this Section, though cumbersome, adequately accommodates details of work through which the presence of new amino acids bound up into amides of varying structures, and esters and close analogues, has been revealed.

The dipeptide (10) incorporating a novel adenine derivative has been isolated from the fungus *Taloromyces* NK 374200.[45] 2-Oxohistidine has been established to be a constituent of oxidatively-modified proteins,[46] and β-hydroxyhistidine is only one extraordinary feature of exochelin MN (11), the extracellular siderophore from *Mycobacterium neoaurum* that transports iron into *Mycobacterium leprae*.[47] Phosphocysteine is a constituent of a PTS-protein from *Staphyllococcus carnosus*.[48] A β-hydroxy-γ-chloroproline residue is a notable feature of the cyclic pentapeptide astin I (from *Aster tataricus* roots),[49] while trans-2,3-cis-3,4-dihydroxyproline (12) occurs repeatedly in the sequence of the byssus protein from the marine mussel *Mytilus edulis*.[50] The trichlorovaline subunit present in dysidenin (13) from the marine sponge *Dysidea herbacea* is also represented in a novel chlorinated ketide amino acid, herbamide A (14), from the same source.[51]



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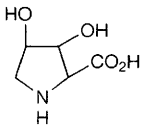

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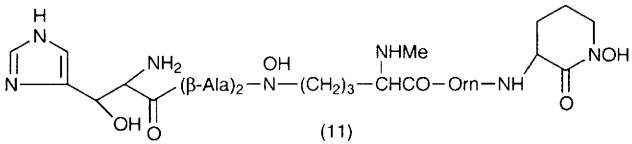

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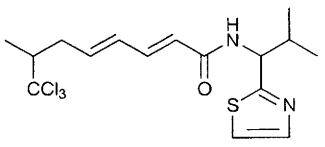

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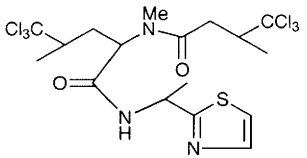
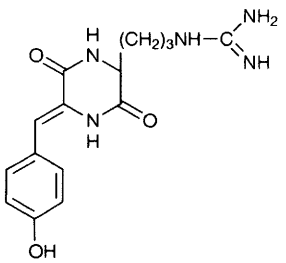


(14)

A new metabolite of the cyclosporin-producing fungus *Tolypocladium terricola* is identical with cyclosporin D except for the presence in this cyclic peptide, of hydroperoxy-MeBmt (i.e. 3-hydroxy-7-hydroperoxy-4-methyl-2-methylamino-5E-octenoic acid).[52]

Dioxopiperazine-2,5-diones (*alias* cyclic dipeptides) have featured in this section over the years, and have continued to show increasingly surprising structures, and they often have useful medicinal properties. Recent examples range from the dehydrotyrosine derivative (15) (together with new pipecolic acid derivatives) from the sponge *Anthosigmella aff. raromicrosclera*,[53] the tryptophan derivatives maremycins A and B (16) from a marine *Streptomyces sp.*,[54] leptosins K, K_1, and K_2,[55] and tryprostatins A and B (17; R = H, OMe, respectively) from *Aspergillus fumigatus BM939*.[56] Dehydrogenated derivatives macrophominol (18)



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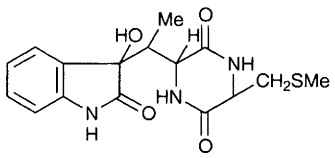

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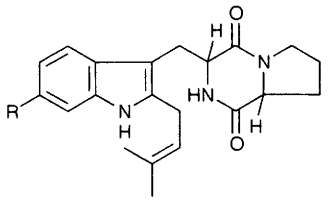

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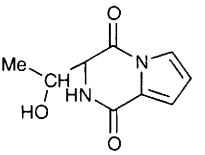

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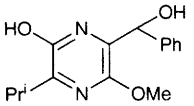

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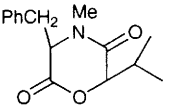


(20)

from *Macrophomina phaseolina,*[57] terezines A – D (19) from *Sporormiella teretispora,*[58] and the morpholin-2,5-dione bassiatin (20) from *Beauveria bassiana K-717*[59] are notable new naturally-occurring amino acid derivatives.

4 Chemical Synthesis and Resolution of Amino Acids

4.1 General Methods for the Synthesis of α-Amino Acids – The methods that have been used for many years are well trusted, and sufficiently broad in their scope to accommodate new and current needs. However, innovation in organic synthesis continues at its usual rapid pace within the amino acids field, as elsewhere, and some new ideas (as well as fresh-looking results that are in fact extensions of existing knowledge) have been a feature of this Section over recent years. The elaboration of side-chains of the readily available α-amino acids is increasingly being chosen for the synthesis of other amino acids, and this literature is covered in a later Section (Section 6.3; Specific Reactions of Amino Acids).

Advances in methodology are more noticeable in aspects of asymmetric synthesis (see next Section), and in some cases these can also be considered to be advances in general methods of synthesis.

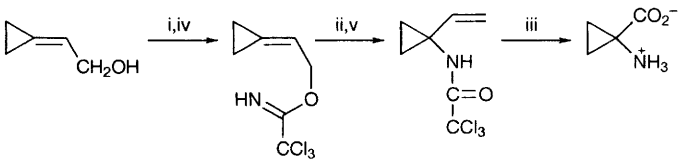
Reviews have appeared covering α-cation equivalents of amino acids[60] and the 1994 literature on the synthesis of amino acids, amides, and peptides.[61] Synthesis of α-amino acids from higher fatty acids,[62] and synthesis through photolysis of chromium(II)-carbene complexes in the presence of nucleophiles (see Vol 27, p. 15, and Ref. 118 for further examples), have been reviewed.[63]

Standard routes illustrated in the 1995 literature are the Bucherer-Bergs synthesis (αα-disubstituted α-amino acids from 3-substituted cyclopentanones;[64] see also Ref. 718) and its near relative seen in the preparation of phenylglycine from $PhCHO/CHCl_3/NH_3$ in aqueous NaOH containing Bu_4NBr.[65] The Ugi

synthesis continues to provide a direct approach in suitable cases (Refs. 258, 295). The Strecker synthesis has been used for the preparation of 3,5-dimethoxyphenylglycine constituents of vancomycins.[66] α-Bromo-substitution (CBr_4) of the 2-methoxycarbonylpyran α-proton can be readily achieved after anion formation using lithium bis(trimethylsilyl)amide, if substituents elsewhere in the ring place this proton in the equatorial plane; ensuing azidolysis and reduction to the αα-disubstituted glycine proceeds normally.[67] Substitution of triflate by azide, and elaboration into α-substituted α-amino acids, has also been used in this study of the preparation of glucofuranose- and glucopyranose-based hydantoins[68] and related dioxopiperazines[69] as analogues of the potent herbicide, hydantocidin.

Conversion of chiral α-hydroxy acid derivatives into amino acid analogues can be accomplished by the Mitsunobu protocol, illustrated this year for N-alkylation of 2-(trichloroethyloxycarbonylamino)thiazole by ethyl (S)-lactate.[70]

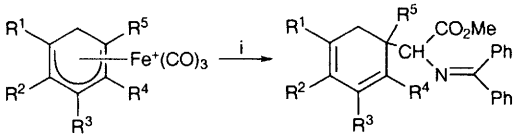
There are numerous examples elsewhere in this Chapter, of applications of classical rearrangements that deliver a nitrogen grouping to carbon, in a manner appropriate for amino acid synthesis [Curtius (e.g. Refs. 183, 207, 287), Hofmann (Ref. 265), Beckmann (Ref. 423) and Schmidt rearrangements among others], and a further example of the aza-Claisen rearrangement of an allyl trichloroacetimidate has offered a new entry to 1-amino cyclopropanecarboxylic acids (Scheme 1).[71]



Reagents: i, Cl_3CCN,NaH; ii, 100 °/toluene, 48 h; iii, aq $NaIO_4$, $RuCl_3$; iv, alternatively *p*-$MeOC_6H_4CCl{=}NH$; v, alternatively, $PdCl_2(PhCN)_2$

Scheme 1

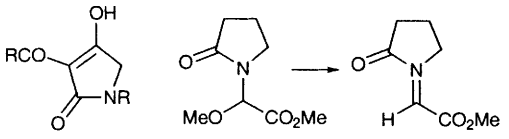
Examples of alkylation of familiar glycine synthons illustrating general routes are found in the phase-transfer catalyzed alkylation of diethyl acetamidomalonate with weakly electrophilic alkyl halides[72] [see also Ref. 296; diethyl formamidomalonate (Ref. 286) and ethyl acetamidocyanoacetate (Ref. 285) have also been used for similar purposes], phase transfer-catalyzed Michael anti-addition of $(MeS)_2C{=}NCH_2CO_2R^1$ to αβ-unsaturated esters[73] (see also Ref.132), and alkylation of $Ph_2C{=}NCH_2CO_2R$ with cyclohexadienyliron π-complexes (Scheme 2)[74] and with 1,ω-dichloroalkanes [giving bis(α-amino acids)].[75] Condensation of diazoacetylglycine methyl ester with an aldehyde, and Bu_4NF cyclization of the resulting β-keto-amides, gives a 3-acyltetramic acid (21; these can be categorized in other ways, as cyclized δ-amino acids for example).[76] Alkylation of the α-bromoglycine derivative $R^1R^2NCHBrCO_2R^3$ by alkyl nitronates gives α-halogeno-, β-nitro-, and αβ-dehydro-amino acid derivatives.[77] Ammonia reacts with methyl N-benzoyl-2-bromoglycinate to yield trimethyl



Reagents: i, $Ph_2C{=}NCH_2CO_2Me$, then LDA

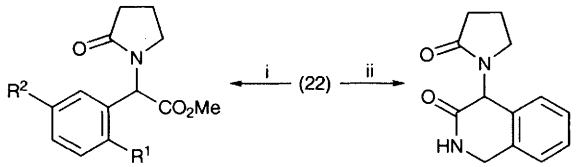
Scheme 2

2,2′,2″-nitrilotris[2-(benzoylamino)acetate] as a 6:1-mixture of diastereoisomers, which can be separated by crystallization.[78] α-Methoxyglycine derivatives (*alias* glyoxylic acid – amine adducts) continue to reappear in different applications, e.g. the 2-pyrrolidinone adduct (22) or its pyroglutamic acid analogue, that is susceptible to arylation *via* its N-acyliminium ion (Scheme 3).[79] An example of an unfamiliar glycine synthon is carbethoxyformonitrile oxide, used as Michael donor to alkylate a vinyl ester in a synthesis of the racemic form of the natural sweetener, monatin (Scheme 4).[80]



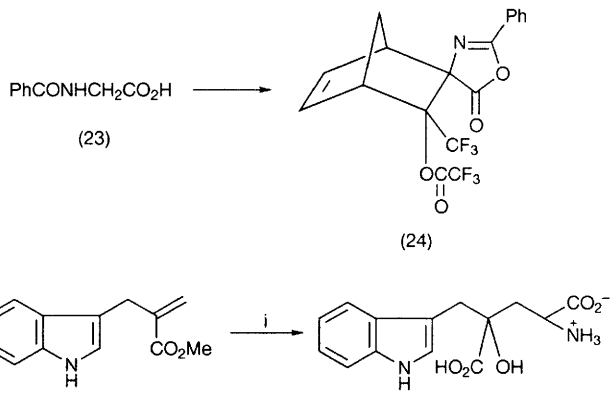
(21) (22)

The oxazolone (*alias* azlactone) route, based on alkylation at C-4 of a 2-substituted oxazol-5(4H)-one obtained by dehydrative cyclisation of an N-acyl-α-amino acid, has been used for the synthesis of γ-(1,2,4-triazin-5-yl)butyrines,[81] β-phosphonioethylglycines $[R^1CONHCR^2(CO_2R^3)CH_2CH_2PPh_3{}^+Br^-]$[82] and novel open-chain and constrained tyrosine analogues (see Refs. 191,192). Hippuric acid undergoes unconventional alkylation with trifluoroacetic anhydride and cyclopentadiene (23→24).[83]



Reagents: i, H_2SO_4, $R^1C_6H_4R^2$; ii, $PhCH_2NH_2$, then methanesulfonic acid, 6 days

Scheme 3

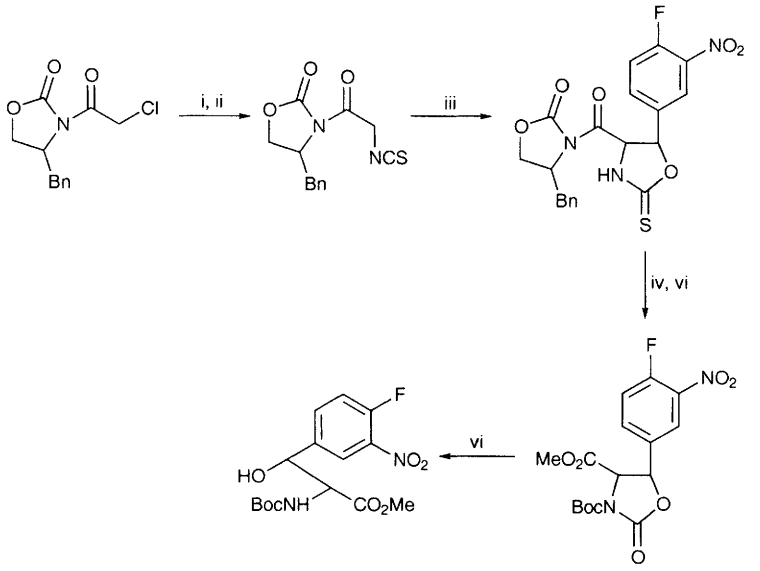


Reagent: i, $EtO_2CCOCNO$, then hydrolysis

Scheme 4

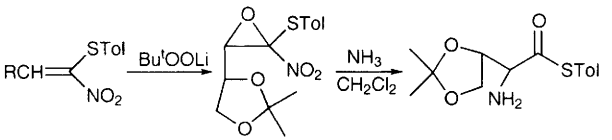
4.2 Asymmetric Synthesis of α-Amino Acids – The 1995 literature covering the asymmetric synthesis of α-amino acids mainly amounts to a consolidation of existing methods, though the modifications introduced by some workers when revisiting established protocols occasionally provide valuable steps forward.

Direct applications of some of the general methods of DL-α-amino acid synthesis covered in the preceding Section, so as to give an α-amino acid enantiomer, continue to be studied. One such example is a highly diastereoselective Strecker synthesis employing α-phenylglycinol as chiral auxiliary.[84] This procedure has also been used for a synthesis of the 3,4,5-trihydroxyphenylglycine moiety of vancomycin.[85] The same paper describes a chiral auxiliary approach (see later discussion) to the vancomycin constituent (2S,3R)-β-(4-fluoro-3-nitro)-phenylserine in which the appropriate aldehyde is reacted with a chiral isothiocyanate (Scheme 5). α-Aminonitriles serve as starting material in some versions of the Strecker synthesis, and have been prepared by addition of trimethylsilyl cyanide to a chiral nitrone with essentially total syn-stereoselectivity.[86] Further illustration by way of a stereocontrolled Strecker synthesis, in which ammonia is reacted with 2-(arylthio)-2-nitro-oxiranes to give α-amino thioesters in good yield, has been published.[87] The examples of synthesis targets given, β-hydroxy-α-amino acids and α-amino dicarboxylic acids, include the γ-hydroxythreonine derivative (25) and polyoxamic acid. Intramolecular amidoalkylation of 2- or 3-vinyl- or phenylsiloxyaminoacetals, occurring after their conversion into imines or iminium ions with Lewis acids, has been exploited for syntheses of Z-allo-L-threonine and N-ethoxycarbonyl anti-γ-hydroxy-DL-norvaline.[88] N-Alkyl α-amino acids have been obtained through reductive alkylation of optically-pure α-azido acids using bromodimethylborane.[89] This exemplifies a fresh approach to the standard synthesis of amino acids involving amination of a halide, in this



Reagents: i, NaN_3, Bu_4NHSO_4, $CH_2Cl_2 - H_2O$; ii, CS_2, PPh_3; iii, $Sn(OTf)_2$, base, 4-fluoro-3-nitrobenzaldehyde; iv, MeMgBr, then Boc_2O/DMAP; v, H_2O_2; vi, $CsCO_3$, MeOH

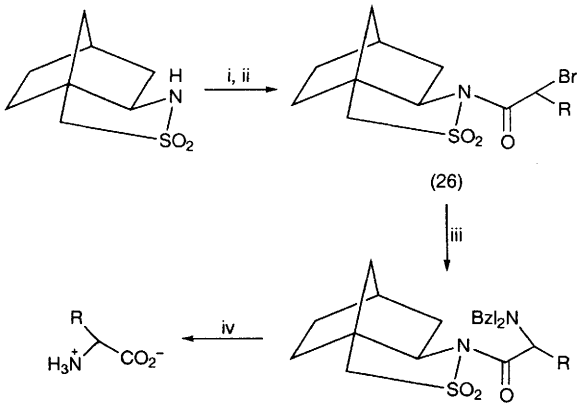
Scheme 5



example taking advantage of newly-introduced reagents. This is also seen in α-stannylation of optically-active enecarbamates with tri-alkylstannyl chlorides, after α-lithiation, then palladium-catalyzed coupling with an acid chloride to give an α-keto enecarbamate, or carboxylation ($BuLi/CO_2$) to give an optically active carbamatoacrylate, leading to an enantiomer of an α-amino acid through addition of a Grignard reagent.[90] Enantiospecific carboxylation using CO_2 is a rare event in the laboratory, and has been accomplished with N-Boc-N-methyl-benzylamine after enantioselective deprotonation with the Bu^sLi/(-)-sparteine complex, to give Boc-N-methyl-D-phenylglycine.[91]

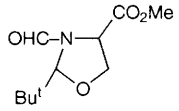
Uses of 'chiral glycine' derivatives continue to provide the greatest volume of literature in this area, with 8-(-)-phenylmenthyl N-Boc-α-bromoglycinate acting as substrate for S_H2' alkylation by allyl tri-n-butylstannanes, a process that

incorporates high diastereoselectivity.[92] Conjugate addition of a chiral glycine synthon [the bis-lactim ether lithium salt; see (30) in Scheme 11] to β-substituted vinyl sulfones starts a route to threo-(2S,3R)- or erythro-(2S,3S)-3-methylphenylalanine and tryptophan.[93] (R,R)-(-)- and (S,S)-(+)-Pseudoephedrine-derivatized glycinamides have been prepared[94] and used[95] for asymmetric synthesis of L- and D-amino acids respectively, through highly diastereoselective alkylation. Surprisingly, there is no need to protect the functional groups in these glycinamides, but it is not obvious why substantial N- and O-alkylation does not accompany the intended C-alkylation. α-Benzamidocinnamic esters of N-methylephedrine or of mandelic acid[96] have been shown to undergo highly diastereoselective alkylation. In the last-mentioned example, the alkylation step took the form of pyrazoline ring formation with diazomethane, and ensuing pyrolysis to give (1R,2R)-, (1S,2S)- or (1S,2R)-1-amino-2-phenyl- or alkyl-cyclopropanecarboxylic acids. Several other established substrates have been employed in this approach, and Oppolzer's chiral sultam has become increasingly popular (see also Refs. 97, 98, 101, 193, 194, 334); starting from DL-α-bromoalkanoic acids (see also Ref. 106). α-Amino acids have been secured in diastereoisomerically-enriched form from this synthon *via* α-bromoamides after nucleophilic substitution by dibenzylamine (Scheme 6).[97] More conventional use of this lactam (26 in Scheme 6; H in place of Br) is illustrated in Pd-catalyzed allylation by $R^5R^3C{=}CR^4CR^1R^2OR$ in a preparation of α-allyl-α-amino acids[98] and in the preparation of the currently-in-vogue (p-phosphonomethyl)-L-phenylalanines (see also Section 4.12).[99] The fully-protected methyl (2S,4R)-oxazolidine-4-carboxylate (27) has been used for a synthesis of components of mycestericins E and G,[100] and using Seebach's imidazolidin-4-one approach for the synthesis of β-{1,2-dicarba-closo-dodecaborane(12)cage}-substituted alanines [the (S)-config-



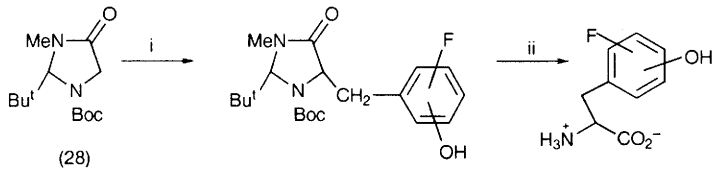
Reagents: i, TMSCl, then NEt_3; ii, RCHBrCOCl, $CuCl_2$; iii, 10 equiv. BzI_2NH; H_2/Pd–C, and hydrolysis

Scheme 6



(27)

uration was assigned to the (+)-enantiomers that were obtained in e.e. >98%].[101] 1-Benzoyl-2-alkyl-3-(1′-(R)- or (S)-methylbenzyl)imidazolidin-4-ones have been shown to undergo highly-diastereoselective alkylation, leading to corresponding α-amino acids by acid hydrolysis.[102] Alkylation of this synthon with heteroaryl halides gives the corresponding α-amino acids.[103] The 2-tert-butyl imidazolidin-4-one [(28) in Scheme 7; it has become abbreviated '(S)-Boc-BMI'] has been used for syntheses of (S)-2- and 4-fluoro-m-tyrosine and of fluoro-tyrosine.[104] A new chiral auxiliary to add to this family is tert-butyl 2-tert-butyl-5,5-dimethyl-4-oxoimidazolidine-1-carboxylate, prepared from L-alanine, α-aminoisobutyric acid and pivaldehyde,[105] and used in syntheses of hindered α-alkyl α-amino acids.



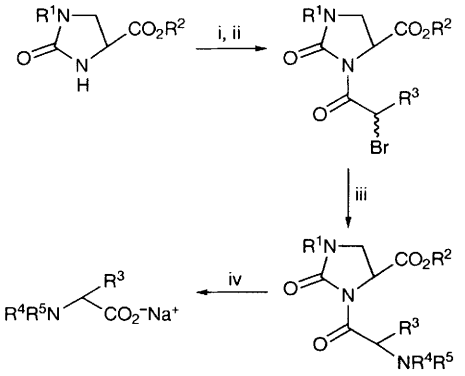
(28)

Reagents: i, F,OH-C_6H_3-CH_2Br, base; ii, hydrolysis

Scheme 7

A variant of this procedure, employing an N^1-alkylimidazolidin-2-one-4-carboxylic acid as novel chiral auxiliary, has been used for stereospecific amination of the derived N-[α-bromoacylated] derivative (Scheme 8), a useful feature being the dynamic kinetic resolution due to epimerization at the side-chain chiral centre that accompanies the amination step, enhancing the yield of the favoured diastereoisomer in some cases,[106] and this feature has been given a particularly thoughtful study for the corresponding auxiliary (S)-tert-butyl 1-methylimidazolidin-2-one-4-carboxylate[107] from which N-benzyl L-amino acids could be prepared after N-acylation by a DL-α-bromoacyl chloride.

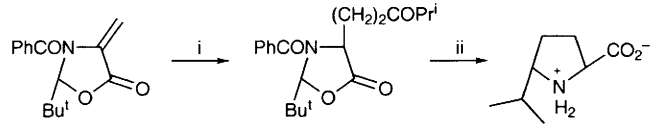
The related oxazolidin-2-one approach is illustrated by syntheses of N^{α}-Fmoc-N^{ε}-Boc-α-methyl-D-ornithine using the oxazolidinone prepared from Z-L-alanine.[108] Preparation of an appropriate oxazolidinone involves potentially hazardous borane reduction and the intermediacy of water-soluble amino alcohols, thus reducing the yields, and these drawbacks are avoided in a thoroughly researched protocol for the synthesis of the (4R)-(-)-phenyloxazolidinone from D-phenylglycine.[109] 4-Diphenylmethyl oxazolidin-2-one is another new chiral auxiliary given thorough testing through representative protocols.[110] A useful variant leading to β-substituted alanines has been given further study leading to (5R,2S)- and (5R,2R)-5-isopropylprolines through conjugate addition



Reagents: i, KOBu^t, THF, –50 °C; ii, $R^3CHBrCOBr$; iii, R^4R^5NH; iv, MeONa, MeOH

Scheme 8

of enamines (Scheme 9).[111] 1,3-Dipolar cycloaddition to this chiral synthon of azomethine ylides derived from N-benzylidene-amino acid esters leads to polyfunctional 2,3-disubstituted prolines of high enantiomeric purity.[112] Use of the norleucine-derived oxazolidinone with N-bromomethylphthalimide as alkylating agent has given (S)-α-aminomethyl-norleucine.[113] Diastereoselective radical addition to these methyleneoxazolidinones is favoured by certain N-protecting groups, and for certain radical species.[114]



(major product)

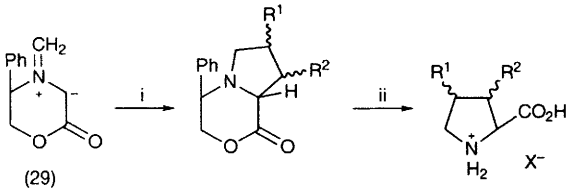
Reagents: i,  , 16 h, RT; ii, Reflux, 6M HCl, then H_2/Pd–C

Scheme 9

The N-acylation of homochiral oxazolidinones, pioneered by Evans (see also Refs. 324, 384) and used by Hruby's group (e.g. to prepare all four isomers of β-methyl-2′,6′-dimethylphenylalanine[115]), has been thoroughly exemplified in preceding Volumes of this Specialist Periodical Report; as has Hruby's work, already committed to the literature in detailed preliminary communications for this compound, and for syntheses by his group of β-methyl-phenylalanine[116] and β-methyl-2′,6′-dimethyltyrosine.[117] Further details of the photolysis of the homochiral N-[(pentacarbonyl)chromium(II)ketene]-oxazolidinone (see also Ref.

63) in the presence of free amines and free, highly-hindered amino acids, leading to amino acid amides, have been given.[118]

Analogous use of the Williams chiral glycine template, benzyl (2R,3S)-2,3-diphenyl-6-oxomorpholine-4-carboxylate, has been reported (see also Ref. 289),[119] also a synthesis of γ-carboxy-L-glutamic acid in excellent (>99%) enantiomeric excess through Michael addition to di tert-butyl methylenemalonate (this paper also provides details of a synthesis of the morpholinone from benzoin oxime).[120] A simpler analogue of this chiral auxiliary (29 in Scheme 10), readily available from (S)-2-phenylglycinol, has served in a chiral synthesis of functionalized prolines through cycloadditions to the derived azomethine ylide.[121] Enantio-



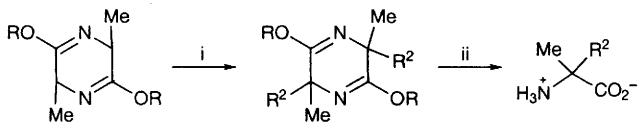
(29)

Reagents: i, $R^1CH{=}CHR^2$; ii, H_2/Pd-C, H_3O^+

Scheme 10

selective synthesis of arylglycines from arylacetic acids proceeds *via* the chiral oxazoline formed with (R)-(-)-2-aminobutanol, but leads only to low optical yields (5-10%).[122]

The piperazinedione prototype that paved the way for all these heterocyclic chiral auxiliaries is illustrated for a synthesis of α-trifluoromethyl-α-amino acids, starting from the 3-hydroxy-3-trifluoromethylpiperazinone.[123] It is now more often seen in the form of its bis-lactim ether derivative (30 in Scheme 11); a typical use (see also Refs. 128, 174, 291, 294, 323, 335, 336, 350) is demonstrated in the preparation of α-methyl-D-serine and its homologues[124] and of L-(-)-6-chloro-5-hydroxytryptophan (a constituent of keramamide A) and its D-(+)-enantiomer,[125] as well as 2-bromo-7-hydroxy-L-tryptophan (a constituent of konbamide and jaspamide).[126] Radical addition to (S)-3-methyl-6-methylene-piperazine-2,5-dione is accompanied by excellent diastereofacial selection,[127] and this may become a favoured method if the product yields can be raised.

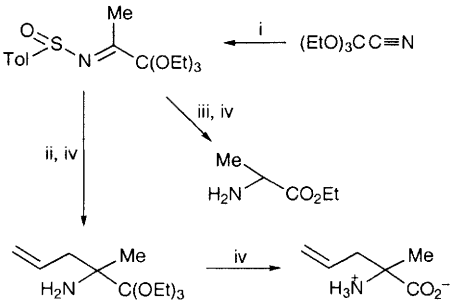


(30)

Reagents: i, R^2X, various catalysts; ii, H_3O^+

Scheme 11

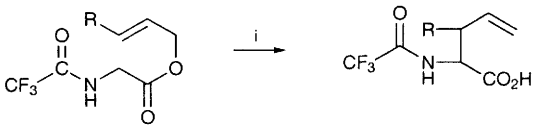
New routes to homochiral α-amino acids based on additions to imines involve the sulfinamide moiety as enantioselector (Scheme 12),[128] and a more conventional approach is offered by the cyanosilylation of imines derived from (S)-TBDMS-lactaldehyde, leading to threonine,[129] and by asymmetric reduction [2-(N-arylimino)-3,3,3-trifluoropropanoic acid esters by the (S)-oxazaborolidine – catechol – borane system],[130] and asymmetric hydrophosphonylation [catalyzed



Reagents: i, MeLi, (+)-*R*-[(−)-menthyl]toluene-*p*-sulfinate; ii, $CH_2{=}CHCH_2$ MgBr; iii, 9-BBN (stereoselective reduction); iv, TFA, H_2O–MeCN

Scheme 12

by La/K-BINOL complexes, to give α-aminophosphonates in 96% e.e.].[131] The presence of a chiral ester component in a glycine-derived imine represents one of the longest-running substrates for the synthesis of homochiral α-amino acids, illustrated with surprising results for 8-phenylmenthyl esters [$(MeS)_2C{=}NCH_2CO_2R$; there is a non-asymmetric version of this process described in the preceding Section, by the same authors (Ref. 73)]; the enolate undergoes alkylation leading to the (S)-α-amino acid series when an alkyl halide – Bu^tOK system is employed, but to the (R)-series when using an alkyl triflate with LDA or tert-butyllithium.[132] Where the ester moiety is derived from a chiral allyl alcohol, Claisen rearrangement (Scheme 13) leads to either one of two possible homochiral outcomes depending on the catalyst used [quinine leads to (2R,3S)-allylglycines as shown in the Scheme, while quinidine leads to the (2S,3R)-series].[133] α-Cyanoalkanoates derived from (1S,2S,4R)-10-(dicyclohexylsulfa-

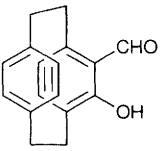


Reagents: i, $LiN(SiMe_3)_2$, quinine

Scheme 13

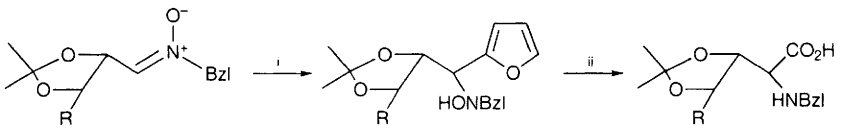
moyl)isoborneol undergo highly diastereoselective alkylation, Curtius rearrangement of the products leading, for example, to (R)-α-methylvaline and to (R)-α-phenyl-α-amino acids.[134] Schiff bases of α-aminonitriles carrying a chiral β-chloroalkyl side-chain can be cyclized with 100% stereoselectivity to 1-aminocyclopropanecarboxylic acids using a mild base (K_2CO_3).[135]

Almost equally long-running in the present context are the Schiff bases derived from N-benzyl-L-prolyl o-aminobenzaldehyde and glycine (see Vol 27, p. 12, and earlier Volumes) which can participate as their nickel(II) complexes in aldol reactions leading to syn-(2S)- and syn-(2R)-β-alkylserines,[136] and as Michael donors towards ethenesulfonate esters leading to fluorinated esters of (S)-homocysteic acid.[137] A novel variant is radical addition to the dehydroalanine version of this Schiff base, radical formation being initiated by 2,2′-azabutyronitrile and tributyltin hydride.[138] The resolution of the aldehyde (31) *via* the Schiff base formed with (R)-α-phenylethylamine, and its use for amino acid synthesis in



(31)

analogous alkylations, leads to β-hydroxy-α-amino acids and α-methylphenylalanine in 45-98% enantiomeric excess (see also Vol 27, p. 12).[139] The Schiff base N-oxides (Scheme 14), available from homochiral α-alkoxyaldehydes, can be carboxylated using α-lithiated furan as a masked form of the carboxy group.[140] A 2-silylated pyrrole, N-Boc-2-TBDMS-pyrrole is a masked glycine anion analogue,

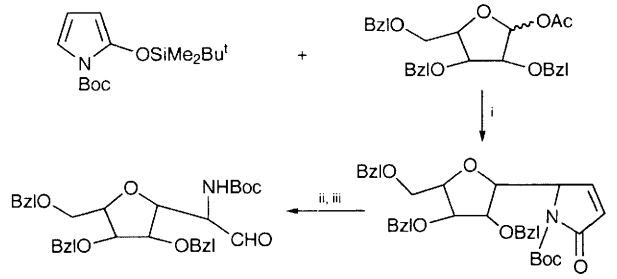


Reagents: i, α-Lithiofuran; ii, $TiCl_3$; then RuO_4

Scheme 14

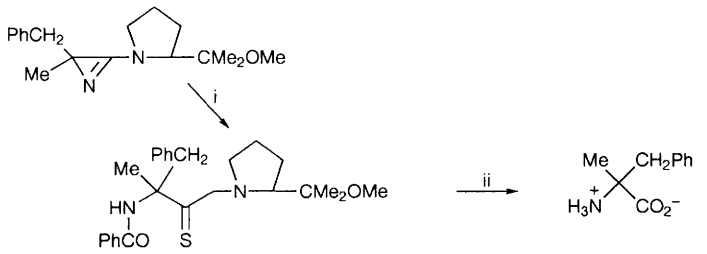
used for diastereospecific α-C-glycosylation of α-C-arabinofuranosylglycine (Scheme 15),[141] and in a synthesis of β-hydroxy-α-amino acids.[142]

Pd-Catalyzed allylation of achiral α-phthalimidoacetates can lead to surprisingly high enantiomeric purity (96% e.e.) when a chiral ligand is incorporated into the catalyst.[143] Ring-opening of a homochiral azirine (Scheme 16)[144] provides an α-methylphenylalanine enantiomer using a sulfur nucleophile, while



Reagents: i, Trityl perchlorate, 0 → 20 °C during 48h; ii, $KMnO_4$, 18-crown-6-ether, then $NaIO_4$; iii, 1M-LiOH/THF (to be followed by $NaClO_2$: CHO → CO_2H)

Scheme 15



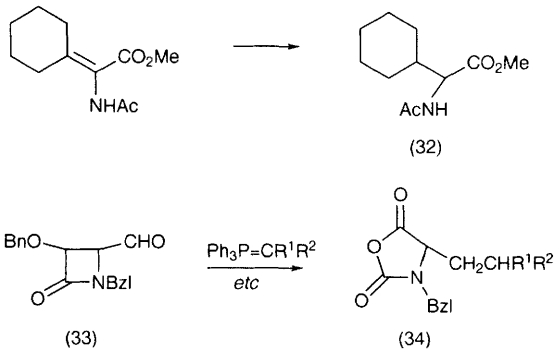
Reagents: i, PhCOSH, Et_2O; ii, H_2O_2, H_3O^+

Scheme 16

ring-opening of (S)-(-)-N-acetyl-2-methoxycarbonylaziridine[145] using a Broensted acid (or using a nucleophile in the presence of a Lewis acid) gives a mixture of α- and β-amino acid derivatives. Excellent regiospecificity has been found, giving optically-pure α-amino acids where the substrate carries one chiral centre, in ring-opening of toluene-p-sulfonylaziridine-2-carboxylic acid salts by carbon nucleophiles.[146] Lithium trimethylsilylacetylide alone, among the nucleophiles studied, led to a high yield of the target α-amino acid (as opposed to an α-amino acid – β-amino acid mixture) when the aziridine carried two chiral centres.

Uses for enzymes are continually being found for the synthesis of unusual amino acids (uses in synthesis of protein amino acids are covered in the next Section), recent examples being preparations of (S)-3-(2-chlorophenyl)- and (S)-(3-hydroxyphenyl)alanine from corresponding 3-arylacrylates, ammonia, and red yeast,[147] and hydration of α-aminonitriles catalyzed by immobilized pronase, to give a mixture of L-α-amino acid and D-α-amino acid amide.[148]

Enantioselective catalytic hydrogenation of α-amino acrylic acid derivatives, a



long-running topic of study that continues to turn up novel features, has nearly reached its peak of perfection in delivering a 99.5% enantiomeric excess of α-cyclohexylglycine (32) using supercritical carbon dioxide as solvent.[149] Better than 96% e.e. is secured for such β-branched compounds using benzene as solvent (H_2 at 90 psi pressure, Me-DuPHOS-Rh catalyst),[150] and the important role played by solvent is well appreciated, seen especially in Rh(II)/achiral phosphine-catalyzed hydrogenation of menthyl α-(N-benzamido)cinnamate.[151] Hydrogenation of enamides catalyzed by the last-mentioned catalyst leads to amino acid derivatives suitable for use in palladium-catalyzed cross-coupling reactions.[152] Rhodium(II)-catalyzed hydrogenation of α-acetamidoacrylates leads to 77-88% e.e. when trans-chelating chiral diphosphine ligands are incorporated into the catalyst,[153] and 86-93% e.e. has been reported for a closely-related system.[154] D- and L-β-(Heteroaryl)alanines have been prepared through Rh(II)/chiral diphosphine-catalyzed hydrogenation of appropriate αβ-dehydro-amino acids.[155] A broad range of rhodium(II)-chiral phosphine-catalyzed hydrogenation studies of αβ-dehydro-amino acids is reaching its culmination.[156] Asymmetric hydrogenation of methyl tetrahydropyrazine-2-carboxylate and the N-tert-butylamide, catalyzed by a Rh(II)/chiral biphosphine, leads to the corresponding (S)-piperazic acid derivatives needed for an HIV protease inhibitor synthesis (Indinavir).[157] Conventional (H_2/Pd-C) hydrogenation of 'dehydroamino acids' in the form of dipeptides prepared with L-proline N-methylamide is accompanied with high enantiomeric bias, as illustrated in a synthesis of (R)-neopentylglycine.[158]

The contraction of a β-amino acid enantiomer to give an α-amino acid is not too exaggerated a description of the route from the 'β-alaninyl cation equivalent' (33) *via* Wittig olefination, MCPBA oxidation and Baeyer-Villiger rearrangement to give the α-amino acid after hydrolysis of (34).[159]

4.3 Synthesis of Protein Amino Acids and other Naturally Occurring α-Amino Acids – The structural features present in individual α-amino acids of natural origin offer a particularly stringent challenge, in some cases, to test the available synthetic methods, while in other cases, structural features are benign but still

offer attractive targets to test synthetic methodology since the properties of the products are so well understood. Syntheses of natural α-amino acids of these types are also described in the preceding two Sections and in later Sections, especially in Section 6.3.

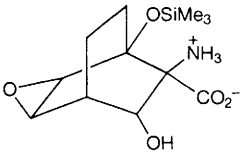
As usual, enzymic catalysis blithely ignores stringent challenges when exploited in syntheses of natural α-amino acids relevant to the specificity of particular enzymes, and representative citations from the rapidly expanding literature on this topic deal with L-tyrosine and its 2- and 3-fluoro-, 3,5-difluoro-, 2-chloro-, 2-methyl- and 3-methyl-derivatives prepared from ammonium pyruvate and the appropriate phenol using *Citrobacter intermedius*, a source of tyrosine phenol-lyase;[160] with L-phenylalanine from N-acetamidocinnamic acid, using a *Corynebacterium sp.*;[161] with L-tryptophan using *Corynebacterium* mutants;[162] and with a hydrogenase – glutamate dehydrogenase cocktail for the reductive amination of 2-oxoglutaric acid to give L-glutamic acid.[163] [^{15}N]-L-Valine can be produced from [^{15}N]-ammonium sulfate through the agency of *Corynebacterium pekinense*.[164] Section 16 of *Chemical Abstracts*: Fermentation and Bioindustrial Chemistry (and other Sections also, Section 10: Microbial, Algal, and Fungal Biochemistry in particular) give access to the full literature (including the patent literature) on this topic, which can only be hinted at here. A review of the production of microbial amino acids using heterotrophic bacteria has appeared.[165]

Glutamic acid, alanine and glycine are generated in low yield in aqueous solutions of corresponding keto-acids, ammonia and reducing agents,[166] and the keto-acids required for the genesis of branched-chain amino acids are suggested to evolve through reductive carboxylation of alkanoic acids.[167] This extends the scope of the prebiotic scenario (see the later Section 4.6) by demonstrating that the usual assumption, the need for some energy source with such reactions to make them plausible prebiotic drivers along the road to Life, is not necessarily valid. Photolysis of lactic acid and ammonia in a cadmium(II) sulfide suspension generates alanine (see also Refs. 608, 730).[168] A laboratory synthesis that is closer to the biogenetic pathway is reflected in the enantioselective generation of threonine and allothreonine from glycine and acetaldehyde, catalyzed by a synthetic cyclophane-embedded pyridoxal (see also Ref. 301).[169]

Stereoselective syntheses of peptide constituents (2S,3R)-3-hydroxyleucine (from lysobactin),[170] of (2R,1'R,2'R)-3-(trans-2'-nitrocyclopropyl)alanine (from hormaomycin) and its 2S-epimer (also present in hormaomycin),[171] of (4R)-4-[(E)-2-butenyl]-4-(NN-dimethyl)-L-threonine (*alias* '4MeBmt' from cyclosporin),[172] and of (3S,4S)-4-hydroxy-2,3,4,5-tetrahydropyridazine-3-carboxylic acid (from luzopeptin A)[173] demonstrate representative current methodology. Full details (see Vol 27, p. 19) of a stereocontrolled synthesis using the bis-lactim ether auxiliary have been published of the recently-revised structure for anticapsin,[174] as well as details of retroaldolization of (35) with Bu_4NF to give some near-relatives of anticapsin;[175] and a further synthesis of 'Adda' starting from L-threonine, have been described.[176] Asymmetric total synthesis of the potent immunosuppressive agent ISP-I (*alias* myriocin or thermozymocidin) has been accomplished without recourse to aldoses or ketoses as chiral starting points;[177]

instead, the optimum method for aldolization of the bis-lactim ether synthon was found that successfully introduced the long-chain moiety with the correct stereochemistry. That simple statement hides a considerable amount of development of the reaction conditions needed for understanding the factors determining diastereoselection in this synthesis, and in the synthesis of α-hydroxyalkyl-L-serine.[178]

Cyclopropyl side-chains have been built on to chiral α-aminopentenoates[179] and on to the azlactone (36),[180] in routes to (-)-allocoronamic acid, (-)- '2,3-methanohomoserine' and methyl (2S,3R)-1-aminocyclopropane-1,2-dicarboxy-



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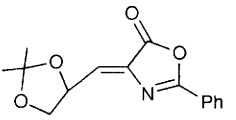
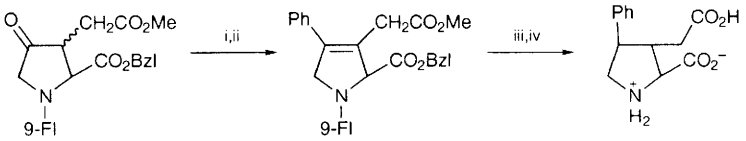


(36)

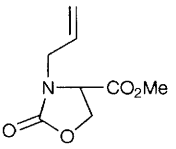
late ('cyclo-Asp-OMe'; Ref. 179); and to (-)-(1S,2R)-allonorcoronamic acid (Ref. 180). D-Glyceraldehyde-derived starting materials were used in these syntheses. (1S,2S)-Coronamic acid and its enantiomer, and (1S,2S)-norcoronamic acid have been obtained through a route involving diastereoselective cyclization of 2-(benzylideneamino)-4-chlorobutyronitriles,[181] and all four stereoisomers of coronamic acid have emerged from another classical synthesis starting from enantiomers of the cyclic sulfate of butan-1,2-diol, used for alkylation of the dibenzyl malonate anion; this stage is followed by partial hydrolysis and Curtius rearrangement.[182] All four stereoisomers of '2,3-methanoleucine' starting from (R)- or (S)-2-hydroxy-3-methylbutan-1-ol, have been prepared from D- and L-valine.[183] The cyclopropane moiety was created from the derived cyclic sulfate through reaction with dimethyl malonate or with dimethyl gluconate, Curtius rearrangement giving (E)- and Z-products respectively. N-Boc-cis-(2S,3R,4S)-'3,4-Methanoproline' and N-Boc-(2S,3R,4S)-'3,4-methanoglutamic acid' have been synthesized by a six-step route starting with condensation of the anion of N-Boc-β-(benzenesulfonyl)-L-alaninol tetrahydropyranyl ether with (2R)-glycidyl triflate.[184] A related approach to stereoisomers of '2,3-methanoglutamine' employs cyclopropane analogues of a γ-lactone and of an α-alkylated malonate to arrive at the requisite cis and trans stereochemistry, respectively, through cautious and traditional functional group development.[185]

The kainic acid family continues to attract attention, and synthesis at its simpler level in this area is represented for arylkainoids (of interest as simple analogues of acromelic acid); phenyl-[186] and substituted phenyl-kainoids[187] have been synthesized, with (2S)-benzyl 4-oxo-N-[9-(9-phenylfluorenyl)]prolinate serving as starting material in one of these routes (Scheme 17). D-Serine starts a route to conformationally restricted kainic acid analogues, *via* the derived N-allyl-5-tert-butoxycarbonylvinyl-lactone (37).[188] Acromelic acid analogues and their C-4 epimers have been reached, starting from trans-4-hydroxy-L-proline



Reagents: i, $KN(SiMe_3)_2$, Tf_2NPh; ii, $PhB(OH)_2$, $(Ph_3P)_4Pd$; iii, NaOH, EtOH; iv, H_2–Pd/C

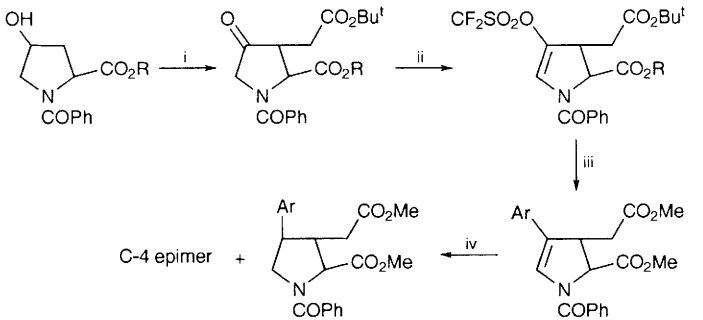
Scheme 17



(37)

through a short and versatile route (Scheme 18) that is suitable for larger scale synthesis.[189]

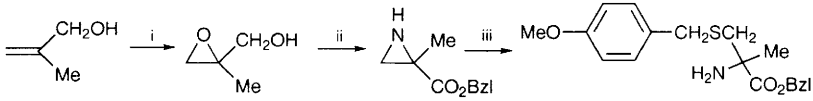
4.4 Synthesis of α-Alkyl Analogues of Protein Amino Acids – The topic is gaining in importance since, among other reasons, it meets the demand for pharmaceutically-active compounds destined to join the high-profile examples recently publicised (α-difluoromethylornithine has emerged as an anti-cancer agent). Together with papers mentioned elsewhere (in preceding Sections of this Chapter) that have described syntheses of α-alkyl-α-amino acids and alicyclic analogues, the current overall picture is completed with a number of papers collected here.



Reagents: i, $RuO_2/NaIO_4$, then pyrrolidine, then $BrCH_2CO_2Bu^t$; ii, $LiN(SiMe_3)_2$, then $PhN(SO_2CF_3)_2$; iii, $ArB(OH)_2/Pd(PPh_3)_4$; iv, Et_3SiH

Scheme 18

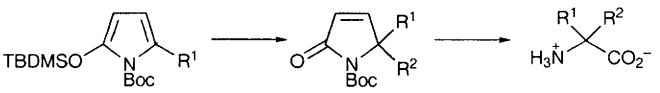
A standard application of methyl acetoacetate, ethyl nitroacetate and ethyl N-(diphenylmethylene)glycinate as anionic glycine equivalents for the synthesis of bicyclic and tricyclic αα-disubstituted glycines,[190] and a standard oxazolone 4-alkylation procedure[191,192] to generate (R)- and (S)-enantiomers of α-methylglutamic acid, α-methylaspartic acid and α-isobutylaspartic acid, illustrate routine methods in this area. One of these routes from Obrecht's group employs L-phenylalanine cyclohexylamide for resolution through aminolysis of the substituted oxazolone and separation of the resulting diastereoisomer mixture. Methylation of $ClC_6H_4CH{=}NCHRCOS^*$ (S* = Oppolzer's camphorsultam chiral auxiliary) employing methyl iodide with a strong base has received a detailed study[193] (see also Refs. 97, 98), and the equivalent substrate ($MeS_2C{=}$ in place of $ClC_6H_4CH{=}$) has been given wide-ranging application including ω-haloalkylation with assistance from phase-transfer catalysis and ultrasound irradiation.[194] Esters formed between benzoylalanine and chiral alcohols are readily alkylated after chiral di-anion generation using LDA, in an enantiospecific approach; the (-)-8-phenylmenthyl esters offer the highest diastereofacial bias to the di-anion, judging by the results obtained.[195] Chiral aziridines (Scheme 19) have been used in a lengthy route to α-methylcysteines.[196]



Reagents; i, Bu^tOOH, (D)-DET, $Ti(OPr^i)_4$; ii, NaN_3, then $OH \rightarrow OMs$, $CH_2OH \rightarrow CO_2Bzl$, and base; iii, $BF_3{\cdot}Et_2O$, *p*-$MeO\text{-}C_6H_4CH_2SH$, CH_2Cl_2

Scheme 19

An extension of the 2-silyloxypyrrole method (see Refs. 141, 142), based on double γ-alkylation *en route* to αα-disubstituted glycines (Scheme 20),[197] and efficient alkylation of α-iminoesters derived from α-amino acids by condensation with a novel pyridoxal-5′-phosphate model carrying a Li^+ ionophore,[198] have been employed. The oxazolidin-4-one chiral auxiliary (Section 4.2) offers a route to α-alkyl analogues of protein amino acids if the auxiliary is synthesized with one of the two required α-alkyl groups already in place; this strategy has been used in a synthesis of α-methyl-L-tryptophan (starting from L-tryptophan)[199] and (R)-α-methyl-phenylalanine (starting with L-alanine, and employing a novel



Reagents: generally as in Scheme 15

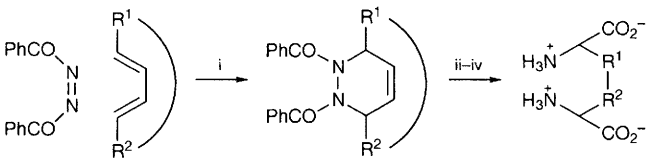
Scheme 20

2-ferrocenyl-substituted chiral oxazolidinone).[200] All isomers of α-methyl-L-serine and α-methyl-L-threonine have been prepared, representing a further stage of Ohfune's long synthesis quest through analogues of the protein amino acids.[201] More innovative procedures have been described, one employing a Pummerer rearrangement *en route* to (S)-α-trifluoromethylserine,[202] another involving conversion of 2-methyl-2-vinyl-3-alkyloxiranes (prepared the Sharpless-Katsuki way) through 1,2-alkyl migration with inversion of configuration into 2-methyl-2-vinylalkanals, followed by functional group manipulation to give (R)-α-methyl-phenylalanine and N-protected α-methyl-α-aminoaldehydes.[203] Chiral N-(β-trimethylsilylethanesulfonyl)aziridinemethanol prepared from (S)-(-)-2-methyl-glycidol (cf. Ref. 196) undergoes nucleophilic ring-opening to give (S)-(+)-α-methyl-serine.[204]

4.5 Synthesis of α-Amino Acids Carrying Alkyl Side-Chains, and Cyclic Analogues – This section catches those papers covering synthesis of aliphatic α-amino acids that do not qualify for location elsewhere in this Chapter, although the synthesis targets are frequently very close structural relatives of the common α-amino acids. Indeed, one common objective for work described here, is the synthesis of analogues of biologically important compounds; the overwhelming emphasis in the recent literature, on alicyclic compounds in this category, reflects the growing interest in the synthesis of conformationally-constrained analogues for biological testing.

Acyclic examples include the synthesis of all four diastereoisomers of 4-methyl glutamic acid.[205] An extraordinary route illustrated for the synthesis of meso-αω-di-amino-dicarboxylic acids (Scheme 21) is largely limited in scope by its dependence on the supply of appropriately substituted dienes, and it remains to be seen whether a particularly useful potential application of this method, the synthesis of αα′-dialkylated analogues, can be achieved.[206]

A continuing flow of new routes to cyclopropane-based amino acids, adding to

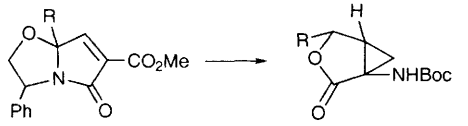


Reagents: i, CCl_4; ii, RuO_2(cat.) aq $NaIO_4$; iii, 6M HCl–AcoH/100 °C, iv, H_2/Pt

Scheme 21

those studies discussed in preceding Sections, includes cyclopropanation of the fused oxazolidinone (38) followed by removal of the chiral auxiliary and Curtius rearrangement.[207] The preparation of 1-aminocyclobutane-1,3-dicarboxylic acids involving degradation of benzenesulfonylbicyclobutanes has been thoroughly documented,[208] and cis- and trans-3-substituted-1-aminocyclobutane-1-car-

boxylic acids are covered, including 3-(2'-phosphonoethyl) derivatives that are effective at the NMDA receptor.[209] This work includes a synthesis of 3-hydroxymethyl-1-aminocyclobutane-1-carboxylic acid; 4-carboxymethyl-1-aminocyclobutane-1,3-dicarboxylic acid has been obtained through a standard hydantoin synthesis employing bicyclo[3.2.0]heptan-2-one followed by oxidative ring-opening.[210] X-Ray structure determination was employed to assign relative configuration to this product. 3-Aminobicyclo[3.3.0]octane-1,3-dicarboxylic acid (39) has been obtained through a lengthy sequence from readily available diketone (40).[211]



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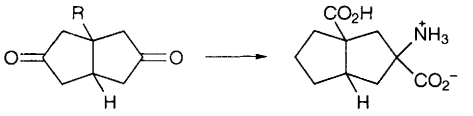
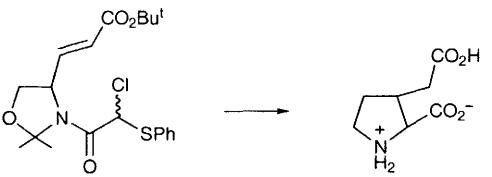


(40) (39)

(S)-1-Phenylethylamine acts as chiral auxiliary as well as nitrogen donor in a route to (R,R)-azetidine-2,4-dicarboxylic acid.[212]

Numerous avenues to substituted prolines are represented in the recent literature:- a general route illustrated by cyclization of 5-(N-phosphinyloxy-amino)valerate esters after generation of the α-anion;[213] a route to (2S,3R)-3-carboxyproline and its 3-amino analogue through stereospecific alkylation of (4S)-N-(TBDMS)-azetidin-2-one-4-carboxylic acid in several ways, best through the use of the cyclic sulfate of ethyleneglycol and elaboration of the resulting β-hydroxyethylated substrate;[214] to 4-hydroxy-3-phenylproline through hydration and hydrolysis of 1-acetyl-2,2-diethoxycarbonyl-2,3-dihydro-3-phenyl-1H-pyrrole;[215] to methyl 3-amino-3-pyrrolidine 3-carboxylate *via* addition of acrylates to the azomethine ylide formed from $PhCH_2N(CH_2OBu)CH_2SiMe_3$, *en route* to cucurbitine;[216] to the simple kainic acid analogue (41);[217] and to

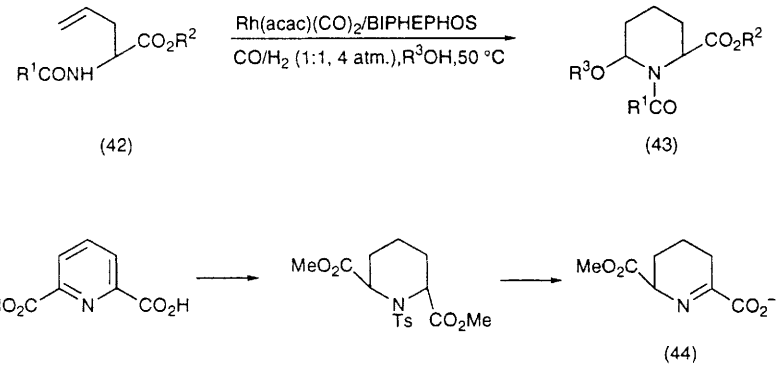


(41)

substituted pyrrolidinecarboxylic acids starting from Diels-Alder adducts of maleic anhydride with alkadienes.[218] Methyl L-prolinate has been used as chiral reagent for ring-opening of meso-norbornene-derived anhydrides, leading to amido acids with excellent levels of asymmetric induction, and thence to valuable synthons.[219]

Bicyclic proline analogues can be constructed through methylenation of pyrrolines using ethyl diazoacetate/Rh(II) tetra-acetate, an approach used with 3,4-dehydroproline[220] and with 2,3-dihydropyrrole-2,2-dicarboxylic acid.[221] Instead of having the proline moiety in place as with these two examples, the synthesis target can be approached by building on to a cyclic template, as in the cycloaddition of ethyl glyoxylate to the azomethine ylide derived from (S)-5-phenylmorpholin-2-one (see also Vol 27, p. 15).[222] Of course, the Diels-Alder addition fits into this category, and has been illustrated again for the addition of cyclopentadiene to a chiral glycine imine, (R)-PhCHMeN=CHCO$_2$Bzl, to give conformationally-constrained proline analogues after hydrogenolysis (cleavage of the chiral auxiliary) and hydrogenation of the C=C bond.[223] [60]Fullerene undergoes 1,3-dipolar cycloaddition to N-benzylideneglycine methyl ester to provide the fullerene-fused 5-phenylproline ester,[224] while C-N bond breaking accompanies the corresponding reaction with sarcosine esters, yielding both fullerene-pyrrolidines and methanofullerenes.[225]

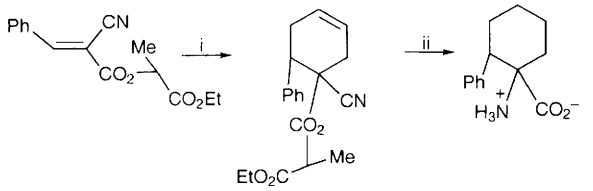
6-Alkoxy-5,6-dehydropipecolic acid esters have been prepared from allylglycine esters through an intramolecular cyclohydrocarbonylation sequence (42 → 43).[226] Conversion of pyridine-2,6-dicarboxylic acid (dipicolinic acid) into dimethyl cis-piperidine-2,6-dicarboxylate, and further conversion into the racemic methylpyrrolidinedicarboxylate (44), could be described as an approximate



(42) (43) (44)

reversal of a common biosynthetic pathway;[227] a similar approach to cis-6-hydroxymethylpipecolic acid involves catalytic hydrogenation of 6-hydroxymethylpyridine-2-carboxylic acid.[228] Aza-Diels-Alder routes, one employing the 8-phenylmenthyl ester of (R)-PhCHMeN=CHCO$_2$H,[229] and another example (heterogeneous catalyzed) employing the (-)-menthyl ester of an N-acetyl-αβ-dehydroamino acid,[230] lead to pipecolic acid derivatives. The double chiral

tagging approach, illustrated in one of these studies, has been used before in unrelated amino acid syntheses but not to such a spectacular outcome as far as diastereoisomeric excess (>95%) is concerned. In a different asymmetric Diels-Alder approach [in which two new chiral centres, 1S and 2R, are generated when butadiene reacts with a chiral (E)-2-cyanocinnamate ester formed with (S)-ethyl lactate], the adduct is converted into 1-amino-2-phenyl-1-cyclohexanecarboxylic acids by routine functional group transformations (Scheme 22).[231] Diels-Alder addition [Danishefsky's diene to (Z)-4-benzylidene 2-phenyloxazol-5(4H)-one]



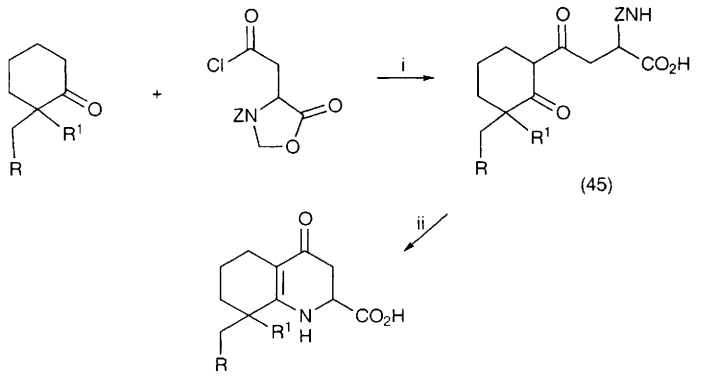
Reagents: i, butadiene, $TiCl_4$, CH_2Cl_2; ii, H_2–Pd/C, then Curtius rearrangement, and CN→CO_2H

Scheme 22

followed by standard operations gives 1-amino-4-hydroxycyclohexane-1-carboxylic acid from which exo-2-phenyl-7-azabicyclo[2.2.1]heptane-1-carboxylic acid, a new constrained proline analogue, was obtained by intramolecular cyclization.[232] N-Protected 2-keto-aziridines react with Ph_3PCH_2 and BuLi,[233] with the same outcome seen for aza-[2,3]-Wittig rearrangements of N-tert-butoxycarbonylmethyl vinylaziridines, giving cis-2-substituted 3,4-dehydropipecolic esters (preserving stereochemistry for homochiral examples);[234] carboxy group manipulations and cyclization leads to indolizidines. The alternative thermal homodienyl-[1,5]-hydrogen shift of the same aziridines can also be brought about quantitatively, to give N-alkylglycine Schiff bases, and consideration has been given to the effects of substituents on these processes.[235]

cis-(2R,3S)- and trans-(2S,3S)-Piperidine-2,3-dicarboxylic acids are of interest as cyclic analogues of N-methyl-D-aspartic acid, and have been synthesized from the morpholinone obtained by condensation of (2S)-phenylglycinol with dimethyl acetylenedicarboxylate followed by annulation with acryloyl chloride, and straightforward steps thereafter.[236]

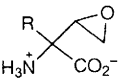
Synthesis of saturated heterocyclic analogues of common amino acids provides another minor component to complete this section, with cyclization of 4-oxo-alkanoic acids (45 in Scheme 23) yielding quinolinic acid analogues.[237] Bischler-Napieralski cyclization of an N-acyl-(L)-3,4-dimethoxyphenylalanine attached to chloromethylated poly(styrene) resin has been investigated;[238] reduction with $NaBH_3CN$ was successful in providing resin-bound 1-substituted isoquinolinic acids, which could be stripped from the resin in the usual way. A synthesis of free



Reagents: i, Mesityl-Li, –78°C, then 1M KOH; ii, HCO_2^- NH_4^+, Pd–C

Scheme 23

α-oxiranyl α-amino acids (46) from Z-DL-vinylglycine through MCPBA oxidation, or *via* the derived diol, is notable.[239]



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4.6 Models for Prebiotic Synthesis of Amino Acids – Standard themes continue to be played or this topic, updated from time to time by new ideas. This year, further account is taken of the proposed genesis of amino acids in submarine thermal vents, an idea that has been undermined by the claims that 'amino acids are irreversibly destroyed at 240°' and that quasi-equilibrium calculations, advanced by others to give credence to experimental observations, are not applicable to high temperature systems involving organic compounds such as amino acids.[240] What is controversial then, is the paper showing that the thermodynamics of the Strecker reaction running at $\geq$400° are in favour of the abiotic synthesis of amino acids in thermal vents,[241] but pyrolysis studies of amino acids over many years have shown that simple α-amino acids can be recovered from self-condensation products formed at these temperatures.[242] α-Amino acids are indeed formed in simulated thermal vent environments;[243] this article also reviews other proposals for the abiotic synthesis of amino acids. Another experiment in which high energy plasma and heterogeneous salt interfaces act on the standard $CH_4/NH_3/H_2O$ mixture, to mimic the marine system, has found the usual products including amino acids, but also polycyclic aromatic compounds including azulenes and long-chain alkanes.[244]

The role of elementary particles in driving the enantioselective synthesis of amino acids has been reviewed.[245] Irradiation at 10K by high energy protons (to simulate cosmic radiation) of aqueous $CO/CH_4/NH_3/H_2O$ mixtures (the likely composition of cometary ice) generates glycine and alanine,[246] a result also achieved using mildly oxidized gas mixtures.[247]

The role of ferrous sulfide (pyrite) in mediating such reactions is, not surprisingly, controversial. Amino acid synthesis does not occur in systems where $CO_2$is reduced by ferrous sulfide + H_2S (a strongly reducing combination) in a reverse citric acid cycle;[248] however, reductive amination of α-ketoacids can be accomplished in a system in which oxidative formation of pyrite is occurring, and involves CO_2 as catalyst.[249] The roles of different prebiotic raw materials, with special emphasis on minerals, has been reviewed.[250]

4.7 Synthesis of α-Alkoxy α-Amino Acids and Analogous α-Hetero-atom Substituted α-Amino Acids – The applications of α-methoxy α-amino acids (Refs. 79, 427) and α-bromo-analogues (Ref. 77), seem to have been more important this year than the routine methods by which they are synthesized.

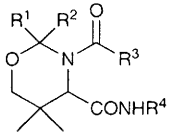
4.8 Synthesis of α-(ω-Halogenoalkyl) α-Amino Acids – A review of synthetic methods has been published as part of a major treatise on fluorinated amino acids.[251]

Fluorination (F_2) of 4-alkylidene-oxazolin-5(4H)-ones and alkaline hydrolysis of the difluorinated adducts gives β-fluoro-α-keto-acids that can be returned to the α-amino-acid family through standard reductive amination to β-fluoro-α-amino-acids.[252] Claisen rearrangement of trifluoromethyl allyl ethers $RCH{=}CHCH_2OCCl(CF_3)CO_2R^1$ gives δε-unsaturated ββ-difluoro-α-keto-esters $CH_2{=}CHCHRCF_2COCO_2R^1$ that can be elaborated into the ββ-difluoro-α-amino-acids through standard reductive amination methods ($NH_3/NaBH_4$).[253]

Simple ω-iodo-α-aminoalkanoic acids, such as β-iodoalanine, continue to show useful applications in synthesis (e.g. Refs. 288, 707). Reliable synthetic methods have been described for higher homologues.[254]

4.9 Synthesis of α-(ω-Hydroxyalkyl)-α-Amino Acids – One of the simplest modifications of a glycine anion, through aldol addition, results in α-(α-hydroxyalkyl)ation and has been regularly featured in the literature. Together with citations in other Sections of this Chapter concerned with simple synthetic applications of aldol reactions (e.g. Section 4.2), the recent crop of papers collected here concentrates especially on stereochemical aspects of aldolization. syn- or anti-Selectivity is shown in the formation of α-amino-β-hydroxyacid esters in this reaction, applied to ethyl N-methyl-N-benzylglycinate and its borane adduct,[255] while titanium enolates of N-benzyloxycarbonylamino acid esters yield alkyl anti-α-amino-(β-hydroxy)alkanoates, best results accruing for bulky aldehydes reacting with sterically-hindered amino acid derivatives.[256]

Homoserine and structural analogues have been laboriously synthesized (homoserine betaines in nine steps from ethyleneglycol[257]) and innovatively synthesized *via* 1,3-oxazines, either through the Ugi reaction applied to 3-



(47)

hydroxy-2,2-dimethylpropanal[258] giving (47), or from a dihydro-1,3-oxazine, giving 1-amino-3-hydroxycyclohexane-1-carboxylic acid as a conformationally constrained homoserine analogue.[259]

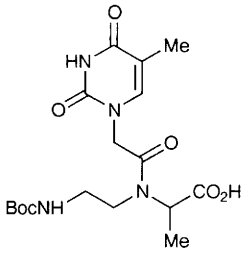
L-Threonine aldolase (*Candida humicola*) has been shown to be relatively unspecific, in catalyzing the addition of a wide variety of aldehydes to glycine to give L-α-amino-β-hydroxy acids; however, it is dismissive of the need to deliver a particular configuration at the β-chiral centre.[260] The same protocol has been used for a synthesis of (2S,3R)-2-amino-3-hydroxybutyrolactone starting from glycine.[261]

4.10 Synthesis of N-Substituted α-Amino Acids - This Section may seem unnecessary, since routine preparations of these derivatives starting from amino acids are located in the later Section 6.2. However, it is appropriate to provide space to cover syntheses from other starting materials, and to acknowledge the growing importance of studies of modified peptides, e.g. those that mimic polynucleotides (these mimics are known as 'PNA's) which has called for the supply of appropriate amino acids for their synthesis. One example is N-(2-Boc-aminoethyl)-N-(thymin-1-yl)acetyl-L-alanine (48) that is synthesized in a straightforward way starting from alanine.[262] Benzyl N-Boc 4-bromo-2-aminobutanoate, prepared from α-benzyl N-Boc-glutamate *via* photolysis of the 1-hydroxy-2-thiopyridyl ester in CCl_3Br, reacts readily with the nucleotide bases adenine, thymine, cytosine or guanine to give useful building blocks[263] for PNA synthesis.[264]

Novel building blocks for PNAs with an N-(aminomethyl)-β-alanine backbone ('retro-inverso PNA') have been synthesized by a route starting with a Hofmann rearrangement [$H_2NCOCH_2NH(COCH_2B)CH_2CH_2CO_2Et \rightarrow H_2NCH_2NH(CO\text{-}CH_2B)CH_2CH_2CO_2Et$; where B is one of the polynucleotide bases].[265]

Modified peptides now known as peptoids, are prepared from N-substituted glycines; a synthesis of one member of the peptoid family calls for N-Fmoc-protected amino acids, prepared from ethyl bromoacetate, aminolyzed by alkylamines representing Phe, Leu, Lys(Boc) and Met side-chains, was then saponified and converted into the Fmoc derivative (to give 'Fmoc-NPhe-OH', etc; 49) in preparation for standard solution-phase peptide synthesis.[266]

4.11 Synthesis of α-Amino Acids Carrying Unsaturated Aliphatic Side-Chains – The 'dehydro-amino acids' are the simplest representatives of this class, and one of the most easily synthesized and also useful in general amino acid synthesis



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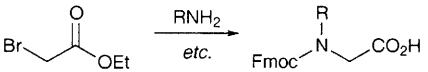
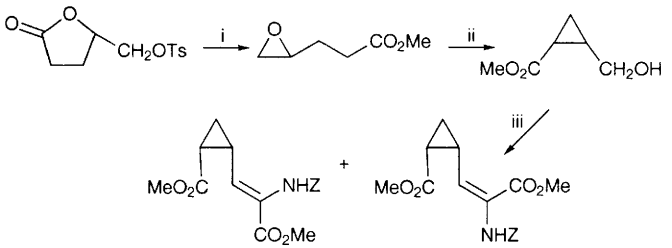


(49)

(Refs. 81, 305), despite lacking the α-chiral centre. These are easily prepared through condensation of an aldehyde or ketone corresponding to the intended side-chain with an anionic glycine synthon, usually an oxazol-5-(4H)-one, and $(MeO)_2P(O)CH(NHZ)CO_2R^1$ is a useful alternative synthon, used in a synthesis of cyclopropylmethyleneglycines (Scheme 24).[267] β-Hydroxy α-amino acids are efficiently dehydrated to 'dehydro-amino acids' using dichloroacetyl chloride and a tertiary amine.[268] 2-Azidoacrylates undergo per-rhenate-catalyzed decomposition in $ClCO_2CCl_3$ or $COCl_2$ followed by NEt_3-induced dehydrochlorination, to give 2-isocyanoatoalken-2-oates.[269]



Reagents: i, MeONa, MeOH; ii, 1.2 equiv. LDA, THF, – 50 °C; iii, CrO_3–py, then $(MeO)_2P(O)CH(NHZ)CO_2Me$

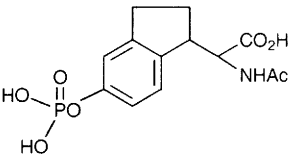
Scheme 24

Vinylglycine represents the archetypal βγ-unsaturated α-amino acid, and has been newly synthesized by Neber rearrangement of the chloroimidate derived from allyl cyanide.[270] This method is successful for the synthesis of (E)-2-aminopent-3-enoic acid and its 3-methyl homologue. Even more importance is acquired for vinylglycine through this work, since it can be used to synthesize other amino acids (see Refs. 239, 710, 711). Mitsunobu synthesis by the condensation of phthalimide with chiral secondary allylic alcohols and (R)-isopropylideneglyceraldehyde leads to βγ-unsaturated α-amino acids [the use of ethyl (S)-lactate in the process leads to αβ-unsaturated γ-amino acids].[271] (E)- and (Z)-β-(Fluoromethylene)-substituted m-tyrosines have been prepared (see also

Ref. 281) and resolved, with configurational details settled through considerable effort (CD and X-ray analysis).[272] Vinylglycine homologues have been prepared starting from homochiral 5-vinyl oxazolidin-2-ones, effectively a protected form of the parent amino acid that can tolerate chain extension by organocuprates.[273]

α-Substituted (E)-βγ-dehydroglutamic acids have been prepared through establishing a simple Michael-type addition process between N-diphenylmethyleneglycine ethyl ester and ethyl propiolate.[274] The initial mixture of E- and Z-adducts was easily separated on the basis of 3,4-dehydropyroglutamate formation by the Z-isomer during work-up.

4.12 Synthesis of α-Amino Acids with Aromatic or Heteroaromatic Groupings in Side-Chains – Phenylalanine and tyrosine analogues synthesized through applications of standard methodology include Fmoc-L-p-azidophenylalanine (from L-p-aminophenylalanine),[275] Tyr[P(O)(NMe$_2$)$_2$]OH as its N-Fmoc derivative[276] (see Vol 27, p. 33); conformationally-constrained analogues of phosphotyrosine (e.g. 50);[277] dimethoxy-L-phenylalanines (a lengthy route starting with chlorocyanoethylation of anisole by 2-chloroacrylonitrile/TiCl$_4$ and routine amination and hydrolysis steps);[278] 2,6-difluoroDOPA (starting from 2,6-difluoroveratraldehyde);[279] and β-(4-diazocyclohexa-2,5-dienonyl)-L-alanines (starting by alkylation of chiral glycine synthons by benzyloxy-nitrobenzyl iodides).[280] 4′-Fluorination of (R)- or (S)-β-(fluoromethylene)-m-tyrosine (see Ref. 272) has been accomplished with AcOF.[281] Ring-substituted α-(4-hydroxyphenyl)-βββ-trifluoro-alanines have been obtained by amination of quinomethides (51),[282] and



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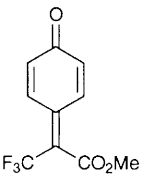
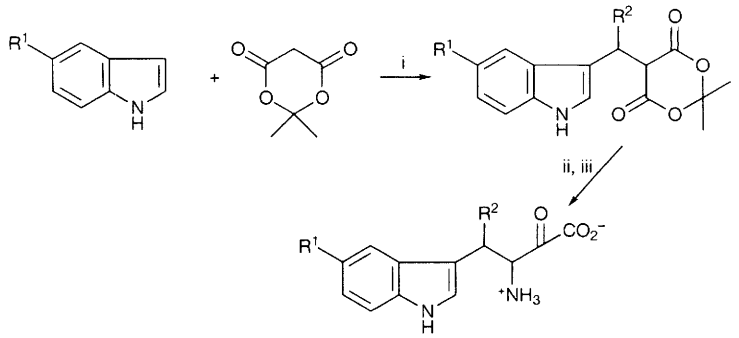


(51)

an 18-crown-6-ether-benzo-substituted L-phenylalanine has been synthesized.[283] Hydroxylation of phenylalanine by H_2O_2 is catalyzed by a remarkable manganese – fluorinated porphyrin, whose effectiveness (and, perhaps, also its selectivity) can be appreciated by the fact that the ring in toluene is also hydroxylated by this system.[284]

Benzo-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acids represent newcomers to the well-known group of conformationally constrained analogues of phenylalanine, and the three possible versions have been synthesized by alkylation of ethyl acetamidocyanoacetate (see also Refs. 298, 803).[285]

Enantiomerically-pure 4-alkyltryptophans have been prepared through a standard formamidomalonate synthesis, using lithiated N-TIPS-protected gramines as alkylating agents, followed by penicillin G acylase resolution.[286] β-Substituted



Reagents: i, R^2CHO; ii, EtOH, py/80 °C; iii, half-ester → NHZ with DPP (Curtius rearrangement), then $H_2NCOOBzl$; iv, H_2/Pd

Scheme 25

tryptophan esters can be prepared in four steps from indoles, aldehydes and Meldrum's acid (Scheme 25; see also Vol 27, p. 41).[287]

Heteroaromatic targets closely related to protein amino acids have been achieved:- L-β-(5-hydroxy-2-pyridyl)alanine (using the β-iodoalanine synthon $BocNHCH(CH_2I)CO_2Bzl$ by coupling with 5-methoxy-2-iodopyridine followed by BBr_3 deprotection),[288] and 'L-azatyrosine' through standard use of Williams' morpholinone chiral auxiliary;[289] 2-substituted tryptophans [Lewis acid-catalysed Michael addition of 2-substituted indoles to $Ph_2C{=}NC(=CH_2)CO_2R^1$];[290] 5-methoxy-D- and L-tryptophans through standard enantiospecific protocols;[291] thia-analogues of tryptophan (β-3-thieno[2,3-*b*]- and [3,2-*b*]pyrrolyl-L-alanine from thienopyrroles, L-serine, and tryptophan synthase from *Salmonella typhimurium*);[292] and β-(3-N-ethylcarbazolyl)-L-alanine (from the corresponding heteroaryl aldehyde through the Erlenmeyer azlactone synthesis followed by acylase resolution).[293] 3-Methyl-5-(arylthio)-L-histidines have been prepared, as models for the Starfish alkaloid imbricatine, through the bis-lactim ether protocol starting off a 10-step route with 4(5)-bromoimidazole.[294]

Heteroaromatic targets synthesized as analogues of other α-amino acids known to be physiologically-active, include:- the NMDA receptor agonists 2-amino-2-(3-hydroxy-5-methyl-4-isoxazolyl)acetic acid (Ugi synthesis and resolution with cinchonidine),[295] and its racemic 5-isopropyl analogue (acetamidomalonate synthesis);[296] (R)- and (S)-homoibotenic acid (use of Boc-L-phenylalanine as chiral auxiliary);[297] (3SR,4aRS,6SR,8aRS)-6-(1H-tetrazol-5-yl)decahydroisoquinoline-3-carboxylic acid [competitive NMDA and AMPA receptor antagonists {i.e. 2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)acetic acid analogues}, synthesized from 6-formyl-decahydroisoquinoline-3-carboxylic acid];[298] and α-(isoxazol-3-yl)glycine and its isoxazol-5-yl and N-phenylpyrazol-3-yl analogues [ozonolysis of α-(cyclohexa-1,4-dienyl)glycine and construction of the hetero-

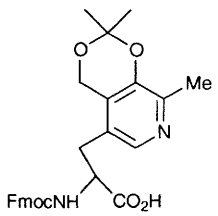
cyclic moiety with hydroxylamine or phenylhydrazine].[299] Synthesis of β-(2-substituted pyrimidin-4-on-5-yl)-L-alanines, shown to possess useful glutamate receptor activity, has followed a 'ring-switching' strategy exemplified earlier, in which a pyroglutamate 4-aldehyde equivalent is converted into a substituted imine, which then opens the pyroglutamate through intramolecular attack at the ring carbonyl group, thus completing a heterocyclization.[300]

In keeping with a theme of a preceding paragraph, where descriptions are given of α-amino acids carrying familiar metabolically-important structures in amino acid side-chains, the 'pyridoxal coenzyme amino acid' (52) has been described (see also Ref.169).[301] The purpose of preparing these amino acids is for their use in peptide synthesis, and in metabolic studies.

4.13 Synthesis of α-(N-Hydroxyamino) Acids – Mitsunobu synthesis involving α-hydroxyacids and N-alkoxycarbonyl-O-alkoxycarbonyl-protected hydroxylamines provides N-hydroxyamino acids in high enantiomeric purity[302] (see also Ref. 380).

4.14 Synthesis of α-Amino Acids Carrying α-(ω-Aminoalkyl) Groups, and Related Nitrogen Functional Groups, in Side-Chains – The contents of this Section overlap considerably with other Sections covering categories of structure with side-chains that extend outwards from the glycine moiety through a nitrogen atom. β-Enamino esters $R^1NHC(SLi)=C(NR_2)CO_2Et$ are formed from the lithium enolate of an NN-diprotected glycine ester and an isothiocyanate,[303] and N^{β}-alkyl diaminopropionic acids are formed by ring opening of tert-butyl N-toluene-p-sulfonylaziridinecarboxylate with a primary amine; treatment of the product with an alkyl iodide in the presence of Cs_2CO_3 creates the selectively N^{α}-alkylated compound.[304]

The addition of nitrogen-centred nucleophiles to dehydroalanine derivatives provides the products of β-addition (leading to β-aminoalkyl α-amino acid derivatives) in competition with α-imine capture.[305] No such competition is seen in the corresponding reaction with methyl 2-acetamidoacrylate catalysed by $FeSO_4$ that leads to β-dialkylamino-α-alanines.[306] An α-amino acid with a 'half-EDTA' side-chain, *viz.* $FmocNHCH[(CH_2)_nN(CH_2CO_2Bu^t)_2]CO_2H$ has been synthesized in a form ready for use in peptide synthesis,[307] and 'azaSAM' (53),



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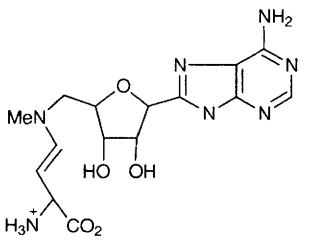


(53)

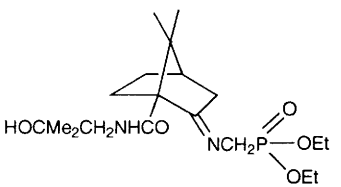
the 5'-methylamino analogue of S-adenosyl-L-methionine, has been synthesized.[308]

Synthesis of the novel natural azoxy compound azoxybacilin (see Vol 27, p.3), starting from α-tert-butyl N-Boc-L-aspartate proceeds *via* the sequence $RCO_2H \rightarrow RCH_2OH \rightarrow RI$ $RN(O)=NMe$, the latter step employing $MeN=NO^-K^+$as reagent.[309]

4.15 Synthesis of α-Amino Acids Carrying Sulfur- or Selenium-Containing Side-Chains – Most of the published routes claiming the synthesis of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid) in fact yield the 7,7-dimethyl isomer; the only effective synthesis is a low yield route.[310]

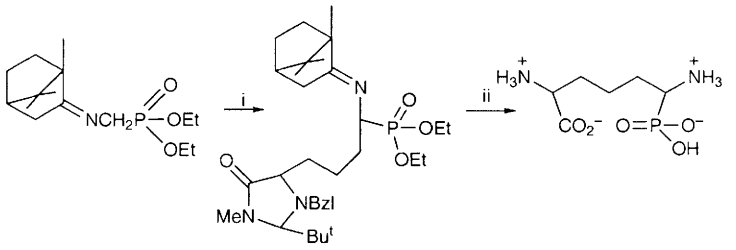
4.16 Synthesis of α-Amino Acids Carrying Phosphorus Functional Groups in Side-Chains, and α-Amino Phosphonic Acids – Whereas common amino acids that are derivatized by phosphorylation are not covered thoroughly in this Chapter [but this year, some citations (Ref. 277) do cover such compounds in passing], the growing importance of alkane- and arene-phosphonates for their potential physiological activity justifies highlighting, through brief separate coverage here.

The asymmetric synthesis of phosphorus analogues of the amino acids has been reviewed.[311] The well-known equivalent of the Strecker synthesis in phosphorus chemistry has been illustrated in a general asymmetric synthesis version, i.e. the preparation of α-amino phosphonic acids from an aldehyde and (R)- or (S)-α-methylbenzylammonium hypophosphate in refluxing ethanol, and bromine water oxidation of the resulting phosphonous acid.[312] (R)- and (S)-2-Amino-5-phosphonopentanoic acids have been prepared through a modified Seebach approach (alkylation of the Li enolate of a chiral imidazolidin-4-one),[313] and the same approach using chiral imidazolidin-4-one 5-phosphonates provides 1,2-diaminoalkane-2-phosphonic acids.[314] Analogous extension of standard practice in the carboxylic acid field is shown in the use of the chiral Schiff base (54),[315] and in stereoselective electrophilic amination of chiral non-racemic α-alkyl



(54)

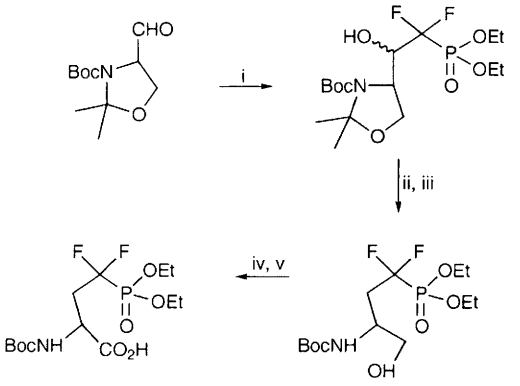
phosphonamides to give α-alkyl-α-aminophosphonic acids.[316] Also, Ru(II)-BINAP-catalyzed hydrogenation of configurationally-labile α-amido β-keto-phosphonic acid esters leads to the (R,R)- or (S,S)-β-hydroxy analogues, the stereochemical outcome indicating the highly enantio- and diastereoselective basis of the process.[317] Synthesis (Scheme 26) and uses of analogues of pimelic acid in which one carboxy group is replaced by the phosphonic acid moiety, have been described.[318]



Reagents: i, BuLi,chiral imidazolidinone; ii, 12 M HCl

Scheme 26

The Garner aldehyde (see also Section 6.3) from D-serine provides the starting point for a synthesis of N-Boc-2(S)-amino-4-(diethylphosphono)-4,4-difluorobutanoic acid (Scheme 27).[319] Synthesis of diphenyl N-(Z-L-α-aminoacyl)pyrrolidine-2-phosphonates, i.e. substituted phosphonic acid analogues of proline, involves cyclization of N-(Z-L-α-aminoacyl)-4-aminobutanal in the presence of $P(OPh)_3$ to give easily separated diastereoisomer mixtures.[320]



Reagents: i, Diethyl difluoromethanephosphonate/LDA/–78 °C; ii, ClCSOPh/DMAP; iii, Bu_3SnH, AIBN; iv, HCl–EtOH; v, $RuCl_3/NaIO_4$

Scheme 27

cis-4-Phosphonomethylpiperidine-2-carboxylic acid has been synthesized from ethyl isonicotinate by reaction with $HCHO/H_2O_2/FeSO_4$.[321] The β-alanine analogue prepared from $PhtNCH_2CH{=}CHP(O)(OEt)CH(OEt)_2$ through catalytic tritiation and deprotection, binds strongly to the GABA-B receptor.[322]

4.17 Synthesis of α-Amino Acids Carrying Boron Functional Groups in Side-Chains, and α-Amino Boronic Acids – (S)-β-(o-Carboranyl)alanine has been synthesized through a standard Schollkopf bis-lactim ether synthesis (see Section 4.2) through propargylation and reaction with 6,9-bis(acetonitrile)carborane, followed by routine work-up.[323]

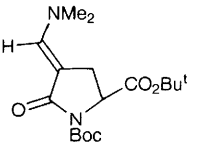
4.18 Synthesis of α-Amino Acids with Silicon Functional Groups in Side-Chains – The Evans oxazolidinone approach (see Section 4.2) is applicable to the preparation of β-trimethylsilyl-L-alanine and $PhSiMe_2$- and $MePh_2Si$- analogues.[324] After uneventful acylation of the chiral auxiliary with $Me_3SiCH_2CH_2$-COCl and analogues, the route involved α-azidation, reduction and cleavage.

4.19 Synthesis of Isotopically Labelled α-Amino Acids – Reviews cover the preparation of ^2H-labelled amino acids[325] and the provision of ^{13}C- and ^{15}N-labelled protein amino acids.[326] A Symposium Proceedings Volume covers most of the salient features concerning the synthesis of labelled amino acids and their current applications,[327] including stereospecific synthesis of ^2H-labelled amino acids[328] (illustrated with D-propynylglycine[329] and ^2H-proline[330]), enzymic synthesis of labelled amino acids[331] and the synthesis of ^{11}C-labelled amino acids[332] including N^{ω}-nitro-L-arginine [^{11}C]methyl ester.[333]

As well as examples given in the preceding paragraph, several other stereospecific syntheses of labelled amino acids have been carried out using standard glycine synthons; thus, alkylation of Oppolzer's camphorsultam (see Section 4.2) and of $Ph_2C{=}NCH_2CO_2R$,[334] base-catalysed deuteriation of the bis-lactim ether chiral auxiliary to give (R)- and (S)- ^2H-labelled α-amino acids[335] and construction of the [^{15}N,$^{13}C_2$]-bis-lactim ether and the corresponding camphorsultam in preparation for the asymmetric synthesis of labelled α-amino acids, e.g. $H_2{}^{15}N{}^{13}CHR{}^{13}CO_2H$.[336] [3-$^2H$]-Labelled phenylalanine stereoisomers have been prepared through deuteriolysis ($^2H_2$/Pd-C) of side-chain brominated phenylalanine derivatives.[337] Catalyzed addition [Pd-C or $RhCl(PPh_3)_3$] of $^2H_2$to dehydroamino acids gives [2,3-$^2H_2$]-labelled α-amino acids,[338] and [1-^{13}C-, 2,3-$^2H_2$]-threo- and erythro-L-α-amino acids have been prepared similarly, involving an enzymic resolution stage. The latter products were used to assist the assignment of ^{13}C-NMR features.[339] L-O-Phosphohomoserine and its two C-3 ^2H-isotopomers have been prepared through standard functional group elaboration of L-aspartic acid and its (2S,3R)- and (2S,3S)-[3-^2H]isotopomers.[340]

Two independent syntheses have been reported of (2S,4S)- and (2S,4R)-[5,5-$^2H_2$]-5,5′-dihydroxyleucine, one from the enaminone (55) from L-pyroglutamic acid [used in a number of previous syntheses of stereospecifically-labelled protein amino acids] through reduction with $NaB(CN)H_3$, and the other involving hydroboronation (disiamylborane) of 4-methyleneproline derivatives.[341] The former route was inefficient though gave the former target, while the latter route was suitable for the latter target. A new synthesis of (2R,3S)-[4-$^2H_3$]valine has been reported.[342] Specific ^3H-labelling of the arene protons of Boc-D-tyrosine in the form of its ethyl ether has been accomplished through iodination – dehalogenation in $^3H_2$[343] (see Ref. 322 for a preparation of tritiated β-aminophosphonic acid).

