SPECIALIST PERIODICAL REPORTS

# Amino Acids, Peptides, and Proteins

## VOLUME 26

THE ROYAL SOCIETY OF CHEMISTRY

A Specialist Periodical Report

# Amino Acids, Peptides, and Proteins

## Volume 26

A Review of the Literature Published during 1993


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# *Preface*

In this, the 26th volume in the series, the task of compiling the chapters has, in the main, been in the hands of authors already deeply rooted in the annual gathering of references. The swathe of material harvested is now vast and most reporters once again report expansion in the publications within their particular expertise. The first five chapters represent the current continuum from volume to volume. It is the turn of the β-lactams to be included for biennial coverage as the 6th Chapter and we are greatly indebted to Drs Schofield and Westwood for reviewing the literature for 1992 and '93. It is very revealing how the β-lactam ring has extended its boundaries beyond its 'enchanted ring' status in the antibiotic field, into the domain of enzyme inhibition and as a synthetic intermediate. Hence a change of chapter name to β-Lactam Chemistry is justified to reflect the wider scope.

While the use of sophisticated technology in structural elucidation is becoming routine in many publications, there seems to be continuing expansion, development and optimisation of solid phase peptide synthesis. The current scene is so different from the embryonic years so modestly described by Bruce Merrifield in his book 'Life During a Golden Age of Peptide Chemistry' (published by the American Chemical Society, 1993). Multiple syntheses, and the construction of large peptide libraries, have capitalised on the continuing development of the solid phase approach, but shifting the emphasis from the chemistry into bio-assaying with minute amounts of peptidic mixtures.

A continuously expanding field also demands an international forum for communication. We have recently seen the launching of new Journals in the area of Protein and Peptide Science, e.g. Journal of Peptide Science (J. Wiley & Sons), Letters in Peptide Science (ESCOM), and Protein and Peptide Letters (Bentham Scientific Publications). Based on the 1993 list of publications, Bioorganic & Medicinal Chemistry Letters (Pergamon) has established itself already as a popular forum for papers discussing pharmaceutically relevant peptides. These augment the previously established Journals, and the valuable books produced from the International symposia of the various Peptide Societies. One hopes that the confidence in the expansion of peptide science can be sustained, at a time when our team of Reporters all report frustrations in the availability of original source references due to library cut-backs. Hopefully an equilibrium will be achieved sometime in the future.

Sadly, one of this year's authors, Christine Bladon, has decided to make this her last report for the time being. Our thanks go to Christine for re-formatting the chapter on peptide analogues, which will no doubt remain an attractive template for next year's reporters to work on. To all my fellow Reporters and Janet Freshwater of the RSC, I offer sincere thanks for their unstinting efforts once again to get this volume into print.

John S. Davies
University of Wales, Swansea

# Contents

# *Abbreviations*

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

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

This year 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-[(cysteinylglycin)-*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 to include new abbreviations in their texts. However with the ever increasing diversification in structures, the author of Chapter 3 (Christine Bladon) compiled her own list, which has been included here, since it has relevance to other chapters as well.

**List of Abbreviations**

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

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

| | |
|---|---|
| Cpp | 1-mercaptocyclohexaneacetic acid, *or* β-mercapto-β, β-cyclopentamethylene propionic acid, *or* Pmp (*below*) |
| *c*PzACAla | *cis*-3-(4-pyrazinylcarbonylaminocyclohexyl)alanine |
| Dab | 2,4-diaminobutyric acid |
| Dap | 2,3-diaminopropionic acid |
| Dbf | 3-(2-dibenzofuranyl)alanine |
| Dip | 3,3-diphenylalanine |
| Dph | α-α-diphenylglycine |
| Dpr | 2,3-diaminopropionic acid |
| Gly(Ph) | phenylglycine |
| Har | homoarginine |
| Hib | α-hydroxyisobutyric acid |
| Hyp | *trans*-4-hydroxyproline |
| Iva | isovaline |
| Mpt | *trans*-4-mercaptoproline |
| 1-Nal | 3-(1-naphthyl)alanine |
| 2-Nal | 3-(2-naphthyl)alanine |
| Nap | β-(1′-naphthyl)alanine |
| Oic | octahydroindolecarboxylic acid |
| Opt | *O*-phenyltyrosine |
| 3-Pal | 3-(pyridyl)alanine |
| Pen | penicillamine |
| Phg | phenylglycine |
| Pip | pipecolic acid |
| Pmp | β,β-pentamethylene-β-mercaptopropionic acid, *or* Cpp (*above*) |
| Qal | 3-(3-quinolyl)alanine |
| Qua | quinoline-2-carboxamide |
| Sar | sarcosine |
| Thi | β-thienylalanine |
| Tic | 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid |

# 1
# Amino Acids

By GRAHAM C.BARRETT

## 1 Introduction

The 1993 literature covering the chemistry and biochemistry of the amino acids, is dealt with in this Chapter. The approach taken in all previous Volumes of this Specialist Periodical Report continues to be relevant, and therefore the coverage in this Chapter concentrates on the literature covering the natural occurrence, chemistry, and analysis methodology for amino acids. Routine literature covering the natural distribution of well-known amino acids is excluded.

Patent literature deals with material that also finds its way into the conventional literature, and is therefore almost wholly excluded from this Chapter. It is easily reached through the appropriate sections of *Chemical Abstracts* (Section 34 in particular).

The flow of Journal papers and secondary literature continues to accelerate, as far as the amino acids are concerned. The coverage in this Chapter is arranged into sections as used in all previous Volumes of this Specialist Periodical Report, and major Journals and *Chemical Abstracts* [to Volume 120 (1994), issue 11] have been scanned to provide the material surveyed here. Where it is helpful to refer to earlier Volumes of this Specialist Periodical Report, the formula "(Vol. XX, p. YY)" is used.

For most of the papers cited, description is brief so that adequate commentary can be offered for particular papers describing significant advances in synthetic and analytical methodology, with mechanistically-interesting chemistry being given prominence.

## 2 Textbooks and Reviews

IUPAC/IUB Nomenclature Recommendations ("Nomenclature and Symbolism for Amino Acids and Peptides, 1983"; see Vol.16 of this Specialist Periodical Report, p.387) have recently been seen to contain three errors (one, in the systematic name for leucotriene D; another, the omission of indication of cyclization through side-chains in the peptide Ala-Thr-Gly-Asp-Gly; and the third, a typographical error),[1] and textbook representations of more subtle stereochemical details of protein amino acids are almost always erroneous.[2] Broad coverage of the recent literature on the chemistry of the amino acids has appeared in a classic organic chemistry series.[3]

Reviews have appeared covering synthetic applications of L- or D-amino

acid esters as chiral auxiliaries,[4] properties and synthesis of 1-aminocyclopropanecarboxylic acids,[5] uses of α-amino-β-hydroxy acids in the total synthesis of aminosugars,[6] synthesis of non-natural amino acids,[7] uses of pyroglutamic acid in the synthesis of near relatives,[8] the reaction of aldehydes with tryptophan giving toxic derivatives (causing eosinophilia-myalgia syndrome; see Vol. 24, p. 58),[9] and the role of β-methylamino-L-alanine in neurodegenerative disorders.[10] The effects of thiol-containing amino acids and peptides in interacting with food toxicants, has been reviewed.[11] Many relevant reviews have appeared in a Conference Volume, including the origin of life and the role of amino acids,[12] recent advances in the biochemistry of amino acids,[13] post-translationally-modified amino acids as constituents of proteins,[14] aldosine (a new crosslink in collagen and in elastin) and oxodesmosine (a new crosslink present in elastin, derived from deaminated lysine residues of tropelastin)[15] and the presence of o-bromophenylalanine in sea urchin eggs, in free form and as its m- and p-isomers in peptides, and bromohistidine in the same source.[16]

## 3 Naturally Occurring Amino Acids

### 3.1 Isolation of Amino Acids from Natural Sources

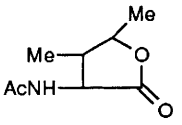
All fermentative processes for the production of amino acids involve routine isolation of the product; or the separation of mixtures of amino acids, as the concluding stage, but this section does not cover this topic, it is intended to deal with rather more subtle aspects, particularly those unexpected outcomes of otherwise straightforward techniques.

Protein hydrolysates may deliver partly-racemized components, which can be eliminated ($< 0.002\%$) through partial chemical hydrolysis (6M HCl/15 min/80-90°C) followed by enzyme-catalysed lysis, first by pronase at 50°C during 12-16h, then leucine aminopeptidase and peptidyl D-amino acid hydrolase during 24h.[17] Microwave heating completes 6M HCl hydrolysis of proteins within 10-20 min as the first stage in an automated protein analysis system.[18] Cystine-containing proteins yield cysteine when subjected to reductive hydrolysis (6M HCl/110°C/0.1% phenol/5% thioglycollic acid/18h), and special attention has been given to ion-exchange chromatographic separation of cysteine from proline in such hydrolysates.[19]

Isolation in the form of its lactam, of γ-(N-propylamino)but-3-enoic acid, employs matrix–solid phase dispersion.[20] Advantages of displacement ion-exchange chromatography of amino acid mixtures have been reviewed, and persuasively illustrated for the preparative-scale separation of valine from isoleucine by displacement with aqueous ammonia from strong acid cation exchangers.[21] On a production scale, a multi-stage fluidized ion exchange bed has been described for amino acid separation.[22]

### 3.2 Occurrence of Known Amino Acids

This Section is restricted to unusual and/or significant results, to the exclusion of the vast continuing literature covering the familiar amino acids.



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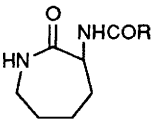
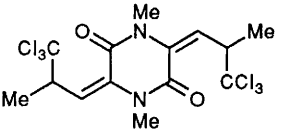


(2)

Caprolactin A:
R = $-(CH_2)_5CHMe_2$
Caprolactin B:
R = $-(CH_2)_4CHMeEt$

(3; R = $-CHCl_2$ or $-CCl_3$)



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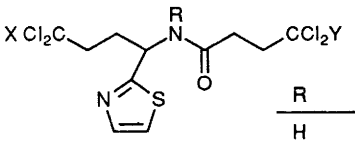
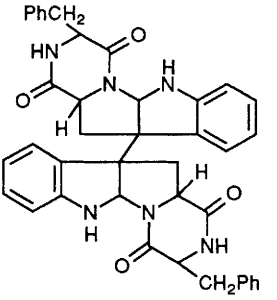


(5)

| R | X | Y |
|---|---|---|
| H | Cl | Cl |
| Me | Cl | Cl |
| Me | Cl | H |
| H | Cl | H |
| Me | H | H |



(6)

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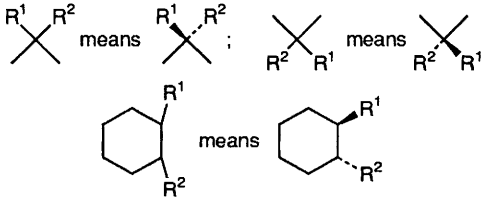
Three-dimensional features at chiral centres of structures depicted in this chapter follow the convention:–

(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:

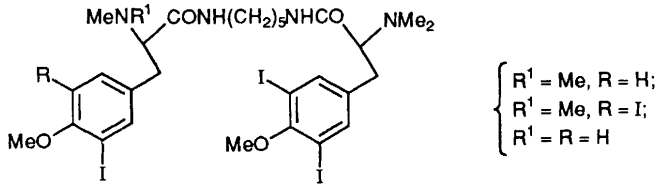


The family of aplyorines, potent anti-tumour compounds from the sea hare *Aplysia kurodai*, carry esterified NN-dimethylserine O-methyl ether and NN-dimethylglycine within their structures.[23] The aerial parts of *Desmodium styracifolium* contain desmodilactone (1).[24] Arthonin, a lichen metabolite of *Arthonia endlicheri*, has been formulated as the ester of N-benzoyl leucinol with N-benzoyl-L-isoleucine, while isoarthonin is the corresponding amide.[25] Further newly-located, though known, compounds have been established in that far more familiar plant source, garlic, now seen to contain the glycoside (-)-N-(1'-deoxy-1'-β-D-fructofuranosyl)-S-allyl-L-cysteine sulfoxide as well as (+)-S-allyl-, (+)-S-methyl-, and (+)-S-(trans-1-propenyl)-L-cysteine sulfoxide.[26] It is comforting to those who enjoy this food accessory, and seek medical rather than aesthetic reasons to justify its inclusion in their diet, that the glycoside showed some inhibition of platelet aggregation *in vitro*. 4-Chloro-L-tryptophan has been located[27] in immature seeds of *Pisum sativum*, and accompanied by its N-malonyl derivative (formerly assigned the D-configuration). Other stereochemical re-assignments concern the polyoxin constituent polyoximic acid (whose side-chain has the cis-configuration rather than trans),[151] and anticapsin (whose C-4 configuration is S).[28]

Protein constituents arising through post-translational modifications have been surveyed (see also reviews cited in Section 2).[29] These include glycosylated, phosphorylated, and sulfated derivatives of well-known protein amino acids, and desmosine, allo-desmosine, hydroxylysylpyridinoline, 3-hydroxypyridinium compounds, cyclopentenosine, and other modified lysines, dityrosine, and the novel tyrosine-derived pulcherosine. o-Tyrosine and the aromatic ether, dityrosine, arise in proteins during radiolysis and through $H_2O_2/Cu^{++}$ oxidation,[30] and evidently survive long storage, since dityrosine has been identified in the collagen content of the Dead Sea Scrolls.[31] Lysinoalanine formation has been reviewed.[32]

### 3.3 New Naturally Occurring Amino Acids

Previously unknown close relatives of the familiar α-amino acids include the antifungal antibiotic β-cyano-glutamic acid, from *Streptomyces* sp. K749-42, particularly effective against *Candida albicans*,[33] and $N^2$-(2-carboxyethyl)-arginine and $N^2$-(2-carboxyethyl)-3-hydroxyarginine, produced by a blocked mutant of *Streptomyces clavuligerus* dclH65.[34] The novel arginines are possibly intermediates in the biosynthesis of clavulanic acid. Caprolactins A and B (2) are new caprolactams from an unidentified gram-positive bacterium, showing anti-viral and cytotoxic properties.[35] Another common type of cyclized aliphatic amino acid is the di-oxopiperazine family, represented in dysamides A-C (3) and corresponding dehydro-amino acid analogue (4) from the marine sponge *Dysidea fragilis*,[36] and in corresponding compounds from *Tolypocladium* sp., in which α-(methylthio)glycine and O-(3-methylbut-2-enyl)-α-(methylthio)-D-tyrosine are condensed together,[37] and dysideathiazole (5), in which the α-carboxy group has been modified to the thiazole moiety, from *Dysidea herbacea*.[38] The previously-known $N^1$-methyl albonoursin, a weakly antibiotic factor from a *Streptomyces* sp. from perennial rye grass,[39] and the $C_1$-symmetric WIN 64821

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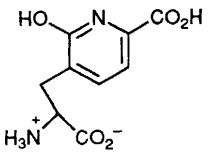


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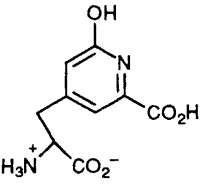


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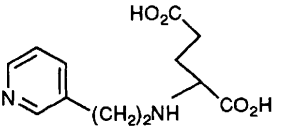


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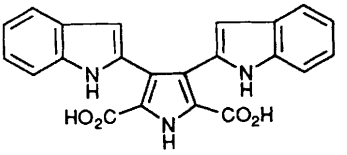


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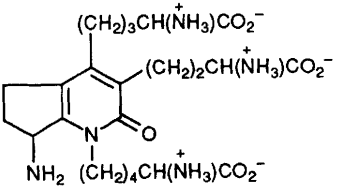


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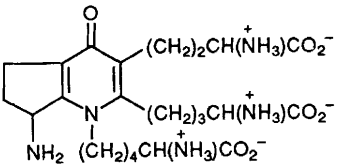


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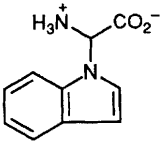


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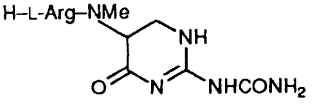


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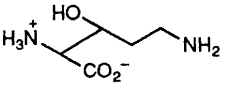



(16)

(6), a new competitive antagonist for Substance P, from *Aspergillus*,[40] have been reported.

Other new aromatic and heteroaromatic α-amino acids (unusually abundant in this year's literature), are the tyrosine derivatives (7) from *Aplidium* sp. (colonial ascidians),[41] and pyridyl-L-alanines (8, 9) and -L-glutamic acid (10) from *Clitocybe acromelalga*, whose existence is consistent with the proposed biogenesis of acromelic acids.[42] threo-β-Hydroxy-L-histidine has appeared as a component of a new pyoverdine-type siderophore (Vol.24, p.5) from the culture filtrate of *Pseudomonas fluorescens* 244, functioning as a bidentate ligand for ferric ions.[43] Chromopyrrolic acid (11) from a *Chromobacterium violaceum* mutant, is a new tryptophan metabolite.[44]

### 3.4 New Amino Acids from Hydrolysates

This section encompasses natural products from which new amino acids can be released by hydrolysis or similarly simple chemistry.

Two new crosslinking α-amino acids, oxodesmosine (12) and iso-oxodesmosine (13), from bovine aorta elastin, contain the oxopyridine moiety but are otherwise closely similar to the well-known desmosines from the same source.[45] These are probably metabolic intermediates *en route* to the major pyridinium crosslinks of elastin.

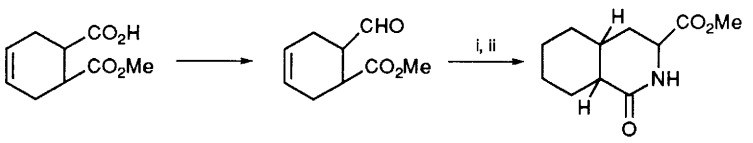
Novel amino acid residues with nitrogen functional groups in side-chains have been reported; the novel α-aminoglycine derivative (14) in lyciumins A-D, cyclic peptides from *Lycium chinense Mill.* (Solonaceae)[46] and the unusual component of the dipeptide antibiotic TAN-1057A (15) isolated from *Flexibacter* sp. PK-74.[47] The oxidative ozonolysis product of cylindramide, a novel cytotoxic tetramic acid lactam from the marine sponge *Halichondria cylindrata*, has been shown to include (2S,3S)-erythro-β-hydroxy-L-ornithine (16).[48]

New β-amino acids have been reported, (2S,3R)-2-methyl-3-aminopentanoic acid as a component of the cyclic depsipeptide metabolite majusculamide C from the alga *Lyngba majuscula*,[49] and a complex β-tyrosine constituent of Antibiotic C-1027.[50] Dolastatin D, a new depsipeptide from *Dolabella auricularia*, contains (2R,3R)-3-amino-2-methylbutanoic acid, not previously found in Nature.[51]

## 4 Chemical Synthesis and Resolution of Amino Acids

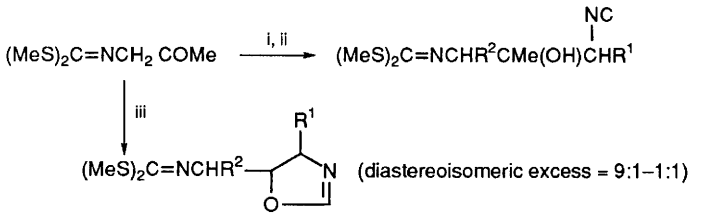
### 4.1 General Methods for the Synthesis of α-Amino Acids

The term "general methods" has been attached to a group of reactions that have become familiar through use for many years; these are covered in this Section. Relatively few novel ideas have been introduced under this heading in recent years, and those that have, have been concerned with the burgeoning area of "Asymmetric Synthesis". Although given a Section of their own, asymmetric synthesis methods are nearly always "general methods of synthesis" too, and so are reactions by which one amino acid is used as starting material for the



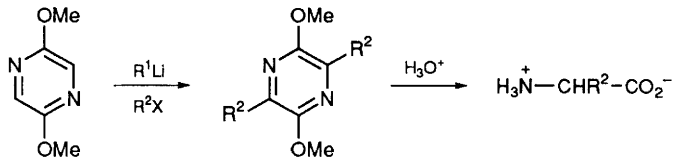

Reagents: i, PhCH=NCH=C(OMe)OLi; ii, $H_2$/Pd

**Scheme 1**




Reagents: i, $R^1CH_2NC$, $KOBu^t$/THF; ii, MeOH; iii, $CNCH_2CO_2Et$

**Scheme 2**

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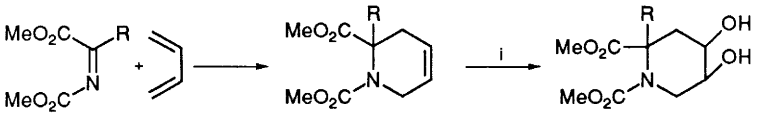



Reagent: i, $OsO_4$

**Scheme 3**

synthesis of another (these reactions are mostly covered in the later Section 6.3: Specific Reactions of Amino Acids).

The acetamidomalonate synthesis [$AcNHCH(CO_2Et)_2$ + RX → $AcNHCR(CO_2Et)_2$ → $H_3N^+CHRCO_2^-$] remains the most popular of the glycine alkylation methods, described in the recent literature. Examples of novel variants include new synthesis of 5-bromotryptophan (from 5-bromo-3-methylindole, after $N^{im}$-benzenesulfonylation and conversion into the bromide with NBS),[52] similar preparation of 3-(carbazol-2- or -3-yl)-DL-alanines from 2- or 3-methylcarbazoles,[53] and Mn(III) acetate-induced addition of conjugated alkenes.[54] The recent literature has many illustrations of forays by Chinese workers into phase-transfer-catalysed examples of this process.[55] N-Boc-L-(2-Bromoallyl)glycine has been prepared by the acetamidomalonate method.[56] The related formamidomalonate route has led to a carboranyl-substituted phenylalanine through alkylation by a 1,2-dicarba-*closo*dodecaborane-substituted benzyl bromide.[57] Ref.162 describes a similar use of isocyano-acetates, and uses for α-bromoglycine are continuing to be favoured (e.g. Ref.189).

Preparation of α-acylamino-β-oxocarboxylic acid esters can be achieved through the above route [$AcNHCH(CO_2Et)_2$ + RCOX + 2 BuLi][58] and also through acylation of glycine Schiff bases [$PhCH=NCH_2CO_2Me$ + RCOX + $KOBu^t$] (see also Refs.169, 208).[59] This is the basis of several related syntheses, both of a simple nature (alkylation by an alkyl halide is complete in 1 minute by a microwave-mediated solid-liquid phase-transfer-catalysed system without solvent),[60] and for more complex targets (Scheme 1).[61] Di-alkylation of the Schiff bases is easier than has been supposed under phase-transfer catalysis.[62] The analogous imines $(MeS)_2C=NCH_2CO_2Me$ undergo alkylation by isonitriles (Scheme 2) with an unusual outcome.[63] Hidden versions of the same procedure include alkylation of 2,5-dimethoxypiperazines (17) derived from glycine methyl ester.[64] The last-mentioned route is illustrated through a "difficult" synthesis, of t-leucine ($H_3N^+CHBu^tCO_2^-$).

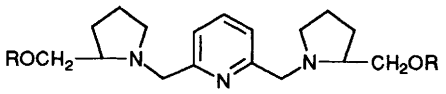
The other version of imine alkylation that can be envisaged for α-amino acid synthesis [alkylation of $R^1N=CHCO_2R^2$ + RZnBr → $R^1N=CRCO_2R^2$ ($R^1$ = $Me_3SiOCH_2CHEt$-)[65]] has also been put to use. An aza-Diels-Alder process using an aldimine (Scheme 3) starts a route to (±)-baikiain.[66] The applications of the nucleophilic alkylation of imines for the synthesis of uncommon amino acids has been reviewed.[67]

Hippuric acid alkylation, *via* its cyclized form [2-phenyloxazol-5(4H)-one], using 4-formyl(2,2'-bipyridine)[68] or 3,4-dimethoxybenzaldehyde,[69] provides 2-amino-3-(2,2'-bipyridin-4-yl)propanoic acid and DOPA, respectively.

The hydantoin synthesis (see also Refs. 168, 233) is particularly suited to the preparation of -α-disubstituted α-amino acids, illustrated for the preparation of geometrical isomers (the trans-isomer receives its first synthesis) of 1-amino-1,2-cyclopentanedicarboxylic acid fortuitously facilitated by epimerization during the hydrolysis of the Bucherer-Bergs reaction product.[70]

The Strecker synthesis is represented in later Sections (Refs. 78, 161, 270).

Amination procedures continue to come into their own, illustrated by azidolysis of methanesulfonyloxy-amides,[71] and of 1-alkenylcyclopropyl toluene-



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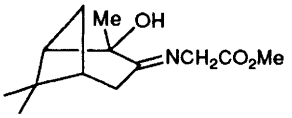

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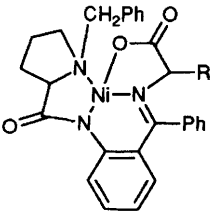

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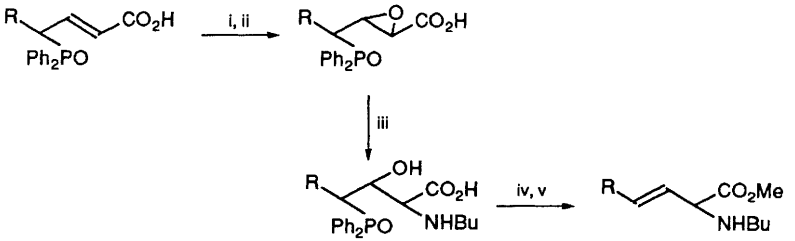
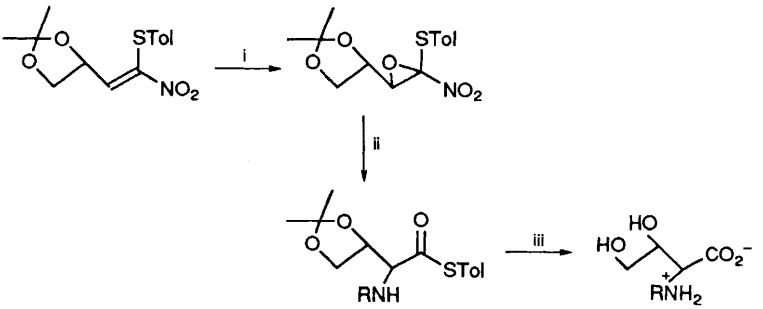


Reagents: i, $Bu^tOOH/Ti(OPr^j)_4$; ii, $RuO_4/NaIO_4$; iii, $BuNH_2$; iv, KOH; v, $CH_2N_2$

**Scheme 4**



Reagents: i, $Li^+ {}^-OOBu^t$ ⟶ *syn* ; KH-$Bu^tOOH$ ⟶ *anti* (preferentially);
ii, $RNH_2$; iii, $H_3O^+$

**Scheme 5**

p-sulfonates [Pd(0)-catalysed, leading to "2,3-methano-amino acids", *alias* 1-aminocyclopropane carboxylic acids; see later Section 4.5][72] and reductive amination of glyceric acid (Ru-Pd/C) to give serine.[73] An extraordinary amination procedure using a molybdenum nitride complex, trans-[MoCl(N)$(Ph_2PCH_2CH_2PPh_2)_2$], has been used to prepare correspondingly-complexed glycine and alanine ester ylides by reaction with α-iodoalkanoates, the amino acid ester being released by electrochemical Mo-N cleavage.[74] Pd(0)-Catalysed amination of allyl acetates [RCH=CHCH(OAc)R' → RCH=CHCH$(NR_2)$R' → $MeO_2CCH(NR_2)R$] followed by ozonolysis at -78°C in MeOH gives methyl esters of α-amino acids.[74a] Many more examples of amination, and of other general methods of amino acid synthesis (e.g. the Gabriel synthesis[97, 272]), are located in the following section.

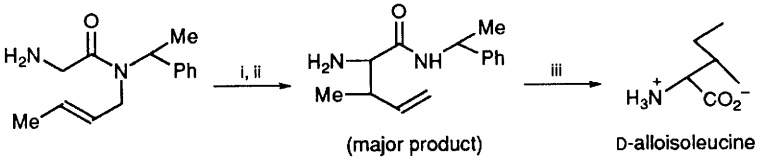
### 4.2 Asymmetric Synthesis of α-Amino Acids

Activity in this area continues to increase, both in the provision of new methodology and in the development of established methods, including well-known standard general methods of synthesis, some of which are described in the preceding section, and re-presented here in "asymmetric versions".

In the last-mentioned category, amination reactions in the presence of homochiral species are represented in a synthesis of L-phenylalanine from phenylpyruvic acid and a mixed ligand copper(II) Schiff base complex formed between pyridoxamine and (18),[75] and in a simple, intriguing synthesis (RCHO + $CHCl_3$ + aqueous $NH_3$ in the presence of β-cyclodextrin)[76] with enantiomeric excesses at a disappointing level (2.6% for L-phenylglycine and 28.2% in favour of D-phenylalanine) and unpredictable direction of the stereochemical bias.

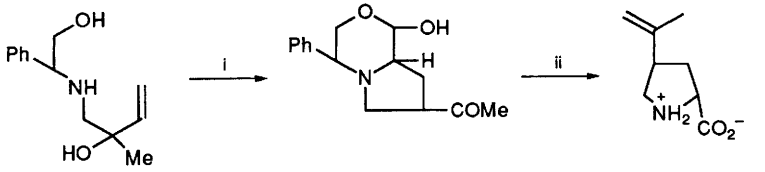
The conventional aldol synthesis of β-hydroxyalkyl-α-amino acids is given an asymmetric bias in an example (PhCHO + glycine → β-phenylserine) conducted in the presence of chiral supramolecular assemblies $[Me(CH_2)_{15}]_2NCO$-Ala-$NHCO(CH_2)_5N^+Me_3$ $X^-$/N,N-bis(hexadecyl)pyridoxal/$Zn^{2+}$].[77]

A one-pot asymmetric Strecker synthesis uses a chiral primary amine [RCHMe$NH_2$ (R = Ph or 2-naphthyl) or 1-amino tetra-O-pivaloyl-D-galactose] as aminating agent with 2,2-dimethylcyclopropane hemiacetal as masked aldehyde, in a synthesis of 2,3-methanovalines in high enantiomeric excess,[78a] and a very similar principle underlies the use of a homochiral α-aminonitrile formed by using a monoterpene ketone as a relay in an otherwise conventional Strecker synthesis of D-α-amino acids from aldehydes.[78b] Other amination reactions of homochiral species leading to homochiral α-amino acids, in which the chirality of the substrate is "transferred" to the α-carbon atom, are increasingly attracting new adherents, for example the route (Scheme 4) starting with an allyl alcohol.[79] Other examples of the genre include nucleophilic epoxidation and aminolysis (Scheme 5)[80] involving treatment of the homochiral epoxide from an allyl alcohol with $BocNH_2$ and $RuCl_3/NaIO_4$ oxidation of the resulting glycol to give the Boc-amino acid.[81] Aza-Claisen rearrangements (Scheme 6) exemplified in a synthesis of D-alloisoleucine, show excellent syn:anti (98:2) and facial (89:11) selectivities,[82] and an aza-Cope rearrangement combined with Mannich cyclization (Scheme



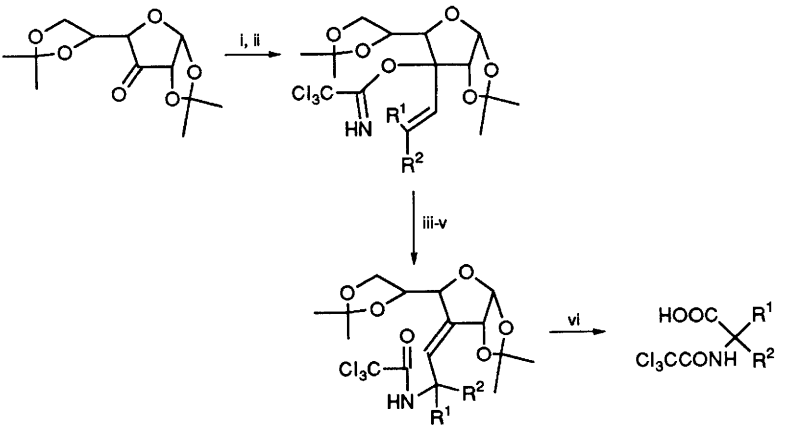

Reagents: i, LHDMS/–78 °C, ii, Δ; iii, $H_2$/Pd-C, then $H_3O^+$

**Scheme 6**




Reagents: i; OHCCHO; ii, $H_2$/Pd-C

**Scheme 7**




Reagents: i, $HC{\equiv}C^-M^+$; ii, TMSCl, then MeI/BuLi; desilylate; iii, $LiAlH_4$; iv, $H_2O$; v, Overman protocol; vi, Sharpless oxidation

**Scheme 8**

7),[83] yield 3-substituted prolines. The Overman rearrangement has been used (Scheme 8) for asymmetric synthesis of D- and L-alanine and chirally deuteriated glycine,[84] and for D-valine and for a more ambitious purpose in a synthesis of thymin polyoxin C.[85]

The nitrone from D-glyceraldehyde [CHO → $C{=}N^{+}(O^{-})CH_2Ph$], protected as the isopropylidene derivative, reacts with a 2-metallated thiazole to give the corresponding α-(N-benzyl-N-hydroxyaminoalkyl)thiazole. This is the basis of an interesting α-amino acid synthesis, since the thiazole grouping is readily degraded to the required carboxy group; 4-O-benzyl-2,3-isopropylidene-L-threose used in this way leads through routine subsequent steps to 5-O-carbamoylpolyoxamic acid [$H_2NCO_2CH_2CH(OH)CH(OH)CH(NH_2)CO_2H$].[86] It is possible to start with a racemic α-bromoalkanoic acid, aminolysis of the derived (R)-pantolactone esters giving homochiral α-amino acid esters; the reaction appears to incorporate a kinetic resolution so leading to efficient delivery of one enantiomer, though this may need verification.[87] An "asymmetric Gabriel synthesis" has been performed with bornyl esters of 2-bromoalkanoic acids.[97] Enolates of N-acyl sultams undergo stereobiased hydroxyamination [$R^1CH_2CONR^2R^3$ → $R^1CH(NROH)CONR^2R^3$, where $-NR^2R^3$ is an isobornylsultam moiety].[88]

An approach using the same principle, applied to the alkylation of glycine derivatives carrying chiral auxiliaries, continues to find favour, illustrated in the successive bromination (NBS) (Vol.25, p.15) and reduction ($Bu_3Sn^2H$ or ($Bu_3SnH$) of (-)-8-phenylmenthyl esters of Boc-glycine or Boc-2,2-dideuterioglycine to give both (S)- and (R)-2-$^2$H-glycine in 90% optical yields.[89] (-)-Menthyl N-Boc-α-bromoglycinate acts as radical source in reacting with $Co(Acac)_2$ to give α-(acetylaceton-3-yl)glycine, on which, various five-membered heterocyclic side-chains were constructed, and from which, L-norvaline was obtained to demonstrate the potential of the method.[90]

Both enantiomers of 2-amino-2-methylbutanoic acid ("isovaline") are available through diastereoselective alkylation of (1S,2R,4R)-10-dicyclohexylsulfamoyl isobornyl esters of cyanoacetic acid.[91] The same moiety attached as an amide to N-benzylideneglycine provides the template for synthesis of L-α-(indan-1-yl)glycine and L-α-(benz[*f*]indan-1-yl)glycine.[92] Schiff bases formed between glycine methyl ester and a chiral amine undergo diastereoselective alkylation and aldol reactions, the latter principle illustrated in aldolization of (19) with protected ribose or galactose in the first asymmetric synthesis of glycosyl-β-hydroxy-(S)-α-amino acid esters.[93] The related alkylation of chiral Schiff bases has been thoroughly studied by Belokon's group in the context of the nickel(II) prolylglycine complex (20) and its prolylalanine analogue, with new results for the preparation of fluorinated (S)-phenylalanines in greater than 90% enantioselectivity,[94] and of (S)-2-amino-4-phosphonobutyric acid and (S)-2-amino-5-phosphonovaleric acid.[95] Conference Reports covering this work have appeared.[96] Further examples (see Vol.25, p.20 and preceding Volumes) of the "double asymmetric induction" procedure, in which phase-transfer-catalysed alkylation of a glycine ester Schiff base in which amino and carboxy groups both carry homochiral substituents, have been published.[97,98]

Glycine Schiff bases yield azomethine ylides with DBU/AgOAc, that add

to chiral enones to yield homochiral prolines (Scheme 9).[99] $SnCl_4$-catalysed asymmetric ene reactions involving (-)-8-phenylmenthyl esters of glycine imines, give L-enantiomers (21) preferentially, considered to be due to blockage of the *re*-face of the imine by the phenyl group.[100]

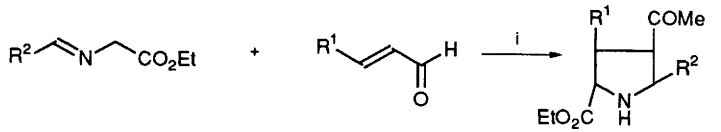
The Schollkopf piperazinedione alkylation procedure, and its more recent variants, are used year after year both by the originators and increasingly by others. The original form of the procedure is now used less for asymmetric synthesis of α-amino acids, but the acetylated synthon (22 in Scheme 10) shows wider usefulness, for example in allowing α-bromination (NBS)[101] and in facilitating aldolization with PhCHO *en route* to 2,3-methanophenylalanine methyl ester.[102] An extraordinary variant involving alkylation (RBr/LiHDMS/THF) of the analogous bis[N-(S)-phenylethyl]-(3S)-3-methyl piperazinedione gives better than 98% diastereoisomeric excess in up to 96% reaction yields.[103] Curiously, the (3R)-epimer performs less well. The bis-lactim ether used in a popular variant of this procedure was chosen for syntheses of β-trimethylsilyl-D-alanine (Scheme 11),[104] L-2-amino-4-phosphonobutanoic acid (see also Ref.255),[105] and anticapsin.[29] The mild conditions (aqueous TFA) for ring-opening with release of the amino acid in the form of its ester were exploited in a synthesis of D-phenylalanine benzyl ester (better than 95% enantiomeric excess).[106]

The continuing interest in enantioselective homogeneous-catalysed hydrogenation of α,β-unsaturated α-amino acids has been demonstrated recently in results for enantiomeric excesses of a modest level (43% for N-benzoyl-L-phenylalanine ethyl ester using $H_2$/BICHEP-Ru(II) complexes[107] to very high levels with related chiral phosphines for cinnamates with Rh complexes[108,109] or Rh or Ni analogues[110] and analogous acylamino(thienyl)acrylic acids,[111] and ferrocenylalanine[112] using Rh-chiral phosphine complexes. Rh-Cyclo-octadiene complexes catalyse asymmetric hydrogenation of N-acyl dehydro-amino acids when in the presence of 2,3-bis(O-diphenylphosphinyl)-D-glucose ethers.[113] The general topic has been reviewed.[114]

Extending the principle to β-keto-esters through subjecting them to asymmetric hydrogenation (chiral Ru complexes) and amination with di-t-butyl azodicarboxylate leads to anti-N-Boc-α-hydrazino-β-hydroxyesters (23).[115] Cyclic α-hydrazino acids are formed diastereoselectively, by aza-Diels-Alder addition (Vol.25, p.17) of azodicarboxylates to homochiral esters ROCH=CHCH=$CHCO_2R'$ (R' = tetra-O-acetyl-D-glucopyranos-1-yl).[116]

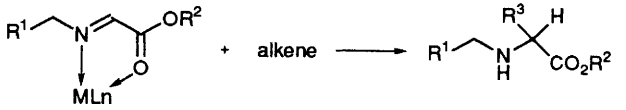
Nucleophilic addition to chiral imines provides a near analogy to the hydrogenation process, but previous results have not been as encouraging as those (90-96% enantiomeric excess) for additions of organolithium or Grignard reagents ($CeCl_3$ catalysis) to (24).[117] Reductive cleavage (Raney nickel) leading to D-alanine is used to illustrate an asymmetric synthesis.

The uses of Evans' chiral oxazolidin-2-ones in the asymmetric synthesis of amino acids have been illustrated in a synthesis of all stereoisomers of O-methyl 2',β-dimethyltyrosine incorporating some beneficial modifications (Scheme 12) to the usual procedure.[118] The same methodology has been used for β-methyltyrosine and has been described as incorporating an asymmetric Michael-like 1,4-



Reagent: i, AgOAc, DBU

**Scheme 9**



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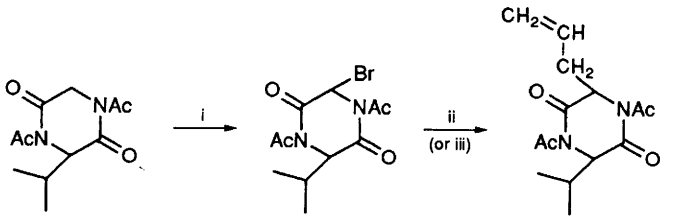
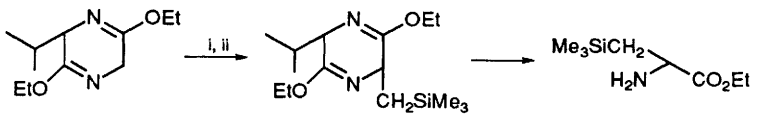


(22)

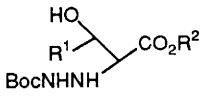
Reagents: i, NBS; ii, $CH_2{=}CHCH_2SnBu^n{}_3$; iii, $^2H_2/PdCl_2 \rightarrow {}^2H$ in place of $CH_2{=}CH\text{-}CH_2$

**Scheme 10**



Reagents: i, BuLi; ii, $Me_3SiCH_2Cl$

**Scheme 11**

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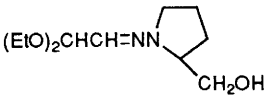


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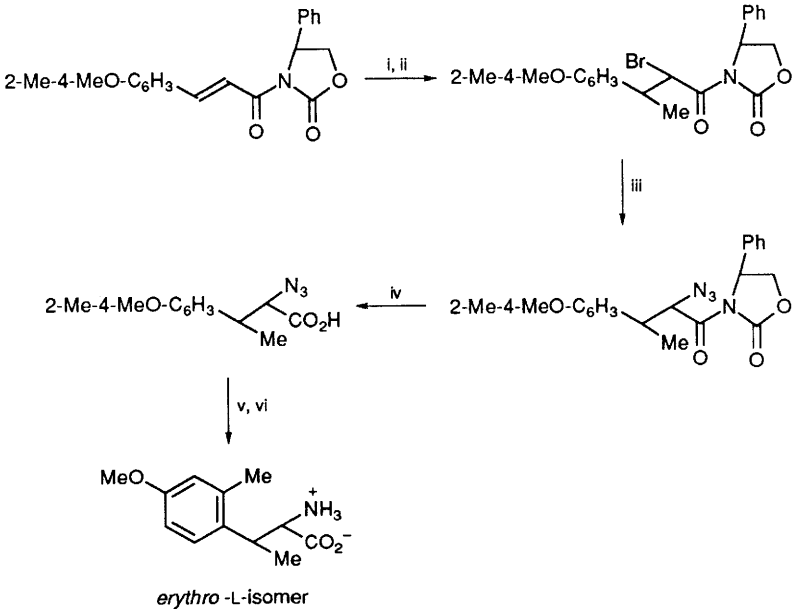



*erythro* -L-isomer

Reagents: i, $MeMgBr,CuBr.SMe_2$; ii, NBS; iii, tetramethylguanidinium azide; iv, LiOH, $H_2O$; v, Pd-C/$H_2$; vi, ion exchange chromatography

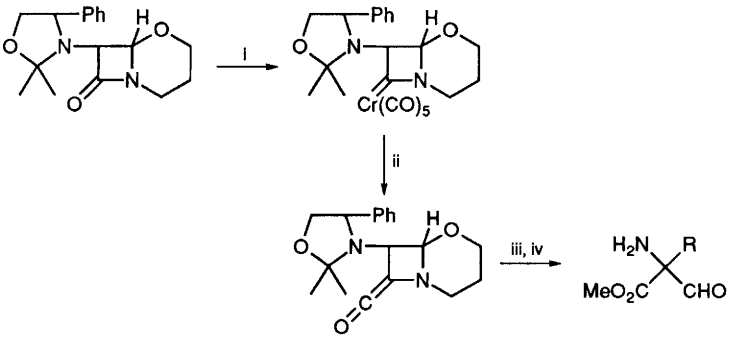
**Scheme 12**

addition.[119] Rather more complicated versions of the procedure are involved in useful asymmetric syntheses of α-alkyl-, -alkenyl-, and -alkynyl-α-amino acids (Scheme 13) through photolytic rearrangement (Vol.25, p.17) of oxazolidine carbene Cr complexes,[120] and in syntheses of polychlorinated threonines (Scheme 14), including the previously known (2S,3S)-4,4-dichloro-2-amino-3-hydroxybutanoic acid.[121] The four stereoisomers of 3-hydroxyleucine have been synthesized starting with Sharpless oxidation of (E)-4-methylpent-2-en-1-ol and $PhCH_2NCO$-induced epoxide opening to give the 4-(2-hydroxy-3-methylpropyl)-N-benzyloxazolidin-2-one.[122] An interesting feature of this synthesis, concluded by Jones' oxidation (leading to recyclization to 4-carboxy-5-isopropyloxazolidinone) and de-protection, is the propensity towards epimerization of the intermediate oxazolidinone. Synthesis of the pyrimidoblamic sub-unit of bleomycin $A_2$ has been modelled by stereocontrolled introduction of the C-2 acetamidomethyl side-chain through alkylation of the stannous (Z)-enolate of the oxazolidin-2-one (25).[123] The "chiral vinyl anion" equivalent, (S)-$MeOCH_2CH_2OCH_2OCHMeCH=CBr_2$, has been used to convert an imine 1,3,5-$Me_3SO_2C_6H_2N=CHR$ into the corresponding homochiral α-amino aldehyde 1,3,5-$Me_3SO_2C_6H_2NHCHRCHO$, for the purpose of synthesis of homochiral oxazolidin-2-ones (26; $R^1$ = 2,4,6-$Me_3C_6H_2$-, $R^2$ = $CMe_2CO_2Me$).[124]

An "Org.Synth." has been published[125] for the standard method for exploitation of oxazolidin-5-ones in this area, based on the earliest report on the introduction of the method[126] The "dehydro-alanine" oxazolidin-5-one (27) undergoes gem-dimethylcyclopropanation with $Ph_3P=CMe_2$ to give unequal proportions of (S)- and (R)-"methanovaline" (*alias* 2,2-dimethyl-1-aminocyclopropane-1-carboxylic acid).[127]

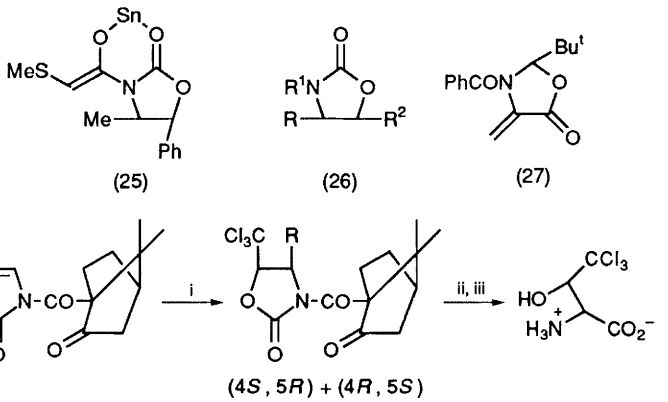
Corresponding uses for imidazolidinones (28 in Scheme 15)[128] and pyrrolidines (29 in Scheme 16)[129] indicate the value of five-membered heterocycles as chiral auxiliaries. Further results (Vol.24, p.12) for the $Hg(OTFA)_2$-catalysed cyclization of homochiral amidals to 2,5-trans-imidazolin-4-ones in a synthesis of D-α-amino adipic acid illustrate the potential of this method.[130] The six-membered analogues have already established a competitive foothold in the same area of applications, with morpholin-2-ones being employed in the enantioselective synthesis of α-alkyl-α-amino acids[131] and in continuing studies (see Vol. 25. p.36) of applications of the cycloaddition reactivity of their derived azomethine ylides with alkenals and alkynals, for the synthesis of prolines of high enantiomeric purity.[132] 2-Substituted pipecolic acids have been synthesised from chiral morpholin-2,5-diones prepared using a chiral α-hydroxyacid (30 in Scheme 17).[133]

Applications of enzymes for the synthesis of α-amino acids can extend beyond the fermentative production methods used for the production of the familiar coded amino acids, covered in the next Section. This small topic area is represented in the recent literature by lipase-catalysed hydrolysis of (±)-3-benzyloxy-4-hydroxy-$\Delta^2$-isoxazoline butyrate and conventional work-up to provide cycloserine enantiomers,[134] and (R)- and (S)-oxynitrilase-catalysed enantioselective addition of HCN to aldehydes, with incomplete enantioselectivity in forming the cyanohydrins, from which α-amino acids are easily accessible.