^{11}C-Labelling continues to stimulate the inventive use of rapid synthetic methods in view of the need to work within the half-life of the isotope (most of the decay occurs in less than one hour). ^{11}C-Labelled alanine has been prepared using $^{11}CH_3I$ for rapid alkylation of N-Boc-3-methyl-4-imidazolidinone (see Section 4.2), and phenylalanine has been prepared in an analogous fashion.[344] $^{11}CH_3I$ has been used for rapid alkylation of L-homocysteine adsorbed on Al_2O_3/KF to give ^{11}C-L-methionine.[345] As an indication of what can be achieved by way of rapid synthesis, this product was ready within 10 minutes after synthesis stages, and including C18-SepPak and alumina SepPak purification. A 40 minute sequence leading to α-[^{11}C]-methyl-L-tryptophan, starting from the enolate of (56), has been described.[346] [2-^{11}C]-α-Aminoisobutyric acid and its N-methyl homologue have been prepared through the Strecker synthesis starting from [^{11}C]-acetone.[347]



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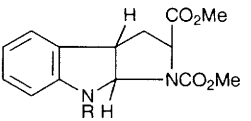

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Alkylation of (S)-N-propanoyl 4-isopropyloxazolidin-2-one and the N-[2-^{13}C]-propanoyl analogue, with $^{13}CH_3I$/NaHMDS and the unlabelled reagent, respectively, gives diastereotopically-^{13}C-labelled L-leucine after routine work-up.[348] The same approach was applied for the synthesis of (2S,4R)-[5,5,5-$^{2}H_3$]leucine.

[2,3-$^{13}C_2$]-4-Hydroxy-L-threonine has been prepared in an 8-step sequence starting from [1,2-$^{13}C_2$]-acetylene, *via* correspondingly labelled 4-benzyloxy-(Z)-but-2-en-1-ol.[349] The relatively much simpler task, synthesis of [2-^{13}C]-labelled amino acids, is exemplified in routes from commercially-available intermediates to [2-^{13}C]-L-lysine (from [2-^{13}C]-glycine through the bis-lactim ether alkylation protocol; see above, and Section 3.2) and to [3,4-$^{13}C_2$]- and [5,6-$^{13}C_2$]-labelled analogues (analogously, from ethyl [1,2-$^{13}C_2$]bromoacetate and [1,2-$^{13}C_2$]acetonitrile, respectively).[350] The same protocol has been used in routes to ^{2}H, ^{13}C- and ^{18}O-labelled L-serine and L-threonine,[351] and to [2′-^{13}C]-, [1′-^{15}N]- and [3′-^{15}N]-L-histidines, though the hard work in these routes was the construction of 1,5-disubstituted imidazoles from labelled toluene-p-sulfonylmethyl isocyanide, 3-phenylpropenal and benzylamine.[352]

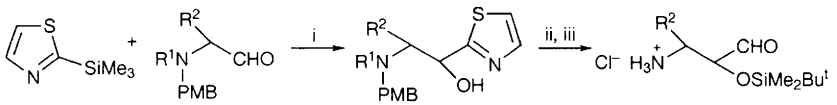
[6-^{14}C]-Vigabatrin, i.e. (RS)-4-aminohex-5-enoic [6-^{14}C]acid, can be obtained through Wittig condensation of 1-(1-butenyl)-2-oxo-5-pyrrolidine carboxaldehyde with methyl[^{14}C] triphenylphosphonium iodide.[353] [U-^{14}C]Glycine has been converted into its N-Z-derivative for use in synthetic operations.[354]

The material in this Section continues to demonstrate the absorption into routine use of asymmetric synthesis methodology employing chiral auxiliaries. The imidazolidinone method has been used for a route to 6-[^{18}F]fluoro-L-DOPA[355] (emphasis is given to the need for care in carrying out the various

stages of this preparation) and the same target has been achieved through the condensation of a chiral methanobenzoxazinone with 3,4-dimethoxy-2-[^{18}F]fluorobenzaldehyde and NaH, followed by L-Selectride-catalyzed hydrogenation.[356]

4.20 Synthesis of β-Amino Acids and Higher Homologous Amino Acids – Reviews of this expanding field cover the general topic of enantioselective synthesis of β-amino acids,[357] and the route involving addition of chiral lithium amides to αβ-unsaturated esters.[358] It is difficult to discern a general falling-into-line behind any particular synthesis method; in this broad area, most of the available methods are being developed successfully.

The Arndt-Eistert homologation of N-alkoxycarbonyl-L-phenylglycine only achieves 9:1-stereoselectivity, while conservation of the initial configuration seems totally assured in the application of this protocol to other L-α-amino acids.[359] This paper also addresses stereoselective α-alkylation of β-amino acids through providing new results. An alternative homologation procedure through the sequence $RCO_2H \rightarrow RCH_2OH \rightarrow RCH_2I \rightarrow RCH_2CN \rightarrow RCH_2CO_2H$ is made somewhat less tedious by the use of polymer-bound triarylphosphine-I_2 complex for one of the steps,[360] and has been employed for the synthesis of several Fmoc, Z and Boc-protected examples.[361] 2-Dibenzylaminobutan-1,4-diol, easily available from L-aspartic acid, is used in the same way, except for the intermediacy of a mesylate in place of the iodide, and has been used for the asymmetric synthesis of representative β-amino acids, including β-proline.[362] Homologation of N-protected α-aminoaldehydes looks more promising as a further use for 2-trimethylsilylthiazole (Scheme 28), giving syn β-aminoalcohols and β-amino-α-



(+25% *syn* -isomer)

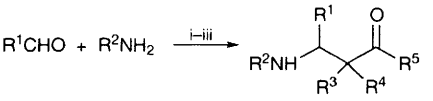
Reagents: i, CH_2Cl_2, –20 °C, then Bu_4NF/THF; ii, Bu^tMe_2SiCl, then CF_3SO_3Me, then $NaBH_4$/MeOH; iii, $HgCl_2$

Scheme 28

hydroxyaldehydes [illustrated for a preparation of (S,S)-3-amino-2-hydroxy-4-phenylbutanol, for use in synthesis of a HIV protease inhibitor] from which β-amino acids may be generated.[363] L-Phenylglycine reacts with the thiazole synthon to lead to the taxol side chain component (2R,3S)-3-phenylisoserine, in the form of its N-benzoyl- and N-Boc-derivatives.[364] Careful study of the chain extension of N-protected α-aminoaldehydes through aldolization with vinyl α-anions derived from acrylic esters has been reported.[365]

The logical deconstructionist view to be taken, in the face of these successful methods, is to bring an aldehyde into touch with an amine together with an

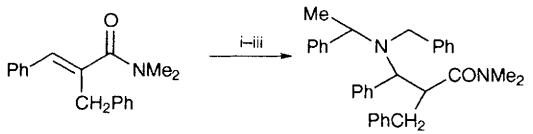
electrophilic synthon that is destined to provide the α-carbon carrying a carboxy group (i.e. a silyl enolate; Scheme 29). In such a way, a one-pot synthesis of β-amino acid esters has emerged after the role for ytterbium(III) triflate as catalyst was established, a method that is also adaptable to β-lactam synthesis.[366] A new, highly regioselective and stereoselective asymmetric oxirane ring-opening brought about with $MgBr_2$ has been established using phenylisoserine as target.[367]



Reagents: i, add $R^3R^4C{=}CR^5OSiMe_3$ after 30 min at room temp.; ii, $Yb(OTf)_3$ 5–10 mol %, CH_2Cl_2; iii, dehydrating agent (molecular sieve 4Å, or $MgSO_4$)

Scheme 29

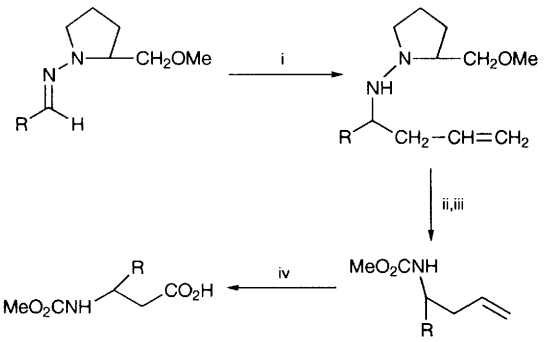
Ytterbium(III) triflate-catalyzed high pressure addition of amines to αβ-unsaturated esters gives moderate to high yields of hindered β-amino esters.[368] Several research groups are extending the general 'asymmetric β-amination of αβ-unsaturated esters or analogues' approach to the synthesis of homochiral β-amino acids in which chiral ammonia equivalents, *viz.* homochiral amidocuprates,[369] the homochiral lithium amides Li (α-S)-(α-methylbenzyl) benzyl allylamide[370,371] and Li α-phenylethylamide,[372] SAMP,[373,374] or TMS-SAMP [(S)-2-methoxymethyl-1-(trimethylsilylamino)pyrrolidine][375,376] are used. Conjugate addition of benzylamine to (S)-5-[(tert-butyl)diphenylsilyloxymethyl]-2(5H)-furanone generated the trans-adduct, a useful synthon for further elaboration by alkylation at C-3 *via* its lithium enolate.[377] High enantioselectivity is achieved in these routes (typical applications are displayed in Schemes 30 and 31), and intermediate enolates can be trapped by quenching with ^2H_2O so as to provide



Reagents: i, (*S*)-[α-Methylbenzyl]benzylamine, ii, $LiNR_2$; iii, H_2–Pd

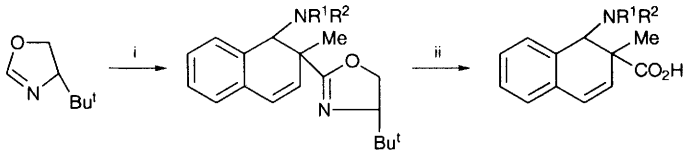
Scheme 30 (ref. 370)

α-^{2}H-β-amino acids (Ref. 369). The use of lithium (α-S)-(α-methylbenzyl)allylamide calls for de-allylation [tris(triphenylphosphine)Rh(I)Cl] to liberate the product (Ref. 371) while the TMS-SAMP procedure ends with N-N cleavage (Ref. 373). The mirror image of this process, carboxylation of enamines, provides an alternative general route, and a veiled form of this has been established in which chiral oxazolines are alkylated (Scheme 32).[378] Another approach to



Reagents: i, Allylcerium chloride, or allyl Grignard reagent; ii, Li/NH_3; iii, MeO_2CCl; iv, O_3

Scheme 31 (Ref.373)



Reagents: i, $LiNR_2$, 2-chloronaphthalene derivative, ii, H_3O^+

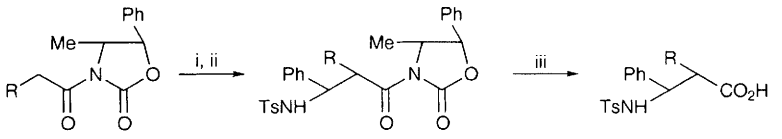
Scheme 32

assembling the components of a target β-amino acid is offered in the Michael-type addition of a carbon radical (alkyl iodide/Bu_3SnH/hν) to α-aminoalkylvinyl esters $ArCH(NHTs)C(=CH_2)CO_2Me$, though syn/anti-ratios are remarkably sensitive to the nature of the o-substituent of the arene moiety.[379]

Michael addition of N-methylhydroxylamine to γ-alkoxy-αβ-unsaturated esters gives β-(N-methyl-N-hydroxyamino) esters.[380]

Synthesis of stereoisomers of 3-amino-2-hydroxydecanoic acid and 3-aminodecanoic acid using the chiral lithium amide strategy, and comparisons of published data for the former β-amino acid (it is the N-terminal amino acid of microginin) with those for the synthesis products, has allowed assignment of the (2S,3R)-configuration to the natural compound.[381] N-Methoxycarbonyl-1-methoxyamines $MeO_2CNHCH(OMe)R$ react with glyoxylic acid derivatives to give syn/anti-mixtures of 3-amino-2-hydroxyalkanoates, appropriate choice of protecting groups allowing control of diastereoselectivity.[382]

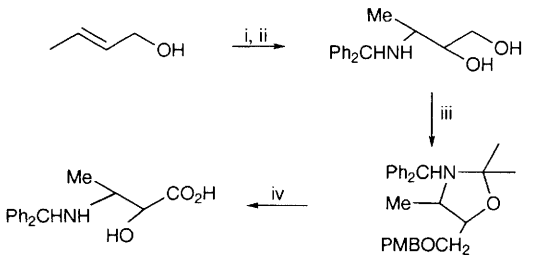
The addition of an α-sulfinyl ester enolate to a benzaldimine carrying an electron-withdrawing group at nitrogen gives a β-amino acid ester in up to 94% e.e., diastereofacial selectivity being widely changeable and determined by the N-



Reagents: i, LDA, −78 °C; ii, PhCH=NTs; iii, H_3O^+

Scheme 33

substituent and additives in the reaction mixture.[383] Lithium and titanium enolates of N-acyloxazolidinones are as useful in β-amino acid synthesis as in their main use in the α-amino acid field, e.g. through addition to imines (Scheme 33). The stereochemical outcome indicates that addition occurs to the *si*-face of the chelated (Z)-enolate.[384] Amidoalkylation of this chiral synthon with 1-(Z-aminomethyl)benzotriazole and routine workup gives (R)-(+)-3-amino-2-phenylpropanoic acid, establishing (by X-ray crystal analysis) the correct absolute configuration for this known compound.[385] Anti-3-amino-2-hydroxybutyrates can be obtained with the aid of the chiral auxiliary anti-$Ph_2CHNHCMe(OH)CH$-MeOH, *via* the oxazolidine derived from it (Scheme 34).[386] Another connection

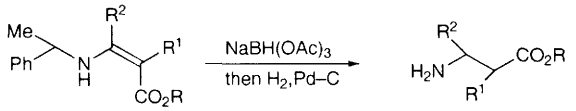


Reagents: i, Bu^tOOH, (D)-DIPT/$Ti(OPr^i)_4$; ii, $Ph_2CHNH_2/Ti(OPr^i)_4$;
iii, PMB-Cl after oxazolidinone formation; iv, H_3O^+ after $CH_2OPMB \rightarrow CO_2H$

Scheme 34

with the α-amino acid field through use of a six-membered analogue of a standard chiral heterocyclic auxiliary (see Section 4.2) is represented in the diastereoselective hydroxylation at C-5, using the camphorsultam-derived oxaziridine, of chiral 6-substituted perhydropyrimidin-4-ones. Enantiomerically pure α-hydroxy-β-amino acids are obtained in this way, illustrated for representative examples,[387] and specifically for preparations of L-isothreonine and D-alloisothreonine[388] and of N-benzoyl-(R,R)-3-phenylisoserine.[389]

Radical cyclization of β-aminoacrylates gives β-proline derivatives.[390] Reduction of homochiral β-enamino esters [N-vinyl (S)-phenylethylamines (57)] using $NaBH(OAc)_3$ gives β-amino acid esters with moderate enantiomeric selectivity.[391]



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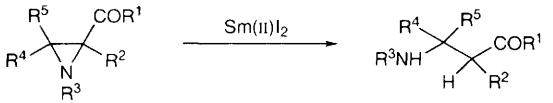
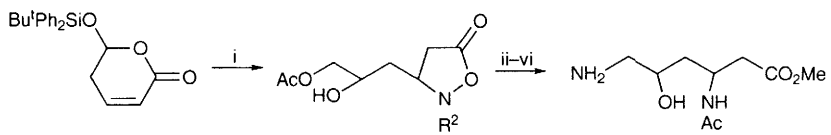


(58)

Small-ring synthons have provided conventional routes to particular targets over the years, and are represented in a synthesis of (2R,3S)-3-phenylisoserine (glycidic ester intermediate),[392] and in oxidative ring-opening of aziridinecarboxylic acids (58).[393] Reductive ring-opening (catalytic hydrogenation) of methyl 3-methyl N-toluene-p-sulfonylaziridine-2-carboxylate gives 3-aminobutanoic acid derivatives, a process that leads to enantiomers when homochiral starting materials (N-α-methylbenzyl and/or non-racemic N-toluene-p-sulfonyl derivatives) are used.[394]

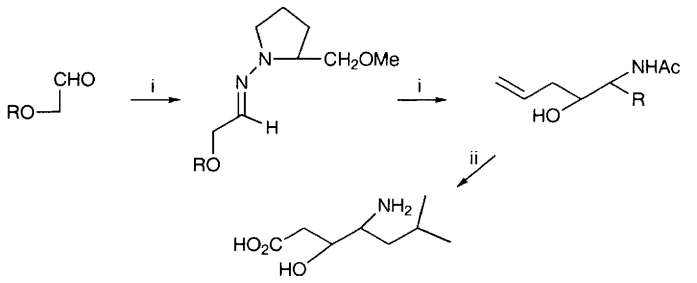
β-Amino acids prepared in other ways include (R)-(+)-β-phenylalanine from (S)-(+)-benzylidene-toluene-p-sulfinimide [S*N=CHPh + CH_2CO_2Me→(S*NH-$CHPhCH_2CO_2Me$, where S* is the chiral auxiliary],[395] allophenylnorstatine [*alias* (2S,3S)-3-amino-2-hydroxy-4-phenylbutanoic acid] in eight steps from L-phenylalanine,[396] and '(R)-(+)-Boc-iturinic acid-(n-C-14)' [a curious choice of nomenclature, intended to convey the meaning 'the unbranched isomer of iturinic acid', i.e. $Me(CH_2)_{10}CH(NHBoc)CH_2CO_2H$] starting from L-aspartic acid, a key step being organocuprate addition to (S)-$BocNHCH(CH_2OTs)CH_2CO_2Bzl$.[397] (2S,3R)-3-Amino-2-methylpentanoic acid is conveniently accessible from aspartic acid *via* (3R)-3-N-toluene-p-sulfonylamino-γ-butyrolactone.[398] Further examples of the use of an easily-available amino acid to synthesize a higher homologue are found later in Section 6.3. A synthesis of negamycin lactone *via* the isoxazoline (59 in Scheme 35) starts from D-glucose.[399]



(59)

Reagents: i, R^2NHOH; ii, mesylation; iii, MeOH, K_2CO_3; iv Bu_4NF, repeat ii; v, NaN_3/DMF; vi, H_2, 10% Pd–C

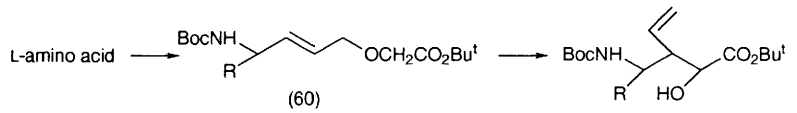
Scheme 35



Reagents: i, SAMP protocol (Scheme 31); ii, O_3

Scheme 36

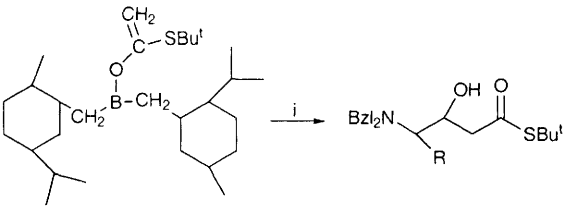
Several important individual examples of free and peptide-bound γ-amino acids provide valid synthetic targets in their own right, irrespective of the general need for reliable synthetic methodology to prepare γ-amino acids to support the burgeoning peptide mimetic field. Some of the current methods are extensions of routes used with lower homologues, such as amination by TMS-SAMP illustrated in an (R,R)-statine synthesis (Scheme 36),[400] and Arndt-Eistert homologation of L-malic acid-derived (S)-N-Boc-2-oxo-oxazolidine-5-carboxylic acid to give (R)-α-hydroxy-γ-aminobutyric acid [(R)-GABOB].[401] Stereoselective Wittig rearrangement of tert-butyl 4-aminoallyloxyacetates (60 in Scheme 37; prepared from L-α-amino acids) leads to α-hydroxy-γ-amino acid esters.[402] Bromination of



Scheme 37

enamines gives an iminium salt [$R_2NCH{=}CR^1R^2 \rightarrow R_2N^+{=}CHCR^1R^2Br$] from which 'αβ-unsaturated GABAs' are obtained by quenching with lithium tert-butyl acetate (see also Ref. 395).[403] Novel lipophilic γ-amino acids (2-amino-methylcyclopropanecarboxylic acids and tricyclic analogues) have been prepared through rhodium(II)-catalyzed cyclopropanation of corresponding alkenes with α-diazoesters N_2CPhCO_2Me and $N_2C(CF_3)CO_2Et$.[404] The formation of azetidin-3-ones from L-α-amino acids *via* corresponding diazoketones has been explored further; reduction of the azetidin-3-ones with complex hydrides or addition of an organometallic reagent leads to γ-amino acids.[405]

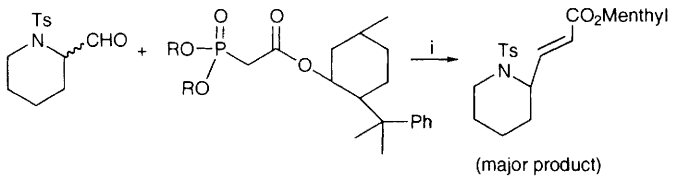
Osmylation of γ-Boc-aminocrotonates has been carried out, routine work-up leading to α-hydroxystatine.[406] N-Aminoacylpyrazoles undergo Reformatzky reaction with β-bromoesters to yield δ-aminated β-ketoesters, from which 4-



Reagents: i, $Bzl_2NCHRCHO$

Scheme 38

(protected amino)-3-oxoalkanoates can be obtained as statine precursors.[407] Stereocontrolled (3S,4S)-statine synthesis from (+)-menthone-derived boron enolates and α-NN-dibenzylamino-aldehydes (Scheme 38)[408] is a notable example of the scope of the aldol family of reactions. DL-α-N-Toluene-p-sulfonylamino aldehydes undergo kinetic dynamic resolution through olefination with a chiral phosphonate in the presence of a slight excess of base (Scheme 39).[409] Much of the interest in an (R)-carnitine synthesis lies in the quinidine-mediated [2 + 2]cycloaddition of keten to chloral that leads to the synthesis intermediate (R)-4-(trichloromethyl)oxetan-2-one.[410]



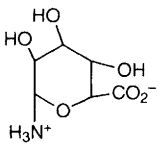
Reagents: i, Slight excess KHMDS

Scheme 39

δ-Amino acids of current interest include baclofen [enantiomers of 4-amino-2-(4-chlorophenyl)butyric acid], accessible from N-toluene-p-sulfonyl-2-(4-chlorophenyl)aziridine *via* ring opening with allylmagnesium bromide and oxidative modification of the allyl group of the resulting pyrrolidin-2-one,[411] also formed from RuO_4 oxidation of dehydroproline formed from trans-4-hydroxy-L-proline,[412] and in enantiomerically pure form through chymotrypsin-catalyzed hydrolysis of dimethyl 3-(4-chlorophenyl)glutarate or through lipase-catalyzed esterification of 2-(4-chlorophenyl)propan-1,3-diol.[413] '(E)-Olefine dipeptide isosteres', i.e. dipeptide mimetics in which the amide grouping is replaced by the (E)-ethene moiety, are also classifiable as αβ-unsaturated δ-amino acids, and a route

(61) (62)

has been established (61→62) involving allylic nitration (NO_2BF_4) of (E)-crotylsilanes followed by reduction.[414] The Overman acetimidate rearrangement route (cf. Scheme 1) is an efficient entry to these αβ-unsaturated δ-amino acids, and has been applied to β-hydroxy γδ-unsaturated acids.[415] Homochiral 4-(α-hydroxyalkyl)-3-(phenyldimethylsilyl)-γ-lactones, which are readily available through stereocontrolled routes, are suitable precursors of αβ-unsaturated δ-amino acids through displacement by azide and ring-opening accompanying desilylation.[416] Hydroxyethylene dipeptide isosteres, i.e. δ-amino-γ-hydroxy acids, have been prepared from α-aminoalkyloxiranes by conversion into homoallylic alcohols $BocNHCHR^1CH(OZ)CH_2CR^2{=}CH_2$ followed by routine elaboration,[417] and from γ-(α-aminoalkyl)lactones prepared from L-glutamic acid.[418] Alkenylaziridines may be prepared by chain extension of β-hydroxy-α-amino acids or from allylic alcohols, and converted into αβ-unsaturated δ-amino acids.[419] The α-carboxy group of L-glutamic acid can, likewise, be extended [after reduction of N-(2,4-dimethoxybenzyl)-L-pyroglutamic acid to the aldehyde] to give 4-amino-5-hexynoic acid.[420] The tetrahydropyran-based amino acids (63) and furan analogues can be viewed, like many other cyclic compounds carrying



(63)

amino and carboxy functions, as conformationally constrained ω-amino acids, and this has attracted interest in their synthesis involving the application of standard operations (azidolysis of sugar-derived epoxides as the crucial step).[421] (S)-5-Aminopiperidin-2-one, which can be categorized as the lactam of either a γ- or a δ-amino acid, has been prepared in enantiomerically pure form from L-pyroglutaminol through ring-opening with methyl carbamate, and amination of the remaining primary hydroxy group through the Mitsunobu method (Ph_3P/diethyl azodicarboxylate).[422]

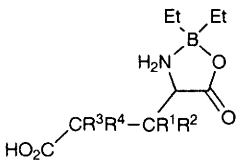
Beckmann rearrangement of alicylic ketones to ω-amino acid salts and then to lactams during workup has a long history, and simple modifications manage to convince Referees of their need for urgent publication (e.g. microwave irradiation of SiO_2-adsorbed reactants, cyclopentanone to cyclododecanone and hydroxylamine-O-sulfonic acid).[423]

4.21 Resolution of DL-Amino Acids – An expanding number of methods for the resolution of DL-amino acids seems to be entering the 'classical' category, due to

the development of new methods over recent years leading to reliable protocols. One of the two archetypal classical non-enzymic resolution protocols, *viz.* formation of diastereoisomeric salts and their separation through crystallization or other physical principles, is regularly represented in this Chapter; this year for DL-amino acids and (-)-1-phenylethanesulfonic acid;[424] for the benzyl ether of (R)-(-)-2-aminobutan-1-ol and the (S)-(+)-enantiomer with N-acyl-DL-phenylglycines and (p-hydroxyphenyl)glycines;[425] and for N-protected α-alkoxyglycines with alkaloids[426] (see also Ref. 295).

When the resolving agent is structurally similar to the enantiomers to be separated, more efficient resolution is achieved because quasi-racemic diastereoisomeric salts are then formed between components of opposite configuration.[427] This is of course, the basis of the long-running preferential co-crystallization method (e.g. D-threonine crystallizes from solutions of its racemate containing L-serine or 4-hydroxy-L-proline[428]), and has also been intuitively felt, and passed on, through generations of organic chemists. However, it is satisfying that the proposal is generally borne out by the results of twelve typical laboratory resolutions, including several common amino acids and their derivatives (described in Ref. 428).

The other archetypal protocol amounts to the conversion of a DL-amino acid into a diastereoisomeric derivative and exploitation of the physical differences between the diastereoisomers; an unusual example is the formation of diastereoisomeric α-boroxazolidinones (64; see also Section 5.1) for the resolution of



(64)

variously α- and β-substituted DL-glutamic acids.[429] The preparation of (R) and (S)-2-(aminomethyl)alanine and 2-(aminomethyl)leucine through reaction of oxazolones of the DL-forms of these amino acids with L-phenylalanine cyclohexylamide also falls within this category (see also Refs.82, 155, 191).[430] Esterification of an N-acyl-DL-amino acid with (+)- or (-)-menthol is also typical (Ref. 426). N-(D- or L-tetrahydro-2-furoyl)ation of DL-amino acid esters gives diastereoisomers that show a difference in boiling points of up to 7°.[431] An inert liquid whose density lies between the densities of a racemate and that of a constituent enantiomer will achieve a separation of the two forms, and L-phenylalanine has been floated off from a mixture with its more dense racemate.[432] This is equivalent to the foam flotation method already described in the recent literature (Vol 27, p. 45).

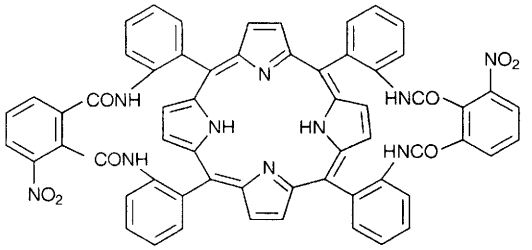
Kinetic resolution of aldehydes, by an asymmetric Wittig-type reaction with chiral 2-phosphonopropionates, has been illustrated for N-toluene-p-sulfonylpipecolic aldehyde.[433]

Enzymes that are shown to be useful in this context are increasing in number and in their diversity. Current standard procedures that represent the culmination of classical methodology for the simplest functional group conversions, illustrated in the use of proteases and lipases for the conversion of DL-amino acid esters into an L-amino acid amide + D-amino acid ester mixture with moderate to high enantioselectivity[434] (D-phenylglycine methyl ester racemizes slowly under these reaction conditions, but enantioselective penicillin G acylase-catalyzed L-mandelylation of DL-phenylglycine and its p-hydroxy analogue seems secure).[435] The use of penicillin G acylase for enantioselective hydrolysis (greater than 95% e.e.) of N-phenylacetyl β-aryl-β-amino acids [(R)-enantiomers are hydrolysed more rapidly][436] is one of several examples of the use of acylases [including also the use of acylase I for the enantioselective hydrolysis of N-acetyl-DL-vinylglycine[437] and the use of aminoacylase for enantioselective acetylation of DL-methionine[438] (see also Refs. 66, 286, 293)]. The use of subtilisin for enantioselective hydrolysis of DL-ββ-difluoro-α-amino acid methyl esters;[439] the use of lipase for enantioselective transesterification of DL-phenylalanine methyl ester with octan-1-ol;[440] and the use of papain for enantioselectively-catalyzed esterification of DL-acids (Ref. 270) illustrate another common approach. A novel procedure is represented in enantioselective alcalase-catalyzed hydrolysis applied to an amino acid ester, parallel with pyridoxal-5-phosphate-catalyzed racemization of the unhydrolysed antipode.[441]

More surprising successes have accrued, for the resolution of representative well-known amino acid pharmaceuticals (aromatic α-methyl-α-amino acids and α-methyl-α-hydrazino acids, using *Candida lipolytica* for ester hydrolysis),[442] and similar results have been secured for αα-disubstituted glycine esters (using *Humicola langinosa*);[443] while the selective hydrolysis of DL-hydantoins so as to liberate D-amino acids (using thermophilic micro-organisms,[444] *Arthrobacter* sp.DSM7330[445] and *Agrobacterium* sp.I-671[446]) represents a more thoroughly-established area. A route to L-tert-leucine from DL-2-phenyl-4-tert-butyloxazolin-5-(4H)-one employs lipozyme present in *Mucor miehei* as catalyst for transesterification with n-butanol, and *Bacillus licheniformis* alcalase for clean hydrolysis, in a new example of the long-established oxazolinone dynamic resolution route.[447]

Destructive 'resolution' procedures, in which the total initial amount of racemate is not returned as separated isomers, are represented by the action of yeast D-amino acid oxidase on a molasses fraction, converting D-α-amino acids into α-keto-acids, to leave L-enantiomers unaffected.[448] An interesting extension of this approach employs genetically-engineered *E.coli* TM93 for the production of D-glutamic acid from its L-enantiomer, involving two successive stages imposed by a glutamate racemase (L→DL) and then enantioselective decarboxylation.[449]

Separations of enantiomers based on chiral recognition principles are represented in a number of papers describing homogeneous solution methods: diastereoselective partition between water and chloroform, of host-guest complexes of phosphorus hexafluorophosphate salts of DL-amino acid esters with poly(1-6)-2,5-anhydro-3,4-di-O-alkyl-D-glucitol,[450] and similar host-guest studies



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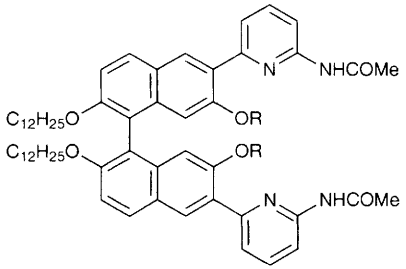

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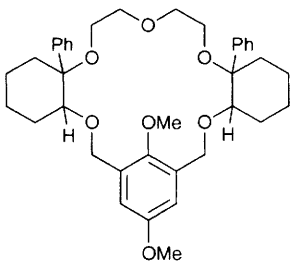

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with an αααα-atropisomer of (65) formed beween meso-tetrakis(o-aminophenyl)-porphyrin and 4-nitroisophthaloyl chloride,[451] and involving Z-aspartic acid, Z-glutamic acid, or Z-kainic acid complexed in C^2HCl_3 with (R)- and (S)-binaphthyls (66);[452] and a FAB-MS evaluation of the host-guest behaviour of the homochiral crown ether (67)[453] (see also Ref. 532). A related NMR study of eleven 1,2-bis(D-hexopyranoside and D-mannitol-derived)-18-crown-6-ethers, showing enantioselection of salts of phenylglycine enantiomers in C^2HCl_3, has been published.[454] Resolution of dinitrobenzoyl-DL-amino acids on a gram scale, by high-speed counter current chromatography using N-decanoyl-L-proline-3,5-dimethylanilide as chiral selector, is a variation of standard chromatographic principles in this area (analogous analytical-scale resolutions are covered in Section 7),[455] and chromatography over poly(acrylate)s imprinted by Boc-L-phenylalanine offers efficient chiral separation of amino acid derivatives[456] (see also Refs. 866, 867). Novel chiral stationary phases (CSPs) have been prepared involving L-phenylalanine or D-phenylglycine residues separated by long spacer units.[457]

Analogous membrane separation studies include ultrafiltration resolution of phenylalanine, tyrosine and tryptophan, employing cellulose acetate derivatized with (-)-menthol;[458] and copper(II)-mediated transport of α-amino acids across a bulk chloroform membrane into aqueous EDTA, by chiral 1,2-diaminoethane derivatives[459] (see also Section 5.4).

Clefts that bind histidine esters tightly are a feature of a homochiral bis(porphyrin) that is structurally a Troger's base analogue, resulting in chiral discrimination between histidine ester enantiomers amounting to 80 – 86% e.e.; only 48% e.e. is achieved with lysine benzyl ester.[460] α-Zirconium phosphate, intercalated by a cationic chiral π-acceptor, selectively binds one enantiomer of 2-naphthyl-DL-alanine methyl ester from solutions.[461]

Speculation about the prebiotic origin of molecular chirality continues to revolve around the effects of the contemporary physical environment on racemic mixtures. 'Symmetry breaking' is the favoured term to refer to the genesis of L-amino acids and D-sugars starting from racemates; lack of rigorous experimental proof for proposed mechanisms[462] is a notable feature of the literature on this topic over many years. The current theme revolving around the parity violation (PV) phenomenon for electroweak interactions,[463] which can be calculated[464] to favour the current scene, has been claimed by Salam (see Vol 24, p. 40) to require that a phase transition will exist at low temperatures that should lead to enantiomeric purity over vast stretches of time. The theory implies that experimental proof will be easier to obtain for the phase transition hypothesis (compared with the difficulty of establishing very small differences in D:L-ratios starting from pure racemates submitted to aggressive radiation treatment), but the vanishingly small energy differences between each enantiomer of a racemate, that PV leads to, may explain why no enantiomeric imbalance could be detected for samples of DL-cystine derivatives held at 0.01K.[465] On the other hand, a specific heat discontinuity found for a single crystal of D-valine held at 272K is not shown by L-valine, and the fact that there is no crystal structure transition for these samples over the temperature range 123 – 293K, is taken to support the conclusion[466] that evidence to support Salam's prediction has become available from this work. Clearly, the failure to try to develop other explanations will cause this conclusion to be queried.

A long-standing critic of the PV hypothesis has continued to maintain that the genesis of molecular chirality in the prebiotic environment must have required the influence of an extra-terrestrial energy source.[467] The proposal, that circularly-polarized UV synchrotron radiation from the neutron star remnants of supernovae caused the enantioselective photolysis of interstellar dust, is based on verified science in all aspects except for decisive laboratory proof of the enantioselective photolysis of amino acid racemates. Some amplification mechanism is required to accompany preferential destruction by radiation of one enantiomer, if a slight chiral excess is to be amplified over time with the extinction of this enantiomer; an amplification mechanism is also needed by other D:L-unbalancing protocols based on the PV hypothesis.[468]

5 Physico-Chemical Studies of Amino Acids

5.1 X-Ray Crystal Analysis of Amino Acids and Their Derivatives – The considerable extent of this topic will be familiar to readers over the years, the sheer volume being partly due to the continual revisiting of particular free amino

acids. Structures for protein amino acids determined recently include DL-proline monohydrate,[469] DL-alanine nitrate,[470] DL-aspartic acid nitrate monohydrate,[471] L-(+)-histidine acetate dihydrate,[472] L-histidine dihydrochloride,[473] anhydrous DL-glutamic acid,[474] DL- and L-lysine formate,[475] ^{1}H and ^{2}H-labelled L-arginine phosphate monohydrate (neutron diffraction),[476] L- and DL-histidine – formic acid complexes[477] and hydrated L-serine – inosine-5′-monophosphate 1:2-complexes.[478] Some of these papers emphasise the hydrogen-bonding features that are revealed, in direct fashion by neutron diffraction, and an unusual version of this approach is used to determine the structure of the hydration sphere surrounding glycine molecules in concentrated (5 mol %) aqueous solution.[479] The overall picture for details of participation of C-H bonds in hydrogen-bonding to oxygen atoms, in a range of molecules including amino acids, has been discussed.[480] Inelastic incoherent neutron scattering of samples of DL- and L-valine,[481] and coherent inelastic neutron scattering features for solid L-alanine,[482] have been assessed. The X-ray structure of the L-proline – 2,5-dihydrobenzoic acid complex provides a rare example of a hydrogen-bonded zwitterion co-crystal, and its non-linear optical properties may be useful in the construction of microelectronic devices (these properties are also shown by other homochiral solids).[483] Re-determination of the structure of DL-norleucine, and observation of changes that occur during heating,[484] of the structure of L-DOPA (for comparison with molecular orbital calculations providing electron distribution),[485] and newly-determined structures for DL-prop-2-ynylglycine,[486] L-nitroarginine monohydrochloride monohydrate,[487] 3-iodo-L-tyrosine methanol solvate[488] and (Z)-β-fluoromethylene-m-tyrosine[489] (see also Refs. 272, 281) cover examples of near relatives of the protein amino acids.

Amino acid derivatives subjected to X-ray crystal analysis include N-benzyloxycarbonylglycine,[490] benzoyl-L-histidine monohydrate,[491] N-[(2R)-bromopropanoyl]-(2S)-proline methyl ester,[492] N-carbamoyl-DL-aspartic acid,[493] NN′-carbonyl bis(L-phenylalanine ethyl ester),[494] N-phthaloyl (E)-α,β-dehydrophenylalanine,[495] the alanine-derived boroxazolidinone (64; Ph in place of Et; Me in place of $CR^1R^2CR^3R^4CO_2H$),[496] (S)-α-ethylphenylalanine N-carboxyanhydride,[497] trans-3-hydroxy-N-methyl-L-proline hydrochloride,[498] methyl N-phenyl-L-tyrosinate,[499] and Boc-N$^\tau$-benzyl-histidine p-nitrobenzyl ester.[500]

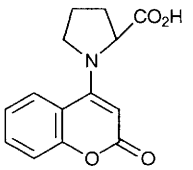
The structure of an unusual condensation product, 1-cyclohexyl-2-cyclohexylaminoimidazol-5(4H)-one, from glycine ethyl ester and dicyclohexylcarbodiimide, has been confirmed by X-ray crystal analysis.[501]

5.2 Nuclear Magnetic Resonance Spectroscopy – Out-of-the-ordinary NMR studies involving amino acids and their derivatives is a rough description of papers selected for this Section, and the term 'out-of-the-ordinary' has been interpreted over the years in this Specialist Periodical Report to cover work with newly-introduced NMR instrumental techniques, and routine techniques applied to structural analysis of unusual substrates.

Solid state ^{1}H-NMR data, from which information of proton relaxation and molecular motion in solid amino acids is available, have been presented.[502] Magic angle spinning HQMC and TOCSY ^{13}C-NMR assessment of N$^\alpha$-Fmoc-N$^\varepsilon$-Boc-

L-lysine bonded to solvent-swollen Wang resin, has shown that on-resin monitoring may be achieved during solid-phase peptide and combinatorial synthesis.[503] Rotational-echo double resonance NMR evidence may be interpreted to indicate an extended conformation for [1-^{13}C,^{15}N]acetyl-L-carnitine in the solid state, which contrasts with the folded conformation revealed by X-ray analysis.[504] More routine conformational studies have involved ^{1}H-NMR measurements for solutions of amino acids and nucleotides in $^{2}H_2O$, purporting to derive association constants by fitting changes in chemical shifts for anomeric and ring protons to an isotherm;[505] the strongest associations occurred between coded amino acids and their respective anticodonic nucleotide sequences, a self-fulfilling outcome that does not in itself verify the somewhat tenuous thermodynamic basis of the study. A study of interactions of amino acids with nucleic acids and caffeine calls on a range of techniques.[506] More narrowly-based studies cover Z-N-methylisoleucine derivatives,[507] N-substituted αω-diamino acids[508] and 1-aminocyclopentane-1,3-dicarboxylic acid derivatives (whose importance lies in their potential as glutamic acid analogues).[509] As is often the case with such objectives, X-ray analytical support was obtained for these last-mentioned derivatives. Tautomeric equilibria established by NMR for simple aliphatic NN-dimethylamino acids in $(C^2H_3)_2SO$ reveals the predominance of the unionised form.[510]

Continuing themes are represented in photo-CIDNP studies of N-acetyl histidine, tryptophan and tyrosine,[511] and for ^{1}H-NMR assignment of enantiomeric purity to α-amino acids, for which purpose N-coumarinyl-L-proline (68) has been proposed as a new chiral derivatization reagent.[512] Diastereoisomers formed between a racemic chiral acid and (R)- or (S)-phenylglycine methyl ester show sufficiently large differences for chemical shifts for particular protons, to allow assignment of absolute configuration.[513] New chiral NN′-disuccinate ligands (69) formed from (5R)- or (5S)-(menthyloxy)furan-2(5H)-one have been used as complexes with Eu salts to create effective chiral shift reagents for the estimation of D:L-ratios for amino acids.[514]



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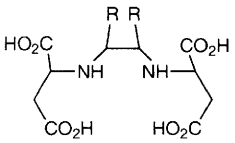

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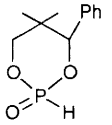

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NMR techniques that deal with higher mass isotopes are featured in an X-ray/^{13}C-MNR study of N-acylated 3-methylaziridine-2-carboxylate esters, showing a 40° tilt in the plane of the amide carbonyl group with respect to the plane of the ring,[515] pH-dependence of the ^{15}N-NMR features of histidine,[516] a study involving ^{1}H-^{15}N-heteronuclear multiple quantum coherence (HMQC) transfer NMR at 200 MHz focussing on the generation of [5-^{15}N]-glutamine after *in vivo* perfusion of $^{15}NH_4^+$into rat brain,[517] ^{19}F-NMR studies of interactions

within complexes of hexakis(2,3,6-tri-O-methyl)-α-cyclodextrin with (RS)-fluorinated amino acid derivatives[518] and an evaluation of ^{13}C- and ^{29}Si-NMR data of trimethylsilyl derivatives of 25 amino acids[519] and analogous TBDMS-amino acids.[520] ^{31}P-NMR has been advocated for providing well-separated signals for diastereoisomers formed from amino acids with the novel chiral derivatization reagent (70).[521]

5.3 Optical Rotatory Dispersion and Circular Dichroism – Further data confirming the anomalous optical rotation behaviour of N^{α}-acyl-L-lysines in water over a wide range of pH (see Vol 27, p.49) have been published.[522] The circular dichroism (CD) characteristics over the wavelength range 200-600 nm, of solutions containing Cu(II) and Ni(II) – L-DOPA complexes can be interpreted to reveal individual stages in the oxidation of the arene chromophore.[523] Among routine assignments of absolute configuration in the recent literature (see also Ref. 272), the interpretation (Ref. 37) of the CD feature at 207 nm indicating the (S)-configuration for cis-2-amino-5-chloropent-4-enoic acid, may be found to be undermined by the mutual influence of the two chromophores absorbing in this wavelength region.

5.4 Mass Spectrometry – The pioneering character assigned to studies of a range of ionization techniques only a few years ago, has been transfigured as they enter into routine laboratory use. FAB and electrospray methods are the most prominent of these newer methods, the former category being represented in a study of phosphoryl derivatives (of 20 common amino acids), in which positive ion analysis is found to be most appropriate for derivatives of the basic amino acids, while negative ion analysis is suitable for the derivatives of the neutral and acidic amino acids, because intense parent ions are produced.[524] Metastable ion fragmentation of cations formed from twelve different copper(I)-amino acids,[525] and a study of molecular radical cations formed from N-toluene-p-sulfonyl-N-alkylamino acid esters embedded in various matrices, have been reported.[526] More fundamental ion-molecule bombardment studies deal with gas-phase protonation of glycine by CH_5^+,[527] and corresponding work with amino acid dimers has been described.[528] Spectra obtained by seeding tryptophan into argon undergoing pulsed supersonic expansion can be linked to particular lowest energy conformations of the amino acid; a straightforward study[529] and a sophisticated version of this approach (IR-laser desorption of tryptophan, and its multiphoton ionization in the gas phase[530]) provide an excellent indication of the application of state-of-the-art MS techniques to fundamental studies of molecules.

Electrospray mass spectrometry of free amino acids can yield isotope ratios for component elements in leucine, arginine and proline, with a standard deviation around 0.1%.[531] This figure was assigned from experiments which yielded accurate diagnostic evidence when amino acid mixtures were spiked with 0.85 mol% of a singly-^{13}C-labelled isotopomer. Further exploitation of the mild nature of electrospray ionization has been reported, leading to spectra for host-guest complexes of amino acids and cyclodextrins.[532]

Other variations of techniques are seen in electron-capture negative ion MS of pentafluorobenzyloxycarbonylamino acids (prominent $[M-181]^-$ ion for the derivatized phenylalanine ethyl ester)[533] and CTC/MS of TBDMS [$^2H_5$]-phenylalanine.[534]

Establishment of absolute configuration for amino acids employing HPLC and mass spectrometry has been addressed, with a modified Marfey derivatization protocol proving useful (with a new reagent, 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide).[535]

5.5 Other Spectroscopic Studies of Amino Acids – Most of the work consigned to this Section deals with frontier-pushing IR/Raman and ESR studies of amino acids and their derivatives. There are other areas of spectroscopic applications, not covered here or in the preceding Sections 5.1-5.4, that have a subsidiary role in analytical applications, and are mentioned later, particularly in parts of Section 7.

Considerable effort has been expended on interpreting IR/Raman data for L-asparagine, employing strategically-labelled substrates (as is typical in such solid-state studies);[536] the straightforwardly-synthesized isotopomer $^2H_3N^+CH\cdot(CO_2^-)CH_2CH_2CON^2H_2$, was used in this particular study. Raman-spectroscopic features have been assigned to different conformations of tryptophan through studies including the [2,4,5,6,7-$^2H_5$]isotopomer,[537] and Stark effect fluorescence spectra of this amino acid have been determined.[538] Photoacoustic spectra have been measured for tryptophan.[539]

Structural features of radicals generated in oxidized samples of phenylalanine, tyrosine, histidine and tryptophan have been assigned through combined FTIR and ESR study.[540] The ESR technique continues to provide decisive information concerning the site of high-energy radiation attack on solid samples of simple aliphatic amino acid derivatives, and on the fate of radicals that are generated in this way. Two radicals have been identified by ESR in samples of N-acetyl-DL-alanine subjected to vacuum UV synchrotron radiation, one being the decarboxylated substrate.[541] Similar studies of a range of solid amino acids subjected to pulse radiolysis have been reported.[542] Aromatic side-chains offer the prime site for radical generation through milder radiative treatment, and hydrogen hyperfine interactions have been determined for the tyrosyl radical through ^2H-electron spin echo envelope modulation spectroscopy.[543] ESR spin-stabilization evidence has been acquired pointing to the formation of o-quinones when DOPA methyl ester and α-methylDOPA undergo peroxide oxidation.[544]

Reference has been made in the mass spectrometry section (Ref. 529), to seeding samples into an inert gas undergoing pulsed supersonic expansion, and the fact that data obtained for the gas-phase amino acid can be linked to its lowest energy conformation. In the context of absorption spectra, this approach allows the determination of spectral features that show vibrational, and even rotational, fine structure,[545] and features of detailed rotational spectra obtained in this way for the $^2H_2$, ^{13}C, ^{15}N-isotopomer have been linked to conformers 1 and 2 of glycine, and to conformer 2 for the N,O-$^2H_3$ isotopomer.[546] The electron density distribution in the highest-occupied molecular orbital of the most stable

conformation of glycine has been determined by multichannel electron momentum spectroscopy.[547]

5.6 Physico-Chemical Studies of Amino Acids – Data measured using standard equipment from within the physical chemistry laboratory continue to accumulate, and enlightenment accrues from some of these studies, on the matter of the behaviour of amino acids in solutions. Thus, calorimetric, densimetric and nuclear relaxation time measurements at 25°C for binary and ternary aqueous solutions of amino acids carrying functional groups in side-chains (racemates and enantiomers of lysine, asparagine, glutamine, serine and homoserine) reveal very high differences, compared with simple aliphatic amino acids that lack side-chain functional groups, between homochiral and heterochiral pairwise enthalpic interaction coefficients.[548] In other words, the chiral recognition shown by a homochiral amino acid towards its enantiomer is strongly enhanced by certain functional groups in side-chains. In the same context, calorimetric data have been used to assess the energies of interaction of amino acids (L-phenylalanine, L-tyrosine, L-tryptophan and L-histidine) with α- and β-cyclodextrins.[549] Side-chain transfer free energy values between amino acids and osmolytes (sucrose and sarcosine) have been computed from measurements for binary mixtures as a function of osmolyte concentration,[550] and enthalpies of interaction of glycine with constituents of aqueous solutions containing DMF, formamide and various ureas have been determined.[551] Various thermodynamic characteristics of common amino acids emerge from interpretations of temperature dependence of partial molar volume and adiabatic compressibility data for dilute aqueous solutions[552] and densimetric and heat capacity measurements for aqueous L-asparagine and L-glutamine, and their dipeptides.[553] Details of the thermodynamics of α-chymotrypsin-catalyzed hydrolysis of ethyl N-acetyl-L-phenylalaninate in water and in organic solvents have been described.[554]

Equilibrium constants for the amino acid – hydroxy acid hydrolysis – aminolysis system have been considered in the wider context of the process.[555]

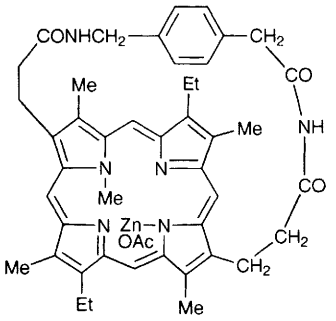
Protonation constants measured for threonine and methionine in aqueous NaCl provide new data that extend the already voluminous literature on this general topic.[556] A down-to-earth exposition[557] shows that three acid-base dissociation constants are needed to calculate the fraction of glycine that is non-ionic in aqueous solutions at the isoelectric point. The effect of micelles on the ionization kinetics of arginine and aspartic acid has been assessed with aid of ultrasonic absorption measurements.[558] Protonation constants of amino acids in salt solutions of various ionic strengths and at various temperatures have been determined.[559]

Hydrophobic parameters and colloidal properties of solutions of N-acylamino acids and their salts have been calculated and compared with data from HPLC,[560] and attention has been given to the means by which the hydrophobicity index for amino acids should be derived.[561] This index is commonly taken to be the value for a particular amino acid, of log $P_{O/W}$(where $P_{O/W}$ is the partition coefficient of derivatized amino acids between water and octan-1-ol), but it has been suggested that retention data for amino acid – o-phthaldialdehyde/N-acetyl-

L-cysteine condensation products (see Section 7.4), as derived by liquid chromatography using micellar eluents, would be a better index (Ref. 561). Four different hydrophobicity scales that have been advocated, based on HPLC data, have been critically assessed.[562] α-Cetylpyridinium bromide has a negligible effect on the hydrophobicity of amino acids in aqueous media, as judged by charge-transfer thin-layer chromatography (the amino acids most affected are alanine, methionine, tryptophan and tyrosine).[563] The large hydrophobicity of phenylalanine is emphasised by its rate-diminishing effect on the hydrolysis of N-acyltriazoles in aqueous media, bearing in mind the rate-enhancing effect of other common amino acids.[564] This general area, the effects of amino acids on other processes, has a literature of its own that can only be hinted at here (e.g. aqueous L-alanine adsorbed from aqueous media on to silica-supported nickel creates a catalyst that brings about asymmetric hydrogenation of methyl acetoacetate[565]). Freezing-point data for aqueous solutions have been interpreted to reveal details of control of local water-structuring by structural features in individual amino acids.[566]

Partition of tryptophan derivatives between sodium dodecanesulfonate micelles and aqueous phases can be assessed easily by UV absorption and fluorescence measurements (fluorescence quenching by succinimide has been noted),[567] and kinetics of mass transfer of tryptophan, partitioned between aqueous solutions and water-in-oil emulsions, have been measured.[568] Partition coefficients have been determined, of amino acids within poly(ethyleneglycol) – aqueous salt solution two-phase systems.[569] Extraction of amino acids from water into CH_2Cl_2 can be achieved through complexation with crown ether – N-methoxy-2,4,6-trinitroanilinium salts,[570] and into $CHCl_3$ by cryptand-2,2,2 analogues.[571] Macrocyclic pseudo-peptides carrying N,N′-ethylene-bridged dipeptide units have been found to show specific transport properties towards salts of amino acid esters across $CHCl_3$ and CH_2Cl_2 – water membranes.[572] New calix[4]arenes carrying carboxymethyl groups have been rendered chiral through coupling to L-phenylalanine, L-phenylglycine or L-tryptophan, and then show chiral recognition towards amino acid esters and Z-amino acids.[573] An optically-pure C_2-symmetric macrobicycle featuring an amidopyridine as a carboxylic acid binding site, and amide functions to provide further hydrogen bonding interactions, is an effective host for binding N-protected amino acids, showing modest chiral differentiation.[574] Highly enantioselective binding of N-Z-, Boc-, 3,5-dinitrobenzoyl- and acetylamino acids is seen for the chiral Zn – porphyrin (71),[575] in which the metal ion binds the carboxylate anion, and the amide groups contribute hydrogen-bonding. Study of the transfer of chiral lanthanide complexes of zwitterionic amino acids from neutral aqueous solutions into CH_2Cl_2 has been described.[576]

Adsorption equilibria involving amino acids have featured in studies of synthetic carbon adsorbents,[577] crosslinked chitosan fibres[578] and talc (which is capable of adsorbing amino acid esters, but not free amino acids).[579] Amino acids are only weakly adsorbed on to silica,[580] but clay to which modified metal complexes are adsorbed is capable of chirality recognition when presented with amino acid derivatives.[581] Blood cell membranes carry about 8% of the total amino acids present in blood.[582] Shifts in membrane potentials measured for



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immobilized γ-globulin membranes accompany the introduction of amino acid solutions into the experimental cell; each amino acid generates a characteristic potential response curve, and a further indication of the possible exploitation of this phenomenon is the finding that potential response curves for the enantiomers of aspartic acid show significant differences.[583]

Sorption and diffusion of DL-tryptophan, DL-phenylalanine and DL-DOPA into divinylbenzene – poly(styrene) resins,[584] and similar non-exchange sorption of amino acids by a weak acid cation exchange resin,[585] have been studied. Ion exchange equilibria involving amino acids have been reviewed,[586] and new work under this heading involves DL-lysine hydrochloride with the ion exchange resin Amberlite IRA-420,[587] and L-glutamic acid with a weakly basic anion exchange resin.[588]

5.7 Molecular Orbital Calculations for Amino Acids – Theoretical studies incorporating molecular orbital calculations provide useful assistance to the interpretation of experimental data in a number of areas of amino acid science, illustrated with ^{13}C-nuclear shielding parameters for solid samples,[589] including solid α-glycine,[590] influence of nearby water molecules on proton transfer energies of glycine and alanine,[591] and verification of the stoichiometry of the dihydrated glycine zwitterion assembly.[592] Other computations for underivatized amino acids include free energy data for transfer of individual amino acid molecules from vapour to water,[593] and consideration of conformational aspects of alanine,[594] glycine and alanine,[595] alanine, serine and lysine,[596] glutamic acid in its non-zwitterionic forms[597] and in its neutral and zwitterionic forms,[598] γ-aminobutyric acid in comparison with β-alanine and glycine,[599] δ-aminopentanoic acid,[600] and ω-amino acids.[601] The structural features of glycine in the gas phase[602] and of the carbon-centred glycine radical in its zwitterionic form,[603] as well as structures and gas phase thermochemistry of glycine and its ions and radicals,[604] have been shown to be a valid focus for molecular orbital calculations.

N-Acetyl-L-amino acid N-methylamides continue to provide the favoured model for calculations of conformational details for individual amino acids in

polypeptides and proteins, and these derivatives of L-alanine[605] and L-proline[606] have been studied this year. Calculations of structural features of complexes of amino acid esters with a porphyrin host have been performed.[607]

6 Chemical Studies of Amino Acids

6.1 Racemization – Major topics under this heading over recent years continue to attract attention, with new themes revealed in papers (see also Refs. 168, 730) on photolytic racemization at wavelengths shorter than 300 nm of L-lysine in the presence of suspended cadmium(II) sulfide particles under anaerobic conditions (L-leucine and L-phenylalanine are also racemized under these conditions, but not L-glutamic acid),[608] and of L-lysine by phosphoric acid and acetic acid.[609]

The extent of racemization of free amino acids under the conditions of acid hydrolysis of peptides (130°; HCl – AcOH = 1:1) has been found to be small for valine (0.1% within 4 hours) and greater (as expected) for aspartic acid (1.5% in 4 hours).[610] This is an important study, confirmed in rather less precise terms for 6M-HCl at 105°,[611] with attention to the effects of other temperatures and acid concentrations, and the effects of irradiation by UV light, etc. As the sensitivity of instrumental analysis methods in this area becomes more and more enhanced, and as statutory requirements for quality control of homochiral pharmaceutical compounds become more stringent, these levels become significant enough to require taking into account when considering the results of routine enantiomeric analysis of samples. This last point provided the stimulus to re-investigate the racemization of L-serine kept in pure water at 100°.[612] In earlier studies, half-life values 400 years for aqueous L-serine at 25°[613] and 4 days at 100°[614] were determined, though the new value (Ref. 612) is 40 days at 100°. Cysteine derivatives protected as alkyl disulfides are easily racemized by 25% piperidine in DMF when the carboxy group is in the form of an ester, but not when it is amidated.[615]

A review has appeared of the post-translational events through which L-amino acids are incorporated into polypeptides as their D-enantiomers, and the role of peptidylaminoacyl L-D-isomerase in some of these processes.[616]

Apart from results for laboratory racemization, and puzzles of a mechanistic nature that arise from them, racemization data obtained by enantiomeric amino acid analysis continue to be exploited in fossil dating (see also Refs. 22, 34). A favoured technique depends on analytical quantitation of L-isoleucine – D-alloisoleucine diastereoisomers, and this has been applied to an eggshell sample of the extinct Pleistocene ratite *Genyornis*, in order to assess variations in ages given by different molecular weight fractions.[617] Results for fossil bones dated in this way have been compared with those obtained through the classical ^{14}C-method,[618] which is not subject to the influences that appear to undermine the amino acid dating method. This is already becoming more than a minor issue, since queries have been surrounding the amino acid racemization dating technique in recent years; thus Otztal Ice Man (the unmolested corpse found at Hauslabjoch, Austrian Tyrol, in September 1991) which has been dated to

4550 ± 27 BC by radiocarbon dating,[619] would have a grossly inaccurate assignment of birthday, based on amino acid data. For younger samples that have been kept in constant conditions, e.g. human tooth cementum, a linear relationship applies for racemization as a function of time.[620]

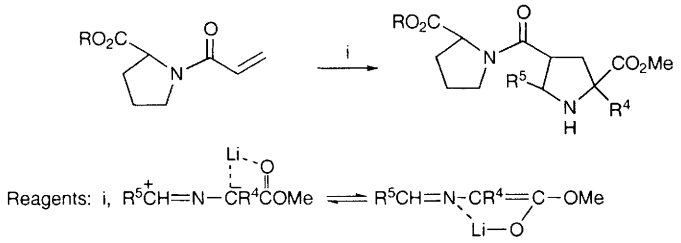
6.2 General Reactions of Amino Acids – Reactions at the amino and carboxy groups of the amino acids are dealt with in this Section as well as reactions at the α-carbon atom of α-amino acids. Although some other papers in this Chapter also qualify for the same narrowly-defined category, these papers are located elsewhere because of priority given to their coverage of another theme.

A long-running project (see Vol 27, p. 53) has established that the oxidative decomposition of alanine, aminoisobutyric acid and proline after N-bromination follows first order kinetics in aqueous alkali.[621] N-Chlorination of optically-active methyl aziridine 2-carboxylates is followed by base-induced elimination to give optically-active 2H-azirine-2-carboxylates, a process that is accompanied by alternative HCl elimination, but Swern oxidation accomplishes the desired change cleanly.[622] Replacement of the amino group by a halogen atom, with retention of configuration, is the best way of preparing homochiral α-halogeno acids, illustrated recently for the conversion of L-isoleucine to (2S,3R)-2-chloro-3-methylpentanoic acid using sodium nitrite and 5M HCl,[623] and for preparing (2S,3R)-2-bromo-3-hydroxybutanoic acid from L-threonine (sodium nitrite and KBr with 1.25M H_2SO_4).[624] The corresponding replacement of the amino group by a hydroxy group employs sodium nitrite and 1.5 equivalents of H_2SO_4 in water.[625] The oxidative decomposition of amino acids by ninhydrin has been shown to offer quantitative release of ammonia, and offers a useful total assay when coupled to an inexpensive flow injection – gas diffusion instrument.[626] Tungstate-catalyzed oxidation of N-alkyl-α-amino acids causes decarboxylation and efficient nitrone formation.[627] Among the vast number of papers on routine oxidative decarboxylation studies of amino acids involving inorganic reagents, is found an account of the first observation of the formation of an initial major reaction product which is neither an aldehyde nor a carboxylic acid. The acetone – potassium peroxymonosulfate reagent used in this work generates dimethyl dioxirane as the effective oxidant.[628] Another component of the classical range of amino acid reactions, the Maillard reaction, has provided yet another fascinating aspect with potential analytical use: the generation of oxygen-dependent chemiluminescence.[629] This, from the 6-aminocaproic acid – D-ribose system, is sufficiently intense to be seen by unassisted eyesight. As far as changes in food are concerned, Maillard degradation of amino acid – carbohydrate mixtures mainly concerns only lysine, histidine and tryptophan.[630] Mechanisms of individual steps in the Maillard reaction have been reviewed[631] (see also Ref. 721). 3-Methylpyrazin-2(1H)-ones are newly-discovered characteristic Maillard reaction products formed from asparagine and monosaccharides.[632] The formation of N-oxides by oxidation of N-benzylprolines is completely diastereoselective;[633] nitrones are formed from esters of the unprotected imino acids when undergoing sodium tungstate-catalyzed oxidation with the urea-hydrogen peroxide complex.[634]

The preparation and reactions of N-acyl-α-amino acids have been reviewed.[635] Reviews in the *ChemTracts* series[636] cover work[637] on the transposition of N-protection; e.g. deprotection of N-allyloxycarbonylamino acids using the Guibe method (tri-n-butyltin hydride as nucleophile; e.g., Ref. 653) in the presence of an active acylating agent; such as Boc-anhydride, to give Boc-amino acids. This becomes a one-step deprotection – coupling procedure leading to peptides when performed in the presence of amino acid pentafluorophenyl esters of amino acids. Palladium(0) – sodium borohydride offers the same de-allylation and protecting group transposition opportunities, as well as the peptide bond-forming option.[638] Palladium(0) – phenyl trihydrosilane or N-methyl-N-(trimethylsilyl)trifluoroacetamide offer effective allyl cleavage protocols.[639] N(O,S)-Isobutyloxycarbonylation of amino acids could become the most favoured protocol as part of the sample preparation procedure for gas-chromatographic analysis of amino acid mixtures, in view of the finding that sonication with isobutoxycarbonyl chloroformate in aqueous alkali completes the derivatization in a matter of seconds.[640] However, the side-reactions (leading to derivatized peptides) of this protocol need to better appreciated. Treatment of an N-alkoxycarbonylamino acid mixed anhydride with a NaH – alkyl chloroformate mixture generates the NN-di(alkoxycarbonyl)amino acid after simple work-up.[641] The bis(N-Boc)amino acids that were studied over several years by several research groups have sustained less interest recently, partly due to some side-reactions and difficulties in preparation; in the latter context, the expected correlation of substrate acidity with ease of introduction of a second Boc grouping catalyzed by 4-dimethylaminopyridine is confirmed, but steric hindrance for some amides does not limit reaction rates as much as might have been expected.[642] N-Acylation of hindered N-alkylamino acids, e.g. $AllocNHCHMeCH_2NHCHBu^iCO_2Me$, is achievable using one of the newer peptide coupling agents, O-(7-azobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.[643]

N-Derivatization of imino acids (L-proline, pipecolic esters) in water has been established to apply to the bismuth(III) chloride – benzotriazole system.[644] Photoinduced Wolff rearrangement (366 nm) of dibenzoyldiazomethane in the presence of an amino acid ester generates the highly electrophilic benzoylketene, which causes N-(α-benzoylphenylacetyl)ation.[645] Mitsunobu processing converts amino acid esters into amidines through use of NN′-bis(benzyloxycarbonyl)acetamidine as reagent,[646] and the α-amino group of lysine has been substituted by a 4-methylbenzylthio-grouping, *via* the pyridinium salt formed through condensation with a pyrylium salt;[647] tryptophan has been converted similarly into pyridinium salts and dihydropyridines, the heteroaromatic nitrogen atom being derived from the α-amino group.[648]

Reliable N-mono-alkylation of amino acids is often achieved only by indirect routes, and the preparation of particular amino acids reversibly derivatized by N-benzylation, e.g. N-tert-butoxycarbonylmethylation of leucine methyl ester using tert-butyl bromoacetate and di-isopropylmethylamine,[649] and preparation of N-(2-hydroxy-4-methoxybenzyl)-L-amino acids, required by some improved peptide synthesis protocols, that illustrate these roundabout routes, have been described.[650] Preparations and uses of NN-dibenzylamino acids and aldehydes have

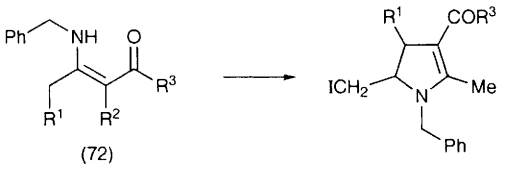


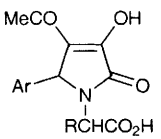
Scheme 40

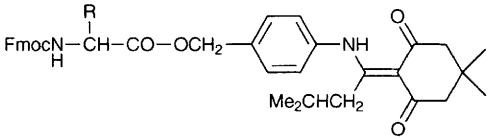
been well-researched already, and further examples have been described.[651] Methylation of glycine by the dimethylchlorinium ion occurs at N and at O in the gas phase (chemical ionization source of a mass spectrometer), while the methoxymethyl cation adds at N under these conditions.[652] Selective deprotection of allylamines using Guibe's palladium(0)/NN-dimethylbarbituric acid/DPPB system, together with 2-mercaptobenzoic acid, has been illustrated for ethyl NN-diallyl L-phenylalaninate.[653]

N-Alkylidenation of amino acids, i.e. Schiff base formation, provides an essential stage of some amino acid synthesis protocols (see Sections 3.1, 3.2), and also extends the uses of amino acids in general organic synthesis, e.g. diastereoselective synthesis of pyrrolidines (Scheme 40).[654] Trimethylorthoformate is an effective dehydrating agent under mild and non-acidic conditions, for imine formation between aldehydes and amino acids.[655] Pyrrolines can be prepared by cyclization of α-alkenyl-β-enamino esters (72),[656] and amino acids react with methyl acetylpyruvate to give pyrrolinones (73).[657] Isoindoles formed from amino acids in reaction with o-phthaldialdehyde/2-mercaptoethanol are of lower stability (as shown by fluorescence decay studies) than those formed from peptides (see also Refs. 20, 658, 874 for studies of this reaction).[658]

Carboxy group manipulations described in the recent literature include all the familiar elaborations of this functional group. Useful modifications and





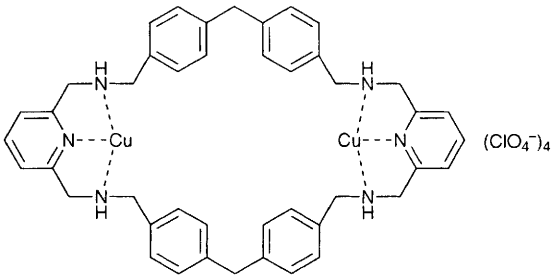


innovative contexts associated with the citations chosen for inclusion, justify the space given here. Reduction to the aldehyde through lithium aluminium hydride reduction of the Weinreb amide [$RCO_2H \rightarrow RCONH(OMe)Me \rightarrow RCHO$] and chain extension through aldolization and standard elaboration is illustrated in a synthesis of keramamide F,[659] and another standard protocol, $NaBH_4/I_2$reduction, has been put to use for the conversion of L-phenylalanine to L-phenylalaninol,[660] *en route* to L-phenylalaninal *via* standard alternative routes. Further results have been reported (see Vol 27, p. 55) on the reduction of N-protected amino acid chlorides to aldehydes using lithium tris(tert-butoxy)aluminium hydride,[661] and further examples have been described of the conversion of N-protected amino acids into amides through the use of di-tert-butyl pyrocarbonate/pyridine/NH_4HCO_3 as condensation reagent.[662] Lithium aluminium hydride reduction of N^{α}-tritylamino acid amides to corresponding amines offers better results for practical reasons than reductions in the presence of other N-protecting groups.[663]

Use of BOP – diethylamine for efficient esterification of Z-L-phenylalanine by methanol has been established;[664] similar reagent systems have not been found to be so effective in the past. Improvements leading to products destined for a role in peptide synthesis include a benefit of phase-transfer catalysis in condensing Boc-L-amino acids with chloromethylpoly(styrene);[665] conversion of Z-L-glutamic acid into a mixture of α- and γ-tert-butyl esters, and separation of the products as their cyclohexylammonium salts;[666] and formation of the pentafluorophenyl ester of Fmoc-L-asparagine using pentafluorophenol with a water soluble carbodi-imide that does not affect the unprotected side-chain.[667] Preparation of N-substituted 4-aminobenzyl esters (74) that fragment readily into the starting acids with 2% hydrazine in aqueous DMF, and therefore provide a useful new protection system for the carboxy group based on the safety-catch principle, has been described.[668] Hetero-Diels-Alder addition to cyclopentadiene, of acylnitroso dienophiles prepared by oxidation of N-protected amino hydroxamic acids, can start routes to other useful products through cleavage of the N – O bond.[669] $Mo(CO)_6$ reduction of the Diels-Alder adducts provides aminocyclopentanols, and Pd-catalyzed allylic substitution of the derived acetates opens up a route to carbocyclic nucleosides or their precursors.[670]

Replacement of the carboxy group by a hydrogen atom is illustrated in a lengthy route to enantiomerically-pure α-methylamines, that involves the transformations [$RCO_2H \rightarrow RCH_2OH \rightarrow RCH_2OMs \rightarrow RCH_2SEt \rightarrow RCH_3$]; the last step features classical Raney nickel reduction, and the route is clearly limited to benign substrates.[671]

Ester hydrolysis studies with novel mechanistic features continue earlier work on systems that promote enantioselectivity. Hydrolysis of the L-enantiomer of N-dodecanoyl-DL-phenylalanine p-nitrophenyl ester by micelles enclosing the peptide Z-L-phenylalaninyl-L-histidyl-L-leucine occurs 77 times faster than that of the D-isomer.[672] Imidazole-appended β-cyclodextrins cause similarly selective enhancement of the hydrolysis of Boc-DL-alanine p-nitrophenyl ester,[673] and an L-histidine-containing polymer has been used in a corresponding way (see Vol 27, p. 55).[674] Clear evidence has been obtained of the cooperation of the two



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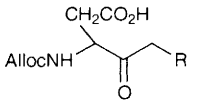
copper atoms complexed by the macrocycle (75), when used as a hydrolysis catalyst for β-alanine p-nitrophenyl ester, but this cooperation does not occur for leucine p-nitrophenyl ester.[675] A water-soluble polymer has been rendered capable of enantioselective hydrolysis of Z-DL-leucine p-nitrophenyl ester through imprinting with Z-L-phenylalanine phosphonate.[676] Nucleophilic cleavage of an N-protected alanine decyl ester can be catalyzed by poly(styrene)-supported ethylenediamine – copper(II).[677] The mimicking of an enzyme, that lies behind some of these studies, still falls behind the achievements of the real thing, especially in the unexpected flexibility shown by familiar enzymes; thus, α-chymotrypsin and subtilisin Carlsberg are effective in organic media (cyclohexane) in catalyzing the transesterification of N-acetylalanine or phenylalanine esters,[678] and porcine liver esterase accomplishes the selective hydrolysis of aspartic acid di-esters into corresponding β-esters.[679]

Preparation of aminoacyl halides has been given new impetus with the finding that their use in peptide synthesis need not be encumbered with side-reactions. New findings for the preparation and uses of N-protected L-pyroglutamyl chlorides[680] (see also Ref. 661) and Fmoc-L-amino acid fluorides[681] have been published.

Photodecarboxylation of N-phthaloylamino acids, in continuation of earlier studies (see Vol 25, p. 69), has led to the discovery that N-{α-[^{2}H]alkyl}phthalimides are formed in this way in ^2H_2O.[682] Decarboxylation is also seen in the photolysis of N-phthaloyl phenylalanine and tyrosine in non-polar solvents,[683] but cis and trans-cinnamic acids are also formed, as well as the benzazepin-1,5-dione in the case of tyrosine by photolysis in MeCN; phthaloylserine and threonine behave similarly, though with a noticeably easy loss of the side-chain.[684]

Other transformations involving the carbonyl moiety of amino acids and their carboxylic acid derivatives include a two-step conversion into hydroxamic esters using organo-aluminium reagent-promoted transamidation (O-benzylhydroxylamine hydrochloride and Et_3Al in benzene) followed by hydrogenation (H_2/Pd-C);[685] and a racemization-free preparation of N-protected α-amino-aldehydes through oxidation of corresponding alkanols with 1,1,1-tris(acetoxy)-1,1-dihydro-1,2-benzo-iodoxol-3(4H)-one ('periodinane').[686] Several papers deal with



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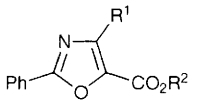


(77)

preparations of α-amino-ketones from α-amino acids, and describe uses of these products; thus, a route to N-allyloxycarbonyl-L-aspartic acid-derived substituted methyl ketones (76) involves the corresponding bromoketone;[687] anions of N-phenylsulfonylamino acids, generated using lithium hydride, are suitable for multigram-scale conversion into ketones through addition of a Grignard reagent;[688] α-aminoalkyl trifluoromethyl ketones are available in excellent yields from oxazolidin-5-ones through cesium fluoride-catalyzed addition of Ruppert's reagent [(trifluoromethyl)trimethylsilane] to the carbonyl group;[689] N-Boc-α-amino ketones are formed efficiently and in high enantiomeric excess through addition of organolithium and Grignard reagents to the pseudoephedrine amide of N-Boc-α-amino acids (see Section 4.2);[690] α-chloro-α′-(dibenzylamino)methyl ketones are the starting material for the highly diastereoselective synthesis *via* reduction and epoxidation, of threo-aminoalkyloxiranes, while a route to erythro-analogues starts with the protected α-amino-aldehyde, which is reacted with *in situ*-generated halomethyllithium.[691] A large-scale preparation of (2S,3S)-N-Boc-3-amino-1,2-epoxy-4-phenylbutane from NN-dibenzyl-L-phenylalaninol proceeds through the classical route *via* aldehyde and chlorohydrin.[692] Preparation of homochiral N-Boc-aminoalkyl oxiranes from anti N-diphenylmethyl-3-amino-1,2-diols has been described;[693] the preparation of protected erythro-α-(aminophenylethyl)oxiranes derived from phenylalanine involves a bromomethyl ketone intermediate,[694] which features as the substrate in a study of their stereoselective reduction by borohydrides to give erythro-Boc-amino alcohols and derived epoxides.[695]

The reactions of amino acids that involve both amino and carboxy groups are maintaining a substantial proportion of the papers selected for this Section, as in previous years. There is more to report this year (see Vol 27, p. 54) on the self-condensation induced in aqueous solutions of amino acids by high concentrations of NaCl and a copper(II) salt, to give peptides.[696,697] The activated species is clearly shown to be the monochlorocuprate(amino acid) complex, and this structural feature is carried through to the products which then continue to undergo condensation that results in chain extension. Stability constants measured for these complexes verify this explanation, since a reaction mixture containing α-, β-, and γ-amino acids tends to generate peptides built up preferentially from α-amino acids, and it is α-amino acids which give the most stable monochlorocuprate(amino acid) complexes.

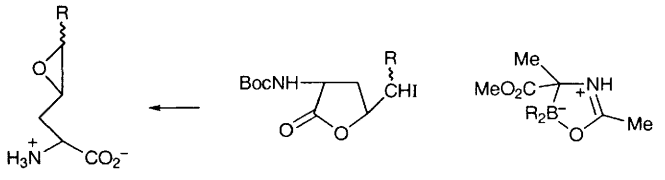
The property of achieving easy cyclization of α-, β-, and γ-amino acids to corresponding lactams is claimed for the N-alkyl-2-benzothiazolylsulfenamide/PPh_3 reagent.[698] Chain extension of the L-proline carboxy group and cyclization of derived N-acetyl- and -propionyl anions gives efficient access to homochiral pyrrolizidines.[699]

The 4,4-disubstituted 2-tert-butoxy-oxazol-5(4H)-one formed by carbodi-imide cyclization of an N-Boc-α-trifluoromethyl-α-amino acid is unusually easily converted into its corresponding N-carboxyanhydride,[700] while corresponding cyclic dipeptides (i.e. dioxopiperazinediones) form unexpectedly easily from the intended preparation of the N-carboxyanhydride of (R)-thiazolidine-4-carboxylic acid.[701] N-Benzoyl-α-amino acids form 2-phenyloxazol-5(4H)-ones easily when treated with a carboxy group-activating reagent, a long known fact but contrasting with the surprising formation of 2-phenyloxazole-5-carboxylic esters (77) in the corresponding reaction with excess oxalyl chloride followed with an alcohol.[702] Esterification accompanies N-ethoxycarbonylation of amino acids treated in MeOH in the presence of K_2CO_3 with ethyl chloroformate,[703] an outcome already established some years ago and explained by the dual role of the reagent (causing carboxyl group activation as well as N-acylation).

6.3 Specific Reactions of Amino Acids – This section covers the reactions of amino acid side-chains that mostly depend on one or both of the amino and carboxy groups being in a protected state. A review covers the uses of amino acids as starting materials for the preparation of enantiopure products.[704]

Among several possible glycine radicals, that in which the unpaired electron is localized on the α-carbon atom is the most stable.[705] A classical radical mechanism accounts for the rearrangement of diethyl 2-acetylamino-2-methylpropanedioate into diethyl 2-acetylaminobutanedioate, that occurs through coordination to a Vitamin B_{12} derivative.[706]

β-Iodo-L-alanine derivatives are proving to be useful synthons, undergoing Pd(0)-catalyzed arylcarbonylation in the form of the derived organozinc reagent RZnI, with an aryl iodide and carbon monoxide (illustrated in a short synthesis of kynurenine).[707] This protocol offers an alternative route to 2-amino-5-oxoalkanoic acids from γ-iodo-L-butyrine derivatives, compounds that are usually approached from the same starting point through reaction with an acyl halide.[708] Carbon-carbon bond formation occurs when the corresponding mixed-metal analogue RCu(CN)ZnI reacts directly with allylic halides and toluene-p-sulfonates.[709] The Heck reaction applied to Z-vinylglycine through reaction with 1-iodo-4-methoxybenzene in DMF in the presence of palladium acetate, illustrates another way of extending the aliphatic side-chain of this useful member of the α-amino acid family.[710] Aza-sugars (i.e. substituted 2-hydroxymethylpyrrolidines) can be synthesized starting from vinylglycine *via* the derived N-allyl 5-vinyloxazolidin-2-one.[711] A route from L-allylglycine and L-crotylglycine derivatives *via* iodolactonisation ends with functionalised 4,5-epoxy-α-amino acids (78),[712] and intramolecular cyclization of βγ-unsaturated acyloxysilanes followed by H_2O_2 ring cleavage are the crucial steps in a route from (S)-3,4-dehydroproline to (2S,3R)-N-Boc-3-hydroxyproline methyl ester.[713] Tributyltin hydride-mediated radical cyclization of N-(α-chloroacetamido) dehydroalanine gives pyroglutamic acid.[714] The amino group is involved, together with the side-chain, when attempted hydroboration of methyl 2-acetamidoacrylate leads to a heterocyclic oxytriorganoborate (79).[715] The nucleophilic character of the α-carbon atom is enhanced in these compounds, as it is in many other heterocyclic compounds



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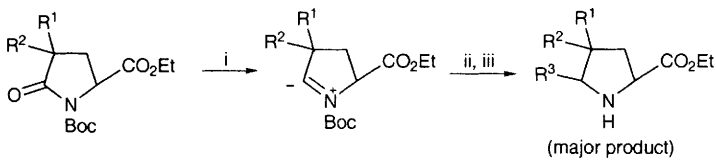
enclosing an α-amino acid moiety, and α-alkylation is possible, though this competes with N-alkylation.[716]

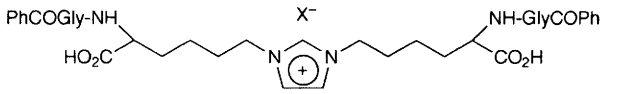
Reactions of cyclic aliphatic α-amino acids most often encountered in this Section over the years are based on proline (and, increasingly often, on pipecolic acid). Natural trans-4-hydroxy-L-proline is used (often, most ingeniously) to synthesize a widening range of substituted prolines, and C-4-inversion to yield the (2S,4R)-epimer has added another useful synthon for this area of synthesis.[717] The spirohydantoin formed from 4-oxoproline through the Bucherer-Bergs amino acid synthesis is easily transformed into 4-amino-4-carboxyproline, and this work has provided all four isomers of this conformationally-constrained glutamic acid analogue.[718] 4-Oxo-L-proline also serves to start a route to the kainoid analogues (2S,3R,4S)-3-benzyl-4-phenylproline and its C-3 epimer.[719] The stereoselective addition of organomagnesium cuprates to the acylium ion from 4,4-disubstituted prolines (Scheme 41) has been developed as a viable stereoselective route to 5-substituted proline analogues.[720]



Reagents: i, $LiBEt_3H$, then TsOH; ii, R^3MgX, CuBr, Me_2S, BF_3–Et_2O; iii, remove Boc

Scheme 41

An N^{α}-protected lysine, namely N^{α}-hippuryl-lysine, reacts with glyoxal to give the bis(4-carboxy-4-hippurylaminobutyl)imidazolium salt (80);[721] this process is an appropriate model for the formation of the Maillard reaction-generated crosslink in proteins, because glyoxal is a retro-aldol cleavage product of many of the common Maillard reaction intermediates (see also Refs. 629-631). There is a quite different course for the reaction between N^{α}-Z-lysine and glyoxal at 37°, which leads to N^{α}-Z,N^{ε}-carboxymethyl-lysine.[722] (E)-4,5-Epoxy-(E)-hept-2-enal, a well-established lipid peroxidation product, gives pyrroles through heating with lysine (see also Ref. 919).[723] Milder conditions for the corresponding reaction of trans-4-hydroxynon-2-enal have confirmed that the Michael addition pathway leads to a 1:2-cyclic hemiaminal with lysine or histidine (formed through the N^{π}-



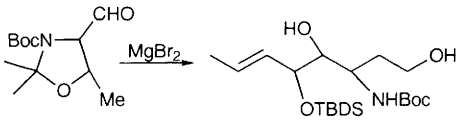
(80)

atom).[724] Thermal rearrangement of (S)- or (R)-hexahydro-1-nitroso-3-phthalimido-2H-azepin-2-one, prepared in known ways from appropriate lysine derivatives, gives the corresponding lactone from which ε-hydroxy- and ε-chloronorleucine enantiomers have been prepared.[725] The side-chain amino group of a protected lysine has been modified to give L-N^{ε}-(1-iminoethyl)lysine, a selective inhibitor of nitric oxide synthase.[726]

Glycosylation of N^{α}-protected β-amino-alanine methyl ester provides a glycosylated 'retro-asparagine', a confusing name if applied to amino and carboxy group derivatives of this isomer, because only one of the three functional groups of derivatives of this protein amino acid is reversed.[727] All diastereoisomers of 2,3-diaminobutanoic acid (*alias* β-aminobutyrine) have emerged from nucleophilic substitution reactions of D- and L-threonine derivatives.[728] Heating N-acetyl-2,4-di-aminobutanoic acid yields ectoine (2,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid).[729] 2,6-Di-aminopimelic acid undergoes photochemical cyclization (λ> 300 nm) into piperidine-2,6-dicarboxylic acid in a deaerated aqueous cadmium(II) sulfide dispersion (see also Refs. 168, 609).[730]

Arginine remains at centre stage because of its importance, among other things, as the source of *in vivo* nitric oxide (released by UV irradiation of squamous cell carcinoma, together with peroxynitrite, ammonia and hydroxylamine),[731] an ESR study of N^{G}-hydroxy-L-arginine revealing that the N-hydroxyguanidine tautomer, not the amino-oxyformamidine form, is the effective source of nitric oxide[732] (addition of hydroxylamine to N^{δ}-cyano N^{α}-Boc-ornithine tert-butyl ester provides the substrate for this study[733]). Methanesulfonylethyloxycarbonyl protection of arginine side-chain nitrogen atoms has been advocated, for easy deprotection and for reliable control of side-reactions originating with arginine during peptide synthesis.[734] Deprotection conditions are mild (aqueous NaOH) for this protection strategy, but analogous Boc side-chain protection has certain benefits, not least for the ease of preparation.[735] Two new close analogues of well-established arenesulfonyl protecting groups (arene = 4-Ph-C_6H_4- or 3-Me_3C-, 4-OMe-C_6H_3-) have been proposed for the arginine side-chain.[736] New details, from the point of view of optimization, of the arginase-catalyzed conversion of arginine into ornithine still come to light.[737]

Preparations of corresponding 4-oxaprolines from serine and threonine enantiomers have familiar precedents,[738] and the well-established, L-serine-derived, Z-α-amino-β-lactone, has been used in a multi-kilogram synthesis of Z-(S-phenyl)-L-cysteine,[739] and in a synthesis of lanthionines through regioselective ring-opening by cysteine derivatives and their ββ-disubstituted analogues.[740] The equally well-appreciated Garner aldehyde obtained from L-serine, and the homologue (81) formed from L-threonine, reacts with γ-oxygenated allylic stannanes through an exclusively syn-selective pathway,[741] and with dimethyl 1-



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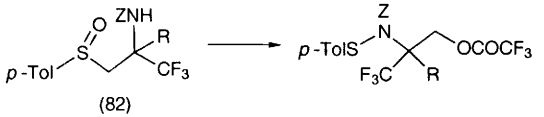
diazo-2-oxopropyl phosphonate to give N-Boc-D-ethynylglycine[742] and its homologues, through metallation (BuLi) and use as nucleophile towards common organic halides.[743] The Garner aldehyde (see also Ref. 319) is the basis of syntheses of (2R,3S)- and (2R,3R)-phenylserine, (R)-3,3-diphenylserine and (R)-3,3-diphenylalanine, through manipulations of the aldehyde function, and using the original serine side-chain to generate the eventual carboxy group.[744] Further examples of the latter device are seen in a stereoselective synthesis of (2S,3S)-3-hydroxyleucine in which the aldehyde group of a version of the Garner aldehyde is elaborated into the eventual side-chain,[745] and in a synthesis of N-Boc-D-diphenylalanine (*via* the oxazolidin-2-one).[746] The conversion of the aldehyde group into an oxirane through ylide epoxidation,[747] and chain extension through aldolization with $THPO(CH_2)_{13}C{\equiv}CLi$, leads on to a sphingosine analogue suitable for use in acylation of aminated glass beads to be used for the purification of sphingosine kinase.[748] Numerous applications outside the amino acid field (e.g. a route to manzamines)[749] have been found for the Garner aldehyde.

A range of Boc-(S-alkyl)-L-cysteine methyl esters has been obtained through treatment of N-Boc-L-serine methyl ester with disulfide/PBu_3reagents.[750] Mitsunobu processing of N-Boc-L-homoserine benzyl ester with N^3-benzoylthymine provides the corresponding γ-N-heteroarylbutyrine,[751] and the analogous transformation of L-homoserine into L-homocysteine derivatives and into L-2-amino-4-phosphonobutanoic acid[752] emphasises the synthetic usefulness of the ω-hydroxyalkyl-α-amino acids.