Reagents: i, $Cr(CO)_6$; ii, *hν*; iii, MeOH; iv, $H_2$/Pd-C

**Scheme 13**

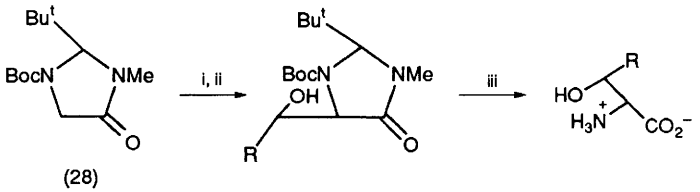


(25) (26) (27)

(4*S*, 5*R*) + (4*R*, 5*S*)

Reagents: i, $CCl_4$ or $CCl_3Br$/radical initiator; ii, separate diastereoisomers; iii, $H_3O^+$

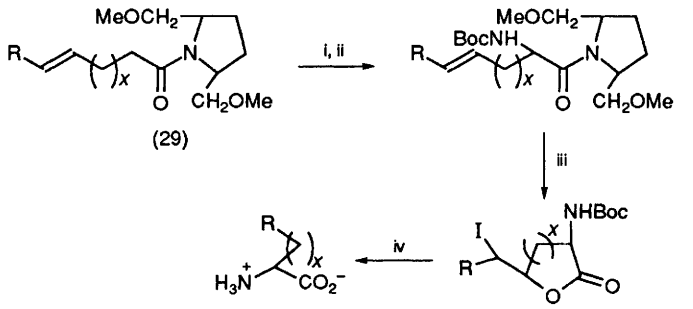
**Scheme 14**



(28)

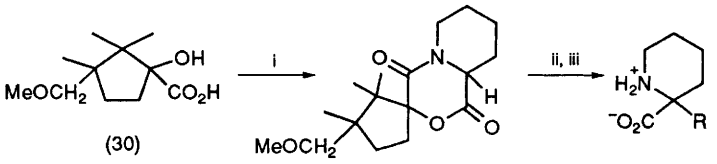
Reagents; i, RCOCl; ii, $LiBHEt_3$; iii, $H_3O^+$

**Scheme 15**



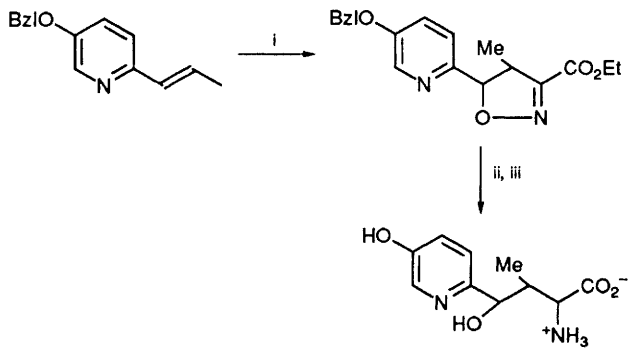

Reagents: i, $I_2$-Collidine; ii, $BocNH_2$; iii, $I_2$-EtOH/$H_2O$; iv, Zn/THF, then routine deprotection steps

**Scheme 16**




Reagents: i, condense with pipecolic acid; ii, $R^1Li$; iii, RBr

**Scheme 17**




Reagents: i, (*Z*)-$EtO_2CCCl$=NOH; ii, Zn, Cu/AcOH; iii, deprotection

**Scheme 18**

Improved optical yields result from the presence of organic solvents in the reaction media.[135]

### 4.3 Synthesis of Protein Amino Acids and Other Naturally Occurring α-Amino Acids

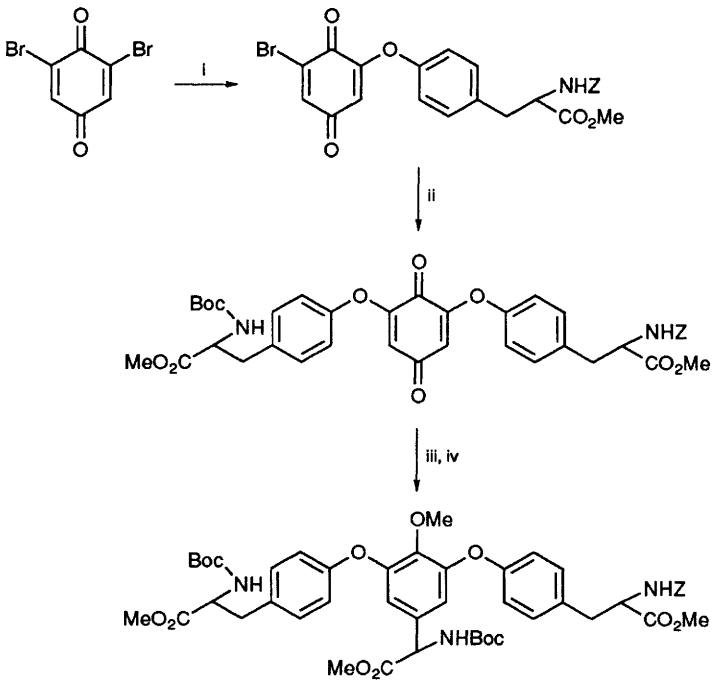
This Section concentrates on laboratory synthesis, but fermentative production of the familiar protein amino acids and near relatives constitutes its opening paragraph, as it has done over recent years and with an ever more perfunctory coverage of the burgeoning literature, now mostly emanating from Pacific Rim countries. This topic has an increasingly routine nature but the full literature can be easily accessed through Section 16: Fermentations and Bio-industrial Chemistry of *Chemical Abstracts*. Some contributions in a recent text deal with this area, represented by a review of membrane bioreactors for the production of L-amino acids,[136] production of L-lysine by asymmetric transformation of α-amino-ε-caprolactam,[137] production of homochiral protein amino acids through the use of aminotransferases,[138] and a review of methods for the production of natural and non-natural homochiral amino acids.[139] Production of L-DOPA continues to be an active area of research, with descriptions of interesting routes from catechol, pyruvate, and ammonia, one using *Escherichia coli* into which had been cloned the gene encoding tyrosine phenol-lyase from *Erwineia herbicola*[140] and the other, an economical and high yielding route using polyphenol oxidase from banana leaf.[141] *Escherichia coli* accomplishes the conversion of pyrrol-1-ine 2-carboxylic acid into L-proline.[142]

Simple syntheses have been described for threonine (copper glycinate and acetaldehyde)[143] glutamic acid (from cyclopentadiene by successive addition of HCl, ozonolysis, ammonolysis, and hydrolysis).[144] A convenient synthesis of L-α-amino-adipic acid starts with (S)-hexahydro-3-phthalimido-2H-azepin-2-one.[145]

A new synthesis (Scheme 18) of the pyridyl γ-hydroxy-α-aminobutanoic acid component of Nikkomycin Z, has been developed by authors who were unable to reproduce a nitrile oxide addition step in a previous synthesis.[146] Further syntheses of antibiotic components include the bicyclo-C,D,E-diphenyl ether component of vancomycin (Scheme 19)[147] and the component ISP-1 (*alias* myriocin or thermozymocodin) of a recently-isolated immunosuppressant (Scheme 20).[148]

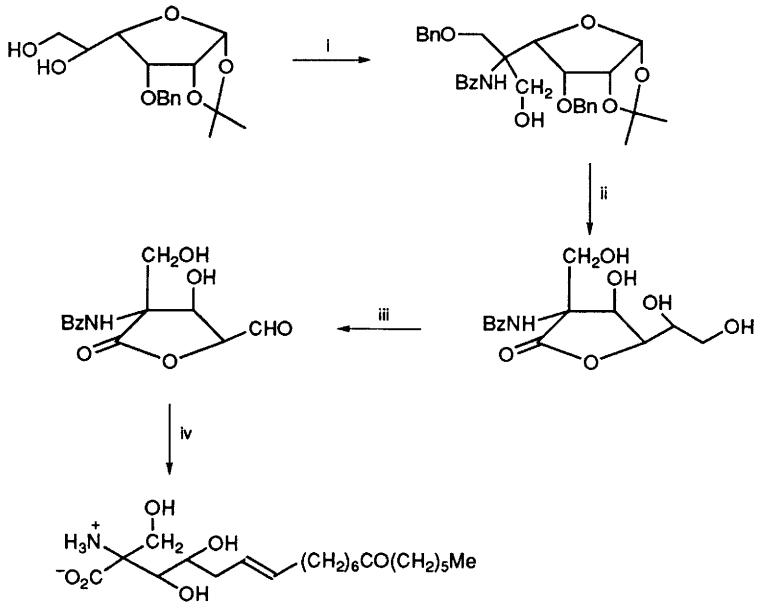
Alicyclic α-amino acids that derive from natural sources include ring sizes from 3 to 6 in this year's literature, which at the small ring end, is represented by 3-(trans-2'-nitrocyclopropyl)alanine (a constituent of the peptide lactone hormaomycin). The (±)-compound has been synthesized in three steps from t-butyl acrylate, and the (1'S,2'R)- and (1'R,2'R)-isomers (31 in Scheme 21) synthesized in six steps from (S)-2,3-isopropylidene glyceraldehyde.[149] Carnosadine (32) has been prepared from the corresponding cyclopropylmethanol.[150]

A notable synthesis from D-serine O-t-butyldiphenylsilyl ether, of cis-polyoximic acid (Scheme 22), now known to be the natural isomer after a correction of the literature, has been reported.[151] The non-natural (-)-isomer of polyoxamic acid has been synthesized starting from the N-benzyl β-lactam (33).[152] A rearrangement in a synthesis (Scheme 23) of (+)-monomorine, an



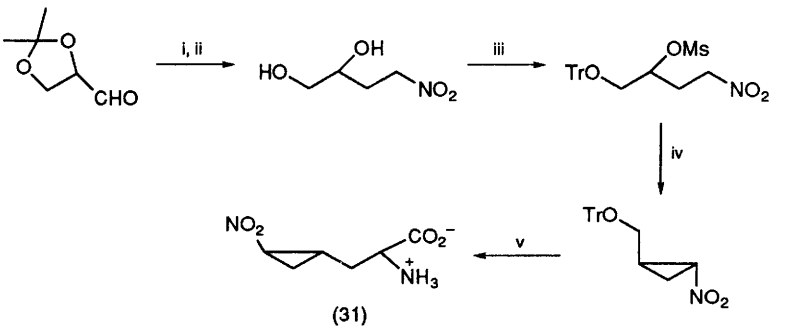

Reagents: i, ii, protected L-tyrosine/6 eq. KF/DMF/90 °C; iii, $Na_2S_2O_4$, selective conversion of OH to $-CH=CH_2$; iv, $CH=CH_2 \longrightarrow$ L-$MeO_2CCHNHBoc$ by standard methods

**Scheme 19**



Reagents: i, extended sequence of standard functional group manipulations; ii, $RuCl_3/NaIO_4$; iii, iv, *J.Chem. Soc., Perkin Trans. 1,* 1983, 1613

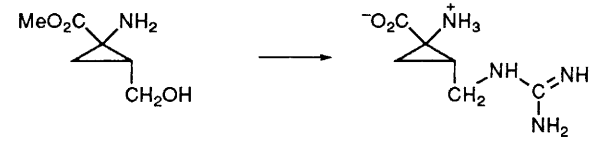
**Scheme 20**



(31)

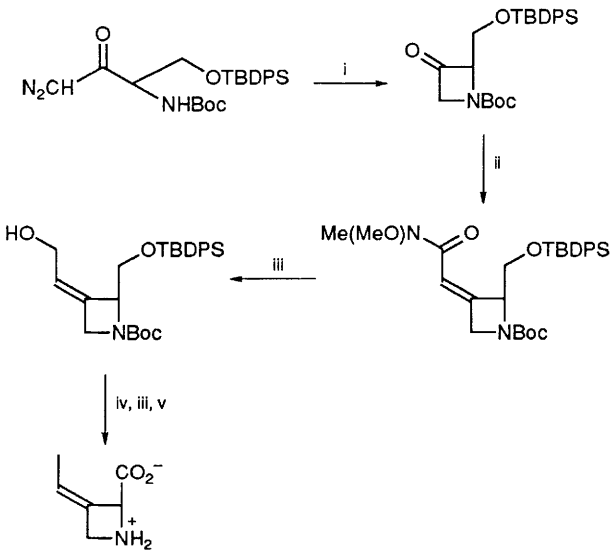
Reagents: i, $MeNO_2/KF$, then $Ac_2O$/DMAP and $NaBH_4$; ii, TsOH; iii, OH group protection; iv, $Na_2CO_3$–toluene/110 °C/15h; v, $-CH_2OTr \longrightarrow CH_2Br$, then $Ph_2C{=}NCH_2CH_2CO_2Bu^t$/BuLi and routine deprotection

**Scheme 21**



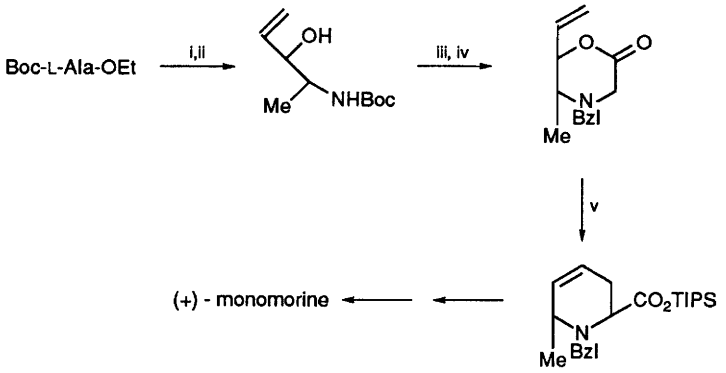
(32)

(33; R = D-mannitol residue) (–)-polyoxamic acid



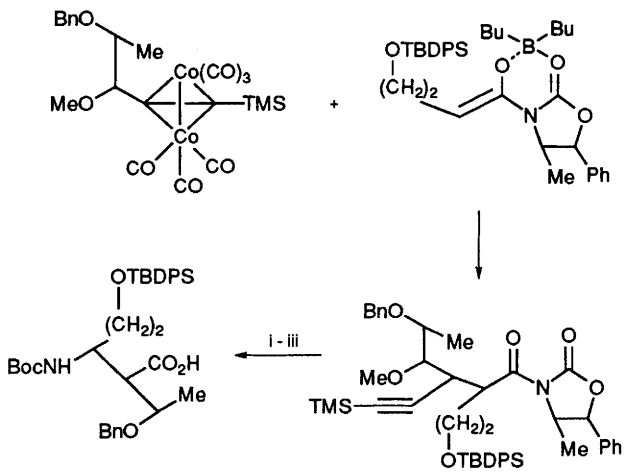
Reagents: i, $Rh_2(OAc)_4$; ii, NaH, (*N*-methoxy-*N*-methyl)-2-(triphenylphosphoranylidene) acetamide ⟶ *trans* :*cis* = 11:89; iii, *cis* ⟶ $LiAlH_4$; iv, $CBr_4$, $PPh_3$; v, remove TBDPS, Jones' reagent, remove Boc

**Scheme 22**



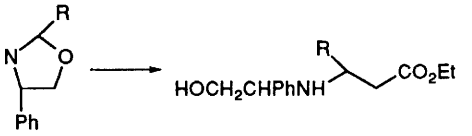

Reagents: i, DIBAL-H; ii, $CH_2$=CHMgCl; iii, protecting group changes; iv, $BrCH_2CO_2Ph$; v, TIPS-OTf

**Scheme 23**

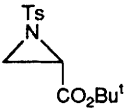



Reagents: i, $H_2O$; ii, Curtius (DPPA/Bu$^t$OH) degradation;
iii, $-C\equiv C- \longrightarrow -CO_2H$ with $OsO_4/NaIO_4$

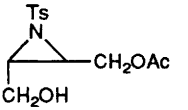
**Scheme 39**



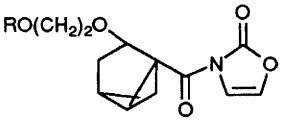

(56)



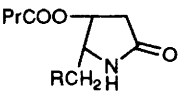

(57)



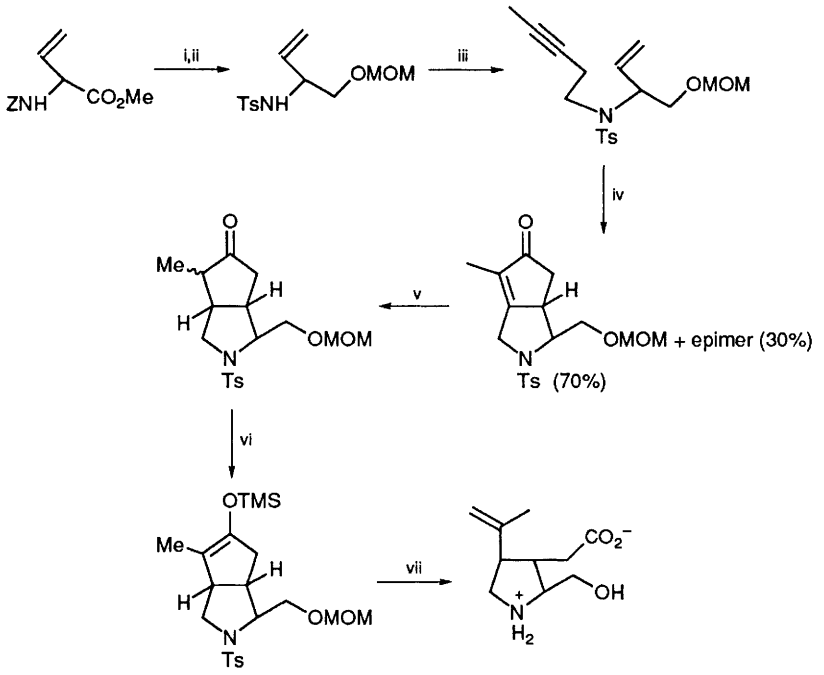

(58)




(59)

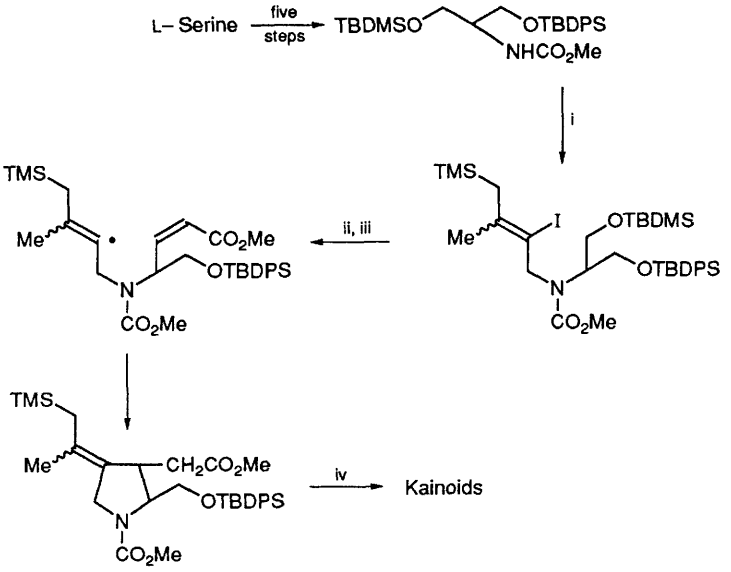



(60)



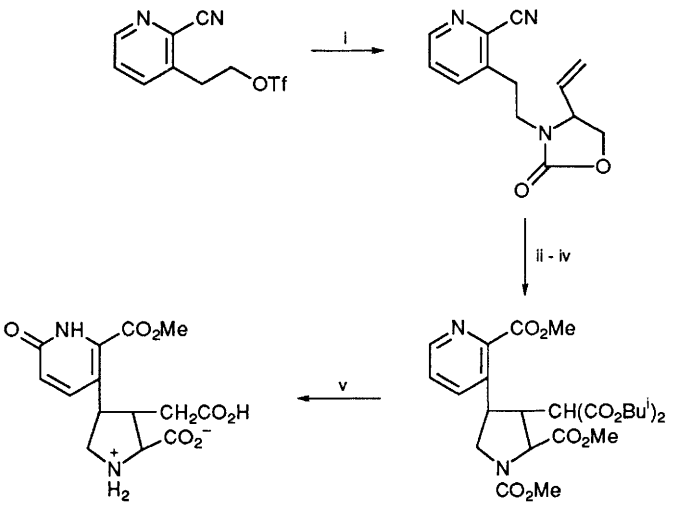

Reagents: i, $CO_2Me \longrightarrow CH_2OH$; ii, protecting group introductions and changes; iii, $MeC{\equiv}CCH_2CH_2Br$; iv, $Co_2(CO)_8/CH_2Cl_2$; v, $H_2$/Pd on epimer mixture; vi, $FeCl_3$/EtMgBr/TMSCl on major isomer; vii, followed by routine development $\longrightarrow$ (–)-kainic acid

**Scheme 24**




Reagents: i, $TMSC(Me)=CICH_2I$ (from $TMSCH_2CMe=C=CH_2$);
ii, deprotection, TsOH, [O], $(CF_3CH_2O)_2P(O)CH_2CO_2Me$
iii, $Bu_3SnH$/AIBN; iv, protodesilylation, then complete deprotection

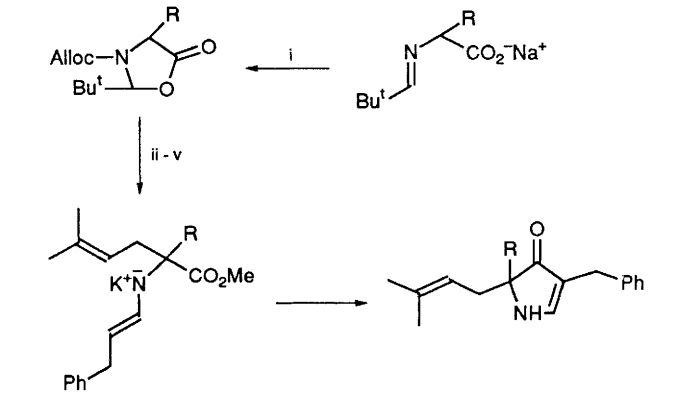
**Scheme 25**




acromelic acid B

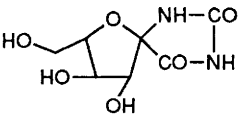
Reagents: i, L-vinylglycinol-derived oxazolidin-2-one;
ii, $-CH{=}CH_2 \longrightarrow -CH(CO_2Bu^i)_2$; iii, $-CN \longrightarrow -CHO$ *etc*.;
iv, MeONa; v, established methods

**Scheme 26**

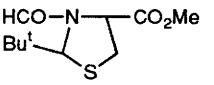



Reagents: i, AllocCl; ii, KHDMS; iii, $Me_2C{=}CHCH_2Br$; iv, $Pd(PPh_3)_4$;
v, $Ph(CH_2)CHO$ then ii

**Scheme 27**



(34)



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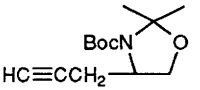

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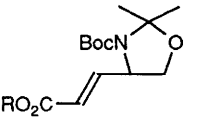

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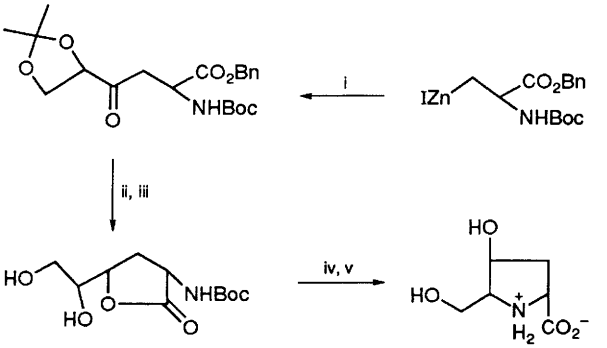
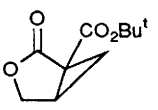


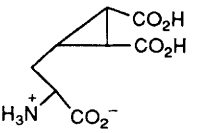
(+) - bulgecinine

Reagents: i, (*R*) - isopropylideneglyceryl chloride/Pd(0); ii, L - Selectride; iii, $I_2$/MeOH; iv, TBDMSCl; v, steps established earlier (*Tetrahedron*, 1987, **43**, 423)

**Scheme 28**



(38)



(39)

$HCONHCMe(SiMe_3)CO_2Et$, and α-acetoxyglycine analogues, yield highly electrophilic iminium ions by electrochemical oxidation, that react with allylsilanes and silyl ethers to give novel α,α'-disubstituted glycines.[170] A notable inclusion in the list of amino acids prepared in this way is the α-phenyl family.

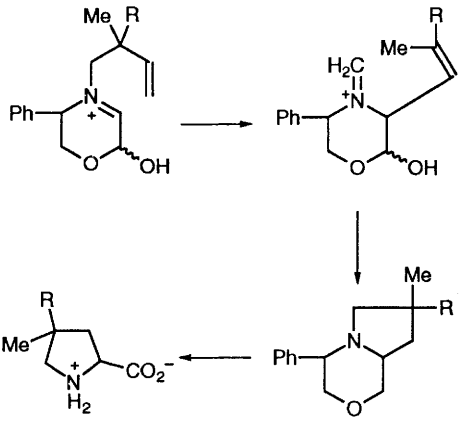
## 4.5 Synthesis of α-Amino Acids Carrying Alkyl Side-chains, and Cyclic Analogues

Close structural analogues of the aliphatic protein amino acids are collected here, together with alicyclic analogues.