Serine and threonine protection as benzyl ethers is retained when hydrogenolysis (H_2/Pd-C) of N-Z-protected benzyl esters of these amino acids is performed in the presence of ammonia, ammonium acetate or pyridine.[753] This will be a useful observation if it can be verified for a wider range of substrates. The tert-butyl ether of Fmoc-L-allothreonine can be prepared more efficiently, through long-established though somewhat drastic methodology (lengthy hydrolytic cleavage of a benzoyl group is needed); inversion of the side-chain chiral centre in this route involves the oxazoline prepared from N-benzoyl L-threonine, and introduction of protecting groups is otherwise straightforward.[754]

Formation of the acid chlorides of serine and threonine calls, not surprisingly, for O- and N-protection through strategies that make stringent demands, and ultimate deprotection after acylation by these synthons must take account of the sensitivity of the β-hydroxy-α-amino acid grouping. Because of this problem associated with unprotected side-chains, N-acetyl 5-chlorocarbonyloxazolidin-2-ones, though not readily purifiable, were studied and were shown to be suitable for introducing serine and threonine residues where standard peptide-forming



(82)

conditions had failed.[755] Conversion of serine derivatives into β-halogeno-alanines by trimethylsilyl halides requires lengthy reflux in MeCN, and fails for the preparation of the fluoro-compound.[756]

O-Glycosylation of α-(ω-hydroxyalkyl)-α-amino acids has generated a sizeable literature, though methods vary from one amino acid to another; a representative example, the preparation of O-glycosylated hydroxy-L-prolines, discusses different approaches and protection strategies.[757]

An alternative route to (S)-2-amino-4-oxo-butanoates (see Refs. 707, 708) involves L-methionine, which requires protection as the phthaloylated methyl ester so that oxidative modifications can be made.[758] The S-p-tolylcysteine sulfoxide (82) undergoes an unusual non-oxidative Pummerer rearrangement with trifluoroacetic anhydride, and (R)- or (S)-α-trifluoromethylserine can be prepared in this way.[759]

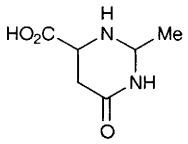
Reactions at the side-chain thiol group in cysteine derivatives are described in papers that deal with kinetics of nitrosothiol formation from N-acetylcysteine and nitric oxide in the presence of oxygen,[760] oxidation of cysteine by copper(II) species and by copper(I) – O_2 adducts,[761] and further studies of the autoxidation of S-aminoethylcysteine ketimine (see Vol 27, p. 67) to give 2,3,6,7-tetrahydro-4H-[1,4]thiazino[2,3-*b*]thiazine, thiomorpholin-3-one and 5,5′,6,6′-tetrahydro-2,2′-dihydroxy-3,3′-bi[(2H)-thiazine].[762] Palladium-catalyzed S-arylation of N-acetyl-L-cysteine methyl ester with an aryl iodide requires mild conditions in leading to moderate to good yields.[763] The reaction of OPA with cysteine-containing proteins at several amino acid residues, in addition to the expected location (the cysteine side-chain), provides a puzzle that will need to be solved if the spectrum of reactivity of this popular reagent (Section 7.5) is to be fully understood.[764] The thiol group in the ovothiols undergoes the expected thiol-disulfide exchange process with glutathione.[765]

The sulfur analogue of the serine-derived Garner aldehyde (see above) has been prepared from cysteine, and used for development of the erstwhile carboxylic acid grouping into a propenyl grouping as part of a synthesis of a model for curacin A.[766]

Aspartic and glutamic acids, and their derivatives, provide the basis of most of the papers in this Section, since valuable synthetic applications originate in the characteristic reactions of the carboxy group and adjacent methylene group in these compounds. Side-chain 2,4-dimethylpent-3-yl ester protection of aspartic acid effectively prevents side-chain involvement (i.e. aspartimide formation) during amidation of the α-carboxy group.[767] Allyl ester protection of the side-chain carboxy group of glutamic acid allows convenient manipulation of the other two functional groups, e.g. to prepare α-tert-butyl N-trityl-L-glutamate.[768] Nucleophilic attack on N-tritylaspartic anhydride occurs at the β-carbonyl

group,[769] to yield asparagines and other familiar derivatives. Elaboration of the side-chain of a protected aspartic acid, leading to N-trifluoroacetyl-5-bromo-4-oxo-norvaline methyl ester and N-trifluoroacetyl-3-formylalanine methyl ester, is uneventful; but the construction of heteroaryl groupings on these modified side-chains, e.g. to give azatryptophan, is notable.[770] However, N-Boc γ-methyl α-tert-butyl-L-glutamate may be elaborated through its side-chain function into the urea (side-chain = $CH_2CH_2NHCONH_2$) *en route* to the corresponding pyrimidinone, built upon the urea nitrogen atoms in the standard way.[771] Asparagine itself can be converted through routine steps *via* a β-homoserine derivative into (S)-3,4-diaminobutanenitriles, through Mitsunobu amination, and thence into 3-aminoGABA.[772] A pathway from Boc-L-glutamine to N^{α}-methyl-arginine and ornithine derivatives proceeds *via* the nitrile.[773] Acetaldehyde reacts with asparagine in aqueous solution at high pH to give the tetrahydropyrimidinone (83; claimed to be novel, but quite well known in the early literature).[774] Side-chain enolates of strategically-protected aspartic acids can be used to synthesise 3-carboxyproline and 5-substituted analogues,[775] and similar manipulation of the L-aspartic acid side-chain after protection using hexafluoroacetone [to give the 2,2-di(trifluoromethyl)oxazolidin-4-one] *en route* to 4-oxopipecolic acid has been described.[776] Sodium borohydride reduction to give cis-4-hydroxy-pipecolic acid and its trans-isomer was also described in this study. α-tert-Butyl Z-L-glutamate gives trans-4-carboxypipecolic acid and a series of analogues, through a route involving electrophilic addition to its γ-enolate.[777] These enolates are formed using lithium bis(trimethylsilyl)amide, and are essentially lithium chelates, a fact that helps to account for the very high diastereoselectivity leading to (2S,4S)- or (2R,4R)-products through electrophilic addition. This was observed in the preceding examples, and with N-(p-nitrobenzoyl)-L- and -D-glutamic diesters.[778] Amidation of Z-L- and D-glutamic diesters catalyzed by the lipase from *Candida antartica* leads to the α-amides with the L-substrates, but to the γ-amides for the D-isomers.[779]

The considerable potential in synthesis already established for pyroglutamic acid and its derivatives continues to be upheld, with syntheses of 4-alkylprolines and 4-alkylglutamic acids by BF_3-catalysed aldolization at C-4 of their lithium enolates[780] and synthesis of (2S,4R)-4-methylglutamic acid,[781] as in the preceding examples. 4-Benzylation in this way, reduction of the ring carbonyl function and functional group manipulation, gives the corresponding 2,3-dehydroproline, a Michael acceptor from which α-allokainic acid analogues were prepared.[782] Aldol reactions with the titanium trichloroenolate of methyl N-ethoxycarbonyl-L-



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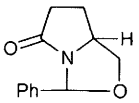

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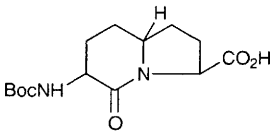


(85)

pyroglutamate give trans-4-(α-hydroxyalkyl)-substituted products, almost exclusively.[783] C-4 Functionalization can be achieved by thio-Claisen rearrangement of S-allylated pyrothioglutamates (5-thioxoprolines) that occurs easily in triethylamine – chloroform;[784] like some other electrophilic C-4 alkylation processes performed with analogous homochiral substrates, this route is not diastereoselective. Carboxy group conversion into the 5-methylisoxazol-2-yl grouping, and modification of other functional groups of (S)-pyroglutamic acid, gives the novel cholinergic channel activator, (S)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole.[785] Stereocontrolled C-2 functionalization of (S)-pyroglutamic acid can be accomplished by the usual alkylation protocols after conversion into its oxazolidinone with pivalaldehyde.[786] Sulfoxide elimination from a protected pyroglutamic acid derivative leading to 3,4-dehydropyroglutamic acid, starts another pathway that has been trodden before, and illustrated recently for a Diels-Alder addition to cyclopentadiene[787] to give the obvious adduct that is certain to be subjected to further elaboration to provide other amino acids carrying alicyclic structures. A new variation of this approach is illustrated in the use of pyroglutaminol N,O-acetal (84), which can be alkylated following the standard practice with pyroglutamate synthons, and leads to 4,4-disubstituted pyroglutamates after conventional de-acetalization and oxidation.[788] Natural amino acids, and pyroglutamic acid in particular, are increasingly used in syntheses of diverse target natural products and their analogues, outside the amino acid field; thorough coverage of this topic is not justified here. A preparation of long-chain N-acylated pyroglutamates has been described.[789] The efficient routes from tert-butyl N-(9-phenylfluorenyl)-L-γ-methylglutamate to the bicyclic alkaloid analogue (85)[790] and to (2S)-2-amino-3-(3-tert-butyl-5-oxo-2H-isoxazol-4-yl)propanoic acid[791] employ standard methodology.

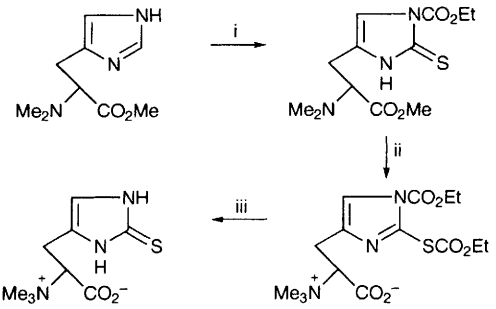
Methyl N-Boc 'pyro-L-aminoadipate' (surely, better named 6-oxopipecolate!) provides 5-alkyl homologues of aminoadipic and pipecolic acids, by stereoselective functionalization in uneventful extensions of the methods used with pyroglutamates, as exemplified in the preceding paragraphs.[792] The (-)-cis-3-hydroxy-6-oxopipecolate analogue features in a route to all four diastereoisomers of 2,6-disubstituted piperidin-3-ol.[793]

Ring contraction and insertion of an isocyanide into a nickelocycle derived from L-glutamic acid anhydride leads to β-methylaspartic acid after functional group conversions (already described in a preliminary communication, Vol 26, p. 67).[794]

Aromatic side-chain construction onto aliphatic amino acids is further illustrated in a synthesis of β-(8-hydroxy-1,4-benzothiazin-6-yl)alanine from S-cysteinylDOPA and H_2O_2 under catalysis by peroxidase.[795] Modifications to existing aromatic moieties in amino acid side-chains are illustrated for the aqueous Heck reaction with arenediazonium salts prepared from 4-aminophenylalanine and 3-aminotyrosine, giving (β-functionalised vinyl) derivatives.[796] L-(O-Malonyl)tyrosine has been prepared as a phosphotyrosine mimic from Fmoc-tyrosine esters by reaction with a di-alkyl α-diazomalonate;[797] further examples of modifications of the tyrosine side-chain are: nitration at pH 5-6 and hydroxylation at pH 2-4, using a peroxynitrite;[798] oxygen-dependent hydroxylation through

γ-radiolysis, effective also with phenylalanine;[799] and m-perfluoroalkylation by a perfluoroalkyl iodide and sodium dithionite.[800] 4-[o,o'-$^3H_2$]Benzoylation of L-phenylalanine can be accomplished through Friedel-Crafts 2,5-dibromobenzoylation followed by ^3H exchange.[801] Breakdown of the phenyl moiety in a protected phenylalanine, through Birch reduction followed by ozonolysis, and condensation of the resulting dialdehyde with appropriate nitrogen derivatives, gives β-isoxazolyl-, (N-phenyl)pyrazolyl-, and pyrazolo[1,5-*a*]pyrimidinyl-alanines.[802] Pictet-Spengler cyclization of 3,3-diphenylalanine is the crucial step in a route to all four isomers of 1,2,3,4-tetrahydro-4-phenylisoquinoline-3-carboxylic acid (see also Ref. 285).[803]

Histidine derivatives have been used in a first synthesis of L-(+)-ergothionene (Scheme 42) that incorporates some useful ring deconstruction – reformation

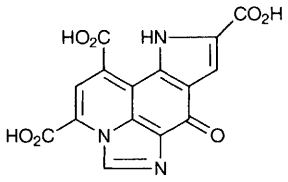


Reagents: i, SCl_2. EtO_2CCl; ii, MeI; iii, deprotection

Scheme 42

operations that will be helpful to others trying to achieve related synthetic objectives (the easy racemization of the betaine, unless acid conditions are used, is noteworthy).[804] Simple imidazole ring modifications include Michael addition to 4-hydroxy-2-nonenal (a lipid peroxidation breakdown product) in confirmation of earlier suggestions that protein modification can occur in this way (see Vol 25, p. 71, and Refs. 805, 919),[805] and N-(O,O-di-isopropyl)phosphorylation of histidine giving derivatives that can cleave supercoiled DNA, a property not shared by either histidine itself, or by its simple dipeptides.[806]

In concentrated H_2SO_4, protonation of L-tryptophan methyl ester occurs at the amino group and also at a ring carbon atom (C-3); then C-5 and C-6 monosulfonation occurs within 2 days, followed by 5,7-, 4,6-, 2,5-, and 2,6-disulfonation.[807] The now notorious toxin, 'peak E', *alias* 1,1'-ethylidene bis(L-tryptophan),[808] that forms in solutions containing the amino acid and acetaldehyde, has been fully documented.[809] 5-Bromocytosine undergoes photochemical coupling with aqueous N^{α}-acetyl-L-tryptophan ethylamide at pH 7, to give the 2-(cytosin-5-yl) derivative.[810] The reaction of an amino acid with the ubiquitous pyrroloquinone PQQ involves decarboxylation, and the side chain is cleaved as a carbanion equivalent; the quinone is converted into an oxazole. A new study of



(86)

this process, using tryptophan, has been shown to give (86) rather than the oxazole isomer previously reported in 1989.[811] Interest in this process has extended to the kinetics of the general PQQ – amino acid reaction, established with the help of CZE monitoring.[812] Acid-catalyzed condensation of N^{α}-Boc-L-tryptophan with aldehydes (the asymmetric Pictet-Spengler reaction) generates trans N^{α}-benzyl-1,2,3,4-tetrahydro-β-carbolines required for syntheses of sarpagine alkaloids, equilibration of the initial cis-trans mixture occurring during removal of the Boc group.[813]

Reactions of the familiar (Vol 25, p. 40; Vol 26, p. 70) N^{im}-benzenesulfonyl hexahydropyrrolo[2,3-*b*]indole methyl ester, formed by cyclization to C-2 through the α-amino group of the corresponding protected tryptophan, now include Barton decarboxylation to give the C^{α}-radical; various reactions illustrate endo face-selective coupling to this radical.[814] Palladium-catalyzed cross-coupling to a 5-iodinated derivative of this cyclic tryptophan has been established as a route to 5-alkyl- and 5-aryl-tryptophans.[815] Tryptophan is the start of numerous biosynthetic pathways, and, not surprisingly, is chosen for equally numerous laboratory syntheses of natural products, e.g. Gyseptin starting from phthaloyl-L-tryptophan methyl ester, a notable step being double oxidative cyclization of the derived dioxopiperazine.[816]

3-Thienylalanine would be a textbook example of an amino acid that an eager research group should find to be worth investigating as an *in vivo* replacement for phenylalanine, and indeed it has been found to be assimilated into protein synthesis by *E.coli*.[817]

6.4 Effects of Electromagnetic Radiation on Amino Acids - Standard photochemical protocols for amino acids under this heading have been used to generate radicals (photochemical decarboxylation of aliphatic amino acids using potassium ferricyanide excited by radiation of wavelengths shorter than 400 nm),[818] and to generate fluorescence (from protoporphyrin IX by δ-aminolaevulinic acid, and potentially useful in medicine for detecting tumours;[819] from dityrosine crosslinks in horse spleen apoferritin using 325nm laser-excitation;[820] or generated in tryptophan and its analogues, interpreted to reveal interactions between the heteroaryl excited state and un-ionised and protonated amino group[821]). Studies of the phosphorescence (i.e. delayed luminescence) of aqueous tryptophan have become possible as a consequence of the availability of sufficiently sensitive detection methods.[822] A 'Proceedings' Volume concentrates on fluorescence studies of protein constituents, e.g.

tryptophan fluorescence in di-octyl sodium sulfosuccinate/iso-octane/buffer reversed micelles.[823]

A rate-enhancing effect has been observed for UV irradiation on the ammonia lyase-induced fragmentation of phenylalanine into trans-cinnamic acid and ammonia.[824]

Rate constants for electron transfer in homogeneous aqueous solutions or within aqueous micelles, between tyrosine or tryptophan and excited states of some sulfonated phthalocyanines, have been determined.[825]

7 Analytical Methods

7.1 Introduction – Reviews of the current status of amino acid analysis have appeared.[826,827]

7.2 Gas-Liquid Chromatography – Fewer papers are appearing under this heading, though not because less use is being made of the method for amino acid analysis; this is a welcome shift away from the repetitive publication of papers describing applications of standard methods, that has been a feature of the analytical literature for amino acids in recent years.

Reliable derivatization protocols [N(O,S)-isobutoxycarbonylation and trimethylsilylation of tert-butyldimethylsilylation] have been illustrated for sample preparation prior to GLC – MS analysis (see Ref. 36). This has provided quantitative data for γ-methylglutamic acid, diastereoisomers of β-hydroxy-γ-methylglutamic acid, and cis- and trans-isomers of 5-hydroxypipecolic acid, as well as constituent protein amino acids, in seeds of *Gymnocladus dioicus*,[828] and for β-methylamino-L-alanine and four non-protein amino acids in *Cycas circinalis*.[829] N(O)-TBDMSylation of amino acids with N-TBDMS-trifluoroacetamide has generated the derivatives of 47 amino acids; their GLC-MS properties compare favourably with those of PTHs, for which extensive data are available and with well-known drawbacks when it comes to definitive identification of some amino acids.[830] N-Ethoxycarbonylation is also a suitable sample derivatization approach.[831]

These methods have continued to be used for the identification of paint binding media in ancient paintings.[832]

A new approach in this area is provided by studies of sample conversion into α-keto acids through digestion with L-amino acid dehydrogenase, derivatization by o-phenylaminediamine yielding stable products.[833] Quantitative analysis of α-amino acids and α-keto acids in human plasma has been explored by GLC – MS, choosing pentafluorobenzyl esterification and N-methoxycarbonylation for sample derivatization.[834]

Enantiomer analysis through separation of derivatized amino acids over chiral stationary phases is also a valuable established application of GLC Modifications to the widely-used Chirasil-Val® stationary phase, through replacement of a proportion of methyl groups of the poly(siloxane) backbone by pentyl and hexyl groups, has been successful in reducing the polarity of the medium, and leading

to shorter retention times for some N(O)-trifluoroacetyl amino acid n-propyl esters.[835]

7.3 Ion-Exchange Chromatography – Papers falling outside the analytical heading have been located elsewhere in this Chapter, and there is some overlap with the HPLC coverage in the later Section 7.5. While there is a considerable volume of routine literature that occasionally provides new material – e.g. shortening of classical analytical protocols by careful choice of buffer constituents,[836] and optimization of the ninhydrin reaction response by deproteinization of samples by passage through a hydroxyapatite cartridge[837] – the non-routine points of interest in the recent literature refer to uncommon analytical targets (underivatized selenium-containing amino acids;[838] diaminopimelic acid;[839] and aspartylglucosamine[840]).

7.4 Thin-Layer Chromatography – Improvements to standard procedures are represented in semi-quantitative assays of amino acids, using mixed natural zeolite and microcrystalline cellulose layers,[841] and using the chromatographic adsorbent RP-18 as stationary phase.[842] Specific attention to the analysis of homocysteine has led to the recommendation that conversion into a disulfide, e.g. through S-(2-hydroxyethylthiol)ation using 2-mercaptoethanol, leads to a reliable assay.[843]

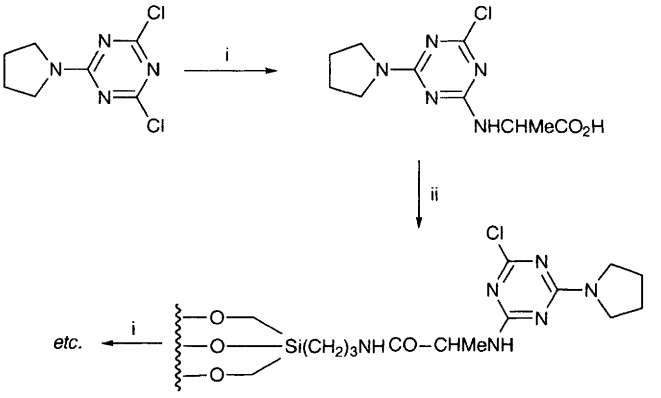
Over-pressured layer chromatography, a simple variant of standard TLC methodology, has been used by the laboratory from which the technique originated, for the separation of betaines of GABA and δ-aminovaleric acid from seaweed extracts.[844]

7.5 High Performance Liquid Chromatography – HPLC methods for the analysis of amino acids[845] and enantiomeric analysis protocols[846] have been reviewed.

An increasing proportion of the published work deals with underivatized amino acids, though this is a distorted reflection of the amount of work actually going on, since some standard derivatization protocols have become routine through regular optimization studies and do not justify further publications. The reason is also ascribable to the advances in instrumentation that favour the analysis of many of the more interesting target amino acids in underivatized form; they have light absorption, electrochemical and other characteristics that permit their detection at low concentrations. Studies cover the crosslinking amino acids desmosine and isodesmosine (detection at 275 nm),[847,848] pyridinoline and deoxypyridinoline (fluorescence and UV absorption; see also Ref. 10),[849] S-adenosyl-L-methionine and S-adenosyl-L-homocysteine (UV absorption at 267 nm),[850] and tyrosine and its 3-amino- and 3-nitro-derivatives (detection at 280 nm),[851] tryptophan (fluorescence,[852] and a similar method for tryptophan in comparison with its neuroactive relatives,[853] or electrochemical detection, and similarly for tyrosine derivatives[854]), ^{11}C-labelled methionine, DOPA and 5-hydroxytryptophan (the need for rapid analysis is met by purification with size-exclusion stationary phases),[855] DOPA and dopachrome,[856] and other protein

amino acids methionine (pulsed electrochemical detection[857] or electrothermal atomic absorption spectroscopy,[858] also applied to selenocystine) and homocysteine (ion chromatography with electrochemical detection).[859] Methionine is retained longer than histidine by poly(acrylamide) containing immobilized silver(I) ions from a buffer at pH less than 5, while the reverse applies at pH 7.[860] Studies of 2-oxo-L-histidine (Ref. 46) and phosphocysteine (Ref. 48), as well as work on the identification of D-amino acids in natural sources (Refs. 33-35), have also been supported by standard HPLC methods.

Determination of enantiomer ratios for amino acids is a continuing interest in increasingly diverse areas, some of which have emerged because the precision and sensitivity of the instrumentation have improved. Newly-proposed CSPs (i.e. stationary phases modified for this application by chiral additives or substitution by chiral groupings) include silica to which L-amino acids are attached (Scheme 43),[861] L-hydroxyproline attached to silica gel[862] and tris-3,5-dimethylphenylcar-



Reagents: i, L-Alanine; ii, *N*-hydroxysuccinimide, aminopropylsilylglass beads

Scheme 43

bamate-derivatized cellulose.[863] Synthesis of poly(siloxane)s carrying N-(3,5-dinitrobenzoyl)-β-amino acid and N-(1-naphthyl)leucine derivatives, and correlation of separation data, has been reported,[864] and optimization of CSP design has been carefully assessed for polymers substituted with chiral N-(1-naphthyl)leucine undecenyl ester and di-n-propylamide groupings.[865] The growing realisation that imprinted polymers are actually capable of doing the job, is emphasised by a growing number of papers from pioneers[866] and new adherents. These CSPs are familiar polymers, prepared from appropriate monomers in the usual way but including a homochiral molecule in the mix that is similar in structure to the analytical target, as illustrated in the trimethylolpropane trimethylacrylate/methacrylic acid copolymer prepared in the presence of a non-racemic dipeptide.[867] A new chiral stationary phase, prepared by attaching an L-tyrosine-

containing cup-shaped macrocycle to γ-mercaptopropylated silica gel, allows separation factors for Boc-DL-amino acids in the range 9 – 43 to be achieved, through elution with organic solvents.[868] An achiral C-18 stationary phase in association with a derivatized β-cyclodextrin component in the eluent[869] achieves the same result, as is the case with a mobile phase containing copper(II) complexes of L-phenylalaninamide used for the analysis of DL-2-hydroxyalkanoic acids.[870]

Standard derivatization methods with occasional new features are described for o-phthaldialdehyde – 3-mercaptopropionic acid (glutamine[871]), and OPA – 2-mercaptoethanol (for arginine and citrulline;[872] and for enantiomer ratio determination with a chiral crown ether-carrying stationary phase[873]). Oppolzer's group has reported a reliable protocol for the determination of D:L-ratios, based on the conversion of analyte into N-[N-(3,5-dinitrobenzoyl)-L-prolyl] derivatives (Ref. 194). Continuation of on-going studies has been described, where the aim has been to detect traces of D-enantiomers in L-amino acids using the OPA – N-isobutyroyl-L-cysteine reagent system[874] (see also Ref. 658; see Ref. 20 for a study of the corresponding OPA – N-acetyl-L-cysteine reagent; these authors found that increasing the time of contact of analyte and reagent from the usual 1–2 min to 15 min achieves enhanced fluorescence). The easy availability of several pharmaceutical amino acid formulations has prompted the need for better quality control of their amino acid ingredients, and some of these products that include racemic amino acids present the same analytical challenge, to establish that the enantiomer ratio is as close to the 50:50 value as is called for to meet the product specification.[875] N-[4-(6-Methoxy-2-benzoxazolyl)]benzoyl-L-phenylalanine or proline have been advocated for use as chiral derivatization reagents for enantiomer analysis of amino acids, through coupling with the 2,2′-dipyridyl disulfide/PPh_3 reagent and HPLC quantitation.[876] A 30 femtomole limit has been assessed for these derivatives ($\lambda_{excitation}$325 nm, $\lambda_{emission}$432 nm).

The effects of concentration and pH on the detector signal intensity have been studied for OPA – 2-mercaptoethanol – amino acid condensation products.[877] An established use for the OPA protocol is based on its exclusive compatibility with primary amino acids; its application to a physiological amino acid mixture, followed by derivatization with Fmoc-chloride[878,879] or DABSYL chloride,[880,881,882] removes primary amino acids from the analysis sample, and heightens the sensitivity of detection for proline and hydroxyproline.

Numerous reports have appeared describing analyses based on DABSYLation (phosphoserine, threonine and tyrosine,[883] amino acids in general,[884] S-sulfocysteine[885]), and on other familiar derivatization schemes: N-phenylthiocarbamoylation (PTC-amino acids in general;[886] S-carboxymethyl- and S-carboxyamidomethyl-cysteine[887]), thiocarbamoylation with a fluorescent chiral isothiocyanate (amino acid enantiomer ratios[888] ; see also Ref. 903) and with 4-(3-pyridinylmethylaminocarboxypropyl)phenyl isothiocyanate,[889] dansylation (amino acid enantiomer ratios through separation of derivatives over cyclodextrin-bonded stationary phases;[890] modification of retention times and enhancement of fluorescence detection, by SDS[891] and by bovine serum albumin[892]), 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl derivatives ($\lambda_{excitation}$315 nm,

$\lambda_{emission}$385 nm),[893] dinitrophenylation (4-hydroxypipecolic acid and pipecolic acid in *Acacia* at 100 pmol levels[894]), and preparation of Marfey derivatives (D- and L-phosphoserine in rat brain;[895] enantiomer ratios for protein amino acids[896]) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole derivatives. The last-mentioned derivatives were prepared from serum samples for analysis over a Pirkle CSP for their D-alanine, D-lysine and D-serine content,[897] and the performance of a variety of other benz-2-oxa-1,3-diazoles was also assessed in this study. In a rare example of post-column derivatization, after separation of enantiomers by ligand exchange chromatography, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole was the chosen reagent.[898]

Specific reactions of side-chain functional groups may be exploited for analytical targetting, as in fluorescent labelling of thiols with N-(1-pyrenyl)maleimide.[899]

7.6 Fluorimetric Analysis – Studies consigned to this section are usually carried out in support of the development of chromatographic and other studies described in surrounding Sections of this Chapter.

N-(Acenaphthene-5-sulfonyl)amino acids show 10 – 25 times greater fluorescence yield compared with their familiar dansyl analogues.[900] Similar explorations of near-relatives of established fluorescent derivatives have been described for naphthalene-2,3-dialdehyde (already well established), its 1-phenyl analogue, and anthracene-2,3-dialdehyde;[901] 7-N,N-dimethylaminosulfonyl-4-(2,1,3-benzodiazolyl)isothiocyanate ($\lambda_{excitation}$387 nm, $\lambda_{emission}$524 nm for arenethiocarbamoyl derivatives of amino acids generated by this reagent)[902] and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole (for fluorescent Edman-type derivatives useful for D:L-ratio determination for amino acids)[903] (see also Ref. 888); and (+)-2-methyl-2β-naphthyl-1,3-benzodioxole-4- and 5-carboxylic acid chlorides ($\lambda_{excitation}$310 nm, $\lambda_{emission}$370 nm for corresponding derivatives of amino acids, with a 0.1 pmol detection limit).[904]

7.7 Capillary Zone Electrophoresis, and Other Analytical Methods – This family of related techniques has developed from its inception in 1988 to a position of considerable importance, a trend that is continuing strongly. Reviews have appeared that give a clear assessment of the scope of current methods.[905,906,907]

The separation by CZE of free amino acids is improved by cyclodextrins as buffer additives.[908] Indirect absorbance detection was used for analyte quantitation in this case, while other studies have employed amperometric detection[909,910] (e.g. selenium-containing amino acids in human milk[911]), electrospray mass spectrometry[912] or post-column derivatization, e.g. by naphthalene-2,3-dialdehyde – 2-mercaptoethanol in a sensitive lysine assay.[913] Samples extracted from rat brain striatum *in vivo* through a microdialysis probe have been assessed for their GABA content by mass spectrometric analysis following CZE separation,[914] and analysis for tryptophan and kynurenine in rat brain samples obtained in the same way can be accomplished at attomole levels.[915]

Many of the standard sample preparation and derivatization procedures have been inherited from HPLC methods, but taking advantage of the greater

sensitivity and resolution that is achievable by CZE; thus, sample preparation by derivatization with naphthalene-2,3-dialdehyde – sodium cyanide (giving N-substituted 1-cyanobenz[*f*]isoindoles) has been illustrated for an assay of α-difluoromethylornithine.[916] PTHs continue to provide a stringent test for CZE methods,[917] seen against the voluminous background of HPLC studies that these derivatives have generated.

The related MEKC technique permits the estimation of tryptophan and related indoles at nanomolar levels based on laser-induced fluorescence detection,[918] and has been applied to the analysis of N^{ε}-pyrrolylnorleucine [the condensation product of lysine with the lipid degradation product, 4,5(E)-epoxy-2(E)-heptenal] after derivatization with diethyl ethoxymethylenemalonate (see also Ref. 724).[919]

CZE of esters of DL-tryptophan in buffers causing them to migrate towards the cathode can become an enantiomer separation procedure if the capillaries are coated with the protein transferrin.[920] The adaptation of CZE techniques for resolution, so as to deliver D:L-ratios, has been taken up enthusiastically; a broad study of the applicability of current derivatization procedures for enantiomeric separation by cyclodextrin-modified CZE, has been described.[921] Enantiomer analysis in the same way, of dansyl-DL-amino acids assisted by β-cyclodextrin[922] and methyl β-cyclodextrin[923] or alkyl glucosides,[924] as buffer additives, and similar studies using a γ-cyclodextrin zinc(II) complex[925] or sodium dodecanoyl-L-amino acid and poly(sodium 10-undecenoyl)-L-valinate micelles (separation of 3,5-dinitrobenzoyl-DL-amino acid isopropyl esters),[926] illustrate clearly-established methods. Amino acid enantiomers derivatized by condensation with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate have been separated by MEKC by including a synthetic homochiral surfactant in the buffer.[927] Enantiomeric separation of Fmoc-DL-amino acids in a cyclodextrin system has been compared with separation of diastereoisomers formed between DL-amino acids and (-)-fluoren-9-ylethyl chloroformate;[928] this, and an identical study using the enantiomeric reagent (the derivatized L-amino acid travels faster than its D-isomer),[929] favour the latter alternative protocol, which can deliver quantitative data for 3×10^{-10}M levels of analyte.

7.8 Assays for Specific Amino Acids – The growth of this topic is following its well-established direction towards ever more diverse biosensor applications for the detection and quantitative analysis of amino acids (the general topic has been reviewed[930]). However, a number of non-enzymic methods that exploit particular structural characteristics in some common amino acids have been explored; thus, the histidine content of samples can be assessed through differential pulse adsorptive stripping voltammetry[931] and cerium(IV)-generated chemiluminescence from tryptophan in a flow injection system is readily related to the concentration of analyte.[932] Use for the analysis of methionine in rat brain, of specific antibodies raised by injecting rats with methionine-bovine serum albumin conjugates,[933] and of ELISA methods (Ref. 849) are examples of techniques that are outside the general run of papers included in this Section over the years.

Use of an enzyme to generate H_2O_2 from L-α-amino acids, and to use this to

generate chemiluminescence in a flow injection system [leucine dehydrogenase and NADH oxidase co-immobilized on aminated poly(vinyl alcohol) beads][934] gives an illustration of developing methods. To describe a specific case in more detail, delayed chemiluminescence developed in luminol by horseradish peroxidase-generated H_2O_2 formed by copper(II)-catalyzed oxidation of L-cysteine with oxygen, may be measured to provide an estimate of the amount of this particular amino acid in complex mixtures.[935]

More conventional uses for enzymes are described for assays of L-glutamic acid and L-glutamine (immobilized glutaminase;[936] glutaminase *in situ* in kidney cortex tissue or in *E.coli*;[937] L-glutamic acid oxidase;[938,939] an L-glutamic acid oxidase – horseradish peroxidase pair, on a tin oxide surface for amperometric detection[940]) and a combined L-glutamic acid, L-glutamine and D-glucose biosensor.[941] Full details of appropriate methods for attachment of enzymes to micro-electrodes (glutaraldehyde condensation to aminopropylplatinized platinum wire; immobilization in an electropolymerized 1,3-di-aminobenzene film) show that the methods are simple, and this will encourage further expansion of interest in these devices. Construction of an L-tryptophan biosensor through immobilizing tryptophan-2-mono-oxygenase on to silica gel and using this in conjunction with an oxygen electrode,[942] and co-immobilization of D-amino acid oxidase and horseradish peroxidase by glutaraldehyde to bovine albumin[943] are also typical procedures.

Garlic tissue cells contain L-asparaginase, and can be used in conjunction with an ammonia gas electrode for an L-asparagine assay.[944] Innovative instrumental methodology continues to emerge in this area, illustrated by the involvement of a surface acoustic wave conductance sensor to detect the frequency shift as conducting ions are produced from the fragmentation of L-arginine by an arginase and urease mixture.[945]

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2
Peptide Synthesis

BY DON T. ELMORE

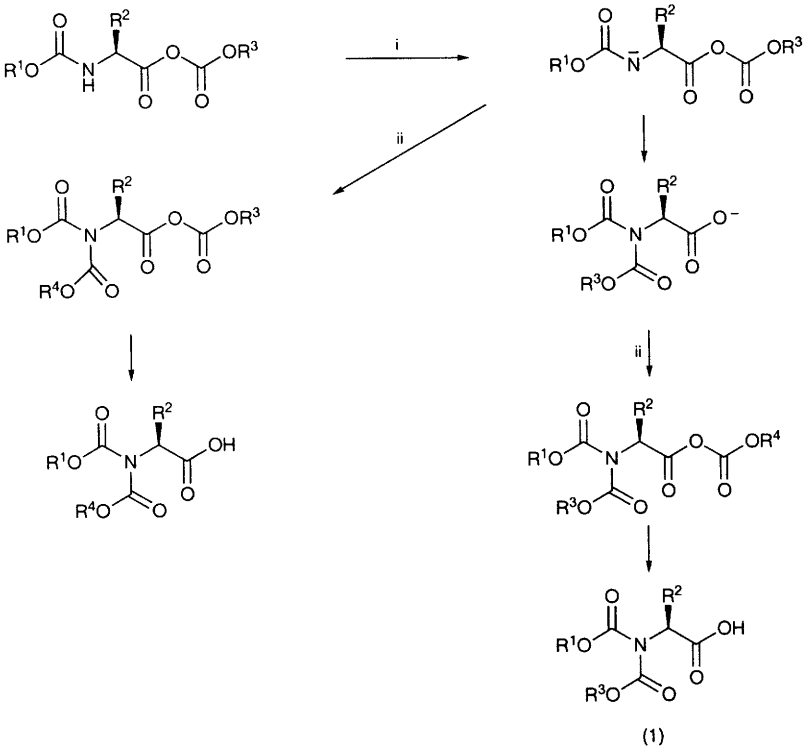
1 Introduction

Although 1995 has seen the publication of more papers concerned with peptide synthesis than in the last Report,[1] there have been fewer advances in fundamental techniques. Some of these, however, are likely to be very important. Space has been saved by citing papers in either Section 2 or 3 but not both.

Several books and numerous reviews have been published, and SPPS and combinatorial library generation have been the most popular topics. Reviews and books relevant to Section 2[2-27] and Section 3[28-48] have been separated in the list of references.

2 Methods

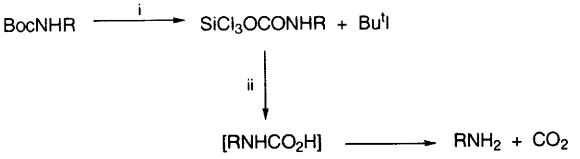
2.1 Amino-group Protection – Esters of pyroglutamic acid can be *N*-protected by Z- or Boc-groups in good yield without racemization using ZCl or Boc_2O with lithium hexamethyl disilazide in tetrahydrofuran at $-78\,^{\circ}C$.[49] *N*-Boc amino acids can be prepared in good yield by reduction of α-azides with n-Bu_3P followed by reaction with Boc_2O.[50] α-Fmoc-alloThr(t-Bu)-OH has been synthesized in five steps from Bz-Thr-OCH_2COPh.[51] α-Fmoc-Lys(Mtt)-OH has been made (42%) from lysine in two steps.[52] The 4-methyltrityl group is very sensitive to acid, being removed by 1% CF_3CO_2H in CH_2Cl_2 during 30 min. *NN*-Dialkyloxycarbonyl amino acids can be generated from the reaction of alkyl chloroformates with unsymmetrical anhydrides of *N*-alkyloxycarbonylamino acids in the presence of NaH followed by acid hydrolysis of the initial product. The reaction follows two possible routes (Scheme 1).[53] The major product (**1**) results from the intramolecular transfer of an alkyloxycarbonyl group. Troc groups can be efficiently removed with a 10% Cd-Pb couple,[54] a mild procedure that might revive interest in this protecting group. Boc groups can be removed without resource to acidic conditions.[55] $SiICl_3$, generated *in situ* by adding $SiCl_4$ to NaI in dry CH_2Cl_2/MeCN, removes Boc groups rapidly (Scheme 2). An S_N2 mechanism involving the strongly nucleophilic I^- ion is postulated. t-Alkyloxycarbonyl groups containing an *N*-methacroylamino moiety (**2**; n=1,2) have been designed[56] as polymerizable protecting groups. Acidic deprotection gives oxazoles (n=1) or oxazines (n=2). *Ras*-proteins have an *S*-farnesyl-Cys residue, and an *S*-palmitoylated-Cys residue is present not far away. These *S*-substituents cannot withstand



(1)

Reagents: i, NaH: ii, R^4OCOCl

Scheme 1



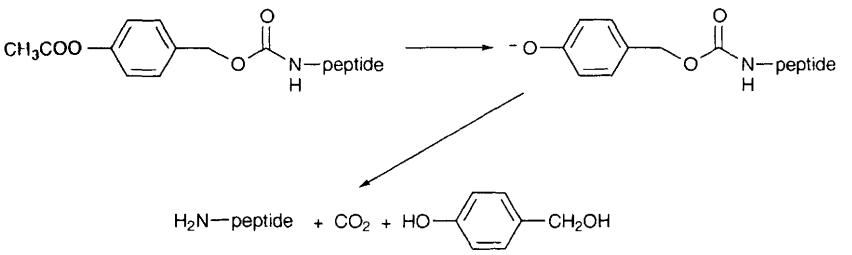
Reagents: i, $SiCl_3$: ii, EtOH

Scheme 2

$$CH_2{=}CCONH(CH_2)_nCMe_2OCO{-}$$

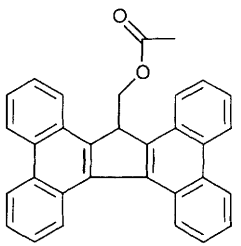
(2)

exposure to acid or base so that deprotection of synthetic derivatives presents a problem. An ingenious solution[57] uses 4-acetoxybenzyloxycarbonyl protective groups. These can be deacetylated at pH 7.0 and 45 °C with acetyl esterase from oranges. The deacetylated product spontaneously fragments (Scheme 3).



Scheme 3

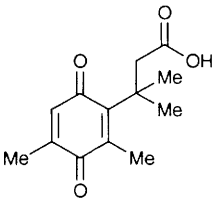
A very hydrophobic derivative of the Fmoc group (**3**), (17-tetrabenzo[a,e,g,i]-fluoroenyl)methyloxycarbonyl (Tbfmoc), has been further examined as a tool for the purification of peptides after cleavage from the support used in SPPS. The Tbfmoc group is incorporated on the *N*-terminal residue at the end of peptide assembly. It then permits affinity binding to porous graphitized carbon or purification by reverse-phase HPLC. The Tbfmoc group can then be removed with 6M-guanidine hydrochloride containing DTT at 37 °C.[58] *N*-Alloc groups can be removed with a Pd(0) catalyst in the presence of $NaBH_4$,[59] $PhSiH_3$,[60] $CF_3CONMe(SiMe_3)$[60] or Bu_3SnH[61] and the amino ester can then be coupled in a one-pot procedure to give peptides. For example, if a mixture of Boc-Ala-OH, EDC and HOBt is added to Alloc-Phe-OMe in THF containing $Pd(PPh_3)_4$ and $NaBH_4$, Boc-Ala-Phe-OMe is obtained in good yield without detectable enantiomerization.[59]



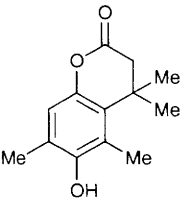
(3)

If the Nps group is used to protect α-groups, it can be removed with HOBt in the presence of a weakly basic nucleophile such as $PhNH_2$ or PhNHMe to which the protecting group is transferred. The deblocked amine is obtained as an HOBt salt which can be acylated without adding a tertiary base.[62] A novel redox-sensitive protecting reagent (**4**) for amines can be removed by reduction with

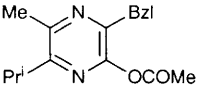
$Na_2S_2O_4$ to the hydroquinone which spontaneously lactonizes to (**5**) due to the 'trialkyl lock' comprising the three methyl groups.[63] Moreover, the product (**5**) can easily be reconverted into (**4**) in high yield. Finally, although acetylation of α-amino groups is not a feature of peptide synthesis except for capping during SPPS, it often features as the last step in the synthesis of some peptide hormones and sometimes in preparing proteinase substrates and inhibitors. A new reagent (**6**) acetylates α-amino groups without affecting hydroxyl groups.[64]



(4)

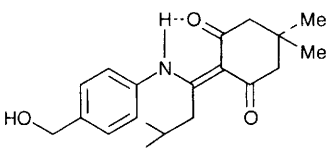


(5)



(6)

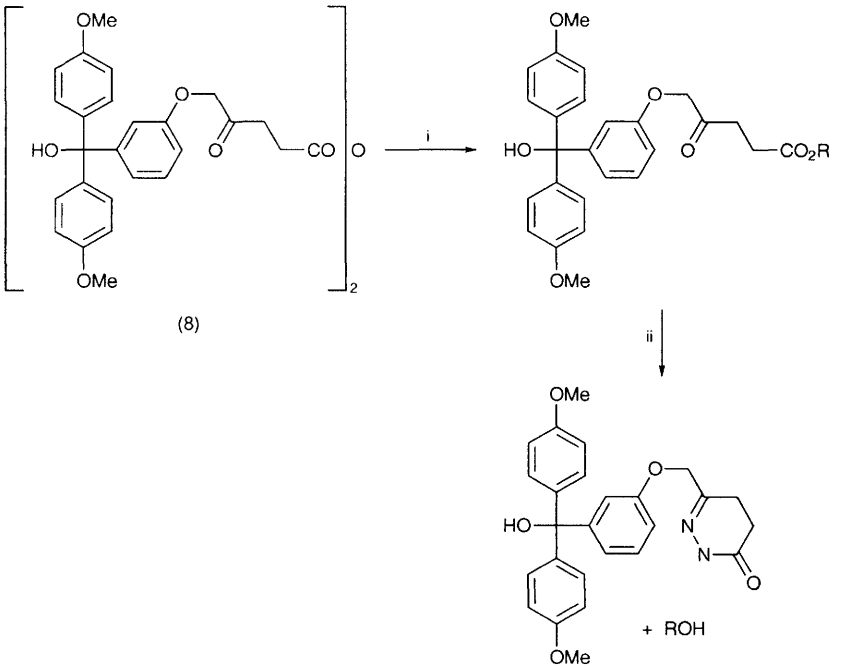
2.2 Carboxyl-group Protection – More methods for the regiospecific protection of carboxyl groups in Asp and Glu have been reported.[65-68] In one of these,[65] the β-2,4-dimethyl-3-pentyl ester is recommended for Asp because it prevents base-catalysed aspartimide formation during SPPS. The t-pentenyl group is recommended for protection of the β-carboxyl group of Asp with Fmoc or Boc chemistries in glyco-peptide synthesis. The group is removed with Pd(0) catalysis. ω-Phenylhydrazide protecting groups on Asp or Glu are stable in peptide synthesis using Fmoc or Boc chemistries.[69] An ingenious method for protecting carboxyl groups employs the safety-catch principle.[70] Esterification is effected with the easily prepared 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}benzyl alcohol (**7**). Treatment with 2% N_2H_4 solution in water at room temperature converts the Dmab ester into a 4-aminobenzyl ester which readily hydrolyses. Fmoc groups are unaffected by this treatment. Finally, Carpino continues to impress us with the fruits of his labours. With a large team of collaborators, he recommends the dicyclopropylmethyl group (Dcpm) for carboxyl group protection, since it can be selectively removed with 1% CF_3CO_2H in CH_2Cl_2 in 15 minutes, conditions that do not affect the t-Bu or *N*-Trt groups.[71]



(7)

2.3 Side-chain Protection – 4-Nitrobenzyl-based side-chain protection for carboxyl, thiol and amino groups has been recommended for SPPS using Fmoc

chemistry on Wang resin.[72] These protecting groups can be removed under mild acidic conditions with $SnCl_2$, PhOH and CH_3CO_2H in $CHONMe_2$. The symmetrical anhydride of 5-[3-(bis-4-methoxy-phenyl)hydroxymethyl]phen oxylaevulinic acid (**8**) is used to protect hydroxyl groups.[73] Removal of the protecting group is effected with $N_2H_4.H_2O$ (Scheme 4). The group has a high UV extinction coefficient which is useful for analytical purposes. 5-*O*-Benzyl-N^{in}-Boc-α-*N*-Fmoc-Trp-OH has been used to construct a library of peptides containing 5-hydroxytryptophan.[74] Ammonia, pyridine or ammonium acetate are effective inhibitors of the hydrogenolysis of benzyl ethers catalysed by Pd-charcoal.[75] Thus, Z- and benzyl ester groups can be selectively removed from derivatives of *O*-benzylserine. The hydroxyl group of Tyr can be protected as its 2,4-dinitrophenyl ether during peptide synthesis and subsequently liberated by thiolysis with $HSCH_2CH_2OH$ or 2-thiophenol.[76]



Reagents: i, ROH, C_5H_5N ; ii, N_2H_4

Scheme 4

Two new protecting groups for the protection of the guanidino function of Arg have been reported.[77] They are 4-$PhC_6H_4SO_2$- (Bip) and 3-t-Bu-4-$MeOC_6H_4$ (Tbs); both are cleavable with CF_3CO_2H or $MeSO_3H$. Extended bicyclic aromatic conjugation as in Bip increases acid lability as revealed by kinetic measurements.

2-Adoc-Cl substitutes in the τ-position in α-Z- or α-Boc-His-OH.[78] Although

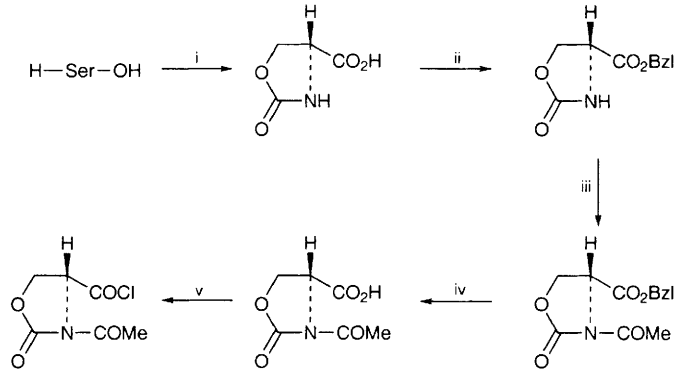
the degree of enantiomerization in peptide synthesis is not disastrously high, neither is it spectacularly low; this is to be expected since it is better if the π-N is substituted. The Im-2-Adoc group is more sensitive than the 1-Adoc isomer to 20% piperidine in $CHONMe_2$ so cannot be used with Fmoc chemistry. The mesitylsulfonyl group (Mts) has been used to protect the indole N-atom of Trp in the synthesis of analogues of melanotropin and CCK. It is detached with HF.[79] Although the Dcpm group can be used to protect amide nitrogen,[71] its removal is slow. Carpino *et al.* have designed and recommended that the dimethylcyclopropylmethyl group (Dmcp) be used. The deprotection of several *N*-protected benzyl derivatives of Asn and Gln by HF has also been investigated.[80] Asn derivatives were more readily deprotected, but four groups, 4-$MeOC_6H_4CHMe$-, 3,4-$Me_2C_6H_3CHMe$-, 2-MeO-1-naphthylmethyl- and 4-MeO-1-naphthylmethyl- could be detached satisfactorily from both Asn and Gln. The hydrogenation of benzyl esters of *N*-protected derivatives of Boc-Asn-OH and Boc-Gln-OH has also been studied.[81]

The stability of *S*-(alkylsulfanyl)cysteine derivatives during SPPS has been examined.[82] *S*-Trt and *S*-t-Bu-sulfanyl derivatives are stable to CF_3CO_2H so that Boc chemistry is available. On the other hand, chirality is lost in 25% piperidine in $CHONMe_2$ so that Fmoc protection is not possible. The *S*-phenylacetamidomethyl group (Phacm), which is compatible with Fmoc and Boc protection, has been tested in SPPS.[83] It is cleaved like Acm and also by penicillin amidohydrolase. Thus, the Phacm group is orthogonal to the 4-MeBzl, Trt and Fm groups. A complication was found using the 4-methoxybenzyl group to protect the thiol group of cysteine in the synthesis of an anti-HIV peptide.[84] During deprotection with 1M $CF_3SO_3SiMe_3$ in CF_3CO_2H, a significant amount of byproduct resulted from the transfer of the 4-methoxybenzyl group from Cys to Trp, possibly located in the 2′ position. This side reaction was efficiently suppressed by adopting a two-step deprotection procedure using either CF_3SO_3Ag or Me_3SiBr followed by $CF_3SO_3SiMe_3$. Finally, it is possible to reduce methionine sulfoxide to Met in peptides with NH_4I in neat CF_3CO_2H at 0 °C leaving disulfide bonds and Acm groups unaffected.[85] Addition of Me_2S accelerated sulfoxide reduction.

2.4 Disulfide Bond Formation – A 22-residue peptide amide resembling the GH loop of the foot and mouth virus protein with protected cysteine residues at positions 3 and 20 has been constructed by Boc/Bzl SPPS. Either Acm or Fm groups were used to block thiol groups. The best method for producing the cyclic disulfide used *S*-Fm protection. Deprotection and cyclization was carried out on the resin with 20% piperidine in $CHONMe_2$ with constant air bubbling.[86] Cysteine can be converted into *S*-sulfocysteine and thence into the Fmoc derivative. The later has been incorporated into the bis-sulfo derivative of Arg vasopressin. Deprotection with Bu_3P followed by oxidation with $K_3Fe(CN)_6$ gave vasopressin.[87] An alternative approach involved assembly of a peptide containing Cys(Acm) residues followed by treatment of the product successively with CF_3SO_3Ag and MeSOMe/HCl.[88] No significant side reactions involving Met, Tyr, or Trp were detected. Urotensin II, tachyplesin I and endothelin I have

been synthesized. The last two peptides contain two disulfide bonds and these were formed regioselectively. It is noteworthy that all three peptides contain Trp and this was unaffected in the oxidative formation of disulfide bonds. An alternative synthesis of endothelin-1 has been reported.[89] Cys^3 and Cys^{11} were protected with t-Bu and Cys^1 and Cys^{15} with Acm.

2.5 Peptide Bond Formation – The acid azide coupling procedure proceeds faster if $Bu_4N^+NO_2^-$ is used in place of $NaNO_2$.[90] Fmoc amino acid fluorides couple efficiently without having tertiary base present[91]; alamethicin F30 and ACP(65-69) were used as test sequences. Acid chlorides of Ser and Thr are accessible by protecting the hydroxyl group in the form of an oxazolid-2-one ring (Scheme 5).[92] The *N*-acetyl group is ultimately removed with pyrrolidine in MeCN at 25 °C.



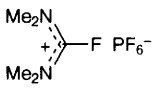
Reagents: i, $COCl_2/NaOH/K_2CO_3$ in H_2O/25 °C; ii, BzlBr/Et_3N in Me_2CO/25 °C; iii, Ac_2O, DMAP (catalytic amount)/Et_3N/CH_2Cl_2/0 °C; iv, H_2/Pd–C; v, $(COCl)_2/CH_2Cl_2$/CHONMe$_2$ (catalytic amount)

Scheme 5

Amide formation is particularly efficient when carried out at 0 °C in a 2-phase system [e.g. CH_2Cl_2/H_2O (1:1 v/v)] in presence of excess $EtN{=}C{=}N(CH_2)_3NMe_2$ (EDC).[93] Enantiomerization is low and 2-hydroxypyridine is reported to be a more effective additive than HOBt or even HOAt. Slightly less spectacular results were obtained using DCCI in $CHONMe_2$ in the presence of an amine salt of *N*-hydroxysuccinimide.[94] One wonders whether N-hydroxypyridine or HOAt would be better than *N*-hydroxysuccinimide? Amide preparation is also recommended to be carried out with Boc_2O in presence of pyridine and NH_4HCO_3.[95] Fmoc-Asn-OPfp can be prepared simply using EDC in dioxan to effect coupling of Fmoc-Asn-OH and C_6F_5OH.[96] Pentafluorophenyl diphenyl phosphate can be used for peptide coupling.[97] For example, H-Gly-Cys(Bzl)-Ser-Gly-Lys-Leu-Ile-Cys(Bzl)-OH (gp41 of HIV) was made by a 5 + 3 route. The HOBt ester of Trt-Glp-OH has been prepared and used to synthesize TRH and some analogues.[98]

Peptide couplings using EDC with either HOAt or 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HODhbt) have been compared.[99] The former was rather better at controlling enantiomerization, except in $CF_3CH_2OH/CHCl_3$ where the opposite was true. Differences were not large. A new coupling agent, 2-hydroxyphenylsulfonyl chloride, can be used in one-pot two-step reactions.[100] Application of the Young and Anteunis tests revealed that very little enantiomerization was caused. A novel method of forming peptide bonds involves formation of the unsymmetrical anhydride of an *N*-protected amino acid or peptide and 3,5-dinitrobenzoic acid.[101] Subsequent treatment with an α-azido ester and a trialkylphosphine affords peptides with insignificant enantiomerization especially in solvents of low polarity. Strangely, addition of HOAt increases enantiomerization as does addition of a small excess of base. The internal anhydride from Trt-Asp-OH reacts with amines and alcohols regioselectively but not regiospecifically.[102] As expected, solvent polarity is the major determinant of the α/β ratio. A rapid one-pot synthesis of Boc-tripeptides is available.[103] An *N*-Z-substituted *N*-carboxyanhydride (Z-NCA) reacts with an amino ester to give a Z-dipeptide ester. A Boc-NCA is added together with 10% Pd on charcoal and the reaction mixture is subjected to hydrogenolysis. As the Z group is removed, the Boc-NCA reacts to give a Boc tripeptide ester. Yields are good, but there is no published information on the chiral purity of the products. *N*-Carboxyanhydrides have been used in the synthesis of peptides containing α-methyl-β-alkyl-serines.[104] A theoretical study has been made of the mechanism of peptide synthesis involving unsymmetrical anhydrides.[105]

Tetramethylfluoroformamidinium hexafluorophosphate (TFFH) (**9**), which is made from the corresponding chloro-derivative, reacts with Fmoc amino acids in presence of i-Pr_2EtN to give Fmoc aminoacyl fluorides.[106] TFFH was used to synthesize satisfactorily H-Tyr-Aib-Aib-Phe-Leu-NH_2 by SPPS. It was also shown that TFFH can be used for segment coupling in the presence of HOAt (1 equiv.) to prevent enantiomerization. A new phosphonium reagent, CF_3-NO_2-PyBOP (**10**), gives very good yields in peptide couplings involving *N*-methyl-amino acids.[107] Improved syntheses of BOP and PyBOP involve the reaction of $(R_1R_2N)_3P{=}O$ [$R_1{=}R_2{=}$Me, $R_1R_2{=}$-$(CH_2)_4$-] with 'triphosgene', CCl_3O-$COOCCl_3$, rather than with phosgene.[108] The coupling of Alloc-NHNHCH(i-Bu)CO_2Me to Fmoc-Phe-OH using HATU/i-Pr_2EtN is another example of a sterically hindered reaction being achieved.[109] The coupling of Z-Ala-Phe-OH with various amino acid esters using 1,1′-carbonylbis(3-methylimidazolium tri-



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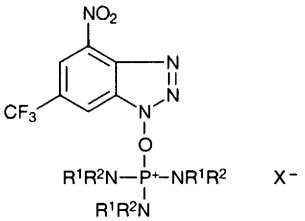
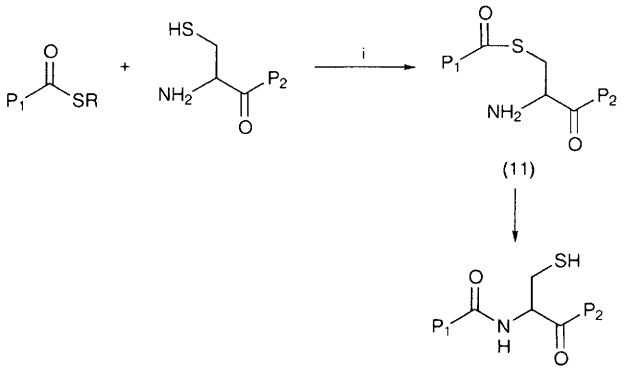


(10)

flate) (CBMIT) usually afforded yields of 81 ± 3% with <0.1% enantiomerization in the presence of Cu^{2+} salts.[110] Enantiomerization increased and yield decreased with hindered nucleophiles such as *N*-methylamino derivatives. α-*N*-CF_3CO-Amino acids are not usually favoured for peptide formation because of the risk of extensive enantiomerization. Better results can be obtained by using 'neutral' conditions such as the use of silylated amino acid derivatives.[111] There are two publications on the use of the 4-component Ugi reaction.[112,113] The more interesting[113] describes the synthesis of glycopeptides with the carbohydrate moiety sited on a peptide *N*-atom. The Arndt-Eistert reaction is useful for preparing homopeptides.[114] At the diazoketone stage, an amino acid ester and $PhCO_2Ag$ (catalyst) are added to complete the reaction. Interestingly, the expected ketene intermediate, $R^1NHCHR^2CH{=}C{=}O$, is rapidly converted into a dihydro-oxazinone which is ring-opened by the amino acid ester.

An ingenious idea for coupling unprotected peptides has been announced.[115] It is site-specific (orthogonal), produces Xaa-Cys bonds and two alternative routes are available. A peptide (P1) terminating in a thiol ester group, reacts with a second peptide (P2) which contains an unprotected cysteine residue at the *N*-terminus (Scheme 6). This reaction is mediated by a trialkyl phosphine and an alkyl thiol. Alternatively, P1 with a free thiocarboxyl group can be alkylated by P2 containing a 3-bromo group. Both methods afford the same thiol ester derivative (**11**) which rearranges rapidly to the P1-Cys-P2 peptide with liberation of the cysteinyl thiol group. The method is notable because P2 can contain an additional unprotected cysteine residue within the body of the chain without any interference with the reaction involving the *N*-terminal cysteine residue. The method of peptide synthesis due to Hegedus involving chromium aminocarbene complexes has been reviewed[25] and extended.[116] It does not seem to have attracted other peptide chemists to use it so far. There has been an IR spectroscopic study of the behaviour of β-sheet-forming peptides in a range of very polar solvents.[117] Although $CF_3CH(OH)CF_3$ is regarded as unsuitable, mixtures of halohydro-carbons and haloalcohols are good solvents and disrupt self-associa-



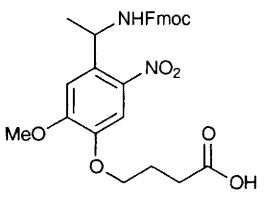
Reagents: i, R_3P, R'SH

Scheme 6

tion; $CHCl_3/CF_3CH_2OH$ mixture is particularly recommended. Finally, *N*-Boc derivatives of α-trifluoromethyl amino acids give 2-t-butoxy-4-trifluoromethyl-5(4H)-oxazolones when treated with DCCI.[118] The product at room temperature, however, undergoes a retro-ene reaction with loss of isobutene yielding an *N*-carboxyanhydride. This route is therefore of limited use.

2.6 Solid-phase Peptide Synthesis – PEG-polyacrylamide resins have been prepared and evaluated experimentally for SPPS.[119,120] The resins have a good capacity and swell satisfactorily in a range of solvents. Moreover, ^{13}C NMR measurements of T_1 relaxation times for appended peptides indicated that the behaviour of the latter closely resembled that in free solution. Resins have also been prepared from hydrophilic components with claimed benefits. Thus, aminomethylated polystyrene/1% divinylbenzene had 98% of its total loading capacity bearing the 5-(2-aminomethyl-3,5-dimethoxy-phenoxy)valeric acid linker and the remaining 2% of the resin was covalently coated with PEG to render the surface hydrophilic.[121] In synthetic tests, peptides were obtained in high yield and purity. Similarly, a resin was prepared by suspension polymerization of styrene and tetraethyleneglycol diacryloate then functionalized with various linkers, some of which permitted photolytic detachment of peptide.[122] Good results were obtained with these resins. Magnetite can be generated in Amberchrom CG-161cd resin so that the beads can be manipulated by small bar magnets.[123]

There has been a substantial addition to our knowledge and choice of linkers. Zn salts of carboxylic acids can be obtained by sonication with $ZnCO_3$.[124] The Zn salts react with Merrifield resin under the influence of ultrasound for conventional peptide synthesis. Although some hydroxymethylphenyl resins react poorly or not at all with $COCl_2$, TentaGel S gives the chloroformate satisfactorily.[125] The latter can be converted into a matrix-linked *N*-carboxyanhydride which reacts with an amino acid ester to form a carbamate-linked dipeptide ester. Treatment with CF_3CO_2H yields the free dipeptide ester. This chemistry might be useful to attach a sterically hindered amino acid to the *N*-terminus of a biologically active peptide to afford protection against aminopeptidases. Fmoc peptides, synthesized on 2-chlorotrityl resin, can be esterified by treatment with 2-chloro-trityl chloride and i-Pr_2EtN after detachment from resin.[126] The α-Fmoc group can be removed and the peptide ester can be coupled in solution to give longer peptides. A fragment of prothymosin α was synthesized by this route. A photolabile linker (**12**) has been designed and tested in the synthesis of a fragment



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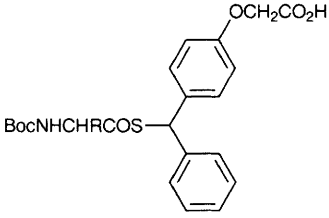

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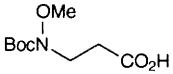
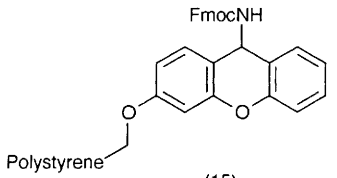


(14)

of CCK.[127] The linker (**13**) provides a convenient route to peptide α-thio-acids using Boc protection and detachment with HF.[128] A linker specifically designed for synthesizing peptide aldehydes (**14**) affords the desired products in moderate yield after detachment with $LiAlH_4$.[129] Although no loss of chiral purity was detected during synthesis, some enantiomer was apparently formed during chromatographic purification of the product. The previously reported glycolamidic ester linker has been further assessed.[130] High yields of very pure products are reported. The polystyrene-linker conjugate (**15**), is useful for the synthesis of peptide amides.[131] Detachment requires only about 8 min. using 1% CF_3CO_2H. Moreover, peptides with *C*-terminal Asn can be acquired if Fmoc-Asp(Ot-Bu) is the first amino acid derivative to be attached. Further, if the linker is first subjected to reductive *N*-alkylation, peptide *N*-alkylamides are easily accessible.[132] If a phenylhydrazide moiety is generated on a support, a peptide chain can be assembled on the hydrazide group.[133] Oxidation in air in presence of $CuSO_4$ and pyridine liberates the peptide without oxidation of Met or Trp residues. Unfortunately, information about the chiral purity of the product is lacking. A novel allylic linker (**16**) permits peptide detachment with Pd(0) in the presence of a scavenger such as morpholine or *N*-methylaniline to trap the allyl moiety irreversibly.[134] This is a useful tool for synthesizing glycopeptides since acidic conditions are avoided. The synthesis of peptide T, a fragment of HIV-envelope protein, gp120, gives it a good start. A new linker (**17**) gives peptide derivatives by SPPS with a finely tuned lability to base.[135] Peptides are not detached by i-Pr_2EtN/CH_2Cl_2 (1:19) during 3h at room temperature but are released by exposure to piperidine-$CHONMe_2$ (7:93) or morpholine-$CHONMe_2$ (1:4). A linker-resin combination that permits attachment of Lys through its ε-amino group has been reported.[136] Peptides are detached by hydrogen transfer hydrogenolysis. A comparative study has been made of several linkers that require acid detachment.[137]

There is little to report on protecting groups. The safety-catch type of protecting group such as 4-(methylsulfinyl)benzyl (Msib) and 4-(methylsulfinyl)-benzyloxycarbonyl (Msz) have been used to synthesize a bombesin analogue and human big gastrin I.[138] The feasibility of using 18-crown-6 as a noncovalent α-*N*-



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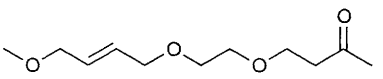

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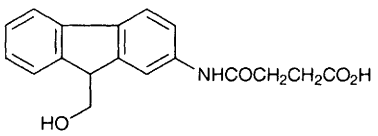


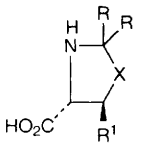
(17)

protecting group has been examined.[139] The use of $CHONMe_2$ as solvent gave rise to more than one product, but a change to CH_2Cl_2 and use of Pro as *N*-terminal residue of the resin-bound moiety greatly improved results. Positive attributes include good results with α-*N*-Fmoc-α-Hmb amino acids and ease of removal of 18-crown-6 with aqueous KCl or 1% i-Pr_2EtN. The Npys group is useful for temporary protection of ε-amino groups during assembly of TASPs.[140]

As expected, Fmoc amino acid fluorides are very efficient for acylating hydroxyl groups in linker-resin conjugates.[141,142] High loading levels are attained with low racemization with DMAP as an exceptionally good catalyst. A series of analogues of alamethicin, which is rich in Aib, and a 22-residue fragment of BOVTESTIS channel protein have been synthesized in excellent purity.[143] For the attachment of Fmoc amino acids to resins with free benzylic hydroxyl groups, $(Boc)_2O$-pyridine-DMAP in CH_2Cl_2 at 0 °C gave high yields with minimal loss of chiral purity. Phase-transfer catalysis is recommended for the attachment of Boc amino acids to Merrifield resin.[144] A crop of papers[145-149] has clearly established Hmb-protection of selected peptide *N*-atoms reported last year as an essential tool for peptide synthesis especially when interchain association is prone to occur. The synthesis of β-amyloid (1-43) used five Hmb goups.[148] In addition, the almost complete suppression of formation of aspartimide in the assembly of a 20-residue fragment of ferredoxin is a further commendation.[149] Other solutions to problems related to chain association and aspartimide formation have been sought. There is a clear correlation between resin swelling and ease of acylation.[150] Coupling efficiency can be increased by using an aprotic, polar solvent such as *N*-pyrrolidinone at 50 °C. A slightly less direct approach involves using pseudo-prolines (e.g. **18**) in place of Pro to increase solubility and prevent aggregation.[151] Fluoride ions have been found to promote the rearrangement of α-Asp and α-Glu peptides to the β/γ isomers.[152] The tendency for aspartimide formation to occur is sequence-dependent and prone to occur if the next residue is Arg(Pmc), Asn(Trt), Asp(Ot-Bu), Cys(Acm), Gly, Ser, Thr(t-Bu) and Thr.[153] Methods are defined that minimize this complication including the use of Fmoc-Asp(Ot-Bu)-OH. It is also claimed that use of 2-chlorotrityl as linker renders this side reaction less likely if Asp is either at or near the *C*-terminus.[154,155] It is probable, however, that well-planned use of the Hmb group will prove to be the most general and effective method for avoiding this difficulty. The need to protect the indole nitrogen atom in order to avoid alkylation has been further underlined.[156] An unexpected complication in SPPS has been described.[157] Commercial samples of linker derivatives based on 5-(4′-aminomethyl-3′,5′-dimethoxyphenoxy)valeric acid were found to contain dimeric linkers.[157] Where peptides are attached to the resin by two linker molecules, acidolytic detachment yields a peptide with one linker molecule still covalently bound at the *C*-terminus. Although coupling methods have been enormously improved and interchain association can be limited, the use of reactive capping reagents to mop up unreacted amino groups is still useful. *N*-(2-Chlorobenzyloxycarbonyloxy)succinimide (**19**) is recommended because it reacts rapidly and the derivatives are stable to reagents used in both Fmoc and Boc chemistries.[158]

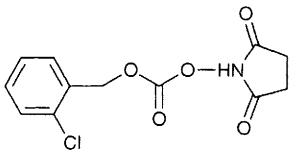
Detachment of peptides from PAM resin can be effected by Me_3SiBr (or

Me_3SiCl and LiBr) with PhSMe in CF_3CO_2H.[159] If Me_3SiCl is used, the reaction must be carried out at 50 °C. The reagents also remove Mts groups from the guanidino group of Arg. Peptides can be cleaved from Merrifield resin using $Me_2NCH_2CH_2OH$ or $N(CH_2CH_2OH)_3$ admixed with 1M NaOH and $CHONMe_2$ with sonication for 20-60 min.[160] Release of peptide amides from resin using the 4-methylbenzhydrylamine linker has been studied in detail.[161] Reagent K removes side chain protecting groups before peptide detachment with CF_3SO_3H. Formation of α-aminosuccinimide residues can be suppressed by working at room temperature. In addition, N→O acyl rearrangement of Thr/Ser residues occurred to only a minor extent. Peptide amides can be synthesized using a photolabile 2-nitrobenzhydrylamino polystyrene support.[162] This is recommended since photolytic detachment (350 nm) gives rise to a 2-nitrosobenzophenone group which does not rearrange to an azobenzene derivative in contrast to polymers yielding 2-nitrosobenzaldehyde as a photoproduct. Finally, some technical improvements have been made to equipment for SPPS.[163,164]



(X = O, S)

(18)



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The remainder of this section is devoted to combinatorial synthesis since this field is dominated by SPPS.[9] The multipin technology still has its adherents,[165,166] but the trend towards the generation of libraries of thousands or even millions of peptides has established dominance, especially since peptides can be stored on beads which are individually tagged by a chemical or physicochemical reagent for purposes of identification and retrieval. The assembly of orthogonal libraries has been described.[167] Using 25 amino acids which can give rise to 15625 different tripeptides, 125 sublibraries of 125 tripeptides were produced. If the 25 amino acids are written in a 5 × 5 matrix, any sublibrary obtained from a horizontal group of 5 and any sublibrary obtained from a vertical group of 5 share only one tripeptide. Introduction of a new, noncoupling operation into a portioning-mixing method allows preparation of binary peptide libraries.[168] Light-directed combinatorial peptide synthesis on microscope slides can be carried out in 10 steps starting with α-6-nitroveratryloxycarbonyl (Nvoc) amino acids.[169] Physical methods continue to be exploited for studying combinatorial synthesis. Thus, matrix-assisted laser desorption/ionization time of flight mass spectrometry is much more sensitive than NMR methods and can be used to identify peptides on single beads. It has allowed side reactions in SPPS to be identified. *N*-Peptidyl-*NN'*-diisopropyl ureas have been identified in preparations in which excess carbodiimide was used.[170] A similar technique applied to combinatorial synthesis on individual beads, which incorporated an intermediate capping procedure, identified not only the full-length peptide but also the intermediate stages.[171] *C*-

Terminal amides that are detached from the support photolytically can be identified by electrospray ionization mass spectrometry.[172] Encoding beads with an easily identified tag uses various methods. A new chemical tagging method uses oligo-nucleotides as identifiers.[173] Most ingeniously, single/multiple addressable RF tags (SMART) which can emit and store RF signals over a range of 150 mm have been used.[174,175] It is possible to store additional data concerning details of synthetic procedures as well as a simple identifier.[175] An obvious application of peptide libraries is the rapid identification of substrate and inhibitors of proteolytic enzymes. A particularly convenient technique uses fluorogenic substrates.[177,178] A synthetic receptor has been developed for studying the binding of small encoded peptides synthesized by combinatorial methods.[178] A library of hexapeptides with defined amino acids at positions 1, 3 and 5 has been tested[179] to identify those peptides that bind Tc.[99] Two peptides, KGHSHV and KAMYHG, had high affinities. Using vancomycin as a model receptor, a library of tetrapeptides was surveyed for binding using a combination of capillary electrophoresis and mass spectrometry.[180] A library of peptides synthesized from D-amino acids to render them resistant to proteolytic enzymes has been assembled and surveyed to identify sequences that bind avidin and streptavidin.[181] Val, Leu or Ile are the favoured *N*-terminal residues with Tyr or Phe at position 2 and Asn at position 3. A library of peptides on cellulose membranes has been assembled and screened to identify sequences able to bind particular ligands such as TGFα and TGFβ.[182] A library of peptides coupled via α, β or γ amino groups with free amino groups acylated by various carboxylic acids has been prepared.[183] Finally, libraries comprising one peptide/bead have been synthesized and the peptides liberated into solution in several steps for screening.[184]

2.7 Enzyme-mediated Synthesis and Semi-synthesis – It is a general tenet that proteinases and peptidases can withstand exposure to organic solvents and nonoptimal temperatures if they are made insoluble either by immobilization or polymerization by a cross-linking agent. Thermolysin has been crystallized from aqueous solution and treated with glutaraldehyde.[185] The modified enzyme had enhanced stability to a variety of organic solvents and inorganic salt solutions and v_0 was not decreased significantly with substrates up to heptapeptides. Conjugation or physical entrapment in PEG-based polymers similarly stabilizes subtilisin and α-chymotrypsin.[186,187] The latter has also been covalently attached to PEG/2-hydroxy-ethyl methacrylate and used in various systems composed of an organic solvent and aqueous buffer.[188] Proteinases have been immobilized on carbohydrates by treating 2-aminohexoses and 2-aminopentoses with CH_2=CMe-COCl, polymerizing with $Na_2S_2O_8$ as initiator and finally allowing the polymer to react with the ε-amino groups of α-chymotrypsin in a ring-opening reaction in presence of $NaBH_3CN$.[189]

The thermodynamic activity of water in mixed solvents is only one factor that determines velocity of peptide bond synthesis. Velocities are markedly different in several solvents in which water activity is unity.[190] Synthesis of *Z*-Phe-Phe-OH and its methyl ester has been studied in various alcohols and K_m and k_{cat} changed

linearly with log P of the solvent.[191] Reactions in water/octane emulsions containing the surfactant, AOT, have been widely studied. Results are generally satisfactory but substrate insolubility causes problems. A recent study has found that addition of EtOAc provides a solution, but reaction rate decreases as the amount of EtOAc is increased.[192] The induction of enzyme memory when α-chymotrypsin is exposed to Ac-Trp-OH in nonaqueous solvent has been further studied by X-ray crystallography.[193] The results may not be the last word on this subject especially since other enzymes merit examination. It may be desirable to wipe the enzyme memory before reusing a proteinase for peptide synthesis. This might be achieved by washing the enzyme with an appropriate solvent. It is relevant to cite another publication[194] reporting successful syntheses without organic solvent and in the presence of small amounts of water and under sonication and fluidization. Another possible approach has been previously examined; supercritical CO_2 and mixtures with organic solvents and small amounts of water could prove to be the solvent of choice as indicated by a recent paper.[195] Supercritical CO_2 brings its own technical difficulties, but then so did HF which has been so widely used as a general deprotecting agent during peptide synthesis.

The structure of substrates used in enzymic syntheses has received further attention. 2,2,2-Trifluoroethyl esters facilitate peptide synthesis controlled by α-chymotrypsin,[196] doubtless because the first step of the Ping Pong mechanism is faster. The use of positively charged, solubilizing protecting groups permits the use of very high substrate concentrations.[197] In a slightly related approach, Co(III) complexes of peptides are more efficient nucleophiles than the free peptides in peptide synthesis.[198] Use of 2-aminoethyl esters of aliphatic acids as nucleophiles showed that synthesis occurred with derivatives of acids containing less than eight carbon atoms but not with longer esters.[199] Thus the depth of the *n* subsite is <1.4455 nm assuming that substrates bind in extended conformation.

There is quite a lot to report under a broad kinetic heading. The thermolysin-catalysed coupling of *N*-blocked Phe derivatives with H-Leu-NH_2 proceeds efficiently in aqueous media even when most of the starting materials and products are out of solution.[200] Such reactions tend to proceed until at least one reactant has completely dissolved. There has been further work on frozen solutions.[201-206] It now appears fairly general that reactions catalysed by enzymes that operate by the Ping Pong mechanism proceed faster in frozen solution and afford higher yields. The results are reflected in increased values of k_{cat}/K_m. Some authors attribute enhanced yields in frozen solution to a decrease in the competing hydrolysis of the ester substrate[206] while others[204-205] think that the acceleration is due to increased nucleophile concentration in liquid micro inclusions in ice. There seems to be no reason *a priori* why both explanations and even others such as changes in solvation state of the enzyme and/or substrates are not involved. If solutions of serine proteinases are frozen in presence of a large excess of 18-crown-6, there is a very large increase in subsequent reaction rate.[207] This treatment does not affect enantioselectivity. Smaller crown ethers evince a similar but smaller effect. Since acetylated trypsin is not similarly activated, the ε-amino groups in the enzyme may be involved in the acceleration process. If accelerated

chymotrypsin is washed with cyclohexane, the behaviour reverts to that of the preactivated enzyme. Alcalase or subtilisin Carlsberg catalyse the coupling of *N*-protected amino acids and secondary amines such as derivatives of Pro.[208] Only L-compounds are accepted at the S1 subsite but stereospecificity is absent at the S1′ site. Kinetic studies showed that k_{cat}/K_m for the imino nucleophiles fell in the range 84-423 min^{-1} M^{-1}. Optimal conditions were found for the synthesis of dipeptide precursors of some renin inhibitors. The kinetics of free and immobilized papain have been compared.[209] Chymotrypsin adsorbed on controlled-pore glass and suspended in anhydrous solvents have been compared by ESR spectroscopy.[210] Addition of a small amount of water to the immobilized enzyme is accompanied by a sharp rise in activity and active-site polarity whereas the similar increase in activity of suspended enzyme is attributed to increased molecular flexibility. The specificity of papain depends strongly on solvent composition. For substrates of the general type Bz-X_1-X_2-NH_2 or Z-X_1-X_2-NH_2, where X_1 is a small amino acid and X_2 has a large hydrophobic side chain, hydrolysis of the X_1-X_2 bond occurs preferentially in aqueous methanol as the water content rises, whereas solvolysis of the X_2-NH_2 bond predominates in methanol containing low concentrations of water.[211] In a study of peptide-bond formation catalysed by thermolysin, a further kinetic complication has emerged.[212] Substrate inhibition by the amino substrate has been observed.[213] When conductometric measurements were used to study the coupling of Z-Ala-OH and H-Phe-NH_2 in the presence of *Pseudomonas aeruginosa* elastase, there was a linear decrease in conductance in the early stages of reaction and this can be used to measure v_0. Clearly, there is much scope for further work which could revitalize the presently unfashionable area of enzyme kinetics.

A few examples of the applications of the use of mutant or chemically modified enzymes will now be given. A mutant of subtilisin BPN′ (S221C, P225A) called subtiligase catalyses peptide-bond formation efficiently in aqueous solution even to the extent of cyclizing linear peptides containing 12-31 residues.[214] A similar variant of subtilisin BPN′ (M50F, N76D, N109S, K213R, N218S) termed stabililigase is useful for the synthesis of proteins including coupling peptides containing mercury or biotin to the *N*-terminus of human growth hormone.[215] Remarkably, the enzyme functioned in a solution that contained 4M-guanidine. Perhaps stabililigase would be a suitable, albeit expensive, component of a biological detergent for removing obstinate protein stains on clothes. Another example of engineered enzyme specificity concerns trypsin. By introducing two His residues at suitable sites (N143H, E151H) and using substrate with a further His residue at P_2', the mutant enzyme will catalyse hydrolysis of the Tyr-Ala bond in AGPYAHSS in the presence of Ni^{2+} or Zn^{2+} ions.[216] Presumably this novel idea could also be applied to peptide synthesis. A mutant of proline-specific endopeptidase (S556C) showed dramatically increased peptide-ligase activity in aqueous solution.[217] A chemically modified proteinase thiol-subtilisin, can use peptide thioesters as substrates.[218,219] A partial sequence of the c-Myc protein that binds DNA has been synthesized[219] in addition to a number of small peptides.[218]

This section ends with some examples of enzyme-catalysed peptide synthesis.

Several peptide esters and 4-nitroanilides containing up to six amino acids have been prepared using gastricsin.[220] Preparative syntheses of the enkephalins are reported.[221] Using four enzymes for the various steps, 'delicious peptide' (KGDEESLA) was synthesized with only one chemical (hydrogenolysis) step.[222] Enterostatin amide has been synthesized in two steps[223] in good yield using the prolyl dipeptidylamino-peptidase from *Lactococcus lactis subsp. lactis NCDO 763*. Salmon calcitonin has been semisynthesized from two fragments (1-24, 25-32) using trypsin to catalyse formation of the Arg^{24}-Thr^{25} bond.[224] Hydrolysis of the trypsin-sensitive Lys^{11}-Leu^{12} and Lys^{18}-Leu^{19} bonds was prevented by operating at pH 8.6. An *N*-acetylated pentapeptide sequence from laminin was synthesized using chymotrypsin and subtilisin to couple the *N*- and *C*-terminal residues respectively to the central tripeptide.[225] Single coupling of *NN'*-bis-Boc-L-cystine can be effected by immobilized ficin by controlling the duration of reaction.[226] Peptides containing *C*-terminal Arg- or Lys-4-nitroanilide residues can be obtained using subtilisin or α-chymotrypsin.[227] Small peptides containing 2-allylglycine can be synthesized using bacterial proteinases and α-chymotrypsin.[228] A very good yield of [B25-Trp] despentapeptide insulin has been obtained by coupling the desoctapeptide derivative and H-Gly-Phe-Trp(CHO)-NH_2 using trypsin to catalyse the formation of the Arg^{B22}-Gly^{B23} bond.[229]

Conversely, peptides with a *C*-terminal Arg or Arg-NH_2 have been made using subtilisin adsorbed on macroporous glass.[230] The products function as substrates for carboxypeptidase B and metalloproteinases, respectively.

2.8 Miscellaneous Reactions Related to Peptide Synthesis – Despite the enormous thrust into solid-phase combinatorial synthesis, a little effort has also been made to develop the complementary approach in the liquid phase.[231,232] It is too early to judge how far this will progress. Some 5-acyl and 5-sulfonyl ornithine derivatives have been used in peptide synthesis and, perhaps not surprisingly, intramolecular cyclization competed with peptide bond formation under basic conditions.[233] A new method of incorporating (S)-isoserine into peptides starting from (S)-malic acid and using CF_3COCF_3 as protecting and activating agent has been described.[234] *N,O*-Acyl migration in linear and cyclic peptides in aqueous acid has been studied.[235] The treatment of a synthetic peptide related to cathepsin D, which contained five Ser residues and one residue of Cys(Acm), with iodine gave some product still containing one Acm group and an unprotected Cys residue.[236] It was suggested that *S,O*-migration had occurred. Dipeptides containing 3-iodoalanine are accessible from the corresponding chloro derivatives by reaction with NaI under carefully controlled conditions to avoid formation of the dehydropeptide.[237] Treatment with activated zinc gave the organozinc compounds which reacted with electrophiles in the presence of Pd(0) catalyst to give the corresponding dipeptide containing a 3-substituted alanine residue without loss of stereochemical purity. Conversely, deliberate β-elimination has been used to synthesize fragments of microcystin that contain *N*-methyldehydroalanine.[238]

3 Appendix: A List of Syntheses Reported in 1995

The syntheses are listed under the name of the peptide/ 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	
Angiotensin	
Antagonists involving changes at position 8	243
IANVNMGE, an antisense peptide	244
Antibacterial peptides	
Fragment of seminalplasmin and analogues	245
Analogues of gramicidin containing Lys in place of Orn	246
Protegrin-1 and related peptides	247,248
Peptides related to gramicidin S	249
δ-Toxin from *Staphylococcus aureus* and analogues	250
Kanamycin and netilmycin derivatives	251
Indolicidin, a Trp-rich peptide from bovine neutrophils	252
Vancomycin carboxamide derivatives	253
Peptide fragment of pseudobactin	254
Tripeptide fragment of nosiheptide	255
Segments and analogues of murine perforin	256
Barnase	
Barnase (1-22) and analogues: complexation with peptide (23-110)	257
Barnase-like domain in DNA-directed RNA polymerase II of *Saccharomyces cerevisiae*	258
Blood-clotting factors	
Analogues of profactor IX (residues −18 to +1) containing photoaffinity peptide inactivators	259
Analogues of thrombin receptor-peptide ligand	260,261
Bombesin	
Pseudopeptides based on bombesin (6-14), some with anti-tumour activity	262
Bradykinin	
Wasp kinins resembling bradykinin and cyclic analogues	263
Analogues of bradykinin and vespulakinin	264
Antagonists	265-267
Calcitonin	
Analogues	268,269
Calcitonin gene-related peptide, CGRP	
N-Terminal analogues	270
Cecropin	
Cecropin A-melittin hybrid molecule	271

3.4 Conformation of Synthetic Peptides

3.5 Glycopeptides

3.10 Purification Methods

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3

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

BY S.C.G. BIAGINI, M. NORTH AND P.J. WILLIAMS

1 Introduction

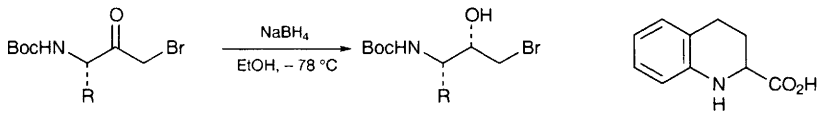
The material for this chapter was obtained by essentially the same method used last year, thus references were initially obtained from the on-line science citation index for 1995 using a series of keywords. These were then whittled down to a more reasonable number by hand, after which the original articles were consulted when they were available in either the UWB or RSC libraries; otherwise an abstract of the paper was obtained. References are restricted to those in the 1995 database which covers a few articles from the end of 1994 (which were not included in last year's review) and most papers published in 1995. However, no 1996 papers have been included, and some papers published in December 1995 will be included in the next annual review. These references were supplemented by those obtained from a manual search of the CA-selects on amino acids and peptides. No work published in patents or in unrefereed form (such as conference proceedings) has been included. The number of references included in this year's review has increased by 30% from 300 last year to almost 400 this year, though the length of this review has been kept constant, and the number of pages of text has actually been reduced by more than 10% to make room for more diagrams. Inevitably, this means that less detail is given in this year's review.

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.

The general format of the review remains unchanged from previous years, though the sections on specific peptides in Section 6 have been modified to reflect current literature reports. In particular, the new sections introduced last year on amino acids with modified side-chains, on RGD containing peptides and analogues, and on helical peptides have been retained. The sections on hydrazine isosteres and cholecystokinin analogues have, however, been deleted this year due to lack of suitable material. The latter section has been replaced by a discussion of neuropeptide analogues.

2 Peptide-backbone Modifications

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 general approach to isosteres of this type based upon the stereoselective reduction of *N*-Boc-bromomethyl ketones has been described, using either K-selectride in THF, or sodium borohydride in ethanol, to form the *erythro*-isomer of hydroxyethylene peptide isosteres in 70-90% yield as outlined in Scheme 1.[1] The pseudodipeptide isostere Pheψ[$CH_2CH(OH)$]Phe has also been prepared from phenylalanine.[2]



R = CH_2Ph, $CHMe_2$, Me (1)

Scheme1

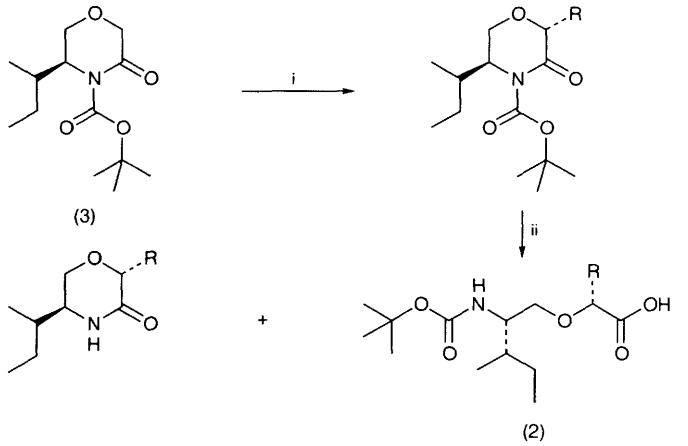
2.1 ψ[NHCO]-Retro-inverso Analogues – A series of retro-inverso peptides, corresponding to peptides known to be recognised by antibodies (lupus mice and autoimmune patients), has been synthesised. The analogues were found to be recognised equally or better than the original peptides.[3] The crystal and molecular structure of the hydantoin 1-[(*S*)-1′-aminoethylmalonyl benzyl ester]-(*S*)-4-methylimidazolidin-2,5-dione derived from the peptide H-Ala-gAla-mGly-OBzl, possessing the retro-inverso modification of the Ala-Gly bond (gAla being the residue with a geminal diamino group, and mGly the malonyl residue), has been determined.[4]

2.2 ψ[CH_2NH]-Aminomethylene and ψ[CH_2O]-Ether Analogues – A short review has been published covering a series of azapeptides and oxapeptide analogues of D-Ala-D-Ala as well as heterocyclic analogues of β-lactam antibiotics. The design synthesis and biological evaluation of the D,D-peptides and β-lactamase inhibitors was discussed.[5]

The pseudotripeptide H-Tyr-Ticψ[CH_2-NH]Phe-OH (Tic=1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid **1**), has been studied using NMR methodology and computer modelling. In particular, the effect of on the backbone conformation was investigated, and the neutral form of the reduced peptide bond was bound to gain rigidity upon protonation.[6] Selective reduction of amide bonds to CH_2NH-isosteres, has been effected upon the 1-12 fragment of motilin, a 22-residue peptide hormone stimulating stomach and intestinal motility. The CH_2NH analogues were studied by NMR and molecular techniques, and tested for biological activity. The *C*-terminal region appeared to be critical for biological activity. Also, conversation of the amide bond rigidity was found to be essential for the activity of non-hydrolysable analogues.[7] Analogues of the *C*-terminal hexapeptide of histone H3 (-Ile-Arg-Gly-Glu-Arg-Ala-OH) have been

obtained by the systematic replacement of each peptide bond with ψ(CH_2-NH). The analogues were found to bind to antibodies raised against the parent peptide.[8]

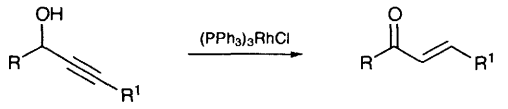
The stereoselective alkylation of morpholin-3-ones has been employed towards the preparation of ψ[CH_2O] pseudopeptides, including previously unavailable ψ[CH_2O] dipeptides.[9] A synthetic approach to methylene-oxy isoteres such as **2**, has been reported. The key reaction involves the stereoselective functionalisation of an isoleucine derived acyl morpholinone **3**, as outlined in Scheme 2.[10] Replacement of the Phe residue of *N*-formyl-methyl-Leu-Phe-OMe with phenylalaninol or its derivatives led to the synthesis of analogues, some of which were found to stimulate superoxide production strongly and showed activity in lysozyme release.[11]



Reagents: i NaHMDS, THF/DME (1:4), – 78 °C, then RBr; ii LiOOH, THF (aq).

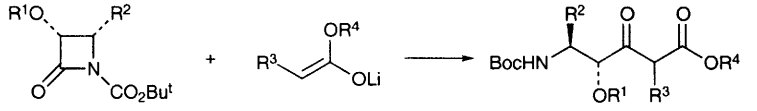
Scheme 2

2.3 ψ[CH=CH] Isosteres and Related Analogues – The preparation of a series of γ-alkylated (*E*)-olefins as peptide bond mimetics has been reported.[12] The stereocontrolled synthesis of *trans*-dipeptide isosteres has been achieved, using the diastereoselective dihydroxylation of ester-allylsilanes in the key step.[13] The isomerisation of hydroxy alkynes to α,β-unsaturated ketones is mediated by tris(triphenylphosphine)rhodium chloride as shown in **Scheme 3**, and has been used to prepare keto vinyl esters and trans-hydroxy vinyl ester dipeptide isosteres.[14]

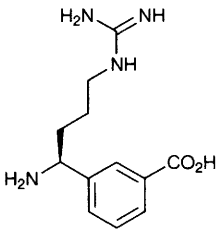


Scheme 3

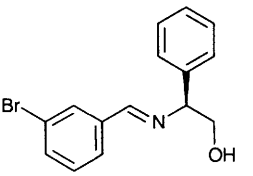
Hydroxy(keto)ethylene dipeptide isosteres have been prepared by the ring opening coupling reactions of (3*R*,4*S*)-1-Boc-3-silyloxy-4-substituted-azetidin-2-ones with lithium enolates of ketones and esters (**Scheme 4**).[15] The synthesis of a phenylogous amino acid **4**, which mimics an extended conformation of the Arg-Gly dipeptide unit has been reported. The synthesis involved several steps, from the starting phenylglycidol derived chiral imine **5**.[16]



Scheme 4

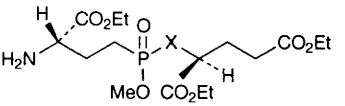


(4)

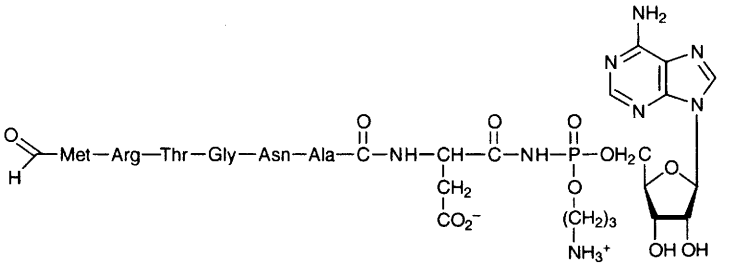


(5)

2.4 Phosphorus Containing Peptide Bond Isosteres – An approach to phosphonamidate pseudopeptides has been reported. The methodology is compatible with side chain functionalised amino acids, and is suitable for incorporation in solid phase synthesis.[17] The total synthesis of the phosphonamidate **6** and phosphonate **7** has been reported. The two compounds



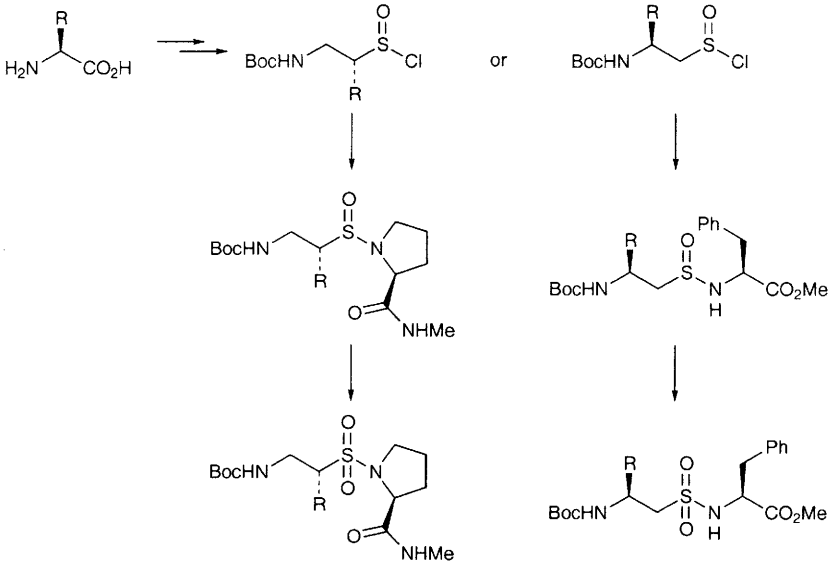
(6) X = NH
(7) X = O



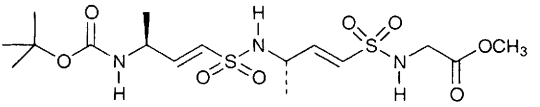
(8)

are isosteres of γ-glutamyl-glutamate dipeptides.[18] The structure of *Escherichia coli* microcin C7 (MccC7) **8**, an antibiotic that inhibits protein synthesis *in vivo* has been determined by the use of ^{1}H homonuclear and heteronuclear NMR techniques and mass spectrometry.[19] The structure consists of a peptide joined to an adenoside via a phosphoramide link.

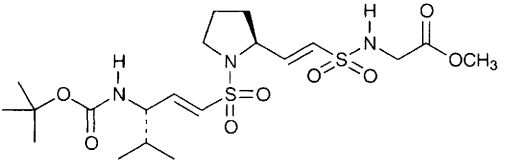
2.5 Sulfur Containing Peptide Bond Isosteres – Sulfinyl chlorides have been used as precursors for the synthesis of homochiral α- or β-substituted sulfinamide or sulfonamide transition state isosteres as shown in **Scheme 5**. The same methodology could be applied to the preparation of peptidosulfonamide peptidomimetics, and a tetrapeptidosulfonamide which can be considered as a biopolymer mimetic.[20] Sulfonamide containing peptides **9** and **10** have been synthesised by using solid phase synthesis in 50-60% yield without any loss of stereochemical integrity at the amino acid α-centres.[21] The same authors also designed the related peptide **11** which was intended to bind to oligopeptides, and was attached to a disperse red (DR) dye to facilitate the detection of binding. In the event, **11** showed low binding with oligopeptides but high selectivity, and the binding strengths were just as good as those of analogous receptors built with natural α-amino acids. It was also shown that the binding selectivity of the sulfonyl peptide based receptors differed from their peptide counterparts.[22] Thus when tested against a peptide library, peptide **11** showed preferential binding to the D-Pro-D-Gln-L-Gln or L-Pro-D-Val-D-Pro sequences whilst the parent peptide showed optimal binding to D-Val-D-Val-D-Gln.



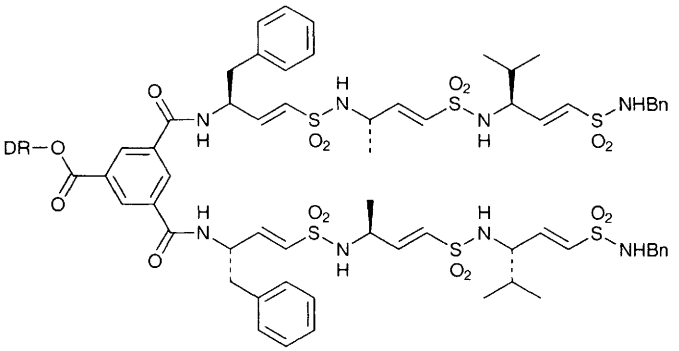
Scheme 5



(9)



(10)



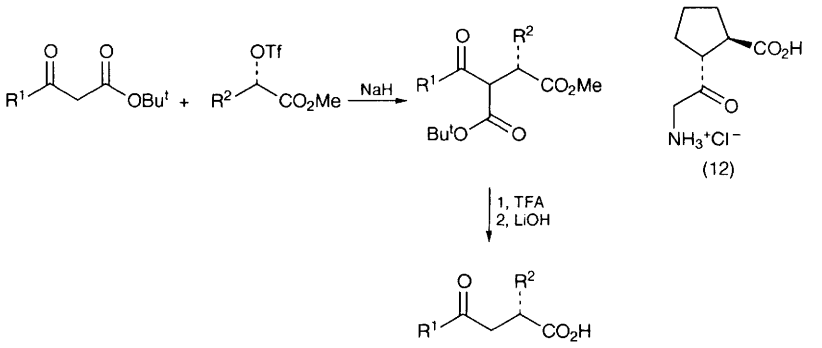
(11)

DR = Disperse red

The sulfonamide isostere containing peptide *N*-acetyl-tauryl-proline methylamide (tauryl = 2-aminoethanesulfonyl) has been prepared, and its conformation was compared with that of the peptide *N*-acetyl-β-alanyl-proline methylamide. It was found that although *N*-acetyl-β-alanyl-proline methylamide existed in two conformations due to rotation about the amide bond, *N*-acetyl-tauryl-proline methylamide existed predominantly in a single extended conformation. This was also confirmed by the crystal structure of *N*-acetyl-tauryl-proline methylamide.[23]

Five peptides of general structure Ala-Xaa-ψ[CS-N]-Pro-Phe-NH-Np (Xaa = Gly, Ala, (*S*)-2-aminobutyric acid, Phe and Leu; Np = *para*-nitrophenyl), each of which contains a thioamide isostere, have been synthesised.[24] The peptides were used to study the mode of action of peptidyl-*cis*/*trans* isomerases. Lawesson's reagent has been used to thionate astin-B, an antitumor cyclic pentapeptide, yielding [Ser_3-ψ(CSNH)-β-Phe_4]astin-B. NMR studies showed that the thioanalogue retained the bio-active conformation of astin-B.[25] A procedure for the solid phase synthesis of peptide-α-thioacids from thioester resin linkers using Boc-chemistry has been reported.[26] The peptide is attached to the resin by a benzhydryl thioester which is cleaved by treatment with hydrogen fluoride.

2.6 Aldehyde and Ketone Containing Isosteres – A simple method for the preparation of ketomethylene peptide isosteres has been reported. This involved the enantioselective synthesis of γ-keto acids and heterocyclic γ-keto acids which possess an alkyl group at C-2. The alkyl group was introduced by chiral alkylation using a scalemic 2-triflyoxy ester as shown in **Scheme 6**.[27] The aminoketone **12**, was found to be a potent competitive inhibitor of porcine kidney prolidase (K_i = 270 nM). A number of related analogues was also prepared, but all were found to be less inhibitory.[28] Ketomethylene tripeptide isosteres have been synthesised using amino acid derived sulfones. Isosteres of the type Xaa-Gly-Xaa', incorporating phenylalanine, tyrosine and valine units, were prepared.[29] *N*-Acyl-aspartic acid ketones have been reported as inhibitors of interleukin-1b converting enzyme (ICE), with the best compound giving a K_i of 3.5 μM versus ICE.[30] The same laboratories have also reported the synthesis and evaluation as ICE inhibitor of *N*-allyloxyaspartyl aryloxymethyl ketones, with K_i values of 90-500 nM.[31]



Scheme 6

A solid phase approach to the synthesis of *C*-terminal peptide aldehydes has been reported. The procedure involved the formation of a linker based upon the Weinreb amide, followed by classical solid phase synthesis, with cleavage by HF and reduction with $LiAlH_4$.[32] Boc- or Z-protected amino acids have been converted to activated mixed anhydrides. Reduction of the anhydrides with either DIBAL or lithium tri-tert-butoxyaluminium hydride yielded the corresponding protected aminoaldehydes.[33]

2.7 α,α-Dialkylated Glycine Analogues – A pyridoxal 5'-phosphate model compound has been used to alkylate α-imino esters, yielding α-alkyl-α-amino esters.[34] In a different approach, ethyl *N*-(diphenylmethylene)glycinate has been used as an ionic glycine equivalent, leading to the preparation of bicyclic and tricyclic α,α-disubstituted α-amino acids.[35] The Ugi reaction has been applied to *N*-benzyl-1,1-dicyclopropylmethanimine in the key step towards the preparation of α,α-dicyclopropylglycine and its peptide derivatives.[36] A series of racemic α-substituted α-amino acids has been synthesised using as the key step the

alkylation of α-substituted pyrrole based silyl dienol ethers.[37] A stereoselective synthesis of α-alkylated γ,δ-unsaturated amino acids has been reported, based upon the ester enolate Claisen rearrangement of chelated *N*-protected amino acid allylic esters.[38] A new enzyme isolated from crude *Humicola langinosa* lipase has been used to resolve a series of α,α-disubstituted amino esters. The amino esters were prepared chemically and included derivatives with aliphatic, aromatic and cyclic substituents.[39]

The conformational properties of α,α-dialkylated amino acid residues possessing acyclic and cyclic side chains have been compared in solution. The peptides used were studied by NMR and CD spectroscopy.[40] The Aib-L-Pro (Aib = α-aminoisobutyric acid) sequence, often found in channel forming antibiotics, has been mimicked by replacing Aib with isovaline (α-ethyl-α-methylglycine, Iva). The replacement of the methyl group of Aib with an ethyl group did not appear to change significantly the β-turn conformation of the dipeptide sequence in the solid state.[41] The (αEt)Phe residue, has been incorporated into a number of oligopeptides (up to pentapeptides), and has been found to be a strong β-turn and helix promoter. The residue was found to follow the trends previously observed for (αMe)Phe, whereby L-amino acids yield left-handed helices.[42] *Para*-BrC_6H_4-CO-[D-(αMe)Phe]$_{4,5}$-$OCMe_3$, and *para*-BrC_6H_4-CO-[D-Iva]5-$OCMe_3$ have been examined by vibrational CD. The (αMe)Phe peptides folded in a right-handed 3_{10}-helix, whilst the D-Iva peptide folded in a left-handed helical sense. The helical senses were consistent with those of α-methylated amino acids and D-amino acids, previously reported.[43]

The X-ray crystal structure of *N*-benzyloxycarbonyl-α-aminoisobutyryl-α-aminoisobutyryl-(*S*)-phenylalanyl-α-aminoisobutyric acid [*N*-Z-Aib-Aib-(*S*)-Phe-Aib], has been resolved. The tetrapeptide was found to fold in an incipient, left-handed 3_{10}-helical structure.[44] An N^{α}-blocked, Aib-rich octapeptide methylamide containing two N^{α}-benzoylated L-Lys residues at positions 3 and 6 has been synthesised by solution state peptide synthesis methodology. The solution and crystal-state conformations were determined by FT-IR, ^{1}H NMR, CD and X-ray diffraction techniques. The results indicated that the peptide was folded into a regular right-handed 3_{10}-helix stabilised by seven consecutive N-H···O=C intramolecular H-bonds of the β-turn III type. The two benzamidobutyl L-Lys side chains were located on the same side of the helix after one complete turn.[45] The crystal structure of *para*-BrBz[D-(αMe)Leu]$_3$-O^{t}Bu, has been examined by X-ray diffraction, and revealed to possess a fully extended (2_5-helix) conformation. This conformation has never been previously assigned unambiguously to a chiral α-methylated amino acid.[46] The solid state conformations of three peptides containing α,α-dipropylglycine (Dpg) and α,α-dibutylglycine (Dbg) residues have been reported. Boc-Ala-Dpg-Ala-OMe and Boc-Ala-Dbg-Ala-OMe formed distorted, type II β-turns, whilst Boc-Ala-Dpg-Ala-NHMe crystallised with two independent molecules, displaying a mixture of β-turns and 3_{10}-helices.[47] The crystal structure of Z-L-Ala-D-(αMe)Trp-NH_2 has been determined. The diastereomeric peptide Z-L-Ala-D-(αMe)Trp-NH_2 has also been prepared and an X-ray structure of the racemate obtained. The authors discussed the use of the (αMe)Trp residue in designing conformationally restricted bioactive peptides.[48]

Two sterically constrained peptides (^{i}Boc-Aib-Aib-Aib-DkNap-Leu-Qx-Ala-Aib-Aib-HNCH$_2$CH$_2$OMe and ^{i}Boc-Aib-Aib-Aib-DkNap-Leu-Aib-Ala-Aib-Aib-HNCH$_2$CH$_2$OMe) containing Aib or related α,α-disubstituted amino acids (DkNap, Qx) in conjunction with selected mono-α-alkyl amino acids and ^{i}Boc and HNCH$_2$CH$_2$OMe as solubilising groups were synthesised using TBTU/HOBt. The peptides possessed a predominantly 3_{10}-helical backbone conformation.[49] An Aib analogue **13** of gramicidin-B **14,** has been prepared and investigated by CD and patch-clamp experiments. The analogue showed a diversity of conducting behaviours and rectifying properties.[50]

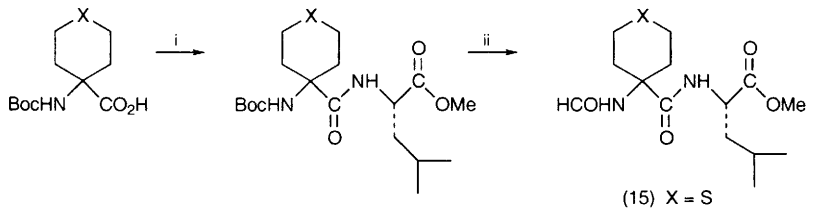
Formyl-L-Val-Gly-L-Ala-Aib-L-Ala-Aib-L-Val-Aib-L-Trp-Aib-L-Phe-Aib-L-Trp-Aib-L-Trp-Glyol

(13)

Formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Phe-D-Leu-L-Trp-D-Leu-L-Trp-Glyol

(14)

Conformational studies (^{1}H NMR and IR) have been carried out on the peptides HCO-Thp-Leu-OMe **15** (where Thp = 4-aminotetrahydrothiopyran-4-carboxylic acid) and HCO-Ac$_6$c-Leu-OMe **16** (Ac$_6$c = aminocyclohexane carboxylic acid) which were synthesised from the constituent amino acids as shown in **Scheme 7**.[51] Especially notable in this synthesis is the one step conversion of a Boc-protecting group into an *N*-formyl group by treatment with formic acid and EEDQ. The two peptides were found to have similar conformational behaviours in solution, i.e. the sulfur atom in peptide **15** does not perturb the conformation. Compound **15** is a conformationally constrained analogue of methionine.



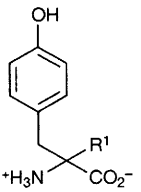
(15) X = S
(16) X = CH_2

Reagents: i Bu^{i}OCOCl, H-Leu-OMe•HCl, *N*-methylmorpholine
ii HCO_2H, EEDQ

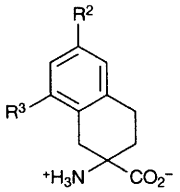
Scheme 7

Conformationally constrained α,α-disubstituted tyrosine analogues **17** and **18** have been synthesised in good yields by a route involving the resolution of racemic precursors by peptide formation.[52] The absolute configuration of compounds **17** and **18** was determined by X-ray analysis of the dipeptides used to resolve them. A procedure for the optical resolution of α-alkoxyglycines, using chiral amines or menthyl esters, has been outlined.[53] A series of α-amino acids in which the α-proton has been replaced by an oxirane ring has been prepared. The compounds were prepared from the corresponding α-vinyl amino acid, either by direct

epoxidation or by hydroxylation, followed by selective primary mesylation and then cyclisation.[54]



(17) R^1 = Me
R^1 = Pr^i
R^1 = Ph



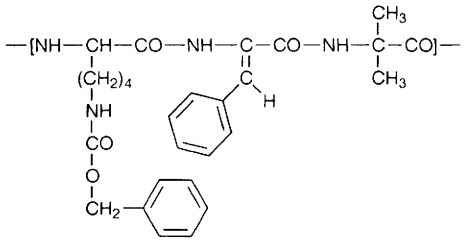
(18) R^2 = OH, R^3 = H
R^2 = H, R^3 = OH

Dipeptide mimetics D-phenylglycine-L-α-methyldopa, D-hydroxyphenylglycine-L-α-methyldopa and L-α-methyldopa-D-phenylglycine have been prepared in an effort to improve intestinal absorption of dopa, and found to exhibit good characteristics as α-methyldopa prodrugs.[55,56] Similarly, the tripeptide mimetic, formed from D-*para*-hydroxyphenylglycine-L-proline and L-dopa, has been prepared and showed an anti-Parkinson effect in biological tests.[57] The dopa containing dipeptides Asp-dopa-OMe, Asp-dopa-OBu and Asp-dopamine have also been synthesised.[58]

2.8 Dehydroamino Acid Analogues – The treatment of serine and threonine derivatives, with dichloroacetyl chloride and a tertiary amine base has been used for the preparation of dehydro amino acids in good yields (58-89%).[59] Treatment of *N,N*-dimethyl-*N'*-heteroarylformamidines with hippuric acid in acetic acid has been used to prepare the corresponding 2-phenyl-4-heteroaryl aminomethylene-5(4H)-oxazolones. The oxazolones, thus prepared, were reacted with amino acids to give a series of dehydropeptide derivatives as products.[60]

The polypeptide poly[Lys(Z)-Δ^zPhe-Aib] **19**, has been synthesised and its conformation studied by circular dichroism in a range of solvents.[61] The pentapeptide Boc-Leu-Phe-Ala-ΔPhe-Leu-OMe has also been synthesised to determine whether the α,β-dehydrophenylalanine (ΔPhe) residue would restrict the peptide backbone to a β-bend conformation. It was found, however, that all the peptide links were *trans* and that the peptide adopted a 3_{10}-helical conformation.[62] The octapeptide Ac-ΔPhe-Val-ΔPhe-Ala-Val-ΔPhe-Gly-OCH_3 (which contains three α,β-dehydrophenylalanine residues) has been synthesised and its conformation determined both in solution and in the solid state by X-ray crystallography.[63] The solid state structure was found to possess an *N*-terminal 3_{10}^R-helix, a central non helical segment and an incipient 3_{10}^L-helix at the *C*-terminal end, and a similar structure was suggested to be formed in solution. The solution and solid state conformation of *N*-Ac-ΔPhe-L-Val-OCH_3 has been investigated. The conformation was found to be invariant for a number of solvents, and corresponded to that obtained for the solid state.[64] The solid state structure of OHC-Met-Leu-Δ^zPhe-Phe-OMe has been determined and compared

with the results of molecular dynamics simulations. The X-ray structure revealed an internal β-turn conformation of the molecule.[65] The crystal structure of (Z)-*N*-phthaloyl-2,3-dehydrophenylalanine methyl ester has been resolved and discussed.[66]



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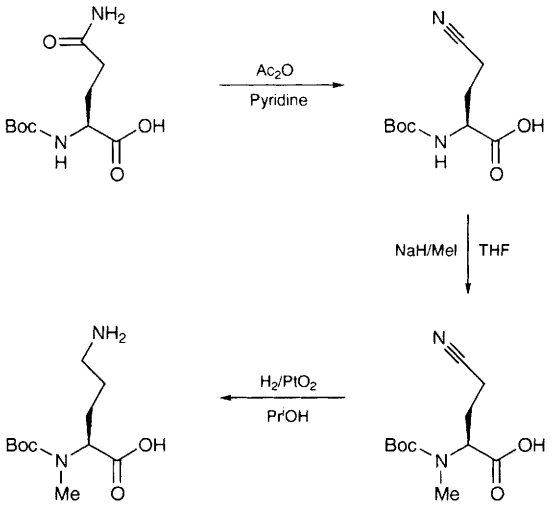
2.9 Miscellaneous – The structure of the hexapeptide Boc-(D-aIle-L-Ile)$_3$-OMe (aIle = alloisoleucine) in which the two amino acids differ in configuration at the α-centre but not at the β-carbon has been determined by X-ray diffraction. The peptide shows a bent U-shape conformation stabilised by three intramolecular hydrogen bonds.[67] X-ray diffraction analyses of three *N*- and *C*-terminally blocked L,D dipeptides namely t-Boc-D-Leu-L-Leu-OMe, t-Boc-L-Ile-D-aIle-OMe and t-Boc-D-aIle-L-Ile-OMe, possessing enantiomeric or diastereomeric amino acid residues have also been performed. The results indicated that the change in chirality in the main chain atoms perturbs the backbone conformation to only a small extent, but has a major influence on the sidechain conformation.[68] In contrast, it was found by Bobde et al., that the diastereomeric tetrapeptides Boc-L-Glu-Ala-Leu-LysNHMe and Boc-D-Glu-Ala-Leu-LysNHMe possessed different conformations in solution, based on the results of NMR spectroscopic studies.[69] Thus, whilst the peptide Boc-L-Glu-Ala-Leu-LysNHMe adopted a random coil conformation, Boc-D-Glu-Ala-Leu-LysNHMe exhibited an ordered 3_{10} type distorted protohelix due to an intramolecular salt bridge under the NMR conditions (DMSO-chloroform mixtures).

The crystal structure of the peptide Boc-Phe-Val-OMe has been solved. It was noted that there were three crystallographically independent peptide molecules in the asymmetric unit and all exhibited an extended conformation.[70] The crystal structure of the peptide Boc-Phe-Met-OMe has also been determined by X-ray diffraction. The peptide backbone was again found to possess an extended conformation with the side chain Phe and Met residues arranged below and above the backbone chain.[71]

An approach to *N*-methyl-arginine and *N*-methyl-ornithine derivatives has been reported, whereby Boc-L-Gln was dehydrated to form the γ-nitrile, followed by *N*-methylation of the α-amine and then reduction of the nitrile to an amine as shown in **Scheme 8**.[72] The same chemistry can also be used with a Z-protecting group on the glutamine.[73]

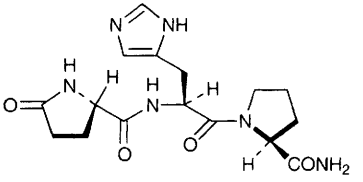
The peptide backbone of thyrotropin-releasing hormone **20**, has been replaced



Scheme 8

by a cyclohexane backbone. A series of mimics thus formed, proved to be active compounds, exhibiting oral activity.[74] The amide bond of a series of dipeptides has been replaced by 2,5,5-trisubstituted imidazoline units. Amide bonds within the CCK-4 analogue Trp-Nle-Asp-Phe-NH_2, and the pentagastrin analogue Gly-Trp-Nle-Asp-Phe-NH_2, were similarly substituted with imidazoline units.[75]

Two procedures for the introduction of stable isotopes in to peptide backbones have been investigated. A number of backbone labelled Boc-derivatives of Ala, Phe and Tyr were prepared.[76] L-*Threo* and L-*erythro*-[1-^{13}C, 2,3-$^{2}H_2$] amino acids **21** and **22**, have been synthesised as probes for NMR studies. The synthesis of the labelled amino acids, involved catalytic (Pd or Rh) deuteration of dehydroamino acids and enzymatic resolution of products.[77]



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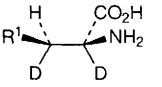

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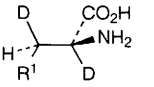

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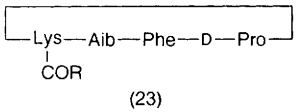
3 Conformationally Restricted Cyclic and Bridged Analogues

A review of biologically active cyclopeptides from marine sources has been produced by Wipf.[78] A review of templates which induce α-helical, β-sheet or loop conformations has also been written by Schneider and Kelly.[79]

3.1 Rings and Bridges formed *via* Amide Bonds – A method for the cyclisation of unprotected peptides in aqueous solution has been reported, based upon intramolecular oxime formation.[80]

The smallest cyclic peptides are diketopiperazines, and the use of solid state ^1H NMR and 2D solid state NMR techniques to study the conformation of *cyclo*-[(*S*)-His-(*S*)-Phe] has been reported. The solid state conformation of this peptide which is a catalyst for the asymmetric addition of HCN to aldehydes was found to resemble the previously determined solution conformation.[81]

A large scale synthesis of *cyclo*-(D-Pro-L-Hyp-D-Pro-L-Hip) has been reported. The structural assignment and solution conformation, were also reported.[82] Cyclo-tetrapeptide analogues of chlamydocin and HC toxin (**23** and **24**) have been synthesised. The peptides were investigated for cytotoxicity, with positive initial results.[83] The conformations of the phytotoxic cyclic tetrapeptide tentoxin [*cyclo*-(L-*N*-MeAla-L-Leu-*N*-MePhe[Δ(Z)]-Gly)] **25** have been studied in aqueous solution by two-dimensional proton NMR at different temperatures. Tentoxin was found to exist in multiple exchanging conformations in water, all of which possessed a *cis-trans-cis-trans* arrangement of the amide bonds.[84] An improved method for the preparation of tentoxin has also been reported. An azalactone is used as a key intermediate, and the methodology allows for the easier preparation of analogues of this cyclic tetrapeptide.[85] The cyclic peptides **26** and **27** have been synthesised, and found to be active as chemoattractants, secretagogues and superoxide anion generating agents. The structure-activity relationship of the peptides was also discussed.[86]



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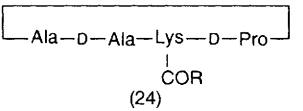

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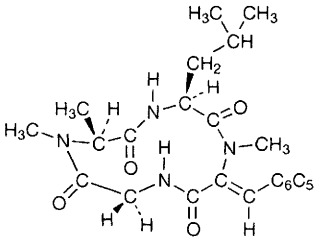

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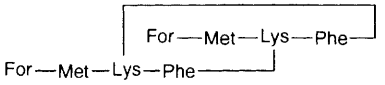

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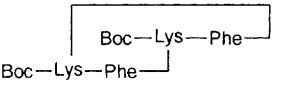

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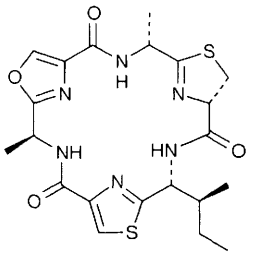
The synthesis of a mixture of cyclic peptides *cyclo*-(Arg-Gly-Asp-Xxx-Aca), (Aca = ε-aminocaproic acid), has been reported using solid phase synthesis

followed by cyclisation with oxime resin.[87] Pentapeptide active esters H-L(or D)-Phe-L-Pro-L(or D)-Val-Orn-L-Leu-ONSu related to gramicidin-S have been prepared and cyclised. CD and NMR studies of the corresponding ethyl esters were also carried out.[88] An evaluation of the effect of ring size upon the secondary structure and antibiotic activity of gramicidin-S has also been effected by the preparation of analogues consisting of 6, 7, 8, 9, 11, 12, 13 and 14 amino acid residues. Generally, the smaller ring sizes showed weak activity, whereas for the larger ring sizes the activity was comparable to or stronger than that of gramicidin-S.[89]

Three new cyclic peptides, segatalins-B, C and D have been isolated and their conformational structure investigated by NMR and computational methods. The estrogen-like activity of segasalin-B *cyclo*-(Gly-Val-Ala-Trp-Ala) was compared with that of segasalin-A *cyclo*-(Gly-Val-Pro-Val-Trp-Ala). The Trp-Ala-Gly-Val sequence and its conformation was thought to play an important role in the activity of the peptides.[90] Cyclic analogues of immunostimulating peptide tuftsin Thr-Lys-Pro-Arg, have been synthesised. The analogues *cyclo*-(Thr-Lys-Pro-Arg-Gly) and *cyclo*-(Thr-Lys-Pro-Arg-Asp) were found to exhibit enhanced phagocytosis activity. This was thought to be due to conformational effects, and to greater resistance to proteolytic degradation.[91] A systematic study of analogues of vasoactive intestinal peptide led to the design and synthesis of a number of analogues. Amongst these, Ac-His-Ser-Asp-Ala-Val-Phe-Thr-Glu-Asn-Tyr-Thr-Lys-Leu-Arg-Lys-Gln-Nle-Ala-Ala-Lys-Lys^{21}-Tyr-Leu-Asn-Asp^{25}-Leu-Lys-Lys-Gly-Gly-Thr-NH_2 (25-21 lactam) proved to be a potent lead compound with good metabolic stability and duration of action. A conformational analysis of the cyclic hexapeptide *cyclo*-[D-Pro^1-Ala^2-Ser^3(Bzl)-Trp^4-Orn^5(Z)-Tyr^6] with and without protecting groups on Ser^3 and Orn^5 has been conducted by NMR spectroscopy. In the protected peptide with Ser^3(Bzl) a βII-turn was observed between Trp^4 and Orn^5, whilst the deprotected compound showed a βI-turn in this region.[92] A series of 15 cyclic (head to tail) hexapeptides which were analogues of the active sequence (Ser^{17}-Trp^{18}-Arg^{19}-Tyr^{20}) of the α-amylase inhibitor protein tendamistat (HOE 467) has been synthesised.[93] A conformational analysis of each of the peptides was conducted using NMR spectroscopy in DMSO-d_6 and constrained molecular dynamics both in vacuo and in DMSO. Some of the cyclic hexapeptides orientated the Ser-Trp-Arg-Tyr tetrapeptide sequence in the same orientation as found in tendamistat, whilst others exhibited no single preferred conformation.[94]

The total synthesis and absolute stereochemistry of dolastatin-E, a cyclic hexapeptide (**28**), has been described.[95] A conformational study of dolastatin-10, a potent antineoplastic pseudopeptide, has been effected using NMR techniques. The results showed two conformations for dolastatin-10, corresponding to a *cis/trans* isomerisation about the dolaisoleucine-dolaproline peptide bond.[96] The solution conformation of patellamides-B and C, cytotoxic cyclic hexapeptides, have been determined using NMR methods and molecular dynamics simulations. The authors also speculated on the structure-activity relationship of the peptides.[97] The structure of patellamide-F (**29**) has been determined, using a combination of spectroscopic and chemical methods. The cytotoxic peptide was

isolated from the tunicate *Lissoclinum patella*.[98] Echinocandin B is a cyclic hexapeptide, whose *N*-terminus is acylated with linoleic acid. A review covering both the primary and patent literature has been published which describes the development of echinocandin lipopeptide antifungal agents.[99]



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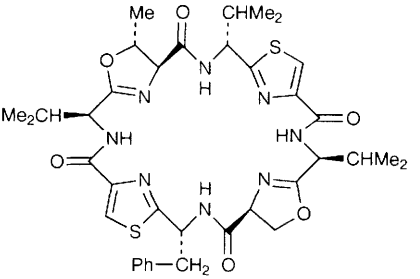

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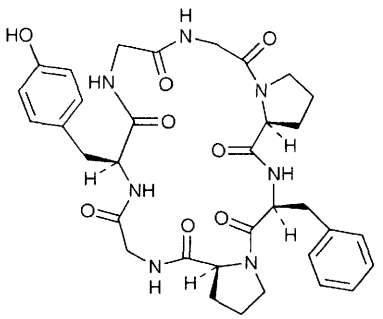
Two novel cyclic peptides Yunnanins-A (**30**) and B (**31**) have been isolated from *Stellaria yunnanensis* (Caryophyllaceae) and their structures elucidated by spectroscopic techniques and chemical degradation.[100] Yannanin-A is a cyclic heptapeptide containing only proteinogenic amino acids, whilst Yannanin-B is a cyclic heptapeptide which contains a δ-hydroxy-isoleucine residue. Solution and solid state structure determination of stylopeptide 1 [*cyclo*-(Pro-Leu-Ile-Phe-Ser-Pro-Ile)] has been achieved using a combination of NMR, X-ray and chiral gas-chromatography analyses.[101] The solid state and solution conformation of the heptapeptide pseudostellarin D, *cyclo*-(Gly-Tyr-Gly-Pro-Leu-Ile-Leu), has been determined. A revised structure showed that the peptide possessed a type II β-turn between Leu^7 and Gly^1 and a type I β-turn between Ile^6-NH and Gly^3-CO. The solution structure, determined by NMR, was found to be homologous with the solid state structure, determined by X-ray analysis.[102]



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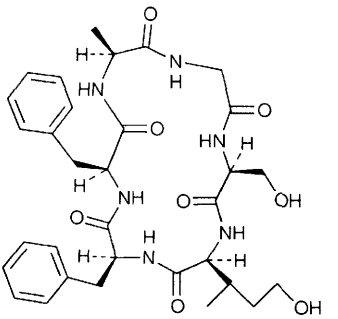
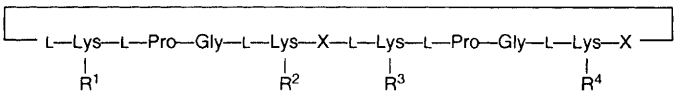


(31)

The preparation, and solid state and solution conformations of *cyclo*-[Pro-Phe-Phe-Ala-Glu-(O^tBu)]$_2$, have been reported. The solution structure of this potent

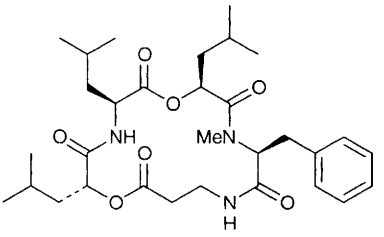
cyclolinopeptide-A analogue was found to be homogenous, symmetrical and similar to the solid state structure.[103] The peptide *cyclo*-[Pro-Pro-Phe-Ac_6c-Ile-DAla-Val][C_8-Ac_6c], where Ac_6c is 1-aminocyclohexane-1-carboxylic acid, has also been prepared as an analogue of cyclolinopeptide-A. The peptide was studied by NMR techniques and found to adopt a rigid structure. The Pro-Pro-Phe segment retained its natural configuration, whilst the bulky Ac_6c residue did not permit the formation of inorganic complexes.[104] [$Aib^{5,6}$-D-Ala^8]-Cyclolinopeptide, *cyclo*-(Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val), has been crystallised and the solid state conformation determined.[105] The cyclic decapeptides **32**, have been prepared by a combination of solid- and solution-phase chemistry. These peptides were synthesised for use as regioselectively addressable functionalised templates (RAFT) for use in template assembled synthetic proteins.[106] The solid state conformational analysis of [Tyr^4]cyclolinopeptide-A has been determined by X-ray diffraction techniques. The crystal structure showed five intramolecular N-H···O=C hydrogen bonds, with the formation of one α-turn, one inverse γ-turn, and two β-turns (one of type III and one of type I).[107]



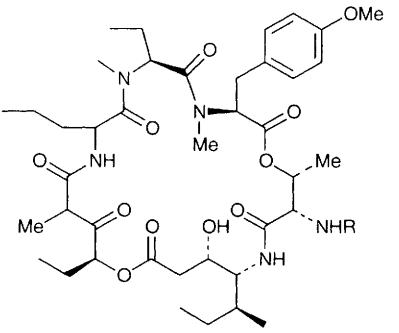
(32)

The mapping of cyclosporin A (a potent immunosuppressant cyclic undecapeptide), binding sites in cyclophilin A (CyP A) has been achieved, using a set of overlapping synthetic octapeptides. Notable binding was observed in N^{α}-acetylated peptides possessing the *N*-terminal amino acids, corresponding to the residues in positions 24-26, 42-44, 69-73, 75, 76, 89-91, 102, 116, 124-131, 144-151 and 152 in human CyP A respectively.[108] A cyclosporin A (CsA) derivative, containing the sterically hindered sequence [-(D)-Ala^8-$MeLeu^9$-$MeLeu^{10}$-$MeVal^{11}$-$MeBmt^1$-], has been synthesised using solid phase techniques. In the same way, [$MeLeu^1$]CsA was prepared and found to inhibit the PPIase activity of cyclophilin with a K_i of 1.8 μM, which when compared with a K_i of 11 nM for [MeLeu(β-hydroxy)^1]CsA, would imply a 180-fold greater binding stabilisation for the latter due to the β-hydroxyl group.[109] (5-Hydroxynorvaline)-2-cyclosporin has been prepared as a CsA derivative with an additional cavity for enhanced binding with cyclophilin A. The binding affinity of the analogue was, however, found to be 8-9-fold lower than for CsA. This was thought to be due to the displacement of a water molecule in the analogue structure, leading to a change in the prebinding equilibrium.[110] Ten analogues of cyclosporin have been described with modifications of the MeBmt (*N*,4-dimethyl-4(*R*)-[2(*E*)-butenyl]-L-threonine) side chain. The best analogues were, however, 2-3-fold less active than cyclosporin.[111] The side chain of cyclosporin-A has been converted to a sulfhydryl group, using Lawesson's reagent. The conformation in solution was found to be analogous to that of cyclosporin-A. The crystal structure of the corresponding acetate was also obtained.[112]

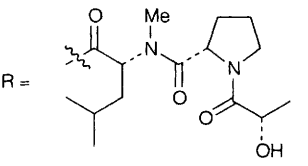
The depsipeptide cereulide, *cyclo*-(D-*O*-Leu-D-Ala-O-Val-Val)$_3$, an emetic toxin, has been conformationally studied as the K^+ complex using NMR methods in chloroform.[113] The solid state structure of the meso-valinomycin analogue *cyclo*-(D-Val-D-Hyi-D-Val-L-Hyi-L-Val-D-Hyi-L-Val-L-Hyi-L-Val-D-Hyi-D-Val-L-Hyi) has been determined by X-ray crystallography direct methods. Two water molecules were found to reside in the cavity of the centrosymmetric depsipeptide ring.[114] An efficient total synthesis of the cytotoxic cyclic depsipeptide arenastatin has been published. Also reported was the structure-activity relationship of the peptide and several of its stereoisomers, in view of its potent cytotoxicity.[115] The total synthesis of the cyclic depsipeptide leualacin **33**, a calcium channel antagonist, has been reported in 15 steps and 25% overall yield from commercially available amino acids using solution phase chemistry.[116] An X-ray analysis on the depsipeptide *N*-{1-{N-{4-{[3-hydroxy-5-methyl-1-oxo-4-(*N*-L-threonylamino)heptyl]-oxy}-2,5-dimethyl-1,3-dioxohexyl}-L-leucyl}-L-prolyl}-*N*,*O*-dimethyl-L-tyrosine hydrobromide hydrate **34** was obtained in order to determine the backbone folding of the macrocycle, and the results compared with those reported for the natural product didemnin B **35**. Two conformations were identified for structure **34**, which possessed regions in the peptide backbone that were more flexible than the corresponding regions in **35**.[117]



(33)



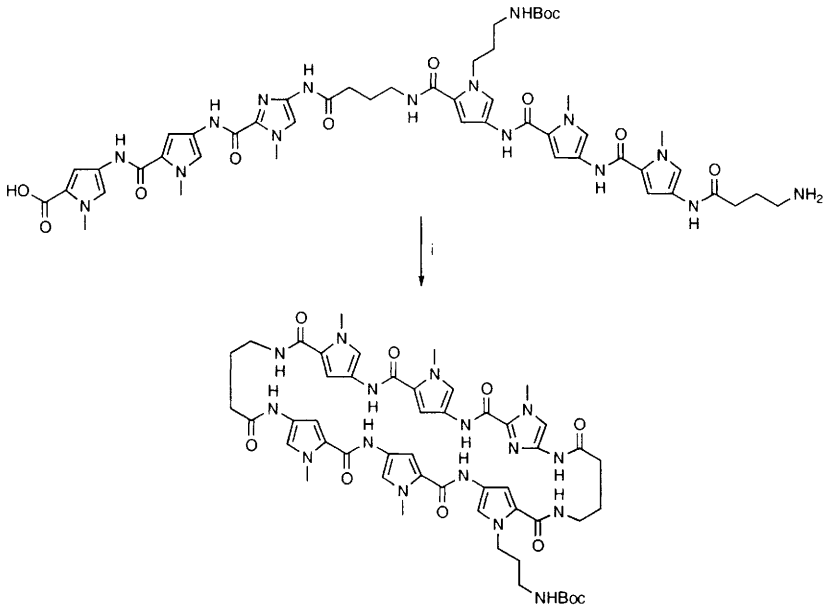
R = H•HBr (34)



(35)

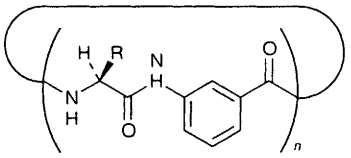
The synthesis of cyclic polyamides containing *N*-methylimidazole and *N*-methylpyrrole amino acids designed to induce specific binding in the minor groove of DNA has been carried out as shown in **Scheme 9**. The key macrocyclisation was achieved using diphenylphosphoryl azide. It has been reported that the crescent-

shaped polyamides bind to DNA as antiparallel dimers with each polyamide making specific contact with the floor of the minor groove.[118] A series of cyclic peptides (**36-39**) incorporating a *meta*-amino-benzoic acid (Aba) residue has been synthesised and were shown to accelerate the hydrolysis of 4-nitrophenyl acetate.[119]



i, Diphenylphosphoryl azide/$NaHCO_3$

Scheme 9



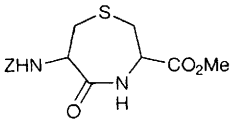
Cyclo(-AA-Aba-)$_n$

AA: Natural Amino Acids, Aba: 3-Aminobenzoic Acid

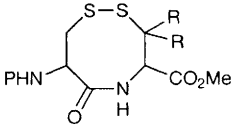
Cyclo(-Ser-Aba-Ser-Aba-Ser-Aba-) (36)
Cyclo(-Ser-Aba-His-Aba-Asp-Aba-) (37)
Cyclo(-Ser-Aba-His-Aba-Asp-Aba-Ala-Aba-) (38)
Cyclo(-Ser-Aba-His-Aba-Ala-Aba-Ala-Aba-) (39)

3.2 Bridges Formed by Disulfide Bonds – The synthesis of all four stereoisomers of the cyclo-lanthionine derivative **40** has been carried out on a Kaiser-oxime resin starting from orthogonally protected lanthionine units. The peptide ring was prepared in 79-85% yield *via* amide bond formation whilst the peptide was still attached to the resin. The conformations of all four stereoisomers were determined by X-ray diffraction techniques and by NMR in DMSO-d_6, and all of the peptides were found to possess a *cis*-amide bond.[120] This result contrasts with the conformations of the corresponding 8-membered ring disulfides **41**, for which

the amide bond geometry depended upon the relative configuration of the two chiral centres.[121] The β-turns of the cyclic peptides Cys-Pro-Xxx-Cys, where Xxx = Gly, Phe or D-Phe have been studied in nonaqueous solvent using IR absorption and vibrational circular dichroism (VCD). The peptides all form 14 membered rings closed by a disulfide linkage and it was shown that the chirality of the residue Xxx in the 3-position dictated whether the peptide formed a type I or type II turn (**Figure 1**).[122]

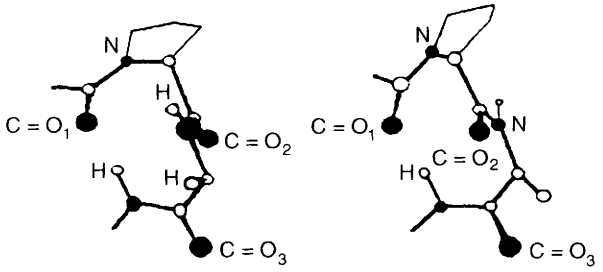


(40)



R = H, Me

(41)

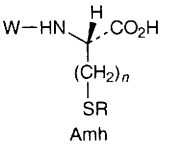


Type I β-turn Type II β-turn

Figure 1

A structural study has been carried out by aqueous NMR on an octadecapeptide sequence containing residues 23-40 of toxin-α of *Naja nigricollis*, cyclised by a disulfide bridge between residues 23-40. The synthetic peptide was found to induce the formation of antibodies which would cross-react with toxin-α. A conformational comparison of the peptide and toxin revealed that residues 23-28 and 37-40 were involved in a β-strand in the toxin but were disordered in the peptide, whilst residues 28-36 were ordered in both cases. However, only residues 30-33 were ordered in the same way (a reverse turn) in the peptide and the toxin, so it is suggested that these three residues may be responsible for the antibody recognition.[123] Conversion of the ε-NH_2 group of lysine to a *para*-methylbenzylthioether **42**, allowed the preparation of peptide **43**, a 22-residue peptide with a 62-atom cyclic disulfide.[124] The synthesis of a 22-residue peptide **44**, with disulfide bridging has also been reported. The synthesis was achieved using solid phase techniques, and the authors favoured the use of the fluorenylmethyl group for cysteine protection rather than ^{t}Bu or benzyl based chemistry.[125]

The cyclic disulfide containing peptide cyclo-Ac[Cys-His-Pro-Gln-Gly-Pro-Pro-Cys] -NH_2, forms cocrystals with strepdavidin. It has been shown that when juxtaposed, the disulfides of neighbouring peptides undergo disulfide interchange to produce a peptide



(42)

H-Thr-Thr-Amh-Thr-Ala-Ser-Ala-Arg-Gly-Asp-Leu-Ala-His-Leu-Thr-Thr-Thr-His-Amh-His-Leu-NH_2

(43)

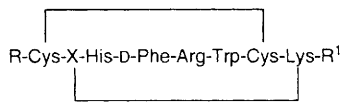
H-Thr-Thr-Cys-Thr-Ala-Ser-Ala-Arg-Gly-Asp-Leu-Ala-His-Leu-Thr-Thr-Thr-His-Ala-Cys-His-Leu-NH_2

(44)

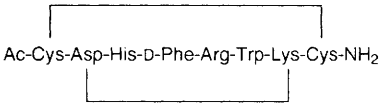
dimer adopting the symmetry of the crystal.[126] The synthesis of iberiotoxin (IbTX), a 37-amino acid containing peptide with three disulfide bridges as well as a series of mono-looped analogues has been achieved using Fmoc chemistry. The analogues were found to be biologically inactive indicating that the three disulfide bridges are necessary for channel blocking activity.[127] The γ-carboxyglutamic acid-rich domain of blood coagulation Factor IX is required for the binding of the protein to phospholipid membranes. To probe the three-dimensional structure of this domain, a synthetic peptide corresponding to residues 1-47 of Factor IX was studied by ^{1}H NMR. The results indicate three major structural elements; there is a short amino-terminal tetrapeptide loop (amino acids 6-9), the disulfide-containing hexapeptide loop (amino acids 18-23) and a carboxyl-terminal α-helix (amino acids 37-46).[128]

The bicyclic α-melanotropin analogues **45** and **46**, have been synthesised and their conformations investigated. The peptides were found to be fully agonistic and were 25-400-fold less potent than α-melanotropin in the frog and lizard skin bioassays.[129] Analogues of protegrin, an 18-residue antimicrobial peptide possessing two intramolecular disulfide bonds, have been synthesised. The analogues contained three types of disulfide bridges; (6-8, 13-15), (6-13, 8-15) and (6-15, 8-13) disulfide bonds. Each analogue showed differing antibacterial action as well as significant anti-HIV activity.[130] The crystal structures of Streptavidin-bound linear and two disulfide-bridged cyclic peptide ligands containing the HPQ sequence have been determined. In this investigation the HPQNT portion of the ligand peptide was found to be well defined and it was also discovered that both the cyclic peptides adopt a β-turn involving a hydrogen-bond between the His main chain carbonyl and the main chain amide NH of the *i*+3 residue. In the Streptavidin bound structure an additional hydrogen-bond indicates an α-helix between the main chain His carbonyl and the main chain *C*-terminal Cys amide NH group.[131]

The connectivity of the disulfide bridges of a cysteine-rich repeat of the low density lipoprotein (LDL) receptor ligand-binding domain have been determined.



(45)



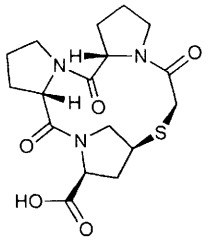
(46)

The LDL receptor is the exemplar of a family of basic related cell surface receptors which contain the RGD sequence, and which intercede the endocytosis of multiple ligands in mammalian cells.[132] The disulfide connectivity places a cluster of negatively charged residues and the RGD sequence within the same loop of the protein. The A chain loop in relaxin has been modified using site-directed sequential disulfide bond formation. The results indicated that structural features of the A chain loop were important for biological activity and that the structural requirements of relaxin and insulin are different.[133] The human glycosylasparaginase (N^4-β-*N*-acetylglucosaminyl)-L-asparaginase (EC 3.5.1.26) possesses five cysteinyl residues (Cys-61, Cys-64, Cys-69, Cys-163 and Cys-179). In order to localise the potential disulfide bonds human glycosylasparaginase was treated with the enzyme α-chymotrypsin and the resulting peptides separated by HPLC prior to and after reduction and *S*-carboxymethylation. The peptide containing the Cys-163 residue (which when replaced in human glycosylasparaginase with serine causes glycoprotein degradation, the Finish-type aspartylglycosaminuria) and the peptide to which it is attached by a disulfide bridge were structurally characterised. It was found that the disulfide bridge responsible for essential biological activity was located close to the carboxyl terminus at positions 163 and 179.[134]

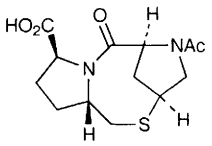
The solution structure and NMR assignments of chlorotoxin (a scorpion toxin) have been determined. The results showed that there were three disulfide bonds cross-linking the α-helix to the β-sheet and a fourth linking the *N*-terminal β strand to the rest of the molecule.[135] The chemical synthesis of ShK-toxin, a 35-residue peptide potassium channel inhibitor containing three disulfide intramolecular bonds, has been reported utilising an Fmoc strategy.[136] The 3D solution structure of ω-conotoxin MVIIA, a 25 amino acid containing peptide antagonist of *N*-type calcium channels was determined by two-dimensional ^{1}H NMR spectroscopy and simulated annealing calculations. The molecular structure consisted of a short triple stranded antiparallel β-sheet, and was similar to that of ω-conotoxin GVIA which has the same disulfide bond combination. Tyr[13] is known to be essential for activity and is in the same conformation in both conotoxins.[137]

3.3 Helices and Helix Inducers – The triproline derivative **47**, has been prepared by Kemp and co-workers as a template for helix nucleation. Both the solid state[138] and solution (in a variety of solvents) conformations of **47** have been determined.[139] Polypeptides containing 9, 12, 16, 19, 23, 26, 30, 33 and 35 amino acids have been synthesised by solid-phase methods and were used to investigate the effects of chain length on the formation and stability of synthetic α-helical coiled coils. The helicity of the peptides increases as the length of the

peptide increases (monitored by CD), and a minimum of six helical turns are needed to form a 2-stranded α-helical coiled coil conformation.[140] NMR, CD and molecular mechanics have been used to determine the conformational properties of **48**, an *N*-terminal helix-inducer template also prepared by Kemp *et al.* The *trans* to *cis* ratio was shown to be a reliable monitor of stability of peptide structure, induced by **48**.[141]



(47)



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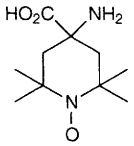


(49)

The pentapeptide Boc-Val-ΔPhe-Ala-Leu-Gly-OMe has been shown by X-ray crystallography to adopt an α-helical conformation in the solid state.[142] Conformational studies have been carried out on the decapeptides Boc-Gly-Dpg-Xxx-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (Xxx = Leu, Pro, or Ala; Dpg = α,α-di-*n*-propylglycine; Aib = α-aminoisobutyric acid) each of which possesses a known helical heptapeptide unit attached to a helix destabilising tripeptide segment. X-ray crystallography indicated that all of the peptides possessed a helical conformation between residues 2 and 9.[143] A study of alanine-based peptides has been effected using electron spin resonance. The investigation focussed on differentiating between the α- and the 3_{10}-conformations of the peptides. The investigation, which used Toac (4-amino-4-carboxy-2,2,6,6,-tetramethylpiperidine-1-oxyl **49**) as a conformationally constrained spin-label, implied that the peptides existed predominantly in the α-conformation, and not in the 3_{10}-conformation, as had been suggested in previous studies.[144] The solid state structure of Boc-Tyr-Pro has been investigated. The compound exhibited an extended *trans*-conformation and was classified as an α-helix type structure.[145]

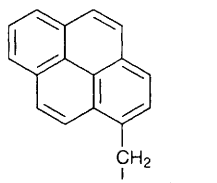
The peptide **50**, has been synthesised by solid phase techniques and was designed to fold into a 'hairpin' helix-loop-helix motif. Peptide **50** was designed to be a basic building block which could be used to construct catalytically active peptides, and two polypeptides derived from **50** have indeed shown catalytic activity.[146] Structural analysis has been performed on the peptide Ac-YMSE-DELKAAEAAFKRHGVP-amide, which contains an *N*-terminal capping structure and a five residue central helix stabilising linker.[147] The conformation of a 15-residue peptide, incorporating numerous α-helix stabilising features such as ion pairs, helix dipole capping, peptide bond capping, and aromatic interactions, has also been studied using CD spectroscopy in three solvents, and by molecular simulation techniques. As expected, both CD and modelling studies showed a decrease in helicity at higher temperatures.[148] A helical hairpin peptide, containing the α-helix/turn/α-helix motif, has been previously reported by Fezoui

and co-workers. In a recent report, the authors fully explain the strategies and rationale in the design of the peptide, and include recent experimental results.[149]

K-A-Aib-Nle-H-E-A-L-A-K-I-A-A-E-Nle-D-A-Aib-N
|
G-P-V-D-Aib-A-Q-Nle-A-E-Q-L-A-K-A-F-E-A-F-A-R-Aib-G

(50)

The L-pyren-1-ylalanine (Pya) residue **51**, can be used as a fluorescence probe and has been incorporated into two 14-residue peptides **52** and **53**, designed to form α-helices.[150] The same research group also inserted the Pya moiety into two 53-residue peptides **54** and **55**, designed to adopt a four α-helix bundle structure. The conformation of these polypeptides was investigated by CD in the pyrene absorption region and the results indicate that the Pya residues are in close proximity confirming the formation of a four α-helix bundle.[151] An antiparallel four α-helix bundle structure incorporating a pair of Pya residues on a cyclic pseudo-peptide template has also been constructed. The final structure was characterised by pyrene excimer emission.[152] L-1-Pyrenylalanine has also been introduced into each segment of a peptide containing two amphiphilic β-strands. CD and fluorescence spectra were used to examine the super-secondary structure of the peptides. The β-strands transformed into α-helical conformations upon the addition of trifluoroethanol.[153]



(51)

H-AlaLeuAlaLysAlaPyaAlaAlaLeuAlaLysAlaLeuAla-NHCH$_3$
(52)
Ac-AlaLeuAlaLysAlaLeuAlaAlaPyaAlaLysAlaLeuAla-OH
(53)

DA P E L L K A X_1 A E L L K-
DA P E L L K A X_2 A E L L K-
DA P E L L K A X_3 A E L L K-
DA P E L L K A X_4 A E L L K G

X_1	X_2	X_3	X_4	
Pya	Phe	Pya	Phe	(54)
Phe	Pya	Pya	Phe	(55)

Four repeating units of a 20-residue peptide with different end and protecting groups (**56**, **57**) were assembled together by S-alkylation to form a four-helix

S—Ad SH
CH$_3$CO—CGGELEELLKKLKELLKGGCY—NH$_2$

(56)

S—Acm
Br—CH$_2$CO—GGELEELLKKLKELLKGGCY—NH$_2$

(57)

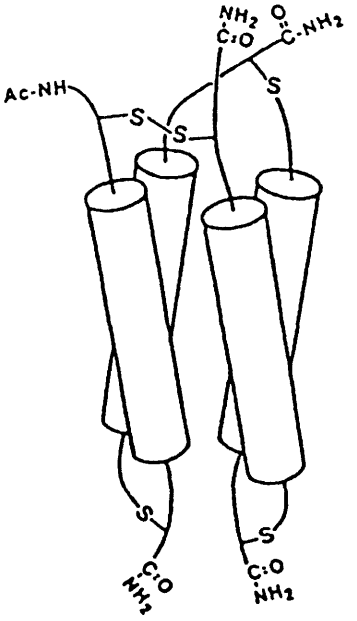
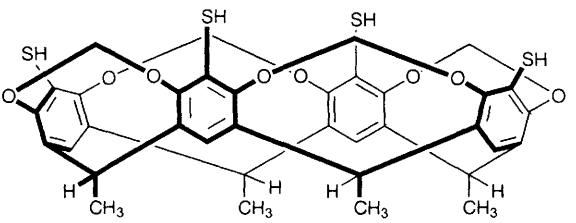


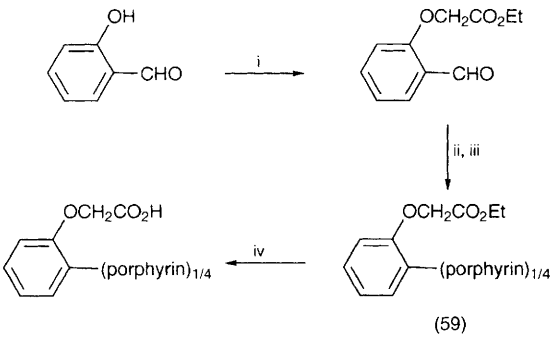
Figure 2

bundle, as shown in **Figure 2**, which was found to be resistant against chatropic and thermal denaturation.[154] A four helix bundle has also been prepared by synthesising the peptide derivative $ClCH_2CO$-(EELLKKLEELLKKG)-NH_2, using Fmoc chemistry, and binding this via the chloroacetate groups to the tetrathiol cavitand **58**.[155] A polypeptide-porphyrin hybrid template {5, 10, 15, 20-



(58)

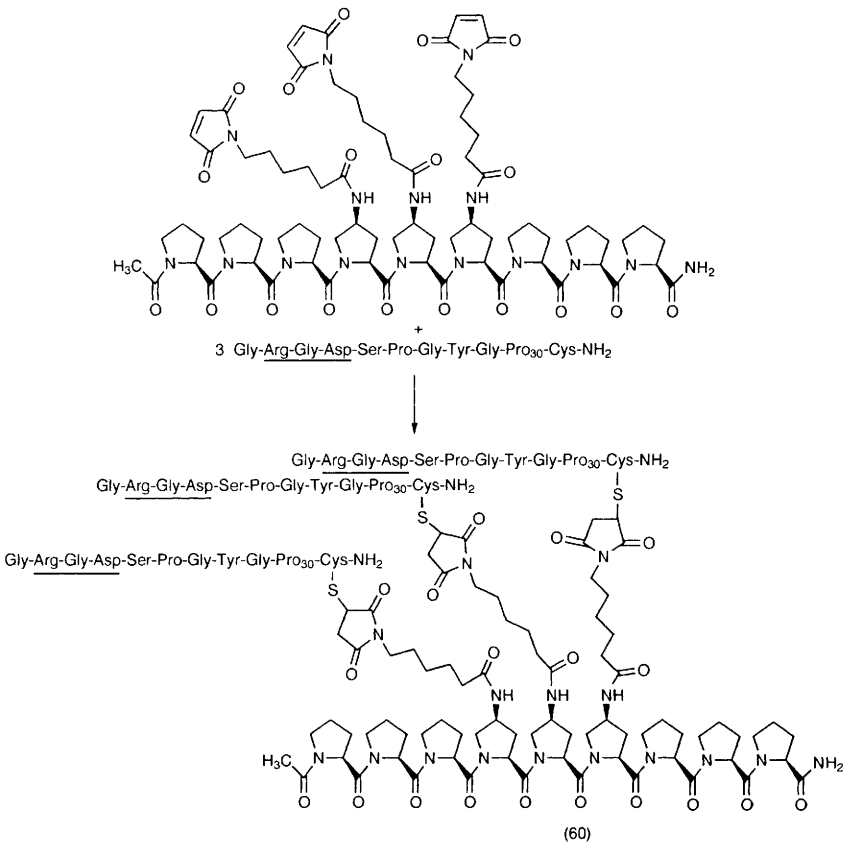
tetrakis[2-(ethoxycarbonylmethoxy)-phenyl]porphyrin} **59** has been synthesised as shown in **Scheme 10**. The ester functionalities were then hydrolysed and four α-helical 21-residue peptides, H-(Gln-Leu-Leu-Gln-Ala-Leu-Ala)$_3$-$NHCH_2CH_2OH$ were attached. The polypeptide-porphyrin hybrid produced was incorporated quantitatively into the egg yolk lecithin membrane.[156] A tripod receptor-adhesive modular protein (RAMP) containing 129 residues **60** has been synthesised by McCafferty *et al.*, utilising the Michael addition of cysteine thiol units onto



(59)

Reagents: i, ethyl bromoacetate, K_2CO_3; ii, pyrrole, $BF_3{\cdot}OEt_2$; iii, chloranil; iv, NaOH aq.

Scheme 10



(60)

Scheme 11

maleimide units to attach the peptide arms onto a modified proline core (**Scheme 11**). Each of the three arms of the RAMP contains an RGD sequence, and the presence of the large number of proline based residues ensured that the tripod arms adopted a type II helix.[157]

A series of amphiphilic peptide analogues [H-(Leu-Aib-Lys-Aib-Aib-Lys-Aib)$_{1,3}$-X, where X = OMe, Ala-N($C_{18}H_{37}$)$_2$], with α-helical structures and terminal long alkyl chains has been prepared as models for biologically active peptides, and their conformational properties have been studied by CD in water and methanol.[158] It has been shown that hydrophobic interactions stabilise helical structures in basic peptides, so a series of small peptides incorporating two ε-(3,5-dinitrobenzyl)Lys residues at numerous positions have been synthesised. The results indicated that in aqueous/organic mixtures, methanol induced helical stability over a wider range and at higher concentrations than trifluoroethanol. It was also noted that in mixtures of trifluoroethanol and water the peptide helical stability was greatest when the modified residues were separated by two or three other resides.[159] The role of amphipathicity on the folding and self-association of melittin, a 26-residue peptide (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2), which is known to adopt an α-helical configuration has been investigated by single residue substitutions of lysine 7. The results indicated that loss of biological activity of melittin analogues arises due to an initial inability to adopt an amphipathic α-helix and to self-associate.[160] CD and NMR techniques have been used to determine the solution conformation and interactions of all-D, retro-all-D and retro-bombolitin III. The results collaborated previous findings, that the ability to form amphiphilic helixes is crucial for biological activity of bombolitins.[161]

The peptides representing the *N*-terminal domain of the cardiac sarcoplasmic reticulum protein phospholamban (residues 1-25[PLB(1-25)]) and a phosphorylated form of this peptide [pPLB(1-25)] have been synthesised and their conformations determined by circular dichroism and nuclear magnetic resonance spectroscopy. CD studies showed both peptides to be disordered in water but in 30% TFE, PLB(1-25) was 60% helical, whilst pPLB(1-25) was only 27% helical. The NMR studies supported this difference, suggesting an α-helix between residues 1-16 in PLB(1-25), but only between residues 1-12 in pPLB(1-25).[162] Model peptides based upon the nucleolin TPAKK motif have been prepared. The conformational properties of these peptides were investigated by CD and NMR spectroscopy. The authors concluded that the Thr-Pro sequence initiates short helical segments which then interact with nucleic acids, probably via the lysine and threonine residues.[163]

It has been discovered from a combination of hydrogen/deuterium exchange and attenuated total reflectance Fourier transform infrared spectroscopy that nicotinic acetylcholine receptor (nAChR) possesses an exchange-resistant core of α-helical peptide hydrogens.[164] The ligand binding domain of the human retinoic acid receptor-γ (RARγ) has been found to be primarily α-helical with a Trp residue in the ligand binding site.[165] A ^{1}H NMR (D_2O-H_2O mix) and CD study on the *C*-terminal fragment (residues 385-411) of human fibrinogen γ-chain i.e. KIIPFNRLTIGEGQQHHLGGAKQAGDV has been carried out. It was found

that by gradually decreasing the pH using trifluoroethanol, the helical content of the peptide was increased.[166] A tricosapeptide representing the *N*-terminal extension of yeast cytoplasmic aspartyl-tRNA synthetase has been synthesised. It was found that under acidic conditions in trifluoroethanol, the conformation of the peptide was predominantly α-helical as determined by circular dichroism.[167]

The structure of human parathyroid hormone residues 1-37 has been studied in a physiological solution of H_2O/buffer (pH 6.0 and 270 mM salt) by CD, ultracentrifugation, NMR and molecular dynamics calculations. The hormone was found to possess a helical structure and showed hydrophobic interactions defining its tertiary structure.[168] A synthetic analogue of alamethicin (a 20-residue peptaibol) called L2, in which all the Aib residues were replaced by Leu, was found to possess similar biological behaviour to the parent peptide. Conformational studies were performed on peptide L2 using FTIR, CD and NMR spectroscopy. The results indicated a predominantly helical structure.[169]

The solution conformation of a 17-residue, essential regulatory RNA-binding peptide for viral replication of the Rev protein of human immunodeficiency virus (type 1) has been determined by NMR. The peptide was found to possess an α-helix in both water and 20% trifluoroethanol (TFE) solutions, and that the α-helix spans the RNA binding site. The formation of this α-helical structure contradicts modelling predictions based upon the sequence of the peptide.[170] There has been an in-depth study using circular dichroism and NMR on the 28-residue synthetic peptide SIV-L which corresponds to the *C*-terminal portion of the SIV transmembrane glycoprotein gp41. The peptide tends to adopt an α-helical structure.[171] Amyloid-β peptide is a 42-residue peptide with a hydrophobic *C*-terminus (residues 29-42) which adopts a β-strand, and an *N*-terminus (residues 10-24) which exists as a dynamic equilibrium between an α-helix and a β-strand. However, a single substitution of valine 18 to alanine changes the conformation of the *N*-terminus to mainly α-helical, as determined by Fourier transform infrared spectroscopy and circular dichroism.[172]

The structural unfolding properties of the spherical, highly α-helical colicin E1 channel peptide has been studied using genetically substituted tryptophan residues and fluorescence techniques.[173] The conformations of three peptide fragments of β-lactoglobulin have been examined by CD and NMR studies. The peptides showed little ordered structure in aqueous solution, but showed high helical propensity when 2,2,2,-trifluoroethanol was added.[174] The three-dimensional solution structure of Bombyxin-II has been derived from NMR data and simulated annealing calculations. Bombyxin-II, is a two chain peptide produced by the brain of the silkworm *Bombyx mori*, and has been structurally compared to insulin and relaxin, hormones to which it is related. Its conformation is highly α-helical and resembles relaxin more closely than it does insulin.[175] A combination of NMR data and molecular modelling was used to determine the three-dimensional structure of Scyllatoxin (leiurotoxin I), a venom peptide, the *N*-terminus of which is α-helical. A comparison was also made with apamin and P05 toxins.[176] ESR, FTIR and CD spectroscopy have all been used to examine the secondary structure of a 20-amino acid containing synthetic peptide analogous to the *N*-terminus of the second subunit of hemaglutinin (HA2) of the influenza

virus A/PR8/34. The techniques were also used to see the interactions of the peptide with a phospholipid bilayer.[177] The *N*-terminus of the peptide is α-helical, and penetrates into the membrane at an angle of about 45°.

The solution structure of the 39-residue peptide margatoxin (a scorpion toxin that blocks the voltage-gated potassium-channel $KV_{1.3}$) has been solved by ^{1}H, ^{13}C and ^{15}N triple-resonance NMR spectroscopy. The secondary structure of the peptide consists of a helix between residues 11 and 20, a loop between amino acids 21 and 24 and an antiparallel β-sheet between residues 25 and 38. Compared to related toxins, margatoxin contains two additional amino acids which are incorporated into the β-sheet, lengthening this structural feature by one amino acid which may explain the channel selectivity.[178]

The helix-sheet conformational transition of the 18-residue amphiphilic peptide H_2N-GELELELEQQKLKLKLKG-COOH has been investigated at various pH, peptide concentrations and ionic strengths using CD in aqueous solutions, and the differing conditions under which the peptide undergoes α-helix to β-sheet conformational transitions were determined.[179] The conformational properties of the peptide Boc-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe have been examined by ^{1}H NMR in $CDCl_3$ and DMSO. The results suggested a multiple β-turn conformation, indicating Leu^3 has a preferred extended or semiextended conformation rather than a helical conformation in $CDCl_3$. A structural transition to a frayed right-handed helix is, however, observed in DMSO.[180] The crystal structure of recombinant neutrophil-activating peptide-2 (NAP-2) in which the methionine at position 6 was replaced with leucine has been determined. NAP-2 is found in the α-granules of human platelets. The structure includes an extended amino-terminal loop, three strands of antiparallel β-sheets arranged in a Greek key fold and one α-helix at the carboxyl terminus.[181]

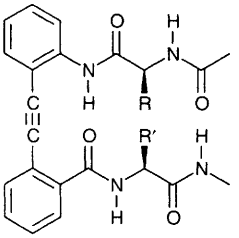
Single point mutations in the *N*-terminal domain (residues 10-24) of the amyloid-β-peptide have been effected in order to affect the equilibrium between α-helix and β-strand conformations. The results led the authors to conclude that promotion of the α-helical conformation may provide control of the amyloid deposition observed in Alzheimer's disease patients.[182]

The 15-residue peptide LAV, part of the CD4 binding domain of gp 120, has been studied using CD and NMR techniques in aqueous and trifluoroethanol solutions. The results showed evidence of formation of a 3_{10}-helix in trifluoroethanol.[183] Two Aib-rich peptides ^{i}Boc-$(Aib)_3$-DkNap-Leu-Aib-Ala-$(Aib)_2$-$NH(CH_2)_2OCH_3$ (Dk^4[7/9]; Dk = diketonaphthyl) and Ac-$(Aib)_2$-β-(1′-naphthyl)Ala-$(Aib)_2$-Phe-$(Aib)_2$-NHMe (Nap^3Phe^6[6/8]), where the brackets indicate the number of Aib-class residues/total number of residues, were studied for helical stability by 1D and 2D NMR spectroscopy over a wide temperature range. The peptide Dk^4[7/9] was studied in $C_2D_2C_{14}$ (up to 120 °C) and the peptide Nap^3Phe^6[6/8] in DMSO (up to 150 °C). It was found that the two peptides retained their 3_{10}-helical conformations over the entire temperature range.[184]

PACAP related peptide, a 29 amino acid region of the PACAP precursor protein, has been synthesised and its structure investigated by CD and NMR techniques. The peptide displayed an α-helical region between residues 1-20 and a

nascent helix after Gly[21]. The biological activity of the peptide was also evaluated.[185] The peptide fragment (1013-1056) of human DNA topoisomerase II-α has been synthesised and forms a stable coiled-coil structure in solution, as determined by experiments utilising cross-linking experiments and CD analysis.[186]

3.4 β-Turn Mimetics and Miscellaneous Bridges – The sheet initiating turn mimetic **61** has been reported by Kemp and Li; the template was studied by ^{1}H NMR and CD techniques but in the absence of X-ray data. The precise sheet conformation remains to be resolved.[187] The conformation of the proline containing analogue **62** was, however, determined to be an antiparallel β-sheet in both DMSO-d_6 and CD_2Cl_2.[188] The achiral dipeptide mimetic trans-5-amino-3,4-dimethylpent-3-enoate, has been designed as an achiral unit that promotes β-hairpin formation. Incorporation of the mimic into dipeptide and tetrapeptide residues followed by conformational analysis using NMR and IR spectroscopy showed that β-turn and β-hairpin folding were, respectively, induced in solution.[189]



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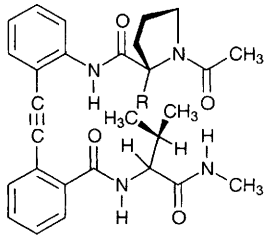
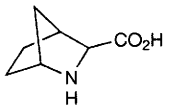


(62)

The dipeptides Ala-Gly, Ala-Ala and Gly-Gly have been cyclised with each of the stereoisomers of 6-amino-3,5-dimethylcaproic acid and 6-amino-3-methylcaproic acid. The resulting compounds were examined by CD, NMR, and in many cases by X-ray crystallography. The results indicated that both type I and type II β-turns were present to varying degrees in the solid and solution states.[190] Peptides containing the terminally blocked (L-Pro-Aib)$_n$ sequence, known to form β-bend ribbon spirals, have been investigated conformationally by electronic and vibrational CD and by IR. The results were also compared with previous related studies.[191] The solid state and solution conformation of an 18-membered ring cyclic pseudopeptide, containing alternating glycine and *N,N'*-ethylene-bridged-alanyl-alanine units has been determined, using X-ray crystallography, NMR and computational methods.[192]

The tripeptide L-leucyl-L-leucyl-L-leucyl has been crystallised and shown to adopt a twisted β-sheet structure rather than a helical conformation. A review of tripeptide conformations, was also included in this paper.[193] The tetrapeptide Boc-D-Val-Ala-Leu-Ala-OMe with a D-residue at the beginning of the sequence has been studied by crystallography. The peptide has an extended β-conformation and the packing is stabilised by four N-H···O hydrogen bonds in an antiparallel

β-sheet arrangement.[194] Both enantiomers of 2-aza-bicyclo[2,2,1]heptane-3-carboxylic acid **63** have been used as conformationally constrained analogues of proline at residue 30 of synthetic peptides based on the B-loop β-sheet sequence of human transforming growth factor-α. The synthetic peptides were found to induce DNA synthesis (EC_{50} 130-330 μM), and the results accentuated the requirement of appropriate conformation of peptides for recognition.[195]



(63)

A series of analogues of β(25-35) amyloid peptide, where each amino acid was replaced by alanine, have been prepared. The structure-neurotoxicity profiles indicated that peptide aggregation from intermolecular β-sheet formation was necessary for the neurotoxicity of the amyloid β(25-30)peptide.[196] The peptide β(34-42) from the *C*-terminus of the β-amyloid protein has been subjected to conformational studies; the results indicated the presence of a pleated antiparallel β-sheet characterised by a specific intermolecular alignment.[197] Replacement of each amino acid of amyloid β(25-35) with Ala has been effected, in order to study the structure-neurotoxicity relationships of the peptide. Replacement of Asn^{27} led to a more hydrophobic but less toxic analogue, which was the opposite effect observed for replacement of Met^{35}. Peptide aggregation via β-sheet formation was also found to be essential for neurotoxicity of amyloid β(25-35).[198] The β-amyloid peptide fragment β(12-28), VHHQKLVFFAEDVGSNK, has been studied by NMR and CD, in water and trifluoroethanol-water solutions. The conformational behaviour of the fragment was found to be consistent with the aggregation behaviour reported for the parent β-amyloid peptide.[199]

The structural characterisations of the neuropeptide tyrosine (NPY) and its agonist analogue [Ahx^{5-17}]NPY have been accomplished by two-dimensional NMR techniques in DMSO and molecular modelling studies. The results indicated that both of the peptides were folded in the centre of their chain and that two β-turns were found in NPY whilst only one was present in [Ahx^{5-17}]NPY.[200]

The 33-amino acid containing β-sheet domain of native platelet factor 4 (PF4), residues 23-55, has been synthesised and studied by CD and NMR techniques. It was found that at low temperatures (10 °C) in aqueous solutions the peptide showed random-coil distribution, but as the temperature or concentration was increased the structural analysis indicated the formation of multiple native-like anti-parallel β-sheet conformations.[201]

The four residue sequence Asn-Pro-Asp-Gly has been used to replace the five residues Thr-Leu-Thr-Gly-Lys of ubiquitin, a 16-residue peptide with a monomeric β-hairpin. The modified peptide formed a β-hairpin with a one frame-shift realignment relative to the original peptide.[202] The folding of protein G B1 domain has been investigated by studying the isolated fragments 1-20 (β-hairpin),

21-40 (α-helix) and 41-56 (β-hairpin) employing NMR and CD techniques. The results were also compared to the structurally related ubiquitin.[203]

The human calcitonin (hCT) analogues, *cyclo*^{17,21}-[Lys^{17},Asp^{21}]hCT, *cyclo*^{17,21}-[Asp^{17},Lys^{21}]hCT and *cyclo*10,14-[Lys10,Asp14]hCT have been synthesised by solid phase peptide synthesis in order to investigate the structural and conformational requirements for hCT activity. The secondary structure of each of the peptides was investigated by CD, and *cyclo*^{17,21}-[Lys^{17},Asp^{21}]hCT was found to give a very similar CD spectrum to that observed for hCT, suggesting the adoption of the same β-sheet/disordered conformation. The CD spectrum of *cyclo*^{17,21}-[Asp^{17},Lys^{21}]hCT, however, suggested a much more ordered (but not helical) conformation, whilst *cyclo*^{10,14}-[Lys^{10},Asp^{14}]hCT was found to be α-helical. Of the three peptides, *cyclo*^{17,21}-[Asp^{17},Lys^{21}]hCT had 80 times the biological activity of the other two analogues and it is suggested that this peptide contains a type I β-turn which is important for activity.[204] Analogues of calcitonin gene related peptide have been prepared by replacements in the α-helical region with a linear ethylene glycol containing amino acid, or with a β-strand forming sequence. In tests, the analogues showed noticeable affinity for the receptor.[205]

A conformational analysis of tetragastrin (Trp-Met-Asp-Phe), the *C*-terminal tetrapeptide of gastrin which possesses the same biological activity as gastrin, has been carried out using Monte-Carlo simulations in DMSO and aqueous solution. In water the peptide was found to adopt an extended conformation (**Figure 3a**), whilst in DMSO a more compact, folded structure was predicted (**Figure 3b**).[206]

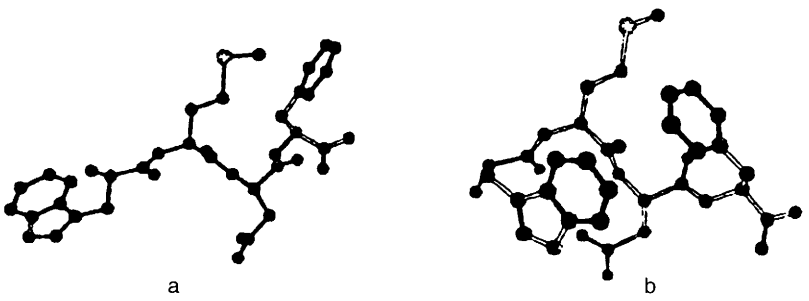
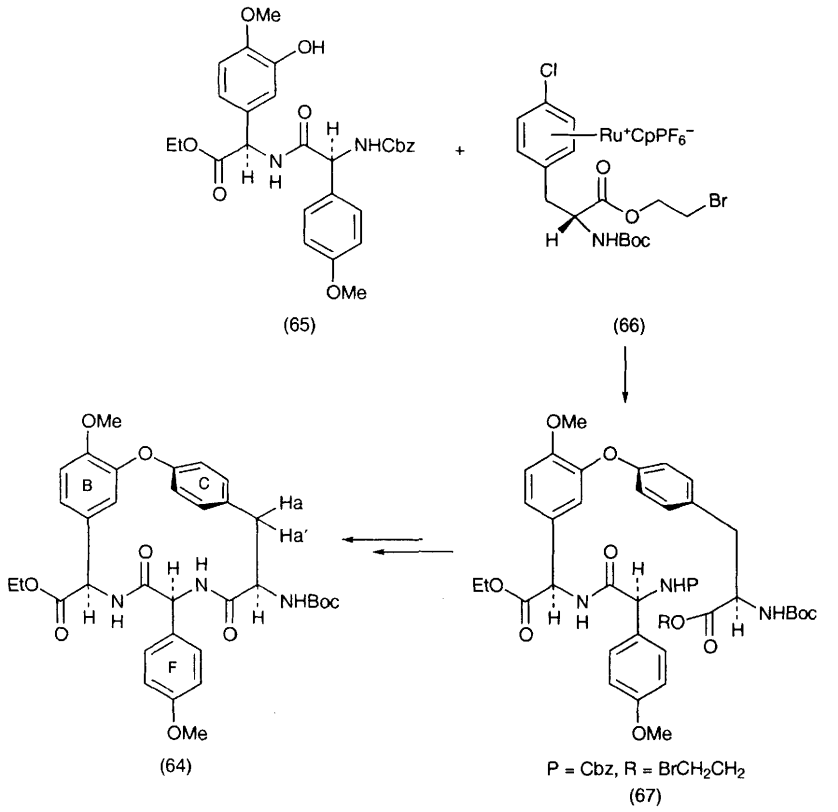


Figure 3

The conformational structure of neuromedin-C has been studied in the absence and presence of lipid membranes. In solution, the peptide conformation is an equilibrium of ordered and disorded states, whereas it is ordered in the presence of a lipid membrane.[207] [D-Ser^{8}]-cyclosporin has been converted into [η-amino-MeBmt]^{1} and [*O*-(carboxymethyl)-D-Ser]^{8}-cyclosporin derivatives, and subsequently cyclised to yield bridged cyclosporin lactam and lactone analogues.[208] A macrocycle analogue of the vancomycin binding pocket has been synthesised, employing a biaryl ether cyclisation of the corresponding linear tetrapeptide. The compound in acetone was found to adopt the correct conformation for carboxylate anion binding.[209]

The cyclic peptide **64**, which is a model for the B/C/F ring system of ristocetin

B has been synthesised by the coupling of the phenolic dipeptide **65** and *para*-chlorophenylalanine-RuCp cationic complex **66** as shown in **Scheme 12**. Subsequent demetalation gave diaryl ether **67**, which could be deprotected and cyclised to give **64** in 14% yield as a mixture of atropisomers.[210]

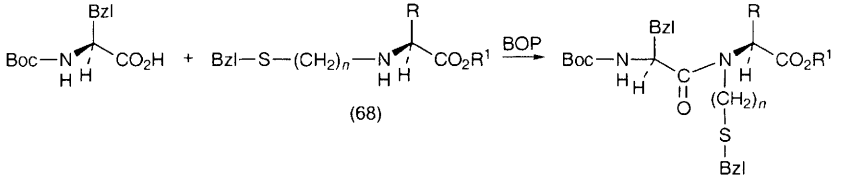


Scheme 12

4 Amino Acids with Modified Side-chains, including Sidechain to Backbone Cyclisations

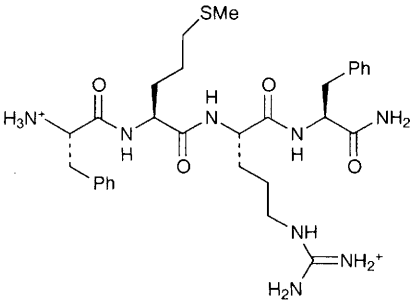
α-Substituted amino acids are included in Section 2.7. Cysteine *S*-sulfonate derivatives Boc-Cys(SO_3Na)-ONa and Fmoc-Cys(SO_3Na)-ONa have been synthesised, and the Fmoc derivative was used to prepare the peptide Arg^8-vasopressin by solid-phase methodology.[211] Amino acids which contain an ω-thioalkylene group attached to the N^{α}-amino nitrogen have been synthesised.[212] The synthesis was achieved by the alkylation of ω-thioalkylamines with triflates of α-hydroxy acids, and the resultant protected N^{α}-(ω-thioalkylidene)amino acids **68** were utilised to form dipeptides (**Scheme 13**) *via* solution phase chemistry using BOP as

the coupling reagent. Dipeptides containing a *C*-terminal homocysteine and an *N*-acylated hydrophobic amino acid have been found to inhibit peptidylglycine α-amidating mono-oxygenase, with IC_{50} values in the nanomolar range.[213]



Scheme 13

The methionine residue within the peptide Phe-Met-Arg-Phe-NH_2 **69** has been replaced with (2*S*,3*S*)-2,3-methanomethionine to give peptide **70** in order to determine whether the incorporation of a conformationally constrained amino acid would have any conformational effects on the peptide as a whole. The results indicated that the (*E*)-cyclo-Met residue could induce the formation of turn or 3_{10}-helical structures.[214]



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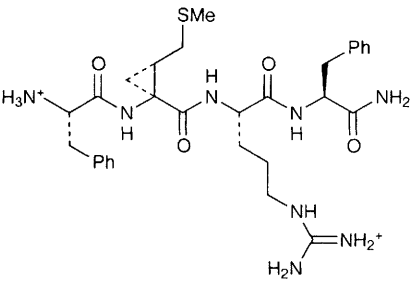
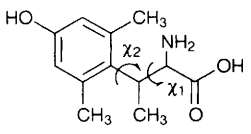


(70)

Phosphorylated pentapeptides derived from Tyr^{751} of the PDGF-β receptor ($pTyr^{751}$-Val-Pro-Met-Leu, pTyr = phosphotyrosine) have been synthesised to examine their ability to inhibit the association of the *C*-terminal SH2 domain of the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) with the PDGF-β receptor. The peptidic analogues were prepared to examine the importance of the amine and carboxy terminus, and of specific amino acids *via* alanine/D-amino acid scans and site specific modifications. It was shown that neutralisation of the amine and carboxy terminus gave analogues with enhanced activity, and only small modifications were allowed for pTyr and Met while other positions were tolerant to modifications.[215] An *O*-phosphorylated tyrosine analogue of a decapeptide has been prepared using Fmoc strategy. The peptide showed a high propensity to adopt an α-helix in various solutions. The phosphorylation of tyrosine was thought to increase this propensity.[216]

A six step synthesis of *N*-Boc-D-diphenylalanine, from L-serine methyl ester,

has been described.[217] The aromatic α-amino acid 2′,6′-dimethyl-β-methyl tyrosine **71** has been prepared as a conformationally restricted tyrosine analogue. The methyl groups hinder rotation around both χ_1 and χ_2. All four stereoisomers of **71** have been synthesised, though incorporation of this amino acid into a peptide had not been reported.[218] Solid-phase synthesis has been used to incorporate two heterocyclic tyrosine analogues **72** and **73** into decapeptides. The peptides were



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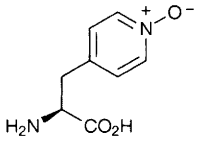

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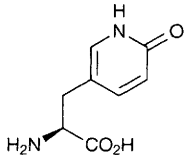
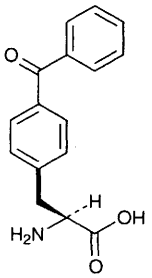


(73)

found to inhibit epidermal growth factor tyrosine kinase.[219] The non-phosphorus containing phosphotyrosyl mimic L-*O*-(2-malonyl)tyrosine, has been incorporated in to various peptides.[220] The preparation of 2- and 6-fluoro analogues of *threo*-3-(3,4-dihydroxyphenyl)serine has been reported. The synthesis involved a $ZnCl_2$-catalysed reaction of a protected glycine trimethylsilylketene acetal with benzyl protected 2- and 6-fluoroprocatechuealdehyde.[221] Boc-substituted phenylalanine derivatives have been prepared and incorporated into analogues of the vasoactive intestinal peptide (VIP), as replacements for tyrosine[22]. The analogues were, however, generally 300-fold less potent than the parent analogue (Ac-[Lys[12], Ndl[17], Tyr[22], Val[26], Thr[28]])-VIP.[222] The use of 4-benzoyl-L-phenylalanine **74** and ε-para-benzoylbenzamido-L-lysine **75** as photoreactive benzophenone analogues for photoaffinity labelling has been reported. Using these two amino acids, the bimolecular interface between the parathyroid hormone (PTH) and its receptor has been studied to identify the contact points between the ligand and receptor.[223] The synthesis of tritiated 4-benzoyl-L-phenylalanines has also been achieved by the hydrogenation [$Pd(OH)_2$] of the brominated amino acids **76** and **77**.[224] The enkephalin analogues research conducted by Pitzele and co-workers, involving *O*- and *N*-alkylated derivatives of the dipeptide amide L-2,6-dimethyltyrosyl-*N*-(3-phenylpropyl)-D-alanimide has been reviewed.[225] The enkephalin analogue DAGO, has been synthesised using solid-phase methodology.[226]

Endothelin analogues with 2-substituted D-tryptophan replacements have been prepared. All derivatives showed potent affinity for the ET_B receptors, wheras ET_A affinity was dependent upon the substituent on the D-tryptophan residue.[227] The synthesis of the antibiotic L-azatyrosine (L-β-(5-hydroxy-2-pyridyl)-alanine, has been reported in four steps starting from 3-hydroxypyridine.[228] A Lewis acid catalysed Michael addition of dehydroalanine derivative $Ph_2C{=}NC(CO_2Me){=}CH_2$, to 2-substituted indoles has been utilised in the preparation of 2-substituted DL-tryptophan derivatives.[229] A general procedure for the synthesis of β-substituted tryptophan esters has been reported. The key step involved the initial condensation of indoles, aldehydes and Meldrum's acid. This was followed by half ester formation and Curtius rearrangement.[230] The intermediate isocya-



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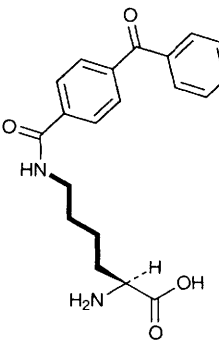

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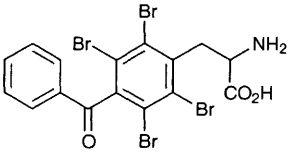

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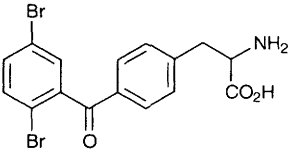
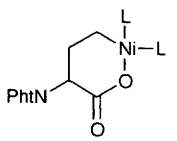


(77)

nates were then converted into benzyl carbamates. 4-Alkylsubstituted tryptophans have been prepared. The key steps involved the introduction of alkyl substituents at the 4-position, using organolithium reagents, and enzymatic resolution of tryptophan derivatives using penicillin-G acylase.[231]

N-Trityl aspartic acid has been dehydrated using DCC. The resulting anhydride was used in a variety of reactions involving nucleophilic attack at the β-carbonyl function, including reaction with Wittig and Grignard reagents to give a variety of unnatural amino acids.[232] Protected 3-methyl aspartic acid derivatives have been synthesised by ring contraction of nickelacycle **78**, followed by insertion of isocyanides.[233] The asymmetric synthesis of both diastereomers of (2*S*)-3-carboxyproline **79** as well as a range of protected derivatives suitable for use in peptide synthesis has been reported starting from aspartic acid. Compound **79** is a conformationally constrained aspartic acid derivative, and can also be considered as an acidified proline residue.[234] Similar methodology starting from glutamic acid has been used by the same authors to prepare a range of γ-substituted glutamic acid analogues, including the conformationally constrained dehydroproline derivative **80**.[235] A diastereoselective synthesis of 4-alkyl-threo-



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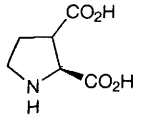

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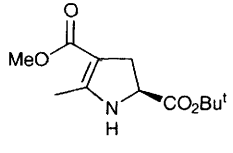


(80)

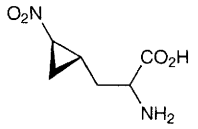
glutamic acids has been achieved by the reaction of alkyl halides with the γ-enolate of *N-para*-nitrobenzoyl-D/L-glutamic acid diesters.[236]

Ethyl diazoacetate was reacted with 3,4-didehydroproline derivatives to yield the corresponding 6-carboxy-3,4-methanoprolines, as rigid analogues of glutamate. The analogues were also tested for displacement of receptor binding to inotropic and metatropic (mGluR1α) glutamate receptors.[237] The synthesis of conformationally constrained analogues of glutamic acid based on 1-amino-cyclopentane-1,3-dicarboxylic acid, as antagonists of metabotropic receptors, has been reported. The analogue (±)-ABHD-I was found to be a competitive antagonist at the mGluR1α receptor, with a K_B of 300 μM.[238] *N*-[4-(2-*trans*-[(2, 6-Diamino-4(3*H*)-oxopyrimidin-5-ylmethyl)-thio]cyclobutyl)benzoyl]-L-glutamic acid has been reported as a potent dihydrofolate reductase inhibitor (K_i = 12 nM) with good *in vitro* culture growth inhibition (IC_{50} = 29 nM).[239] The four stereoisomers of 4-methylglutamic acid have been prepared and tested for their affinities at the kainic acid receptor. The potency of the (2*S*, 4*R*) isomer was comparable to that of kainic acid.[240] The preparation of enantiomerically pure (*R*)- and (*S*)-2-(aminomethyl)alanine and (*R*)- and (*S*)-2-(aminomethyl)leucine has been described. L-Phenylalanine cyclohexylamide was employed as a chiral auxiliary for the resolution of the racemic amino acids. The 2-(aminomethyl)alanines were also derivatised with Boc- and Z- protecting groups, for incorporation into peptides.[241] Modification of the Lys^3 residue of bombesin with a fluorescent marker, fluorescein 5'-isothiocyanate, has been effected with no apparent alteration to the biological activity of the hormone.[242]

Ring closing olefin metathesis has been utilised to prepare a small number of constrained amino acids and peptides. The suitably derived precursors, were ring closed, using a well defined ruthenium complex.[243] Jackson and co-workers have continued the development of serine zinc/copper reagents, and have applied them to the synthesis of enantiomerically pure unsaturated amino acid derivatives.[244] A procedure has been reported for the preparation (trialkylsilyl)-alanines in overall reasonable yields. The silyl-amino acids were also coupled with other amino acids using solution-phase chemistry.[245] (2*S*,3*R*)-3-Hydroxy-leucine has been synthesised stereoselectively in several steps from a xylose derivative.[246]

Peptide nucleic acids (PNA) are peptides derived from amino acids which contain nucleic acid bases in their sidechains or linked to the amide nitrogens. The peptides form base-paired helical duplexes, and the helicity of the PNA is determined by the chirality amino acid attached to the C-terminus. It has been shown by Wittung *et al.* that the induced helicity, measured by circular dichroism is dependant on the nucleobase sequence proximal to the chiral centre.[247]

The synthesis of (2*R*/*S*,1'*S*,2'*S*)- and (2*R*/*S*,1'*R*,2'*R*)-3-(*trans*-2'-nitrocyclopropyl)alanine [Ala(3-Ncp)] **81** has been reported. The synthetic amino acids were used to determine the absolute configuration of the two **81** residues in the naturally occurring depsipeptide hormaomycin **82**. It was found that the natural product **82** contained one molecule each with (2*R*,1'*R*,2'*R*)- and (2*S*,1'*R*,2'*R*)-configuration, as established by comparison of hydrolysate from peptide **82** and the synthetic amino acids.[248]



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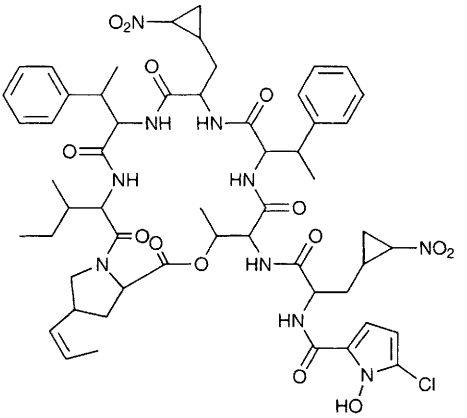
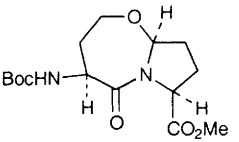


(82)

The synthesis of highly constrained 7,5-bicyclic dipeptides (1-aza-6-oxa-2-oxobicyclo[5.3.0]decane) ring skeletons **83** and **84** has been accomplished on a mmol scale in ~50% yield by a one step electrochemical cyclisation using the dipeptides Boc-L-homoserine-L-proline-OMe (Boc-Hse-Pro-OMe) and Boc-Hse-D-Pro-OMe.[249] The 3,6-dioxoperhydropyrrolo[1,2-*a*]pyrazines **85** have been synthesised by Martin-Martinez *et al.*[250] for use as peptidomimetics. The heterocycles are prepared by reductive amination of 4-ketodiesters derived from dipeptides (**Scheme 14**). The amino acid side chains in **85** can also be used as a template for peptide construction.