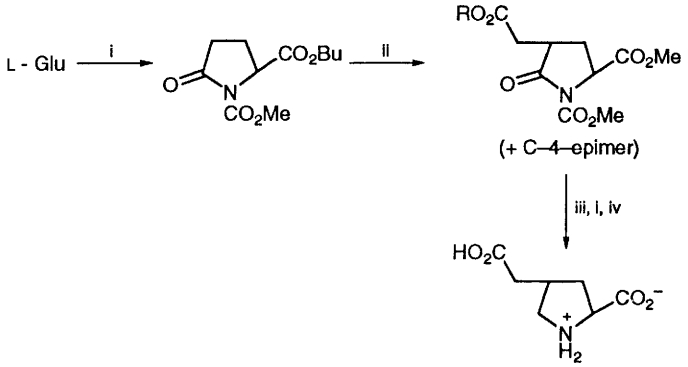
Acyclic α-amino acids fulfilling the title of this Section include new types of 4,4-disubstituted L-glutamic acids (4-methylene-, 4,4-dimethyl-, and the cyclopropyl analogue incorporating C-4, prepared as conformationally constrained L-glutamic acid analogues from Boc-L-aspartic acid γ-benzyl ester *via* (36), which is subjected to aminocarbonylation.[171] Three diastereoisomers of L-2-(2-carboxy-4-methylenecyclopentyl)glycine have been prepared (one of which is a potent kainoid receptor agonist), through the use of chiral oxazolines (37) and similar heterocyclic auxiliaries, starting with alkylation by 2-[(trimethylsilyl)methyl]prop-2-en-1-yl acetate.[172] More pedestrian syntheses of 4-methyl- and 4-ethyl-L-glutamic acids from corresponding glutaric acids are mediated by glutamic oxalacetic aminotransferase.[173] Further synthetic targets for the L-serine-derived zinc reagent shown in Scheme 28 include α-(4-oxo-alkyl)-α-amino acids (elaborated into (+)-bulgecinine precursors),[174] and alkylation of the synthon by $C_6H_5{}^+Fe(CO)_3\ PF_6^-$ gives cyclohexadienylalanine;[175] reactions with chloroformates[176] and with acyl chlorides and allylic chlorides[177] have also been described, the last-mentioned study covering uses of the glutamic acid-derived organocopper analogue. The γ-oxoalkyl α-amino acids have been approached in another way, from nucleophilic ring-opening of activated chiral α-alkoxycarbonyl β-lactams by $Me_2S^+(O)CH_2^-$, by lithiated sulfones, or by $Bu_2Cu(CN)Li_2$.[178]

α-Amino acids with alicylic structures in the side-chains continue to attract attention as conformationally-constrained mimics of the physiological action of the familiar acyclic protein amino acids, and the lactone (38) is a useful cyclopropyl chiron for the synthesis of 2,3-methano-amino acids[179] (several other recent papers describe synthesis of members of this class: Refs. 72, 78, 102, 342). A simple synthesis of 1-aminocylopropanecarboxylic acid starts with the conversion of a chelated homoserine into 2-amino-4-bromobutyrate.[180] Further potential glutamic acid agonists, (39) and its stereoisomer with reversed chirality for the ring $CO_2H$ groups, have been prepared following the synthetic methodology reported earlier by the same workers (Vol. 24, p.22).[181] Alkenoic esters prepared from (1S,2R)-PhCHRCHPhOH [cleavable by $Pb(OAc)_4$], have been used for the preparation of (1S,2R)-1-amino-2-phenylcyclopropane carboxylic acid.[182]

Cyclic α-imino acids, the family of alicyclic α-amino acids that enclose the amino group as a member of the ring, are represented in a synthesis of (-)-trans-azetidine-2,4-dicarboxylic acid,[183] in a synthesis of 5,5-dimethyl-DL-proline (prepared by addition of HCN to 5,5-dimethylpyrrolideine N-oxide,[184] and in an interesting aza-Cope rearrangement process (Scheme 29).[185] A more conventional proline synthesis employs L-pyroglutamic acid (Scheme 30).[186]




**Scheme 29**




Reagents: i, conventional methodology; ii, LiHDMS/$BrCH_2CO_2R$; iii, separate epimers; iv, deprotection

**Scheme 30**

Six-membered ring α-imino acids approached through unusual routes include 4- and 5-substituted (S)-pipecolic acids, formed by ring-expansion of 4-oxo-L-proline with $N_2CHCO_2Et$.[187] The 4-oxopipecolic acids on reduction and sulfation give homochiral products exhibiting potent NMDA receptor agonist activity. NMDA Antagonism is shown by 3-(β)-phosphonoalkyl-substituted pipecolic acids prepared by established methodology.[188] Aza-Diels–Alder cycloaddition methods providing pipecolic acid derivatives involve N-camphorsulfonylimines $RSO_2N=CHCO_2Et$ (prepared from the corresponding α-bromoglycine derivative) with Danishefsky's diene,[189] and N-arylidene α,β-dehydro-α-amino acid esters $ArCH=NCH(=CH_2)CO_2Me$ (prepared by either a long-known method from cysteine methyl ester, or from serine methyl ester) with electron-deficient alkenes (an interesting aza-Cope rearrangement concludes one of these syntheses).[190]

Conventional Diels-Alder addition of cyclopentadiene to either isomer of 4-benzylidene-2-phenyloxazol-5(4H)-one provides all four racemates of 2-amino-3-phenylnorbornane-2-carboxylic acids.[191]

## 4.6 Models for Prebiotic Synthesis of Amino Acids

Conventional studies of the formation of α-amino acids in activated mixtures of simple compounds [A2]aqueous ammonium acetate subjected to high energy LET particle [$^{10}B(n,\alpha)^7Li$] irradiation, or $^{60}Co$-γ-irradiation, giving aspartic acid, serine, glycine, alanine, valine, β-alanine and γ-aminobutyric acid, etc),[192] or UV irradiation of a gaseous mixture of $H_2$ and HCN,[193] as reported in this Section over the years, are accompanied by results of investigations into intermediate stages involved in abiotic synthesis of the starting compounds. Formation in the early non-reducing atmosphere, of ammonia or fixed-nitrogen compounds required by theories of prebiotic α-amino acid synthesis, must have proceeded *via* nitric oxide, thence to nitrous and nitric acids whose reduction in water at pH 7.3 at temperatures above 25°C can be accounted for by the oxidation of ferrous salts to ferric compounds.[194]

Aqueous solutions of ammonia and 2-aminopropionitrile, a putative alanine precursor plausibly formed in an HCN-containing atmosphere, react to give 2,2'-iminodipropionitrile, N-(cyanoethyl)alaninamide, and alanine. 3-Aminopropionitrile reacts similarly to give β-alanine, among other products.[195]

## 4.7 Synthesis of α-Alkoxy-α-Amino Acids and Analogous α-Heteroatom-substituted α-Amino Acids

Several papers have appeared dealing with members of the easily-prepared α-hydroxyglycine family and analogues that are also mentioned in other sections of this Chapter. N-Z-[-(Diethoxyphosphonyl)]glycine has been prepared from Z-α-hydroxyglycine (i.e. $ZNH_2$ + $OHCCO_2H$) using $PCl_5/P(OEt)_3$, and deprotection and Schiff base formation have been demonstrated.[196]

Growing interest in protected α-aminoglycines (Scheme 31) and sulfur analogues for use in peptide synthesis is supported by relevant preparative methods.[197]

### 4.8 Synthesis of α-Halogenoalkyl α-Amino Acids

Amino acids carrying fluoroalkyl side chains have yielded rewarding results as far as their enzyme inhibitory properties are concerned, and their synthesis continues to be studied, in some cases providing novel mechanistic insights.

A γ-fluorine substituent increases the propensity for 1,4-addition during the ammonolysis of α,β-unsaturated α-bromobutenoic acid esters, leading to aziridines and lowering the yields of the intended reaction product, the trans-β,γ-unsaturated γ-fluoroalkyl-α-amino acids.[198] New fluorinated analogues of (S)-norvaline (4,4-difluoro-, 4,4,5,5,5-pentafluoro-) and of (S)-norleucine (5,5-difluoro-, 5,5,6,6,6-pentafluoro-, and 4,4,5,5,6,6,6-heptafluoro-) have been prepared by standard methods,[199] also illustrated for the synthesis of a 1:1-mixture of (2S,4S)- and (2S,4R)-5,5,5-trifluoroleucine from 5,5,5-trifluoro-4-methyl-2-oxo-pentanoic acid by enzymatic transamination using *Alcaligenes faecalis* IAM 1015.[200]

A review of synthetic approaches to 4-fluoroglutamic acid,[201] and methods for the preparation and separation of cis- and trans-4-fluoropyroglutamic acid, have been published.[202] All four stereoisomers of 4-fluoroglutamic acid are accessible from (-)-trans-4-hydroxy-L-proline through inversion at C-4 ($Ph_3P$/DEAD), substitution of the hydroxy group by diethylaminosulfur trifluoride, and $RuO_4$ ring-opening.[203] DL-3,3-Difluoroglutamic acid is accessible from 3-hydroxyprolinol in a very similar way.[204] 4,4-Difluoro-L-arginine has been prepared from Boc-D-serine *via* the Garner aldehyde (37; CHO in place of -CH=$CHCO_2R$) through reaction with ethyl bromodifluoroacetate and routine elaboration to incorporate the guanidine grouping.[205] A combination of side-chain fluorination and phosphonation to provide potential pharmacological activity is involved in 4-phosphono(difluoromethyl)-DL-phenylalanine, a target reached through a synthesis starting from 4-(diethoxymethyl)benzaldehyde and its reaction with ethyl α-azido-acetate.[206] A protected L-tyrosine O-trifluoroacetate is the starting material in an independent synthesis of the L-analogue, involving carbonylation [$CO/Pd(OAc)_2$], conversion into the triethoxyphosphonylcarbonyl-L-phenylalanine, and fluorination with diethylaminosulfur trifluoride.[207]

### 4.9 Synthesis of α-(ω-Hydroxyalkyl) α-Amino Acids

This, one of the particularly variegated families of modified α-amino acids, is accessible through a range of mechanistically-interesting synthesis methods. β-Hydroxyalkyl-α-amino acids are easily prepared from glycine derivatives by aldol reactions using aliphatic aldehydes unless steric hindrance is involved; in which case, titanium enolates formed through transmetallation of lithium enolates using dichloro-di-isopropoxy-titanium are useful. They react well with N-alkylideneglycine esters preferentially yielding the anti-isomer under kinetic control, and have provided anti-2R-products with glycinamides in which the amide moiety is a chiral oxazoline.[208] Separable mixtures of diastereoisomeric racemates of β-hydroxyalkyl-α-amino acids are obtained when glycine enolates [$Cl_3CCONRCH_2CO_2Me + CF_3SO_3SiMe_3 \rightarrow Cl_3CCONRCH=C(OMe)OSiMe_3$] react with aldehydes.[209]

Ethylene oxide is a valuable vinyl cation equivalent for use in the synthesis of γ-hydroxyalkyl-α-amino acids through alkylation of di-anions formed from N-benzoylglycine esters using LDA (Scheme 32),[210] and thence to α-vinyl-α-amino acids. Nitrogen functions can be introduced stereoselectively into D-ribonolactone to yield 4,5-dihydroxy-D-erythro-norvaline and 4,5-dihydroxy-L-threo-norvaline.[211] Monosaccharide derivatives (40) and (41) have been used in sophisticated syntheses of hydroxylated 1-aminocyclopentanecarboxylic acids[212] and furan analogues,[213] the routes incorporating mechanistically-interesting ring-contractions.

N-Z-O-TBS-L-Serinal yields (5S)-Z-amino-(4R)-hydroxy-6-TBSO-hex-1-ene through highly stereoselective addition of allyltrimethylsilane, elaboration giving (2R,3S)-3-hydroxyproline.[214]

N-Protected L-aspartic acid α-esters are useful starting points in the synthesis of α-(ω-hydroxyalkyl)-α-amino acids, through subjecting them to side-chain elaboration, and they have been used in a synthesis of RI-331 [(-)-5-hydroxy-4-oxo-L-norvaline];[215] and in a similar way using hexafluoroacetone as protecting agent for both amino and α-carboxy groups.[216] 2-Amino-5,6-dihydroxy-5-(acetamidomethyl)hexanoic acid is an interesting putative biosynthetic precursor of oxapenam antibiotics, that has now been established to be represented by (42) through synthesis from N-Z-β-iodo-L-alanine by free-radical alkylation with $HOCH_2C(=CH_2)CH_2SnR_3$, followed by Sharpless epoxidation and routine elaboration.[217]

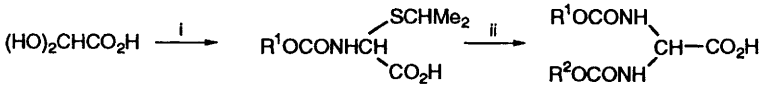
## 4.10 Synthesis of α-Amino Acids with Unsaturated Aliphatic Side-chains

In addition to standard methods of synthesis involving elimination reactions [of α-vinylglycine, Ref.210 and preparation from L-methionine *via* the sulfoxide[218] and of α,β-dehydroamino acids, $(MeS)_2C=NC(CO_2Me)=CHR$ starting from β-hydroxy-α-amino acids,[219]], some unusual approaches provide useful new methodology. (2S,3S)-2-Amino-3-methylpent-4-ynoic acid has been prepared starting with 3-chlorobut-1-yne,[220] and the serine-derived organozinc synthon (cf.Refs.174-177) has proved useful *via* transmetallation [→ $IZn(CN)CuCH_2CH(NHBoc)CO_2Bn$] for synthesis of allenic amino acids through reaction with toluene-p-sulfonyloxymethyl alkynes $RC\equiv CCHR'OTs$.[221]

α-Allylglycine has been prepared from methionine by the application of the Ramberg-Baecklund rearrangement.[222]

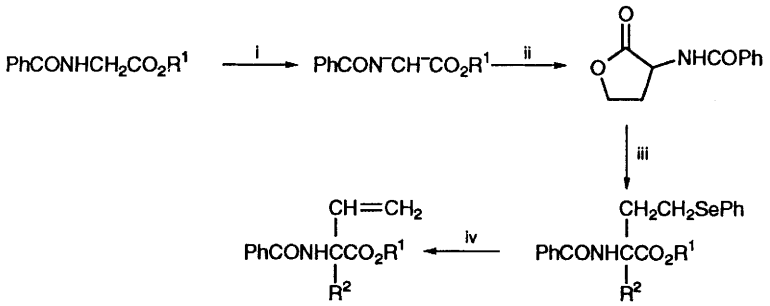
## 4.11 Synthesis of α-Amino Acids with Aromatic or Heteroaromatic Groupings in Side-chains

Active research topics providing routes to near relatives of the aromatic and heteroaromatic protein amino acids are reported in several recent papers collected here. Standard methodology for the preparation of phenylalanine analogues is illustrated in a route to 3'-azidotyrosine employing 3-azidophenol, pyruvic acid, and tyrosine phenol-lyase.[223] Preparations of conformationally-constrained L-phenylalanine analogues, one using Evans' methodology,[224] and



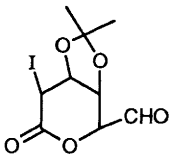
Reagents: i, $R^1OCONH_2$, $Me_2CHSH$; ii, $R^2OCONH_2$/NBS

**Scheme 31**



Reagents: i, LDA; ii, ethylene oxide; iii, LDA, $R^2X$, then $(PhSe)_2$/$NaBH(OMe)_2$; iv, $O_3$, $R^1OH_2$, Δ

**Scheme 32**



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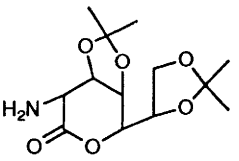

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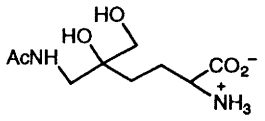

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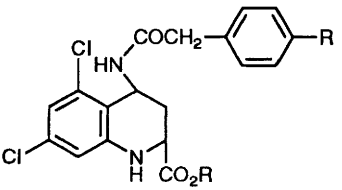


(43)

another developing existing routes to trans-2-carboxy-4-substituted tetrahydroquinolines (43) that are showing promise as glycine-site NMDA antagonists (see also Vol.25, p.40).[225] Simple transformations through nucleophilic substitution of p-iodophenylalanine derivatives lead to new phenyl-modified analogues, e.g. p-(tri-n-butylstannyl)phenylalanine.[226] A new synthesis of actinoidic acid (44; $H_3N^+CHCO_2^-$ in place of CHO) involves an efficient biphenyl-forming step (44 + 45 → 46) followed by Strecker synthesis.[227]

Tryptophan analogues continue to predominate in the heteroaromatic category, with new examples prepared through familiar routes. L-4-Aza-tryptophans are accessible through the condensation of a 4-aza-indole with serine, mediated by tryptophan synthase,[228] and chlorotryptophans have been prepared similarly,[229] while N-1- and C-2-substituted tryptophans, and 5-substituted analogues, are available from the corresponding indoles through alkylation by $BrCH_2C(=NOH)CO_2Et$ and conventional elaboration.[230] Pd-Catalysed annulation of substituted 2-iodoanilines with δ-silylated propargylglycines gives substituted tryptophans.[231] α-Substituted 5-hydroxytryptophans have been obtained through alkylation of homochiral pyrroloindoles (Vol.25, p.40) (LDA, bromoalkane) with retention of configuration.[232] 1,2,3,4-Tetrahydro-2-amino-2-carboxycyclopent[*b*]indole is a new conformationally-constrained tryptophan analogue prepared through the hydantoin synthesis from the corresponding ketone.[233]

Side-chain pyridinium salt moieties are accessible from the corresponding β-(pyrid-3-yl)alanines through alkylation using a halogenoalkane in the presence of $Ag_2O$.[234] A general route to such β-(heteroaryl)alanines has been fully documented for the case of N-Z-β-(pyrazol-1-yl)-L-alanine, prepared from N-Z-L-serine through conversion ($Ph_3P$/DEAD) into the β-lactone.[235] Boc-L-Serine methyl ester has been converted into novel amino acid nucleosides *via* its methylthiomethyl ether followed by reaction with silylated N-benzoyl purine and pyrimidine bases.[236] Vederas' 1988 route to these compounds using N-Boc-L-serine-β-lactone continues to be used by others.[237]

Further examples have been provided of preparative methods leading to new β-(heteroaryl)alanines and homologues containing two or more nitrogen atoms. β-[(3-Phosphonoalkyl)quinoxalin-2-yl]alanines[238] present a familiar general disposition of functional groups (47) for potential NMDA receptor affinity. γ-(3,5-Dimethylpyrimidin-2-onyl)-L-butyrine has been prepared from L-glutamic acid *via* the corresponding ureide.[239] ω-(Tetrazol-5-yl)alkyl analogues have been prepared as potential NMDA antagonists,[240] and trans-4-(tetrazol-5-yl)-L-proline (LY300020) has been announced as a novel systematically-active AMPA agonist, prepared from N-Z-hydroxy-L-proline *via* nucleophilic displacement by $CN^-$ on the O-toluene-p-sulfonyl derivative followed by tetrazole construction with $Bu_3SnN_3$.[241]

3-(Thiazol-4-yl)alanines and selenium analogues have been prepared by conventional Hantzsch synthesis from 2,2-bis(trifluoromethyl)-4-(3-bromo-2-oxopropyl)-1,3-oxazolidin-5-one, readily obtained from aspartic acid protected by condensation with hexafluoroacetone.[242] Thiazol-2- and 4-yl analogues and homologues have been reported independently.[243]

### 4.12 Synthesis of α-Aminoalkyl α-Amino Acids

Derivatives of the basic protein amino acids showing useful pharmacological potential include the cyclized ornithine derivative (48), already known to act as a partial agonist of the glycine site of the NMDA receptor. A series of β-substituted analogues has been prepared by the previously-established methodology.[244] Ring-opening by hydroxylaminolysis, of the pyrrolid-1-ine carboxylic acid obtainable from hydroxy-L-proline, gives the oxime of the α-keto-acid corresponding to (4R)-hydroxyornithine.[245] One-pot Schiff base alkylation and amination of (Z)-$AcOCH_2CH=CHCH_2OCO_2Et$ by BocONHBoc or $Me_2NH$, respectively, gives 1,4-adducts with $Ph_2C=NCH_2CO_2Et$ from which, by hydrogenation, DL-$N^6$-hydroxylysine and DL-laminine can be obtained.[246] The same target, but the L-(+)-enantiomer, has been synthesized starting from L-allylglycine, through a sequence resulting in hydroxymethylation at C-5.[247]

Mitsunobu processing of N-Fmoc-L-threonine and -allo-L-threonine N-Boc-hydrazides, giving (2S,3R)- and (2S,3S)-$N^{\alpha}$-Fmoc-$N^{\beta}$-Boc-α,β-diamino acids, can be operated on a multigram scale.[248] The greater confidence with which α-amino aldehydes are being used is illustrated in a synthesis of homochiral 2,4-di-amino acids (49 → 50).[249]

### 4.13 Synthesis of α-Amino Acids Carrying Sulfur- or Selenium-containing Side-chains

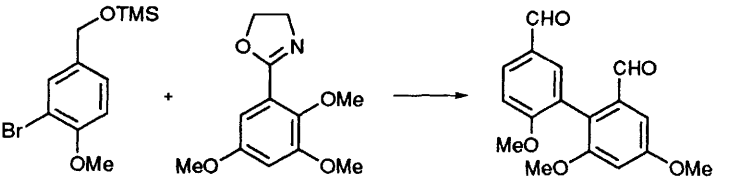
Cysteine homologues $RNHCH_2S(CH_2)_2CH(NH_2)CO_2H$ and $MeS(CH_2)_3CH(NH_2)CO_2H$ that are, from another point of view, also lysine and methionine analogues respectively, are accessible from L-methionine by $Na/NH_3$ cleavage and S-alkylation by $AcNHCH_2OH$, and from L-ornithine through nucleophilic substitution of the derived pyridinium salt by methanethiolate.[250]

Isothiazolidine-1,1-dioxide 3-carboxylic acid[251] can also be viewed in two ways; as a proline analogue or as a homocysteine analogue. A near analogue (51) has been unintentionally prepared, in addition to the expected sulfonamide, through reaction of aspartic acid diesters with arenesulfonyl chlorides.[252]

### 4.14 Synthesis of α-Phosphonoalkyl α-Amino Acids and α-Amino Acids Carrying Other Phosphorus Functional Groups in Side-chains

Representing the simplest trivalent phosphorus derivatives, N-protected 3-(triphosphonio)alanine esters are valuable in synthesis for preparing "vinylglycines" with high optical purity through ylide formation and reaction with carbonyl compounds [with PhCHO → (S,E)-$PhCH=CHCH(NHR)CO_2R'$].[253]

Another simple representative of this family, (2R)-2-amino-5-phosphonopentanoic acid, is available through an interesting new route from (S)-serinal that involves addition of the trimethylsilylethyne carbanion, dehydration-rearrangement to the allene, and (after reductive de-silylation) routine completion of the synthesis.[254] Its near relative, (E)-$H_2O_3PCH_2CH=CHCH(NH_2)CO_2H$, a constituent of plumbemicine, has been prepared through standard bislactim ether methodology or from ethyl 3-ethenyloxazoline 4-carboxylate.[255] The 4-oxo analogue of (2R)-2-amino-5-phosphonohexanoic acid has also generated interest as a receptor antagonist, and homologues carrying methyl substituents at other

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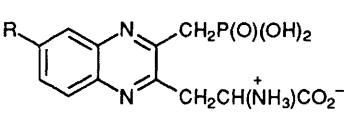


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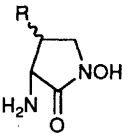


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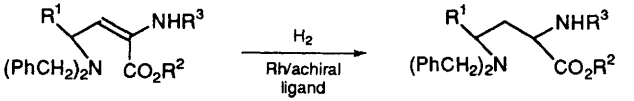



(49) (50)


(51) (52)

side chain locations, have been prepared (they are less strongly bound to receptors).[256]

Synthesis of (4'-phosphonodifluoromethyl)phenylalanines has been covered in the earlier Section 4.8.