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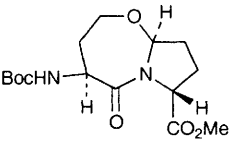

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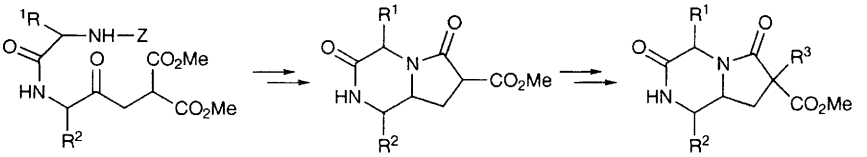
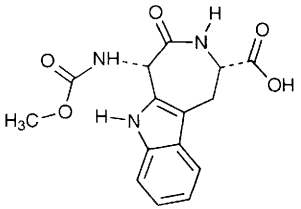


R^1, R^2, R^3 = amino acid side chains (85)

Scheme 14

A new diastereoselective synthesis of a cyclic dipeptide template **86** has been reported. The template is related to H-Gly-Trp-OH, but with the incorporation of an intramolecular bond between the α-C of Gly and ring position 2 of the Trp unit using a Pictet-Spengler type electrophilic aromatic substitution. The absolute

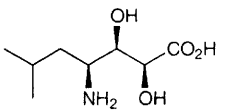
configuration of dipeptide analogue **86** was determined by X-ray diffraction and found to be (2*S*, 5*S*).[251]



(86)

5 Enzyme Inhibitors

5.1 Renin Inhibitors – A dansylated inhibitor of renin has been used as a fluorescent probe in analysing ligand exchange reactions. The on and off rate constants (k_{on} and k_{off}), were determined for a series of peptidomimetic, amido diol transition state inhibitors of human renin.[252] The α-hydroxy statine derivative **87** was synthesised and incorporated into a peptide framework which was found to be a potent renin inhibitor.[253] A general method for the asymmetric synthesis of statine **88** and statine analogues has also been published.[254] Radiolabelled compounds, related to renin inhibitor compounds have been synthesised. The compounds were evaluated for their physicochemical parameters in respect of oral availability.[255] Pyrrolinone rings have been incorporated into the peptide backbone of peptidomimetic inhibitors of renin. Compound **89**, was found to have IC_{50} values of 0.6 - 18 μM.[256]



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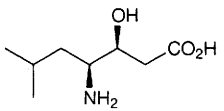

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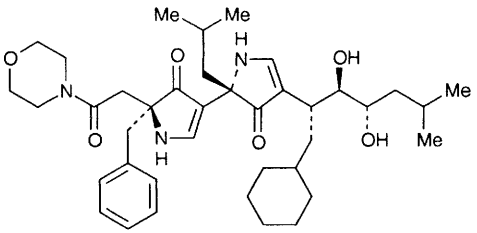

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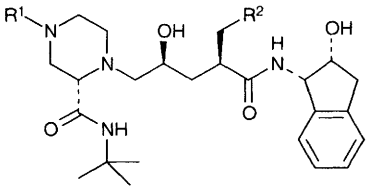

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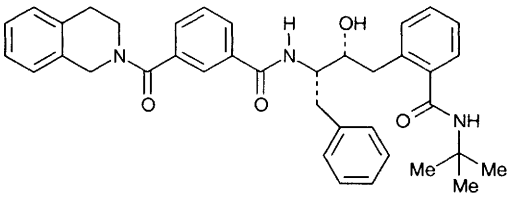


(91)

5.2 HIV-1 Protease Inhibitors – The peptomer containing a duplicate copy of the amino acids from residues 419-436 of the C4 region of the glycoprotein 120 (gp120) of the human immunodeficiency virus type 1 (HIV-1) envelope was synthesised for use as a conformationally constrained immunogen. Circular dichroism studies revealed the polymerised peptide peptomer-(419-436), possessed a predominantly α-helical structure.[257] It has been discovered that the spread of the AIDS virus in animals can be halted by a set of two HIV protease inhibitors possessing a hydroxyaminopentanamide **90** transition state isostere.[258] A new series of HIV-1 protease inhibitors has been developed incorporating substituted isophthalic acid derivatives as amino acid surrogates. Compound **91** was the optimised structure from which a number of non-peptide HIV-1 protease inhibitors with notable antiviral activity could be produced.[259]

Hydroxyethylene dipeptide isosteres of Leu-Leu and Phe-Phe have been prepared from L-Leu and L-Phe using in the key steps a thiazole-aldehyde synthesis, followed by Wittig olefination and reduction of the double bond. The isosteres were prepared as inhibitors of renin and HIV-1 aspartic protease.[260] Tripeptide analogues containing a dioxyethylene moiety between proline and phenylalanine residues have been prepared. The analogues were designed as mimics of HIV-1 protease inhibitors RPI-856 A, B, C and D, and showed high inhibitory activity.[261] Peptide aldehydes have been tested as inhibitors of hepatitis-A virus 3C proteinase. For example, Acetyl-Leu-Ala-Ala-(*N,N'*-dimethylglutaminal) was found to be a reversible inhibitor with a K_i of 4.2×10^{-8} M. The peptide aldehydes were prepared by the reduction of a thioester precursor.[262]

5.3 Inhibitors of Other Protease Enzymes

5.3.1 Serine protease inhibitors – A linear analogue of the squash trypsin inhibitor EETI II (*Ecballium elaterium* trypsin inhibitor) has been prepared. The

three disulfide bridges present in the native EETI II were eliminated by replacing the six cysteine residues with serines. CD, IR and NMR studies indicated that many of the secondary structures were still populated in the absence of disulfide bonds.[263] During the purification of the trypsin-modulating oostatic factor (TMOF) of *Neobellieria bullata*, a new factor with oostatic activity was discovered. The amino acid sequence of this factor was found to be H-SIVPLGLPVPIGPIVVGPR-OH. Owing to structural sequence similarity with parts of known collagens and its oostatic activity it was called Neb-colloostatin.[264]

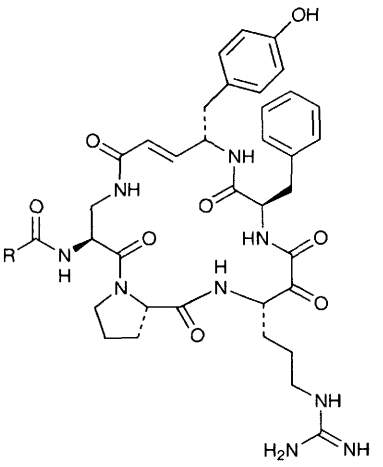
Two primary serine proteinase inhibitors in goat plasma have been isolated and characterised. The *N*-terminal sequence of the purified proteins revealed that they are structurally very similar to each other and highly homologous to human α_1-anti-chymotrypsin. However, it was found that the goat inhibitors differed from each other and from anti-chymotrypsin in their inhibitory ability, one was found to inhibit contrapsin, and the other to inhibit elastasin. This was attributed to the difference in amino acid sequence at the reactive site of the proteins.[265]

^{3}H-labelled Z-Ala-Ala-Pro-Phe-$COCH_2Cl$ has been synthesised. Cucumisin, a serine protease, was inhibited by this peptide mimic, and the reactive site histidine of cucumisin was identified.[266] The conformation of four peptides which together represent the entire sequence of bovine pancreatic trypsin inhibitor (BPTI) have been studied using CD and NMR techniques, with trifluoroethanol acting as a cosolvent.[267]

The total synthesis of cyclotheonamide A **92**, a cyclic peptide isolated from the marine sponge *Theonella sp.*, which is a serine protease inhibitor, has been reported. The synthesis was achieved by a convergent, [3+2] fragment-condensation route.[268] A unique class of mechanism-based inhibitors of serine proteases have been reported. Thus, tripeptide boronates esterified by an alcohol or alcohols at the boronic acid **93** act as transition state analogues of, and possess a tight binding to trypsin comparable enzymes. The ability to vary the structure of the peptide can be used to obtain selectivity between different proteases. The most structurally adept inhibitors are either selected by binding to, the enzyme (epitaxial selection) or assembled by epitaxial reaction on the enzyme surface.[269] Proline boronic acid has been linked with Ala, Pro and Val to yield dipeptides, which are potent inhibitors of dipeptidyl peptidase-IV, and are also immunosuppressants.[270]

A series of pseudo-peptide bond and peptide-aldehyde inhibitors of atrial granule serine proteinase have been prepared. Bz-APR-ψ-SLRR (IC_{50} = 250 μM) was found to be the most potent pseudo-peptide inhibitor, which was five fold less potent than EACAAPR-CHO the best peptide-aldehyde inhibitor.[271] A hydrazine isostere has been employed to mimic the Gln-Gly sequence within a small peptide. Thus compound **94** was synthesised in five steps from *N*-Z-phenylalanine, and was found to be a potent inhibitor of human rhinovirus (HRV) 3C protease (IC50 = 48 nmol dm^{-3}).[272]

5.3.2 Cysteine protease inhibitors – A large scale procedure for the preparation of ketones **95** and **96** has been reported. These two compounds are prodrugs of two



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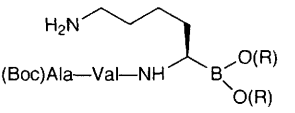

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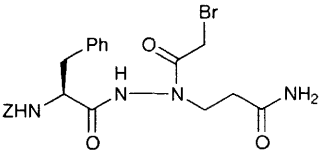
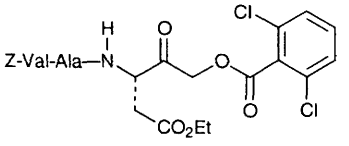


(94)

potent Interleukin-1β-converting enzyme (cysteine protease) inhibitors. Both **95** and **96** showed potent effects (ED_{50} <0.1mg kg^{-1} p.o.) in models of acute inflammation such as LPS-pyrexia and Carrageenan oedema, although the specific inhibitory effects upon Interleukin-1β-converting enzyme have yet to be determined.[273] Diacylhydrazines ([azaAsp^1]) have been prepared and tested for inactivation of interleukin-1β-converting enzyme (ICE). The inactivation rates were generally 10-fold slower than their α-substituted methylketone congeners.[274] The peptide derivative ^{i}Noc-Gln-Val-Val-Ala-Ala-pNA (^{i}Noc = isonicotyl oxycarbonyl) has been designed and prepared, but its potency against thiol proteases was found to be mediocre.[275]



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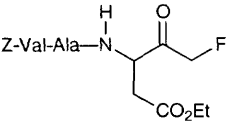


(96)

5.3.3 Metalloprotease inhibitors – The phosphinic peptide Z-(L,D)Pheψ-(PO_2CH_2)(L,D)Ala-Arg-Phe has been reported as a potent (K_i = 70 pM) and

specific inhibitor of the endopeptidase (24.15), a zinc metalloprotease.[276] A series of cyclic and acyclic dipeptidyl acetals has been synthesised. In particular, *N*-[*N*-benzyloxycarbonyl-(*S*)-prolyl]-(*S*)-prolinal di-methyl acetal was found to be a potent inhibitor of prolyl endopeptidase.[277] The synthesis of dicarboxylic acid dipeptide neutral endopeptidase (24.11) inhibitors, has been reported. The authors of this work also described the biological activity of the inhibitors, the most potent dipeptide displaying an IC_{50} of 50 nM, both *in vivo* and *in vitro*.[278] The model tetradecapeptide used in the purification of the RXVRG-endoprotease from *Xenopus laevis* skin exudate has been studied by two-dimensional NMR, correlation (COSY) and NOE (NOESY) spectroscopy. The peptide had the 5-9 consensus sequence (RXVRG) along with an acidic moiety (1-4) and a hydrophobic domain (10-14).[279] A series of α-thio dipeptides containing central non-natural amino acids has been prepared. Many of the compounds were found to inhibit neutral endopeptidase and angiotensin-I-converting enzyme in *in vitro* tests and also in *in vivo* experiments.[280]

Two hexapeptide derivatives *cyclo*-(Gly^1-Pro^2-Phe$^3\psi$[PO_2-CH_2]Gly^4-Pro^5-Nle^6] and *cyclo*-(Gly^1-Pro^2-D-Phe$^3\psi$[PO_2-CH_2]Gly^4-Pro^5-Nle^6], have been studied in aqueous solution using NMR spectroscopy and molecular dynamics simulations, and their 3D structures have been resolved. The peptides, are inhibitors of bacterial collagenase, a zinc metalloprotease, and their structure-activity relationships are discussed.[281] A range of *N*-carboxyalkyl dipeptides has been prepared and found to show good inhibition of human stromelysin-1 and gelatinase (K_i = 2-40 nM), but weak inhibition of collagenase.[282]

5.4 RGD Containing Peptides – A review has been published outlining antithrombic agents and focusing in particular on the RGD motif.[283] Another review covers the literature (from 1963-1992) on the use of peptides containing the RGD sequence as inhibitors of platelet aggregation.[284]

Degradation of fibrinogen led to peptide fragments with antithrombic properties. One fragment containing the RGD sequence efficiently inhibited fibrinogen binding and platelet aggregation (IC_{50}: 20-50 μM) *in vitro*.[285] Continuing the studies on benzamidinopentanoyl (BAP) receptor antagonists, the Searle laboratories have introduced an amide bond adjacent to the benzamidine. This novel series of inhibitors, with an (aminobenzamidino)succinyl-Arg-Gly surrogate, possessed higher *in vitro* potencies than the BAP antagonist. The compounds were also evaluated in oral studies, and a pharmacore model based on inhibitors from benzamidino classes was developed.[286]

Cyclo-(D-Val-Arg-Gly-Asp-Asn) and Ac-D-Val-Arg-Gly-Asp-β-Ala-NH_2 have been prepared and their inhibitory capacities on cell adhesion tested against a fibronectin coated surface. The octapeptide H-Gly-Gly-Gly-Arg-Gly-Asp-Ser-Pro-OH has been derivatised at its *N*-terminal, with 4-azidobenzyloxysuccinimide. This was followed by adsorption and UV irradiation onto polyvinyl alcohol (PVA) to create a two-dimensional tissue. Bovine endothelial cells were found to adhere and spread upon the surface in a biologically specific manner.[287] A tetrapeptide containing the RGD motif, has been bound to a sialyl-Lewis-glycoconjugate, and found to be a highly active ligand for P-selectin.[288]

A series of cysteine containing decapeptides, containing the sequence RGDFPASS, has been prepared by solution phase methods. The peptides were shown to inhibit platelet aggregation strongly.[289] Analogues of the type Arg-Xaa-Asp-Ser have been prepared, as well as *N*-terminally modified Arg-Xaa-Asp-Ser derivatives. The analogues retained good antimetastatic and anti-cell adhesive properties.[290] A series of 88-residue proteins containing an α-helical coil, a spacer and a ligand with the RGD sequence has been synthesised. The proteins were tested for their receptor-adhesive modular properties.[291]

5.5 Miscellaneous Enzyme Inhibitors – A series of gonadotropin-releasing hormone (GnRH) antagonists, homologous to azaline B ([Ac-D-Nal[1]-D-Cpa[2]-D-Pal[3]-Aph[5](Atz)-DAph[6](Atz)-Ilys[8]-DAla[10]]GnRH) with substitutions at the Atz positions, has been synthesised and biologically evaluated. Seven of the twenty analogues tested had potency and activity comparable to azaline B.[292] Buserilin, [D-Ser(tBu)[6], desGly[10]]GnRH-Et, a gonadoliberin agonist has been synthesised by connecting the fragments Glp-His-Trp-Ser-Tyr-$NHNH_2$ and Z-D-Ser(CMe_3)-Leu-Arg-Pro-NHEt. Both segments were prepared in good yield without protection of the side chain functional groups.[293]

Hruby and co-workers have reported the synthesis of the glucagon antagonist H-Ser-Gln-Gly-Thr-Thr-Ser-Glu-Tyr-Ser-Glu-Tyr-Ser-Lys-Leu-Asp-Ser-Arg-Arg-Ala-Gln-Asp-Phe-Val-Gln-Trp-Leu-Met-Asn-Thr-NH_2, employing solid-phase methodology. The peptide was found to be a pure antagonist, and displayed a binding potency IC_{50} value of 48 nM.[294] Thiol-, aminophosphonic acid-, and hydroxamic acid-collagenase inhibitors containing the lactam and azalactam P_2'/P_3' substituents, have been prepared. The hydroxamic acid derivative showed an IC_{50} of 12 nM.[295]

Pseudodipeptide amides, which correspond to truncated versions of the CAAX motif, have been shown to be inhibitors of protein farnesyltransferase. Also, despite the lack of the X residue, the pseudodipeptides were over 100-times more selective for protein farnesyltransferase over type 1 protein geranylgeranyltransferase.[296] The unit 3-amino-1-carboxymethyl-2,3-dihydro-5-phenyl-[1]H-1,4-benzodiazepin-2-one (BZA), has been introduced as a mimic of the central part of the Cys-X-X-Cys motif. The analogue Cys(BZA)Met was found to be of modest potency towards inhibition of Ras farnesyltransferase (IC_{50} = 400nM). *N*-Methylation of the cysteine amide was found to increase the potency 1000-fold; this was thought to be due to conformational changes.[297] The peptide kelatorphan (an inhibitor of enkephalin degrading enzymes) and related analogues have been reported as inhibitors of leukotriene A_4 hydrolase.[298] The glutathione analogue γ-(L-γ-azaglutamyl)-L-cysteinyl-glycine, has been synthesised and found to be a competitive inhibitor of hog kidney γ-glutamyltransferase.[299]

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

6.1 Peptides with Opioid Characteristics – Analogues of dynorphin A H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH, truncated at the 11-residue [Dyn A(1-11)-NH_2] have been synthesised. In particular, substitution at the 3-position with D- and L-alanine resulted in potent and selective agonists for the κ-receptor.[300] The lactam *cyclo*-[D-Asp^2,Dap^5]dynorphin A and two related homologues have been prepared as conformationally constrained dynorphin analogues. The constraints were envisioned as mimicking the required conformation for recognition at κ-receptors. Potency at κ-receptors was indeed achieved, although only slight selectivity over μ-receptors was observed.[301] A series of cyclic opioids related to dermorphins and deltorphins have also been synthesised. The conformations were studied by NMR and the compounds were found to be highly active at both the μ and δ-opioid receptors.[302] A series of deltorphin analogues has been synthesised, in which Leu^5 and Met^6, were replaced by t-Leu(Tle) and/or N^{α}-alkylated glycine. Some of the compounds, such as [Tle^5]deltorphin, were found to be potent δ-receptor antagonists, but generally the analogues exhibited mixed receptor selectivity properties.[303]

The isostere ψ(CH_2NH), has been incorporated into eight analogues of dynorphin A (1-11)-NH_2. The results indicated that changes at the address site, do not generally affect binding at the κ, μ and δ receptors. Changes at the message segment, however, did decrease binding at all three receptors.[304] The phenylalanine residue of deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH_2), has been replaced by ring-substituted and heterocyclic amino acids. The substitutions adversely affected δ binding, but preserved selectivity over κ and μ receptors. The best δ binding was with the 3-(2-thienyl)alanine residue (K_i = 1.38 nM). Lypophilicity was concluded to be more important than electronic character when comparing the relative opioid bindings of similar sized heterocyclic replacements.[305] Analogues of [D-Leu^8]Dynorphin(1-8)-NH_2 have also been prepared. Each peptide bond was systematically replaced with the ψ(CH_2NH) isostere using solid-phase methods. The ^{3}ψ^4-analogue showed high κ-receptor selectivity (μ/κ K_i ratio = 339, δ/κ K_i ratio = 24104), whilst introduction of the CH_2NH isostere at Tyr^1-Gly^2 greatly enhanced the enzymatic stability of the peptide.[306]

The requirement of His^4 in deltorphin for δ-selectivity has been examined by systematic replacements. MeHis^4, MePhe^4 and Phg^4, all decreased δ-selectivity, and reduced δ-binding. Other modifications increased μ-receptor affinity. Imidazole methylation of His^4 doubled δ-selectivity. The authors speculated that a spatial orientation of the His^4 imidazole parallel to the phenolic side-chain of Tyr^1, contributed to high δ-affinity and selectivity.[307] The *N*-terminal dipeptide Tyr-D-Ala of a μ-selective agonist, demorphin tetrapeptide (DT, H-Tyr-D-Ala-Phe-Gly-NH_2) and δ-selective agonist deltorphin C (DEL-C, H-Tyr-D-Ala-Phe-Asp-Val-Gly-NH_2) have been changed into a aminodiacyl moiety. However, it was found that all of the analogues prepared in this way showed a reduction in opioid receptor affinity.[308] The synthesis and tritium labelling of the selective δ-

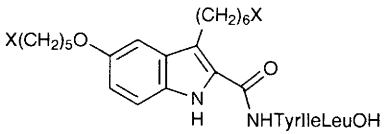
opioid antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP; Tic = tetrahydroisoquinoline-3-carboxylic acid), and its analogue H-Tyr-Ticψ[CH_2-NH]Phe-Phe-OH, have been reported. Both labelled analogues showed K_d values below the nanomolar range.[309] The δ-receptor selective dermorphin gene associated peptide and five analogues with modifications at positions 2, 4 and 5 have been synthesised *via* solid phase methods. The analogues were found to be δ-receptor selective with μ/δ ratios of 19.5-236.3.[310]

Comprehensive proton assignments of the μ-selective dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and δ-selective deltorphin-I (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) were carried out by two dimensional NMR techniques in order to allow the conformations of these peptides to be studied in the membrane-mimetic micelles of perdeuterated dodecylphosphocholine.[311] Two dermorphin and deltorphin peptides, H-Tyr(SO_3H)-D-Ala-Phe-Gly-NH_2 and H-Tyr(SO_3)-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, each containing the Tyr(SO_3)-residue, have been prepared. In bioassays, only the latter peptide retained significant activity.[312]

A series of dimeric enkephalin analogues has been synthesised, whereby two identical tetrapeptides, were connected at their C-termini by α,ω-diamino-α,ω-dideoxyalditols varying in length by 3-6 carbons. The spacer length was found not to affect δ-receptor affinity, but did affect κ- and μ-receptor affinities.[313] A series of N^{15}-labelled Leu enkephalins has been prepared using solution methods.[314]

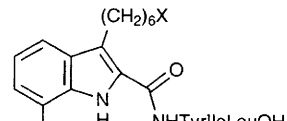
The X-ray structures of the tetrapeptide TIPP, H-Tyr-Tic-Phe-Phe-OH (Tic = 1,2,3,4-tetrahydroquinolone-3-carboxylic acid), and of RTI02, *N*,*N*-diallyl-(*O*-*tert*-butyl)-Tyr-Aib-Aib-Phe-Leu-OMe (Aib = α-aminoisobutyric acid), have been determined. The latter peptide is a protected derivative of the δ-opioid antagonist ICI 174,864. TIPP exhibited high affinity, selectivity and antagonism for the δ-receptor.[315]

6.2 Neuropeptide Analogues – The Arg^8-Arg^9Pro^{10} region of neurotensin (NT, pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) has been replaced by substituted indole-2-carboxylates to yield compounds **97** and **98**. The mimic **97**, was found to be an antagonist of NT, whereas **98** was found to be an antagonist only at low concentrations; at higher concentrations (10-100 μmol dm^{-3}) **98** was found to be an NT agonist.[316] The *C*-terminal region of NT (NT_{8-13}) has been cyclised and related cyclic hexapeptides were also prepared. The NT analogues showed nanomolar binding affinities for the NT receptor. Structure-activity



X = $NHC(NH_2)NH$

(97)



X = $NHC(NH_2)NH$

(98)

investigations were also effected using NMR techniques.[317] A neurotensin analogue has been described, whereby the Pro-Tyr sequence of neurotensin(9-13) was replaced with a urea linkage.[318]

A series of analogues of neuropeptide Y (NPY) **99** have been synthesised. The analogues, including shortened and elongated cyclised derivatives, were found to be able to bind selectively to Y_1 and Y_2 receptors. Residues 10-17 of NPY were postulated as not directly involved in Y1 or Y2 recognition or activation. The selectivity of NYP receptors was, however, determined to be sensitive to subtle conformational changes of NYP.[319] The synthesis of porcine neuropeptide Y(pNPY) *N*-terminal fragments was completed by solid-phase techniques and the solution conformational properties studied by CD and ^{1}H NMR spectroscopy. The conformation of the peptides varied according to the solvent and/or percentage of trifluoroethanol.[320] A series of truncated analogues of neuropeptide-Y has been prepared. The *N*-terminal segment was maintained, and was linked to the *C*-terminal segment which was systematically varied in length using 6-aminohexanoic acid. The affinity to the Y receptor was investigated and the results indicated one optimal length for the *C*-terminal segment. The authors speculated, therefore, that the three-dimensional arrangement and orientation of the amino acids was more important for high receptor affinity than the amino acids themselves.[321]

Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Tyr-Ser-Ala-

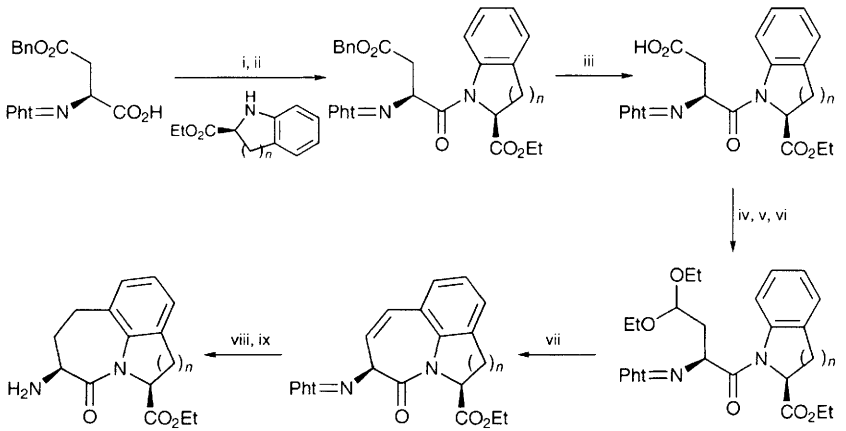
Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2

(99)

The 30-residue human neuropeptide galanin has been investigated by NMR techniques in aqueous solution. Regions of the peptide were shown to adopt short range structures, including nascent helices, in rapid equilibrium with random coils.[322] The binding characteristics and agonistic and antagonistic actions of the chimeric peptide M35 galanin(1-13)-bradikinin(2-9)amide, upon Rin m 5F insuloma cells have been investigated. Radiolabelled M35 (^{125}I) was used for the binding-affinity studies. The results indicated that the mixed agonist/antagonist properties of M35 derived from the chimeric nature of the peptide acting solely at the galanin receptors.[323] Eight FMRFamide-like neuropeptides have been reported. The peptides range from 8-14 amino acid residues. The peptide AF4, Gly-Asp-Val-Pro-Gly-Val-Leu-Arg-Phe-amide, was found to induce a strong, long-lasting contraction on dorsal muscle strip.[324]

6.3 Angiotensin Analogues – A diuretic peptide from the leech *Erpobdella octoculata* has been characterised as an angiotensin-II amide. The peptide, was found to be immunoreactive to an antiserum against angiotensin-II. Molecular recognition theory has been used to design a peptide strand complementary to angiotensin-II.[325] The 'antisense' peptide Ile-Ala-Asn-Val-Asn-Met-Gly-Glu was synthesised by solid phase methods, and the binding affinities with angiotensin-II and related analogues were evaluated.[326]

A metalloendopeptidase, isolated from rabbit liver microsomes and identical to soluble angiotensin-binding protein present in the cytosol, has been found to be inhibited in a competitive manner (K_i = 7.6 μM) by the angiotensin antagonist [Sar^1,Ala^8]-angiotensin II.[327] Synthetic methodology for the preparation of benzo-fused, 7,5- and 7,6-fused azepinones as dipeptide mimetics has been reported. Intramolecular addition of an oxonium ion to the proximal indoline/tetrahydroquinoline aromatic ring was involved in the key step as shown in **Scheme 15**. The mimetics were applied in the synthesis of inhibitors of neutral endopeptidase and angiotensin converting enzyme.[328] This work was further extended by the incorporation of mercaptoacetyl or mercaptopropanoyl groups into azepinonethiazolidine and azepinonetetrahydrothiazine carboxylic acids to provide conformationally restricted peptidomimetics of Ala-Pro exhibiting powerful dual activity *in vitro* against angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP).[329]



Reagents: i, cyanuric fluoride; ii, amine (2,6-di-tert-butyl-pyridine); iii, H_2, $Pd(OH)_2$/C; iv, EtSH, EDAC, DMAP, CH_2Cl_2; v, Et_3SiH, Pd/C; vi, $CH(OEt)_3$, TsOH, EtOH; vii, PPA, 100 °C; viii, H_2, Pd/C; ix, $H_2N—NH_2 \cdot H_2O$

Scheme 15

6.4 Oxytocin and Vasopressin Analogues – A series of compounds has been prepared, whereby a D-Cys^6/L-Cys^6 substitution was effected upon known vasopressin and oxytocin antagonists. The interchange resulted in reduced antagonist values in assays, but gave possible leads for orally active oxytocin and vasopressin antagonists.[330] The copper complexes of oxytocin, 4-Glu-oxytocin, 5-Asp-oxytocin and Gly-Gly-Gly-Lys^8-vasopressin have been studied using potentiometric and spectroscopic methods. The formation of 4N-coordinated complexes was found to be characteristic of all ligands.[331] A solid phase procedure, which allows the replacement of a disulfide bond with a thioether has been used to synthesise C_1-oxytocin.[332] A tritiated propionic acid residue has been introduced into an oxytocin antagonist, leading to [^{3}H][1-(β-mercapto-β,β-

cyclopentamethylenepropionic acid), 2-(*O*-methyl)-tyrosine,4-threonine,8-(N^{δ}-propionyl)-ornithine,9-tyrosylamide]vasotocin. The labelled analogue was obtained in good yield with high specific activity (100 C_i $mmol^{-1}$).[333] End-group modified retro-inverso isomers of MYF and LYF (tripeptide oxytocin analogues) have been prepared. The mimics were found to possess similar affinities to the parent tripeptides. The results suggested that only the correct orientation of the side group and of the α-amine play a role in the recognition process.[334]

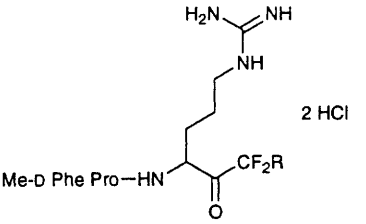
The drug [1-des-amino,8-D-arginine]vasopressin has been modified by glycosylation of the serine at position 4. The glycosylated derivatives were found to possess low agonist and antagonist properties at the vasopressin V2-receptor. Conformational studies, however, did not reveal a major modification of the peptide backbone to be caused by the glycosylation. Steric hindrance was therefore suggested as the cause for the low activity of the derivatives.[335] Seven analogues of Aaa-D-Tyr(Et)-Phe-Val-Asn-Pro-Arg-Arg-NH_2, a potent antagonist of V2 receptors, have been synthesised. Two of the analogues were also found to be potent antagonists.[336] L-Tyr has been replaced by L-Tyr(Me), L- or D-Phe(pMe), L- or D-Phe(pEt), and arginine by homoarginine in 8-D-homoarginine-vasopressin analogues. Tests, showed dissociation of Factor VIII and tPA activating properties, or a decrease in Factor VIII and tPA in the blood of squirrel monkeys.[337] Four vasopressin analogues, modified at position 3 with β-thienylalanine, have been synthesised using Boc methodology. The agonists were found to possess similar activity to the parent compounds.[338]

6.5 Thrombin Binding Peptides – A series of analogues of the *N*-terminal region of the tethered peptide ligand Ser-Phe-Leu-Leu-Arg-Asn, has been synthesised and subjected to conformational studies. A model for the receptor bound structure was suggested on the basis of the results.[339] The human thrombin receptor agonist SFLLRN was subjected to residue substitutions, including SFLLEN, and tested as an agonist against the thrombin receptor. The results identified the Glu^{260} and, to a lesser extent, the Phe^{87} sites of the receptor as important determinants of agonistic specificity.[340] The agonist H-Ala-Phe(*para*-F)-Arg-Cha-HArg-Tyr-NH_2 has been reported to exhibit a thrombin receptor activation of EC50 = 0.01 μM. The iodinated derivative also exhibited a potent activation of EC50 = 0.03 μM.[341] A series of thrombin inhibitors based upon peptide boronates has been reported. The compounds, based upon the previously reported Z-D-Phe-Pro-boroMpg-OPin, displayed K_i values between 10 and 100 nM. ^{11}B NMR showed the interaction between the boron atom and the active site.[342] Four more boron containing peptides have been prepared and tested for activity against thrombin inhibition; these were Ac-D)Phe-Pro-boroLys-OH, Ac-(D)Phe-Pro-boro-homoLys-OH, Ac-(D)Phe-Pro-boroOrn-OH and Ac-(D)Phe-Pro-boro-*n*-butylamidinoGly-OH. The kinetic and crystallographic data of these peptides were reported.[343] A series of thrombin inhibitors has been reported, based on structural variations of P2 and P3 residues in tripeptidic boroargenine inhibitors.[344]

The synthesis of fluorinated ketone thrombin inhibitors of general structure **100** in which the *C*-terminal arginine residue has been converted into a fluorinated ketone has been reported.[345] Peptide derived transition state analogue inhibitors

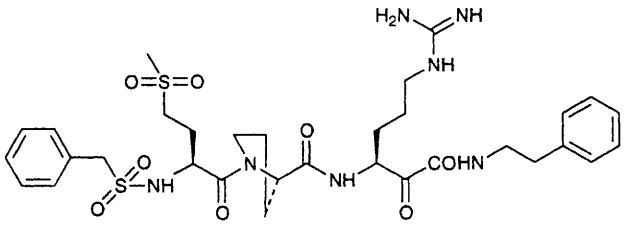
of thrombin have been synthesised and assessed for biological activity. A series of tetra to hexapeptide (H-D-Phe-Pro-Arg-Gly-Phe-Lys-OH) sequences which spans the catalytic site of thrombin was prepared in which the Arg-Gly amide bond was replaced with the ketomethylene, the hydroxymethylene and the hydroxyethylene amide bond isosteres. The hexapeptide containing a ketomethyl isostere was found to be the most potent thrombin inhibitor.[346] The crystal structure of a complex between human α-thrombin and a hydrolysis product of CVS-1347 **101**, an α-keto amide inhibitor of this enzyme, has been determined.[347]

A series of thrombin inhibitors based upon chloromethyl ketones has been



R = F, CF_3, $CH_2CH_2CH_3$

(100)



(101)

investigated. The tripeptide D-Phe-Pro-Arg-$CHCl_2$ was found to be the best inhibitor ($k_{obs}/[I] = 10^7\ M^{-1}s^{-1}$). However, modifications such as one amino acid extension (Phe or Gly) or addition of a reversed amino acid residue resulted in reduced inhibition of thrombin.[348] Analogues of D-Phe-Pro-Arg-H, namely D-hydroxyacyl-prolyl-arginyl derivatives, have been synthesised and tested. Thrombus formation in various thrombosis rabbits was prevented by a single oral dose of 5 mg kg^{-1}.[349] Isosteres of the guanidine or arginine moiety have been used to prepare analogues of a series of *N*-arylsulfonylarginine amides. The compounds were evaluated as potential active site inhibitors of thrombin and showed a similar SAR to that of simple arginine compounds.[350] A series of tripeptide arginine aldehydes, where the P2 proline has been replaced by *N*-substituted glycines, has been prepared and tested for thrombin inhibition.[351] A series of constrained tripeptides, based upon the arginal thrombin inhibitors has been reported in a structure-activity study. Included in the study were compounds of the type D-MePhe-Pro-Arg-H, Boc-Phg-Pro-Arg-H and D-1-Tiq-Pro-Arg-H (Tiq = 1,2,3,4-tetrahydroisoquinolin-1-ylcarbonyl). The results led to the preparation of other

analogues such as TFA-D-Phg(αEt)-Azt-Arg-H with improved selectivity properties for thrombin versus plasmin.[352]

The solid phase synthesis of a fragment of hirudin (fragment 1-47 bearing a Tyr^3-Tyr exchange, Y3W analogue) was carried out in ~35% yield. The fragment possesses three disulfide bridges between the Cys residues, Cys^6-Cys^{14}, Cys^{16}-Cys^{28} and Cys^{22}-Cys^{37}, which naturally form in the same order as for hirudin. CD studies showed the analogue to possess the same conformation as hirudin, but the Tyr^3 analogue is five times more active.[353]

The fourth EGF-like domain of thrombomodulin (TM4), residues E346-F389, has been synthesised and the structure investigated by NMR techniques. The TM4 fragment was tested for anticoagulant activity but was found to be inactive, despite being essential for TM cofactor activity.[354] The solution phase structure of a 19-residue peptide has been investigated. The sequence of this peptide was based on the C-loop residue (371-389) of the fourth epidermal growth factor like domain of thrombomodulin, a protein which acts as a cofactor for the thrombin activation of protein C. Despite its small size, the peptide was found to adopt a compact structure held together by hydrophobic interactions and containing two β-turns.[355]

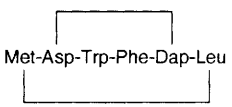
The total synthesis of endothelin has been reported. Acetamidomethyl and ^{t}Bu groups were utilised as orthogonal protecting groups for the thiols. The disulfide formation was effected by successive oxidation with iodine and $MeSiCl_3$-Ph_2SO.[356] Endothelin-1 (ET-1), a 21-residue vasoconstricting peptide, has also been extended at the *N*-terminus by addition of Lys-Arg. This addition was found to decrease the biological activity of the peptide, and to increase the formation of native disulfide bridges. The conformation of KR-ET-1 was studied by CD and NMR methods.[357] The conformational preferences of endothelin-1 have been studied using high temperature molecular dynamics/annealing and Monte Carlo/minimisation search in torsion angle space.[358] *Cyclo*-(D-Trp^1-D-Glu^2-Ala^3-D-Val^4-Leu^5) and *cyclo*-(D-Trp^1-D-Glu^2-Ala^3-D-$AlloIle^4$-Leu^5) have been used as initial models to probe the structure-activity relationship of endothelin-A receptor antagonists.[359] The conformation of the endothelin-B receptor selective antagonist RES-701-1, which consists of 16 amino acid residues linked between the β-carboxyl group of Asp^9 and the α-amino group of Gly^1, has been investigated by NMR methods. The structure obtained from the results indicated that the region consisting of Thr^6, Ala^7, Tyr^{14} and Tyr^{15}, and/or the region Asn^2, Tyr^{14} and Tyr^{15} are involved in binding with the endothelin receptor.[360]

The synthesis and thrombin inhibition of a number of cyclotheonamide derivatives, has been reported. Comparison with other inhibitors and kinetic studies was carried out and the structure-activity relationship of the analogues was also investigated.[361] Thrombin inhibitors, derived from 3-amidino-phenylalanine whereby both the N^{α}- and *C*-terminal substituents were varied, have been reported. Also reported were the pharmacokinetic properties of the compounds.[362]

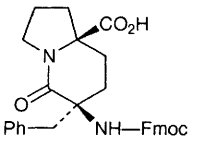
6.6 Tachykinin Analogues – Radiolabelled analogues of tachikinin substance P (H-RPKPQQFFGLM-NH_2) have been prepared in order to study contact sites between substance P and its receptor. A model for the agonist peptide-binding sites was proposed on the basis of the results.[363] The Phe^7 and Phe^8 residues of

substance P have been substituted with (2*S*,3S)-β-methylphenylalanine and/or (2*S*,3*R*)-β-methylphenylalanine. The peptides were found to be agonists of substance P in the smooth muscle contraction assay, with EC_{50} values between 0.15 and 10 nM. Structure-activity implications were also discussed.[364] Analogues of the *C*-terminal hexapeptide and heptapeptide of substance P (SP), namely $[Glp^5,Glu(OBzl)^{11}]SP_{(6\text{-}11)}$ and $[Glp^5,Glu(OBzl)^{11}]SP_{(5\text{-}11)}$, have been synthesised and tested for activity. Both analogues were found to be potent (higher than SP) and selective agonists on GPI through the NK-1 receptor.[365]

The bicyclic hexapeptide *cyclo*-(Met^1-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6)*cyclo*(2β-5β) **102**, has been synthesised by Pavone and co-workers, using a combination of solution and solid phase chemistry. The peptide **102** is a neurokinin A antagonist which, compared with the parent peptide, has a 10-100 fold greater affinity for the NK-2 receptor of different species in *in vivo* experiments. Both the solution and solid state conformations of peptide **102** were determined.[366] A second generation growth hormone-releasing peptide KP-102 was found to bind to a receptor like molecule resembling neurokinin receptor.[367]

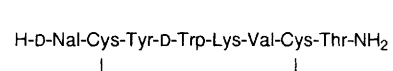


(102)

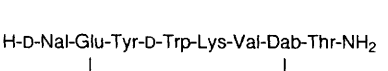


(103)

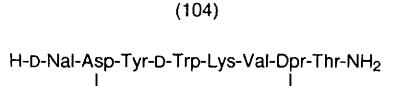
6.7 Somatostatin Analogues – The synthesis of a *cis*-Phe-Pro dipeptide mimetic **103**, has been described. The α-positions of the Phe and Pro residues were joined by a CH_2CH_2 bridge, thus fixing a type-VI β-turn conformation. The dipeptide unit **103** was subsequently incorporated into the cyclic somatostatin analogue *cyclo*-(Phe=Pro-Phe-D-Trp-Lys-Thr), and showed a high affinity for the somatostatin receptor on rat brain cortex membranes (pIC_{50} 8.6).[368] Two lysine homologues, 2,3-diaminopropanoic acid (Dpr) and 2,4-diaminobutyric acid (Dab), have also been used to prepare somatostatin analogues. The cystine bond of the known analogue angipeptin **104**, has been replaced by an amide linkage between the lysine homologues, and the side chain of aspartic or glutamic acid, giving analogues **105-107** which differ in the size of the macrocyclic ring they contain. Binding affinities for the somatostatin receptors were found to be low; however one of the analogues inhibited neointima formation induced by balloon injury in rats (100 μg kgd^{-1}).[369]



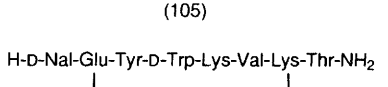
(104)



(105)



(106)



(107)

Cyclo-(Ala^6-Tyr^7-D-Trp^8-Lys^9-Val^{10}-Phe^{11}-ψ-[CN_4]), a cyclic hexapeptide analogue of somatostatin incorporating the 1,5-disubstituted tetrazole as a *cis*-amide bond isostere, has been synthesised and its solution structure investigated by NMR. The results indicated a similarity between the tetrazole geometry and the *cis*-amide solution conformation. The peptide possessed 83% of the activity of somatostatin.[370] The synthesis of octreotide and its D-Phe^1 analogue has been reported using solid-phase methods, involving Fmoc-protection. The cleavage from the resin was achieved by aminolysis with threoninol.[371]

6.8 Bradikinin Analogues – The synthesis by solid-phase Fmoc-chemistry of two bradikinin-like kinins isolated from *Vespa analis* (H-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-Val-Ile-OH, VSK-A) and *Vespa tropica* (H-Gly-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg-Val-Val-OH, VSK-T) and their cyclic analogues have been reported. The conformational properties were determined by CD measurements in trifluoroethanol and guanidinium chloride. The cyclic analogues were found to be 50-100 times less potent than their linear analogues.[372] The synthesis of cyclic analogues of Thr^6-bradikinin, N^{ε}-Lys-bradikinin and *endo*-Lys^{8a}-vespulakinin 1 were prepared by solid-phase Fmoc chemistry. The peptides and glycopeptides were characterised by amino-acid analysis, optical rotation, analytical HPLC and FAB-MS.[373] Bradikinin analogues have been reported whereby the modifications involved cyclisation of the backbone. For example, lactam bridges were obtained using *N*-(CH_2CO_2H)Phe and *N*-($CH_2CH_2NH_2$)Phe residues. The high biological activities of some of these analogues implied that the receptor bound conformation of bradikinin agonist does involve a β-turn in the *N*-terminal sequence.[374]

6.9 Miscellaneous Examples – The X-ray crystal structure (at 2.0 angstroms resolution) of recombinant human interleukin has been determined.[375] The production of interleukin-1 from human peripheral mononuclear cells has been induced from three synthetic cyclic peptides, each containing a γ-glutamyl residue.[376] The solution structure of the B-chain of bovine insulin has been determined by ^{1}H NMR spectroscopy combined with simulated annealing calculations. A detailed description of the conformational results is given.[377]

The *C*-terminal region of the human C5a anaphylatoxin (C5a65-74) has been mimicked by a series of conformationally restricted decapeptide analogues. The analogues behaved as agonists and provoked human neutrophil response, implying that the C5a receptors respond to topochemical features presented by the agonist peptide ligand.[378] The 835-846 region of the chicken gizzard myosin heavy chain has been suggested as important for the formation of the 10*S* folded conformation of smooth muscle myosin. This was concluded based upon the result of studies effected upon two, 12-residue synthetic peptides, corresponding to the 821-835 and 835-846 regions of the myosin heavy chain.[379] The structural determination of tick anticoagulant peptide (TAP) was determined using NMR techniques including homo- and heteronuclear two-dimensional NMR spectra and constant time heteronuclear single quantum correlation spectroscopy.[380]

Site-directed mutagenesis and crystallographic analysis have been utilised in identifying the catalytic and oligosaccharide recognition residues of the peptide N^4-(*N*-acetyl-β-D-glucosaminyl)asparagine amidase F (PNGase F). This is a peptide which removes asparagine-linked oligosaccharide chains from glycoproteins and glycopeptides.[381] Two new echistatin variants (echistatin β and γ) have been isolated from the venom of *Echis carinatus leakeyi*. These proteins were found to be similar in amino acid sequence to echistatin α as shown in **Figure 4**. It was found that all the echistatins were adept at inhibiting the aggregation of platelets from human and other mammals but with different potencies.[382] The glycopeptide antibiotic teicoplanin has been investigated by NMR and molecular modelling techniques. Two conformer structures were deduced, which possessed different binding affinities for cell wall analogues.[383] The synthesis of four major histocompatibility complex (MHC) class I binding glycopeptides and two peptide analogues from a cytotoxic T-lymphocyte (CTL) epitope of Sendai virus nucleoprotein have been described. Also, solution conformation studies using NMR on K3 H-Phe-Ala-Pro-Ser-Asn-Tyr-Pro-Ala-leu-OH, K3-O-GlcNAc and K3-O-GalNAc showed two conformations, due to *cis-trans* isomerisation about the Tyr-Pro amide bond.[384]

Fragments of the vasoactive intestinal polypeptide, a 28-residue linear peptide, have been synthesised. In tests, residual activity was observed for some of the fragments.[385] A series of analogues of kanamycin-A and netilmicin, involving mainly residue substitutions, has been prepared. The compounds were analysed by NMR techniques, and the conformational differences were thought to account for the lack of activity displayed by the analogues.[386] Tests on analogues of the autoinhibitory element of phosphatase calcineurin, formed by residue substitutions, or fragmentation, indicated that most of the 25 amino acid portion is required for inhibition.[387] Site directed mutagenesis has been applied to generate biologically active mutants of insulin that remain monomeric at millimolar concentrations in solution, and that are therefore not affected conformationally by aggregation. The conformation of these analogues has been studied by NMR and compared with solid state conformations.[388]

Over a hundred analogues of *N*-Ac-D-Nal-D-Nal-D-Cpa-D-Pal-Ser-Lys(Nic)-D-Lys(Nic)-Leu-Lys-Pro-D-Ala-NH_2 antide, antagonist of LHRH, have been reported. Analogues included modifications of the Nic group with changes in hydrophobicity or basicity. Also, cyclic 5/6 analogues were prepared. The most potent compounds resulted from substituting the Nic group at positions 5 or 6 with 8-Qis or 2-Pyc moieties.[389] The structural requirements for the IKVAV sequence of lamini-I involved in cell adhesion, neurite outgrowth and tumour growth have been investigated by the preparation of various 12-residue synthetic peptides, including analogues containing D-amino acids. The results indicated that the lysine and isoleucine residues are crucial for biological activity of the IKVAV peptide.[390]

The peptide poly(Ser-Pro-Thr-Ser-Pro-Ser-Tyr), whose sequence corresponds to the *C*-terminus of a subunit of RNA polymerase, and is crucial for *in vivo* activity, has been synthesised. The repeat unit was investigated by CD and theoretical conformational analysis. The authors speculated that the character-

istic repetitive turn structure, is critical for its function mechanism.[391] Analogues of α-factor H-Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-OH have been synthesised using solid-phase methods, with the photoactivable *para*-benzoylphenylalanine (Bpa) group and 3-(mono- or diiodo-4-hydroxyphenyl)propanoic acid or biotin as a tag. Two of the analogues, were found to exhibit an order of magnitude lower affinity for the α-factor receptor. The analogues were also much less active than α-factor, indicating that they would be suitable as agonist probes.[392]

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4
Cyclic, Modified and Conjugated Peptides

BY J.S. DAVIES

1 Introduction

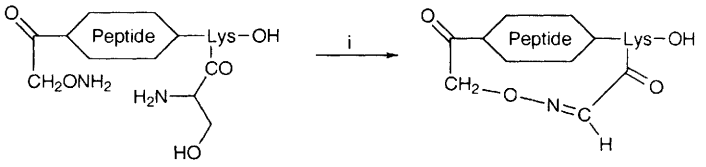
The coverage of the literature allocated to this Chapter is very similar to recent Chapters in this series and has not necessitated any great change in the sub-divisions within the Chapter. The emphasis again has been on naturally occurring molecules, their stucture elucidation, their synthesis and conformation and an increasing interest in rationalising interactions with receptor molecules. Cyclic analogues used as constraints for biologically active linear domains are, in the main, covered in Chapter 3, although some overlap here is inevitable. Phosphorylated and glycosylated peptides continue as major areas of endeavour. All sections of the Chapter are testament to the impressive use of advances in NMR and X-ray techniques. Almost all the structural elucidations reported now use this 'integrated' approach, involving an array of techniques, often benefiting from high performance liquid chromatography in the purification stages. Many congeners within molecular families are being revealed as a result of the improved technology available.

The total number of papers reviewed has not varied much over recent years, and again has reached over 200 using CA Selects[1] on Amino Acids, Peptides and Proteins (up to Issue 13, June 1996) as the main source of retrieval. Manual scanning of indices of mainstream journals made the retrieval more comprehensive, although patents and conference proceedings have not been included. Academia contributed about 80% of the publications. Less than 20% of the papers were retrieved from peptide/protein journals.

2 Cyclic Peptides

2.1 General Considerations – Cyclopeptides from marine sources have been a growth area in recent years, and a review[2] of the synthetic methods used to confirm the structures of marine cyclopeptides and depsipeptides assists greatly in focussing on what has been achieved within the various families of molecules discovered. In a tribute[3] to Professor K.M. Sivanandaiah's (Bangalore) contributions to peptide chemistry on the Indian sub-continent, a Chapter on the solid phase synthesis of cyclic peptides has been included. While this approach to synthesis, or more often the building up of a linear precursor sequence on a

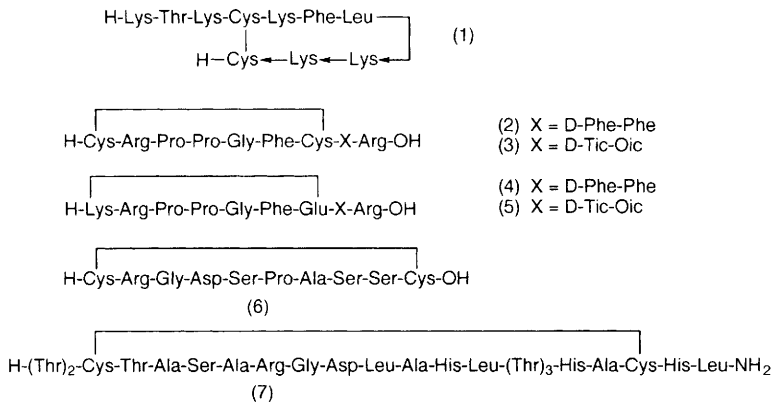
polymer followed by solution phase cyclisation using chemical activation is the norm, other options have also been reported. Enzymatic cyclisation[4] using a mutant of subtilisin BPN' (subtiligase) has yielded cyclic peptides containing between 12 and 25 residues. The linear peptide precursors were cyclised as C-terminal glycolate phenylalanyl amide esters and the best yield (85%) was obtained with the longer peptides, presumably due to the increased flexibility of the longer chains. A practical method[5] for cyclisation of totally unprotected peptides in aqueous buffer, as shown in Scheme 1, produces a metabolically



Reagents: i, aq IO_4^- /pH 6.8

Scheme 1

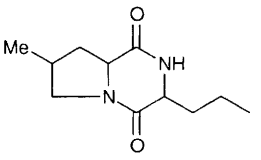
stable oxime bond in a very chemoselective manner. Although this Chapter has not regularly reviewed comprehensively, the role of disulfide links in constraining conformational freedom, there is no doubt that authors in general refer to cystine containing peptides as cyclic peptides. Thus when three different cyclic peptide libraries were constructed[6] in an M13 phage display system, the presence of flanking cysteine residues allowed efficient disulfide formation and cyclisation during assembly. Sequences were discovered (all containing HPQ) which could bind to streptavidin more efficiently than their linear counterparts. The bound cyclic peptides, cyclo(Ac[CHPQGPPC]-NH_2) and cyclo(Ac[CHPQFC]-NH_2) were also shown[7] to adopt a β-turn. Cyclic disulfide peptides having structures cyclo(Cys-Pro-Xxx-Cys) with Xxx = Gly, Phe or D-Phe have been studied[8] by vibrational circular dichroism (VCD) and show solvent-dependent conformational characteristics with some evidence of β-turns. A rectangular shape, stabilised by hydrogen bonds and the five hydrophilic lysine residues has been deduced[9] using 2D-NMR and distance geometry calculations for the polymyxin analogue (1). A comparison has been made[10] between disulfide and lactam bridges at the N-terminal sequence, providing a new bradykinin antagonist. The synthesis of (2) and (3) involved closing the disulfide bridge with iodine while the peptide was on a solid support, whereas the deprotected side chains of Lys and Glu were coupled to form (4) and (5) using TBTU. Cyclic analogues were mildly active antagonists with the lactam bridge being more stable than the disulfide bridge. Iodine oxidation of linear precursors also provided[11] analogues, based on changing residues at positions 5 and 6 in the highly active platelet aggregation inhibitor (6). Phe inserted in position 5 provided the highest activity. In a comparison[12] of methodologies for the synthesis of a mimic (7) of the G-H loop of foot and mouth disease virus the most successful route involved on-resin



disulfide formation with the thiol group protected by the fluorenylmethyl group giving better results than S-Acm. However, S-Acm cysteine units can form disulfide bonds directly[13] if treated with silver trifluoromethane sulfonate and DMSO/HCl.

2.1 Dioxopiperazines (Cyclic Dipeptides) – The Caribbean sponge, *Calyx cf. podatypa* has yielded[14] a new dioxopiperazine (8) amongst its half-dozen proline-derived cyclic dipeptides. Similarly, the sponge *Dysidea fragilis* is a rich source of polychlorinated dioxopiperazines, with compound (9) being one of the latest identified.[15] The structures of the first sulfur-containing dioxopiperazines from a marine species (*Streptomyces* sp B9173) have been established[16] and named maremycin (A) (10) and B (11).

The amorphous nature of cyclo[(*S*)-His-(*S*)-Phe] (12) has prevented its conformation being determined by X-ray techniques. However, a solid state



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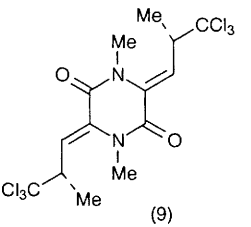

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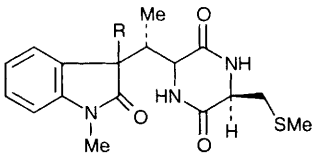
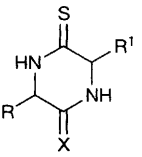
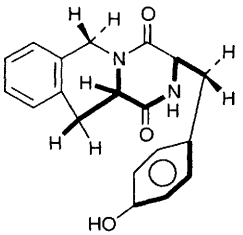


(10) R = ---OH (11) R = —OH

NMR study[17] has revealed that the catalytically active form of (12) in the asymmetric addition of HCN to aldehydes is the same as its previously reported solution phase conformation. An X-ray determination[18] was possible for cyclo(Ac-L-Ala-L-MeAla), which was shown to be a boat conformation; the alanyl side-chains occupy pseudo-axial orientations and the dioxopiperazine ring is folded by an angle of $-29.1°$ between the planes containing the endocyclic amide bonds. Quantitative HPLC techniques, CD and NMR studies have confirmed[19] extensive racemisation during the formation of mono- and di-thiones (13) from the corresponding dioxopiperazines. The solution and solid state conformation of cyclo(Tyr-Tic), where Tic is the constrained analogue of phenylalanine has been defined[20] as (14). This is quite different from the biologically active linear dipeptide form. The formation of dioxopiperazines containing the Tic (tetrahydroisoquinoline-3-carboxylic acid) residue has also been studied kinetically[21] and has been found to be catalysed by the presence of buffers and carboxylic acids.



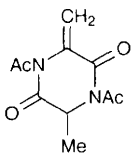
(13) X = O or S



(14)

The methylene dioxopiperazine (15) has been synthesised[22] and shows excellent chiral induction when side-chains are added to the exocyclic double bond using $R^1HgCl/NaBH_4$. Enantiometrically pure epoxides can also be obtained[23] from (16) when the dioxopiperazines are reacted with 3,3-dimethyloxirane. A specific inhibitor of glycogen phosphorylase, with the spirodioxopiperazine structure (17) has been synthesised[24] in a chirally pure form. The spiro compound showed no inibition of α- and β-glucosidases, galactosidases, β-N-acetylglucosaminidase, pectinase, xylanase or cellulase. Confirmation of the stereochemistry of the cycloadducts in a series of 2-azabicyclo[2,2,1]hept-5-enes has come from their coversion[25] to dioxopiperazines having structures such as (18). A detailed analysis[26] of the positive ion mass spectra of dioxopiperazines from FAB and collision-induced dissociation has been reported.

2.3 Cyclotripeptides – A series of template molecules based on the triprolyl sequence (19) has been prepared[27] as potential N-terminal template to induce helix nucleation. The macrocyclisation step was carried out at point 'a' in the structure using a *p*-nitrophenylester/HOBt coupling. X-ray analysis confirmed a very strained conformation with the *cis-cis-trans* conformer being the most stable, but an NMR determination in solution showed two forms equilibrating slowly with



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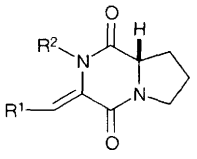

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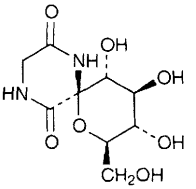
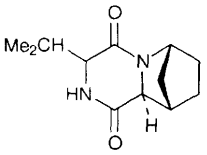
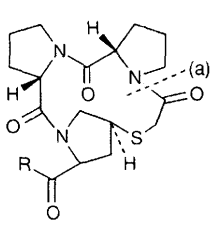


(17)

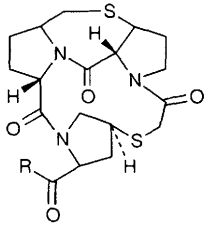


(18)

evidence that only one form brings about nucleation with di- and tri-Ala conjugates. A molecular modelling-based redesign[28] of (19) introduced an extra -CH_2-S- bridge, as seen in (20), to restrict the number of *cis-trans* amide conformations available. The all-*trans* conformer proved to be an optimal initiation site for an α-helix, and a *cis-trans-trans* conformer an initiator of a 3_{10}-helix.

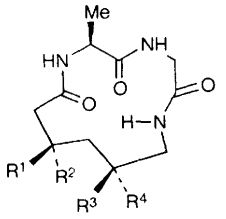


(19) R = OH or MeNH

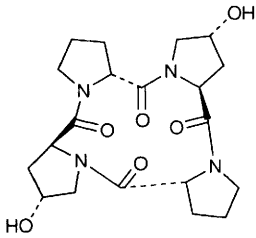


(20) R = OH or R = $(Ala)_n$–OR^1

In what can be defined as the formation of 'stretched' cyclotripeptides, a series of substituted 6-amino caproic acid containing tripeptides has been cyclised[29] at the C-terminal caproic acid residues using diethylcyanophosphonate for the cyclisation step to give the series of β-turn models (21) – (25). Depending on the substituents on the caproic acid, different proportions of type II and type I β-turns were in evidence. Compounds (21) and (23) favoured type II turns, while (22) favoured type I.



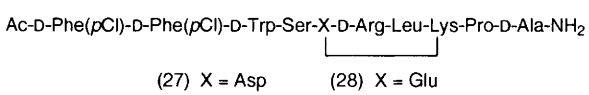
	R^1	R^2	R^3	R^4
(21)	H	H	H	H
(22)	H	Me	H	Me
(23)	Me	H	Me	H
(24)	Me	H	H	Me
(25)	H	Me	Me	H



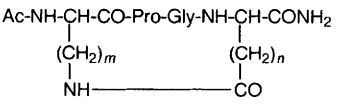
(26)

2.4 Cyclotetrapeptides – To overcome the lack of reactivity of dehydroamino acid units, an azlactone intermediate has been used in an improved synthesis[30] of tentoxin *cyclo*(MeAla-Leu-MeΔPhe-Gly). Cyclisation by activation of the glycine residue of the linear precursor with diphenyl phosphoryl azide (DPPA) gave a 17% yield under high dilution. A convergent solution phase synthetic route[31] to the dihydroxy cyclo(D-Pro-L-Hyp-D-Pro-L-Hyp) (26) also used DPPA for the cyclisation step. 2D NMR showed the favoured conformation as having the two hydroxyl groups pointing upwards out of the pseudo plane of the macrocyclic ring.

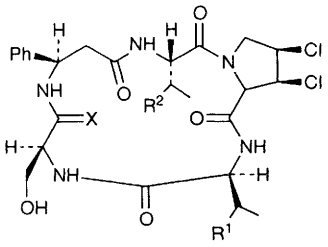
Although in excess of 3000 analogues of LHRH have been tested, there is still activity and interest as exemplified[32] by the conformational restriction of a known antagonist by incorporating a bridge between side-chains of residues 5 and 8. Both of the compounds (27) and (28) were more potent antagonists than their linear analogues and showed evidence of an unusual type II′ β-turn involving residues 3 to 6. The conformational consequences[33] of the 1 → 3 residue side-chain cyclisation in the models (29) have been investigated as a means of exploring biologically active cyclic analogues of the mating pheromone α-factor. Analogues (29) were synthesised by on-resin (4-methylbenzhydrylamine resin) cyclisation of appropriately protected linear precursors using the BOP reagent. A major side-product identified in each case was the cyclic dimer. Orthogonally chosen Fmoc, Boc, 9-fluorenylmethylesters (OFm) and t-butyl esters were used as protecting groups. Compound (29) $m = 2$, $n = 2$ assumed a type II β-turn while the others displayed distorted versions of this conformation.



(27) X = Asp (28) X = Glu



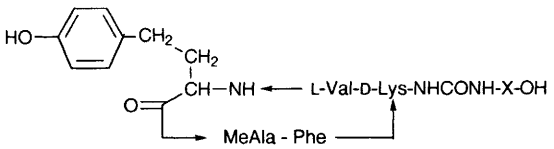
(29) m = 1–4, n = 1–2



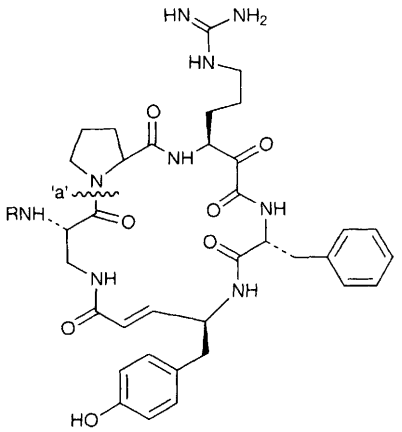
(30) R^1 = H, R^2 = OH X = O
(31) R^1 = OH, R^2 = H X = O
(32) R^1 = H, R^2 = H X = O
(33) R^1 = H, R^2 = OH X = S
(34) R^1 = OH, R^2 = H X = S
(35) R^1 = H, R^2 = H X = S

2.5 Cyclopentapeptides – A full report[34] of the structural elucidation of the astins A (30), B (31) and C (32) has appeared together with a conformational analysis[35] of astins A and C. The backbone conformations deduced from NMR and computational methods for these two congeners are different from their astin B analogue, which could be an indication as to why the latter has the highest anti-tumour activity of the three. Thionation of astins (30) – (32) with Lawesson's reagent has yielded[36] the corresponding thio-analogues (33) – (35) which showed a more promising antitumour activity than their oxygen counterparts. Two unidentified peaks[37] in the HPLC of an extract from the cyanobacterium *Anabaena flos-aquae* NRC 525-17, have been identified as being due to the cyclic peptides anaebonopeptins A (36) and B (37). These structures have a resemblance to kermamide A and konbamide isolated from Okinawan marine sponges.

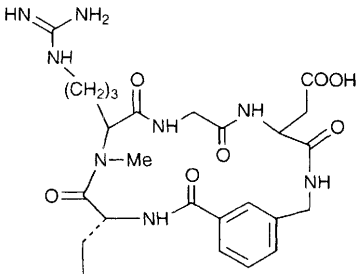
Three new cyclic peptide segetalins B-D have been identified[38] in the seeds of the higher plant *Vaccaria segetalis*. Segetalin B has been shown to be cyclo(Gly-Val-Ala-Trp-Ala) and C and D were found to be the cycloheptapeptides cyclo(Gly-Leu-His-Phe-Ala-Phe-Pro) and *cyclo*(Gly-Leu-Ser-Phe-Ala-Phe-Pro), respectively. Only segetalin B had estrogen-like activity similar to the previously reported segetalin A and their common feature is a Trp-Ala-Gly-Val sequence. The fresh roots of *Stellaria yunnanensis* have also yielded[39] stellarins F and G,



(36) X = Arg (37) X = Tyr



(38) R = CHO
(39) R = Ac

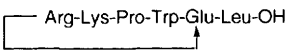


(40)

the former being identified as cyclo(Gly-Ala-Gly-Ser-Pro-Trp-Phe-Pro) and the latter cyclo(Gly-Ala-Tyr-Leu-Ala).

Their potential as thrombin inhibitors has initiated great interest in the cyclotheonamides as potential antithrombotic agents. Two research groups have independently reported convergent [3+2] fragment-condensation routes to cyclotheonamides A (38) and B (39) using cyclisation at point 'a' as almost the final major step. In the US-based work,[40] BOP-Cl under high dilutions gave a 65% yield in the cyclisation step to form (38) and (39), while the Netherlands group[41] cyclised a linear precursor to form (39) using TBTU/HOBt/DMAP. Both sets of researchers claim flexibility in their methodology for the preparation of more analogues. The antithrombotic cyclic peptide antagonist (40) of glycoprotein IIb/IIIa has also been synthesised.[42] The N-methylated argininyl side-chain was only produced in the later stages from guanylation of the corresponding N-Me-ornithine analogue using Z-NH-C(SMe)=N-Z.

The naturally occurring endothelin receptor antagonist cyclo(D-Trp-D-Asp-Pro-D-Val-Leu) has been subject to a structure-activity relationship study[43] through the synthesis of analogues such as cyclo[D-Trp-D-Glu-Ala-D-Val(or D-alle)-Leu]. It appears that the DDLDL chirality sequence is critical to inhibitory activity, and the most potent and selective analogue turned out to be cyclo(D-Trp-D-Asp-Pro-D-Thg-Leu) where Thg = 2-(2-thienyl)glycine. Structure-activity relationships of C-terminal cyclic neurotensin analogues revealed[44] that analogue (41), synthesised using a Boc-strategy on resin with side chain carboxylates

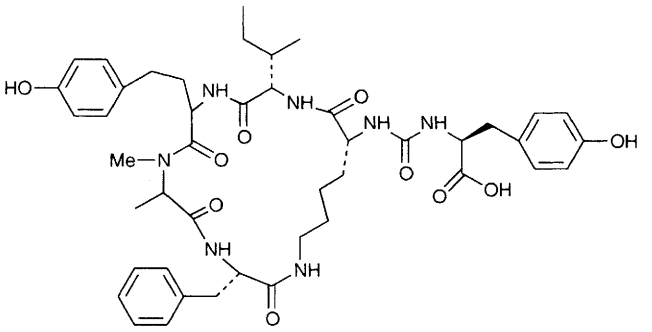


(41)

protected as 9-fluorenylmethyl esters, showed agonist activity *in vitro*. Cyclic analogues[45] of the immunostimulating tetrapeptide tuftsin, H-Thr-Lys-Pro-Arg-OH, have been synthesised to a design indicated by molecular modelling studies. Both cyclo(Thr-Lys-Pro-Arg-Gly) and cyclo(Thr-Lys-Pro-Arg-Asp) were synthesised with the former showing better potency in phagocytosis assays, possibly due to conformational effects. This enhanced potency has also been one of the conclusions of a molecular dynamics study.[46]

The 4-methyltrityl group, as an acid-sensitive lysyl side-chain protection, has been used[47] in the synthesis of side-chain to side-chain cyclic peptides and oligolysine cores suitable for solid phase synthesis of MAPs and TASPs (template-assembled synthetic proteins). The group can be removed in 1% trifluoroacetic acid, conditions that do not cleave t-butyl esters, 2-chlorodiphenylmethyl- and the Wang-resin. In the continuing demand for peptide library mixtures, the use of amino-caproic acid (Aca) at the C-terminal position linked to an oxime resin has released[48] a mixture of cyclic pentapeptides based on the sequence cyclo(Arg-Gly-Asp-Xaa-Aca), where Xaa was permutated between Ser, Lys, Gln, Ala, Glu, Pro, Val, Tyr, Phe and Trp(CHO). The mixtures produced were well-separated on HPLC, to an extent that it was possible to distinguish between monomer and dimer formation. The best ratio of monomer to dimer achieved was 91:9. Energy minimisation studies on hydrogen-bonded all *trans* cyclopentapeptide backbones have been carried out.[49]

2.6 Cyclohexapeptides – A toxic strain of freshwater *Oscillatoria agardhii* has yielded[50] oscillamide Y (42) which strongly inhibits chymotrypsin activity at 1×10^{-5} M. Although β-carbolines derived from Trp have familiar structures in marine alkaloids, the marine sponge *Cribrochalina olemda* has biosynthesised an unprecedented fused tetracycle[51] involving both Phe and Trp in the structure of kapakahine B (43). The compound is moderately cytotoxic against P388 murine leukemia cells. Key steps in the total synthesis[52] of RA-VII (44) and deoxybouvardin (45) have been the regioselective synthesis of the 14-membered biaryl ether. The synthesis of the biaryl ether was carried out by thallium trinitrate-mediated oxidation of tetrahalo-aryl substituted precursors. In an exhaustive study[53] of the effect of the N-methyl substituents at positions R^6, R^7 and R^8 in the cyclohexapeptide ring backbone, a complete set of seven N-desmethyl analogues of RA-VII (44) has been synthesised. The conformation of each analogue has been studied by 1- and 2D NMR, and it is interesting to note that the *cisoid* conformation of the N-methylated amide bond involved in the 14-membered diaryl ether ring is maintained in the N-desmethyl analogue [R^6 = H in (44)]. The conformations of other desmethyl analogues, *e.g.* [$R^6 = R^8$ = H in (44)] are also very similar to the single conformation seen for the native RA-VII. It is also revealed that it is the N-methyl group at position R^7 in (44) that dictates the *cisoid* conformation of the tertiary amide involved in the diaryl ether ring (*i.e.* position R^6). These observations therefore contrast with previously held views that it was the R^6-methyl which held the key to the *cisoid* conformation. In another study[54] the effect of modifying the 18-membered polypeptide ring of RA-VII has been assessed. Substitution of $(Gly)_4$ and $(Gly)_3$ for the D-Ala-Ala-



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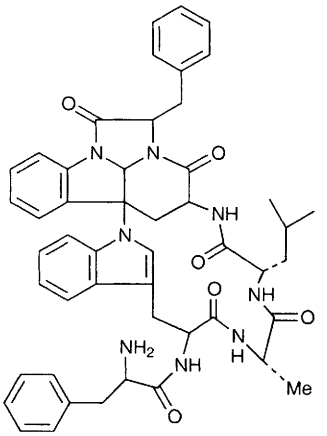
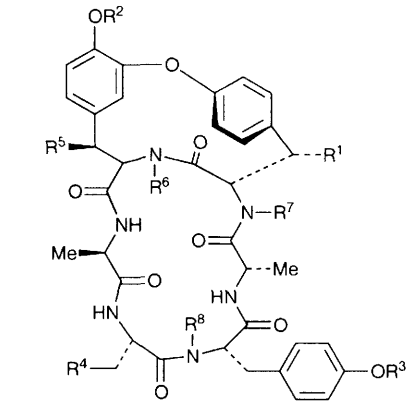


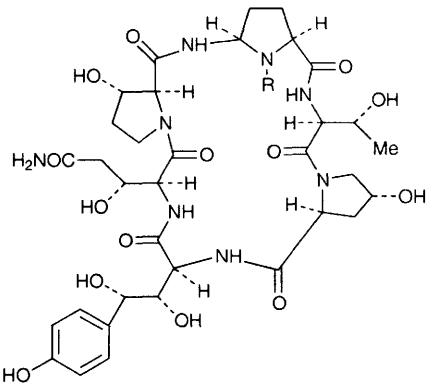
(43)

MeTyr(OMe)-Ala unit changes the conformation of the central amide bond in the cyclodityrosine subunit to the biologically inactive *trans* form. The conformational equilibrium of RA-VII (44) in d_8THF has been found[55] to be equivalent to that observed in conventional NMR solvents. However, on addition of one equivalent of LiCl, a single dominant conformation, corresponding to the major conformation in other solvents, was detected. This conformation closely resembles the X-ray structure previously published.

Structure (46) has been identified[56] as the acid-catalysed, internally cyclised dehydration product of pneumocandin B_0, a member of the echinocandin class of fungicidal cell-wall active lipopeptides. NMR techniques and restrained molecular dynamics simulations have been applied[57] to ascertaining the biologically active conformation of patellamides B and C from the marine tunicate *Lissoclinum patella*. At least two more conformers, types I and III as shown schemati-

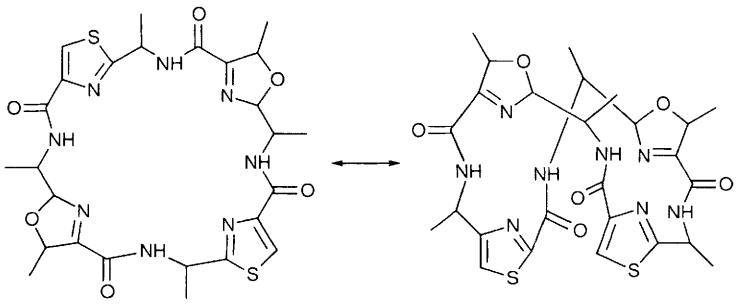


	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
(44)	H	Me	Me	H	H	Me	Me	Me
(45)	H	H	Me	H	H	Me	Me	Me



(46) R = 10,12-dimethylmyristoyl

cally in Figure 1, have been identified, with the tendency for patellamides B and C to show type III conformation in chloroform solutions. However, type I is more likely to exert biological activity since ulithiacyclamide (which has a disulfide bond across the ring), the most potent cytotoxic cyclopeptide, can only exhibit type I. In a conformational analysis[58] of segetalin A, cyclo(Gly-Val-Pro-Val-Trp-Ala), using X-ray methods, high field NMR and molecular dynamics, two β-turn structures have been identified. In the solid state the molecule showed type I and type VI β-turns, fixed by two transannular H-bonds between Gly^1 and Val^4, while in the solution state the β-turns were of type II and type VI. Fifteen cyclic hexapeptides have been used[59] to mimic the conformation of the active



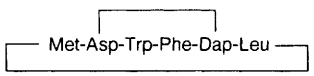
type I type III

Figure 1

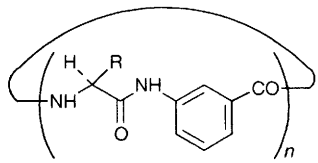
tetrapeptide sequence -Ser[17]-Trp[18]-Arg[19]-Tyr[20], found at the first hairpin loop of the α-amylase inhibitor tendamistat (HOE 467). Ten of the cyclic peptides without the Arg residue were synthesised on solid phase (Boc strategy) and cyclised *via* the azide method, while a convergent strategy in solution was used for Arg-containing analogues. Three cyclopeptides cyclo(D-Pro-Ala-Ser-Trp-Arg-Tyr), cyclo(Pro-D-Ala-Ser-Trp-Arg-Tyr) and cyclo(D-Ala-Pro-Ser-Trp-Arg-Tyr), were in agreement with the native conformation, and all three showed a small but significant α-amylase inhibition. However, there could be more surface residues of tendamistat involved in further bonding to α-amylase. A relatively new spectral technique, vibrational circular dichroism (vcd) has been applied[60] to the ion-carrying cyclic hexapeptide model cyclo(Pro-Gly)$_3$. An advantage of the technique is its sensitivity to short range structural order.

Cyclo(His-Gly-Asp-Ser-Gly-Asp) has been synthesised[61] on a solid support using the BOP reagent for ring closure. The α-carboxyl group of Asp had been protected as a fluorenylmethyl ester. On the basis of the bioactive conformation of the known neurokinin A receptor antagonist cyclo(Met-Gln-Trp-Phe-Gly-Leu), a much more potent antagonist (47) has been designed and synthesised.[62] Both the solid and solution state conformations are almost identical, characterised by quite a rigid conformational profile. Potent, cyclic hexapeptide analogues of somatostatin are believed to adopt a II′β-turn at one end and a type VI turn with a *cis* amide bond at the other. To confirm the need for the *cis* amide a constraining tetrazole surrogate has been introduced[63] by the synthesis of cyclo(Ala[6]-Tyr[7]-D-Trp[8]-Lys[9]-Val[10]-Phe[11]-ψ[CN$_4$]) which was shown to have 83% of the activity of somatostatin. All conformational data confirmed the similarity of the tetrazole geometry to that of a *cis* amide in solution.

The rigid 3-aminobenzoic acid residue (Aba) has been used[64] to control the orientation of amino acid residues and to act as a spacer group in the design of functional peptides. A typical general formula to represent a series of compounds synthesised is (48), where the natural amino acid component was L-Ser, L-His or L-Asp residues. The cyclohexapeptides, cyclo(Ser-Aba-Ser-Aba-Ser-Aba), and



(47) Dap = L-2,3-diaminopropanoic acid



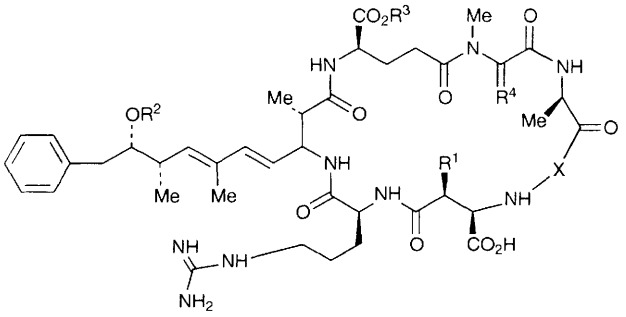
(48)

cyclo(Ser-Aba-His-Aba-Asp-Aba), were synthesised by stepwise synthesis on an oxime resin with cyclisation being achieved when the deprotected loaded resin was treated with acetic acid/triethylamine for one day. A cyclic octapeptide with an alternating Ser, His, Asp, Ala and Aba proved to have the highest hydrolysis activity for *p*-nitrophenylacetate in a 2nd order reaction. The neutral analogue cyclo(Ala-Aba)$_3$ bound with 4-nitrophenylphosphate with a very high binding constant.[65]

2.7 Cycloheptapeptides – Marine sources have provided a rich source of microcystins whose structural family continues to expand[66] as a result of larger scale isolations. Thus, in the water bloom of *Microcystis* spp. five previously reported microcystins have been characterised together with seven more family members. It is therefore opportune to list the current members as in Figure 2. *Oscillatoria agardhii* has also yielded[67] a microcystin RR (in Figure 2, X = Arg, R^1 = H, R^2 = Me, R^3 = H, R^4 = CH-Me). The sequence of the cyclic heptapeptides cyclo(Asn-Pro-Phe-Val-Val-Pro-Val) axinastatin 1 (or pseudoaxinelline) cyclo(Asn-Pro-Pro-Phe-Val-Val-Val), called malaysiatin, have been queried.[68] Both have been synthesised using TBTU/HOBt for cyclisation and analysed by physical techniques, and the results confirm that the structure of axinastatin was correct but the structure assumed for malaysiatin differed significantly from the natural product. A weakly cytotoxic compound from the marine ascidian *Didemnum molle* has been given[69] the structure (49). Cyclo(Pro-Leu-Ile-Phe-Ser-Pro-Ile) has been shown[70] by extensive physical methods to be the structure of stylopeptide 1 isolated from Pacific Ocean sponges *Stylotella* sp. and *Phakellia costada*.

Segetalins C and D from the seeds of *Vaccaria segetalis* have been shown[38] to be cyclo(Gly-Leu-His-Phe-Ala-Phe-Pro) and cyclo(Gly-Leu-Ser-Phe-Ala-Phe-Pro), respectively. A crystal and solution structure have been assigned[71] to pseudostellarin D, cyclo(Gly-Tyr-Gly-Pro-Leu-Ile-Leu). A type II β-turn between Leu^7 and Gly^1 and a type I β-turn between Pro^4 and Leu^5 have been identified both in the solid and solution states. Stellarin A[72] from the fresh roots of *Stellarina yunnanensis* has previously been characterised as a cycloheptapeptide, but further studies have revealed the B and C congeners in the family to be two cyclohexapeptides cyclo(Gly-Ser-HOIle-Phe-Phe-Ala) and cyclo(Gly-Ser-HOIle-Phe-Phe-Ser), respectively.

2.8 Cyclooctapeptides – A combination of 2D NMR and mass spectrometry techniques have confirmed[39] the structure of stellarin G from the roots of



	X	R^1	R^2	R^3	R^4
microcystin LR	Leu	Me	Me	H	CH_2
DM Adda 5 microcystin LR	Leu	Me	H	H	CH_2
microcystin RR	Arg	Me	Me	H	CH_2
microcystin AR	Ala	Me	Me	H	CH_2
microcystin FR	Phe	Me	Me	H	CH_2
[LMeSer[7]] microcystin LR	Leu	Me	Me	H	H,CH_2OH
[D-Glu-OC_2H_3(Me)OH)[6]]microcystin LR	Leu	Me	Me	C_2H_3(Me)OH	CH_2
microcystin H_4 YR	X^1	Me	Me	H	CH_2
[D-Asp[3]] microcystin LR	Leu	H	Me	H	CH_2
microcystin Hil R	X^2	Me	Me	H	CH_2
MeLan[7] microcystin LR	Leu	Me	Me	H	H, Z (R,R)

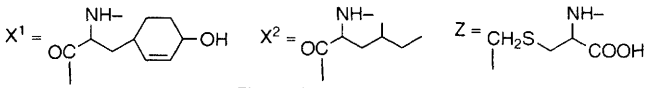
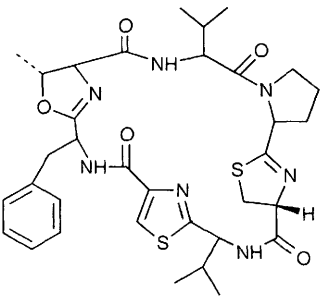
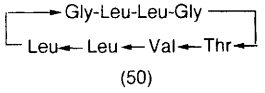


Figure 2

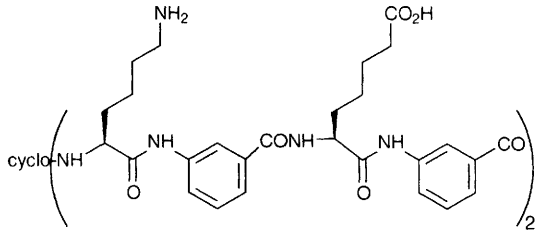


(49)

Stellaria yunnanenis as cyclo(Gly-Ala-Gly-Ser-Pro-Trp-Phe-Pro). The latex of *Jatropha curcas* has yielded[73] curcacycline A (50) which displays a moderate inhibition of classical pathway activity of human complement and proliferation of human T-cells. The cyclo pseudo-octapeptide (51) has been synthesised[74] from a linear precursor (Lys was N-terminal) in the solution phase using benzotriazol-



(50)



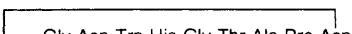
(51)

1-yloxytris(trimethylamino)phosphonium hexafluoro phosphate/HOBt in DMF for 30 min. A 4α-helix bundle structure was built on to the Lys and amino pimelic side chains, and after treatment with methanol single α-helices could be deduced from the fluorescence of pyrenylalanines built into the chains.