Intense synthesis activity must be an accurate description of work leading to the identification and stereospecific synthesis of (2R,4R,5S)-2-amino-4,5-(1,2-cyclohexyl)-7-phosphonoheptanoic acid, the potent, selective, and competitive NMDA antagonist (52), previously prepared in admixture with other stereoisomers (NPC 12626). An efficient nine-step synthesis incorporates the hydantoin method to construct the α-amino acid moiety on the 4,5-bis(carboxymethyl) cyclohexene monomethyl ester.[257]

### 4.15 Synthesis of Isotopically Labelled α-Amino Acids

Direct synthesis of $^2$H-labelled α-amino acids based on enzyme-catalysed exchange in $^2H_2O$ represents the simplest approach, and as illustrated in experiments with *Escherichia coli* cystathionine γ-synthase, can be operated on a gram scale with several protein amino acids.[258] The procedure is most effective with arginine, glutamic acid, histidine, homoserine, and lysine; less so for asparagine, glutamine, methionine, ornithine, and S-methylcysteine; and of insignificant use for other amino acids. Some β-exchange can also be detected. Improved chemical catalytic exchange methods (better than 95% exchange) have been described as applied to syntheses of [2,3,5,6-$^2H_4$]tyrosine and [2,3,4,5,6-$^2H_5$]phenylalanine, as part of an account of new syntheses that include [2,3,5,6-$^2H_4$]phenylalanine and [2,3,6,7-$^2H_4$]tryptophan.[259] $^2H$–$^1H$ Exchange kinetics in $^2H_2O$–$^2HCl$ and $^2H_2O$–$^2H_2SO_4$ for L-phenylalanine, L-tyrosine, and L-tryptophan, and for [2,4,5,6,7-$^2H_5$]L-tryptophan and [3,5-$^2H_2$]L-tyrosine in aqueous HCl, yield activation energies for direct exchange at various atoms in these amino acids.[260]

Stereospecific syntheses in the $^2$H-labelling category have been reported for (2S,3R)-[3-$^2$H]-3-methylaspartic acid,[261] and for (2S,3S)-[2,3-$^2H_2$]-L-ornithine and (2S,3R)-[3-$^2$H]-L-ornithine, by asymmetric homogeneous-catalysed reduction [(R)-Rh-Prophos] of the protected α,β-dehydro-α-amino acid PhCONHC($CO_2Me$)=$CR(CH_2)_2NPht$.[262] Labelled pyroglutamic acids formed by intramolecular ketene trapping in diazomethyl ketones originating in labelled glutamic acids, can be reduced ($BH_3$-$Me_2S$) to (2S,3S)-[3-$^2$H]- and (2S,3R)-[2,3-$^2H_2$]prolines[263]

Tritiated analogues of GABA and β-phenylGABA, respectively, can be secured either through addition of $^3H_2$ to $PhtNCH_2C{\equiv}CCO_2Me$, catalysed by tris(triphenylphosphinerhodium(I) chloride, and work-up to give (E)- and (Z)-$H_2NC^3H=C^3HCO_2H$,[264] or by Pd/C-catalysed addition of $^3H_2$ to the homochiral ester $PhtNCH_2CPh=CHCO_2R$.[265]

Among carbon isotopes, the most interesting synthetic challenges are provided by the need to introduce the short-lived $^{11}$C label within the shortest possible time. There have been many impressive successes in this respect, reviewed recently.[266] New results include an interesting route to α-[3-$^{11}$C]aminoisobutyric acid (Scheme 33) with an unusual N-protection strategy,[267] and independent reports on the preparation of [$^{11}$C]-L-methionine[268] and its DL-analogue.[269]

Strecker methodology leading to DL-[1-$^{13}$C]valine from isobutyraldehyde and K$^{13}$CN followed by resolution using a D-amino acid oxidase–branched amino acid aminotransferase enzyme cocktail illustrates a typical approach to a simple case.[270] L-Glutamic acid can be prepared carrying $^{13}$C at any position through enzyme-mediated syntheses employing appropriately-labelled pyruvic acid, acetic acid, and sodium bicarbonate.[271] $^{14}$C-Vigabatrin has been synthesized.[320]

Multiple labelling is carried out increasingly confidently, illustrated by a one-pot preparation of DL-[2-$^{15}$N,5-$^{13}$C]glutamic acid from 2-bromobutyrolactone using potassium [$^{15}$N]phthalimide and K$^{13}$CN,[272] L-[3,4-$^{13}C_2$]proline and L-[$^{15}$N]proline from correspondingly-labelled L-glutamic acids *via* the appropriate 5-oxo-L-prolines (reduction of the amide grouping *via* the thioamide using $Bu_3SnH$).[273] Stereoselective synthesis and applications of L-[$^{15}$N]- and -[$^{13}$C]-amino acids, prepared either from labelled L-serines produced through bacterial synthesis, or using 1-chloro-1-nitrosocyclohexane as electrophilic amination agent for chiral enolates, has been reviewed.[274]

$^{15}$N-Enriched amino acids can be prepared through use of a coupled enzyme system, e.g. $(^{15}NH_4)_2SO_4$ with α-ketoglutaric acid and glutamate dehydrogenase.[275] Direct chemical synthesis of labelled asparagine and glutamine, based on [$^{15}$N]ammonolysis of the benzyl esters of the N-Boc-amino acids, is straightforward.[276]

$^{18}$F is another short-lived isotope whose properties lead to important medical uses when it is incorporated into an α-amino acid. The topic has been reviewed.[277] The chemistry of fluorination and the need for relatively swift working, in view of the short half-life of the isotope, lead to choice of the tyrosine family as substrates, and there have been new syntheses in this category. 6-[$^{18}$F]- and 4-[$^{18}$F]Fluoro-m-tyrosines have been prepared through $^{18}$F-destannylation (using [$^{18}$F]$^{18}F_2$ and [$^{18}$F]acetyl hypofluorite), of (3'-acetoxy-5'-trimethylstannyl)-phenylalanine, protected as its N-trifluoroacetyl ethyl ester derivative.[278] 6-[$^{18}$F]Fluoro-L-DOPA is accessible (in 110 minutes' reaction time) from 6-nitroveratraldehyde, subjected to nucleophilic [$^{18}$F]fluorination and further elaboration into 2-[$^{18}$F]fluoro-4,5-dimethoxybenzyl bromide and presentation to the Li enolate of the 1-(S)-camphorimine of glycine t-butyl ester or (S)-(-)-1-Boc-2-butyl-3-methyl-4-imidazolidinone, giving *ca.*85% enantiomeric excess.[279]

6-[$^{77}$Br]BromoDOPA is accessible through direct bromination of DOPA.[280]

### 4.16 Synthesis of β-Amino Acids and Higher Homologous Amino Acids

Arndt-Eistert homologation is a standard general approach in this area, and bearing in mind the easy availability of α-amino acids, it is used for the synthesis of β-amino acids more often than for the higher homologues. Its use is illustrated for homologation of cis-4-hydroxy-L-proline (Scheme 34).[281] A new approach to homologation of α-amino acids in which derived ketones are subjected to the Wittig reaction followed by diastereoselective hydroboration, oxidation and de-protection [MBzlNTsCHR'C(=$CH_2$)R → MBzlNTsCHR' $CHRCO_2H$] has been investigated.[282] A novel diastereoselective rearrangement

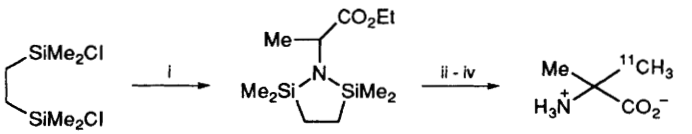
of O-prop-1-enyl α-N-acyl(methylamino)alkyl ethers, leading to β-(acyl-N-methylamino)aldehydes (Scheme 35) amounts to a synthesis of a β-amino acid from an equivalent α-amino acid.[283] A use of α-N-Boc-amino aldehydes for the synthesis of β-(N-Boc-amino)-α-keto acids employs 2-trimethylsilylthiazole to supply the extra carbon atom, through the formation of the aldol-type adduct, the corresponding 2-(β-N-Boc-amino-α-hydroxy)thiazole.[284] Selective reduction of the cyano-group of 1-cyanocyclopropanecarboxylic acid benzyl ester has provided the 1-aminomethyl analogue, required for study as a mechanism-based inactivating agent for mono-amine oxidase.[285]

The addition reactivity of imines has been as useful for β-amino acid synthesis as for the preparation of their α-amino acid analogues, though with the unique benefit of being able to base the method on mild Lewis acid-catalysed addition of ketene acetals (Scheme 36).[286] With the inclusion of vinylic ketene acetals in one of these studies, it was possible to demonstrate the effectiveness of the method for the synthesis of δ-amino acids.

α,β-Unsaturated β-amino acids are produced by "allylic" acylation of imines by carbonyldi-imidazole [$R^1N{=}CR^2CH_2R^3 + Im_2CO \rightarrow R^1NHCR^2{=}CR^3COIm$],[287] and base isomerization of N-benzylimines of β-fluoroalkyl-β-keto-esters gives high yields of corresponding N-benzylidene β-amino acid esters.[288] Addition of malononitriles to imines [$RCH(CN)_2 + R'N{=}CHAr \rightarrow R'NHCHArCR(CN)_2$] calls for high pressures.[289]

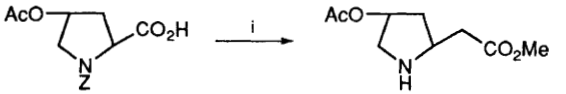
The growth in interest in enantioselective methods for β-amino acid synthesis is now very noticeable. The recent literature includes descriptions of several new procedures as well as extensions of methods used in the asymmetric synthesis of α-amino acids. The imine addition theme is given an interesting variation in a synthesis of (R)-β-amino acids using (S)-prolinol-based hydrazones that can be alkylated by an organometallic reagent (Scheme 37). The synthesis target is released by reductive N-N cleavage followed by ozonolysis.[290] 99% Diastereoisomeric excess is claimed in the addition of a chiral imine (Scheme 36; $R^2$ = (R)-PhChMe-, $R^1$ = Ph) to silyl acetals mediated by an *in situ*-generated homochiral borate complex.[291] The Staudinger reaction (ketene + imine → β-lactam) applied to the homochiral imines $ArCH{=}NCHMePh$ with $AcOCH_2$-$COCl/NEt_3$ gives an unequal mixture of cis-adducts from which, after separation and HCl ring opening, phenylisoserine esters are obtained.[292] A similar approach using the homochiral Evans-Sjogren ketene (from an N-$ClCOCH_2$-oxazolidin-2-one) has been used.[293]

Asymmetric Michael addition has been used previously in the β-amino acid field, usually to establish chirality at the β-carbon atom. Addition of lithium (R)-(–methylbenzyl)benzylamide to t-butyl cinnamate and its 2-methyl analogue gives β-phenylalanine (95% e.e.) and its α-methyl homologue, as well as corresponding β-lactams.[294] Addition of a chiral azepine to t-butyl crotonate followed by hydrogenolysis gives a mixture of erythro- and threo-α-substituted β-amino acid esters,[295] while addition of lithium enolates to homochiral 2-aminomethyl acrylates (53) is exceptionally effective (better than 99% diastereoselectivity) in establishing α-chirality.[296] It would be interesting to see the method extended to acrylates carrying a homochiral 2-(aminoalkyl) grouping, with or without the



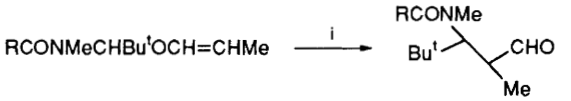
Reagents: i, L–alanine; ii, BuLi + 2,2,6,6 - tetramethylpiperidine; iii, $^{11}CH_3I$; iv, 5M aq. HCl

**Scheme 33**



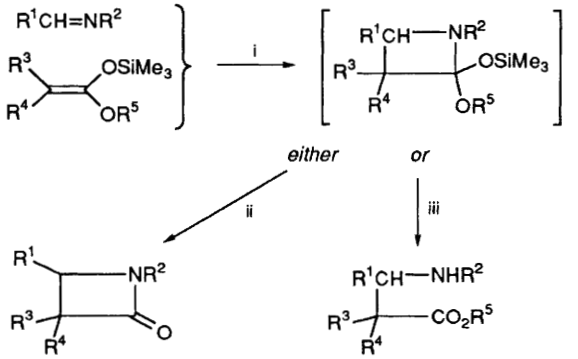
Reagents: i, Bu$^i$OCOCl, NMM; ii, $CH_2N_2$; iii, BzOAg/MeOH, 40 °C; iv, deprotect

**Scheme 34**



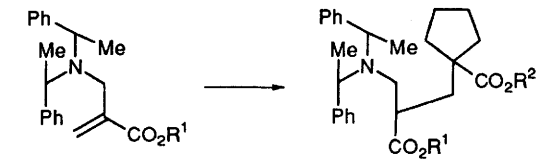
Reagent: i, TMS triflate

**Scheme 35**



Reagents: i, metal salt of Lewis acid coated on dry montmorillonite; ii, $-R^5OM$; iii, $H_2O$

**Scheme 36**



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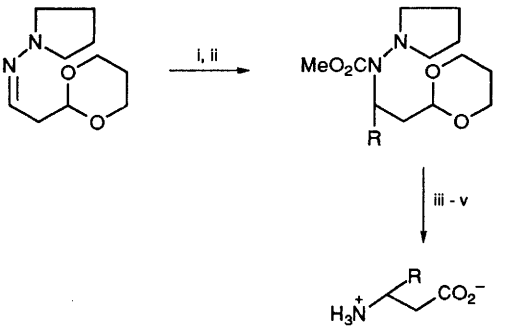
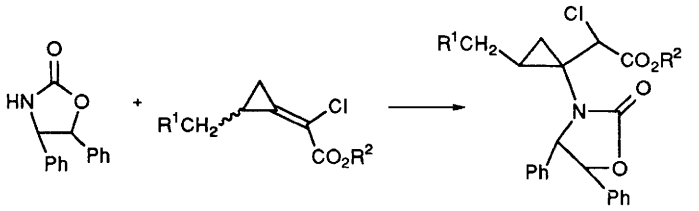


Reagents: i, RM; ii, $MeO_2CCl$; iii, [H](N—N cleavage); iv, $O_3$; v, deprotection

**Scheme 37**



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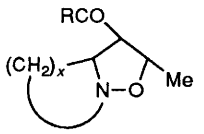
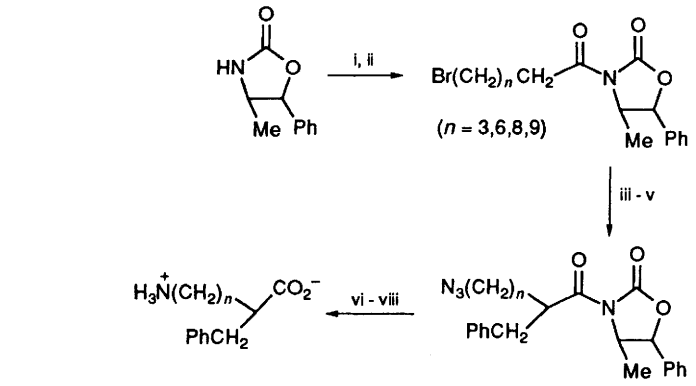


(55)

homochiral N-substituents. Michael addition through the nitrogen atom of homochiral oxazolidin-2-ones to α-cyclopropylidene α-chloroacetates in the presence of 10 mol% KH, mediated by an 18-6-crown ether, yields β-amino acids carrying a β-cyclopropyl function (54).[297] Lewis acid catalysed 1,4-addition of O-benzylhydroxylamine to N-(alk-2-enoyl)oxazolidin-2-ones is a similar example of the genre, leading to homochiral α-substituted β-alanines, with a curious dependence of stereochemical pathway on the Lewis acid ($TiCl_4$ and $Me_2AlCl$ provide opposite diastereoselectivity).[298] $I_2$ catalysed 1,3-dipolar addition of cyclic nitrones to homochiral bornane-1,2-sultam esters of crotonic acid gives β-amino acid derivatives (55) with high diastereoselectivity, from which piperidine and pyrrolidine alkaloids, (+)-sedridine and (+)-hygroline respectively, were obtained by further elaboration.[299] Intramolecular nitrone–alkene cycloaddition involving homochiral $PhCH_2N^+(O^-)=CH(CH_2)_3CH=CHR$ [R = (S)-CHMeOH] yields the naturally-occurring β-amino acid cis-pentacin, after hydrogenolysis and oxidative elaboration of the cycloadduct.[300] Acryloyl chloride $CH_2=CHCOCl$ reacts through the Michael addition pathway in aza-annulation of enamines formed between $BuNH_2$ and β-ketoesters to give δ-lactam epimer mixtures,[301] and a straightforward Michael reaction gives β-amino-β-(pyrimidin-5-yl)propanoic esters.[302]

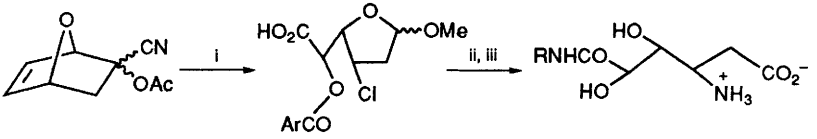
Several research groups have established the merits of Evans methodology for the asymmetric synthesis of β-amino acids using chiral oxazolidin-2-ones (already illustrated in this Section to put stereochemical bias on to the Michael addition route). The acylation–azidation–alkylation sequence shown in Scheme 38 is not quite the way things are done for α-amino acid synthesis![303] The Nicholas reaction applied to boron enolates of N-acyloxazolidin-2-ones (Scheme 39) leads to excellent diastereoselectivity.[304] The closely-related approach employing alkylation (LDA/MeI or $PhCH_2X$) of (S)-1-benzoyl-3,6-dimethylperhydropyrimidin-4-one (prepared by cyclization of the Schiff base of (S)-3-aminobutanoic acid)[305] is a hidden form of a general approach in which one homochiral β-amino acid is used to synthesize another, also illustrated in uses for L-aspartic acid *via* its N-toluene-p-sulfonyl anhydride and thence *via* the protected 4-iodo-3-amino acid synthon to (R)-γ-alkyl-β-amino acids[306] and their α-hydroxy-analogues.[307] This route,[306] which can be operated on a multigram scale, exploits regioselective $NaBH_4$ reduction of the anhydride, $Me_3SiI$ lactone cleavage, and substitution of the iodo-atom by $R_2CuLi$, leading to (R)-γ-alkyl-β-amino acids after somewhat fierce de-toluene-p-sulfonylation process (refluxing in 47% aq HBr/PhOH). (R)-S-Methylcysteine is used as starting material for a homochiral 4-methylene-oxazolidin-5-one, from which 1-aminobicyclo[2.2.1]heptane-2-carboxylic acids can be prepared through Diels-Alder addition.[308] C-2-Alkylation of the homochiral N,O-acetal (56) using $Bu_3SnCH_2CO_2Et$ followed by $Pb(OAc)_4$ cleavage of the resulting N-[(S)-1-phenyl-2-hydroxyethyl] β-amino acid, is offered as a new enantioselective approach to these amino acids.[309]

Nucleophilic ring-opening of N-toluene-p-sulfonylaziridines (57) using a Grignard reagent with CuBr-$SMe_2$/THF-HMPA has been established to give modest (0–55%) yields of (R)-N-toluene-p-sulfonyl-β-amino acids.[310] The aziridines can be prepared from an L-serine ester in seven steps. Chiral aziridines (58)



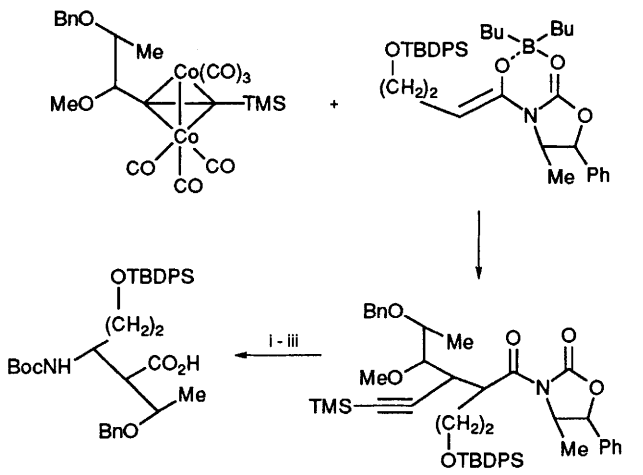

Reagents: i, BuLi; ii, $Br(CH_2)_n CH_2COCl$; iii, $NaN_3$/DMF; iv, $NaN(SiMe_3)_2$; v, $PhCH_2Br$; vi, [H]; vii, $PhCH_2OLi/PhCH_2OH$; viii, various protection-deprotection steps

**Scheme 38**



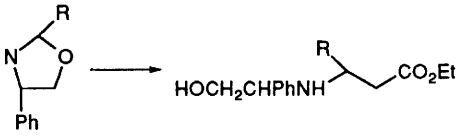

Reagents: i, MeOH – $Cl_2$; ii, $NH_3$, iii, [O]

**Scheme 40**




Reagents: i, $H_2O$; ii, Curtius (DPPA/Bu$^t$OH) degradation;
iii, $-C\equiv C- \longrightarrow -CO_2H$ with $OsO_4/NaIO_4$

**Scheme 39**

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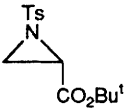
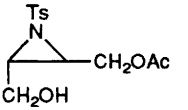



(57)

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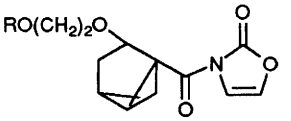


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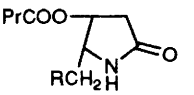



(60)

have been used to synthesize enantiomers of $ZNHCH(CH_2Ph)CH(OH)CO_2Me$, which are key intermediates for bestatin synthesis.[311] The use of lipase for enantioselective transesterification of methyl trans-β-phenylglycidate and successive ring-opening with HBr, azidolysis, and routine elaboration provides the (2R,3S)-enantiomer of the taxol side-chain, phenylisoserine.[312]

Corresponding ring-opening processes are well-established for azetidinones, the interest residing as much in the methods of synthesis of the four-membered rings as in the β-amino acids. Recent examples are C-4-alkylation, using titanium enolates, of azetidinones formed by cycloaddition of chiral imines derived from (S)-mandelic aldehyde or (R)-glyceraldehyde,[313] and C-4-deuteriation of homochiral 3-trimethylsilyl-4-phenylthio-azetidinones leading to stereospecifically-C-3-deuteriated β-alanines.[314]

Azidolysis of the methanesulfonate of homochiral 4-hydroxy-3-methylhex-1-enes, and alkene → $CO_2H$ conversion, provides (2R,3R)- and (2R,3S)-isomers of 3-amino-2-methylpentanoic acid, verifying through comparison with moieties from majusculamide C and dolastatins that these contain the (2S,3R)-isomer.[315]

Unusual synthesis methods for β-amino acids, whose course is determined by the particular synthetic target, have been described for a synthesis of N-alkylamides of the β-amino acid component of the gastroprotective agent AI-77B (Scheme 40),[316] and for (+)-megamycin and its 5-epimer [a fifteen-step synthesis involving Pd(II)-assisted alkylation of a homochiral ene carbamate followed by carbonylative coupling to a trialkylvinyltin].[317] The (9R)-isomer of the 3-amino-10-phenyl-2,5,9-trihydroxydecanoic acid known as "Ahda" has been synthesized by a route starting with a C-C bond-forming step involving the appropriate aldehyde and $(MeO)_2P(O)CH_2COCH_2CH(NHBoc)CH(OR)CH_2OR$.[318] A C-C bond-forming step is involved ($N^{\alpha}$-Boc-$N^{\delta}$-Z-ornithine + ethyl lithioacetate) in a synthesis of (2R,3S)-3-amino-2-carboxymethylpiperidine.[319]

γ-Amino acids are of increasing general interest because of pseudopeptide field, and for the growing number of members of the family contributing useful physiological properties. Standard methods are illustrated for $^{14}C$-vigabatrin (4-amino-5-hexenoic acid), prepared from 5-hydroxymethyl-pyrrolidin-2-one toluene-p-sulfonate and $Na^{14}CN$, and reduction of the nitrile in the presence of $Me_2NH$.[320] and the natural (S)-isomer, synthesized from either L-glutamic acid through elaborating the α-carboxy group ( → $-CH_2OH$ → $-CHO$ → $-CH=CH_2$)[321] or from D-methionine through a similar sequence but using $(EtO)_2P(O)CH_2CO_2Et$ for the Wittig reaction step, and converting the methylthioethyl side chain into the vinyl moiety.[322]

Vinylogous α-amino acid esters, $R_2NCHR'CH=CHCO_2Et$, undergo Michael addition ($MeNO_2$/DBU) to give β-substituted γ-amino acid esters.[323] A useful route to a synthon for homochiral vinylogous esters involves homogeneous metal-mediated hydroformylation (CO, $H_2$) of (R)-2-t-butyloxazoline.[324]

(R)-(-)-Baclofen has been prepared by Evans' methodology (cf. Scheme 12) through alkylation of the chiral enolate with $BrCH_2CO_2Bu^t$ and routine elaboration.[325]

(R)-(-)-GABOB (γ-amino-β-hydroxybutyric acid) and (R)-carnitine have been prepared by catalytic asymmetric dihydroxylation of allyl bromide using

$K_3Fe(CN)_6/K_2OsO_2(OH)_4$ in the presence of a dihydroquinidine-derived ligand, conversion of primary OH to CN, and routine elaboration.[326] A more traditional approach to GABOB and to isoserine involves enzymatic kinetic resolution of acetylated racemates formed from cyanohydrins $EtO_2C(CH_2)_nCH(OH)CN$.[327]

A new homologation employs the aluminium acetals that have recently been discovered as intermediates in the low temperature DIBAL reduction of esters; these add to silylketene acetals and allylstannanes in the presence of a Lewis acid, to give γ-amino-β-hydroxy esters.[328]

As in recent years, considerable interest is being sustained in statine synthesis, including routes leading also to stereoisomers and analogues. The chiral synthon (59) is amenable to electrophilic addition [59; R = Me + $Br_2$/ $MeC(OMe)_3$ → N-substituted (4S,5S)-4-bromo-5-methoxyoxazolidin-2-one → allyl replacing Br with retention of configuration using $CH_2=CHCH_2SnMe_3$/ hν],[329] and Ru(II)-catalysed intramolecular cycloaddition [59; R = $COCHR^1R^2$, $R^1$ = $R^2$ = Cl or F],[330] giving enantiomerically-pure products.