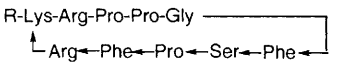
A deletion analogue of the cyclolinopeptide has been designed and investigated[75] by 2D NMR. The cyclooctapeptide, cyclo(Pro-Pro-Phe-Phe-Ac_6c-Ile-D-Ala-Val) includes the bulky 1-aminocyclohexane-1-carboxyl residue (Ac_6c) and a D-Ala residue to study their effect on the conformation of the Pro-Pro-Phe-Phe sequence, when compared to cyclolinopeptide A. Only one set of sharp signals was observed in both acetonitrile and chloroform, and the conformation could be interpreted as the Ac_6c reinforcing ring rigidity, keeping the Pro-Pro-Phe-Phe segment as in the natural cyclolinopeptide A. Arrangements for the formation of parallel and anti-parallel β-sheets have been studied[76] following the synthesis of cyclo[(L-Phe-D-MeAla)$_4$] from H-(L-Phe-D-MeAla)$_4$-OH using 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/HOBt for 12 h (70% yield). Under appropriate conditions the N-methylated cyclooctapeptide self assembled into a discrete soluble cylindrical dimer (non-polar solvents) or into a unique porous crystalline form having an ordered parallel array structure.

2.9 Cyclononapeptides and Cyclodecapeptides – The crystal of [Aib^{5,6}, D-Ala^8] cyclolinopeptide A, having structure cyclo(Pro-Pro-Phe-Phe-Aib-Aib-Ile-D-Ala-Val), grown from benzene/acetonitrile mixtures[77] has undergone X-ray analysis, and the conformation is similar to that obtained from MeOH/H_2O. The Pro^1-Pro^2 unit was in the *cisoid* form with all other amide bonds *trans*.

A novel and selective endothelin type B receptor antagonist from *Streptomyces* spII, given the code RES-701-2 has been shown[78] to incorporate a cyclic nonaresidue structure (52). The availability of multi-gram quantities of the previously reported RES-701-1 (53), has provided[79] the opportunity of reassessing its inhibition of ET_B receptors. It is now reported that it possesses only micromolar affinity for either human ET_A or ET_B receptors. Cyclic analogues of Thr^6-



(52) R = Trp-Phe-Phe-Asn-Tyr-Tyr-Htp-OH Htp = 7-hydroxytryptophan
(53) R = Trp-Phe-Phe-Asn-Tyr-Tyr-Trp-OH

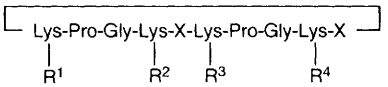


(54) R = H
(55) R = H-Thr-Ala-Thr-Thr-$(Arg)_3$-Gly-
(56) R = H-Thr-Ala-$[Thr(\beta\text{-Gal})_2]_2$-$(Arg)_3$-Gly-

bradykinin, N^{ε}-Lys-bradykinin and endo-Lys[8a]-vespulakinin have shown[80] significantly less potency than their corresponding linear analogues when tested on smooth muscle preparations. In this work, Fmoc-based linear assembly on solid phase followed by cyclisation with DPPA yielded compounds such as cyclo(Phe-Arg-Arg-Pro-Pro-Gly-Phe-Thr-Pro) and compounds (54) – (56).

Antamanide and cyclolinopeptide A share the same postulated active sequence -Pro-Pro-Phe-Phe-, and have justified a further study of the conformation of analogues incorporating this sequence. Thus, cyclo[Pro-Phe-Phe-Ala-$Glu(OBu^t)_2]_2$ has been synthesised[81] as a cyclolinopeptide analogue, and X-ray diffraction and NMR studies have shown that the conformation (both in solid and solution) allows all amides to be *trans* with two β-turns (one type I and one of type II). The analogue shows similar biological activity to the natural compound. It has already been established that reducing the size of the gramicidin S ring reduces antibiotic activity. When residues were added[82] to the ring, as in cyclo(Val-Orn-Leu-Leu-D-Phe-Pro-Val-Orn-Leu-D-Phe-Pro), the activity was twice that of gramicidin S against *S. epidermidis* Sp-al-1 and *B. subtilis* ATCC 6633.

Cyclic decapeptides of the general structure (57) incorporate four orthogonally protected lysine residues and represent[83] Regioselectively Addressable Functionalised Templates (RAFTs) for use in the TASP concept. Final macrocyclisation to form the cyclopeptide was achieved using PyBOP at high dilution.

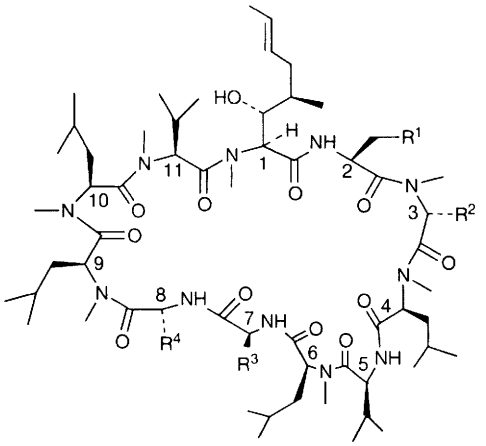


(57) R^1 = Boc, Fmoc; R^2 = Alloc, Boc, Dde; R^3= Boc;
R^4 = Alloc, Boc; X = Ala, Lys (Alloc)

2.10 Cyclododeca- and Cyclotetradecapeptides – It has been reported previously that gramicidin S analogues $cyclo(Leu\text{-}Orn\text{-}Leu\text{-}Orn\text{-}D\text{-}Phe\text{-}Pro)_2$ and $cyclo(Leu\text{-}Orn\text{-}Leu\text{-}Orn\text{-}Leu\text{-}D\text{-}Phe\text{-}Pro)_2$, inhibit bacterial growth with high potency. Two further analogues, where the Orn residues were replaced by Lys residues, have now been synthesised[84] but neither showed antimicrobial activity although there were no differences in their conformation. It is speculated that the hydrophobic/hydrophilic balance might have changed in the replacement. Two cyclic analogues of wasp kinins from *Vespa analis* and *Vespa tropica* have been synthesised.[85] The linear analogues H-Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-

Arg-Val-Ile-OH (VSK-A) and H-Gly-Arg-Pro-Hyp-Gly-Phe-Ser-Pro-Phe-Arg-Val-Val-OH (VSK-T), were first synthesised using Fmoc-based procedures on solid phase followed by cyclisation using DPPA/K_2HPO_4. The cyclic(VSK-A) and cyclic(VSK-T) were much less potent than their linear counterparts and behaved like kinase inhibitors by preventing the degradation of straight kinins.

2.11 Higher Cyclic Peptides – Apart from one reference in this section, this year is dominated by studies of the cyclosporins. But considering that they have been subject to over 20,000 publications already, the trawl of papers this year seems about normal. It is known that the contact surface between cyclosporin A (58) and its intracellular receptor protein cyclophilin A has a unique cavity between the molecules in the vicinity of the Abu residue [residue 2 in (58)]. To provide additional interactions in this pocket analogue (59) has been synthesised[86] and compared with (58), (60) and (61). Analogue (59) had lower binding affinity for cyclophilin A. Modifications in residues 3, 7 and 8 have also been made[87] to study the effect of multiple residue replacement adjacent to the effector region. Analogues (62) – (65) were synthesised for this purpose, with cyclisation being effected between residues 7 and 8 by propylphosphonic anhydride/DMAP in 30-70% yield. Although the changes in position 3 and 7 proved detrimental to activity, when all three modifications were made as in (65), this analogue bound



(58) cyclosporin(CsA), R^1 = Me, R^2 = H, R^3 = Me, R^4 = Me

(59) [(5-hydroxynorvaline^2) CsA, R^1 = CH_2CH_2OH, R^2 = H, R^3 = Me, R^4 = Me

(60) [norVal]^2 CsA, R^1 = Et, R^2 = H, R^3 = Me, R^4 = Me

(61) [norVal^2],[DMeSer^3] CsA, R^1 = Et, R^2 = CH_2OH, R^3 = Me, R^4 = Me

(62) [Phe^7] CsA, R^1 = Me, R^2 = H, R^3 = $PhCH_2$, R^4 = Me

(63) [DMeAla^3, Phe^7] CsA, R^1 = Me, R^2 = Me(D), R^3 = $PhCH_2$, R^4 = Me

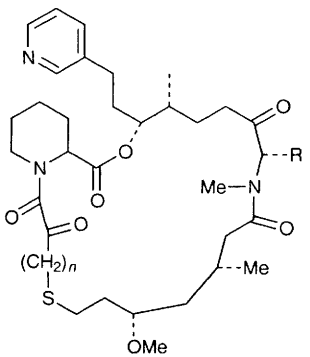
(64) [D-Ser^8, Phe^7] CsA, R^1 = Me, R^2 = H, R^3 = $PhCH_2$, R^4 = CH_2OH

(65) [D-MeAla, Phe^7, D-Ser^8] CsA, R^1 = Me, R^2 = Me(D), R^3 = $PhCH_2$, R^4 = CH_2OH

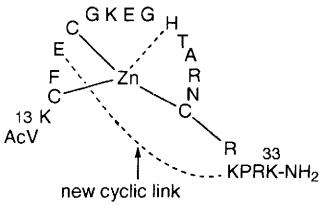
more tightly (K_i value of 3 ± 1.5 *versus* 6 ± 2 nM). Synthesis of such a combination of N-methylamino residues as in (58) has proved difficult in the solid phase, but with the availability of HOAt or HATU solid phase synthesis[88] has been achieved with better coupling yields using double couplings. A linear undecapeptide required as a precursor to [MeLeu^1]CsA has been synthesised using these reagents and cyclised at -Ala^7-D-Ala^8- using propyl phosphonic anhydride/DMAP in 10-15% yield.

A new metabolite of *Tolypocladium terricola* has been shown[89] to have a cyclosporin D backbone but the first residue (MeBmt) is present as a hydroperoxy derivative, 3-hydroxy-7-hydroperoxy-4-methyl-2-methylamino-5*E*-octenoic acid. PSC 833, [3-*O*-didehydro-MeBmt^1, Val^2] cyclosporin has previously been found to be sensitive to multi-drug resistant cells *in vitro* and is ten times more active than (58). An X-ray analysis[90] of PSC-833 showed that it has a similar backbone conformation to cyclosporin. The relevant features of cyclosporin and FK506 have been combined[91] in (66) with both the compounds (n = 1 and 2) showing binding to FKBP12 with moderate affinity, but showing no affinity for calcineurin. Several analogues utilising modification of residue 1 (MeBmt) in cyclosporin have been prepared. Thus, replacement[92] of the OH group in MeBmt with SH using Lawesson's reagent reduced activity by 40-100 times. Other modifications[93] also proved unsuccessful. However, a series[94] of *O*-alkylated ethers of the serine residue in [D-Ser^8] cyclosporin A further modified to form cyclic ethers at position 8 did show some enhancement of activity *in vitro*. But when a bridged lactone was formed[95] between MeBmt^1 and Ser^8, as in [3(*R*),6-dihydroxy-4(*R*)-methyl-2(*S*)-(methylamino)hexanoic acid^1]-[O-(carboxymethyl)-D-Ser^8] cyclosporin, this again was considerably less active than the parent compound. Additional methods[96] using FAB/linked-scan mass spectrometry have been developed to distinguish between isobaric residues, *e.g.* between Ala and MeSar.

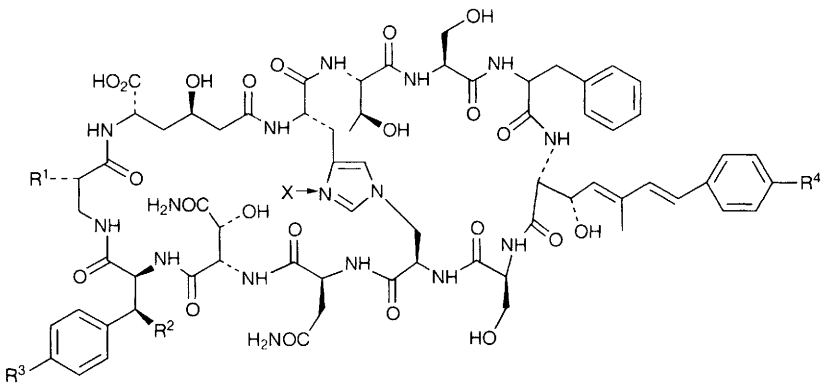
A cyclic peptide analogue[97] of (67), cyclo[Glu^{17},Lys^{30}(13-33)NCp7] of the proximal zinc finger of nucleocapsid protein NCp7 of HIV-1 virus, has been synthesised in the solid phase using Fmoc/Boc chemistry. Structures of the cyclic and native peptides complexed with Co^{2+} and Zn^{2+} have been studied by 2D NMR and visible spectroscopy. Results show that the cyclic peptide retained



(66)



(67)

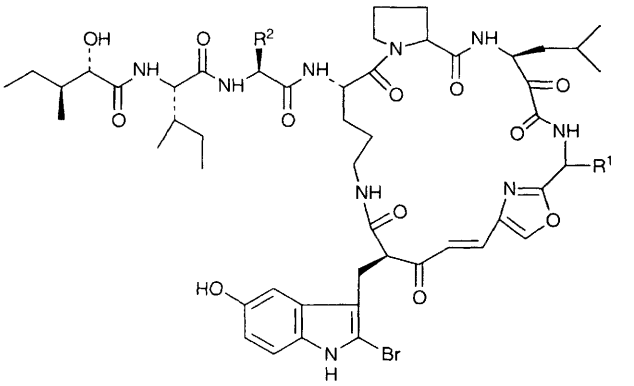


	R¹	R²	R³	R⁴	X
Theonellamide F	H	H	Br	Br	–
Theonellamide B	OH	Me	Br	H	–
Theonellamide C	H	H	H	Br	–
Theonellamide A	OH	Me	Br	H	β-D-Gal
Theonellamide D	H	H	Br	Br	β-L-Ara
Theonellamide E	H	H	Br	Br	β-D-Gal

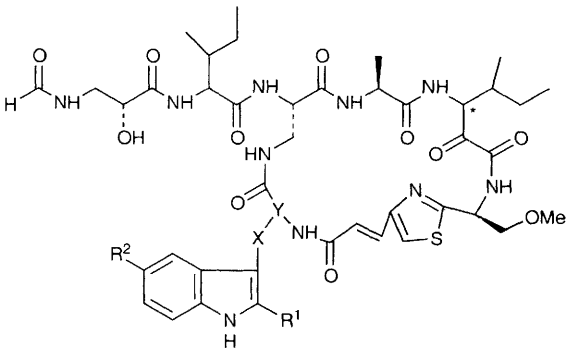
Figure 3

the highly folded structure of the native form and exhibited enhanced activity for metallic ions. Five new cytotoxic bicyclic peptides have been characterised[98] from a marine sponge *Theonella sp.* Named theonellamides A-E, the family structures are summarised in Figure 3 comparing them with the known theonellamide F.

2.12 Peptides Containing Thiazole/Oxazole Rings – Further investigations of extracts from the *Theonella* sponge have revealed[99] four new cyclic peptides, kermamides E, G, H and J to augment the previous congeners A-F. Kermamide E (68) had quite distinct structural differences when compared with F (69), G (70), H (71) and J (72). Three fragments useful for the synthesis of (69) have been synthesised.[100] *Dolabella auricularia* has yielded[101] dolastatin E (73) to add to the many other bioactive compounds isolated from this sea hare. A total synthesis[102] has also been completed, initially forming (74) by cyclisation at point 'a' using DPPA (22% yield, 50% polymerisation), and then treatment of (74) with diisopropyl azodicarboxylate/Ph_3P to form (73). As well as the known cyclic peptides patellamide B, ulithiacyclamide and lissoclinamide 3, a new cytotoxic compound, patellamide F (75) has been characterised[103] from the tunicate *Lissoclinum patella.* Lissoclinamide 4 (76) from the same tunicate has also been synthesised[104] with the thiazoline subunits being formed by the dehydration of serine-derived hydroxythioamides using the Burgess Reagent ($MeO_2CNSO_2NEt_3$). A sequence and stereochemical reversal of two thiazolyl amino acids in the antibiotic MDL 62,879 has been put forward[105] from degradation studies which now favour the structure (77) as the revised form.

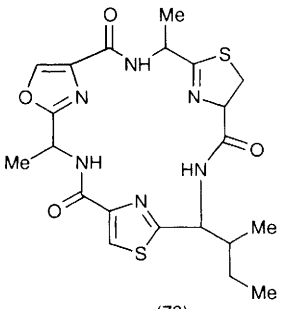


(68) Kermamide E, R^1 = Me, R^2 = Pr

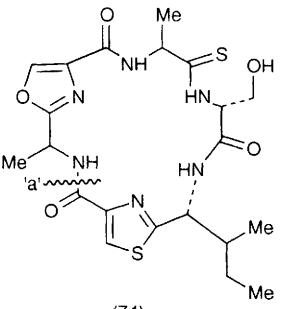


(69) $R^1 = R^2 = H$ X–Y = CH=C * *S* configuration
(70) $R^1 = R^2 = H$ X–Y = CH=C * *R* configuration
(71) R^1 = Br, R^2 = OH X–Y = CH_2CH * *S* configuration
(72) $R^1 = R^2 = H$ X–Y = CH_2CH * *S* configuration

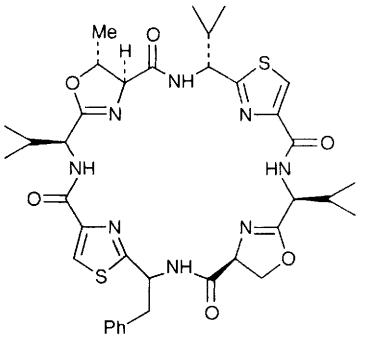
2.13 Cyclodepsipeptides – Marine sponges continue to be a rich source of novel cytotoxic cyclodepsipeptides, as exemplified[106] by the extracts from *Cymbastela* sp. yielding (78). Extracts from the sponge *Neosiphonia superstes* have yielded[107] neosiphoniamolide A (79) which is related to jaspamide and the geodiamolides previously isolated. Antifungal and cytotoxic properties have been found in the halicylindramides A-C (80-82) isolated[108] from *Halichondria cylindrata*. The isolation[109] of seven new didemnins, M, N, X and Y, nordidemnin N, epididemnin A, and acyclodidemnin A, from the Caribbean tunicate *Tridemnum solidum* using the usual cascade of physical techniques provides us with a welcome opportunity to summarise the structures of the



(73)



(74)



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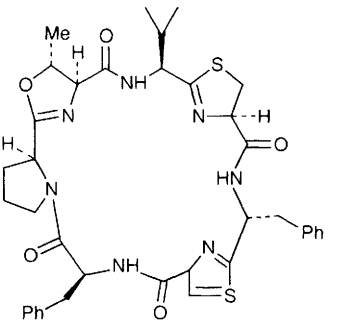

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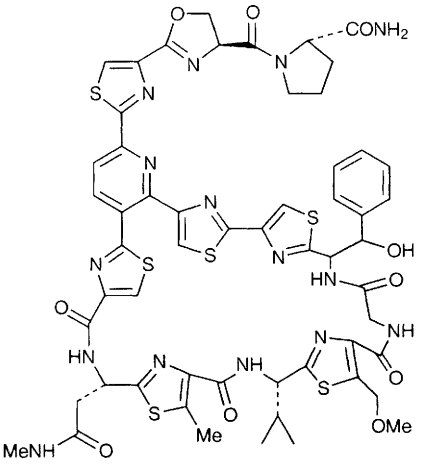

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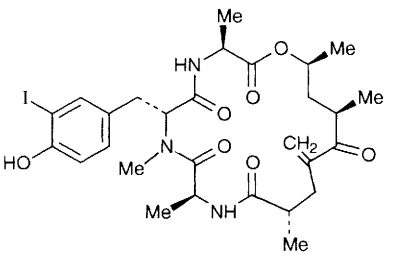

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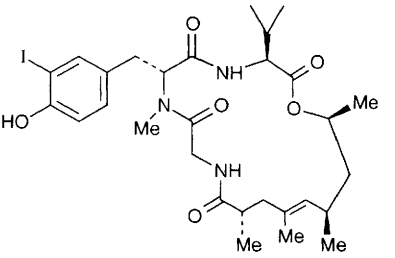

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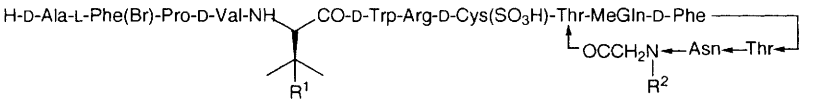
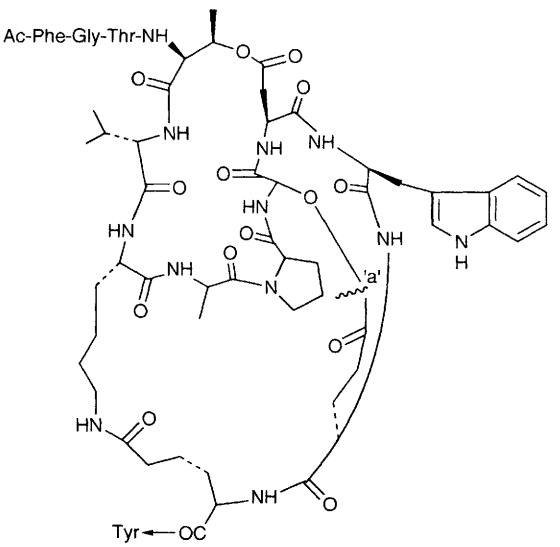
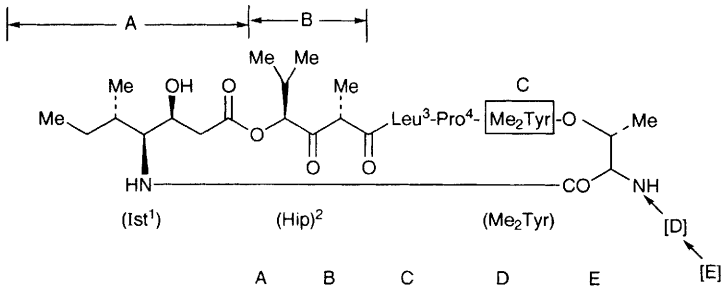


(80) R^1 = H, R^2 = Me (81) R^1 = Me, R^2 = H (82) R^1 = Me, R^2 = Me

family as given in Figure 4. Further examination of *Tridemnum cyanophorum* has provided[110] two new didemnins [Tyr^5]didemnin B and [D-Pro^4]didemnin B. The cyclic depsipeptide backbone of the didemnins has been explored[111] using an X-ray determination of N-{1-{N-{4-{[3-hydroxy-5-methyl-1-oxo-4-(N-threonyl-amino)heptyl]oxy}2,5-dimethyl-1,3-dioxohexyl}-L-Leu}-L-Pro}-N,OMe$_2$Tyr hy-



(83)
(84) with OH/COOMe at 'a'



		A	B	C	D	E
α-Didemnins	A	Ist	Hip	Me_2Tyr	D-MeLeu	H
α-Didemnins	B	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac
α-Didemnins	C	Ist	Hip	Me_2Tyr	D-MeLeu	Lac
α-Didemnins	D	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac(Gln)$_3$ *p*Glu
α-Didemnins	E	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac(Gln)$_2$ *p*Glu
α-Didemnins	G	Ist	Hip	Me_2Tyr	D-MeLeu	CHO
α-Didemnins	M	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac-Gln-*p*Glu
α-Didemnins	N	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac
α-Didemnins	X	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac-(Gln)$_3$-Hydec
α-Didemnins	Y	Ist	Hip	Me_2Tyr	D-MeLeu	Pro-Lac-(Gln)$_4$-Hydec
Nordidemnin	A	Norsta	Hip	Me_2Tyr	D-MeLeu	H
Nordidemnin	B	Norsta	Hip	Me_2Tyr	D-MeLeu	Pro-Lac
Nordidemnin	N	Norsta	Hip	Tyr	D-MeLeu	Pro-Lac
Methylenedidemnin	A	Ist	Hip	Tyr	X	H
Epididemnin	A_1	Ist	epi Hip	Tyr	D-MeLeu	H
Acyclodidemnin	A_1	Ist	Hip	Me_2Tyr	D-MeLeu	H

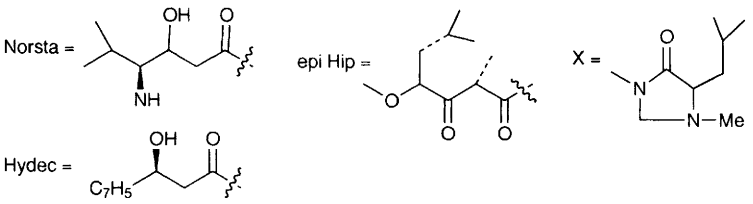
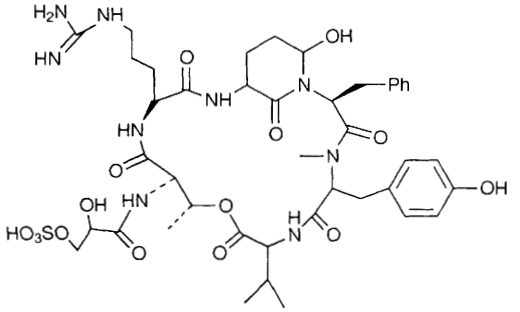


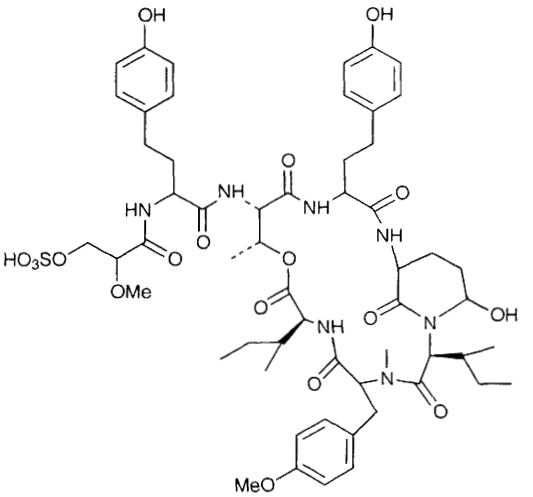
Figure 4

drobromide. The transannular H-bonds in this structure seemed stronger than in didemnin B.

Microcystis aeroginosa, a blue green alga cultured in freshwater has been shown[112] to be a source of new elastase inhibitors, microviridins B (83) and C (84) which join another forty or so microcystin type structures already reported. Isolated[113] from the same source also is a plasmin and trypsin inhibitor, micropeptin 90 (85). Oscillapeptin (86) from *Oscillatoria agardhii* (NIES-204) has been characterised[114] using extensive 2D NMR and chemical degradation and shows similarities to (85). Inhibition of elastase and chymotrypsin with IC_{50} values of 0.3 and 2.2 μg ml^{-1} by oscillapeptin was instrumental in its discovery from its cyanobacterium source. *Pseudomonas* sp. is a source of a cyclodepsipeptide pholipeptin (87)[115] an inhibitor of phosphatidylinositol, and an extremely



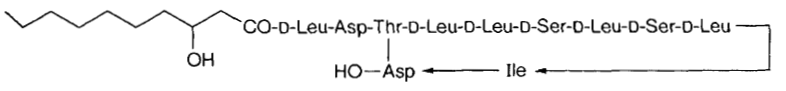
(85)



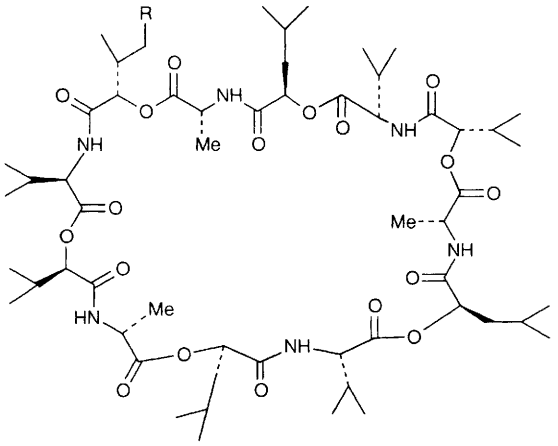
(86)

potent cytotoxic compound homocereulide (88), and its analogue cereulide (89) have been isolated[116] from the marine bacterium *Bacillus aureus*. In a separate study[117] on cereulide (89) using NMR and a calculated modelling of the 1:1 K^+ complex (cf. valinomycin), it was shown that the main chain shows a hexagonal cylinder-like framework which is similar to the known bracelet-like structure of the valinomycin-K^+ complex.

Total synthesis[118] of cryptophycins C (90) and D (91) have led to the confirmation that the methylated tyrosine residue has the D-configuration, and

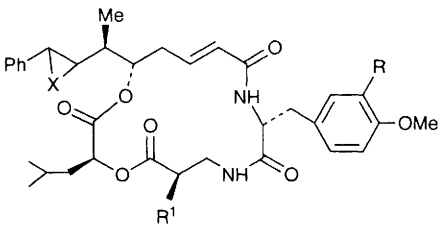


(87)

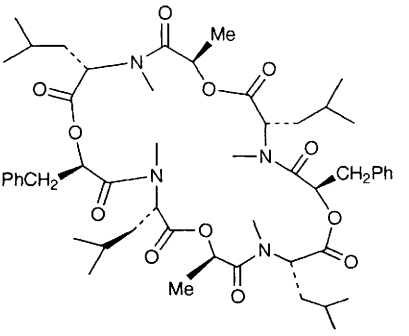


(88) R = Me
(89) R = H

an improved total synthesis[119] of the related arenastatin A (92) has been reported. The last stage carried out was the epoxidation of the double-bond, while the macrocyclisation stage was carried out between the carboxyl group of the methylated tyrosine residue and the β-alanyl amino group using diphenylphosphoryl azide. A third synthesis which affords gram scale quantities of the anthelmintic cyclooctadepsipeptide PF 1022A (93) from *Mycelia sterilia* has been reported.[120] A high 85-90% yield at the cyclisation stage between the NH of MeLeu and the carboxy group of Phlac using BOP-Cl completed the synthesis, with the BOP-Cl reagent showing up well in other couplings involving N-methyl amino acids. Synthesis of fragments containing hydroxymethylbutyric acid (Hmb) has enabled[121] evidence to be gathered to suggest that the natural product from the mushroom *Sarcodon scorbrosus* is cyclo(Hmb-Ile-Hmb-Ala-Thr). The potent calcium channel antagonist leualacin (94) has been synthesised[122] in 15 steps in the



(90) R = Cl, R^1 = Me, X = alkene
(91) R = H, R^1 = Me, X = alkene
(92) R = H, R^1 = H, X = O



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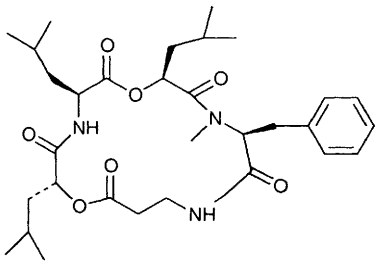
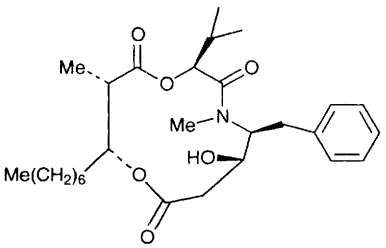


(94)



(95)

solution phase which is flexible enough to incorporate replacements such as mimics and isosteres. The final macrocyclisation was between the carboxyl of MePhe and the NH_2 of β-alanine using diphenylphosphoryl azide. The solution phase was also chosen for the synthesis[123] of the multidrug resistance-reversing agent hapalosin (95). After convergence of two fragments the penultimate step was a macrolactonization using modified Mukaiyama conditions.

The valinomycin template still commands interest. A crystal and molecular structure has been put forward[124] for the centrosymmetric meso-valinomycin analogue cyclo(D-Val-D-Hyi-D-Val-L-Hyi-L-Val-D-Hyi-L-Val-L-Hyi-L-Val-D-Hyi-D-Val-L-Hyi). In contrast to mesovalinomycin this analogue does not adopt an octahedral cage bracelet conformation. It has an unusual centrosymmetric elongated form with two type II terminal β-bends formed by 4 → 1 type intramolecular H-bonds. Molecular recognition of K^+ and Na^+ by valinomycin has been examined[125] using potential of mean force, free energy perturbation and molecular dynamics simulations. For Na^+ there was reasonable agreement between free energy of binding (from -1.1 to -2.5 kcals mol^{-1} *vs.* the experimental value of -1.2 ± 0.4 kcals mol^{-1}). The calculated free energies of the K^+ complex were overestimated when compared to the experimental value.

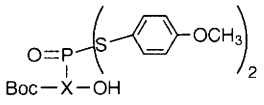
3 Modified and Conjugated Peptides

Non-peptidic conjugates of peptides have been an expanding area of this Chapter over the years. Again the phospho- and glyco-conjugates dominate the section, and for anyone requiring a background to recent developments on their solid phase synthesis and conformation, a review with over 100 references has appeared.[126]

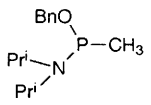
3.1 Phosphopeptides – The importance of protein phosphorylation in the regulation of cell growth and differentiation is again emphasised in the considerable interest in the facile synthesis of peptides with selectively phosphorylated residues. A review[127] of synthetic protocols has appeared and an evaluation[128] has been made of the approaches to the phosphorylation of hydroxyl groups in HGly-Gly-X-Ala-OH, where X was varied between Ser, Thr and Tyr. Two post-synthetic phosphorylation methods were assessed, **(i)** the

tetrazole catalysed phosphitylation by di-t-butyl N,N-diethyl phosphoramidate followed by oxidation and, **(ii)** phosphorylation by dibenzylphosphochloridate. For Ser and Thr, both gave comparable yields, but method (i) proved better for Tyr. However, this method was not as good as using Fmoc-Tyr(PO_3Me_2)-OH as a building block for the synthesis. It was also confirmed in this work that a diagnostic fragmentation in the mass spectra of phosphorylated peptides is due to loss of the phosphate group to yield dehydroamino acid residues. However, if Fmoc-Tyr(PO_3H_2)-OH is used[129] to make peptides related to autophosphorylation sites of the epidermal growth factor receptor, some phosphorylated peptides are made, but a side reaction giving pyrophosphate derivatives is also a serious problem. Less pure phosphopeptides using Fmoc-Tyr(PO_3H_2)-OH have also been reported[130] in another study, which established that in a comparison of Fmoc-Tyr($PO_3Bu^t_2$)-OH and Fmoc-Tyr(PO_3Bzl_2)-OH in solid phase synthesis, the former derivative gave the product with highest purity. Yet for the continuous-flow Fmoc-solid phase synthesis of an *O*-phosphorylated analogue of a heptadecapeptide the dibenzyl derivative was favoured.[131] In a CD spectroscopic study of the products the *O*-phosphorylated peptide had a longer helix chain length than the non-*O*-phosphorylated peptide. Both phospho-Ser and phospho-Thr containing peptides have been synthesised[132] successfully using Fmoc-(monobenzylphosphono)-Ser and -Thr building blocks.

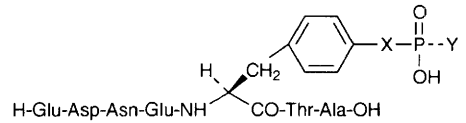
In the Boc-mode of solid phase syntheses several Boc-derivatives of Ser, Thr and Tyr have been assessed as building blocks. The dimethyl-protected Boc-Ser(PO_3Me_2)-OH and Boc-Thr(PO_3Me_2)-OH have been successfully applied[133] in a two-step deprotection protocol consisting of high acidic (S_N1/S_N2) and low acidic (S_N2) reagents. It has also been reported[134] that this procedure can be carried out in one pot as exemplified by the synthesis of a 19-residue AP-kinase peptide possessing vulnerable Met and Trp residues as well as two phosphoamino acids. Phosphoserine protected peptides corresponding to the B-cell epitope sequence in αS1-casein have been made[135] on an oxime resin using either Ser(PO_3Bzl_2) or Ser[PO_3(cHex)$_2$] for protection. It has also been reported[136] that the building block for the latter [Boc-Ser(PO_3cHex_2)-OH] and Boc-Thr(PO_3-$cHex_2$)-OH can be made as crystalline compounds as well as their di-4-nitrobenzylphosphono equivalents. Mono- and multi-phosphopeptides related to Src-protein kinase were the test-beds[137] for assessing syntheses using trichloroethyl as a phosphate protecting group. Its stability in acidic conditions was seen at its best in several liquid phase syntheses, which utilised a previously communicated method of making Boc-Ser[PO(S)(OCH_2CCl_3)]-OH and Boc-Tyr[PO_2-(OCH_2CCl_3)]-OH using methylthiomethyl esters. Boc-technology in the liquid phase using the building blocks based on (96), whose side chains can be deprotected by silver nitrate, have yielded[138] H-Ser(PO_3^-H)-Pro-Leu-OH, H-Thr(PO_3^-H)-Pro-Arg-OH and H-Ser(PO_3^-H)-Pro-Tyr-Lys-OH, models for sites phosphorylated by cdc2 kinase. Phosphorylated RB protein fragments have been synthesised[139] on Boc-solid phase protocols using Boc-Ser(PO_3Allyl_2)-OH and its threonyl analogue. Benzyloxy(diisopropylamino)methyl phosphine (97) has been shown[140] to be an efficient reagent for introducing the methylphosphorylated group on to amino acid and peptide side-chains. The development of



(96) X = Ser, Thr or Tyr



(97)

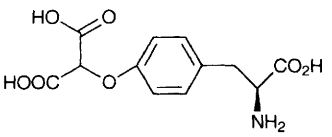


(98) X = CH_2, Y = OH or X = –, Y = OH

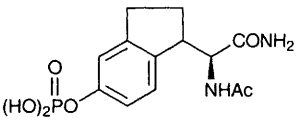
Fmoc-Tyr[P(O)$(NMe_2)_2$]-OH as a versatile phosphotyrosine building block has also been reported[141] and used in the solid phase preparation of B-cell immuno receptor tyrosine activation motifs.

Stable mimetics of phospho tyrosyl residues have been identified as having potential as inhibitors of kinases and phosphatases. Thus (98) represents[142] a successful attempt at incorporating the non-hydrolyzable 4-phosphonomethyl-Phe and 4-phosphono-Phe into the main autophosphorylation site of Src tyrosine kinases. These analogues, while not significantly affecting the activity of CSK, PTK-IIB and TC-PTP (non-receptor tyrosine kinases), served as efficient inhibitors on Lyn, influencing both exogeneous phosphorylation and autophosphorylation. The malonyl mimic (99) can be introduced[143] into peptides utilising Fmoc-Tyr[$CH(CO_2CMe_3)_2$]-OH, and is stable to protein-tyrosine phosphatases and the diester malonyl derivative improves passage across cell membranes. Based on the traditional approach that constraining phosphotyrosine to its bound conformation could increase binding affinity, analogues such as (100) and (101) have been synthesised[144] to give a variety of torsion angles. When examined for inhibitory activity when inserted into various domains, analogue (100) showed a roughly (2-3)-fold increase in potency relative to phosphotyrosine but a racemic form of the benzazocaine (101) retained full binding potency of the L-form of (100). Problems with enzymatic resolution of the 4-phosphonomethyl analogue (102), have initiated a successful asymmetric synthesis[145] using camphorsultam as a chiral auxiliary. Although not themselves synthesised as phosphopeptides, cyclo(Glu-Asp-Asn-Glu-Tyr-Thr-Ala) and its dimer have been synthesised[146] as substrates of the oncogene Lyn, to detect Src-like tyrosine kinase activity. The dimer displayed very favourable kinetic constants and was a powerful inhibitor of phosphorylation activity and enzyme autophosphorylation. In the making of the cyclic analogues, DPPA/K_2HPO_4 was used for cyclisation.

3.2 Glycopeptide Antibiotics – The clinical importance of the vancomycin group of glycopeptide antibiotics, in blocking cell wall synthesis, and therefore providing a feasible treatment for methicillin resistant *Staphylococcus aureus*



(99)



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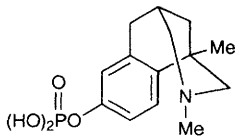

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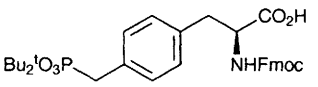
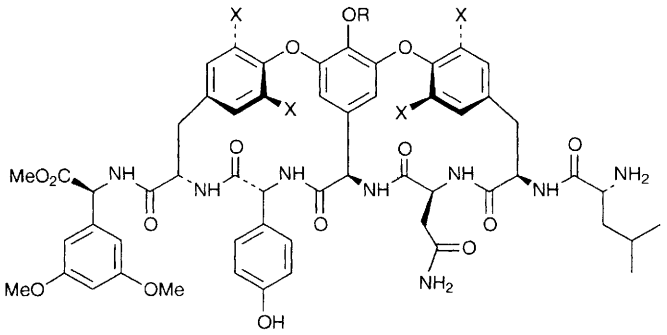


(102)

(MRSA), has spurred on the interest in these molecules as well as the search for synthetic analogues. The trend up to date has been summarised in a text[147] useful for anyone involved in their syntheses.

The power of 2D NMR spectroscopic techniques is amply demonstrated in the interactions studied between the vancomycins and bacterial wall substrate ligands. Having already assigned the full ^{1}H spectrum of vancomycin, a full and unambiguous assignment of the 66-atom ^{13}C-spectrum has now been reported[148] which will assist in further interaction analyses. Association constants for a number of ligands which bind weakly to the antibiotic have been determined[149] using ^{13}C NMR, and it has been found that they compare favourably with other methods. A tighter complex forms with N-acetyl-D-Ala than with acetate. IR studies[150] on vancomycin in D_2O at different pD values and in the presence of N-Ac-D-Ala-D-Ala and N,N^{1}Ac$_2$-L-Lys-D-Ala-D-Ala show substantial increases in intensity of a signal at 1588 cm^{-1} due to the asymmetric stretch of the peptide carboxylate group on binding to vancomycin. Conformational analyses[151] using NMR techniques such as DQFCOSY, HOHAHA and ROESY combined with molecular modelling using QUANTA(ver. 4.0) and CHARM(ver. 23.0) have been carried out on aglucovancomycin and synthetic analogues SAV-1 and SAV-2 (103). The latter have become the basis[152] of an investigation of their binding with Ac-D-Ala-D-Ala as well as with aglucovancomycin. The binding model suggested appears as Figure 5. NOe constraints from NMR data and energy minimisation and molecular dynamics calculations on a dimer complex of ristocetin A[153] with a bacterial cell wall analogue show that the dimer is asymmetric, in which the conformation of the two monomeric units differ in the parallel head to head alignment of the tetrasaccharide unit attached to the aromatic ring residue 4. Similar association seen in Figure 5 can also be seen in the summary diagram for ristocetin in Figure 6. NMR studies[154] on teicoplanin, the only member of the vancomycin family which does not seem to form an asymmetric dimer, have shown that it exists as two conformers which have different binding affinities for cell wall analogues. The two conformers are closely analogous to those adopted by each half of the asymmetric dimers of the other



(103) SAV 1 (R = X = H), SAV 2 (R = H, X = Cl)

vancomycin group members. The role of the phenolic groups in vancomycin-type antibiotics has been examined[155] to find out whether the groups interact with the carbonyl groups of the binding peptide. High field NMR of ψ-aglycoristocetin and its complexes with bacterial cell wall mimetics as given in Figures 5 and 6 at varied pH showed no evidence of strong H-bonds, but at high pH the decreased peptide binding is due to repulsion by the phenolate anions.

The total synthesis of the vancomycin group still remains a great challenge. The progress made up to 1995 has been thoroughly reviewed[156] together with comments on biosynthesis and mode of action. The vancomycin analogues (103) have been synthesised[157] for the association studies with bacterial cell wall analogues as discussed above. Both the diaryl ether rings were constructed using the previously published thallium(III) nitrate cyclisation of iodinated precursors. Analogues (103) are the first examples of analogues carrying the full peptide chain plus a diaryl ether moiety. Generally, the year under report generated most of the synthetic papers on functionalised ring systems capable of further elaboration as exemplified by the synthesis of the CD and DE rings.[158]

Two papers have reported the use of ruthenium π-arene complexes for the intramolecular S_NAr construction. In one example[159] the biphenyl ethers (104) with R^1 = Boc, R^2 = Ile, Phe Tyr(Bu^t) side chains, R^3 = Me and R^4 = H were

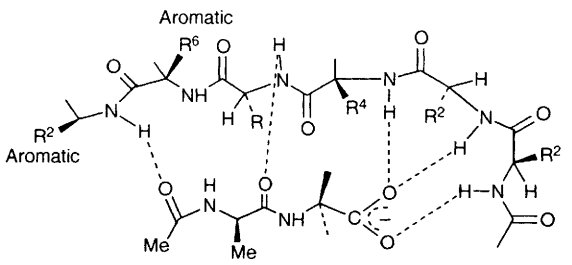


Figure 5

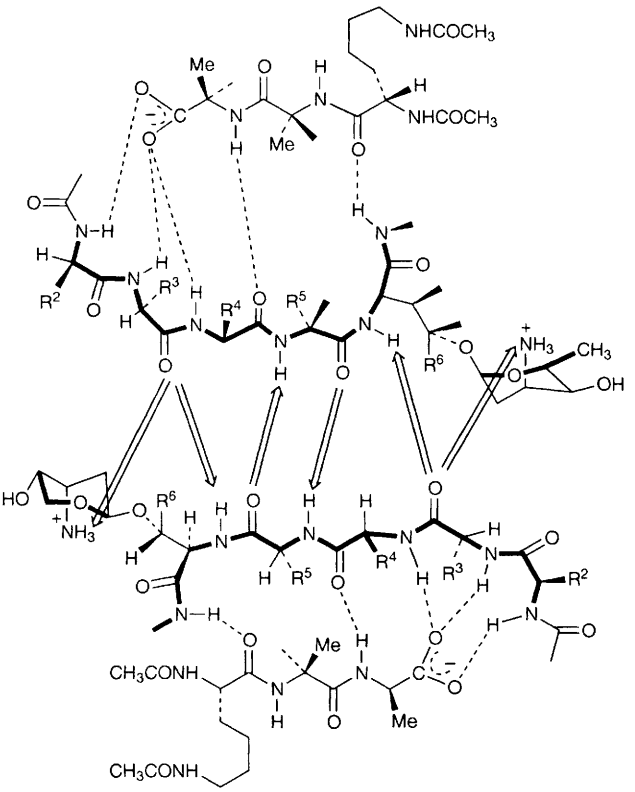
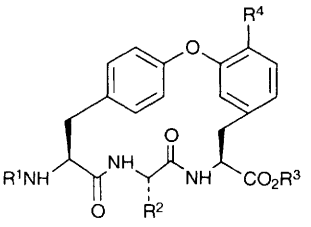


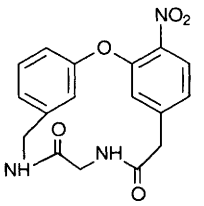
Figure 6



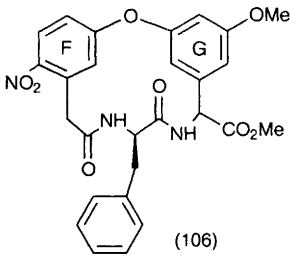
(104)

synthesised as a route to analogues related to the protease inhibitor K-13, while in the other paper[160] a model for the BCF rings of ristocetin A (104) with R^1 = Boc, R^2 = *p*-methoxyphenyl, R^3 = Et and R^4 = OMe was synthesised.

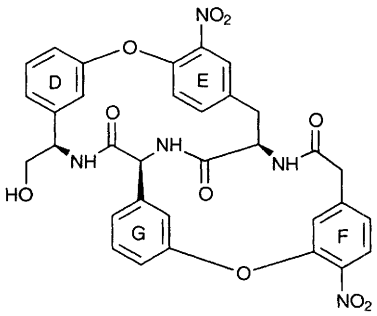
Intramolecular S_NAr reactions involving the displacement of fluoride atoms next to nitro groups by potassium carbonate/crown ethers have been successful in macrocyclisation *via* biaryl ether formation in the synthesis of a model (105) for



(105)



(106)

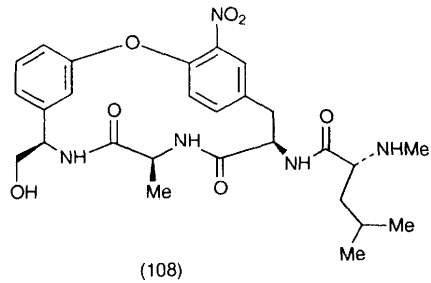


(107)

the F-O-G ring of teicoplanin[161] and an analogue (106) for the same 14-membered ring.[162] The bicyclic D-O-E-F-O-G rings (107) of teicoplanin have also been synthesised[163] *via* sequential intramolecular S_NAr reactions. In the vancomycin series the modified carboxylate binding pocket (108) has been synthesised[164] and the fully functionalised C-O-D ring (109) has been assembled[165] using the same synthetic strategy.

New semisynthetic[166] glycopeptides MDL63,246 and MDL63,042, which are new amide derivatives of the antibiotic A-40,926, have been shown to be active against several strains of *Enterococcus faecalis* and *faecium* which are highly resistant to both vancomycin and teicoplanin. An efficient method[167] for the solution and solid phase synthesis of vancomycin carboxamide has also been reported. Using HBTU, vancomycin carboxamides could be isolated in 55-82% yields, and *via* peptide esters vancomycin peptide conjugates to solid support were produced.

3.3 Glycopeptides – This expanding area of endeavour has again generated useful reviews on various aspects of glycopeptides synthesis. Solid phase synthesis of O-glycopeptides has been the subject of a review[168] (up to 1993), while papers since 1989 on chemical synthesis of glycopeptides[169] and their chemical and enzymatic synthesis[170] have been reviewed. It is still convenient this year again to sub-divide the discussion to O- and N-glycopeptide categories, yet some papers lie outside such a division. For example, the assembly of C-glycosyl amino acid subunits in an enantiospecific manner has been reported[171] with the formation of



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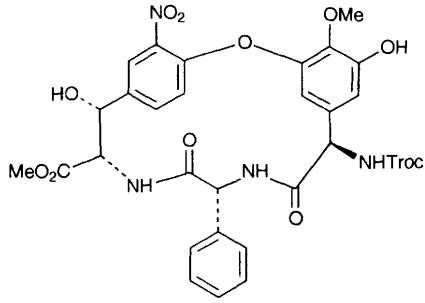
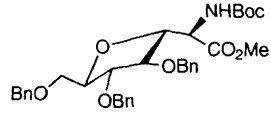


(109)

glycyl derivative (110). Both O- and N-glycopeptides have been prepared[172] as Major Histocompatibility Complex (MHC) class I binding glycopeptides using the building blocks Fmoc-Ser(Ac_3-β-D-GlcNAc)-OH, Fmoc-Ser-(Ac_3-α-D-$GalN_3$)-OPfp, Fmoc-Asn(Ac_3-β-D-GlcNAc)-OH and Fmoc-Asn(Ac_3-β-D-GalNAc)-OH. Evidence of *cis-trans* isomerisation about the Tyr-Pro amide bond was obtained for the K3 peptide FAPSNYPAL and its glycosidic analogues FAPS(OGlcNAc)NYPAL and FAPSN(GalNAc)YPAL. Glycodendrimers have been synthesised[173] by coupling various thiolated glycosides to preformed N-chloroacetylated dendritic lysine cores such as $(XCH_2CO\text{-}Gly\text{-}Gly)_8\text{-}Lys_4\text{-}Lys_2$-Lys-β-Ala-O-WangResin. A lipophilic derivative of GMDP, N-acetylglucosaminyl-(β1→4)-N-palmitoylmuramoyl-L-Ala-D-$GluNH_2$, has been synthesised[174] *via* coupling of the carboxyl group of the protected muramic acid and H-L-Ala-D-Glu-NH_2. To further assist with the understanding of immunostimulator's action, GMDP has been attached[175] either to the N-terminus of tuftsin or to the



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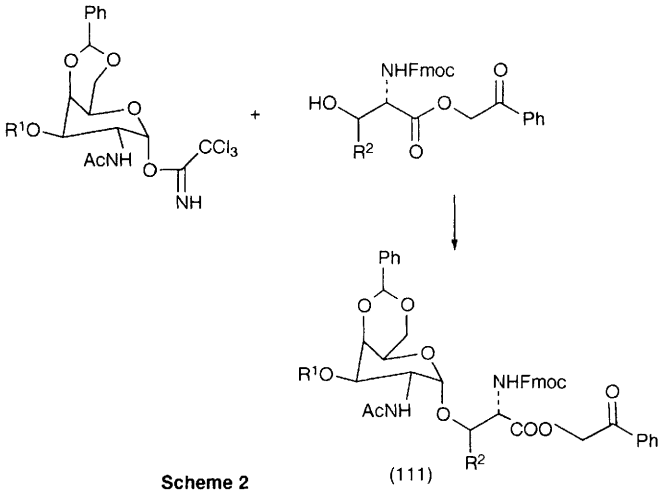
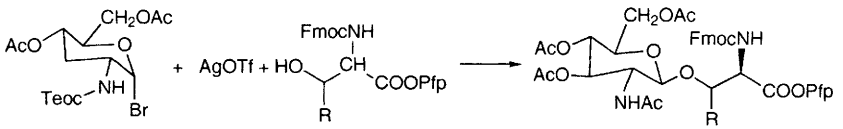


Scheme 2

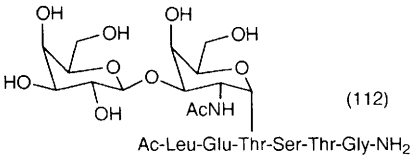
(111)

ε-NH_2 of its Lys residue. The best activity came from H-Thr-Lys(GMDP)-Pro-Arg-OH.

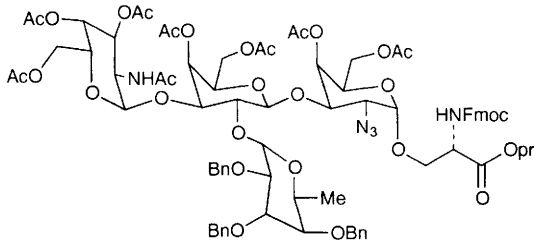
O-Glycopeptides – The vast majority of papers this year are geared towards the 'building block' approach on solid phase as the most popular mode of synthesis. Hence most papers concentrate on the making of suitable protected building blocks. Stereochemical control in the making[176] of (111) has been provided by the 4,6-cyclic acetal protected N-acetylgalactosamine described in Scheme 2. The use of the 2-trichloroethoxycarbonyl amino (Teoc) protecting group, which is compatible with the use of OPfp esters, has allowed an efficient synthesis of Ser/Thr derivatives to be developed[177] as summarised in Scheme 3. Comparisons have been made[178] between a protected [Fmoc-Ser(α-L-$FucAc_3$)-OH] fucosylated seryl derivative and an unprotected form [Fmoc-Ser(α-L-Fuc)-OH] in the synthesis of a fragment of the EGF domain Human Factor IX55-65. The unprotected form gave the best overall yield although the protected form had advantages during the acid cleavage from the resin. Glycopeptide libraries have been generated[179] utilising activated building blocks such as Fmoc-Ser-OPfp derivatives glycosylated directly with dimannosyl bromides, and twenty core 5- and 7-mucin O-glycopeptides have been synthesised[180] *via* the building blocks Fmoc-O-[α-D-Ac_3GalN_3p-(1→3)-α-D-Ac_2GalN_3p]-Thr-OPfp and its (1→6) analogue. Eight triply glycosylated mucin peptides with T^n and T antigenic structures have been synthesised[181] using Fmoc-Ser[Ac_4-β-D-Galp-(1→3)-Ac_2-α-D-$GalN_3$-



Scheme 3



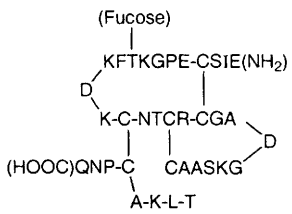
(112)



(113)

p]OPfp and its Thr equivalent on multiple columns. The synthesis[182] of hexapeptide (112) of human sialophorin in high purity is a convincing demonstration that glycosylation using trichloroacetimidates in the presence of trimethylsilyltrifluoromethane sulfonate is compatible with the use of Fmoc-pentafluorophenyl esters for building block protecting/activating groups. The prize for the most impressive serine derivative of the year must go to the acetamidodeoxy oligosaccharide[183] (113) which has been used for the synthesis of one of the predominant substructures present in human blood group A ovarian mucin. Highly nucleophilic Schiff bases of Ser and Thr have been successful[184] in giving a stereoselective synthesis of N-Fmoc, 2-acetamido-2-deoxy α- or β-glycosides of Ser/Thr. Fmoc-Thr(α-Ac_3Fuc)-OH was the key building block[185] used in the solid phase glycopeptide synthesis of the O-fucosylated serine protease inhibitor (114) from *Locusta migratoria*.

The need to protect the α-carboxyl groups of Fmoc amino acids during glycosylation has been dispensed with[186] in the O- and S-glycosylation of side chains catalysed by $BF_3.Et_2O$ or tin(IV)chloride. The protection of the side-chain carboxyl of Asp as the benzyl ester has made possible[187] the synthesis of [α-Glc(1→6)-β-Glc(1→6)-α-Glc(1→Asn-Pro$^{(a)}$-Leu-Phe-Gly-Ile-Ala-Gly-Glu-Asp$^{(b)}$-



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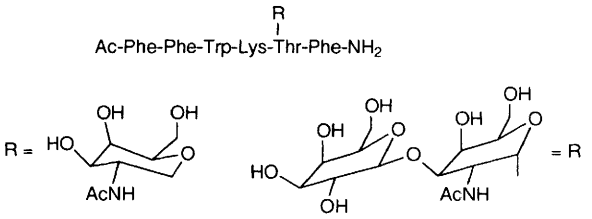
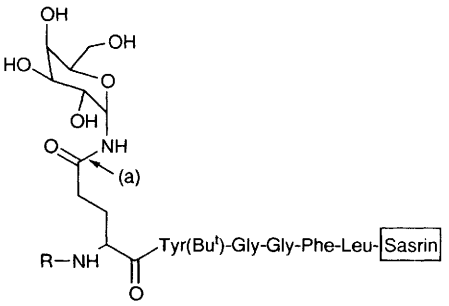


(115) (116)

Gly-Pro-Thr-Gly-Pro-Ser-Gly-Ile-Val-Gly-Gln] without aspartimide formation, using fragments which were attached at points (a) and (b). Direct glycosylation of the O-trityl serine derivative in Fmoc-Ala-Ser(Trt)-Gly-OBn has been carried out[188] by 3,4,6-Ac_3-1,2-O-(1-cyanoethylidene)-α-D-galactopyranose in the presence of $TrClO_4$ in CH_2Cl_2. Glycosylate analogues linked to Ser^4 in the antiduretic drug [1-desamino,8-D-Arg]-vasopressin (DDAVP) have been shown[189] to have improved bioavailability and enzyme stability over non-glycosylated [$D\text{-}Tyr^2$,Ser^4] DDAVP. No major change in conformation could be detected in comparison of NMR data. However, NMR data[190] derived from the two glycopeptides (115) and (116) suggested that the glycosylations cause the peptide backbone to bend, with the extent of the turn being dependent on the size of the sugar unit.

N-Glycopeptides – Unit (117) has been assembled[191] on a solid phase resin with the glycosyl residue being introduced while the peptide is on the resin *via* the coupling of the pentafluorophenyl ester of the γ-carboxyl group of Glu with glycosylamine. A pentasaccharide azide, reduced to its corresponding glycosyl amine, has also been condensed[192] with an Asp acid derivative to furnish an Asn-linked oligosaccharide. Examples[193] of some of the largest N-linked glycopeptides synthesised to date have been accessed *via* attachment of an oligosaccharide terminating in a glycal to a solid support and converting it to a solid bound oligosaccharide bearing a terminal 2N-acetyl-1β-aminoglucosamine which can then be readily coupled to, for example, the side chain of Asp in a peptide from which the peptide can be 'grown'. Retroisomers (118 and 119) of N-glucoaspar-



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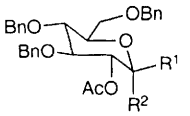
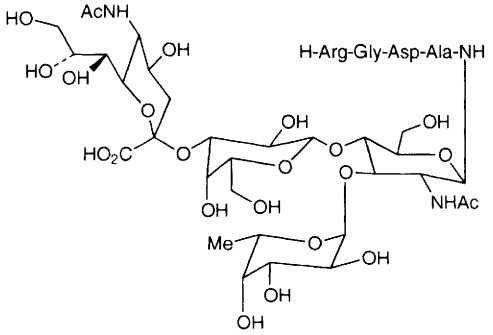
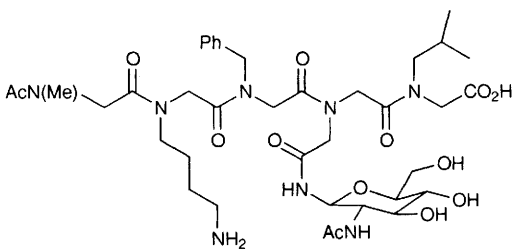


(118) $R^1 = CONHCH_2CH(NHZ)CO_2Me$, $R^2 = H$
(119) $R^2 = CONHCH_2CH(NHZ)CO_2Me$, $R^1 = H$



(120)

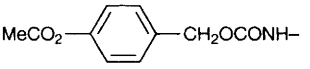
agine have been stereoselectively synthesised[194] *via* coupling of the heptonic acid derivatives with protected β-aminoalanine methyl ester. The compounds (118) and (119) are in essence C-glycosidic retro equivalents. The trimethylsilyl group has been used successfully[195] in protecting the hydroxy groups of sugar units during coupling to Fmoc-Asp(Cl)OPfp to form building blocks for solid-phase synthesis of N-linked glycopeptides. The structural elements of Arg-Gly-Asp and those of the sialyl Lewis^x ligand have been combined[196] to make the most potent antagonist (120) of P-selection yet reported. The conformations[197] of the glycopeptide Manα1→6(Xylβ1→2)Manβ1→4GlcNAcβ1→4(Fucα-3)GlcNAc-β1→N(Asn-Glu-Ser-Ser), prepared from pineapple stem bromelain, have been analysed by molecular dynamics simulations and NOESY NMR spectroscopy. The tetrapeptide moiety appeared to be in quite a mobile conformation. Families[198,199] of N-substituted oligoglycine bearing N-linked carbohydrate units such as in (121) have been synthesised as the first examples of this class of



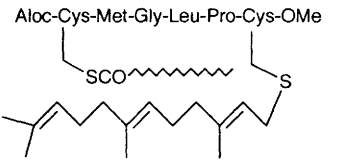
(121)

glycopeptidomimetics. Compound (121) can be considered to be a mimic of H-Ala-Lys-Phe-Asn(D-GlcNAc)-LeuOH.

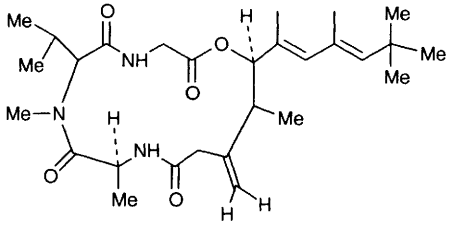
3.4 Lipopeptides – To overcome the inherent sensitivity of side linkages containing S-palmitoylated and S-farnesylated in lipopeptides to the normal conditions of solid phase syntheses, a strategy[200] involving the use of the enzymatically cleaved urethane group (122) has achieved a synthesis of the C-terminal lipohexapeptide (123) of the human N-Ras protein. Antillatoxin (124), from the tropical cyanobacterium *Lyngbya majuscula*, has been characterised[201] and found to be one of the most toxic compounds (LD_{50} = 0.05 μg ml^{-1}) isolated from a marine plant. N-Bromoacetic distearoylphosphatidylethanol amine conjugates of H-Ser-Phe-Leu-Arg-Asn-(β-Ala)$_3$-Tyr-$NHCH_2CH_2SH$ have been purified[202] by normal phase liquid chromatography.



(122)

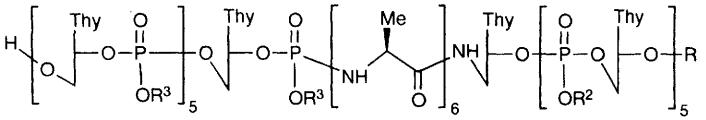


(123)



(124)

3.5 Miscellaneous Conjugates – In order to test the hypothesis that monomethoxy poly(ethyleneglycol) (PEG) modified peptides and proteins have increased resistance to proteolytic degradation, several sites of pegylation have been assessed[203] using the potent analogue of growth hormone-releasing factor [Ala^{15}]-hGRF(1-29)-NH_2. Regardless of the size of PEG(*e.g.* PEG_{2000} or PEG_{5000}), N-terminal pegylation was no worse in reducing activity than the acetyl group. Pegylation at Asp^8 or Lys^{12} decreased potency, but at Lys^{21} and Asp^{25} and the C-terminal carboxyl group, the modification had no effect on biological activity. A series of linear and cyclic Arg-Gly-Asp-containing peptides from human bone sialoprotein has been synthesised[204] incorporating a bromoacetyl diaminopropionic acid residue for site specific conjugation of the cyclic peptides on to protein carriers or to glass. Results showed that conjugates

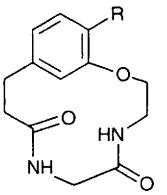


(125)

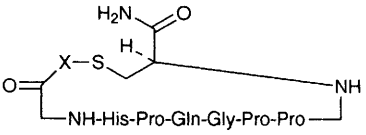
containing linear or cyclic forms of Glu-Pro-Arg-Gly-Asp-Asn-Tyr-Arg supported cell adhesion when immobilised on a support. Directly-linked peptide-oligodeoxynucleotide hybrids of the general type (125) have been synthesised[205] on a controlled pore glass support employing phosphoramidite chemistry for the oligonucleotide part and Fmoc chemistry for peptide assembly.

4 Miscellaneous Structures

A few papers annually do not exactly fit into the sub-divisions chosen for a Chapter. This section records such examples. The core structure (126) of the phomopsin-ustiloxin class of antibiotics has been synthesised[206] and the merits of thio-ether cross linking, instead of disulfide links, have been assessed[207] from the synthesis of streptavidin analogues (127). The advantages seen are lack of disulfide interchange/dimerisation reactions and the availability of additional



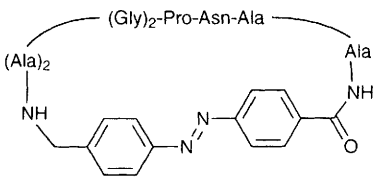
(126) R = OH, H



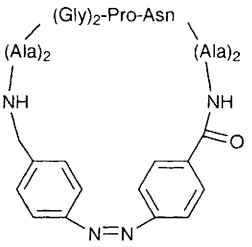
(127) X = $(CH_2)_6$, p-$C_6H_4CH_2$

sulfoxide diastereoisomer analogues. Biological activity 'on demand' is an interesting possibility as a result of the photochemically catalysed[208] flip over of *trans*-azo form (128) to *cis*-azo form (129), with the latter form being the only one to template a β-turn. The 20-membered cyclic moiety (130) has been synthesised[209] as an analogue of For-Met-Leu-Phe-OMe, and proved to be active as a chemoattractant and superoxide anion generator when tested on human neutrophils. A 60-atom cycle is produced[210] by the cyclisation of linear precursor to give (131) representing the primary structure 134-155 residues of VP1 from foot and mouth disease virus. Cyclisation yields in solution were consistently better than on solid phase though the latter offered fewer purification steps.

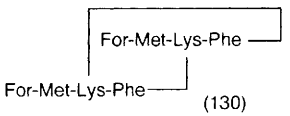
Peptide ionophores still generate a great deal of interest. Bicyclic compounds (132), representing a family of polycyclic ionophores and characterised by C_2 symmetry have been synthesised[211] using solid phase protocols. Complexation with alkali and alkaline earth ions gives an equilibrium between inclusion (1:1)



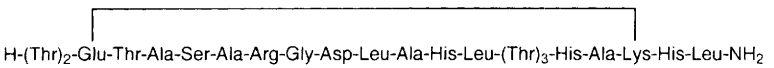
(128)



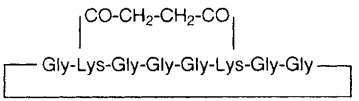
(129)



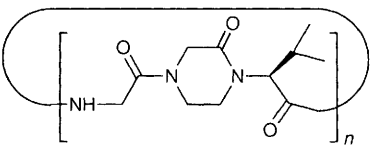
(130)



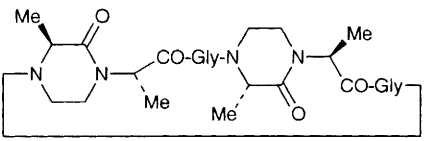
(131)



(132)

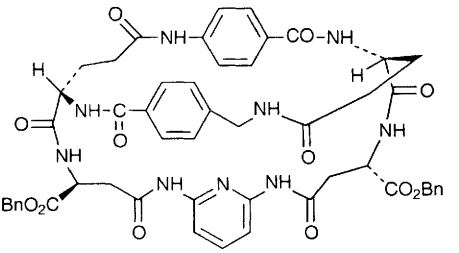


(133) n = 3 or 4



(134)

and sandwich (2:1) complex models with affinities in the $10^6 M^{-1}$ and $10^{-11} M^{-2}$ range, respectively. Enantioselective transport of amino acid ester salts by macrocyclic pseudopeptides have been shown[212] to occur with the cyclic examples such as (133). Another member (134) of the same family has been analysed[213] by X-ray crystallography, ^1H NMR and molecular mechanics calculations but showed no intramolecular H-bonds. In a series of papers[214-217] Kilburn *et al.* have set out to design and synthesise macrocyclic receptor molecules, with one of the most impressive structures being (135) which exists[217] as two interconvertible atropisomers of in-out L-Glu, D-Glu.



(135)

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5
β-Lactam Chemistry

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1 Introduction

Undoubtedly the major point of interest in the field of β-lactam research during the period reviewed (1994-1995) is the threat posed by resistance caused by the continued use of many antibacterials. Epidemiological studies have demonstrated the very real clinical problem of resistance to β-lactam antibiotics and considerable efforts have made been towards understanding the processes by which resistance mechanisms are generated and transferred. Crystallographic analyses coupled to kinetic and mutagenesis studies have provided detailed insights into the mode of action of β-lactams and mechanisms of β-lactamase-mediated resistance. It will be of interest to see if the detailed structural-mechanistic information accumulated will lead to the (semi) 'rational' design of new types of inhibitor: β-lactams or otherwise. Major advances in biosynthetic studies have also come from crystallographic studies on both isopenicillin N synthase and the penicillin acylases and with studies on the genetics of β-lactam biosynthetic pathways.

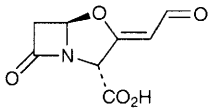
The number of synthetic studies on β-lactams has decreased somewhat since the last review period, but is still considerable. The commercially used total syntheses of the carbapenem antibiotics represent major synthetic achievements and this remains an area of significant interest. Perhaps surprisingly the carbacephems, also prepared by total synthesis, have received relatively scant synthetic attention. There is probably room for more synthetic efforts towards these antibiotics, which appear to be of increasing clinical importance. A gap in academic synthetic studies remains an asymmetric synthesis of clavulanic acid.

A list of review articles concerning β-lactam chemistry published during the period reviewed is given in the Appendix.

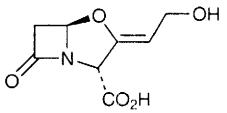
2 New Natural Products

Clavulanate-9-aldehyde (1) has been isolated from *Streptomyces clavuligerus*.[1] (See Section 3.2 for details of the biosynthetic significance of this compound.) This aldehyde is a significantly more potent inhibitor than clavulanic acid (2) of 'serine' β-lactamases, but is highly unstable with a half-life of *ca.* one hour in aqueous solution. The aldehyde was observed to undergo racemisation in

solution, consistent with its proposed intermediacy in the biosynthetic pathway to clavulanic acid (2).



(1)

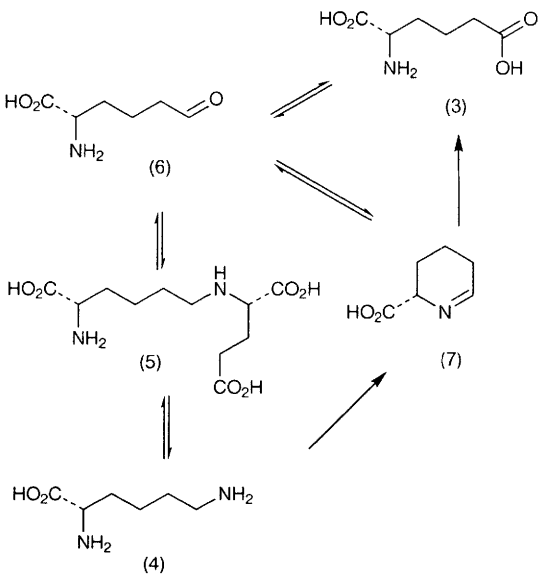


(2)

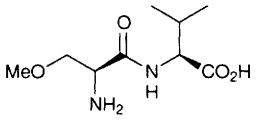
3 Biosynthesis

3.1 Penicillin and Cephalosporin Biosynthesis

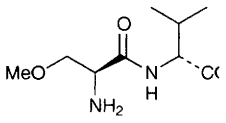
3.1.1 Early Stages – Studies using a *Penicillium chrysogenum* lysine auxotroph indicated the operation of two pathways leading to (L)-α-aminoadipate (3) from (L)-lysine (4). In addition to the normal metabolic route *via* saccaropine (5) and α-aminoadipic semialdehyde (6) it is believed that a route involving conversion of (4) to 1-piperideine-6-carboxylic acid (7) before oxidation to (L)-α-aminoadipate (3) also operates.[2] A broad range disulfide reductase isolated from *P. chrysogenum* is believed to be responsible for maintaining (L)-δ-(α-aminoadipoyl)-(L)-cysteinyl-(D)-valine (ACV) in its monomeric thiol form as required for isopenicillin N synthase (IPNS). The enzyme is heterodimeric consisting of a 72 kDa NADPH reductase and a 12 kDa general disulfide reductant.[3]



3.1.2 ACV Synthetase and Related Enzymes – Substrate analogue studies on ACV synthetases from *Cephalosporium acremonium* and *S. clavuligerus* have been carried out.[4] Two assays were used: amino acid stimulated ATP/PPi exchange, and a peptide formation assay. Analogues of (L)-α-aminoadipate (3), (L)-cysteine, and (L)-valine were all examined. It was found that (L)-α-aminoadipate (3) could be efficiently replaced by (L)-carboxymethylcysteine to give an isolable tripeptide product. Similarly (L)-cysteine could be replaced by allyl glycine or vinyl glycine to give isolable tripeptide products implying that binding to sulfur is not required for peptide formation. Use of (L)-*allo*-isoleucine but not (L)-isoleucine in the place of (L)-valine was tolerated by the enzyme. (L)-*O*-Methyl serine was active in the place of (L)-cysteine in the ATP/PPi and crude peptide formation assays, but instead of giving a tripeptide product, two dipeptides (8) and (9) were isolated. The apparently broad substrate specificity of ACVS was used to explain the isolation of several unusual tripeptides and penicillins from fermentations. On the basis of this work a revised mechanism for formation of ACV has been proposed, in which the cysteinyl-valine bond is the first formed peptide bond, contrary to previous proposals.[4] Crude ACVS has been used in a semi-continuous mode for the *in vitro* biosynthesis of ACV.[5]



(8)



(9)

3.1.3 Isopenicillin N Synthase – After many years of effort the X-ray crystal structure of IPNS has been determined.[6] Initial X-ray diffraction studies on crystals of recombinant IPNS from *C. acremonium* were reported, however a structure solution was not possible.[7] Subsequently, use of recombinant IPNS from *Aspergillus nidulans*[8], crystallised with manganese[9] in the place of iron led to the solution of the IPNS structure. The structure reveals the presence of 10 α-helices and 16 β-pleated strands. Eight of the β-strands fold to form a jelly roll motif, but unlike other jelly roll enzymes, one end of the barrel structure is open. The active site of IPNS is buried inside the open end of the barrel. The IPNS structure is from the family of non-haem ferrous dependent oxygenases which catalyse a wide range of oxidative reactions. Information obtained from the IPNS structure has led to new mechanistic proposals for IPNS and the 2-oxoglutarate dependent oxygenases.

Mutagenesis studies on IPNS from *C. acremonium* led to the conclusion that His-272 is essential for catalytic activity, and is probably involved in iron binding at the active site as demonstrated by the crystal structure of the IPNS-Mn(II) complex.[10] Work has also been carried out on the over-production of IPNS from various sources[11] including *Lysobacter lactamgenus.*[12] Studies on IPNS isozymes have provided further information on the lax substrate specificity with respect to unnatural substrates of IPNS.[13]

3.1.4 Cephalosporin Biosynthesis – Work on the later cephem biosynthetic genes has resulted in the cloning of 3-hydroxymethylcephem-*O*-carbamoyl transferase from *Norcada lactamdurans.*[14] The side chain specificity of crude deacetoxycephalosporin C (DAOC) synthase from *S. clavuligerus* has been reported to be very tight,[15] in contrast to DAOC/deacetycephalosporin C (DAC) synthase from eukaryotic sources which has a wider substrate specificity. More recent work with purified enzyme has however demonstrated that DAOC synthase from this source can utilise non-natural substrates such as adipoyl-6-APA.[16]

The genes encoding a two protein component system that converts cephalosporin C to 7α-methoxy cephalosporin C (cephamycin C) has been identified in *N. lactamdurans.*[17] Both proteins are required for the hydroxylation and methylation steps and are synthesised in a coordinated fashion. The first bears a resemblance to cholesterol monoxygenase and *O*-methyl transferases, the second is thought to act as a coupling agent, required to aid efficient hydroxylation in a manner similar to that seen in the two component system of *Pseudomonas putida* *p*-hydroxyphenylacetate-3-hydroxylase.

3.1.5 Penicillin Acylases – The crystal structure of penicillin G acylase from *E. coli* has been solved.[18] The kidney shaped protein is a heterodimer of 80 kDa. It is biosynthesised as a single unit with cleavage of a 26 amino acid signal peptide and a 54 amino acid spacer generating the two sub-units of 209 and 556 amino acids. The nucleophilic active-site seryl residue is located at the mouth of the binding pocket and unusually is at the *N*-terminus. In contrast to the catalytic triad unit of the serine proteases there is no adjacent histidine residue so the nucleophilicity of this seryl residue must be promoted by an alternative mechanism. The postulated mechanism[18] invokes activation of the reactive hydroxyl group by the seryl amino group (acting *via* a bridging water molecule) to direct attack onto the amide carbonyl group.

Two isozymes of penicillin G acylase from *P. rettgeri* have been purified and their X-ray crystal structures determined.[19] In common with the acylase from *E. coli,* the *P. rettgeri* acylase is also produced as a propeptide and cleavage generates two subunits of 23.7 and 62.2 kDa, and although there are slight differences in cleavage position there is a 75% homology between the two enzymes. Site-directed mutagenesis studies have supported the involvement of the *N*-terminal serinyl residue in the catalytic mechanism of this acylase.[20]

Extensive studies continue on the commercial use of penicillin acylases/acyltransferases[21-23] including attempts to change the substrate tolerance of penicillin G acylase to accept cephalosporin type substrates.[19] Other studies have been directed at changing the substrate selectivity of the acylases by selective pressure based on growth requirements. The work has generated mutants that hydrolyse adipyl-, glutamyl-, valeryl-, caproyl- and heptanoyl-(L)-leucine side chains better that the native enzyme.[24] Another approach uses immobilised penicillin acylases modified by the inclusion of inhibitors during the enzyme immobilisation process.[25] It is claimed that these inhibitors promote a specific active site conformation which is retained after completion of the immobilisation process and removal of the inhibitor. It is proposed that this process may be used

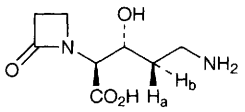
to generate altered enzyme substrate selectivities. It has been reported that benzyl alkylketones are exceptionally potent irreversible competitive inhibitors of penicillin acylase.[26]

The reasonably wide substrate selectivities of the penicillin acylases continue to be exploited for a variety of synthetic uses, for example the resolution of racemic mixtures of β-fluoro-alkyl-β-amino acids[27] by hydrolysis of their phenyl amide derivatives. An alternative approach to selective hydrolysis of amides for resolution of such compounds is the selective *synthesis* of their phenyl amides using penicillin G acylase so removing the need for chemical formation of the racemic phenyl amide derivative before the resolution step.[28]

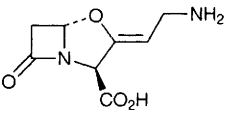
3.1.6 Cephalosporin Acylases/Acyltransferases – Methods for the production of 7-aminocephalosporins continue to be developed. The DAOC synthase gene from *S. clavuligerus* has been cloned into *P. chrysogenum* under the transcriptional and translational control of the *P. chrysogenum* aminohydrolase/acyl transferase gene promoter. This sequence allows for simultaneous expression of expandase and acyltransferase genes. Combined with the use of carboxymethylthiopropionic acid in the fermentation media this allows facile recovery of 2-(carboxyethylthio)acetyl- and 3-(carboxymethylthio)propionyl-7-ADCA as part of a process for production of 7-ADCA.[29] A similar approach utilises adipic acid to allow isolation of adipoyl-7-ADCA, a useful intermediate for production of 7-ADCA.[30] An alternative, widely investigated approach to 7-ADCA is *via* the oxidation of the (D)-α-aminoadipate side chain of cephalosporin C using a (D)-amino acid oxidase prior to decarboxylation with peroxide to yield a glutaryl side-chain, which is subsequently removed by an acylase. Stabilised permealised cell systems containing both cephalosporin acylase and (D)-amino acid oxidase clones have also been developed for commercial use.[31] Further studies on a number of acylases from various sources for this purpose have been carried out.[32] A small scale approach to the generation of 6-amino penicillins (and in principle 7-aminocephalosporins) is *via* replacement of (L)-α-aminoadipoyl side chain of tripeptide substrates with the 5-amino-5-carboxy-2-oxo-pentanoyl side chain in the IPNS catalysed formation of penicillins from tripeptides. Treatment of the penicillins or cephems so produced with a (D)-amino acid oxidase followed by peroxide leads to *in situ* decarboxylation and decarboxylative elimination of the side chain.[33] Work has also been carried out with the aim of altering the substrate selectivity of cephalosporin acylases with the aim of improving the cleavage of the (L)-α-aminoadipoyl side chain.[34] The gene encoding a cephalosporin C deacetylase from *Bacillus subtilis* has been cloned and sequenced.[35] The deduced amino acid sequence indicated that this enzyme has an activated serine residue in the active site, in common with many esterases and proteases. Work continues to be carried out on developing stabilised forms of cephalosporin acylases.[31]

3.2 Clavam Biosynthesis – Further evidence based on feeding studies for a shared biosynthetic origin of clavulanic acid and clavams has been reported.[36] Significant advances in the identification of the genes encoding the various biosynthetic steps have been achieved. The use of disruptants has led to the

assignments of genes encoding proclavaminate amidino hydrolase (PAH), clavaminic acid synthase (CAS), and proteins with identities to ornithine acyltransferase and a peptide transport protein. The function of the latter two and two other genes remains unknown.[37] The identification[38] of the gene encoding the enzyme PAH, responsible for removal of the guanidino group from the biosynthetic precursor to proclavaminic acid (10) was accomplished and its expression[39] together with cloned CAS has allowed a 4-step biosynthetic conversion to clavaminic acid (11) to be carried out *in vitro*.[40] Incubations of (4*R*)- and (4*S*)-[4-$^2H_2$]-proclavaminic acid (12, 13) with clavaminic acid synthase resulted in stereoselective removal of the deuterium and hydrogen respectively.[41] It was thus suggested that the conversion of dihydroclavaminic acid (14) to clavaminic acid (11) is achieved *via* an enzyme catalysed *syn* elimination. The expression and purification of recombinant CAS isozymes from *S. clavuligerus*[42,43] and further work on wild-type CAS from *S. antibioticus*[44] has been reported and initial mechanistic studies made.[43] Interestingly, the two CAS isozymes in *S. clavuligerus,* which appear to have the same function, are differentially regulated depending on the fermentation media used.[45]



(10) H_a=H_b=^1H
(11) H_a=^2H, H_b=^1H
(13) H_a=^1H, H_b=^2H



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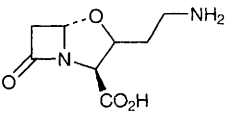
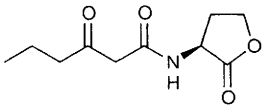


(14)

Clavulanate-9-aldehyde (1), a postulated precursor to clavulanic acid, has been isolated from *S. clavuligerus*. The facile racemisation of synthetic samples of (1) indicates that this is a likely point for the required double epimerisation process which must occur during clavulanate (2) biosynthesis.[1] A clavulanic acid (2) biosynthesis regulatory gene has also been cloned[46] and the gene encoding for the enzyme catalysing the dehydrogenation of (1) to yield clavulanic acid (2) has been sequenced.[47]

3.3 Carbapenem Biosynthesis – Autoinduction of antibiotic production by *N*-(3-oxohexanoyl)-(L)-homoserine lactone (15) has been shown to operate for carbapenem biosynthesis in *Erwina cardovara*. The production of (15) was found to be cell density dependent, and when produced to a critical level antibiotic production is triggered.[48] Similarly, studies on non-thienemycin carbapenem producing strains of *S. cattleya* have identified the action of a small molecule which appears to regulate expression of the gene cluster.[49]



(15)

3.4 Regulatory and Related Studies – Activity continues to be directed towards the identification of regulatory factors in penicillin and cephalosporin biosynthesis. Metabolic flux studies on penicillin V biosynthesis in *P. chrysogenum* have demonstrated substantial leakage of biosynthetic intermediates into the medium.[50] The cloning of multiple copies of IPNS and acyl CoA:6-APA acyltransferase (AAT) genes into *A. nidulans* greatly increased the total *in vitro* activities of IPNS and AAT. The forced expression of IPNS however only resulted in a small increase in antibiotic production whilst for AAT a slight drop was observed implying that neither of these steps is rate limiting.[51] The use of halogenated phenylacetic acid derivatives as inhibitors for both of these enzymes has been reported.[52] The introduction of multiple copies of DAOC/DAC synthase into *C. acremonium* led to a reduction in leakage of intermediate, implying that this step is rate limiting under some conditions.[53] However, cloning extra copies of the lysine-6-aminotransferase (LAT) gene into *S. clavuligerus* led to increased LAT activity and cephalosporin production and it was concluded that the rate limiting step of antibiotic production is temporally controlled, requiring the optimisation of several genes to maximise output.[54] Similar analysis of penicillin production in *P. chrysogenum* has demonstrated a shift of the rate limiting step from that catalysed by ACVS to that catalysed by IPNS.[55] Cloning of the *S. clavuligerus* DAOC synthase gene into *P. chrysogenum* resulted in greatly increased expandase activity, reportedly up to 75% of that observed in industrial strains of *C. acremonium*.[56]

Studies on the expression of the early penicillin biosynthetic genes from *S. clavuligerus* imply that LAT, ACVS and IPNS function as an operon regulated by the LAT promoter,[57,58] indeed it is reported that all three are transcribed on a single piece of RNA of 13kb.[59] Studies in *P. chrysogenum* show a similar clustering of ACVS, IPNS and AT which are expressed as a single transcript.[60] However, studies in *A. nidulans* rule out the sequential induction of the IPNS gene and the AT gene in this organism.[61] In *C. acremonium* ACVS and IPNS are in a separate cluster from the later cephalosporin genes but both clusters are regulated by methionine,[60,62] known to stimulate β-lactam production. The analysis of high producing *P. chrysogenum* strains has demonstrated the presence of multiple linked clusters of the penicillin biosynthetic genes.[63]

A study has demonstrated that alanine inhibits antibiotic production in resting cells and is an inhibitor of ACVS, IPNS, and DAOC/DAC but not the enzyme epimerase. It is therefore postulated that the regulation mechanism for cephalosporin production involves alanine as well as ammonium ions.[64] The growth of *P. chrysogenum* in a low glucose medium has demonstrated a close relationship between glucose levels and metabolic capacity (as measured by RNA levels).

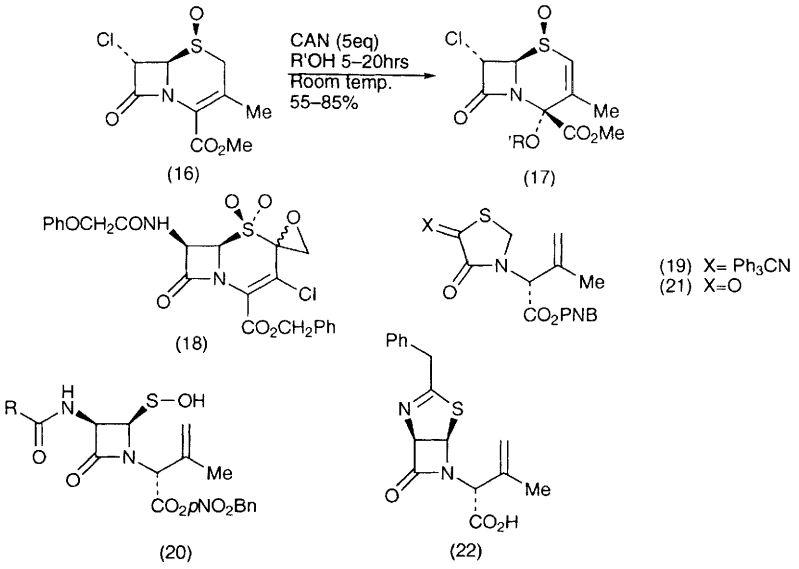
Continued growth in low glucose environments leads to loss of production due to the evolution of mutants which have lost the genes encoding for ACVS, IPNS and AT.[65]

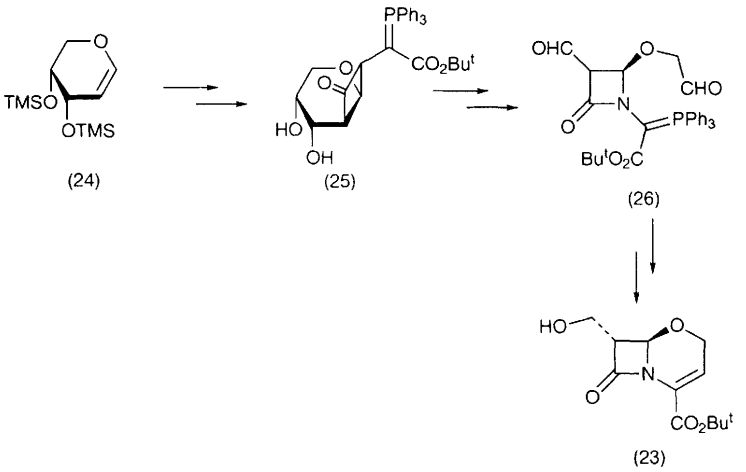
4 Penicillins and Cephalosporins

Whilst oxidation of cephalosporins with ceric ammonium nitrate (CAN) in alcohols is known to result in 2-alkoxylation, the use of CAN on cephem 1β-sulfoxides, *e.g.* (16) results in 4-α-alkoxy-Δ^2-cephem-1-β-sulfoxides (17) in high yield and with good regio- and stereoselectivity.[66] Interestingly, the corresponding 1α-sulfoxide resisted oxidation, whilst the sulfone underwent slow reaction resulting in a mixture of C-4 epimers. Dimethyldioxyrane in acetone oxidises cephalosporins to their sulfones in high yield without Δ^2/Δ^3 isomerisation.[67] Use of 1 equivalent of CAN resulted in a mixture of α and β-sulfoxides. Unsaturated C-3 side chains are compatible with excess oxidant, however 2-methylene cephalosporins are oxidised to the epoxide-sulfone derivative (18).