Other statine syntheses have been developed; using pyrrolidin-2-ones (60) prepared from methyl (E)-4-chloro-3-methoxybut-2-enoate and incorporating lipase-mediated kinetic resolution,[331] using (4R,5S)-oxazolidin-2-ones (61) prepared in 8 steps from D-glucosamine, leading to natural statine and analogues,[332] or using tetramic acids (62) prepared from (S)-4-N-Z-3-oxo-alkanoate esters through DMAP-catalysed cyclization, and used in a syn-statine synthesis.[333] Pd-Catalysed [3.3]-sigmatropic rearrangement of trichloroacetimidates prepared from homochiral vinylogous α-amino acid esters through reduction [$-CO_2Et$ → $-CH_2OH$ → $-CH_2OC(=NH)CCl_3$] leads to 3-aminodeoxystatines.[334]

N-Protected L-serine and (S)-prolinol undergo DABCO-catalysed Baylis-Hillman addition to methyl acrylate *en route* to the novel sphingosine analogue (63).[335] The increasing use of homochiral aldoses for amino acid synthesis is illustrated in the use of the protected furanose (64) in stereospecific azidolysis and elaboration into 2,3-dihydroxy-4-aminobutanoic acid and its 5-aminopentanoic acid analogue.[336] L-Glutamine acts as starting material for a synthesis of 1-aminoalkyl-4-carboxy-3,4,5,6-tetrahydropyrimidines, pyoverdin constituents that are formally δ-amino acids.[337] Homochiral 5-amino-2-hydroxy-4-oxoalkanoic acid derivatives have been prepared starting from L-α-amino acid-derived $\Delta^2$-1,2-oxazetidines.[338]

All four stereoisomers of 2-methyl-4-hydroxy-5-aminopentanoic acid are obtainable from D- or L-glutamic acids *via* the lactone (65) and its enantiomer;[339a] 5-amino-4-hydroxyalkanoic acids and 3-amino-2-hydroxyalkanoic acids can be approached starting from D-isoascorbic acid.[339b] Synthesis of δ-phthalimido-γ-keto esters has been illustrated in a specific case, employing methyl 3-iodo-2-methylbutanoate and the acid chloride of phthalimido-L-phenylalanine.[340]

New homochiral 7-aminoalkanoic acids are accessible in the form of N-protected δ-amino-γ-lactones, from analogues of (65), through toluene-p-sulfonylation, azidolysis, and conventional elaboration.[341]

## 4.17 Resolution of DL-α-Amino Acids, and Assignments of Absolute Configuration to Enantiomers of α-Amino Acids

The increasing number of papers on this topic collected here for discussion, include several that are cross-referenced to other sections of this Chapter, since resolution is often a routine terminal step in a synthesis route.

Conventional procedures based on diastereoisomeric derivatives have been used for 2,3-methanopyroglutamic acid (salt formation with L- or D-leucinamide).[342] Correlation of the enantiomers with 2,3-methanoproline, whose absolute configuration was previously established by X-ray crystallography, shows that (-)-2,3-methanopyroglutamic acid is the (2S,3S)-isomer. New variants of this classical resolution procedure include O-benzyl derivatives of (S)-(+)- and (R)-(-)-2-aminobutan-1-ol (resolution of N-acetyl α-phenylglycine and α-(4-hydroxyphenyl)glycine),[343] and moderately-effective mutual resolution of amino acids (81% and 74% enantiomeric excesses, respectively, for phenylalanine and α-phenylglycine) and mandelic acid complexed with Cu(II) ions.[344] Resolution of α-methyltryptophan by co-ordination to Co[(R,R)-N,N-di(2-picolyl-1R,2R-diaminocyclohexane] has been described.[345]

Studies of the underlying physical basis of resolution using these principles are illustrated by an estimation of interactive forces between enantiomers (L- + D-pairs of alanine and phenylalanine have lower energy than L-L- and D-D-pairs).[346] This study has also demonstrated enantiomeric molecular recognition between 4-nitrobenzoylamino acids and N-butyroylvaline t-butylamide. A detailed X-ray study of interactions at the molecular level between one of the classical resolving alkaloids, brucine, and N-phthaloyl threo-β-hydroxy-D- or -L-leucine, reveal hydrogen-bonding between the carboxy and hydroxy groups and the methoxyindole moiety of the alkaloid, as well as electrostatic and van der Waals interactions.[347]

Asymmetric transformations of a traditional nature involve tartaric acid and salicylaldehyde [applied to (R,S)-1,3-thiazane-4-carboxylic acid and leading to enantiomers of homocysteine],[348] and carboxylic acid-catalysed racemization and asymmetric transformation of "unwanted" enantiomers formed during resolution of (R,S)-N-methyl-2-phenylglycine with (1S)-camphor-10-sulfonic acid and of N-ethyl-N-methyl-2-phenylglycine with (R)-phenylethylamine.[349]

Growing interest in the use of homochiral macrocyclic hosts for the resolution of amino acids and derivatives has been reviewed.[350] Research papers illustrating well-established principles concentrate on acetylated and methylated cyclodextrins,[351] a homochiral 18,6-crown ether synthesized from D-mannose,[352] and 36-membered ring pseudopeptides prepared from alternating glycine and (2S,3'S)-4-methyl-2-(2'-oxo-3'-isobutyl-1'-piperazinyl)pentanoic acid moieties.[353] The last-mentioned study includes 24- and 27-membered ring analogues, which are more effective than the larger ring in the resolution of (R,S)-alanine N-methylanilides. Similarly exquisite tailoring of the helicity of the tetrakis (o-aminophenyl)porphyrin ring by connecting the amino groups through different bis(acyl) chains is rewarded with significant chiral recognition in the formation of 1:1-adducts of Zn complexes with amino acid esters.[354] Proline (S)- or (R)-phenylethylamides and (S)- or (R)-lactate esters respond enantio- and diastereo-

selectively to new examples (see Vol.25, p.54) of conformationally homogeneous host podand receptors (66 and hexacyclic analogues) by undergoing enantiopreferential complexation and partition into chloroform.[355]

Selective transport of L-enantiomers of phenylglycine, phenylalanine, and tryptophan has been observed through membranes prepared through crosslinking of poly[γ-(2-chloroethyl)-L-glutamic acid] with diethylenetriamine.[356] Similar discrimination occurs in crown ether-mediated transport of amino acids through supported liquid membranes containing o-nitrophenyl octyl ether.[357]

Chromatographic separations of racemic mixtures based on heterogeneous processes based particularly on the chiral stationary phase (CSP) approach, continue to be studied in detail. The notion of "entangled pairs" has been advanced for enantiodiscriminating interactions between racemic solutes in aqueous media with CSPs using N-(undec-10-enoyl)-L-valine t-butylamide and N-(hex-5-enoyl)-L-valine t-butylamide.[358] Equally subtle design leading to chiral brush-type CSPs has been described, providing unexpected consequences; the separation efficiency and even the order of elution of enantiomers are temperature-dependent.[359] Conventional applications of commercially-available Chirasil-Val CSPs and oligopeptide analogues, continue to be described in the research literature.[360] α-Cyclodextrin dodecabenzoate-modified silica gel has been advocated for chromatographic resolution of p-nitrophenyl esters of Z-amino acids.[361] Insoluble proteins offer readily-available chiral surfaces, and immobilized human serum albumin has been studied in this context for the resolution of DL-tryptophan.[362] In establishing baseline resolution in the HPLC mode in less than 2 min elution time, these workers found that the L-enantiomer binds to the indole site of the protein while the D-enantiomer has no interaction with this site but is attracted indirectly to the warfarin site.

The recent excitement (Vol.25, p.51) generated by molecularly-imprinted stationary phases seems to have subsided as measured by the volume of the associated literature, but no doubt much is going on in research laboratories in view of the potential benefits. An indication that the potential of such phases in related areas is being recognized, is the finding that a silica-alumina surface imprinted with bis(N-benzyloxycarbonyl-L-alanyl)amine, $(Z\text{-L-Ala})_2NH$, exhibits discrimination towards L-, D-, and meso-isomers of the structurally similar anhydride $(Z\text{-Ala})_2O$.[363]

The preferential crystallization technique has been established as a simple, effective large-scale method for the separation of an enantiomer from a racemic amino acid, and the range of examples susceptible to this technique is being steadily extended over the years; the accessibility of D-allo-threonine in this way has been established recently.[364]

Enzyme-catalysed enantioselective hydrolysis and related processes with DL-amino acid derivatives are casually referred to as "resolution", though in many instances only one enantiomer of the racemate is accessible. α-Chymotrypsin continues to be a popular choice in this context, with studies of DL-phenylalanine esters in a liquid/liquid/solid three-phase system indicative of the potential of large-scale working.[365] Studies include α-chymotrypsin-catalysed hydrolysis of α-alkenyl-DL-α-amino acid esters.[366] N-benzylidene DL-amino acid

esters,[367] and β-(isoxazol-4-yl)-DL-alanine esters[368] represent more conventional laboratory studies. In contrast to proteases, carbonic anhydrase-catalysed hydrolysis of N-acetyl DL-amino acid esters favours the D-enantiomer.[369] Penicillin acylase-catalysed hydrolysis has been applied to N-phenylacetyl derivatives of threo-β-(4-fluorophenyl)serine and (2-, 3-, or 4-fluoro- and 2,3,4,5,6-pentafluorophenyl)alanines[370] and to N-phenylacetyl-DL-β-amino acids[371] and to analogous γ-ethynyl-, γ-allenyl-, and γ-vinylGABAs.[372] D-Aminoacylase from *Alcaligenes faecalis* releases D-enantiomers from N-benzoyl- and -benzyloxycarbonyl-DL-amino acids,[373] while the more common aminoacylases, immobilized by bonding to alginate, effectively catalyse the hydrolysis of the L-enantiomer of N-acetyl DL-phenylalanine.[374]

Lipase-catalysed hydrolysis (see also Refs.312, 331) has been used with fluorinated 3-acetoxy-2-(methoxyimino)butanoates, syntheses of enantiomers of mono-, di-, and tri-fluorothreonines and allo-threonines being completed by hydrogenation of the methoxyimino group.[375] Non-protein amino acids, derivatized as 2,2,2-trifluoroethyl esters, can be resolved by lipase in organic solvents by enantioselective transesterification with methanol.[376] A combination of lipase (from *Pseudomonas cepaeia*) for ring-opening of oxazol-5(4H)-ones and thiazol-5(4H)-ones into N-benzoyl- and -thiobenzoyl-L-amino acids, and protease-catalysed kinetic resolution, is advocated for efficient production of L-amino acids.[377]

Exploitation of the propensity of alcalase to tolerate organic solvents as operating medium is seen in its use as catalyst for the hydrolysis of DL-amino acid esters, leading to precipitation of the L-enantiomer.[378] High enantiomeric excess and effective use of the technique with several "unnatural" amino acids is dependent upon the lowest possible water content in the medium consistent with a reasonable reaction rate.

The use of *Arthrobacter* D-amidase for the preparation of D-alanine from DL-alaninamide has been described.[379]

Conversion of L-glutamic acid into D-glutamic acid qualifies for inclusion in this Section of this Chapter. Successive reactions of glutamate racemase (from *Lactobacillus brevis* ATCC 8287) and glutamate decarboxylase (to break down any remaining L-glutamic acid) can be operated efficiently on a large scale.[380]

Whole-bacteria applications have been described for the conversion of DL-5-substituted hydantoins into L-amino acids using *Pseudomonas* sp.strain NS671,[381] and for soil bacteria immobilized in poly(acrylamide), acting on the same substrate to give D-9-hydroxyphenyl)glycine.[382] A common problem in such processes is the inefficiency associated with the consumption by the bacteria of the released amino acids, but the method is viable for the production of L-methionine.

The evolution of the L-amino acids over geological time is a topic under the heading of "resolution" that has been a source of speculation informed by advances in physics and in organic chemistry for many years. One approach–the preferential destruction of D-amino acids in racemates–has been encapsulated as the Vester-Ulbricht theory, and another claim[383] that positron annihilation brings about this result in crystalline leucine must be balanced against the many

opposite assertions in the recent literature. Chiral amplification over time, of any microscopic bias in the L:D-ratio, is also the subject of speculation, and a modification of the respected Frank hypothesis has been proposed.[384] The hypothesis proposes that two types of reaction are involved, autocatalytic generation of L- and D-amino acids and an interaction between them by which they eliminate each other, and it has been suggested that instead of both steps being irreversible, the first step could be considered reversible. The racemization process that opposes the effect of any chiral amplification mechanism, has been considered[385] within the context of the open-chain non-equilibrium model proposed by Kondepudi and Nelson.[386]

Keeping all options open, the prior genesis of homochiral carbohydrates could explain the predominance of L-amino acids on the basis of "hetero-pairing"–as known for many years, the complexing of L-amino acids with nucleic acids involving D-ribose is energetically more favourable than L–L (and D–D) complexation.[387]

## 5 Physico-chemical Studies of Amino Acids

### 5.1 X-Ray Crystal Structure Analysis of Amino Acids and Their Derivatives

Although the usual style for this section continues, with papers that report factual material predominating over studies with a deeper penetration, there are some more interesting insights than usual in this year's literature.

Structures for amino acids themselves and their salts have been reported for diglycine hydrochloride,[388] DL-proline hemisuccinate,[389] L-leucine nitrate,[390] sodium cysteine-S-sulfonate,[391] the copper(II) chloride complex of 3,5-di-iodo-L-tyrosine,[392] abrine (*alias* N-methyol-L-tryptophan),[393] and GABA.[394] L-Alanine crystals involve a strong hydrogen bond and significant methyl-methyl interactions, as determined by coherent inelastic neutron scattering data which also provide a measure of vibrational details on a picosecond timescale.[395] Comparison of X-ray details for L-tryptophan picrate and its DL-tryptophan analogue reveals three different sorts of indole–picric acid stacking modes.[396] X-Ray study of DL-histidine–succinic acid (1:3) crystals into which aqueous MeCN is diffused adopt DL-histidine hemisuccinate dihydrate stoichiometry in contrast to the L-histidine system in which the trihydrate is the final state.[397]

N-Substituted amino acids reported on recently are N-di-t-butoxy-carbonyl-L-alanine,[398] N-acetyl-L-homocarnosine monohydrate,[399] N-Z-DL-2-amino-4-phosphonobutanoic acid monohydrate,[400] and $N^2$-toluene-p-sulfonyl-L-glutamine.[401] N-Substituted amino acid esters (N-diphenylmethylene-L-threonine methyl ester,[402] N-acetyl-L-tyrosine ethyl ester monohydrate,[403] N-phthaloyl β-phenylserine methyl ester [shown to be the (2S,3R)-isomer],[404] and N-t-butoxy-carbonyl-L-valine N-hydroxysuccinimide ester[405] have been studied.

### 5.2 Nuclear Magnetic Resonance Spectroscopy

Those papers under this heading that are aimed at more than routine data-collecting are given space here. $^1$H-NMR spectra for Boc-L-valine N-acylurea in

DMSO-$^2H_6$ reveal an intramolecularly hydrogen-bonded ureide NH proton,[406] while studies of pH-dependent chemical shifts for N-acetyl-L-aspartic acid suggest that awareness of the phenomena observed could avoid mistaken interpretations of spectra of similar solutes.[407] In particular, the corresponding signals for N-acetyl-L-aspartic acid overlap those of acetate at pH 4.7, but in more acidic solutions the acetate signals are further downfield. Another well-established principle underlies the use of $^1$H-NMR in assessing the optical purity of α-N-Boc-amino aldehydes through the slightly differing chemical shift of the Boc resonance seen for each diastereoisomer for semicarbazones formed with (S)-PhCHMeNHCONHNH$_2$.[408]

More common practice now, is to call upon interpretations of NMR spectra derived from two or more nuclei to obtain secure information, for example the 56:44 ratio of conformers of trans-4-hydroxy-N-Fmoc-L-proline in solution determined through $^1$H–$^{13}$C studies,[409] and the likelihood of indole ring distortion in 4-methyltryptophan in 0.1M NaO$^2$H/C$^2$H$_3$O$^2$H.[410] A combination of $^1$H–$^{13}$C NMR with molecular orbital calculations and X-ray crystallographic analysis has led to identification of an exclusive chair conformation for 1-aminocyclohexane-1,3-dicarboxylic acid diastereoisomers.[411] Routine stereochemical information can be obtained by NMR measurements through the Mosher approach, derivatization with (S)-[methoxy(trifluoromethyl)phenylacetyl] chloride,[412] and Eu(hfc)$_3$ shift studies, illustrated for the latter case for enantiomeric purity determinations with N-phthaloyl 2-cyanoglycine.[413]

Applications of $^3$H-NMR to tritiated amino acids have been reviewed.[414]

$^{13}$C-NMR has been applied to a precise determination of $^{14}$N/$^{15}$N equilibrium isotope effects on the acid-base chemistry of the amino group of amino acids in solutions, through determining chemical shift data for the carboxyl carbon atom as a function of pH.[415] Sophisticated applications of solid state $^{13}$C-NMR are becoming more frequent, with correlations of protonation state with shielding of the carboxy groups in microcrystalline amino acids,[416] studies of inter- and intramolecular interactions in crystalline amino acids in which the asymmetric unit cell contains three L-isomers and one D-isomer,[417] and measurements of $^{13}$C-chemical shift anisotropies of solid amino acids involving spinning side band separation of protonated and non-protonated carbon atoms in slow spinning conditions *via* dipolar dephasing.[418] The interpretations of NMR spectra for nitrogen nuclei in amino acids remain divided between the acquisition of fundamental physical data, such as the quadrupole coupling tensor for the $^{14}$N nucleus in a single crystal of L-alanine by the overtone NMR approach,[419] and their use for establishing particular structural features, such as the existence of individual tautomeric forms of histidine in aqueous ethanol at −55°C, with only a very weak hydrogen bond between the π-NH group and the α-amino group (previously claimed to be a more significant structural feature).[420]

$^{31}$P-NMR features of phosphonamides (67, for the L-amino acid) formed by derivatization of partly-resolved amino acid esters, provide accurate estimates of enantiomer ratios.[421]

### 5.3 Optical Rotatory Dispersion and Circular Dichroism

Those early applications of these complementary techniques that were used to assign absolute configuration to amino acids, based on the sign of a particular Cotton effect, are now rarely used. The revised geometry for polyoximic acid (Scheme 22) does not query the absolute configuration originally assigned by o.r.d. methods. The unique spectroscopic basis of the techniques can be exploited to follow the course of a chemical change, as in the case of electrochemical oxidation of L-tryptophan.[422]

Architectural features of complex systems, and changes occurring within them, can also be picked out, as for the identification, based on the large positive CD centred at 213–215 nm, of micellar aggregation of N-palmitoyl- and N-stearoyl-L-serines in aqueous solutions.[423] The CD arising through coupled amide chromophores in a regular array around the micelle surface, is largely lost by disintegrating the micelles in 50% aqueous ethanol. A different explanation has been given for the same strong CD feature, seen for methanol solutions of N-dodecanoyl derivatives of L-glutamic acid and L-valine, together with a smaller negative CD peak at 240 nm.[424] These results are now interpreted to indicate dimerisation (supported by IR evidence) and the presence of two different rotamers, and in this respect these workers have replaced a previous interpretation involving hydrogen bonding between carboxy groups and NH moieties.

### 5.4 Mass Spectrometry

All the papers from the 1993 literature discussed here deal with spectra generated for the amino acids themselves using the more sophisticated instrumental variants. Interpretation of spectra obtained for derivatized amino acids through standard ionization techniques now generally amounts to a routine exercise, and papers covering this approach are mostly excluded from this review.

$^{252}$Cf-Plasma desorption MS of glycine–alkali metal salt mixtures [425] and of mixtures of 3, 4, or 5 amino acids,[426] in both positive ion and negative ion modes, have been interpreted. Strong $MH^+$ and $[M - H]^-$ parent ions are formed. The negative ion mode responds most easily to interpretation. Plasma desorption MS provides more prominent parent ions with a range of energies, and compares favourably with ammonia and methane CIMS for the leucine-and-isoleucine test case.[427]

Aqueous solutions of amino acids, sampled by the atmospheric pressure electrospray technique, yield positive ions in intensity order alanine, leucine threonine, serine, aspartic acid, glutamic acid.[428]

Cycloalkane-based β-amino acids have been shown to conform to the general pattern for primary amines in favouring α-cleavage at nitrogen after ionization in the mass spectrometer.[429]

Techniques leading to significant fragmentation can occasionally provide useful stereochemical information, as revealed in an interesting FAB-MS distinction between the N-benzyloxycarbonyl derivatives of γ-hydroxyornithine diastereoisomers due to the faster side-chain dehydration shown by the negative ion of the threo-isomer.[430]

## 5.5 Other Spectrometric Studies of Amino Acids

This section exists to acknowledge the variety of relevant work on amino acids involving spectrometric techniques in addition to those already covered in preceding sections, but again, excludes routine material.

Rotational spectra for alanine have been interpreted in terms of dipole moment data showing the presence of two conformers corresponding to those already demonstrated for glycine.[431] Another example of extensions of earlier work describes IR spectra of $CCl_4$ solutions containing N-Boc-L-proline N-methylamide and phenol, interpreted to reveal the formation of hydrogen-bonded complexes involving the amide carbonyl group.[432]

At first sight, ESR spectra of $CaCO_3$ and hydroxyapatite doped with amino acids represents a routine study in giving the expected signals for radicals derived through side-chain cleavage.[433] However, the septet for isopropyl radicals derived from L-valine is accompanied by signals for the t-butyl radical, indicating the involvement of potentially interesting heterogeneous chemistry. More conventional ESR research is illustrated by monitoring $^2H$–$^1H$ exchange processes occurring in a γ-irradiated single crystal of L-alanine.[434]

Electronic absorption spectra of analogues of phenylalanine and tyrosine constrained within a supersonic jet have been obtained using laser-induced fluorescence measurements.[435]

## 5.6 Other Physico-chemical Studies of Amino Acids

A number of novel strands of research have developed in recent years under this heading, and most of them continue to be pursued. Membranes capable of penetration by amino acids have been of considerable interest, especially when they show enantioselective transport properties (see the earlier Section 4.17 Resolution), and a novel twist is shown in a property of some membranes to allow the transport, by ε-Schiff bases formed between $N^{\alpha}$-Z-L-lysine methyl ester and copper(II) or nickel(II)-3-substituted salicylaldehydes, of Li, Na, K, Cs, Ca and ammonium ions.[436] Gels made up of micellar rods and vesicular tubules form from aqueous solutions of L-lysine derivatives $H_3N^+CH[(CH_2)_4NHCO(CH_2)_{11}NH_2]CO_2^-$.[437]

Thermodynamic data accumulated over recent years, feature enthalpies of solution of amino acids in the 0.005–0.07 mol $Kg^{-1}$ concentration range,[438] enthalpies of dilution of aqueous solutions of β-alanine, α-aminobutyric acid, γ-aminobutyric acid, ε-aminocaproic acid, α-aminovaleric acid, and threonine,[439] and enthalpic pairwise interaction coefficients of N-acetyl-L-leucinamide and N-acetylglycinamide in concentrated aqueous tetramethylurea and in urea.[440] The last-mentioned study indicates structure-dependent coefficients, suggesting that protein denaturation in these media is a complex process. Calorimetric studies have been extended to amino acids with heteroatoms in their side-chains.[441]

Partial molar characteristics (apparent molar volumes, apparent compressibilities, etc) have been determined for aqueous solutions of glycine and alanine under high pressures, by ultrasound methods.[442] More conventional studies have been reported for apparent molar volumes of amino acids in aqueous solutions of

varying KCl concentrations,[443] for limiting partial molar volumes obtained from density measurements of aliphatic amino acids in water containing various admixtures of HCl and NaOH,[444] and for partial molal isothermal compressibilities of glycine and alanine in aqueous solution.[445]

On a simpler conceptual level, the cryoprotectant role of amino acids *in vivo* is reflected in studies of aqueous glycine in canine renal tubules,[446] and separations of amino acid mixtures are represented in measurements of crystal growth kinetics of L-alanine from solutions containing L-phenylalanine or L-leucine.[447]

Hydrophobicity values for tryptophan deduced from partition coefficient data, and hydrophilicity and lipophilicity values for the same amino acid determined from vapour-to-solvent coefficients, have been reported.[448]

Stability constant data for proton- and metal-ion-complexation equilibria for aliphatic amino acids have been reviewed,[449] accompanying new data for mixed ligand complexes of lysine + aspartic acid, lysine + succinic acid, and glycine + malonic acid.[450]

The effect of ionic strength on the acid-base stoichiometric ratios for L-valine[451] has been determined, as has the role of urea (1–8 mol $dm^{-3}$) in suppressing the first ionization constant of amino acids.[452] Fingers have been wagged at those who draw titration curves incorrectly for amino acids to show the change of charge distribution as a function of pH–graphs can be mis-shapen, or the axis of the graph can be mis-labelled.[453] The extraordinary development of scanning tunneling microscopy has been extended in the amino acid field with visualization of individual molecules of glycine, alanine and phenylalanine adsorbed on graphite.[454]

### 5.7 Molecular Orbital Calculations for α-Amino Acids

As usual, the papers collected for this section defy tidy classification, although all are aimed in one way or another at assisting understanding of amino acid structures and properties.

Extensions have been published to a series of papers (Vol.25, p.60) advocating a molecular connectivity model for describing physico-chemical properties of α-amino acids.[455]

Conformational assignments and energies of intramolecular interactive forces are frequently represented in papers under this heading, and N-formyl L-valinamide[456] and other N-formyl amino acid amides[457] and N-acetyl-L-alanin-amide[458] have received detailed attention in this context. *Ab initio* IGLO (individual guage for localized orbitals) calculations for N-acetylglycine N-methylamide have been aimed at relating isotropic $^{13}C$ chemical shifts to putative conformations.[459] These amino acid derivatives are obviously chosen as models for residues in proteins, so that meaningful statements about the behaviour of amino acids in this context may be made, but more direct models have been used, to assess the effect of change of configuration on the phenylalanine residue at the active site of thermolysin,[460] and to assess the influence of neighbouring side-chains on particular amino acid residues in proteins.[461]

For the amino acids themselves, calculations have been reported for

interaction energies of the 20 coded amino acids,[462] for ground state geometries and energies of first excited singlet states of phenylalanine and tyrosine,[463] and for hydrophobicity characteristics derived from calculations of electrostatic fields at points on the van der Waals surfaces of amino acids.[464]

Calculated proton affinities of lysine and histidine show the considerably higher relative basicity of lysine.[465]

## 6 Chemical Studies of Amino Acids

### 6.1 Racemization

A 1989 report that racemization accompanies microwave heating of aqueous L-proline solutions (Vol.23, p.51) has been disputed repeatedly, and most recently through experiments involving aqueous solutions of L-alanine, L-glutamic acid and L-proline. These are unchanged after 30 min heating either on a hotplate or in a microwave oven.[466] Much more drastic treatment, $^{60}Co$ γ-irradiation, of L- or D-leucine, or DL-leucine, fails to cause racemization even though some degradation occurs, into $H_2$, $CO_2$, and $NH_3$.[467]

Time-honoured methods for bringing about amino acid racemization depend upon derivatization, such as dissolution of esters in ketones containing acetic acid; the best reagent is acetone containing 15% acetic acid.[468] Hydantoins are readily racemized through contact with an anion exchange resin (Q-Sepharose) at pH 6–13.5.[469]

Little that is new, has appeared in the scientific literature covering fossil dating through measurement of enantiomer ratios of indigenous amino acids.[470]

### 6.2 General Reactions of Amino Acids

This substantial section of this Chapter deals with reactions involving (a) the amino group; (b) the carboxy group; (c) both amino and carboxy groups. Reactions at the α-carbon atom of α-amino acids have mostly been covered in earlier sections covering synthetic methods. The next section 6.3, covers reactions involving amino acid side-chains.

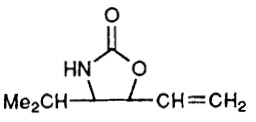
One of the simplest reactions at the amino group, often taking place without being appreciated as such, is carbamate formation in solutions of amino acids and peptides ($H_3N^+CHRCO_2^- + CO_2 \rightarrow H^+ + {}^-O_2CNHCHRCO_2H$).[471] However, amino acids with $pK_a > 9.5$ do not form significant amounts of carbamate in neutral aqueous solutions. Another simple reaction, N-chlorination, continues to receive detailed mechanistic study (Vol.25, p.63), recent results indicating that protonation of N-chloro-α-amino acids takes place at lower pH than previously thought ($pK_a < 1$ for the -NHCl moiety) and that this step is crucial in promoting the decomposition of these species.[472] The decomposition of N-chloro-glutamic acid and of N-chloro-threonine is a first order process, and is independent of pH over the range 5–10.[473]

N-Oxide formation with N-benzyl-L-prolinamide is completely diastereo-selective.[474] N-Alkoxycarbonyl oxaziridines are effective new electrophilic

aminating agents, bringing about the conversion of amino acids (as tetra-alkylammonium salts) and their esters into N-alkoxycarbonylhydrazino acids.[475]

Reductive alkylation of α-imino acids, e.g. L-proline, is the outcome of reaction with ketones in the presence of $H_2$/Pd-C [$HNR^+CHR'CO_2^-$ + MeCOR → (S,S)-$RCHMeNH^+CHRCO_2^-$].[476] This recipe is involved in a classic Z-protecting group removal procedure, and is responsible for inadvertent N-methylation in $H_2$/Pd-C/MeOH treatment of Z-amino acids due to Pd-catalysed oxidation of solvent to formaldehyde.[477] This side-reaction can be avoided by including at least 5% water in the solvent, or changing solvent to isopropanol (or, of course, by ensuring the absence of oxygen!). The Pictet-Spengler reaction is well-known for the preparation of isoquinolines from indolylethylamines, and when applied to a mixture of an aldehyde and an N-[2-(indol-3-yl)ethyl]-L-amino acid ester, it results in enantiospecific ring closure on the secondary amine.[478] Mono-N-methylation of amino acids can be accomplished by cyanoborohydride reduction of N-(o-nitrobenzylidene)amino acid esters followed by photolytic cleavage at 350nm.[479] The same process is used to cleave N-(o-nitrobenzyl)amino acid amides.[480] bis-N-Alkylation of (4'-nitrophenyl)alanine by $BrCH_2CH_2N(CH_2CO_2R)_2$ has been reported.[481] Release of the homochiral β-amino-β-phenylalkanoic acid ester from the product of a classical asymmetric synthesis protocol ( → $EtO_2CCH_2CHPhNHCHPhCH_2OH$ → $EtO_2CH_2CHPhNH_2$) would be accomplished by hydrogenation were it not for the bis(benzyl)amine character of the compound. An alternative $Pb(OAc)_4$ cleavage procedure is effective, without causing racemization.[482] Removal of N-benzenesulfonyl or -toluene-p-sulfonyl groups from alanine or phenylalanine has been long known to be achievable electrochemically, and a recent study throws light on the nature of the three cathodic reduction steps that are involved.[483]

A large crop of papers covering amino acids carrying familiar N-acyl and similar groups has emerged in the 1993 literature. N-Stearoyl-, -oleyl-, and -ricinoleyl-L-leucines have been prepared as potential antibacterial agents.[484] N-Boc amino acids can be prepared through acylation of amino acid salts by Boc-imidazole.[485] The recently disclosed bis-N-Boc amino acids can be converted into their N-Boc analogues by $Mg(ClO_4)_2$/MeCN, and this leaves the t-butyl ester moiety unaffected when applied to $(Boc)_2$-aspartic acid β-t-butyl ester α-methyl ester.[486] Solid phase N-(9-fluorenacetyl)ation of amino acids on a hydrophobic polymeric support has been explored,[487] and insignificant effects have been established, of constituents (salts, buffers, surfactants) in the reaction medium on the course of N-(9-fluorenylmethoxycarbonyl)ation (by Fmoc-Cl), N-phenylthiocarbamoylation, and cyanoisoindole formation (by naphthalene-1,2-dicarboxaldehyde/$CN^-$).[488] Replacement of N-Fmoc by N-Z in good yield can be achieved for the protected amino acids, by using N-Z-5-norbornene-2,3-dicarboximide/KF/$Et_3N$.[489] Optically-pure N-Fmoc amino acids can be obtained by mild [$Ti(OPr^i)_4$] cleavage of N-acylsultams where the acyl group is $(MeS)_2C$=NCHRCO-.[490] Rapid (5 min) N-allyloxycarbonyl group cleavage from N-Alloc-amino acids can be accomplished by Pd(0)-catalysed allyl transfer to diethylamine, and even the most severely hindered cases (e.g. N-Alloc-N-Boc-anilines) are cleaved within 45 min.[491]

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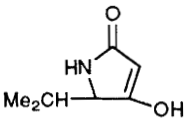


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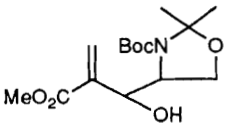


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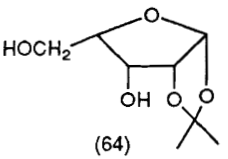


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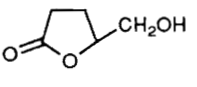


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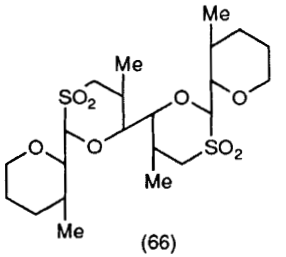


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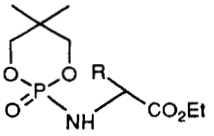


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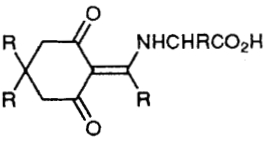
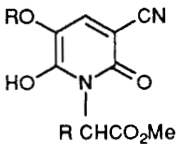



(68; R = H,Me)

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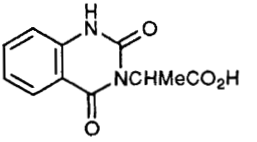


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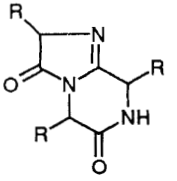


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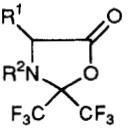


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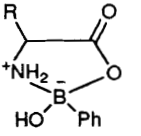



(73)

An N-protection strategy, enamine formation ( → 68 and methyl homologues, deprotected by hydrazine at room temperature)[492] is particularly useful in peptide synthesis since it provides a compatible side-chain protection strategy for lysine.[493] Enamines $MeCOCMe=CMeNHCHRCO_2Me$ have been prepared from a mixture of 1,3-diketone, amino acid ester hydrochloride, and KF in dry conditions, under microwave irradiation.[494] The 3-(3',6'-dioxo-2',4',5'-trimethylcyclohexa-1',4'-diene)-3,3-dimethylpropanoyl grouping used as an N-protecting group leads to redox-sensitive, coloured derivatives, that can be deprotected by aqueous sodium dithionite.[495]

New examples of reactions at nitrogen, that result in this function becoming enclosed within a heterocyclic ring, have been described for methoxymethylene malononitrile ( → 69)[496] and o-methoxycarbonylphenyl isocyanate( → 70).[497]

Enzyme-catalysed de-amination of amino acids is represented here by unusual examples, (R,S)-2-methyl- and (S)-2,2-dimethyl-1-aminocyclopropanecarboxylic acid (by bacterial ACC deaminase),[498] and $N^{\gamma}$-Z-L-lysine to give the α-hydroxy acid (by L-amino acid oxidase from *Providencia alcalifaciens* together with L-2-hydroxyisocaproate dehydrogenase).[499]

Reactions at the carboxy group of an amino acid generate at least as much research interest as the corresponding processes at the amino group, and new methods have been reported, as well as the development of established methods. Tetra-n-butylammonium salt formation has been adopted as both a useful solubilizing technique for taking up amino acids into organic solvents, and as a transient carboxyl protection strategy, and further practical details have been published on the procedure.[500] Sodium L-prolinate–borane complexes have been advocated [501] for asymmetric reduction of aromatic ketones, though they are not so effective as the NN'-dibenzoyl-L,L-cystine–$LiBH_4$–ROH complex. Recently the stability and usefulness of suitably N-protected amino acid fluorides was established, and N-bis(Boc)amino acid fluorides have been added to the list.[502] They are prepared using cyanuric fluoride in $CH_2Cl_2$/py from −30 to −20°C.

Acid anhydrides feature in several studies, in a conventional preparation of Fmoc amino acid p-nitroanilides involving isobutyl chloroformate activation [i.e., unsymmetrical (*alias* mixed) anhydride formation] of Fmoc amino acids,[503] and in a corresponding preparation of Z-amino acid active esters.[504] In the course of the last-mentioned study, it was noticed that some mixed anhydrides disproportionate in $CH_2Cl_2$ during 24h to give the symmetrical anhydride (depending on the alkyl group of the chloroformate) and the amino acid ester.[505] In an alternative but otherwise equally conventional activation of the carboxy group of a Boc amino acid using N-ethyl-N'-(3-dimethylaminopropyl)carbodi-imide, and presentation to p-nitrophenol in an intended conventional esterification protocol, 8–25% of the corresponding dipeptide p-nitrophenyl ester was formed (Boc-$aa^1$-OH → Boc-$aa^1$-$aa^1$-ONP).[506] The side reaction can be prevented by the presence of an equivalent of N-methylmorpholine, and the side reaction is explained by partial Boc breakdown, seen elsewhere (Vol.25, p.67) after cyclization to the 2-t-butyloxyoxazol-5(4H)-one, leading to the amino acid N-carboxyanhydride, an effective acylating agent that reacts with p-nitrophenol and is then in possession

of a free $NH_2$ group that is acylated by the activated Boc amino acid. Di-alkyl pyrocarbonates in the presence of $NEt_3$ have been advocated for symmetrical anhydride formation, and esterification of N-protected amino acids.[507]

Esters can be prepared as described in the preceding paragraph and by other time-honoured methods, applied for the in-vogue synthesis of esters using 1,9-(4-hydroxycyclohexano)buckminsterfullerene.[508] A novel method with alkyl trichloroacetimidates $ROC(=NH)CCl_3$ as esterifying agents, has been used to prepare Fmoc amino acid 2-phenylisopropyl esters.[509] These can be cleaved acidolytically under mild conditions so as to leave N-Boc protection and t-butyl ethers and esters unaffected. Butyl esters of Z-amino acids can be prepared by reaction with $Bu^tBr/K_2CO_3/PhCH_2N^+Me_3\ Cl^-$/N,N-dimethylacetamide.[510] Vinyl esters can be prepared through mild oxidation of N-protected amino acid (2-phenylselenenyl)ethyl esters.[511] De-protection of benzyl esters can be accomplished in dry conditions (microwave irradiation of samples on an alumina surface),[512] and phenacyl esters can be cleaved while leaving benzyl and 4-nitrobenzyl esters unaffected, by using tetra-n-butylammonium fluoride hydrate in the presence of 10 equiv 1-octanethiol.[513] Aminoacylimidazoles are capable of more than the well-known mono-esterification of ribonucleotides, since bis(2',3'-diesters) and mixed anhydrides involving phosphate are also formed.[514] The bis(2',3'-diesters) of 5'-AMP are hydrolysed at different rates at different pH, and N-acetyl-L-phenylalanyl diesters are hydrolysed 1.7–2.1 times faster than their D-analogues possibly due to "protection" of the latter by an association with the adenine ring.[515] When N-acetyl-DL-valine is esterified by ribonucleotides, esterification rates are faster for the D-enantiomer.[516] Similar enantioselectivity has been noted frequently with reactions of amino acid esters, and non-polar L-compounds are hydrolysed twice as fast as their D-analogues in the presence of [trans-5,15-bis(2-hydroxyphenyl)-10-[2,6-bis(methoxycarbonylmethyl)phenyl]-2,3,17,18-tetraethylporphyrinato]zinc(II) (though interestingly, the reverse is the case for serine benzyl ester).[517] Aromatic amino acid octadecyl esters undergo polycondensation much more rapidly in the monolayer state.[518] Racemization occurs during the aminolysis of amino acid active esters by amino acid anions in aqueous DMF, i.e. under basic conditions, though the finer details show that the extent of the side reaction is dependent upon the amino acid and on the base used. It is particularly noticeable for valine and $NaHCO_3$, and can be minimized by working with a 50% excess of the amino acid, and with $Na_2CO_3$ as base.[519] More conventional studies of amino acid esters involve hydrolysis kinetics of phenylalanine methyl ester in comparison with those for aspartame,[520] and ammonolysis through the use of diaminomethane dihydrochloride as a convenient *in situ* ammonia release agent.[521]

Research interests employing amino acid amides often have similar objectives to those described for esters, and an especially notable result is the enzyme-like properties of the synthetic lipids $Me_3N^+(CH_2)_5CO$-L-Ala-$N[(CH_2)_{15}Me]_2$ $Br^-/Cu^{2+}$ that catalyse the condensation of DL-serine with indole to favour L-tryptophan as product, when the L-alaninamides are formed into a hybrid bilayer membrane structure.[522]

Reduction of amino acids to 2-amino alkanols with $NaBH_4$–$I_2$ in THF is

also appropriate for the corresponding process for N-acylamino acids.[523] The direct reduction of L-proline to L-prolinol can be effected in 85% yield with $LiAlH_4$ in THF at 85°C during 3h.[524] $NaBH_4$ Reduction of mixed anhydrides formed from Boc-amino acids gives the corresponding alkanols within a 1h reaction period, and these have been used in a synthesis of homochiral N-Boc-aziridines.[525] Other uses for 2-amino alkanols include a synthesis of α-methyl-amines [L-histidinol → (R)-histamine *via* the chloromethyl analogue and reduction with ammonium formate/Pd-C],[526] and an important role in the preparation of corresponding aldehydes, with Moffatt-Swern oxidation giving good yields of optically-pure products from 2-(Boc-amino)alkanols[527] and applicable also to N-protected N-allylaminoalkanols.[528] Full details of the preparation of Garner's widely-used N,O-protected (S)-serinal (37, CHO in place of $CH=CHCO_2R$) in which DIBAL-H reduction of the protected methyl ester is employed, are available.[529] New methods, illustrated with 3-(N-Boc-amino)-1,2-propanediol giving N-Boc glycinal in 76% yield when cleaved with aqueous $KIO_4$,[530] and reduction of S-benzyl thioesters with $Et_3SiH$/Pd-C.[531] The aldehydes can be used in the Wittig alkene synthesis, and thence to a variety of destinations; in a Diels-Alder reaction with Danishefsky's diene to give dihydropyrones,[532] and in an evaluation of a stereospecific synthesis of pyrrolidines and piperidines.[533] A conversion of N-Boc-valine into $BocNHCH(Pr^i)CH=CHCH_2OH$ is notable.[534] Weinraub amides are a convenient source of the aldehydes in particular cases, and preparation and use in pseudopeptide synthesis, of a fully-protected arginine, $N^{\alpha}$-Boc-Orn[NZC(NHZ)=NH]-NMeOMe,[535] and preparation of bis(N-benzyl)-L-phenylalaninal and its use in the synthesis of (4S,5S)-4-hydroxy-5-amino-6-phenylhexanoic acid,[536] have been described.

γ-Lactams may be obtained by Mg/MeOH treatment of γ-amino acid derivatives $RO_2CNHCHRN^1R^2CH=CHCO_2R^3$ formed in this way.[537]

Amino acid esters react with arylmagnesium halides, to give the expected diarylcarbinols,[538,539] those derived from L-valine methyl ester yielding homochiral 1,2-diamines through routine elaboration.[539] α-Aminoglyoxals have been obtained by reaction of dimethyldioxirane with α-diazoketones derived from amino acids.[540]

Sulfur analogues of the carboxy group are of continuing interest and thiono- and dithio-esters, have been prepared in the conventional manner from nitriles through the Pinner reaction with alkanols and thiols respectively, followed by thiohydrolysis.[541] Reductive acylation of L-α-amino thiocarboxylic acids,[542] and preparations of Boc- or Z-L-amino acid thionimides[543] have featured in recent papers.

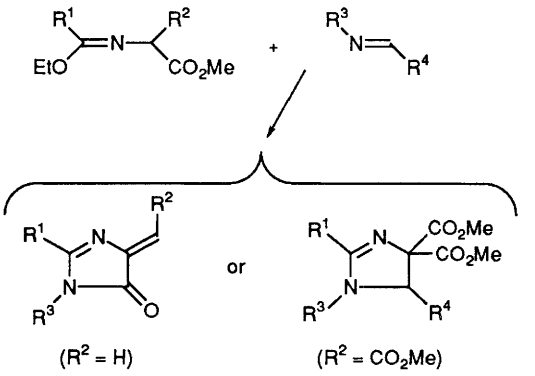
More extensive modification at the carboxy group and the neighbouring α-carbon atom is involved in the numerous oxidative processes that will be familiar to readers of this Chapter over the years. D-Amino acid oxidase reacts slowly with glycine, serine, arginine, histidine, tryptophan, norleucine, and aspartic and glutamic acids. The expected total inability of the enzyme to catalyse the oxidation of L-amino acids has been confirmed in this study.[544] Two citations are offered to represent the numerous mechanistic studies of oxidative decarboxylation of amino acids with simple oxidants (dichloramine-B;[545] N-

bromoacetamide[546]) that continue to appear in the literature. The role of Cu(II), Fe(II), and Mn(II) ions in oxidative modifications of amino acids and proteins has been reviewed.[547] Rates of oxidation of amino acids by the superoxide anion have been measured based on the accompanying chemiluminescence.[548]

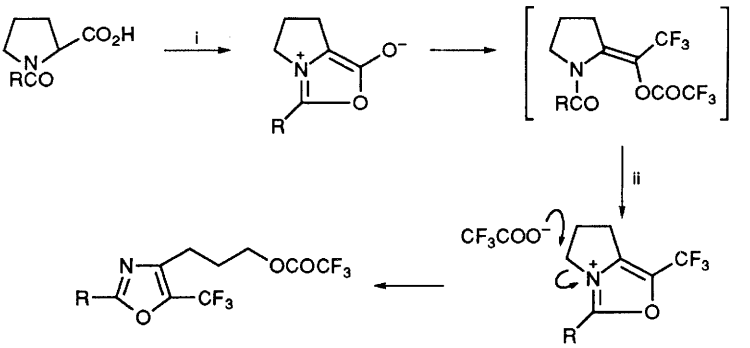
One of the best-known, but only recently properly understood, oxidative decarboxylative modifications of amino acids is that brought about by ninhydrin. Further indications of the synthetic potential of the early azomethine ylide-forming stage of the racemization-free process involving proline or sarcosine are given in the trapping of these transient intermediates by cycloadditions.[549] An account of sensitive colour-forming reactions with ninhydrin and analogues, and corresponding fluorimetric procedures, has been published.[550]

The self-condensation of amino acids takes many pathways, and of course these have considerable importance in geological and biological fields as well as for their essential chemistry. The products of repeated sublimation of simple aliphatic amino acids on to silica and alumina surfaces at 220–240°C have been separated and analysed, and shown to include short peptides, di-oxopiperazines and bi- and tri-cyclic amidines (71) derived from the di-oxopiperazines.[551] Simple additional by-products indicate these products to suffer further degradation.[552] Further results (Vol.25, p.62) for the extraordinary condensation of amino acids into peptides in aqueous solutions with NaCl and $CuCl_2$ have been provided. Successive evaporation and dissolution cycles generate peptides in 1–3 days from glycine, alanine, and aspartic and glutamic acids.[553] Mixtures of glycine, alanine and valine yield mainly N-terminal glycyl dipeptides in the early stages of this process.[554] Rates of formation of multi-component mixtures through heating aspartic acid with proline in aqueous solutions suggest an autocatalytic character to the process.[555] Di-oxopiperazines are formed from 2,2-bis(trifluoromethyl) oxazolidin-5-ones (72) in methanol at room temperature, understood on the basis of the easy hydrolysis of the heterocycle; and the N-carboxymethyl analogues (72; $R^2$ = $-CH_2CO_2H$) have been prepared from N-carboxymethylamino acids.[556]

Heterocyclic compounds enclosing the -HNCHRCO- moiety are most commonly formed through reactions involving both $NH_2$ and $CO_2H$ groups of amino acids, and new examples with interesting properties are still being discovered, such as the relatively lipophilic arylboronic acid chelates (73) whose structure, with a little licence, could be categorized as heterocyclic.[557] Imidazolinones are formed through cycloaddition of stabilized ylides derived from Schiff bases of α-amino acids (Scheme 41).[558] N-Acylamino acids often exhibit reactions that are explicable on the basis of initial cyclization to oxazol-5(4H)-ones, as in the case of N-acylproline ring cleavage with trifluoroacetic anhydride (Scheme 42).[559] Undoubtedly, the course of the reaction of N-acylamino acids with the Vilsmeier reagent ($POCl_3$/DMF) leading to (74) and (75) [and (76) from homocysteine thiolactone], can be explained from the same starting point.[560] Reversal of the cyclization is represented in α-chymotrypsin-catalysed hydrolysis of oxazol-5(4H)-ones to N-acyl-L-amino acids,[561] and basic hydrolysis of analogous 2-anilinothiazol-5(4H)-ones to N-phenylthiocarbamoyl amino acids.[562] Hydrogenolytic cleavage of 4-substituted oxazolidin-5-ones using $Et_3SiH$/

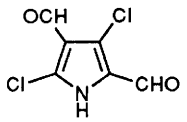



**Scheme 41**




Reagents: i, TFAA, py., DMAP(trace), 80 °C; ii, $-CF_3COO^-$

**Scheme 42**

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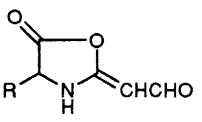


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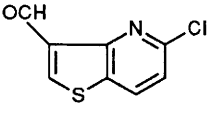



(76)

$CF_3CO_2H$ is a useful, clean, preparation of Z-N-methylamino acids starting from the Z-amino acids ($H_2$/Pd cleaves the methyl group as well as the Z group).[563]

The Maillard reaction represents a long and complex pathway leading to mixtures of heterocyclic compounds, starting with a Schiff base formed between an α-amino acid and an aldose or ketose. An up-to-date review of the process from the food processing perspective is available,[564] and a representative paper covering the reaction in the physiological context establishes that 2-amino-6-(2-formyl-5-hydroxymethylpyrrol-1-yl)hexanoic acid, *alias* pyrraline, is not a major intermediate or advanced glycation end-product formed from amino acids under physiological conditions, as recently claimed.[565]

β-Amino acids have available to them the monocyclization pathway to azetidinones and this can be brought about using (3-nitropyridyl) dialkyl phosphates[566] or alkylaluminium compounds e.g. $Bu^s_3Al$.[567] 5-Amino-2-oxo-pentanoic acid exists partly in cyclized form (pyrrol-1-eine-2-carboxylic acid) in aqueous solutions, in proportions dependent upon pH.[568]

### 6.3 Specific Reactions of Amino Acids.

"The side-chain chemistry of the amino acids" would be a suitable alternative title for this section, and there is some overlap with earlier synthesis sections to the extent that familiar amino acids are chosen increasingly often as the starting point for the synthesis of other amino acids by side-chain modification. Some of this chemistry involves the amino and/or carboxy groups of the amino acids, as well as their side-chains.