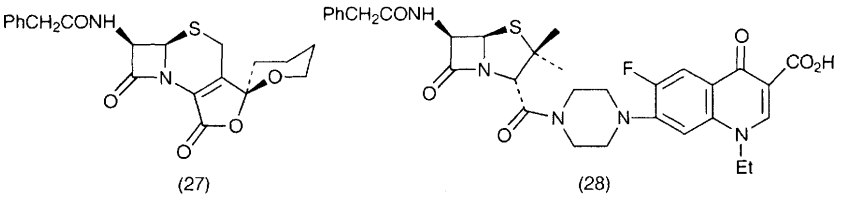
Application of Kamiya's thermal ring opening of penam sulfoxides to 6-tritylamino penams resulted in exclusive isolation of the 5-trityliminothiazolidinone (19), *via* scission of the original C-5 to C-6 penam bond, rather than the expected sulfenic acid (20).[68] (19) may be hydrolysed to the corresponding 4,5-dioxothiazolidinone (21). An improved one-pot production of thiazoline azetidinones such as (22) from penicillin G sulfoxide has also been reported.[69]

The oxacephem 7-hydroxymethyl-1-oxa-3-cephem-4-carboxylate (23) has been synthesised from arabinal (24) through metaperiodate cleavage of the diol (25) and Wittig closure of the resultant dialdehyde (26) (Scheme 1).[70] Further





Scheme 1

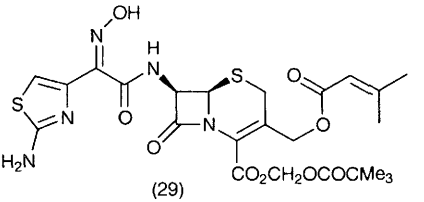


2-oxaisocephems utilising variations of the aminothiazol-hydroxyimino sidechain have been synthesised and show potent antibacterial activity.[71]

C-3-*epi*-Penicillin has been synthesised in homochiral form in four steps from 6β-phthalimidopenicillanic acid in good yield.[72] Cephem based spiroacetal-lactones such as (27) have been synthesised from cephem-3-triflates but show no biological activity.[73] Cephalosporins and penicillins such as (28) linked *via* an amide bond to 4′ and 3′ antibacterial fluoroquinolyl side chains respectively have been synthesised and show significant biological activity. It was proposed that this activity resulted from action of the intact bifunctional molecule as opposed to dual action following cleavage of the amide linker.[74] The relative instability to ester hydrolysis at neutral and low pH of Δ^2 cephem esters compared to Δ^3 cephem esters has been utilised to remove Δ^2 cephem ester impurities resulting from isomerisation of the alkene during cephem synthesis.[75,76]

The replacement of the 3-acyl sidechain of cefotaxime with an α,β-unsaturated unit *e.g.* (29) has resulted in high activity cephalosporins which display improved resistance to degradation by esterases.[77] Replacement of the C-3 side chain with benzotriazolium units as in (30) also gives rise to biologically active species.[78] Similarly, the use of quaternary ammonium side chains based on hydroxylated cyclic amines such as (31)[79] or an azoliomethyl group (32)[80] has resulted in cephems with biological activity. Other heterocyclic groups such as

(33) have been used as side chains with some success.[81-83] Many cephems with 3-(*N*-substituted carbamoyloxy)- and 3-(*N*-substituted carbamoyloxy propenyl)-side chains (34, 35) have been synthesised and show good antibacterial activity.[84-86] The effect of oxime substitution in the 7-sidechain was also examined.[85]



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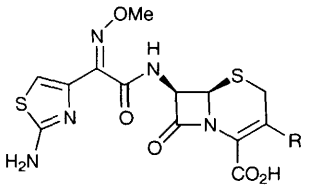
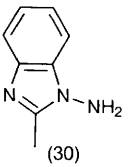


R=



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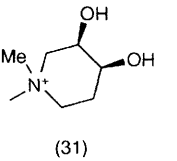

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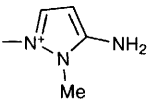

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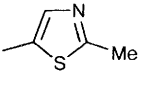

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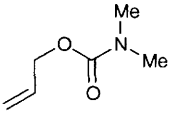

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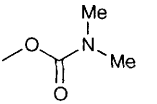
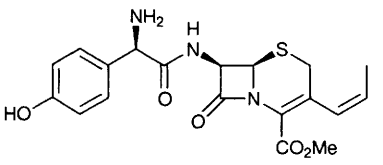


(35)

A novel approach to cephems substituted at C-7 and C-3 such as cefzil (36) has been achieved *via* cuprate mediated cyclisation of an allenylazetidinone (37) obtained from penicillin sulfoxide.[87] An improved system for achieving such cyclisations has been reported based on a coupled catalytic system utilising aluminium, lead bromide and $NiCl_2$(bipy) generating cephems with a wide range of carbon based C-3 side chains (Scheme 2).[88] Utilisation of a lead bromide/aluminium catalytic system with the allene (38) synthesised from the enol triflate (39), leads to production of 6-substituted 2-*exo*-methylenepenam (40) in high yield.[89] Use of the free enol as opposed to the triflate (39) leads to production of 3-hydroxycephems (41).[90] Cephems with a 3-(1,3-butadienyl) sidechain (42) were synthesised *via* base mediated elimination of an allylphosphate precursor (43)[91]



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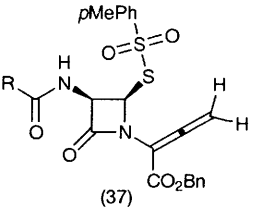

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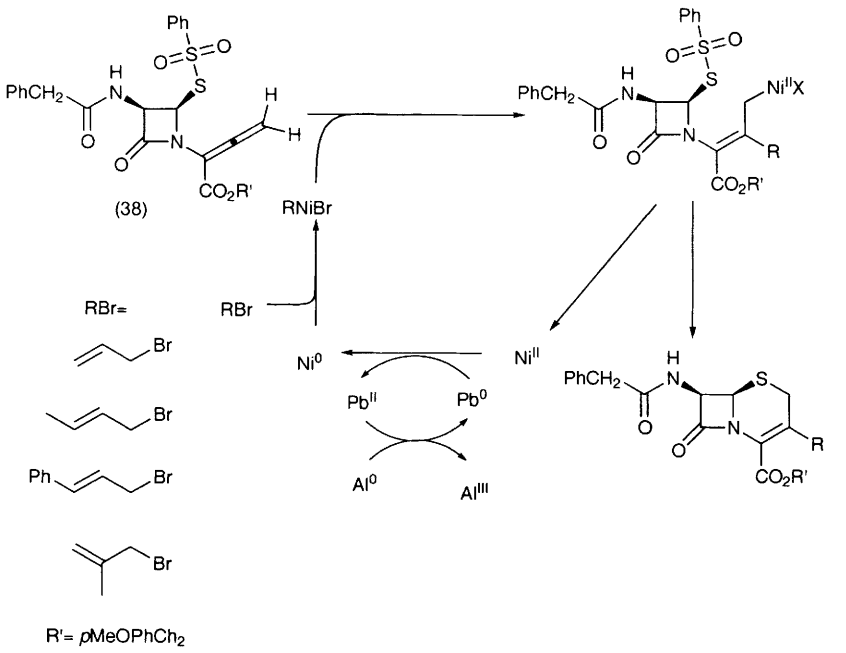
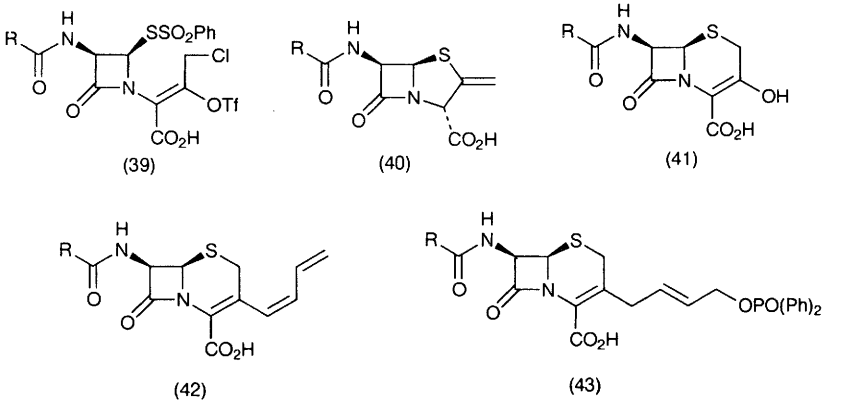
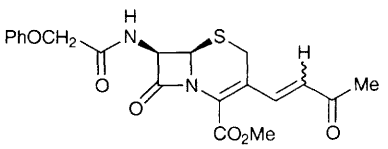


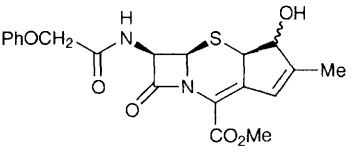
Scheme 2



obtained from 3-chlorocephems. 3-Chlorocephems have themselves been synthesised by treatment of the 3-mesyloxy derivative with LiCl; a subsequent oxidation/reduction procedure was required to convert the resulting Δ^2/Δ^3 mixture to the Δ^3 form only.[92] C-3 Cephem phosphoranes have been converted into 3-alkenylcephems (44) along with tricyclic cephems (45) by Wittig reaction with α-diketones.[93] Protection of the 7-amino group of cephems to allow C-3 elaboration



(44)



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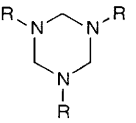
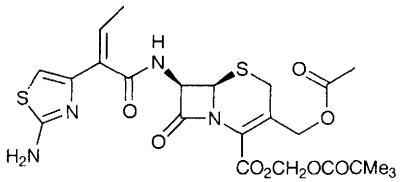


(46) R= 7-ADCA

has been achieved by reaction with formaldehyde as the tri-cephem substituted triazine (46).[94]

Further syntheses of the aminothiadiazol-oxime sidechain used in many fourth generation cephalosporins have been reported.[95] Replacement of the oxime moiety of the corresponding thiazol based sidechain with substituted alkenes *e.g.* (47) provides cephalosporins with good antibacterial activity.[96] Wittig methodology has been used to synthesise α-vinyloximinoacetic acids for coupling to 6-APA and 7-ACA and has provided a range of potent antibiotics, especially when utilising the thiazole moiety as an alkene substituent (48).[97]



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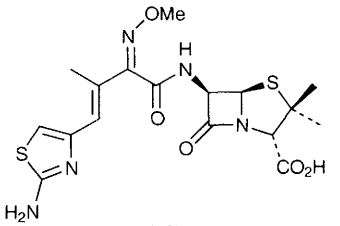
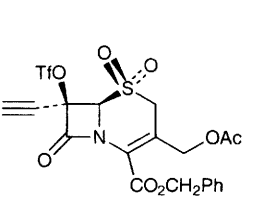
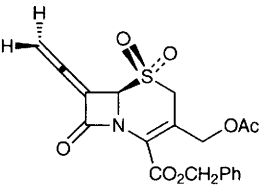


(48)

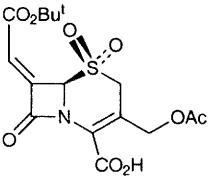
7-Vinylidenecephalosporins have been prepared from 7-ACA *via* treatment of the propargylic triflate (49) with organocopper reagents as reported for previous penicillin congeners. Subsequent oxidation of the sulfur yielded the sulfone (50) which is a progressive inhibitor of the class C β-lactamase from *Enterobacter cloacae*.[98] 7-(Carboxymethylene)cephalosporinates (51) and 6-(carboxymethylene)penicillinates (52) have been synthesised and their sulfone derivatives show potent β-lactamase inhibitory activity.[99] Similarly, sulfonylamido-penicillinic acid sulfones (53) have been synthesised from 6-APA and show potent inhibition of the RTEM β-lactamase.[100]



(49)



(50)



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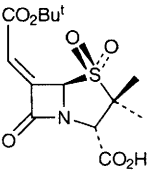
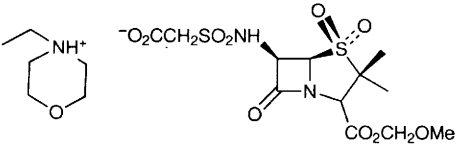


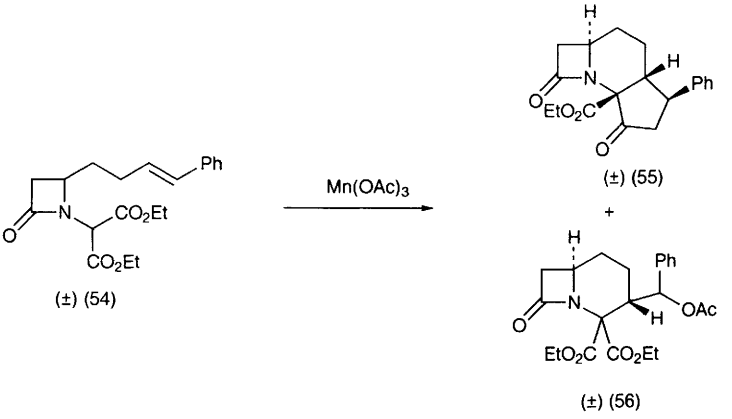
(52)



(53)

5 Carbacephems

Although the clinical use of carbacephems continues to attract interest,[101] compared to the last review period relatively few reports describe synthetic efforts towards these β-lactams. A new approach to the synthesis of the carbacephem skeleton utilises an oxidative free radical cyclisation for annulating bicyclic β-lactams (Scheme 3).[102] Treatment of racemic precursor (54) with $Mn(OAc)_3$



(±) (54)

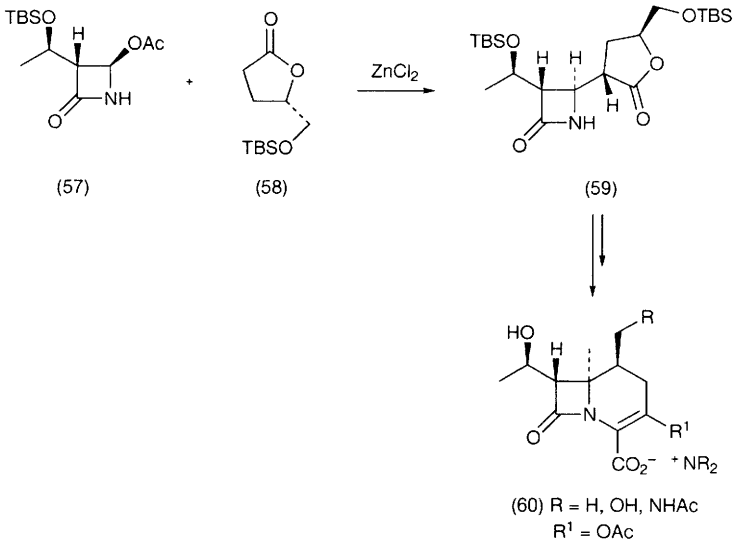
(±) (55)

(±) (56)

Scheme 3

gave (55) as major diastereomer (3:1) (58-68%) together with (56) (13-15%). Attempts were made to combine structural features of different classes of β-lactam antibiotics and create new hybrids with improved activities. Thus, compound (60) incorporates the C-7 hydroxyethyl side chain characteristic of penems together with the carbacephem nucleus.[103] A key step in the synthesis of (60) was addition of acetoxyazetidinone (57) to the zinc-enolate of lactone (58) (Scheme 4). The condensation product (59) was obtained stereoselectively in good yield.[103] In an extension of the general concept (Scheme 5)[104] zinc-mediated condensation of 2-trimethylsilyloxy furan and azetidinone precursor (57) gave (61) which was converted into the desired tricyclic target compound (62). The hybrids (60) and (62) did not display significant antibiotic activity.[105,106]

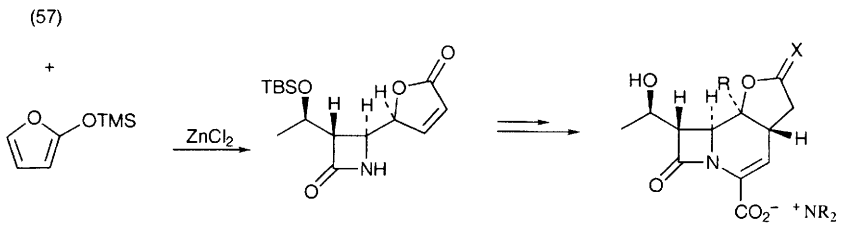
Kinetic data for the kinetic resolution by enzymatic acylation of Loracarbef precursor (63) by penicillin G amidase has been reported.[107]



(57) (58) (59)

(60) R = H, OH, NHAc
R^1 = OAc

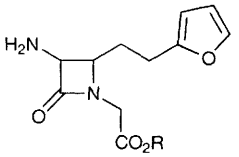
Scheme 4



(61)

(62) R = H, OH, NHAc
R^1 = OAc

Scheme 5

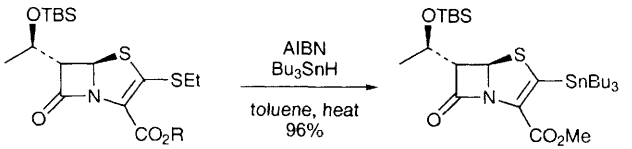


(63) R= H, Me

6 Penems

Attempts at improving the antibiotic activity and hydrolytic stability of the penems have been made by introducing new substituents at the C-2, C-3, and C-4 positions of the penem nucleus.

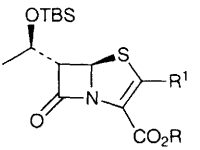
C-2 Substituted penem stannanes were obtained in excellent yield *via* a desulfurative stannylation reaction from the corresponding vinylthioethers (64) (Scheme 6). Palladium mediated cross-coupling with aryl and heteroaryl halides



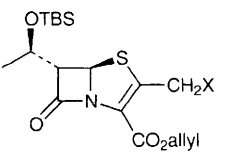
(64) R= allyl, Me

Scheme 6

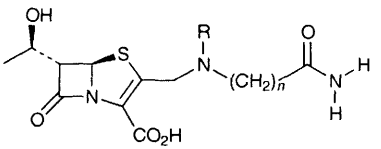
gave access to C-2 substituted penems (65).[108] Displacement of penem mesylate (66) by protected amino acid esters led to 2-substituted penems with amino acid amide related side chains of which MEN 10700 (68) was shown to exhibit significant antibiotic activity.[109,110] Detailed SAR studies of penem derivatives



(65) R = allyl, Me
R^1= aryl, heteroaryl

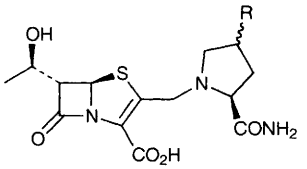


(66) X = SO_3Me
(67) X = I

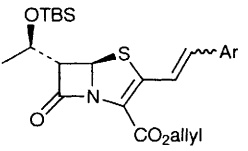


(68) R = Me, *n* = 1

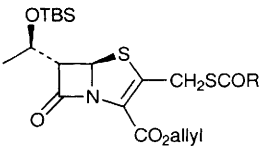
with proline-related substituents in the C-2 position (69) obtained *via* the same route have been carried out.[111] 2-Iodomethyl penems (67) have been reported to give access to 2-arylethenyl penem derivatives (70) by Wittig-olefination in moderate yield and with good *E/Z* selectivity.[112] Mild base hydrolysis of thioester (71) and trapping of the free thiol *in situ* with ethyl isocyanate gave a mixture of (72) with a carbamate protected thiomethyl group in the 2-position and exomethylene compound (73).[113] Introduction of a bicyclic imidazole system in the 2-position of penems by known methodology was shown to result in compounds with good antibiotic activity and resistance to hydrolysis by renal dehydropeptidase-1 (DHP-1).[114]



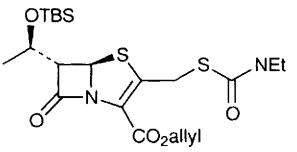
(69) R= H, OH



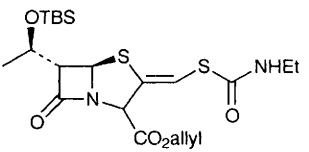
(70)



(71)

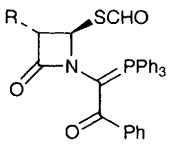


(72)

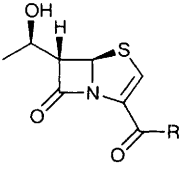


(73)

The effect of altering the carboxylate substituent at the C-3 position of normal penems has been investigated.[115] Condensation of phenyl glyoxal with azetidinones followed by conversion into phosphoranes (74) and cyclisation gave access to 3-benzoyl penems. The 6-unsubstituted derivatives were unstable, but 3-benzoylpenems (75) and (76) bearing a hydroxyethyl side chain in the 6-position were stable and shown to possess moderate antibacterial activity.[115]



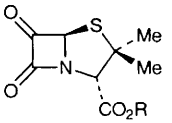
(74) R = H, Et, MeCHOTBS



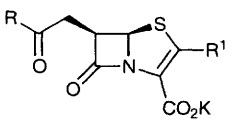
(75) R = Ph
(76) R = NR_2

Introduction of an alkylcarbonylmethyl group in the 6-position of the penem nucleus led to *in vitro* antibacterial activity against Gram-positive, but not against Gram-negative strains of bacteria. Despite their greater chemical stability with respect to imipenem the new substances were more susceptible to hydrolysis by

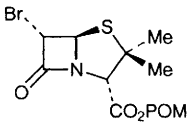
DHP-1.[116] 6-Oxopenicillinate (77) proved a useful intermediate in the synthesis of 6β-alkylcarbonylmethyl substituted (78), whereas 6-bromo-penicillinate (79) served as precursor in the synthesis of the 6α-alkylcarbonylmethyl epimer (80).[116] The effect of 6,6-disubstitution on antibiotic potency and hydrolytic stability has been investigated.[117] Addition of diethyl cadmium to 6-oxopenicillinate (77) followed by acetylation gave 6α-ethyl-6β-acetyl penicillinate (81) which was converted into penem (82) *via* known methodology.[117] Introduction of a 6β-methoxy substituent to give (83) was achieved by ethyl magnesium bromide mediated bromine diplacement of 6-bromo-substituted penicillinate (78).[117]



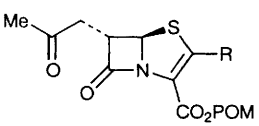
(77) R = $CH_2CO_2Bu^t$, CH_2CCl_3



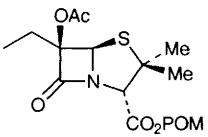
(78) R = alkyl, aryl
R^1 = Ph, CH_2OPh



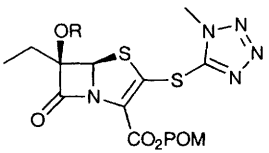
(79)



(80) R = CH_2Cl, CH_2Ph



(81)

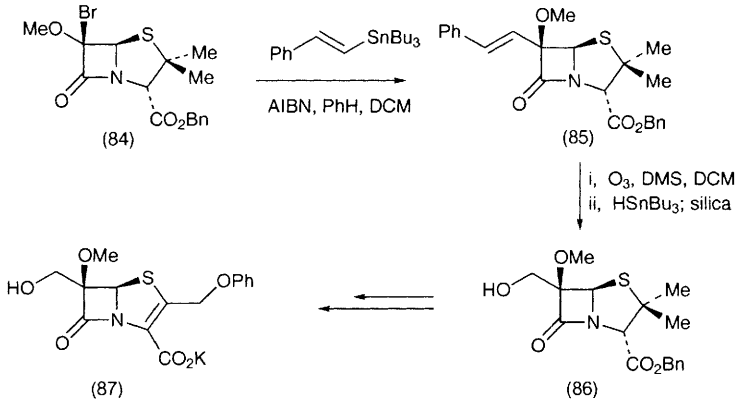


(82) R = Ac
(83) R = Me

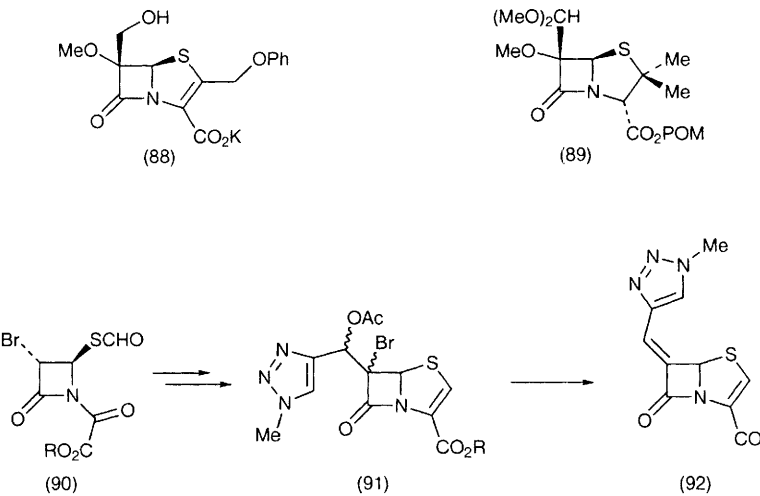
Radical vinylation of 6-bromo-substituted penicillinate (84) gave intermediate (85). After ozonolysis, reduction with tributyltin hydride afforded (86) which was converted into 6β-methoxy-6α-hydroxymethyl penem (87) (Scheme 7).[118] The synthesis of 6α-methoxy-6β-hydroxymethyl epimer (88) of (87) was accomplished following a similar overall-route using 6α-methoxy-6β-dimethoxymethyl penicillinate (89) as the key intermediate. This intermediate (89) was prepared by boron trifluoride mediated addition of methyl orthoformate to 6-diazopenicillinate.[119]

Potent β-lactamase inhibitor (5*R*)-(*Z*)-6-(1-methyl-1,2,3-triazol-4-ylmethylene)-penem-3-carboxylate (92) has been synthesised *via* trialkyl-phosphite mediated closure of the 6-APA derived azetidinone (90) and stereoselective elimination of the bromoacetate (91) (Scheme 8).[120]

Investigations into improved procedures for the preparation of penem precursors continue. Treatment of oxalimides (93) with methyl diethylphosphite and



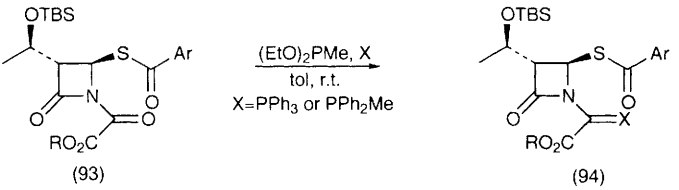
Scheme 7



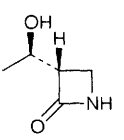
Scheme 8

triphenylphosphine or diphenyl methylphosphine afforded phosphoranes (94) in good yield (Scheme 9).[121] Chiral penem precursor (95) was obtained by esterase mediated kinetic resolution of diastereomeric dihydrooxazines (96) followed by ring-closure. The carboxylesterase NP exhibited poor diastereoselectivity, but good enantioselectivity.[122]

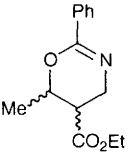
Structural investigations of the penem nucleus using mass-spectrometry[123] and X-ray single crystal analysis[124] have been reported.



Scheme 9



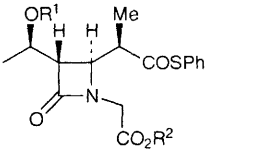
(95)



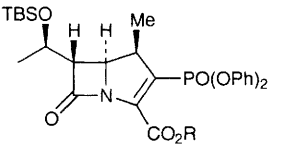
(96)

7 Carbapenems, Carbapenams, Carbacephems and Related Systems

A new synthesis of the 1β-methylcarbapenem skeleton has been reported in which the Dieckmann cyclisation of 1-(2-oxazetidinyl)acetate (97) using sodium hydride is the key step. Trapping of the sodium enolate with diphenylchlorophosphate yielded known enolphosphate (98) without epimerisation of the 1β-methyl substituent. Conversion to meropenem (99) could be carried out in a one-pot procedure from (97) in good overall yield.[125] The same authors have investigated the effect of the C-2 side chain substituent on neurotoxicity of carbapenem antibiotics.[126] A synthesis of key 1β-methylcarbapenem intermediate (100) from enaminoketone (101) *via* a stereoselective reduction followed by several steps to establish the requisite stereochemistry has been reported.[127] Several other reports reflect the importance of (100) as a key intermediate in 1β-methylcarbapenem synthesis. Thus, a diastereoselective acid catalysed decarboxylation of malonate



(97) R^1, R^2= protecting group



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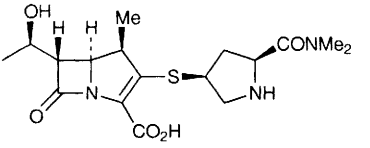

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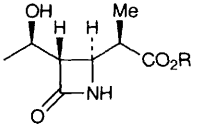
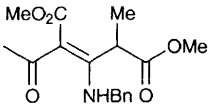
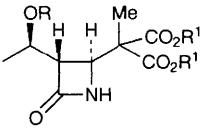


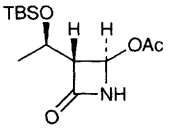
(100) R = H, Me



(101)



(102) R = TBS, R^1 = H
(103) R = TBS, R^1 = allyl



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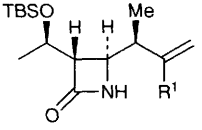
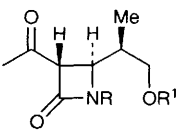


(105) R^1 = H
(106) R^1 = CO_2Et



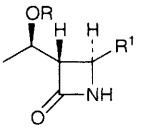
(107) R = CH(*p*-$MeOC_6H_4$)$_2$
R^1 = $PhCH_2$

derivative (102), obtained from commercially available 4-acetoxyazetidinone (104) and Meldrum's acid, has been successfully employed in its preparation.[128-130] Diallyl malonate (103) was converted into (100) by palladium-mediated deallyloxycarbonylation.[131] A convenient synthesis of (100) utilises oxidation of alkylidene precursors (105) and (106) with acidic permanganate solution.[132] Novel methodology based on the palladium catalysed asymmetric acetalization of alkenes has been developed to provide enantiomerically pure carbapenem precursor (107).[133] A copper mediated displacement of the thiophenyl moiety of 4-phenylthioazetidinone (108) by a number of synthetically useful groups has been reported.[134] This procedure overcomes the difficulties posed by the poor ability of the phenylthiol moiety as a leaving group and provides access to synthetically useful intermediates such as (109) and (110).

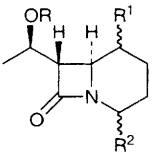
The well known carbene cyclisation strategy to form the carbapenem five membered ring has been used to prepare 1β-hydroxymethyl carbapenem derivative (111).[135] 1β-Carboxyethyl carbapenem (114) was prepared analogously from precursor (113) which was accessible by base catalysed equilibration of isomers (112) and (113).[136]

New methods for the closure of the five membered ring of the 1β-methyl-carbapenems have been developed. Allyl precursor (115) was cyclised employing π-allyl palladium chemistry.[137] Closure of the five-membered ring was also achieved by an intramolecular addition-elimination strategy using iodovinyl sulfone (116). Treatment with $LiN(TMS)_2$ afforded a 5:1 mixture of Δ^2-exo and Δ^2-endo carbapenems (117) and (118).[128] Another approach utilised an aza-Cope Mannich cyclization. Treatment of chloride (119) with $AgBF_4$ afforded aldehyde (120) *via* an iminiun ion intermediate in 33% yield.[138]

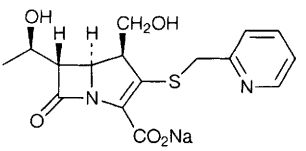
Variation of the carbapenem side chain has been further explored. 1β-Methyl carbapenem FR21818 (121) with a novel pyrazoliomethyl pyrrolidine side chain exhibited excellent *in vivo* activity and was stable to renal dehydropeptidase I.[139]



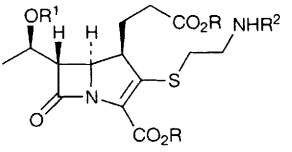
(108) R = TBS, R^1 = SPh
(109) R = TBS, R^1=OAc
(110) R = TBS, R^1 = $CH(CO_2Me)_2$



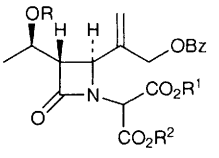
(112) R = TBS; R^1 = α-CO_2Et; R^2 = SPh
(113) R = TBS; R^1 = β-CO_2Et; R^2 = SPh



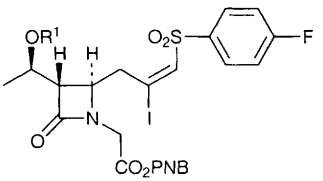
(111)



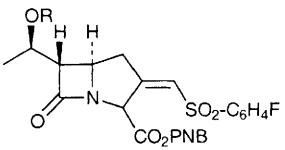
(114) R = allyl; R^1 =TMS;
R^2 = $CO_2CH_2CHCH_2$



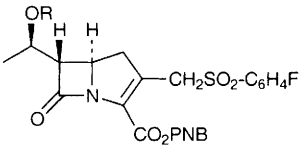
(115) R = TBS; R^1, R^2 = Et



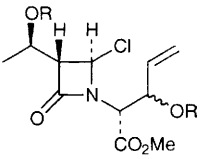
(116) R = TBS



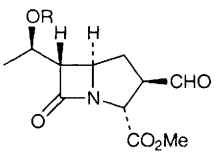
(117) R = TBS



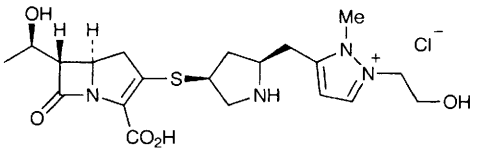
(118) R = TBS



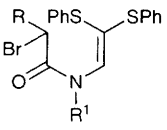
(119) R = TBS



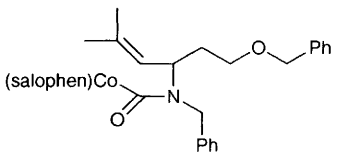
(120) R = TBS



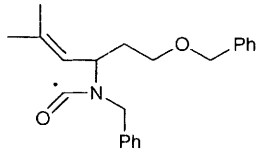
(121)



(122) R = H, Et; R^1 = PMB



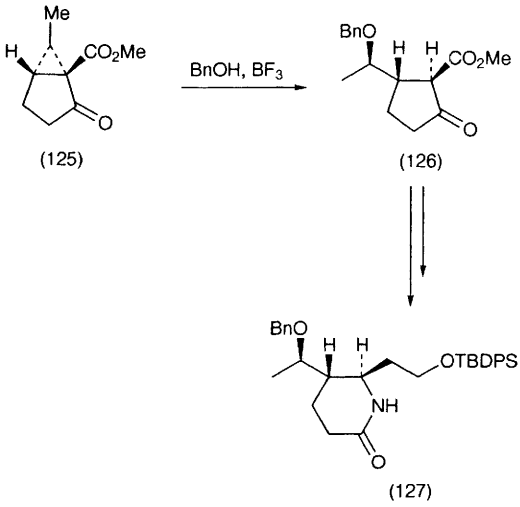
(123)



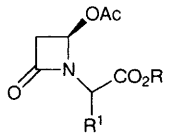
(124)

Radical based chemistry for the formation of monocyclic β-lactam carbapenem precursors has been explored. Sulfur-directed regioselective 4-*exo*-trig cyclisation of the radical *via* carbon bromine bond homolysis of (122) provided access to the β-lactam ring. The phenylthiomethyl group was subsequently used as a handle to introduce the required functionality in a formal synthesis of (±)-PS-5 and (+)-thienamycin.[140] Carbamoyl radicals (124) derived from carbamoylcobalt salophens (123) have been cyclised to give β-lactams in a new formal synthesis of (±)-thienamycin from (123).[141]

Grieco's intermediate (127), used in the synthesis of thienamycin, has been obtained by a Lewis acid catalysed homoconjugate addition of benzyl alcohol to bicyclic precursor (125) to give (126) followed by a Beckmann rearrangement as key steps (Scheme 10).[142]



Scheme 10



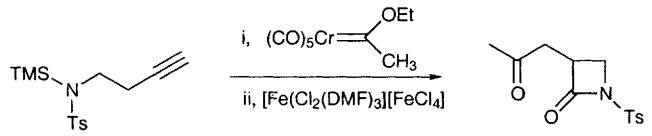
(128)

An overview of the microbiological properties of meropenem[143] and several studies of the activity profile of carbapenems have been reported.[144-147]

8 Azetidinones

Three main areas of interest can be distinguished: new synthetic approaches to azetidinones; modification of azetidinones to generate synthetic precursors for bicyclic β-lactam antibiotics; the application of azetidinones to the synthesis of biologically active compounds, in particular taxol derivatives and precursors.

8.1 Synthesis of Azetidinones – The Ugi reaction has been employed in a synthesis of 4-acetoxy-azetidinone (128) from (L)-aspartate.[148] In an unusual approach vinyl ketenes, obtained by Fischer chromium carbene insertion into alkynes, have been used in the synthesis of β-lactams (129) in satisfactory yields (Scheme 11).[149]

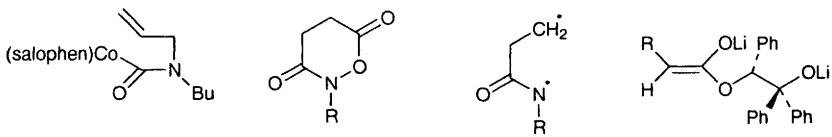


(129)

Scheme 11

Current interest in radical based synthetic methodology is also reflected in the field of azetidinones. Carbamoylcobalt(III) salophen compounds (130) are sources of carbamoyl radicals which were utilised in a 4-*exo*-trig cyclisation to give azetidinones.[150] Photolysis of tetrahydro-1,2-oxazine-3,6-dione (131) gave azetidinones in low yields *via* putative diradical intermediate (132).[151] Copper catalysed reaction of terminal alkynes with nitrones afforded 1,2,4-triphenyl-azetidin-2-ones.[152]

A facile conversion of β-amino acids into β-lactams under neutral and mild conditions has been reported using triphenylphosphine and *N*-alkyl-2-benzothia-

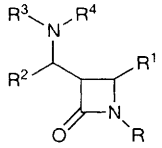


(130) (131) (132) (133)

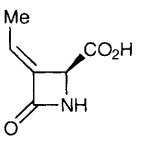
zolylsulfenamides.[153] The synthesis of fluorinated analogues has also attracted interest in the field of azetidinone chemistry. Thus, 3-methyl-3-trifluoromethyl azetidinone has been prepared with the aim of increasing the reactivity of the β-lactam carbonyl group.[154]

The ester enolate-imine condensation route to azetidinones has been exploited further. Potassium *tert*-butoxide has been used for the enolate generation.[155] An enantioselective synthesis of 3,4-*trans*-β-lactams with high e.e. has been achieved from ester enolates (133) using a triphenylglycol as chiral auxiliary. Protection of the tertiary hydroxyl group as a methyl or trimethylsilyl ether reversed the stereochemical outcome of the reaction and yielded 3,4-*cis*-β-lactams in slightly lower e.e.[156] β-Dialkylamino-esters have been coupled with imines to give 3-(dialkylamino)alkyl-azetidin-2-ones (134) which were converted into 3-alkylidene azetidinones under mild conditions. This procedure was employed in a formal synthesis of polyoximic acid (135).[156]

The Staudinger reaction for the preparation of azetidinones continues to attract interest.[157] An extension of this methodology makes use of 2,3-diaza-1,3-dienes (azines) and activated ketenes. This procedure provides an entry to *N*-imino-β-lactams (136) with good levels of *cis*-/*trans*-selectivity and high diastereoselectivities in the case of chiral azines. The *N*-imino group was cleaved by ozonolysis and homochiral β-lactams obtained in good yield.[106]



(134)



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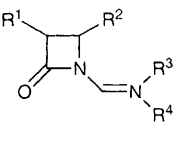
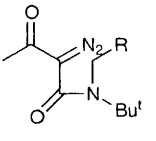


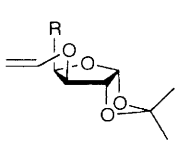
(136)



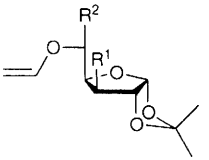
(137) R = Ph, CH_2CO_2Et

Efforts to develop asymmetric syntheses of azetidinones continue. An enantioselective carbene C-H insertion reaction using substrate (137) and a chiral dirhodium carboxylate catalyst exhibited complete 3,4-*cis* selectivity and an e.e. of 74%, which is comparable to the highest values known to date for such systems.[158] The mechanistic basis for the observed stereoselectivity remains unclear. Carbohydrates have been used as chiral auxiliaries in the [2+2] cycloaddition of chlorosulfonyl isocyanate and vinyl glycofuranoses (138) and (139).[158-160] Moderate to good control over the C-4 stereochemistry of the azetidinone ring substitution was achieved depending on the steric bulk of the R and R^1 group of the chiral auxiliaries. Enantioselective preparation of chiral precursors constitutes another approach to the asymmetric synthesis of β-lactams. Thus, stereoselective Michael addition of differentially protected lithium (α-methylbenzyl)allylamide to *tert*-butyl acrylate gave chiral *bis*-protected β-amino acid (140) in very good yield. Selective deallylation of (140) followed by transesterification and cyclisation using standard methodology gave optically pure (141).[161] A similar general strategy was employed in a formal total asymmetric synthesis of (+)-thienamycin.[162] β-Silyl carboxylic acid derivatives

(142) were prepared in enantiomerically pure form by 1,4 addition of higher order cuprates to Oppolzer's *N*-enoyl sultams. Staudinger reaction of (142) with imines gave optically pure azetidinones.[163] In another approach electrophilic amination provided chiral azetidinone precursors (143) in moderate diastereoselectivities.[164] (D)-Serine was employed as chiral starting material in a synthesis of 4,4-disubstituted-3-amino-2-azetidinones. Diastereoselective addition of organometallic reagents to ketones (144) furnished tertiary alcohols (145) which were converted into β-lactams *via* a known four step procedure.[165] The stereoselective enzymatic hydrolysis of *cis*-3-acetyloxy-4-phenyl-2-azetidinone to give enantiomerically pure (146) (>99.5% e.e.) in excellent yields using various lipases has been reported.[166]



(138)



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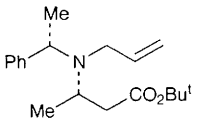

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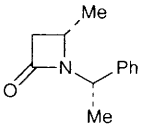
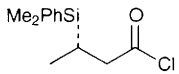


(141)



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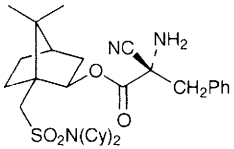
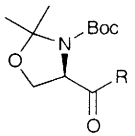


(143)



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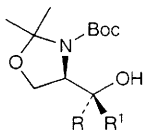
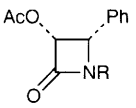


(145)

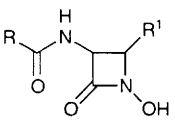
The high profile of azetidinone chemistry is also reflected in publications which investigate mechanistic details of the Staudinger reaction[167] and the [2+2] cycloaddition of alkenes and isocyanates by computational methods.[167-169]

8.2 Chemistry of Azetidinones

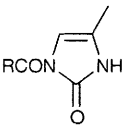
8.2.1 N-*Chemistry* – Deprotection of α-benzylidene-aminotoluene β-lactam (147) can be achieved in high yield under various hydrolytic or reductive conditions.[170] 3-Amido-1-hydroxy-azetidinones (148) were reported to rearrange after *N*-tosylation into imidazolines (149) in moderate yields.[171] Labelled β-lactam derivative (150) was obtained by regioselective deuteration using an iridium catalyst.[172]



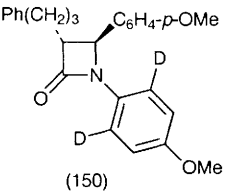
(146) R = H
(147) R = CH(Ph)N=CHPh



(148)



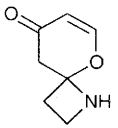
(149)



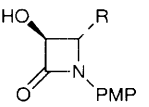
(150)

8.2.2 C-2 and C-2' Chemistry – Azetidinones have been employed as heterodienophiles in a Diels-Alder reaction with Danishefsky's diene. Spirohemiaminal (151) was obtained in poor yield.[173]

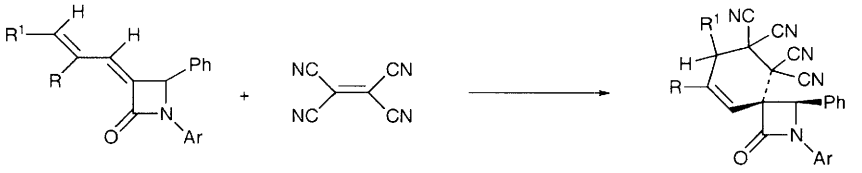
8.2.3 C-3 and C-3' Chemistry – β-Lactam derived enolates underwent electrophilic hydroxylation with *N*-sulfonyloxaziridine to afford *trans*-3-hydroxy substituted azetidinones (152) in low yield.[174] 3-Alkylidene azetidinones were prepared and their use as dienes in Diels-Alder reactions was studied. (*Z*)-Isomers (153) reacted with tetracyanoethylene to give spiro compounds (154) (Scheme 12).[175]



(151)



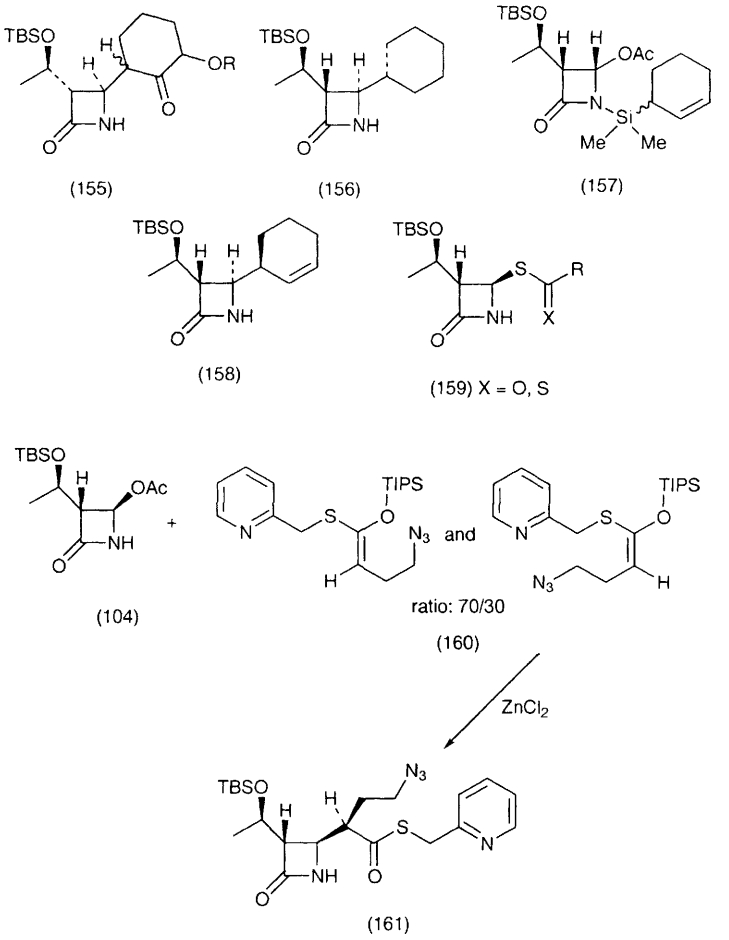
(152)



(153) (154)

Scheme 12

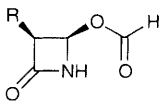
8.2.4 C-4 and C-4' Chemistry – Due to the importance of azetidinones as intermediates in tribactam, penem and penam synthesis, efforts continue to be directed towards selective functionalisation of the C-4 position. The $^{n}Bu_3SnH$ mediated radical reaction of a known seleno-β-lactam precursor with cyclohexenones provided cyclohexanone substituted azetidinones (155) as mixtures of diastereomers in satisfactory yields.[176] A stereoselective synthesis of related compound (156) has been achieved using the amidoalkylation of 4-acetoxy-azetidinones with a chiral allylborane reagent and diethylzinc.[177] Intramolecular Sakurai reaction of (157) furnished a 5:1 mixture of (156) and (158).[178] Azetidinone dithioesters (159, X = S) were obtained by acetoxy group displacement of 4-acetoxy-azetidinones with dithiocarboxylates prepared from copper-grignard complexes and CS_2.[179] Monothiocarboxylates (159; X = O) were accessible by zinc halide mediated nucleophilic attack of thiocarboxylic acid salts onto 4-acetoxy-azetidinones.[180] A stereoselective synthesis of carbapenem precursor (161) which was amenable to kilogram scale has been reported.[181] Stereocontrol was achieved by Mukaiyama-type reaction of silyl-enolethers (160) and 4-acetoxy-azetidinone (104) (Scheme 13). Treatment of β-lactam aldehydes



Scheme 13

with *meta*-chloroperbenzoic acid yielded formate analogue (162) of 4-acetoxy-azetidinones *via* Baeyer-Villiger-oxidation.[182]

Fluorination of 4-trimethylsilyl-azetidinones proceeded regioselectively and in good yield *via* anodic oxidation in the presence of $Et_3N{\cdot}3HF$ to give 4-fluoro-azetidinones (163).[183] Arylation of 4-vinyl-azetidinones employing Heck-type chemistry has been achieved without concomitant ring opening to give (164).[184] Use of a palladium(0)-$^nBu_3P$ catalyst leads to ring opened products in the presence of electron withdrawing substituents on the azetidinone nitrogen.[185] Titanium mediated transesterification reaction allows the efficient conversion of readily available (165) into (166).[186]



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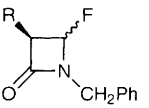
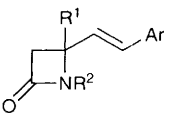
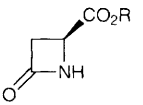


(163)

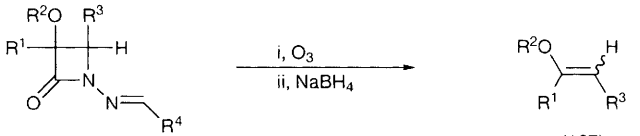


(164)



(165) R = $PhCH_2$
(166) R = alkyl

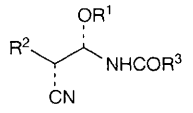
8.2.5 Miscellaneous Reactions of Azetidinones – Ozonolysis of *N*-(arylidene-(or *N*-alkylidene-)amino)-2-azetidinones followed by sodium borohydride work-up afforded vinyl ethers (167) in good yields and with high levels of stereoselectivity (Scheme 14).[187] An unprecedented cleavage of the β-lactam structure was



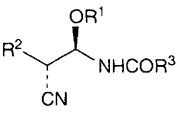
(167)

Scheme 14

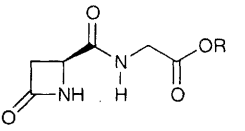
observed on treatment with an alkyl cyanide and trimethylsilyltriflate: 4-alkoxy-azetidinones yielded chiral β-amido cyanides (168) and (169) in ratios of 1:1 to 1:15.[188] Alkylation of β-lactam containing dipeptide (170) at the glycidyl position proceeded in good yields, but with only low degrees of asymmetric induction.[189] The use of azetidinones as monomers in polycondensation reactions has been studied.[190,191] Monodisperse polyamides (171) were obtained by living anionic polymerisation of 4,4-dimethyl and 3,3-dimethyl azetidinones.[192] Acid mediated ring opening of azetidinones furnished β-amino acids which were used in the synthesis of inhibitors of platelet aggregation.[193] The decomposition of azetidinones has been studied both under thermal[194,195] and acidic conditions.[196] A 1,4-diradical intermediate has been postulated for the thermal decomposition.



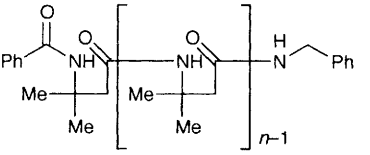
(168)



(169)



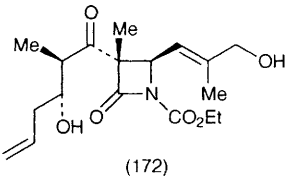
(170) R = Me, menthyl



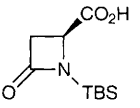
(171)

8.3 Further Use of Azetidinones – Interest in azetidinones as chiral building blocks for the synthesis of natural products continues. Highly functionalised precursor (172) was converted into the δ-lactone fragment of Lankacidin.[197,198] (2*S*,3*R*)-3-Carboxyproline and (2*S*,3*R*)-3-aminoproline were obtained stereoselectively *via* alkylation of chiral template (173).[199] Azetidinone (174) was also used as chiral starting material in an enantiospecific synthesis of the tricyclic guanidine segment of the marine alkaloid Batzelladine A.[200] Further examples for the ring-opening of activated azetidinones in the synthesis of taxol analogues have been reported.[166,170,201-207]

The synthesis and biological activity of structurally modified azetidinones has been the subject of further studies.[208-212] Azetidinones have been used as ligands for lanthanide(III) ion coordination. Complexation was shown to occur *via* the carbonyl oxygen. The complexes exhibited weak antiviral and antifungal activities.[213] The preparation and antibiotic properties of monobactams with heterocyclic groups in the 4-position (175) have been reported.[214,215] 3,3-Dialkylazetidinones (176) were prepared stereoselectively and shown to be highly potent, orally active inhibitors of human leukocyte elastase (HLE)[216,217] and cholesterol absorption.[218,219]



(172)



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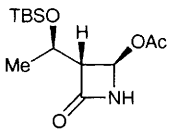
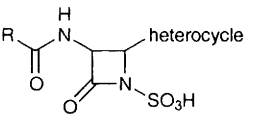


(174)



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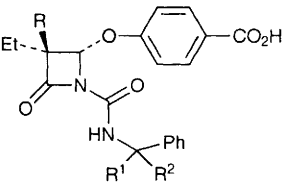
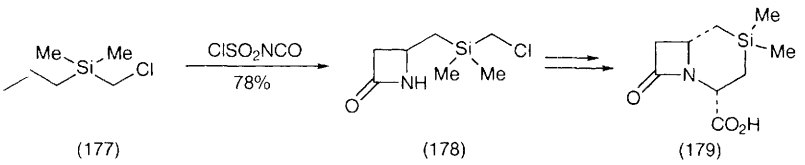


(176)

9 Major Structural Variants

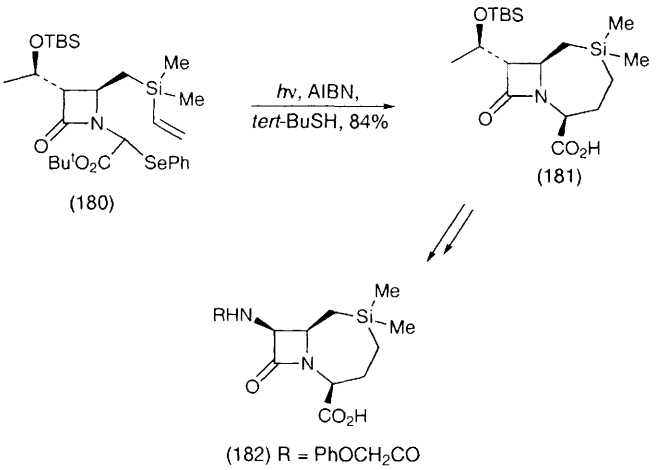
Two main areas of interest in the synthesis of non-traditional β-lactam related antibiotics can be distinguished: the incorporation of heteroatoms like silicon or phosphorus into bicyclic β-lactams and changes in ring fusion or substitution patterns.

2-Sila-1-carba-cephalosporin (179) was obtained by a route in which [2+2] cycloaddition of allylsilane (177) and chlorosulfonyl isocyanate gave silylazetidinone (178) (Scheme 15).[220] Radical cyclisation of chiral azetidinone-vinyl silane (180) gave the fused [4.7.1] ring system (181) in good yield and with



(177) (178) (179)

Scheme 15



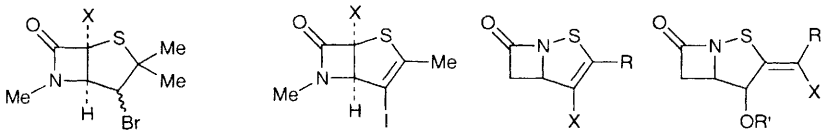
(180) (181)

(182) R = $PhOCH_2CO$

Scheme 16

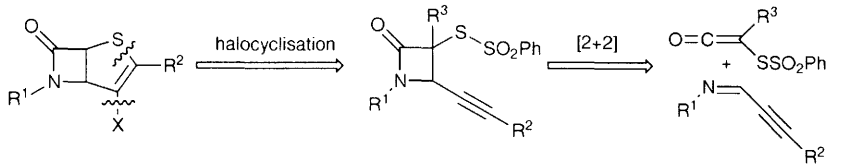
stereoselectivity and figured as key step in the synthesis of silicon containing β-lactam (182) (Scheme 16).[221]

Penem and penam analogues with an altered fusion position of the four- and five-membered ring have been prepared but their biological activity has yet to be determined.[222,223] The syntheses of penicillin analogues (183, 184) and penem analogue (185) were based on a retrosynthetic analysis in which [2+2] cycloaddition and halocyclisation reactions were the key steps (Scheme 17).[222] A similar route was chosen to synthesise inversely-fused compounds (186) and (187).[223] Infra red analysis of the C-fused analogues (184) and (185) demonstrated lower stretching frequencies ($\nu_{C=O}$: 1750-1760 cm^{-1}) than normally observed for penems and penams. However, the stretching frequencies observed for the



(183) X= H
(184) X= SEt

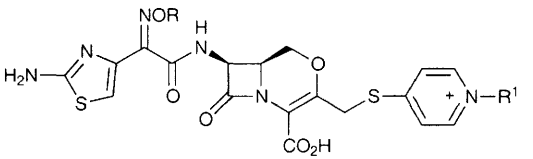
(185) X= H, SEt (186) (187)



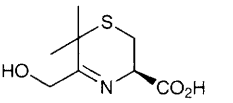
Scheme 17

analogues (186) and (187) in which the sulfur atom was directly attached to the β-lactam nitrogen revealed stretching frequencies comparable to those observed in the penams and penems. Optically active 2-oxa-isocephems (188) were prepared and exhibited higher *in vivo* activity against some methicillin resistant *Stapphylococcus aureus* strains (MRSA) than vancomycin.[224,225]

Other mechanistic studies of the reactions of PBPs and β-lactamases with β-lactam antibiotics have been directed at the design of new agents which remain effective against the resistant strains. Cyclic imines such as (189) displayed biological activity but were chemically labile.[226]

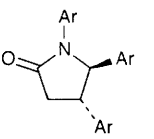


(188) R = Me, cyclopropyl
R^1 = CH_2CO_2Me, CH_2CO_2Et

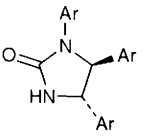


(189)

Gamma lactams and other ring homologues (190-193) of the potent β-lactam cholesterol adsorption inhibitor SCH48461 (194) were synthesised and tested for inhibitory activity. The low activity of these homologues led to the conclusion that the azetidin-2-one ring is required for activity.[227] Analogues such as (196), of the gamma lactam antibiotic lactivicin (195) (isolated from *E. lactamgenus*), have



(190)



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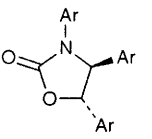

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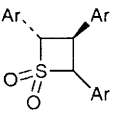

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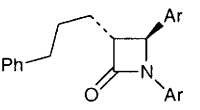

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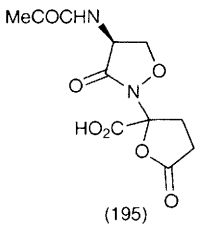

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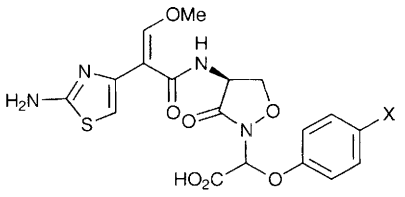


(196)

been synthesised from cycloserine and tested for biological activity. The poor activity observed was attributed to hydrolysis under the assay conditions.[228]

10 Mechanistic Studies

High resolution crystal structures of inhibitor complexes of a (*D,D*)-carboxypeptidase from *Streptomyces* ssp have been reported.[229,230] Crystal structures of cephalothin and cefotaxime bound to Ser 62 of the (*DD*)-carboxypeptidase were acquired and refined to 1.8Å and 2.0Å respectively.[230] In each complex a water molecule was observed within 3.1 to 3.6Å of the acylated β-lactam carbonyl but the water was apparently poorly activated for attack on the acyl intermediate by active site residues. This was interpreted as being a key reason for the efficiency of these compounds as inhibitors. The study also implicated the involvement of Tyr 159 in deacylation of the enzyme-inhibitor complex. Realignment of this residue was suggested as a rationale for the low cefotaxime affinity of an altered penicillin binding protein (PBP) from cefotaxime resistant strains of *Streptococcus pneumoniae*, and the ability of mutant class A β-lactamases to hydrolyse third generation cephalosporins.

Computational models of the active site of penicillin binding proteins has allowed the design of novel antibacterials such as (189) which, although active, suffer from instability.[226] Modelling of the methanolysis of azetidinones, cephems, and penems complexed to PBPs by semiempirical MM and QM methods has provided information on their relative stabilities.[231,232] Δ^3 Cephems are apparently inherently less reactive than penems whilst Δ^2 cephems are less reactive by virtue of a poorer fit into the active site. It was calculated that the mechanism involving *N*-protonation with C-O and N-H bond formation concerted with N-C cleavage was of a lower energy than a pathway involving *O*-protonation (**Figure 1**).

Isolation of 7 penicillin binding proteins from *Shigella dysenteriae* has resulted in the identification of low M_r PBPs which have a low homology to *E. coli* PBPs. It was suggested that the low M_r PBPs may be β-lactam targets.[233]

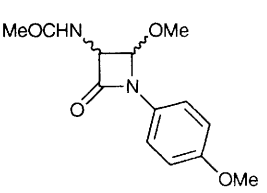
Studies have been carried out with the aim of identifying the general base effecting deprotonation of the nucleophilic Ser 70 residue at the active site of the 'serine' β-lactamases. Whilst replacement of Lys 73 residue with a histidine

N-protonation *O*-protonation

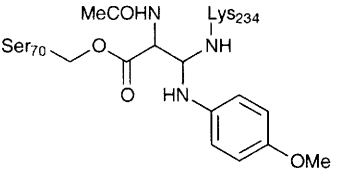
Figure 1

resulted in significant loss in activity,[234] other studies clearly indicated that Glu 166 acts as the general base.[234b] Other detailed studies have aimed at identifying the roles of the other highly conserved active-site residues of the TEM β-lactamases. Substitution of Asp for Asn in position 132 has demonstrated the interaction of this residue with the side chain carbonyl group.[234] This interaction is important for ground state recognition, but also has an indirect catalytic role by modifying the action of Lys 73 and positioning of the 'hydrolytic' water. Multiple substitutions of Glu 104 all yielded active enzymes.[235] The mutant enzymes exhibit no drastic change in kinetic parameters except with in the case of cefaclor, but have an improved affinity for second and third generation cephalosporins. It was concluded that Glu 104 has an indirect role in substrate specificity of the class A β-lactamases.

A study on the binding of a 4-alkoxy-2-azetidinone (197) to a class A β-lactamase from *Bacillus* ssp. has revealed a new mode of inactivation involving cross-linking of Ser 70 and Lys 234 (198).[236] The cross-linked enzyme is irreversibly inhibited whilst its secondary structure is partially stabilised. A novel



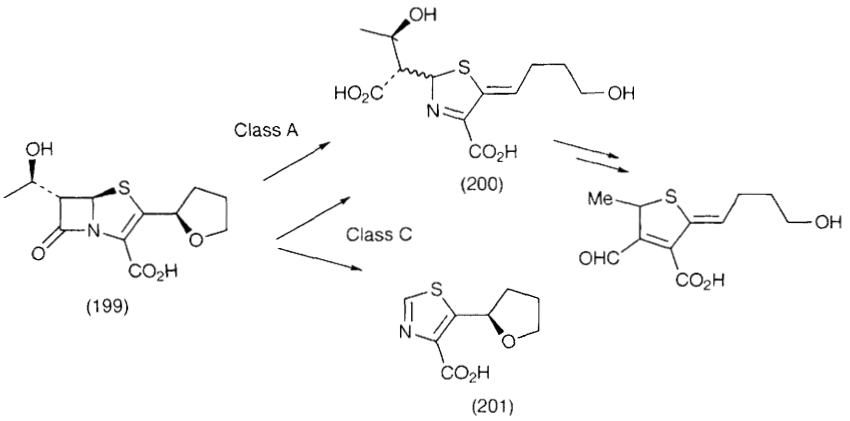
(197)



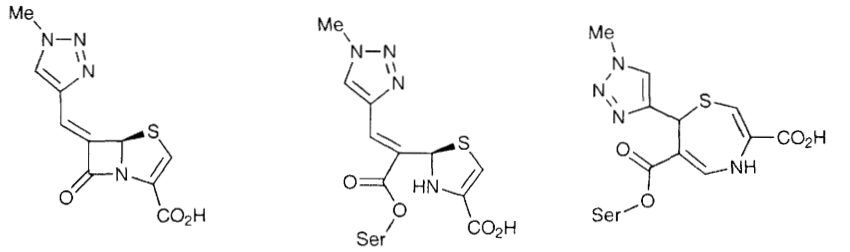
(198)

C-C bond cleavage of a penem antibiotic (199) by class C β-lactamases has been demonstrated to occur in addition to the normal β-lactam cleavage seen for class A β-lactamases which results in (200).[237] Cleavage of the C-5-C-6 bond by the class C β-lactamases resulting in formation of (201) occurs as a result of greater stability of the first formed acyl intermediate in these enzymes. It was suggested that the observed hydrolytic pathway of the penem (199) might be related to the classification of the β-lactamases by amino acid sequence rather than to classification by the substrate specificity.

Studies on the mode of action of penem β-lactamase inhibitor BRL 42715 (202) have been carried out using kinetics and mass spectrometry.[238,239] The penem exhibits rapid stoichiometric binding and forms an initial acyl intermediate (203)

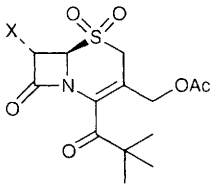


with the active-site serine. Rotation about the penem C-5-C-6 bond allows rearrangement to a dihydrothiazepine species (204). The reversal of inactivation by hydrolysis of an acyl intermediate was shown to be hindered after rearrangement to (204) for class C β-lactamases. It was concluded that the hydrolytic water must approach from the opposite side of the ester carbonyl in class C β-lactamases from that occurring in class A β-lactamases.

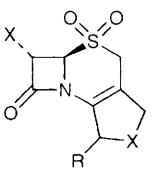


11 New Applications

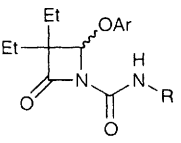
The use of β-lactams for the inhibition of proteases, peptidases and in particular elastases continues to attract interest.[240] Cephemsulfones were shown to be potent inhibitors of human leucocyte elastase (HLE). 7α-Substituted cephem sulfones (205) and (206) exhibited good hydrolytic stability and inhibitory activity.[241] Incorporation of the cephem nucleus into a tricyclic structure as in (207) improved the pharmacokinetic properties further.[242,243] The mechanism of HLE inhibition by azetidinone derivatives (208) and (209) has been investigated using electrospray ionisation mass spectrometry and two-dimensional NMR techniques.[244]



(205) X = Cl
(206) X = OMe



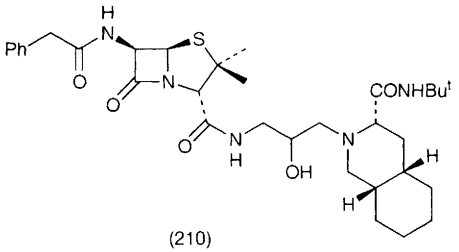
(207) X = S, O, NR
R = Ph, Bu^t



(208) 4*S*
(209) 4*R*

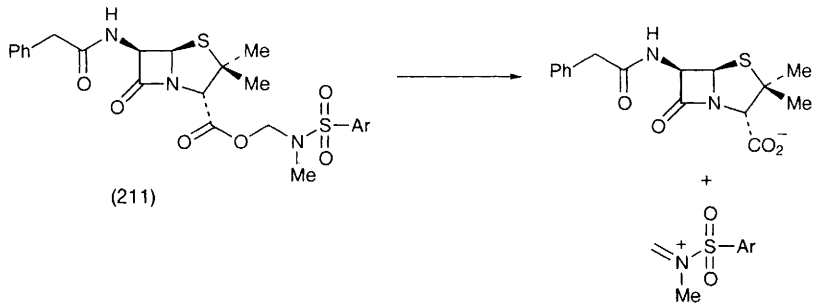
Penems were shown to be excellent inhibitors of the *E. coli* leader peptidase. However, antibacterial activity of the penems against *E. coli* strains was poor, presumably because they are unable to penetrate the bacterial cell membrane and reach the cellular target.[245,246]

Interest in β-lactam derivatives as potential inhibitors of HIV-protease continues.[247,248] Penicillin derived amide (210) was shown to have high inhibitory activity, but its poor pharmacokinetic profile precluded its further development as a potential antiviral.[249]



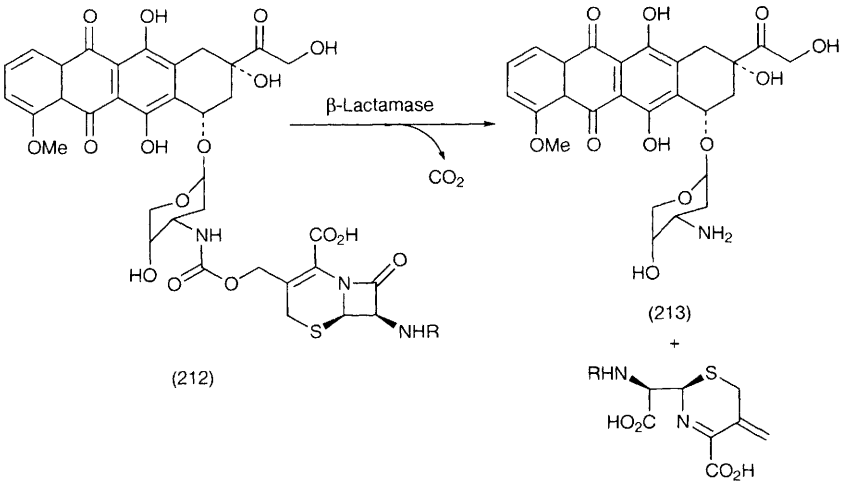
(210)

β-Lactams have been further utilised for the targeted release of therapeutics as prodrugs.[250,251] Thus, acyloxymethyl substituted pencillin sulfones (211) were reported to release the penicillin antibiotic *via* *N*-sulfonyl imminium ion formation (Scheme 118).[252] Activation of cephalosprin containing prodrug (212) by a monoclonal antibody-β-lactamase conjugate has been used to decrease the *in*



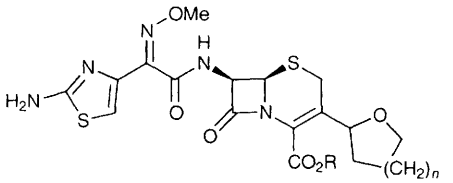
(211)

Scheme 18



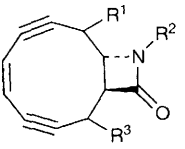
Scheme 19

vivo toxicity of doxorubicin (213) (Scheme 19).[253] Pivaloyloxymethyl prodrug (214) was shown to be a useful cefotaxime analogue with high and prolonged levels of antibiotic activity *in vivo*.[254] Bicyclic ring systems with a azetidinone ring *trans* fused to a ten-membered cyclic enediyne (215), so-called lacten-diynes, have been prepared and shown to be useful as potential prodrugs in the release of enediyne antitumor compounds.[255]



R = $CH_2OCOCMe_3$

(214)



(215)

Acknowledgements: We thank M.D. Lloyd and D.R. Spring for reading the manuscript and apologise for any omissions.

Appendix A: Review Articles

General

1) From molecular genetics and secondary metabolism to molecular metabolites and secondary genetics.[256]

2) Differentiation and secondary metabolism in some prokaryotes and fungi.[257]
3) Penicillin amidase: chemospecificity and stereospecificity.[258]
4) Multiple mechanisms of membrane anchoring of *E. coli* penicillin binding proteins.[259]
5) Strategies in the design of a penicillin acylase process.[260]
6) Metabolic flux distribution in *P. chrysogenum* during batch-fed cultivations.[261]
7) New cephalosporins in development pipelines.[262]
8) Selective activation of anticancer prodrugs by monoclonal antibody-enzyme conjugates.[250]
9) The structures and catalytic mechanisms of active-site serine β-lactamases.[263]
10) Enzymic synthesis of hydrophobic penicillins.[264]
11) Expression of genes and processing of enzymes for the biosynthesis of penicillins and cephalosporins.[265]
12) Genetics of penicillin biosynthesis in *A. nidulans*.[266]
13) Cephalosporin C; mode of action and biosynthetic pathway.[267]
14) Fused-skeleton saturated six-membered 1,3-N,O, N,N and N,S heterocycles. Fused-skeleton aryl-substituted saturated isoindolones.[268]
15) Penicillin amidase: chemospecificity and stereospecificity.[269]
16) The carbacephems.[101]
17) Design, synthesis and evaluation of *D,D*-peptidase and β-lactamase inhibitors: azapeptides, oxapeptides and related heterocycles.[270]

Resistance to β-Lactams

1) Single amino acid replacements at positions altered in naturally occurring extended-spectrum TEM β-lactamases.[271]
2) Val-237 for Ala substitution in the TEM-2 β-lactamase dramatically alters the catalytic efficiencies towards carbenicillin and ticarcillin.[272]
3) Bacterial resistance mechanisms as therapeutic targets.[273]
4) Bacterial resistance to β-lactam antibiotics.[274]
5) β-Lactamases and bacterial resistance to antibiotics.[275]
6) New drugs for tuberculosis.[276]
7) Genetics of extended-spectrum β-lactamases.[277]
8) The β-lactamase problem: New therapeutic options.[278]
9) The structures and catalytic mechanisms of active-site serine β-lactamases.[279]
10) Interaction studies in infectious diseases.[280]
11) β-Lactamases in laboratory and clinical resistance.[281]
12) Contribution of mutant analysis to the understanding of enzyme catalysis. The case of class A β-lactamases.[282]
13) Origin and impact of plasmid-mediated extended-spectrum β-lactamases.[283]
14) The β-lactamase problem: New therapeutic options.[278]
15) Stability in the presence of widespread β-lactamases: A prerequisite for the antibacterial activity of β-lactam drugs.[284]

16) Extended-spectrum plasmid-mediated β-lactamases.[285]
17) TEM- and SHV-derived extended-spectrum β-lactamases: relationship between selection, structure and function.[286]

Appendix B: Additional Articles

Biosynthesis

1) A method for increasing production of microbial metabolites by increasing the maximum rate of the rate limiting step of a metabolic pathway.[287]
2) Method of obtaining penicillin G by biosynthesis process.[288]
3) Molecular cloning of gene and cDNA encoding enzymes associated with biosynthesis of cephalosporin C.[289]
4) Cloning and expression of gene for cystathionine γ-lyase of *A. chrysogenum*.[290]
5) Molecular cloning of genes involved in the biosynthetic pathway of antibiotic carbapenem in *E. carotovora*.[291]
6) *In vitro* activity of biapenem (L-627), a new carbapenem, against anaerobes.[292]
7) Isolation and identification of a new cephem compound from *P. chrysogenum* strains expressing deacetoxycephalosporin C synthase activity.[293]
8) Biogenesis of 6-aminopenicillanic acid (6-APA) and penicillin in *P. chrysogenum*: effect of the biocatalyst chrysin.[294]
9) Use of reporter genes to identify recessive *trans*-acting mutations specifically involved in the regulation of *A. nidulans* penicillin biosynthesis genes.[295]
10) Penicillin amidase: chemospecificity and stereospecificity.[269]
11) Utilization of side-chain precursor for penicillin biosynthesis in a high-producing strain of *P. chrysogenum*.[296]
12) Uptake of phenoxyacetic acid by *P. chrysogenum*.[297]
13) Carbon regulation of penicillin biosynthesis in *A. nidulans*: A minor effect of mutations in creB and creC.[298]
14) Strategies in the design of a penicillin acylase process.[299]
15) A novel 7β-(4-carboxybutanamido)cephalosporanic acid acylase isolated from *Pseudomonas* strain C427 and its high-level production in *E. coli*.[300]
16) β-Lactamase inhibitor FM-41, a new member of the clavam group produced by *Streptomyces* ssp. FM-41 isolated from soil samples in China.[301]
17) Biosynthesis of 5-hydroxy-4-oxo-(L)-norvaline in *S. akiyoshiensis*.[302]
18) Analysis of the regulation of the *A. nidulans* penicillin biosynthesis gene AAT (penDE), which encodes acyl coenzyme A:6-aminopenicillanic acid acyltransferase.[303]
19) Metabolic engineering of cephalosporin biosynthesis in *S. clavuligerus*.[304]
20) Giant linear plasmids of β-lactam antibiotic producing *Streptomyces* ssp.[305]

21) Metabolic control analysis of the penicillin biosynthetic pathway in a high-yielding strain of *P. chrysogenum*.[306]
22) A lacZ reporter fusion method for the genetic analysis of regulatory mutations in pathways of fungal secondary metabolism and its application to the *A. nidulans* penicillin pathway.[307]
23) A homolog of the small subunit of penicillin acylase is encoded in the genome of the extremely thermophilic archaeon *Sulfolobus solfataricus*.[308]
24) Chemo-enzymic approach to the synthesis of each of the four isomers of α-alkyl-β-fluoroalkyl-substituted β-amino acids.[309]
25) On the mechanism of cephalosporin isomerization.[310]
26) Isolation of large linear plasmids from β-lactam producing actinomycete strains.[311]

Penicillins and Cephalosporins

1) Design and synthesis of penicilloyloxymethyl quinolone carbamates as a new class of dual acting antibacterials.[312]
2) 2α-Alkoxymethyl cephalosporins: Reactions of *exo*-2-methylene cephalosporin sulfones with alcohols.[313]
3) Synthesis and *in vitro* antibacterial activities of 3-thiazol-4-yl-1-carba-1-dethiacephalosporins.[314]
4) Synthesis and structure activity relationships of a series of potent *pseudosym.* inhibitors from penicillin.[315]
5) Pummerer-type cyclization of Arnstein tripeptide analogs induced by *O*-silylated ketene acetals.[316]
6) Studies of synthesis and antimicrobial activity of new β-lactam antibiotics.[317]
7) Synthesis, purification and kinetic properties of six fluorescein labelled penicillins.[318]
8) Synthesis, solid-state characterization and *in vivo* cytotoxicity of diorganotin(IV)chloro and triorganotin(IV)chloro derivatives of penicillin G.[319]
9) Synthesis and antibacterial activity of 7β-[3-(un)substituted-2-amino-propionamido]-3-vinylcephalosporins and related compounds.[320]
10) Synthesis and antibacterial properties of 7β-[2-(5-substituted-or unsubstituted-2-furyl)acetamido]cephalosporanic acid derivatives.[321]
11) Synthetic β-lactam antibiotics VII. Antibacterial activity of some 7β-[(*Z*)-(2-amino-thiazol-4-yl)-2-(methoxyimino)-acetamido]-3-(1-azabicyclo[2.2.1] heptanio) methyl-cephalosporins.[322]
12) Recent developments in the chemistry of 3-vinylcephalosporins.[323]
13) Synthesis of ^{14}C-labelled cefluprenam (E1077), a novel parenteral cephalosporin antibiotic.[324]
14) Synthesis of (*O*-vinylphenoxy)methyl penicillin.[325]
15) Synthesis and antibacterial activity of new cephalosporins with lactonyloxyimino moiety.[326]

Carbapenems, Carbapenams, and Carbacephems

1) Preliminary experiments for asymmetric total synthesis of the thienamycin-like γ- lactam.[327]
2) Antibacterial activities of a carbapenem antibiotic, biapenem (L-627), against penicillin-resistant *Streptococcus pneumoniae*.[328]
3) Synthesis and antibacterial activity of new 1β-methyl carbapenem having a thiazolo[3,2]benzimidazole moiety.[114]

Azetidinones

1) Synthesis of 2-cyano-1-oxocarbapenam-3-carboxylate.[329]
2) Three 1,3,4-trisubstituted β-lactam antibiotics.[330]
3) The study of fundamental ring vibrations of the β-lactams moiety of azetidin-2-ones using *ab initio* calculations.[331]
4) Synthesis and biological screening of imidazolyl bisbenzimidazoles and bisbenzimidazolyl azetidinones.[332]
5) Synthesis of 1-arylazetidin-2-ones using calixarenes as phase-transfer catalysts.[333]
6) Synthesis of (3*S*,4*S*)-3-Amino-1-(3,4-dimethoxybenzyl)-4-[(*R*)-2,2-dimethyl-1,3-dioxolan-4-yl]-2-azetidinone.[334]
7) Synthesis of imidazolinones, azetidinones and formazans from hydrazinotriazines as potential antimicrobial agents.[335]
8) New derivatives of 1-hydroxy-2-azetidinone.[336]
9) Stereochemically directed construction of the β-lactam fragment of thienamycin.[337]
10) *trans*-3-Amino-1-methyl-4-phenyl-2-azetidinone.[338]
11) Synthesis and biological activity of 4-thiazolidinones, 2-azetidinones, 4-imidazolinone derivatives having a thymol moiety.[339]

Major Structural Variants

1) Preparation of novel γ-lactams by ring expansion of β-lactams.[340]
2) Synthesis of *tert*-butylcyclopentane-fused 1,3-oxazines and 1,3-thiazines.[341]
3) Construction of α-phosphonolactams *via* rhodium(II)-catalysed intramolecular C-H insertion reactions.[342]
4) Imidazolidinones structurally related to penicillins: synthesis, molecular modeling and biological evaluation.[343]
5) Synthesis of *trans*-β-lactams of estrane series.[344]

Mechanistic Studies

1) Semiempirical molecular orbital calculations of the substituent effects on acylations of 3-cephem analogs.[345]
2) Activity of carbapenem BMS-181139 against *P. aeruginosa* is not dependent on porin protein D2.[346]
3) Molecular structures of penicillin-binding proteins and β-lactamases.[347]
4) Aziridinone and 2-azetidinone and their protonated structures. An *ab initio* molecular orbital study making comparisons with bridgehead bicyclic lactams and acetamide.[348]

5) Revealing active site on the light subunit of penicillin acylase.[349]
6) Study of the activation of penicillin acylase from *B. megaterium* by Co(II).[350]
7) Localization of penicillin binding proteins to the splitting system of *Staphylococcus aureus* septa by using a mercury penicillin V derivative.[351]
8) Nucleotide binding by multienzyme peptide synthetases.[352]
9) On the mechanism of cephalosporin isomerisation.[353]
10) Crystal geometry optimization of β-lactam antibiotics using MMFF parameters.[354]
11) Can penicillins and other β-lactam antibiotics be used to treat tuberculosis.[355]
12) Cluster of genes related to the biosynthesis of cephamycin C and β-lactam resistance in actinomycetes.[356]
13) Molecular characterization of an enterobacterial metallo β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance.[357]
14) The *Bacillus subtilis* dacB gene, encoding penicillin-binding protein 5, is part of a three-gene operon required for proper spore cortex synthesis and spore core dehydration.[358]
15) The asparagine to aspartic acid substitution at position 276 of TEM-35 and TEM-36 is involved in the β-lactamase resistance to clavulanic acid.[359]
16) Effects of Asp 179 mutations in TEMpUC19 β-lactamase on susceptibility to β-lactams.[360]
17) Characterization of TEM-1 β-lactamase mutants from positions 238 to 241 with increased catalytic efficiency for ceftazidine.[361]

New Applications

1) Azetidin-2-one derivatives as inhibitors of thrombin.[362]
2) Selective activation of anticancer prodrugs by monoclonal antibody-enzyme conjugates.[250]
3) Synthetic β-lactam antibiotics VII. Antibacterial activity of some 7β-[(*Z*)-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido]-3-(1-azabicyclo[2.2.1]-heptanio] methylcephalosporins.[363]

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6
Current Trends in Protein Research

BY JENNIFER A. LITTLECHILD

1 Introduction

This chapter reports on some of the advances in protein research carried out in 1995. This area has been covered in Volumes 25, 26 and 27 of this series of Specialist Reports in Amino Acid, Peptides and Proteins. Some highlights of this subject which include protein folding, proteins that bind to RNA and DNA and some important new structures are included in this review.

2 Protein Folding

2.1 New Methods – Our understanding of protein folding increased during 1995. Improved instrumentation has allowed many folding pathways of proteins to be studied in detail. A recently studied protein is an all α-helix containing protein, acyl-coenzyme A binding protein (ACBP).[1] This four helix bundle protein was found to refold in a similar way if denatured by guanidine hydrochloride or acid. This was studied by several stopped and quenched flow techniques as rapid (>5 ms at 25°C) and two-state. Another protein which is all β sheet is the cold-shock protein from *Bacillus subtilus* (CspB).[2] It was found by equilibrium and kinetic studies over several different conditions of protein denaturation that this five stranded β-barrel protein refolded in a two-state process which was fast under optimal conditions, >1.5 ms.

A powerful way to study protein folding is site-directed mutagenesis. This has been a topic of investigation by the group of Fersht for several years. A review of these techniques with reference to barnase and chymotrypsin inhibitor 2 (CI2) makes useful reading.[3] A further paper on CI2 predicts a possible nucleation condensation mechanism.[4] Another approach to monitor protein-folding has recently been used for Staphylococcal nuclease.[5] The folding and unfolding of this protein have been measured by fluorescence spectroscopy after jumps in pressure. The relationship between kinetics and pressure permit the relative activation volumes of native, unfolded and transition states to be determined. These results are interpreted as a structure in which the transition state excludes solvent from its interior while lacking the specificity necessary for a tightly packed compact state. Many proteins refold in a multiphasic manner. Gutin *et al.*[6] have addressed the problem whether hydrophobic collapse is necessary for protein folding.

Many larger proteins fold as domains. NMR has become a powerful technique to study denatured states of proteins. This has been used to compare the refolding of hen lysozyme from dimethyl sulfoxide and guanidinium chloride. The denatured states appear different but the refolding as measured by pulse-labelled hydrogen exchange is the same providing the same media is used.[7] A significant advance is the ability to follow protein folding in real time with NMR techniques.[8] This study concentrated on the refolding of apo-α-lactalbumin and suggested a cooperative transition from the burst state to native state with no evidence for intermediates such as domains having fully native-like packing. Using rapid mix techniques coupled with NMR direct evidence for an intermediate preceding the rate limiting step in the unfolding of ribonuclease A has been determined.[9]

Another advance has been the application of mass spectrometry to protein folding studies. Mass spectrometry rather than observing an average of the species present in a folding pathway can detect the resolution of populations of protein molecules whose mass has changed as a result of hydrogen-deuterium exchange. The distribution of these masses can be used as a measure of cooperativity in protein folding pathways. This approach has been used successfully in 1995 to study a synthetic fibronectin molecule[10] and for avian lysozymes.[11] The concept of domains in protein folding has been studied in cytochrome c by hydrogen exchange studies.[12] The amides can be divided into four groups corresponding to the substructures observed in the native state of this protein.

The molten globule state of a protein has been studied by several groups, including that of Ptitsyn. A review appearing in 1995[13] describes work on this concept where the molten globule has substantial secondary structure but lacks the tertiary contacts necessary for the protein to adopt its native state. Further studies of molten globule state have been reported in 1995 on bovine trypsin inhibitor,[14,15] cytochrome c,[16,17] α-lactalbumin[18,19] and lysozyme.[20] The order of amino acids along the protein chain must dictate the way in which the protein folds. Theoretical approaches to this have been made (reviewed by Dill *et al.*, 1995).[21]

Several theoretical approaches to protein folding have appeared recently. These include a study by Onuchic and coworkers[22] which uses experimental data in conjunction with helix-coil transition theory to characterise the properties of a protein folding funnel. Data derived from lattice models of proteins in the context of experimental observations are discussed by Chan *et al.*, 1995.[23] Shakhnovich and colleagues[24,25] have studied the evolution-like selection of fast folding model proteins and domains in folding of model proteins. Rules have been obtained from the inspection of information present in the protein structures already available (reviewed by Thorton *et al.*).[26] Several groups of researchers have tried to look at the folding of synthetic peptides to understand protein folding.[27,28]

Mutagenesis of proteins has been used to study its effects on folding with the IgG immunoglobulin domain using phage display techniques.[29] Combinatorial mutagenesis has also been used to study the importance of salt bridges for protein

stability.[30] In this study a buried salt bridge in a protein was replaced by hydrophobic residues. In several cases the stability of the protein is increased.

2.2 Protein Folding and Disease – An understanding of protein folding and the principles which guide it is receiving renewed interest due to the realisation that several diseased states are due to proteins that are misfolded. A review of defective protein folding as a basis of human disease was published by Thomas *et al.*[31] The understanding of 3D protein structure and the changes of primary sequence that can affect that structure is gaining considerable significance in diseased conditions such as Huntington's Disease. One trend which is seen in proteins that are thought to be involved in this and other degenerative diseases, is repeats in the sequence of the amino acid glutamine. This phenomena has been under discussion in the last few years and several papers have appeared in 1995. The protein involved is called huntingin, which is found in man and other vertebrates. A paper discussing the four glutamines found in the puffer fish huntingin protein[32] has recently been published. This paper shows that the gene which is mutated in Huntington's disease has a varying number of glutamine residues which suggest that the long glutamine repeat in the human protein may serve no function. However other research work on the polyglutamine expansion as a pathological epitope in Huntington's Disease and fair dominant cerebellar ataxias has been reported.[33] The importance of the huntingin protein has been shown since it is found that mice which lack the gene coding for this protein die as embryos.[34,35] Other papers have appeared in 1995 on the location of the huntingin protein in the cell and it is proposed that it could be a transcription factor. Research in this area is likely to expand in the next few years.

Another disease state associated with glutamine repeats is a spinocerebellar disease called Machado-Joseph Disease. The glutamines in the respective protein vary from 13 to 36 in normal patients and 61-84 in diseased states and are found near the carboxyl end of the protein which shows no obvious homology with any known protein sequence.[36] Other disease conditions also reveal proteins with glutamine repeats and a full discussion of this area is beyond the scope of this review.

The codon for the amino acid glutamine is CAG and replication of double-stranded CAG/CTG triplet repeats *in vitro* have been found to be interrupted by pauses which become more marked the longer the repeats.[37] The pauses may be due to loops or hairpins of the kind previously proposed to cause frameshift mutations by Streisinger and Owen, some 10 years earlier.

The 'knock on' effects of the glutamine repeats have been investigated for huntingin where a search has been carried out for its ability to interact with other proteins.[38] The incorporation of glutamine repeats is thought to make proteins oligomerize and this is the subject of a paper by Perutz and collaborators.[39] Experiments using chymotrypsin inhibitor-2 (Cl_2) with engineered loops containing repeating glutamine residues were carried out. These engineered proteins formed oligomers during the folding of the protein in the *Escherichia coli* cell. Evidence has been presented that the glutamine repeats cause a change in the

protein structure since a monoclonal antibody has been found that specifically recognises the glutamine repeats in huntingin protein from diseased states.[40]

2.3 Chaperons – In the cell proteins are assisted into correct folding pathways by other proteins called molecular chaperons. Research into the way in which this occurs has continued to be a main topic of study in 1995. For earlier discussion on this topic see Vol. 26 and 27 of this series. The hydrolysis of ATP is associated with this process. The nucleotide and protein-binding activities of the chaperons are coupled. Structural rearrangements induced by nucleotide binding with the Dnak chaperon have been followed by intrinsic fluorescence and proteolytic activity.[41] A kinetic model for the coupling described above was reported for the clathrin-associated chaperone by Greene *et al.*[42] A comprehensive study of the overall Dnak chaperon system has been reported by McCarty *et al.*,[43] where the dynamics of peptide, hsp 40 and GrpE binding to the *Escherichia coli* hsp 70 and its relationship to ATP hydrolysis is presented.

A clear discussion of the thermodynamics of the interactions of GrpE and hsp 40 with different conformeric states of hsp 70 has been described by Zeigelhoffer *et al.*[44] The overall quaternary structures involved in the Dnak and GrpE components has been studied by Schonfeld *et al.*[45] NMR has been used to ascribe an unusual secondary structure topology to a 18 kDa peptide-binding domain of hsp 70.[46]

The group of Fersht have continued their studies on the folding of the small protein barnase and GroEL.[47] They find that the folding pathway of barnase is not changed but the addition of ATP leads to a large increase rate of folding ($\times 10$ fold). The dynamics of the ATPase activity of chaperonin has shown that due to its double ring type structure (see Vol. 27 of this series) subunits within a ring show positive cooperativity with ATP binding but the two rings show negative cooperativity to each other. This idea is further developed by Yifrach and Horovitz,[48] Burston *et al.*[49] and Murai *et al.*[50]

Two papers also by the group of Horovitz have looked at the flexibility of the region of the chaperonin where the substrate binds. They show that hydrophobic surfaces which are hidden can be exposed by formation of the assembly competent monomers or by ionic perturbation of the oligomer of cpn 60.[51] The local flexibility of cpn 60 is reduced after binding the unfolded peptide.[52] The dynamics of the chaperonin cycle means that the substrate will spend half of its time in a tightly bound state where bad contacts inhibiting the folding process are disrupted and the other time in a displaced state where the correct contacts are allowed to form. This has been demonstrated with malate dehydrogenase by the group of Clarke.[53]

2.4 Protein Sequence Motifs and Comparisons – A paper providing rules for the minimal making of new protein motifs was described by Bork *et al.*[54] This area is becoming of increasing importance with the human and other genome projects currently underway. SCOP: a structural classification of proteins database for the investigation of sequences and structures has been produced by Murzin *et al.*.[55] This organises protein structures in a hierarchical fashion, identifying likely

evolutionary relationships. SCOP is available on the internet at URL:http://scop.mrc-lmb.cam.ac.uk/scop/. Another network tool for protein comparison was described by Holm and Sander.[56] This tool called Dali is also available on the internet – URL:http://www/embl-heidelberg.de.dali/. Comparisons of proteins using such tools have resulted in interesting similarities of proteins at the structural level that have low amino acid sequence homology.

3 Proteins that Bind RNA

A great deal of information is available for proteins that bind to DNA and this has been discussed on Trends in Protein Research in Vols. 25-27 of this series. The research on proteins that bind specifically to RNA is proceeding at a significant rate. Specific interactions of proteins with RNA play important and vital roles in all living cells. The complexes of aminoacyl tRNA synthetases have been previously discussed and have been the subject of a review in 1995.[57]

3.1 RNP Domain – A common RNA-binding module now found in over 200 proteins is called the RNP domain. A review describing this domain and where it is found has been written by Nagai *et al.*[58] The domain of 70-90 amino acid residues consists of a four stranded antiparallel β sheet with two α helices on one side. There are two short conserved sequence motifs in this structure called RNP1 and RNP2. These are located in the two middle β strands and play a vital role in the binding of RNA (Figure 1).

3.2 Double Stranded RNA Binding Domain – A second RNA binding domain which contains an amino acid sequence motif of 65 amino acids is called the double stranded RNA binding domain (dsRBD). Three papers describing proteins showing this domain have appeared in 1995. These are for the regulatory domain of the protein kinase DA1[59], the dsRNA binding domain of *E. coli* RNase III[60] (Figure 2) and the dsRNA binding domain of *Drosphilia staufen* protein.[61] This latter protein reveals homology to a ribosomal protein S5.

3.3 KH Domain – A third RNA binding domain is called the KH domain. The structure of this domain has been solved from a protein, human vigilin by multidimensional NMR methods.[62] This protein is thought to be involved in tRNA transport and it has been shown to contain a three-stranded antiparallel β-sheet and 3 α helices. Two KH domains have been identified in the α globulin mRNP stability complex.[63]

3.4 Sm Protein Motif – Another RNA binding domain that has received considerable attention in 1995 is the Sm-protein motif. This is found in proteins that are part of small nuclear ribonucleoprotein particles (snRNPs) which are involved in the splicing of mRNA. The motif takes its name from the fact that the Sm proteins are recognised by anti-Sm serotype antisera from patients with autoimmune disorders. This motif has been identified in the SDB23 gene of

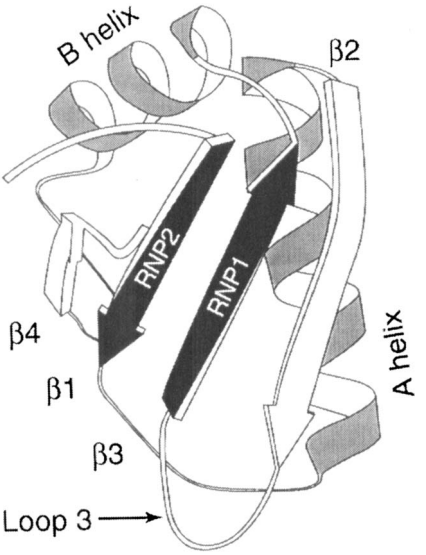


Figure 1 A schematic representation of the RNP domain. The conserved RNP1 and RNP2 motifs are located in the two middle β-strands. Reproduced with permission from reference 58.

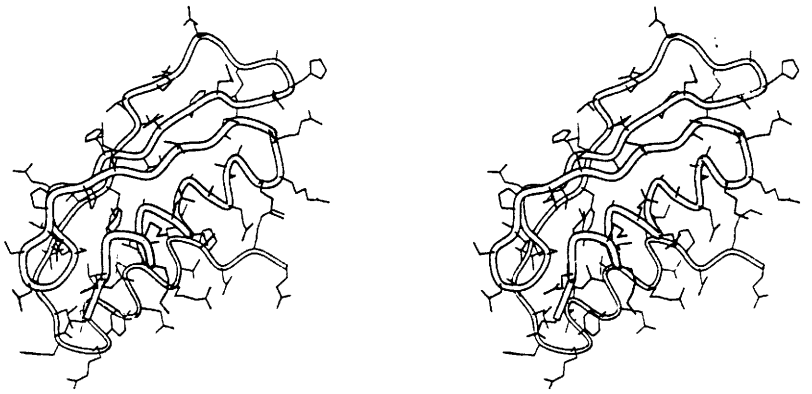
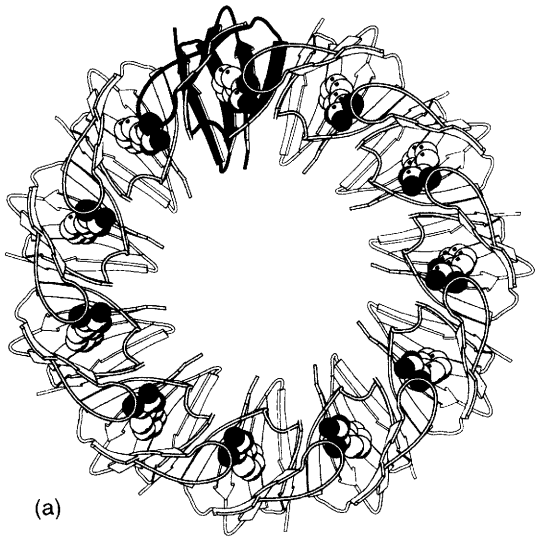


Figure 2 Tertiary structure of the RNase III dsRBD. Stereo pair of the top structure, including side chains. The backbone is represented as a tube. Reproduced with permission from reference 60.

Saccharomyces cerevisiae.[64] Sèraphin[65] described how a common sequence motif was discovered between the Sm proteins by amino acid sequence alignment and Hermann *et al.*[66] have shown that snRNP Sm proteins share two evolutionarily conserved sequence motifs that are involved in Sm protein-protein interactions.

3.5 TRAP Protein – The structure of a novel RNA-binding protein, *Bacillus subtilus* tryptophan (trp) operon RNA binding attenuation protein (TRAP), has been solved in 1995.[67] This is an unusual protein where eleven copies of the TRAP monomer are arranged in a doughnut ring with 11 fold rotational symmetry, with two rings stacked face to face (Figure 3). The indole ring of the tryptophan is buried in a hydrophobic pocket as shown. It is thought that the (U/G)AG triplet interacts with a protein monomer in a specific way since the 5′ end of the polycistronic mRNA of this operon contains 11 tandem repeats of UAG or GAG triplets separated by 2-3 nucleotides. At high concentrations of tryptophan the attenuator protein binds the 5′ of the mRNA and the downstream transcript forms a hairpin and terminates transcription.

3.6 RNA/Protein Complexes – Two important RNA/protein complex structures were reported in 1995: the crystal structure of a complex between U1A protein and hairpin II of U/snRNA[68] and the crystal structure of a RNA bacteriophage coat protein-operator complex.[69] The NMR structure of a 24 nucleotide RNA hairpin has been determined[70] that is closely related to the target of MS2 phage. In these structures binding of the protein to the RNA is often by β sheets as is proposed for the T4regA translational regulator protein whose crystal structure has recently been described.[71] However for other proteins such as Rop encoded by ColEI plasmids recognition is proposed to be *via* a four α helix



(a)

bundle.[72] In RNA protein interactions stacking interactions between RNA bases and aromatic side chains of the protein amino acids seem to be important for recognition. Further RNA/protein crystal structures will support these earlier observations.

4 DNA Binding Proteins

Several structures of proteins involved in binding to DNA have appeared in 1995.

4.1 Homeodomain Proteins – One example is the homeodomain proteins. These proteins are important in development and gene regulation in eukaryotic cells. A knowledge of their structure and how they mediate their effects is of great importance. The homeodomain is a compact 60 amino acid residue domain that has three α helices folded around a hydrophobic core and a flexible N-terminal

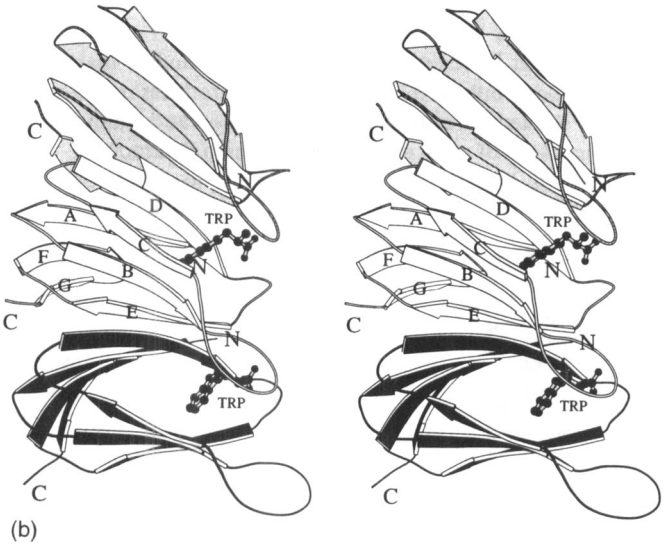
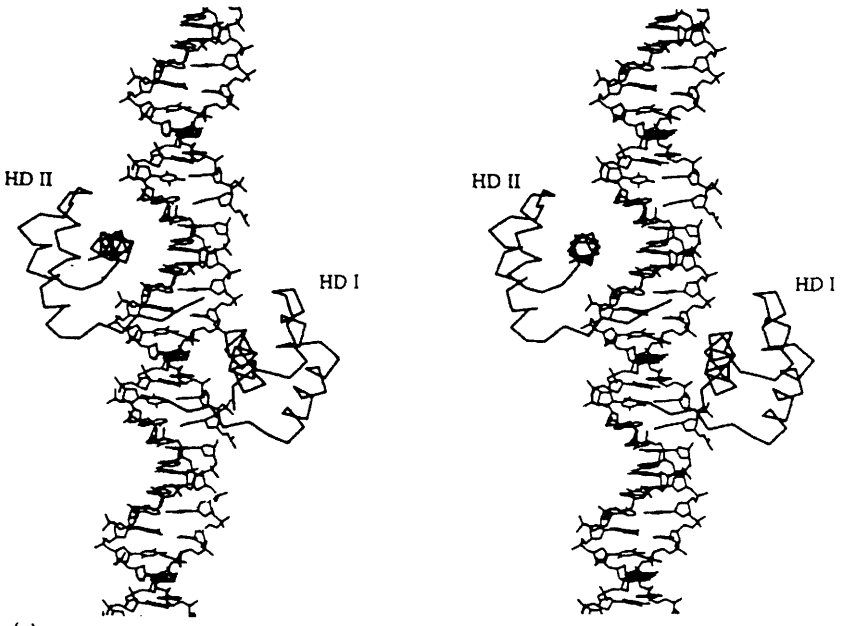


Figure 3 a) A MOLSCRIPT (Kraulis, 1991)[203] representation of the trp RNA-binding attenuation protein reference 67. The L-tryptophan molecules are shown in space filling mode and one subunit is shown in dark shading. b) Ribbon diagram of three adjacent subunits of TRAP. β strands are shown as arrows and L-tryptophan molecules as ball and stick models. Ribbons from different subunits are shown in a different tone. β strands of the middle sub- unit are marked by capital letters in order of polypeptide chain folding. The rings of the β wheel, 7 stranded antiparallel β sheets are formed by β strands G, F, A and D from one subunit and β strands E, B and C from the adjacent subunit in the ring.

arm which only becomes ordered on binding to DNA. These domains often interact with other proteins and bind DNA in a cooperative manner.[73] The structure of a paired homodimer (prd) from *Drosophilia* bound to DNA was published in 1995.[74] Residues in the homeodomain participate in protein-protein contacts. The DNA is bent by 20° which appears to facilitate the observed interaction between two homeodomains. A crystal structure of the MATα1-MATα2 homeodomain heterodimer bound to DNA shows that formation of the dimer depends on a 21 residue tail C terminal to the α2 homeodomain.[75] The tail which is unstructured in the free α2 protein becomes ordered upon association with a1, contacting the homeodomain directly on a surface of the protein not involved in DNA binding. Binding of the heterodimer to DNA induces a 60° bend. Another crystal structure appeared in 1995 of an even skipped homeodomain complexed to an AT rich DNA.[76] This structure shows two homeodomains binding to opposite faces of a single turn of B-DNA (Figure 4a,b,c). The 2.0Å resolution structure shows water mediated hydrogen bonds at the helix 3-DNA interface and reveals a Gln residue that contacts DNA adopting multiple conformations.

Studies on the DNA bending associated with α1 and α2 proteins from yeast have been carried out by solution studies.[77] These confirm that the heterodimer produces the 60° bend but the α2 domain alone introduces only a negligible bend. Jin *et al.*[78] have studied the altered DNA recognition and bending by introducing insertions in the α2 tail of yeast α1/α2 homeodomain heterodimer.

The structure of the 'Paired domain' bound to DNA has recently been determined by Xu *et al.*[79] This is the structure of a subset of the paired class that



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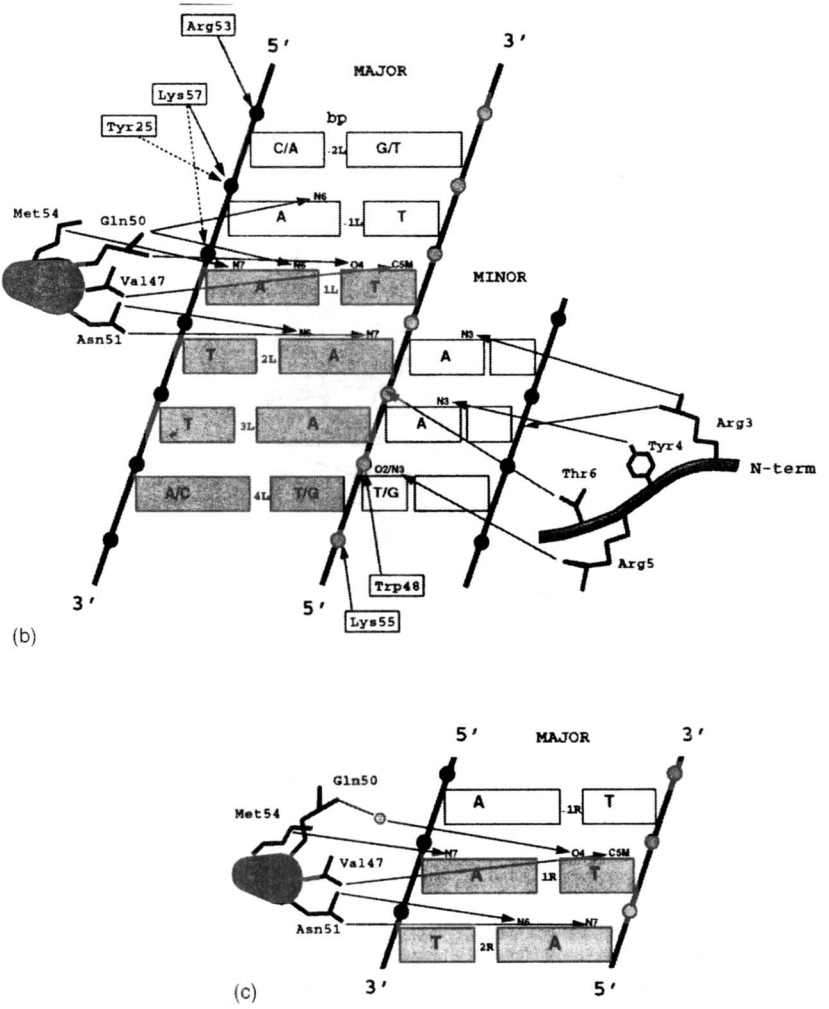


Figure 4 a) Stereoview of the complex of the Eve homeodomain I and II binding in a tandem fashion to opposite faces of the DNA. The recognition helix fits into the major groove and the flexible N-terminal arm lies in the minor groove. Drawn with MOLSCRIPT (Kraulis, 1991). b) Schematic diagram of the contacts between Eve homeodomain I and DNA. Contacts occur in both the major and minor grooves and the phosphate backbone. The core DNA sequence ATTA/C is shown shaded. c) Schematic diagram of the contacts between Eve homeodomain II recognition helix and DNA. Contacts in the minor groove are identical to Eve homeodomain I. Reproduced from reference 76 by permission of Oxford University Press.

has a second DNA binding domain, the 'Paired domain' in addition to the homeodomain (Figure 5). The 'Paired domain' was found to consist of two structurally independent domains both bearing a resemblance to the homeodomain and the Hin recombinase.

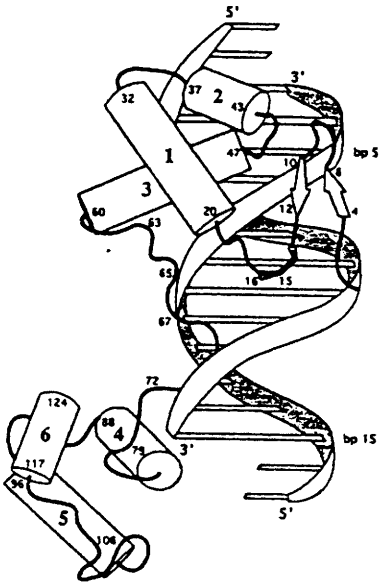


Figure 5 Sketch of the 'Paired' Domain – DNA Complex. The cylinders represent α helices and arrows indicate β sheets. A critical β-turn includes residues 13-16. Reproduced with permission from reference 79.

4.2 Retroviral Integrases – An important group of proteins interacting with DNA are the retroviral integrases. These proteins are responsible for the integration of retroviral DNA into the host genome. The retroviruses are associated with many diseased states including AIDS and leukemia in humans. The design of inhibitors of the integrases are important in treatment of these conditions. The integrases of retrovirus can be divided into three functional domains; an N terminal zinc binding domain, a central core involved in polynucleotidyl transfer which contains an invariant triad of acidic residues, and a C-terminus that binds to DNA. The first structure of the catalytic core domain of HIV-1 integrase was published in 1994[80] and a second from avian sarcoma virus integrase in 1995.[81] The structure showed a similarity to RNase H and Ruv C resolvase. NMR has been used to study the C-terminal domain of HIV-I integrase.[82,83]

The structure of a bacteriophage Mu transposase core was published by Rice and Mizuuchi.[84] This protein showed structural similarity to the catalytic domain of HIV and ASV integrase despite having no obvious similarity at the primary

amino acid sequence level. A review of the similarity among protein structures of the polynucleotidyl transferase superfamily has been written by Yang and Steitz.[85]

4.3 Rel Transcription Factors – These proteins regulate the cellular defence response against stress, injury and external pathogens. They appear to fall into two classes: those that bind DNA as a homodimer or heterodimer and those that bind DNA as a monomer either alone or as part of a multi-component complex. The structures of a homodimer NF-KB 50 homodimer bound to DNA were reported in 1995.[86,87] In the first structure (shown in Figure 6) the p50-p50 dimer is bound to an 11bp DNA and interacts symmetrically with the two half sites in DNA separated by an odd number of base pairs whereas in the second structure binding of DNA is in an alternative mode in which the two half sites are spaced by an even number of base pairs. The Rel homology region (RHR) was found to contain two domains one that makes sequence specific interactions with the major groove of DNA and the other that is involved in dimerisation and some phosphate contacts. Both domains possess a fold that is similar to that found in the immunoglobulin superfamily and bind DNA unlike other proteins where a long loop makes all but one of the sequence-specific contacts with DNA (Figure 6). The overall structure is unusual and extends the use of the Ig versatile fold to nucleic acid recognition.

In the Ghosh structure the DNA is straight and in the Müller structure there is a minor bend of 15° towards the major groove. Falvo *et al.*[88] show that binding of NF-KB can straighten DNA. The ability of Rel proteins to remodel DNA structure is thought to involve higher-order nucleoprotein complexes called 'enhancesomes'. These are necessary for the activation of certain genes such as β-interferon.[89] Interactions of a Rel protein with its inhibitor have been reported by the group of Ptashe.[90] The mutations lie in the canyon formed by association of the dimerization domains.

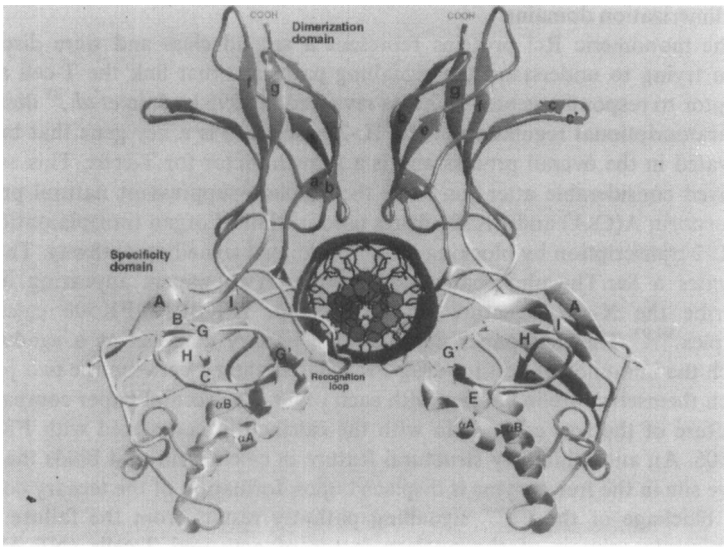
The monomeric Rel proteins represent a second class and were discovered when trying to understand the signalling pathways that link the T-cell antigen receptor to responder genes. This was reviewed in 1995 by Jain *et al.*,[91] describing the transcriptional regulation of the IL-2 gene. IL-2 is a key gene that becomes activated in the overall process and is a growth factor for T-cells. This area has received considerable attention since the immuno-suppressant natural products cyclosporin A(CsA) and FK306 drugs used in clinical organ transplantation lead to IL-2 transcription by blocking a Ca^{++} mediated signalling pathway. The Ca^{++} activates a Ser/Thr phosphatase, calcineurin. Two papers appearing in 1995 describe the X-ray structure of the receptor (FKBP12)-FK506 calcineurin complex.[92,93] The first paper describes the ternary complex as a sandwich in which the immunosuppressant drug FK506 is slathered between the two proteins which themselves are in contact with each other. The second paper compares the structure of the free calcineurin with the calcineurin complexed with FKBP12-FK506. An autoinhibitory structural feature in calcineurin that binds the Zn/Fe active site in the free enzyme is displaced upon formation of the ternary complex. The blockage of the Ca^{++} signalling pathway results from the failure of the cytoplasmic subunit of the nuclear factor of activated T-cells (NF-ATp) to

translocate to the cell nucleus following T-cell stimulation. Cloning of the NF-ATp and related isoforms NF-ATc, NF-ATc3[94,95] show that they could be related to the RHR-related segment. Mutations within the region of the NF-ATp corresponding to the DNA binding loop of RHR disrupt DNA binding.[96]

4.4 *Bam*HI Endonuclease/DNA Complex – The structure of the restriction enzyme *Bam*HI in complex with DNA has been reported.[97] The *Bam*HI binds DNA from the major groove side. The DNA fits into the large cleft and retains a B-type conformation. The *Bam*HI subunit (one of dimer) consists of a large six-stranded mixed β sheet that is sandwiched on both sides by α helices. The enzyme bound to DNA undergoes several conformational changes. The most striking conformational change is the unravelling of C terminal α helices – the arm from one subunit fits into the minor groove of the DNA and the arm from the other subunit follows the DNA sugar phosphate backbone. Tightly bound water molecules play an important part in recognition of the specific hexameric binding site on DNA.

4.5 Other Protein/DNA Complexes – Several other important complexes of proteins with nucleic acids have been reported, and these are summarised below.

4.5.1 ETSI, DNA binding domain/DNA complex – Transcription factors from the ETS family are involved in control of processes such as growth and development, transformation and T-cell activation in different species. The NMR derived structure of this complex[98] show that protein-DNA interactions occur in both the major and minor grooves. This is a novel mode of binding and shows side chain intercalation in the minor groove by the side chain of a tryptophan residue into



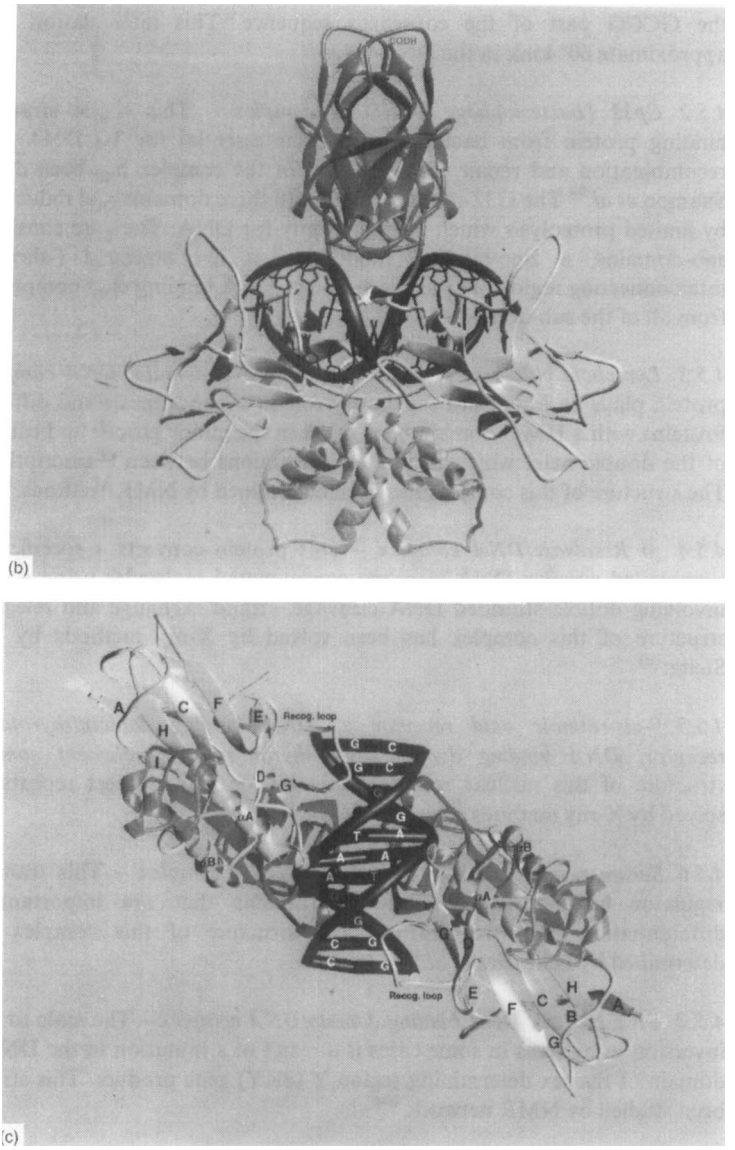


Figure 6 Overall view of the NF-*k*B p50 dimer bound to DNA. Depicted are residues 43-352 of both subunits and the central 11bp of the DNA. Labels A-1 and a-g mark the strands of the N terminal and C-terminal domain respectively a) (p. 346) view along the DNA, with the dyad vertical, b) view perpendicular to the direction of the DNA. The orientation of the dyad is maintained. c) View along the dyad of the complex from below. Reproduced with permission from reference 86.

the GCCG part of the consensus sequence. This intercalation induces an approximate 60° kink in the DNA helix.

4.5.2 Gp32 (bacteriophage T4)/DNA complex – This single stranded DNA binding protein from bacteriophage T4 is essential for T4 DNA replication, recombination and repair. The structure of the complex has been described by Shamoo *et al.*[99] The G32 can be divided into three domains and reduced to a core by limited proteolysis which retains affinity for DNA. The core consists of three sub-domains, a zinc-binding subdomain, a five stranded (-sheet and an interconnecting region. The single stranded DNA binding cleft comprises regions from all of the sub-domains.

4.5.3 Lymphoid enhancer – binding factor, HMG domain, DNA complex – This protein plays an important regulatory role in organogenesis and differentiation. Proteins with a HMG domain bind DNA in the minor groove and cause bending of the double helix which facilitates interactions between transcription factors. The structure of this complex has been determined by NMR methods.[100]

4.5.4 γδ Resolvase/DNA complex – This protein converts a specific negatively supercoiled circular DNA into two concatenated molecules using a mechanism involving double stranded DNA cleavage, strand exchange and relegation. The structure of this complex has been solved by X-ray methods by Yang and Steitz.[101]

4.5.5 9-cis-retinoic acid receptor a, DNA binding domain/thyroid hormone receptor, DNA binding domain/DNA thyroid-response element complex. The structure of this nuclear receptor assembly on DNA direct repeats has been solved by X-ray methods.[102]

4.5.6 Serum response factor core domain/DNA complex – This transcriptional regulator belongs to a family of proteins that are important for cell differentiation and proliferation. The structure of this complex has been determined by Pellegrini *et al.*[103]

4.5.7 SRY protein, DNA binding domain/DNA complex – The male to female sex inversion in humans in some cases is a result of a mutation in the DNA-binding domain of the sex determining region Y (SRY) gene product. This structure has been studied by NMR network.[104]

4.5.8 TF11B/TATA box binding protein/TATA element ternary complex – This is part of the initiation complex which regulates genes transcribed by RNA polymerase II. The structure of this ternary complex has been recently described.[105]

4.6 Exonuclease III – This enzyme is the major apurinic/apyrimidinic (AP) repair endonuclease in *Escherichia coli.* The AP sites are formed in double

stranded DNA *via* oxidative damage, alkylating agents and ionizing radiation. The structure of the enzyme has been solved[106] and shows a pseudo-symmetric α/β sandwich motif containing two six stranded β-sheets flanked by α helices. The active site is in a long groove at one end of the sandwich where the metal ion and dCMP are bound. The catalytic triad of Asp-His-H_2O proposed for P-O3′ bond cleavage reaction is similar to the Asp-His-Ser triad in serine proteases. A positively charged DNA binding site is found near the other site.

4.7 Gal6 Bleomycin Hydrolase – This enzyme is a cysteine protease that detoxifies the anti-cancer drug bleomycin, a small glycometallopeptide that catalytically cleaves double stranded DNA. The structure of the yeast form of the enzyme has been determined.[107] The structure is a toroidal hexamer. Dimer contacts form most of the subunit interactions. A 22Å diameter channel along the threefold axis of the hexamer extends 25Å from both the top and bottom into a central 45Å cavity that is 30Å in length. Sixty lysine residues line the channel which is proposed to be the DNA binding site. The active site triad Cys-His-Asn, important active site residues, superimpose well with papain.

5 Metalloenzymes

5.1 Heme Protein – cytochrome cd_1 – An interesting bifunctional enzyme has been described which is able to reduce oxygen to water and synthesise nitric oxide. This is cytochrome cd_1.[108] A unique noncovalent d_1 heme constitutes the active site of the enzyme. NO has a high affinity for heme and a special mechanism for its release is proposed. The crystal structure suggests that an axial tyrosine ligand is displaced by nitrite. Subsequently the tyrosine would replace the reaction product NO through conformational changes in the enzyme.

5.2 Chloroperoxidase – Chloroperoxidase (CPO) is a haem enzyme whose primary role is the chlorination of aliphatic molecules. The enzyme from *Caldariomyces fumago* is a haem peroxidase-cytochrome P_{450} hybrid. The structure described by the group of Poulos[109] shows the enzyme to have a unique fold consisting of two helical domains that sandwich a haem cofactor. Substrates access the haem iron of CPO *via* a small channel that leads to the distal pocket where a small flexible hydrophobic region appears capable of partially burying aromatic substrates the size of dimethylaniline. These features explain the broad substrate specificity of the enzyme and the selectivity for less bulky substrates. A view of the CPO active site is shown in Figure 7.

5.3 Haemopexin – Haemopexin is a haem binding transport protein that binds haem reversibly in a 1:1 ratio and delivers it to the liver. The protein is made up of two homologous domains sharing 25% sequence identity however they have different functions. The N-terminal domain is responsible for haem binding and the C terminal domain is responsible for modulating haem and receptor binding. The structure of the C-terminal domain has been described.[110] The structural

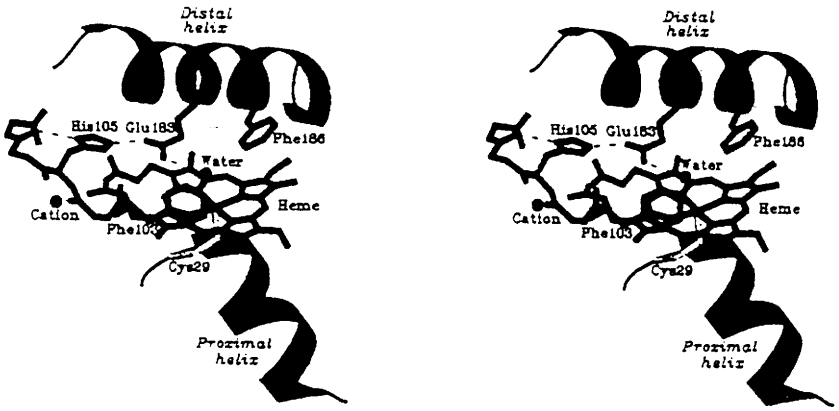


Figure 7 Stereo view of the CPO active site. Hydrogen bonds involving the distal side water molecule, Glu 173, the proposed acid-base catalyst and His 105 are shown as dashed lines. A proposed substrate-binding pocket is bracketed by Phe 103 and Phe 136. Reproduced with permission from reference 109.

elements are β leaflet modules arranged around a pseudo four fold axis. The assembly is held together by a disulfide bond. The structure has a tunnel running through the centre in which ions have been located. It is proposed that the role of the tunnel is to bind to the haem propionate in the anion-binding site. The flat surface of the structure is suited to provide a large surface area that would interact with other proteins.

5.4 Dihydroxybiphenyl 1,2-Dioxygenase – The structure of this interesting biphenyl cleaving extradiol dioxygenase has been described.[111] The enzyme from *Pseudomonas cepacia* LB400 is capable of degrading a wide range of polychlorinated biphenyls (PCBs). The enzyme is a homo-octamer, with each monomer consisting of two domains which are related by pseudo-twofold symmetry. The C-terminal domain houses the ferrous iron atom in the middle of an open cavity surrounded by β strands. The cavity is wide enough to allow entrance of catecholic substrates through the outer opening, while water and dioxygen can approach the active site through an inner opening. A schematic representation of this enzyme is shown in Figure 8.

5.5 Sulfite Reductase – The structure of *Escherichia coli* sulfite reductase has been described at 1.6 Å resolution. An enzyme complex with sulfite and phosphate reveals the structure of the active site and cofactor, a siroheme covalently linked to an Fe_4S_4 cluster[112] (Figure 9). In the form of the enzyme the sirohemes proximal axial co-ordination site is occupied by a cysteine thiolate shared with the Fe_4S_4 cluster beneath, whereas the distal axial coordination site is

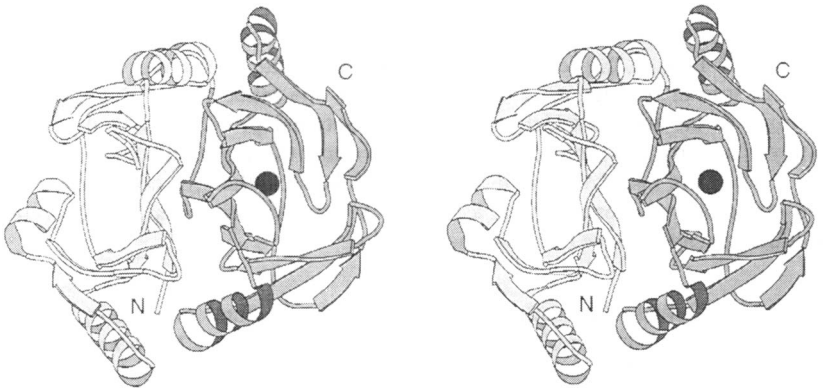


Figure 8 Schematic representation of the backbone fold of the extradiol dioxygenase. N and C terminal domains are as indicated and the iron is represented by a dark sphere. Reproduced with permission from *Science*, 1995, **270**, 976–980. © 1995 American Association for the Advancement of Science.

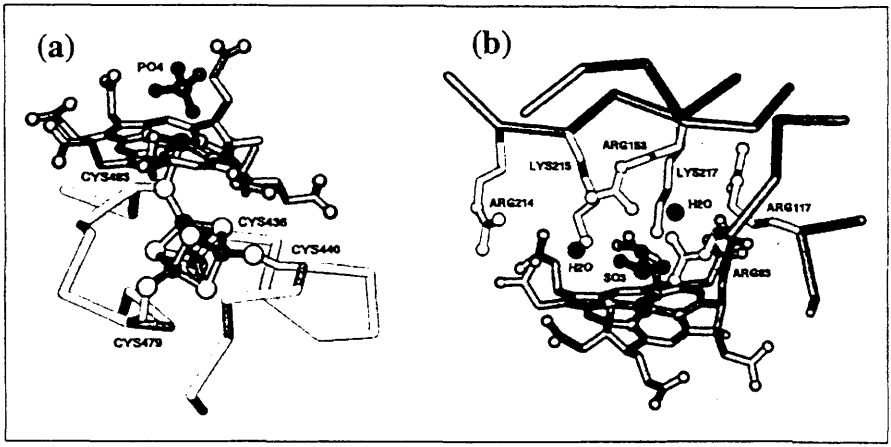


Figure 9 The sulfite reductase active centre a) in oxidised state b) in the complex between oxidised sulfite reductase and sulfite. Reproduced with permission from reference 112.

occupied by exogenous phosphate bound through oxygen. In the complex between the oxidised enzyme and sulfite, the SO_3 binds to the siroheme iron beneath it through a sulfur and interacts with ordered water molecules and positively charged protein side chains. The electronic structure of this unusual chromophore has been studied in the oxidised form of the enzyme.[113] It was shown to mix an excited triplet state into the ground diamagnetic state of the cluster to account for the clusters paramagnetic properties. Site directed

mutagenesis of the spinach enzyme has been carried out by Bellissimo and Privalle.[114] Few of the residues around the active site cluster can be changed without loss of activity, with the exception of one mutant of a glutamic acid changed to an alanine which produced a functional protein with a decreased catalytic activity and an altered optical spectrum. It is suggested that the active site residues are non-catalytic since the free siroheme in the presence of substrate can consume reductant as well as or better than the enzymes. However the products generated have not been characterised.[115]

5.6 Purple Acid Phosphatase – The crystal structure of a purple acid phosphatase containing a dinuclear Fe(III) – Zn(II) active site has been reported by Strater *et al.*[116] The metal ions are located 3 Å apart (Figure 10) in the active site and have an asparate side chain that acts as a monodentate-bridging ligand between the metals. The structures of other related Ser/Thr phosphatases have also been reported.[92,93,117,118]

5.7 Nickel-containing Enzymes

5.7.1 Nickel-iron dehydrogenase – The first structure of a nickel containing enzyme was described in 1995. This was a nickel-iron hydrogenase from

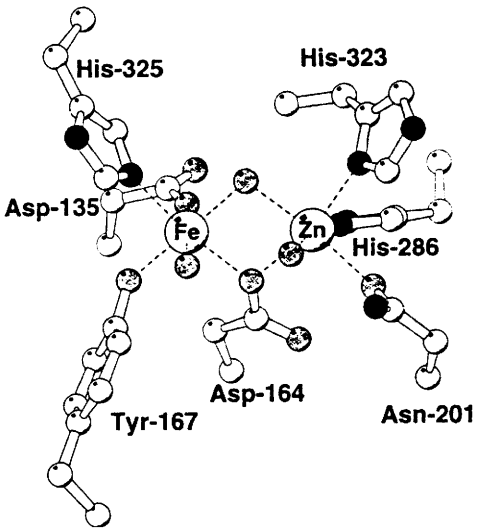


Figure 10 Crystal structure of purple acid phosphatase schematic drawing showing the model of the dinuclear metal binding site. Reproduced with permission from reference 116.

Desulfovibrio gigas.[119] In this enzyme the unexpected presence of a second metal was observed which was postulated to be Fe. The enzyme contains three FeS centres found along a nearly straight line in the small subunit connecting the active site, which is located in the large subunit, to the protein molecular surfaces as shown in Figure 11. The active site is dinuclear containing Ni and Fe.

5.7.2 Urease – The crystal structure of urease, another nickel containing enzyme, has been reported.[120] This enzyme contains a dinuclear Ni^{2+} centre bridged by a carbamate-modified lysine residue, Lys 217 (Figure 12). It is suggested that a carbamate moiety is able to carry higher negative partial charges than a carboxylate which is a feature that is thought to modulate metal ligation. It has been postulated by Lippard[121] that one metal acts as a Lewis acid to polarise the

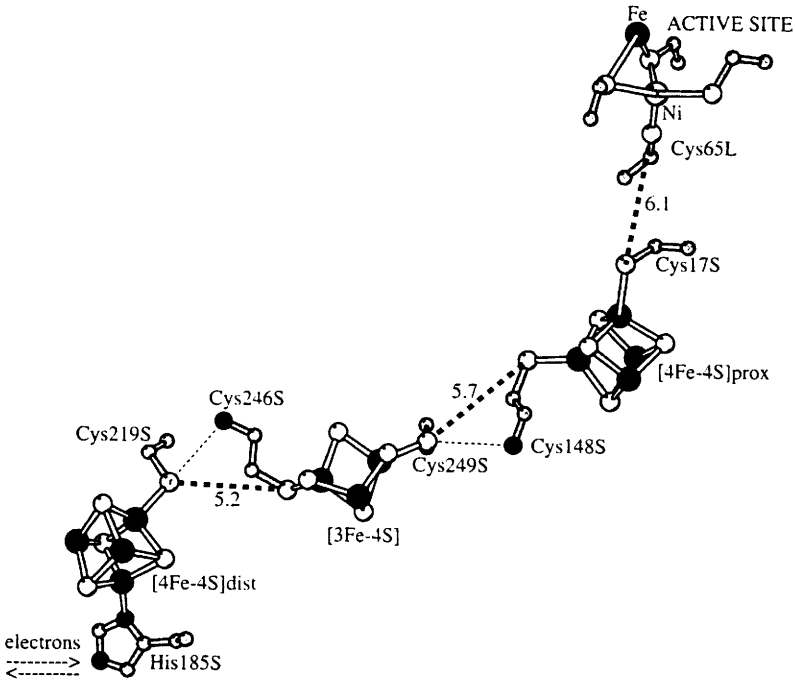


Figure 11 Residues and metal centres involved in a possible electron transfer pathway from the buried active site of the nickel-iron hydrogenase enzyme to the exposed histidine residue. The shortest path would include, besides H bonds (thin dashed lines) and metal ligand interactions, covalent bonds of Cys and His residues as shown. The numbers next to the thick dashed lines give the nearest edge to edge distance (in Å) between subsequent redox centres, assuming that S_γ atoms of cysteine ligands may be counted as part of the respective redox centres. Reproduced with permission from reference 119.

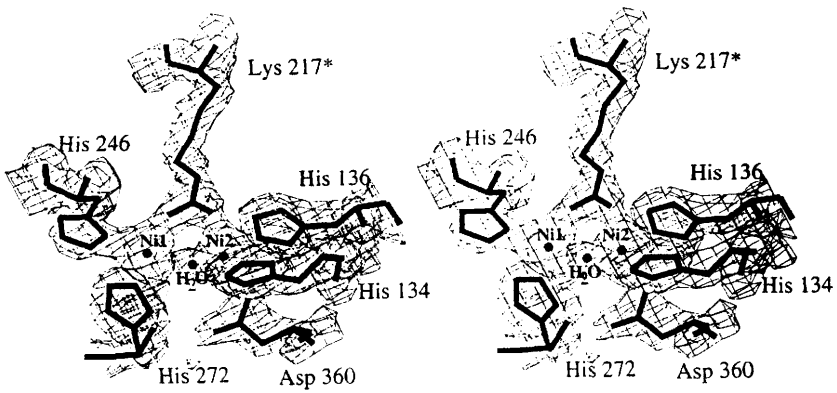


Figure 12 Stereodiagram of the urease bi-nickel centre showing the carbamylated lysine residue (Lys α217). Reproduced with permission from reference 120.

leaving group, whereas the other activates an OH^- group which performs a nucleophilic attack on the substrate as shown in Figure 13. Urease is the only hydrolase known to contain Ni.

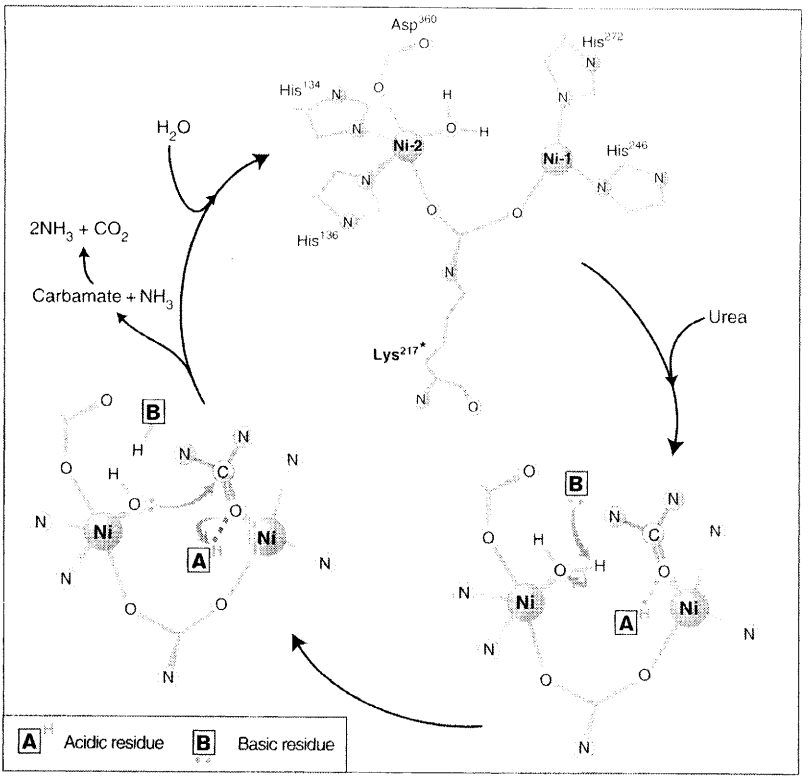
Raman spectroscopic evidence has been presented for a Ni-CH_3 species in the A cluster of the enzyme carbon monoxide dehydrogenase.[122] Freeze-quench resonance Raman spectroscopy has provided evidence for an Fe-CO adduct during acetyl-CoA synthesis and Ni involvement in CO oxidation by carbon monoxide dehydrogenase.[123]

5.8 Zinc-containing Enzymes

5.8.1 Phosphotriesterase – An interesting structure that looks like urease has been reported for the enzyme phosphotriesterase.[124] This enzyme contains Zn instead of Ni in the active site.

5.8.2 RING-finger – RING finger proteins have a novel zinc binding motif that can be defined as Cys-X_2-Cys-$X_{(9\text{-}39)}$-Cys-$X_{(1\text{-}3)}$-His-$X_{(2\text{-}3)}$-Cys-X_2-Cys-$X_{(4\text{-}48)}$-Cys-X_2-Cys where X is any amino acid. A solution structure of the RING finger domain from acute promyelocytic leukaemia protooncoprotein PML has been described.[125] A conserved cross-brace ligation scheme as shown in Figure 14 was also found in the structure of an equine herpes virus protein described a year earlier.

5.8.3 Collagenase – Collagenases belong to a family of zinc-dependent metalloproteinases which are involved in the breakdown of the extracellular matrix. They cleave all three strands of the collagen double helix. Their activity is needed for wound healing, angiogenesis and cell migration. During tumor



Possible mechanism for the chemistry at the catalytic site of urease.

Figure 13 Mechanism of urease postulated by Lippard. Reproduced with permission from reference 121.

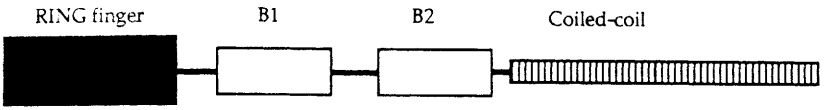


Figure 14 The tripartite motif for PML. B1 and B2 refer to the boxes and the coiled-coil to the predicted α helical coiled-coil domain.

metastasis collagenase activity is uncontrolled resulting in breakdown of the extracellular matrix. The structures of other enzymes of this class are known for the N-terminal catalytic domain but the group of Blow have reported the structure of the full length porcine synovial collagenase.[126] The C-terminal domain is a four-bladed β propeller where each blade is a four stranded, antiparallel β sheet. At the axis of the propeller, a calcium ion is ligated by four carbonyl oxygens. A disulfide between the first and last residues of the domain stabilizes the fold. The complete structure is shown in Figure 15.

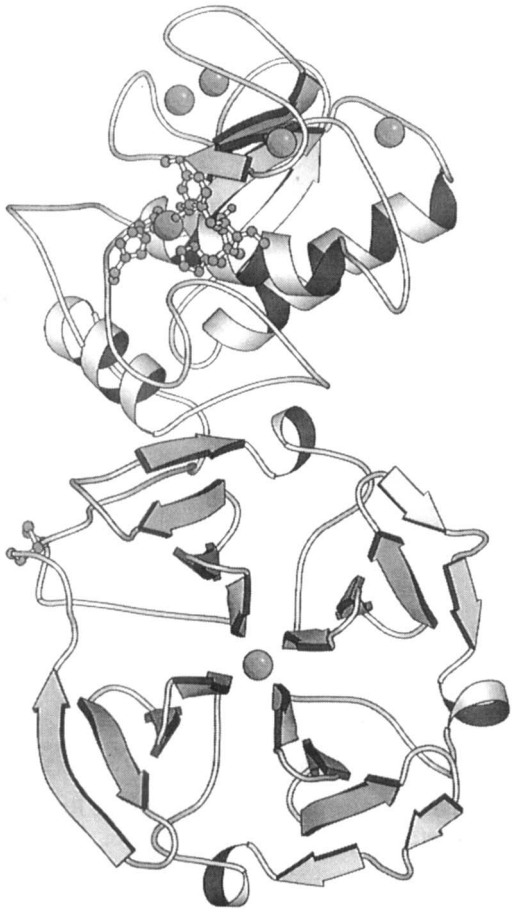


Figure 15 Ribbon tracing of full-length collagenase. The C terminal β-propeller can be seen at the bottom of the figure. Reproduced with permission from reference 126.

5.9 Tungsten-containing Protein

5.9.1 Aldehyde ferredoxin oxidoreductase – Tungsten is used in proteins isolated from some organisms like *Pyrococcus furiosus* which grow under very high temperature conditions. The first structure of one of these enzymes was reported in 1995, and was of the tungstopterin enzyme, aldehyde ferredoxin oxidoreductase.[127] An intramolecular cyclisation of the cofactor is observed which generates a non-planar three-ringed structure as shown in Figure 16. Two

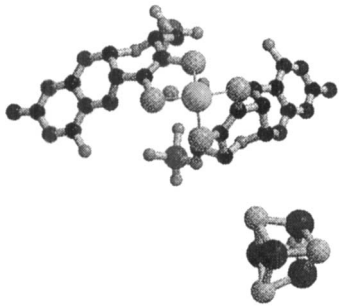


Figure 16 Aldehyde ferredoxin oxidoreductase, showing the intramolecular cyclisation of the cofactor. Reproduced with permission from *Science*, 1995, **267**, 1463–1459. © 1995 American Association for the Advancement of Science

molecules of the tungsten pterin cofactor are observed in the enzyme active site. It appears that tungsten is used in these hyperthermophilic oxidoreductases in place of molybdeum which is found in mesophilic enzymes.

5.10 Molybdenum-containing Protein

5.10.1 *Aldehyde oxidoreductase* – The aldehyde oxidoreductase enzyme structure[128] from *Desulfovibrio gigas* has recently been solved. The cofactor is a single Mo-binding molybdopterin cytosine dinucleotide as shown in Figure 17. The Mo is bound by two dithiolene S ligands and three oxo and/or hydroxo ligands providing a square-planar pyramidal coordination with a vacant axial site that is thought to serve as the binding site for small aldehyde substrates.

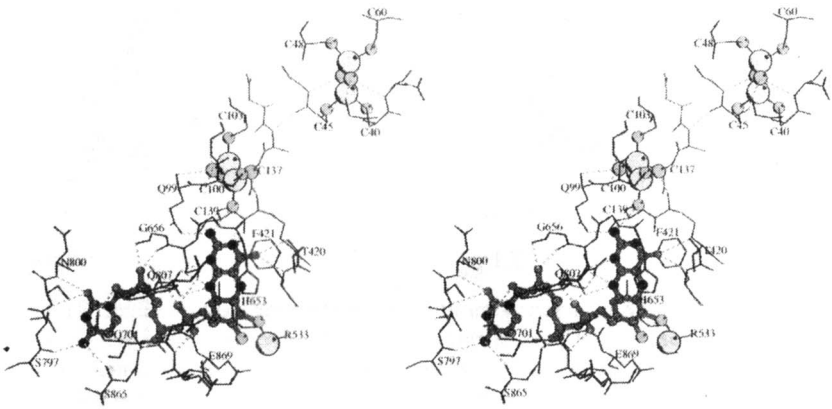


Figure 17 Stereo drawing of the molybdopterin cytosine dinucleotide, the second[2Fe-2S] middle and first [2Fe-2S] cluster (top) and their contacting residues. Reproduced with permission from reference 128.

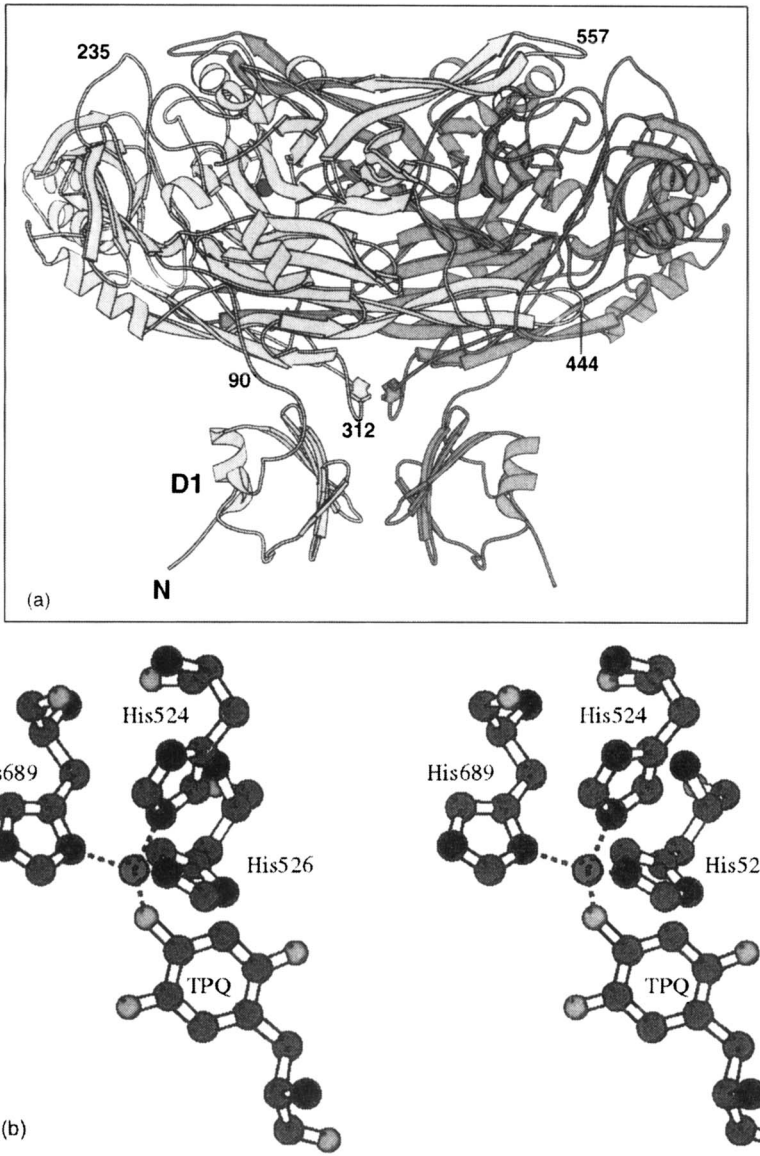


Figure 18 a) Ribbon representation of the copper amine oxidase dimer. The two monomers are in different shading. The residue numbers of the β hairpin turn in the two arms extending from one monomer into the second are labelled. b) Ball and stick representation of the active site in the crystal form 1, in which the copper has distorted tetrahedral coordination. The topa quinone cofactor (TPQ) is shown. Reproduced with permission from reference 130.

5.11 Copper-containing Proteins

5.11.1 Nitrite reductase – The structure of the copper nitrite reductase from *Achromobacter cycloclastes*[129] has been elucidated. The authors propose a simple mechanism for the enzyme that does not require a metal-bound NO intermediate as proposed for cytochrome cd_1.

5.11.2 Amine Oxidase – Another Cu-containing protein, the amine oxidase from *Escherichia coli* uses a new cofactor, topa quinone (see Figure 18 a, b).[130] This enzyme catalyses the oxidative deamination of primary amines to their corresponding aldehydes, with concomitant reduction of molecular oxygen to hydrogen peroxide. The Cu centre of this enzyme binds two water ligands whose possible role in catalysis is as yet undetermined. A mechanism for the formation of the active topa quinone which involves a tyrosine residue has been proposed.[131]

5.12 Manganese-containing Proteins

5.12.1 Isopenicillin N-Synthase – Isopenicillin N-synthase (IPNS) mediates the removal of four hydrogen atoms from a linear tripeptide (L-δ-(α-aminoadipoyl)L-cysteinyl D-valine (ACV)) synthesising the labile and strained ring structure of penicillin in a single step. This represents the first of a new structural family of enzymes and consists of an open jelly roll motif that encloses the active site metal ion in a hydrophobic environment.[132] The manganese bound at the active site has a distorted octahedral geometry with four protein ligands and binds two water molecules. It is thought that ACV and the dioxygen probably replace these molecules during catalysis (Figure 19).

6 Other Protein Structures

6.1 Enolase – The mechanism of the glycolytic enzyme enolase has been elucidated by studying high resolution crystallographic structures and mutant enzymes.[133,134] A site directed mutant of the enzyme where a glutamic acid 211 is changed to a glutamine show a strong UV difference spectrum characteristic of the ionised form of an inhibitor, tartonate semialdehyde phosphate which is interpreted as the first step in the reaction cycle.[135]

6.2 Cholesterol Esterase – The structure of uncomplexed and linoleate-bound *Candida cylindracea* cholesterol esterase has been described.[136] This is one of a group of enzymes that bind steroids. Other members of this group are the steroid dehydrogenase enzymes. The short-chain dehydrogenase/reductase enzymes form a family of related enzymes that have been reviewed in 1995.[137]

6.3 Estrogenic 17β-Hydroxysteroid Dehydrogenase – This human enzyme is important in understanding 'hormone-dependent' breast tumours. The structure was described in 1995 by Ghosh *et al.*[138] This enzyme catalyses the reduction of

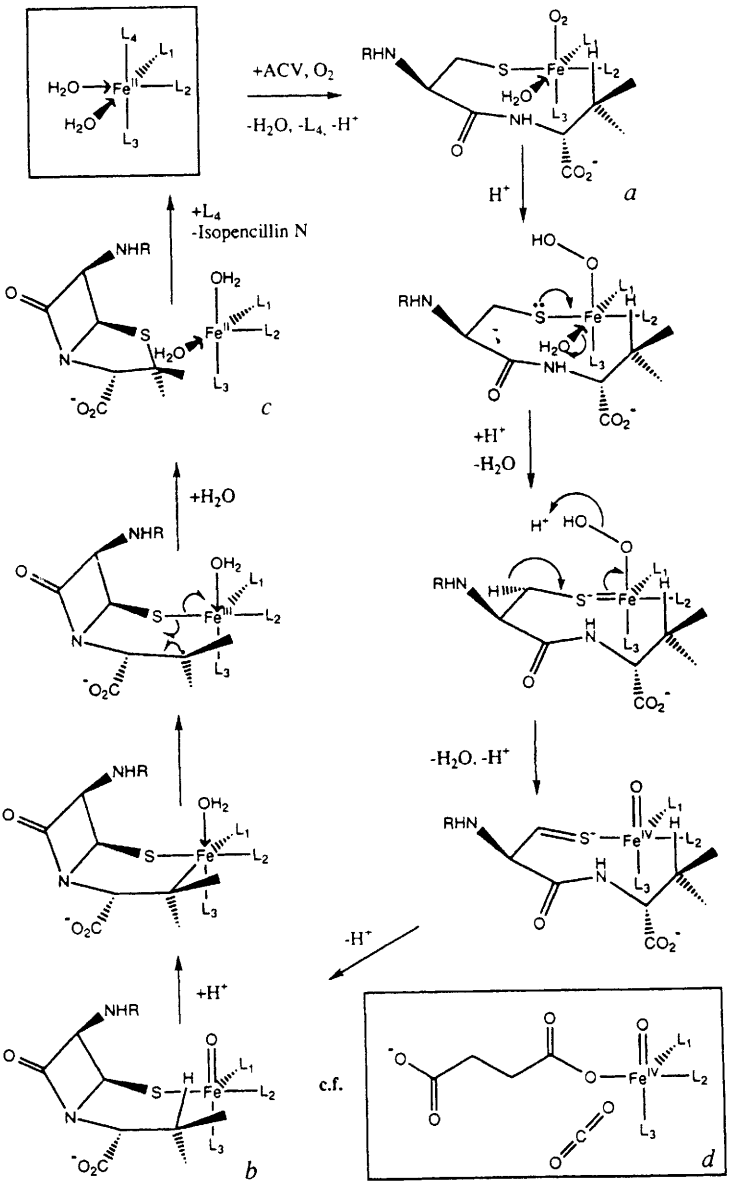


Figure 19 Proposed scheme for the overall reaction catalysed by IPNS. The scheme shows a) the two substrates bound to the iron b) the proposed β-lactam ferryl intermediate c) the enzyme-product complex with IPN and two water molecules. Reproduced with permission from reference 132.

the weak estrogen, estrone, to the potential, estradiol. Excess estradiol is thought to stimulate the development of breast tumours.

6.4 Enoyl Acyl Carrier Protein Reductase – The structures of other members of the steroid dehydrogenase/reductase family were also reported in 1995. These were of the enoyl acyl carrier protein reductase from oilseed rape (*Brassica napus*)[139] and from the enzyme target of isoniazid the antituberculosis drug.[140] The former enzyme was found to show similarity to 3α, 20β-hydroxysteroid dehydrogenase. This allowed a proposal to be made for the catalytic mechanism of these two enzymes as shown in Figure 20.

6.5 Pyruvate Kinase – The crystal structure of *Escherichia coli* pyruvate kinase has given insight into the allosteric nature of this enzyme.[141] This allowed a comparison of the structure in the 'T' state with the muscle 'R' state enzyme. The allosteric activation mechanism results in the simultaneous rotation of all 12 domains that form the tetrameric enzyme.

6.6 Glucosamine 6-Phosphate Deaminase – The enzyme from *Escherichia coli* forms a hexameric structure. A paper describing the structure of this enzyme in the 'R' state proposes a structure based mechanism for the enzyme catalysed reaction.[142] Activation proceeds through a 11° rotation of each trimer about an axis parallel to the threefold axis.

6.7 Fructose 1,6-Bisphosphatase – The structure of the choroplast enzyme has been described.[143] This enzyme is controlled by allosteric effectors and by reduction of cysteines *via* the ferredoxin-thioredoxin f system.

6.8 Bacterial Sialidase – These enzymes catalyse the removal of sialic acid from various glyco-conjugates. The structure of another bacterial sialidase from *Micromonospora virdifacians* shows this enzyme to have a common β-propeller fold and an additional immunoglobulin module and a galactose binding jelly-roll structure.[144]

6.9 Influenza Virus Neuraminidase – The sequence identity between the influenza enzyme and the bacterial enzymes such as that described above is low, however the topology of the catalytic domain is conserved. The influenza enzyme has been studied in great detail in order to design an inhibitor that could be used for the treatment of flu. A high resolution structure (1.8Å) has been described for the influenza virus neuraminidase in complex with 4-guanidino-Neu5Ac2en.[145] Other novel ligands, based on benzoic acid have been described.[146,147,148] A paper describing a strategy for theoretical binding constant calculations for neuraminidase aromatic inhibitors designed on the basis of the active-site structure of influenza virus neuraminidase has been described.[149]

6.10 Glycosyl Hydrolases – The structures of several enzymes falling into this group were described in 1995. This group of enzymes that hydrolyse glycosidic

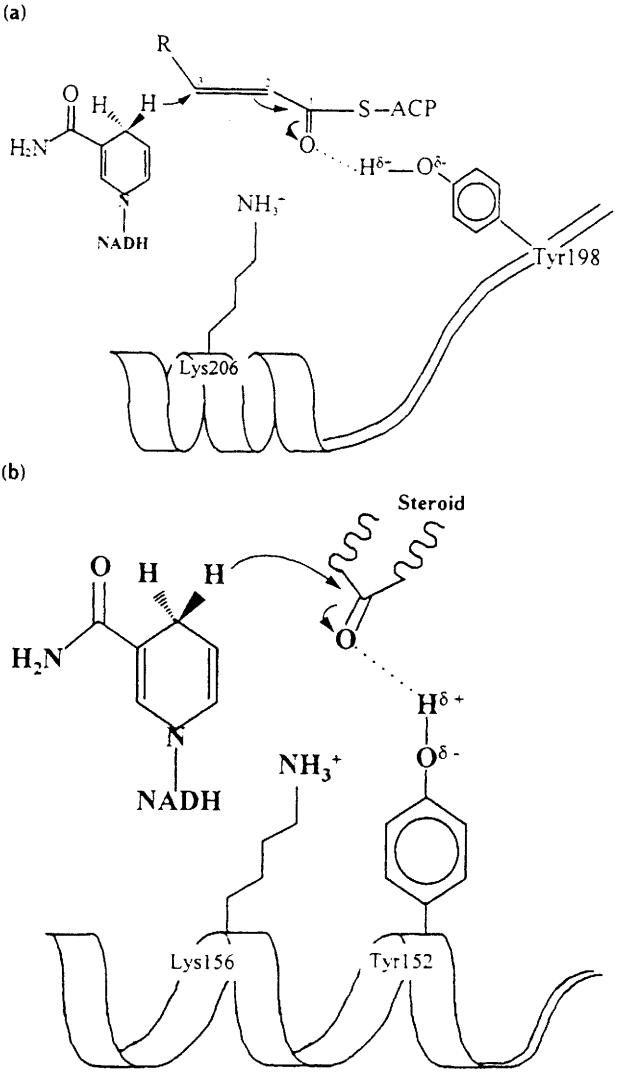


Figure 20 Proposed catalytic mechanisms of substrate reduction by enoyl reductase (ENR) and 3α, 20β-hydroxysteroid dehydrogenase (HSD) a) Reduction of the double bond in an enoyl substrate by ENR b) Reduction of the keto group in a steroid substrate by HSD. Reproduced with permission from reference 139.

bonds form a diverse group that can be classified into 45 families on the basis of amino acid similarities. The cellulases and xylanases are found in 11 of these families. The catalytic domain of a bacterial cellulase belonging to family 5[150] has been described and the cyanogenic β-glucosidase from white clover which is a family 1 glycosyl hydrolase.[151] These enzymes form a barrel structure with the active site at the centre of the barrel. Cyanogenesis in white clover requires the presence of the cyanoglucosides linamarin and lotaustralin that occur in the cell wall. These are both cleaved by the cyanogenic β-glucosidase.

Another member of the glycosyl hydrolases is 6-phospho-β-galactosidase from *Lactococcus lactis*. This enzyme belongs to family 1 and is the first member of the family to be described at a structural level.[152] It hydrolyses phospholactose which is the product of a phosphoenolpyruvate-dependent phosphotransferase system.

6.11 Proteins that Bind Co-enzyme A – Recently several structures of proteins have been reported that bind coenzyme A which is an important cofactor which acts as an acyl group carrier. It is composed of 3′ phosphate ADP, pantothenic acid and β-mercaptoethylamine. The functional group is the thiol group of the β-mercaptoethylamine moiety which combines with acyl groups *via* a thioester bond. Structures reported in 1995 are for *Pseudomonas mevalonii* HMG-CoA reductase,[153] the *Escherichia coli* malonyl-CoA : acyl carrier protein transacylase[154] and the *Megasphaera elsdenii* butyryl-CoA dehydrogenase.[155] The 3-hydroxy-methylglutaryl (HMG)-CoA dehydrogenase is a dimer. The CoA is bound in an extended conformation to a domain with an unusual fold that consists of a central α helix surrounded by three walls comprised of a four stranded sheet, an entirely α helical structure and a mixed αβ unit.

6.12 14-3-3 Proteins – These proteins were initially identified as activators of tyrosine and tryptophan hydroxylases. They function in signal transduction, exocytosis and cell cycle regulation. These proteins have an unusual structure which has been reported by Xiao *et al.*[156] and consists of nine antiparallel helices as shown in Figure 21. These form two domains and dimer contacts are formed between the two N-terminal domains, leaving a central hole 6-8Å across. This channel is suggested to be the binding site for kinases and the conservation of residue implies that it recognises some common feature of the proteins to which it binds. This is reinforced by the structure of a similar protein[157] where it is suggested that an amphipathic helix composed of leucine and asparagine fits well when modelled into the groove.

6.13 D-Amino Acid Aminotransferase – Bacteria have in addition to L-amino acid aminotransferases, an enzyme capable of transaminating D-amino acids. This enzyme catalyses the synthesis of D-glutamic acid and D-alanine which are essential components of bacterial cell walls. They have no sequence homology with other aminotransferases that are specific for L-amino acids. The reported structure[158] shows that the fold of this pyridoxal phosphate enzyme is different from those of other enzymes using this cofactor. Residues from both domains

Figure 21 Orthogonal view of a RIBBONS representation of the τ dimer of the 14-3-3 protein. Reproduced with permission from reference 156.

and both subunits contribute to each of the two symmetrical active sites. Comparisons with the L-aspartic acid aminotransferase show that some features of the active site are common, however there are certain distinct differences.

6.14 Aspartylglucosaminidase – This enzyme is an important lysosomal hydrolase that catalyses the hydrolysis of the N-glycosidic linkage between oligosaccharide and asparagine. Deficiency of this activity through mutation of the human form of the enzyme causes the lysosomal accumulation disease, aspartylglucosaminuria. The structure of this enzyme is a $(\alpha\beta)_2$ heterotetramer and it represents a new structural family of asparaginases.[159] A ribbon diagram of the enzyme is shown in Figure 22 showing the reaction product, aspartate, binding at the catalytic N-terminal end of the β-chain.

6.15 Casein-kinase I – This enzyme is a ubiquitous eukaryotic protein kinase involved in the regulation of DNA repair pathways and cell morphology. This family of proteins have a conserved N-terminal catalytic domain that is linked *via* a linker region to a non-conserved C-terminal domain which anchors the protein to the membrane. The structure of the catalytic domain from yeast bound to ATP has been reported.[160] The fold of this protein resembles that of the cAMP-dependent protein kinase and human cyclin-dependent kinase 2, despite having no significant amino acid sequence similarity.

6.16 Dihydrodipicolinate Reductase – Dihydrodipicolinate reductase (DHPR) is one of the enzymes on the biosynthetic pathway forming L-lysine and diaminopimelate from L-aspartate, specifically converting dihydrodipicolinic acid

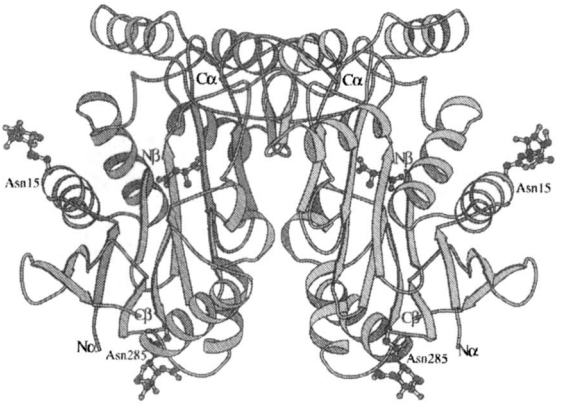


Figure 22 Ribbon diagram of the aspartylglucosaminidase (AGA)-L-asparaginase. Reproduced with permission from *Nature Struct. Biol.*, 1995, **2**, 1102–1108.

to tetrahydrodipicolinic acid in a NAD(P)H linked reduction. This pathway is unique to plants and bacteria and is a potential target for herbicidal or antimicrobial compounds. The structure of the *Escherichia coli* enzyme has been described.[161] A monomer of the DHPR consists of two domains linked by a hinge region. The tetramer which is seen crystallographically and thought to form in solution, is held by a 16 stranded mixed β barrel.

6.17 Dihydrodipicolinate Synthase (DHDLS) – This enzyme catalyses the condensation of pyruvate and L-aspartate-β-semialdehyde. This involves a Schiff-base intermediate between Lys 161 of DHDLS and pyruvate. The enzyme structure has been described[162] and is a homotetramic enzyme where each monomer consists of two domains, an N-terminal TIM barrel and a C terminal α helical domain.

6.18 Luciferase – The luciferase from luminescent bacteria is a flavin monooxygenase that catalyses the reaction of $FMNH_2$, O_2 and a long-chain aliphatic aldehyde to yield FMN, the aliphatic carboxylic acid and blue-green light. The structure reveals that each subunit of the enzyme folds into a TIM barrel motif. The structure of the enzyme is shown in Figure 23.[163]

6.19 NADH Oxidase – This enzyme catalyses the oxidation of NADH by a two electron and two step process. It uses FAD and FMN as cofactors and a large number of electron acceptors as substrates. It shows sequence similarity to nitroreductases and NAD(P)H flavin enzymes from other bacterial sources. Each monomer of the compact dimeric structure reported by Hecht *et al.* has two domains.[164] The N-terminal domain consists of a four stranded antiparallel β-sheet with three helices on one side and two longer helices on the other. The

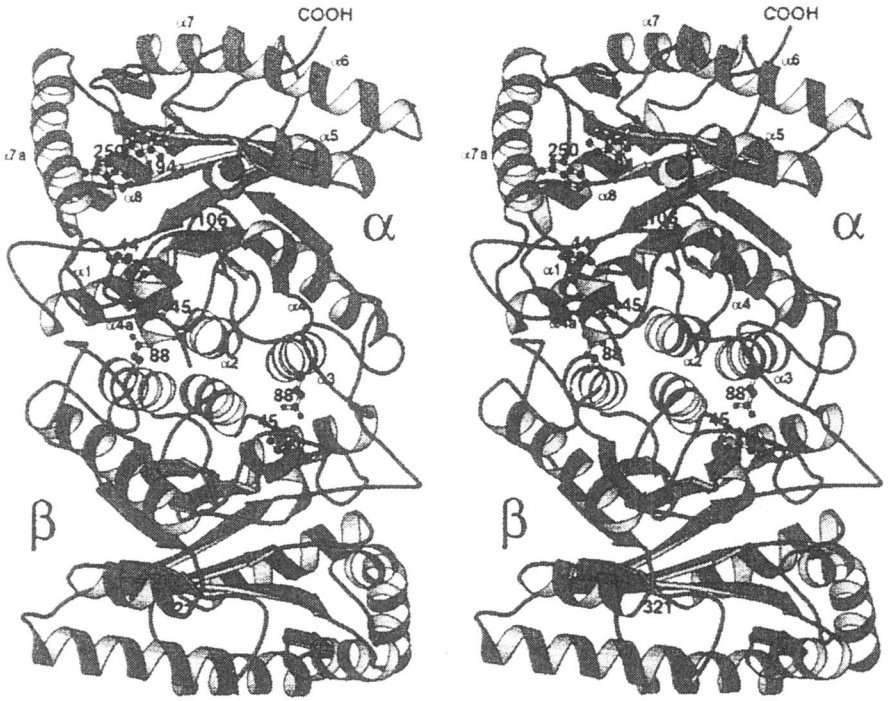


Figure 23 Stereo view of bacterial luciferase. The ordered phosphate or sulfate ion is shown in space-filling representation. Tryptophan residues which interact with the isoalloxazine ring of the flavin and Cys whose modification hinders flavin binding are shown as ball-and-stick models. Reproduced with permission from reference 163

second domain contains two helices one of which is very flexible. Details of the active site bound FMN show contacts on both faces of the flavin ring that force it into a bent conformation even in the oxidised state.

6.20 20S Proteasome – The 20S proteasome is a large protein complex which degrades misfolded and some short lived regulatory proteins without the requirement of an energy source. It is also the core of the 26S proteasome which does require ATP for degradation of ubiquitinated proteins. The complex is a cylinder of four stacked rings made up of two types of subunits α and β. Each subunit consists of a sandwich of two five stranded antiparallel β-sheets flanked by two α-helices on one side and three on the other. This has been solved by X-ray methods to 3.4Å resolution from the archaeon *Thermoplasma acidiophilum*.[165] The interior of the cylinder is accessible only through an aparature at each end which is 13Å in diameter. An inhibitor acetyl-leu-leu norleucine is shown bound to each of the 14β subunits in the interior of the cylinder.

6.21 UDP-N-Acetylglucosamine Acyltransferase – This enzyme catalyses the first step of lipid A biosynthesis in *Escherichia coli*, in which the transfer of (R)-3-hydroxymyristate from its acyl carrier protein thioester to the 3'OH position of UDP-N-acetylglucosamine is carried out. The structure is unusual in that a tandem repeat of an imperfect hexapeptide sequence motif occurs, in which an aliphatic residue (Ile, Val or Leu) occurs at every sixth position and a small residue precedes the hydrophobic residue. This motif occurs 28 times in the N-terminal sequence of this protein and gives the fold of this region of the protein the shape of an equilateral prism. The peptide forms a left handed, parallel β helix with 10 helical turns.[166] The C-terminal domain of the protein forms four α helices and the location of the active site and the substrate binding sites are not known.

7 Membrane Proteins

Many proteins are embedded in lipid membrane of bacterial and animal cells. These have been difficult to study at a structure level but recently several important membrane proteins have been crystallised and their structures elucidated.

7.1 Cytochrome c Oxidase – This protein is the key component of the respiratory chain of most aerobic organisms. The structure of this important protein has been determined to 2.8Å resolution from *Paracoccus denitrificans.* The structure has been solved of the four protein subunits of this enzyme complexed with an antibody F_v fragment.[167] The subunit 1 of this structure shows 12 closely packed helices which adopt a simple anticlockwise sequential arrangement such that they generate three pores. Two of these pores are blocked by heme and the third by aromatic amino acid residues. This subunit is the oxidase complex and is responsible for the oxidation of molecular oxygen to water and the redox-coupled pumping of protons. The second subunit has only two transmembrane helices which bind to subunit 1 and also a polar domain consisting of a ten-stranded β-barrel. This binds the binuclear Cu_A centre. Subunits 3 and 4 can be removed from the core particle by detergents. The transmembrane helices of subunit 3 are arranged in an irregular way and bind at least one lipid molecule. This lipid is thought to help rapid diffusion of oxygen to the heme a_3—Cu_B centre. The subunit 4 whose function is unknown has one transmembrane helix with a small N-terminal extension to the cytoplasmic side.

The structure of the metal sites oxidised in mitochondrial bovine heart cytochrome c oxidase has also been described at similar resolution.[168]

7.2 Quinol Oxidase – The crystal structure of the soluble membrane exposed domain of the wild type subunit II from *Eschericia coli* quinol oxidase and from a mutant with an engineered dinuclear copper centre has been reported.[169] The quinol oxidases show similarities to cytochrome c oxidase but are thought to have lost the Cu_A binding domain during evolution. This has been restored by

protein engineering and the crystal of the mutant enzyme solved to 2-3Å resolution. This demonstrates how protein engineering can be used to build novel multidomain proteins.

Structure of Cu_B in the binuclear heme-copper centre of cytochrome aa_3-type quinol oxidase from *Bacillus subtilis* has been studied by EXAFS and ENDOR spectroscopy.[170] Evidence is presented that the Cu_B site has a low symmetry, tetragonal coordination with two or three histidine nitrogens and one oxygen as ligands. The oxygenous copper ligand seems to possess an exchangeable proton.

7.3 Light Harvesting Complexes

7.3.1 Purple bacteria – The high resolution crystal structure of the purple bacterial light harvesting complex from *Rhodopseudomonas acidophila* has recently been reported.[171] This structure has provided the molecular basis for understanding the mechanism and kinetics of energy transfer. The structure reveals 9 αβ apoprotein dimers packed together to form a double ring of protein around a hollow cylinder with an internal radius of 18Å and an external radius of 34Å as shown in Figure 24. The pigments are held within the proteins with three bacteriochlorophyll molecules (BChl) and two carotenoid molecules per αβ pair. The BChl molecules form two sets with one set of 18 sandwiched between the α and β rings which absorb at 850 nm. Their bacteriochlorin rings lie perpendicular to the plane of the membrane, parallel to the transmembrane α helices. The remaining 9 BChl molecules which absorb at 800 nm lie between the outer β-polypeptides. The carotenoid pigments span the entire complex and make close contact with the rings of both sets of BChls.

A lower resolution 8.5Å projection map of the light harvesting complex 1 from *Rhodospirillum rubrum* reveals a ring composed of 16 subunits.[172] Two reviews published in 1995 have discussed the energy transfer reactions that occur between bacteriochlorophyll and bacteriochlorophyll[173] and between carotenoid and bacteriochlorophyll.[174]

7.3.2 Plant photosynthetic system PSI – This protein complex catalyses the light induced transfer of electrons from plastocyanine or cytochrome c_6 located on the inside of the thylakoids to ferredoxin on the cytoplasmic membrane side. It is composed of 11 (in cyanobacteria) or 13 (in plants) individual proteins. In addition it contains approximately 100 chlorophyll molecules, 20 carotenoids, three [4Fe4S] clusters and two phylloquinones. The present state of crystal structure analysis of this complex was reported by Schubert *et al.*[175]

The structure of cytochrome c_6 has been reported[176] from the green algae *Monoraphidium braunii* at 1.2Å resolution. On the basis of this structure potential electron transfer pathways have been calculated.

7.4 Porins – Porins are membrane channel proteins and their structures have been previously reported. The structure of the first crystal form of an integral membrane porin OmpF was carried out by Cowan *et al.*[177] These proteins are unusual among membrane proteins because they are more polar than water-

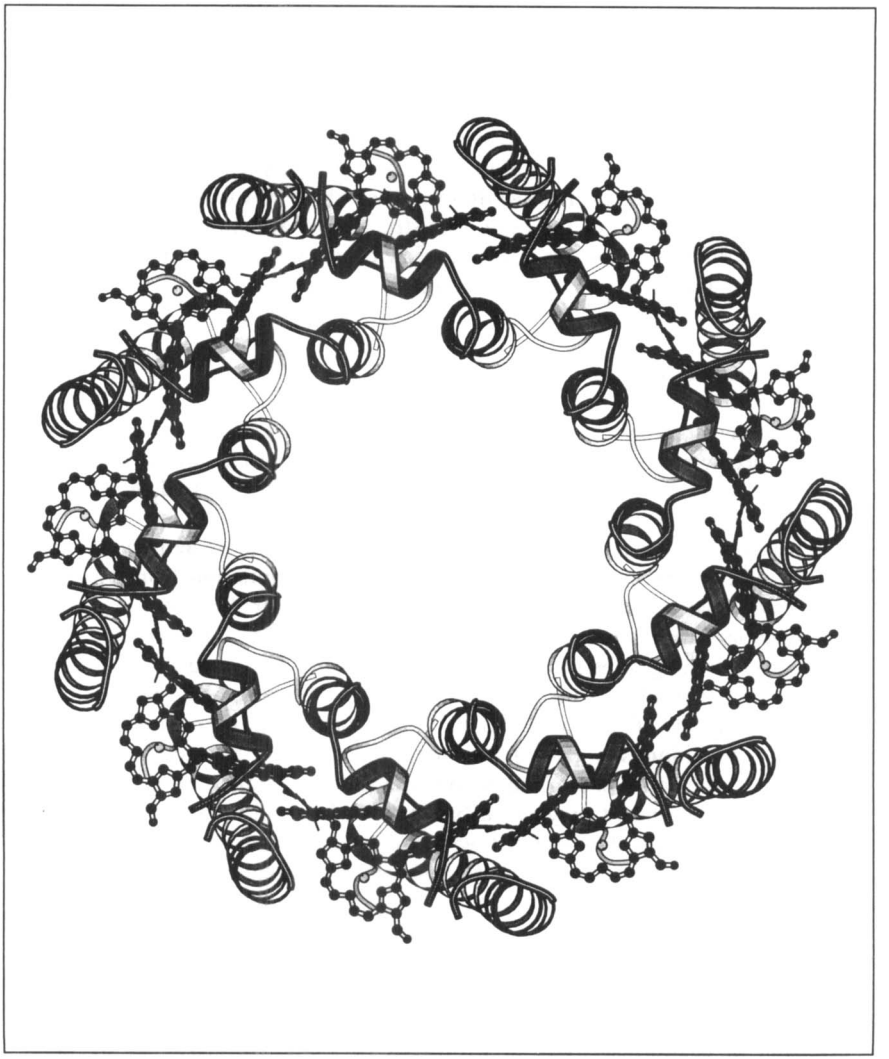


Figure 24 Structure of the integral membrane light-harvesting complex from photosynthetic bacteria. Reproduced with permission from reference 171.

soluble proteins and because they contain β structure and not α helices as found in most other membrane proteins. The forces and factors that contribute to structural stability of membrane proteins have been discussed by Haltia and Freire[178] and modelling of β-barrel pore structures has been carried out by Sansom and Kerr.[179] The basic structure of the porin protein is a homotrimer with each subunit having an antiparallel β barrel in which all strands are H-bonded to their next neighbours along the chain. The polypeptide lining the inner barrel wall restricts the channel width over a length of 10Å to a size that defines the diffusion properties of the pore. The structure of a maltoporin channel has been described[180] which shows a very narrow channel which is just wide enough for a malto-oligosaccharide to pass through.

8 Protein Engineering

Knowing the structure of many proteins has led to a multitude of papers which change specific residues of a protein to understand the mechanism and catalytic properties of an enzyme. The majority of these papers are beyond the scope of this review but a few specific examples are mentioned below.

Papain has been used as a protein in which nitrile hydratase activity has been introduced into the structure by introducing a single mutation from Gln19 to Glu.[181] Modular polyketide synthetases have been engineered in order to generate macrolactones with shorter and longer chain lengths.[182,183] The specificity for histidine-containing substrates has been engineered into trypsin by creating binding sites that allow a metal ion to bridge the substrate and enzyme on the leaving group side of the scissile bond.[184] The conversion of antagonist binding site to a metal-ion site (Zn^{2+}) has been reported in the tachykinin NK-1 receptor.[185] The active site of magnesium-dependent ribonuclease H to form an active metal independent enzyme has been reported by replacing an aspartate and a glutamate residue that interact with the metal ion with arginine.[186] The adenosine binding pocket of the *Escherichia coli* isocitrate dehydrogenase enzyme has been specifically mutated to shift the coenzyme preference of the enzyme from NADP towards NAD.[187] A knowledge of the structure of subtilisin and sequences of eukaryotic homologs known to cleave dibasic substrates has been used in redesigning subtilisin BPN'.[188] The substrate specificity of *Escherichia coli* aspartate aminotransferase has been altered to tyrosine aminotransferase.[189] The inversion of enantioselectivity in hydrolysis of 1,4-dihydropyridines by point mutation of lipase PS has been reported by Hirose *et al.*[190] Thrombin has been converted into an anticoagulant by protein engineering.[191] A mutant of T7 RNA polymerase acts as a DNA polymerase.[192] Relaxing the substrate specificity of an aminoacyl tRNA synthetase allows the synthesis of proteins with unnatural amino acids. This has been used to incorporate p-chloro-phenylalanine into luciferase.[193] Mutagenesis studies with L-lactate dehydrogenase has resulted in improved specificity for substrates with positively charged side chains[194] and a partial reversal of substrate stereospecificity.[195]

Mutagenesis has also been carried out by forced evolution of glutathione-S-

transferase to create a more efficient drug detoxicating enzyme.[196] Glutathione transferases with novel active sites have also been described.[197] A green fluorescent protein has been studied and the area around the tyrosine chromophore region subjected to mutagenesis.[198–201] The use of enzymes in chemical synthesis often depends on their stability and activity in non-aqueous solvents. You and Arnold have directed evolution of subtilisin E in *Bacillus subtilis* to be active in aqueous dimethylformamide.[202]

Those reports listed above provide only a brief outline of the large amount of work being carried out to redesign proteins by a directed mutagenesis approach or by more random approach.

9 Summary

In this review I have tried to summarise some of the wealth of literature which has appeared on proteins in 1995. The research in this area appears to grow at an ever increasing rate and clearly only certain topics and proteins have been discussed.

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