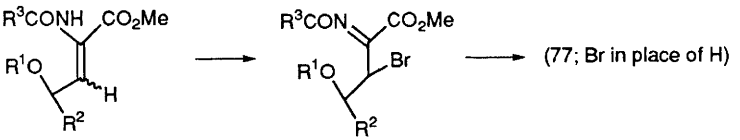
Complete removal of the side-chain could be a way of describing the conversion of protected α-methoxyglycine into highly electrophilic iminium ions [$RO_2CNHCH(OMe)CO_2R' \rightarrow RO_2CNH^+{=}CH(OMe)CO_2R'$] to give versatile glycine cation equivalents that undergo ready alkylation,[569] though α-methoxyglycine and related α-heteroatom-substituted glycines should be appreciated to be a special case. Corresponding alanines react similarly but dehydroalanine formation is a significant side-reaction. Dehydro-amino acid esters, i.e. α,β-unsaturated α-amino acid esters, react stereospecifically with brominating agents to give syn-α-bromo-imines (77 → 78) from which (E)- and (Z)-β-bromo-analogues of the initial reactants can be obtained by base-induced tautomerization.[570]

The side chain ketone function in 3-oxo-L-proline undergoes Baker's yeast-catalysed reduction to give (+)-cis-(2R,3S)-3-hydroxyproline with better than 90% enantiomeric enrichment.[571] Hydroxylation of L-proline catalysed by proline 4-hydroxylase is accomplished with retention of configuration at C-4.[572] The reverse process, the oxidation of the side-chains of the more familiar β-hydroxy-α-amino acids, serine and threonine, to formyl and acetyl respectively, has been accomplished more efficiently than heretofore, after protection of the carboxy groups of the Fmoc-amino acids as the cyclic ortho-ester (79).[573] This useful transformation opens up a further range of applications for synthesising amino acids from serine and threonine (Wittig and similar processes are suggested, and many other functional group transformations could be added), and also allows β-$^2$H labelling. These amino acids are featured in several further citations here as well as in preceding sections of this Chapter, where their uses in

synthesis are described. Full details for the preparation ($Ph_3P$/DEAD) of the β-lactone of Boc-L-serine have been published.[574] The aziridine obtained by cyclization of Fmoc-L-serine benzyl ester can be opened with 3,4-dimethoxy-6-nitrobenzyl alcohol to give the photo-deprotectable O-aryl serine.[575] Serine methyl ester and benzaldehyde gives a more complex equilibrium mixture[576] of ring–chain tautomers (i.e. Schiff base together with oxazolidines) than previously supposed. The formation of the corresponding N-Boc-oxazolidine from N-Boc-L-serine methyl ester and acetone calls for Mitsunobu reaction conditions.[577] N-Trityl-L-serine methyl ester reacts with thionyl chloride to give the cyclic sulfimidate (80), from which the sulfamidate can be obtained by oxidation.[578] O-Allylation of β-hydroxy-α-amino acids can be accomplished using allyl trichloroacetimidate.[579] *Streptomyces amakusaensis* possesses a novel aldolase that catalyses reverse aldol cleavage of β-hydroxy-α-amino acids.[580]

Aspartic and glutamic acids have many synthetic applications, like the β-hydroxyalkyl-α-amino acids, and again, the reader is directed to other sections in this Chapter so as to access the full coverage of the recent literature on this topic. Glutamic acid α-semialdehyde has been prepared by ozonolysis of γ-vinylGABA, and shown to exist in the cyclic form (81), so explaining its unexpectedly high stability; it can be purified by ion exchange chromatography over Dowex 50W-X8, while α-amino aldehydes are generally considered to need careful handling and storage.[581] Several cyclized forms of aspartic and glutamic acids are useful in synthesis; Z-L-aspartic anhydride has been widely used in aspartame synthesis;[582] the 2,2-bis(trifluoromethyl)oxazolidin-5-one (cf. 72) derived from aspartic acid diazomethyl ketone and used in 4-oxo-L-proline synthesis;[583] and similar oxazolidinone formation allowing the side-chain carboxy group of L-glutamic acid to be elaborated into -COSEt and -$CH(OMe)_2$ *en route* to (+)-porothramycins A and B,[584] and allowing side-chain acid chlorides to be elaborated into sensitive functional groups when the oxazolidinone is protected as the N-$Cl_3CCH_2OCO$-group (cleaved by Zn/AcOH).[585] Regioselective ring-opening through amide cleavage of N-Boc-pyroglutamic and "-pyroaminoadipic" acid ethyl esters with nucleophiles ROH, $RNH_2$, $PhCH_2SH$ (KCN catalysis under ultrasound) provides the corresponding γ-carboxy-derivatives.[586]

Enolate di-anions formed with fully protected L-aspartic acid by treatment with $LiN(SiMe_3)_2$ undergo oxygenation by N-(benzenesulfonyl)-3-phenyloxaziridine to give β-hydroxyalkyl-α-amino acids (2S,3S)-$R^1NHCH(CO_2R^2)CH(OH)CO_2R^3$, while with oxydiperoxymolybdenum in pyridine + HMPT they give the 3R-epimer.[587] Acylation of the corresponding glutamic acid enolate leads to δ-oxoalkyl-α-amino acids *via* decarboxylation of the initially-formed β-keto-esters.[588] An improved synthesis of (S)-α-amino adipic acid δ-methyl ester from L-aspartic acid and its elaboration into homochiral 3-aminocyclopentyl-methanols after Dieckmann cyclization, has been reported.[589] Conversion of L-glutamic acid into its "3,4-didehydro-analogue" in the form of its N-(9-fluoren-9-yl) methyl ester, followed by non-stereoselective methylation ($LiMe_2CuR$) to give 3-methylglutamic acid, and conversion by DIBAL-H reduction, carbamoylation and $OsO_4$ cleavage into (+)-5-O-carbamoyl polyoxamic acid.[590] 3-Methylaspartic acid has been prepared from DL-glutamic anhydride in

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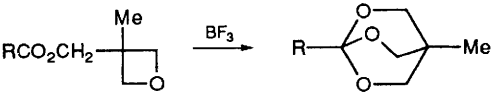


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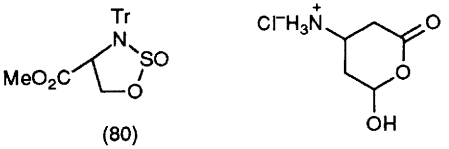


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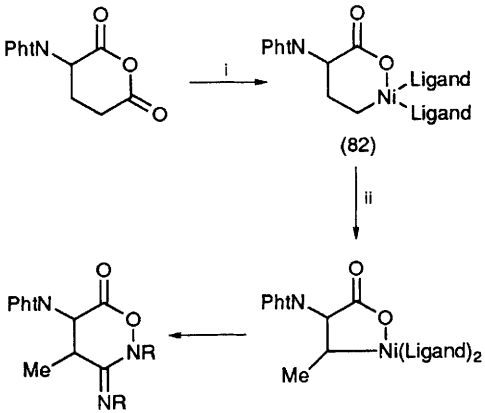



Reagents: i, $Ni(Ligand)_x$, –CO; ii, RNC

**Scheme 43**

a remarkable ring contraction of the derived nickelacycle (82 in Scheme 43) and further unusual steps for which the metal functions as an activating group.[591] Unexpected cleavage of L-pyroglutamates with the α-sulfinyl carbanion (Scheme 44) creates a number of useful opportunities for the synthesis of other amino acids, illustrated for pyrrolidine-2,5-dicarboxylic acids and 5-hydroxypipecolic acids.[592] Development of earlier success in 4-alkylidenation of pyroglutamates has been reported, leading to three naturally-occurring glutamic acids of this class.[593] N- and Side-chain-protected aspartic and glutamic acids have been converted into β-amino- and γ-amino esters, respectively, by substitution of the tosylated α-carboxy group using organocopper reagents.[594] Several synthesis applications for α-(ω-carboxyalkyl)-α-amino acids have been published during 1993, leading away from the amino acid field, and although brief mention of these is made elsewhere in this Chapter, to the extent that routes start with novel functional group modifications, no attempt is made to cover this area in any thorough way.

γ-Carboxyglutamic acid reacts with 4-diazobenzenesulfonic acid to give an intensely red compound ($\lambda_{max}$ 530 nm) resulting from the replacement of both γ-carboxy groups with moieties of the reagent; β-carboxy aspartic acid behaves similarly.[595]

The established side-chain chemistry of lysine and related ω-aminoalkyl α-amino acids is also being extended into new areas, $N^{\varepsilon}$-alkylation by epichlorhydrin being the starting point for a synthesis of naturally-occurring (2S,9R)-hypusine dihydrochloride [Lys($CH_2CH(OH)CH_2CH_2NH_2$] and its (2S,9R)-epimer.[596] $N^{\alpha}$-Fmoc-$N^{\varepsilon}$-bis(t-Butyloxycarbonylmethyl)lysine and ornithine and diaminopropanoic acid analogues have been synthesized from the protected lysine, ornithine and asparagine, respectively.[597] Selective $N^{\beta}$-Boc-protection has been attended to for (S)-2,3-diaminopropanoic acid,[598] and a 100g-scale preparation of $N^{\varepsilon}$-allyloxycarbonyl-L-lysine (85% yield from lysine hydrochloride/$Na_2CO_3$/$CuCl_2$/allyl chloroformate) as its $N^{\alpha}$-Fmoc derivative (Fmoc succinimide) has been described.[599] α-[4-N-(Pyridiniobutyl)]-α-amino acids result from the reaction of $N^{\alpha}$-acetyl-L-lysine with pyrylium salts.[600] Further studies of lysine derivatives carrying redox groupings at the $N^{\varepsilon}$-site (Vol.24, p.58) have been described, the amino acid acting as a vehicle for laser energy conversion by the redox groups (420nm laser pulses generate 1·17 volts energy storage in some cases).[601]

Arginine derivatives that release nitric oxide (other than arginine itself through the action of NO synthase[602,603]) include $N^{\varepsilon}$-nitro-L-arginine and its methyl ester, under the influence of ultraviolet radiation.[604] This may explain the relaxation of smooth muscle that is observed in UV light. The general topic has fascinating physiological implications, and has rejuvenated the study of nitric oxide.[602] The nitric oxide–water system has a short half-life due to oxygenation to nitrite ion (with little or no nitrate ion formed), but the arginine–NO synthase system produces nitrate as well as nitrite if an additional oxidizing species, such as an oxyhaemoprotein, is present.[602]

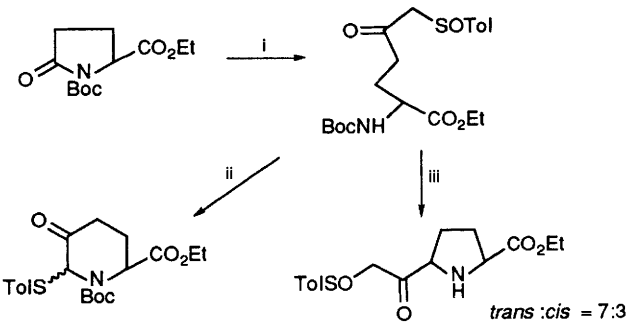
$N^{G}$-Allyl-L-arginine has been prepared in good yield through the standard general route from L-ornithine using an N-allyl-N'-(pyrazol-1-yl)amidine as

amidinating agent.[605] Side-chain protection for arginine using the 2,2,4,6,7-pentamethyl dihydrobenzofuran-5-sulfonyl group is secure under normal peptide synthesis operations and is more easily removed than current alternative protecting groups.[606] Cleavage of the guanidino group of arginine is the consequence of reaction with N-methyl-N-t-butyldimethylsilyltrifluoroacetamide, leading to TBDMS-ornithine and TBDMS-carbodi-imide.[607]

Cysteine side-chain chemistry continues to undergo development in biological contexts, such as the stereospecific conversion of S-allyl-L-cysteine into the (+)-sulfoxide in culture tissues of *Allium sativum*,[608] and conversion of homocysteine into methionine *via* a non-enzymatic transfer in aqueous solution at pH 7, of a 5-methyl group from the 5-methyltetrahydrofolate model (83) that has a positive charge on N-5.[609] Cysteine esters treated with 3 equiv $HNO_2$ are de-aminated, as expected, but also caused to cyclise to thiirancarboxylates, presumably *via* the thionitrite.[610] Of relevance in the food science context, cysteine and dihydroxyacetone react to give a large number of volatile products (specifically thiazoles and pyrazines) in proportions dependent on the relative concentrations of reactants and water.[611] Modifications that are restricted to the the environment of the sulfur functional group of members of the cysteine family protected at amino and carboxy groups, have been reported for N-acetylcysteine methyl ester, which acts as a sulfur transfer agent towards carbodi-imides (84 → 85),[612] and for penicillanic acid derivatives which undergo stereospecific oxidation at S with exclusive exchange of protons with $^2H_2O$ at the adjacent methyl group that is cis to the sulfoxide oxygen atom.[613] Replacement of S-acetamidomethyl-, S-p-methoxybenzyl-, and S-trityl- protecting groups (but not S-benzyl-) from fully protected cysteines, by the S-methylthio-group is achieved using $Me_2S^+SMe$ $BF_4^-$, a well-known disulfide bond-forming process but novel in this context, that yields protected cysteine derivatives amenable to mild reductive S-deprotection.[614]

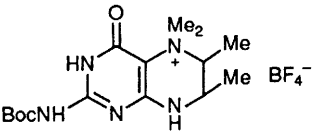
Examples of arene groups undergoing modification have appeared in reports of 4-aminomethylation of protected L-phenylalanine ($Cl_3CCONH$ $CH_2OH$ and $H_2SO_4$, followed by conc HCl),[615] and phenolic O-[4-(piperidin-4-yl)butyl]ation of trimethylsilyl-protected L-tyrosine *en route* to MK-383, a fibrinogen receptor antagonist.[616] Milder than the usual (48% HBr/AcOH) conditions for demethylation of methoxy-substituted phenylalanines, are needed to avoid racemization; and conc aq HBr + NaI/90°C/2 h is effective.[617] A novel [bis(trifluoroacetoxy)iodo]benzene-mediated oxidative hydroxylation of a protected tyrosinal (86 → 87) is a first step in an aranosin synthesis.[618] Even milder processes are involved in dopaquinone formation from L-tyrosine through catalysed oxidation by immobilized tyrosinase, and its detection through fluorescence generated at 480 nm ($\lambda_{excit}$ 350 nm) after reaction with 1,2-diphenylethylenediamine,[619] and oxygenation studies of DOPA in aqueous solutions.[620] Identification of 2,4,5-trihydroxyphenylalanine ("TOPA") as an oxygenation product (0.5% yield) and its conversion into a quinone imply that some properties attributed to DOPA may be those of TOPA/TOPAquinone.

$N^{\alpha}$-Z-Histidine t-butyl ester undergoes Pd(0)-catalysed phenylethynylation at C-4(5) of the imidazole group,[621] a general process that has been used to




Reagents: i, $TolSOCH_2Li$; ii, TFAA/py.; iii, $TFA/CH_2Cl_2$

**Scheme 44**

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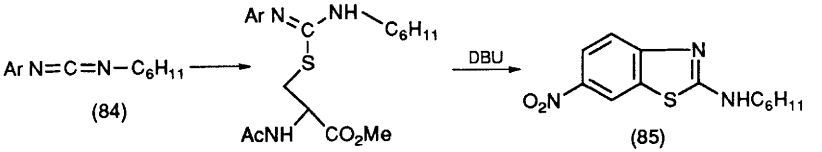


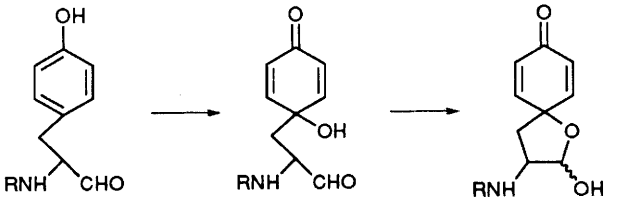

prepare a homologue, diphthine, the major metabolite of diphthamide. Alkanethiols de-iodinate 2'-iodohistidine within 24 h, *via* attack by $RS^-$ on the protonated imidazole.[622] This is thought to account for the limited *in vivo* efficacy of this otherwise promising antimalarial agent.

Regioselective nucleophilic substitution of 1'-hydroxytryptophan in acidic media is the paradoxical result of dissolution in a 10% aqueous sulfuric acid–methanol mixture, leading to 5-methoxy analogues, and offers an attractive model for serotonin synthesis in the central nervous system.[623] Electrophilic substitution at C-5 by NBS is observed during free radical C-3a functionalization of tryptophan (by Br using NBS, by $NO_2$ and OH using ceric ammonium nitrate) in the form of its $N^{in}$-benzenesulfonyl hexahydropyrrolo[2,3-*b*]indole methyl ester derivative (Vol.25, p.40).[624] Stereoselective dioxygenolysis of N-acetyl D- or L-tryptophan methyl esters is catalysed by manganese porphyrins bonded to bovine serum albumin.[625] Reactions of tryptophan have been reviewed.[626]

## 6.4 Effects of Electromagnetic Radiation on Amino Acids

The material traditionally collected here concerns the aromatic and heteroaromatic amino acid side-chains, though some citations that might have been located here have involved additional aspects of chemistry that have caused them to be discussed elsewhere in this Chapter.

Radiolytically-generated hydroxyl and sulfate radicals have been identified as the reagents involved in pulse radiolysis of phenylalanine leading to tyrosine and its isomers.[627] Conversion of L-tryptophan into a hydroperoxide is a well-known example of the role of singlet oxygen, but radiolytic oxygenation has been found to yield two new hydroperoxides where $Cl_3COO^{\bullet}$ can be formed. This adds at C-2 of the indole moiety, and is followed by $O_2$ addition and formation of the epimeric hydroperoxy-oxindolylalanines.[628] A study showing that chloroform at 0.08% levels, modifies the photolysis of tryptophan to give new products showing intense fluorescence in visible light, may prove to involve the same underlying chemistry.[629] Photolysis of flavin-sensitized tryptophan leads to indole-3-acetaldehyde under anaerobic conditions.[630] There are numerous studies describing the search for new radioprotective agents, and the aromatic amino acids feature frequently in these; a representative citation reports the γ-radiation protection possible with a mixture of hydroxylamine, 2-aminethyl isothiouronium bromide hydrobromide and with 5-hydroxy-L-tryptophan as the major component.[631]

A less common type of study in this category involves two-photon-excited fluorescence excitation spectra using circularly-polarized and linearly-polarized light, for phenylalanine, tyrosine and tryptophan in neutral aqueous solutions.[632] Absorption features in the 440–620 nm wavelength range are observed, corresponding to the familiar one-photon excitation absorption features in the 220–310 nm region. Riboflavin-sensitized photochemistry of tryptophan in visible light has been reviewed.[633]

## 7 Analytical Methods

### 7.1 Introduction

Some general reviews have appeared that apply to analysis for particular amino acids in biological samples (homocysteine[634] and tryptophan[635]) and to broader aspects (advances in amino acid analysis[636] and in the analytical chemistry of amino acids, peptides and proteins[637]).

### 7.2 Gas–Liquid Chromatography

The general theme for the 1993 literature is the continuing development of existing methods (derivatization protocols and instrumental variants). The analysis of a mixture of 22 amino acids over a DB-1 capillary column after derivatization with N-methyl-N-(TBDMS)trifluoroacetamide to give N(O)-TBDMS derivatives, illustrates the generally less time-consuming methodology employed for GLC analysis of amino acids, now used in some laboratories.[638] A similar approach employing ethyl chloroformate in EtOH/py to give N-ethoxycarbonyl amino acid ethyl esters for GC–MS analysis[639] seems to risk the introduction of artifacts due to carboxyl activation by the reagent and competition for reaction at the amino group of an amino acid or amino acid ester, to give derivatized di- and polypeptides. Two-step derivatization procedures are illustrated for proline + hydroxyproline analysis as their N-dimethylthiophosphoryl methyl esters, after OPA treatment of the biological sample to remove primary amines,[640] for O-phospho-serine, -threonine, and -tyrosine in urine hydrolysates, as N-isobutyloxycarbonyl methyl esters,[641] and in the specific case of 1-aminocyclopropanecarboxylic acid (1-ACC) in leaf tissue (preparation of the N-benzoylated propyl ester), and use of capillary-GC with an N/P-sensitive detector.[642] The last-mentioned study describes the development of a reliable protocol, and criticizes an established method for 1-ACC analysis that is subject to interference and lacks internal standards.

Enantiomeric analysis by GC continues to be based on either diastereoisomer formation [(N-menthyloxycarbonyl)ation of amino acid esters[643]] or on the separation of amino acids derivatized in simple ways, over chiral stationary phases (packings coated with N-stearoyl-L-valine t-butylamide for the resolution of N-trifluoracetylamino acid isopropyl esters)[644] and closely-related protocols for general amino acid analysis[645] and specifically for selenomethionine[646]

GABOB analysis (urine samples) by GC–MS is complicated by the fact that it co-elutes with leucine in some standard procedures.[647]

### 7.3 Thin-layer Chromatography

A paper in the preceding section[642] refers to the considerable expense of GC–MS instrumentation; the increased activity in TLC analysis of amino acids and their derivatives probably reflects this situation. Densitometric quantitation of ninhydrin-developed hydrophilic TLC plates (silica coated with silicic acid) has provided reliable assays of lysine, homoserine and threonine in culture fluids.[648] Amino acids interact with the non-ionic surfactant, nonylphenyl hexa-ethoxylate, a fact established by charge-transfer reversed-phase TLC that has a negligible

effect on the hydrophobicity of amino acids except for cysteine, glutamic acid, glutamine, hydroxyproline, phenylalanine and tyrosine.[649] These results, although puzzling in terms of the particular amino acids that interact and those that do not, illustrates the usefulness of simple methods in obtaining information of wide applicability in amino acid science.

Routine TLC analysis is well-represented in the literature, as usual (e.g., analysis of dansylamino acids[650]) occasionally employing techniques undergoing evaluation as illustrated by the separation of a mixture of 20 PTH's by automated multiple development over silica gel.[651]

Resolution of enantiomer mixtures of derivatized amino acids, employing chiral stationary phases[652] or mobile phase additives (β-cyclodextrin[653] or bovine serum albumin for the analysis of dansylamino acids,[654] continues to be practised.

## 7.4 High Performance Liquid Chromatography

Some of the derivatization methods encountered in preceding sections are also routinely adopted in HPLC protocols, and the relative merits of the o-phthaldialdehyde/alkanethiol (OPA), N-(fluoren-9-ylmethoxycarbonyl)ation (Fmoc), N-phenylthiocarbamoylation (PTC), and N-dansylation (DNS) methods have been reviewed.[655] HPLC analysis of homocysteine in plasma samples has been reviewed.[656]

HPLC analysis of non-derivatized amino acids (Tyr, His, Phe, Trp) in foods;[657] protein cross-linking amino acids pyridinoline, hydroxylysylpyridinoline, and lysylpyridinoline in urine[658] conventionally involves ion-pair formation with sodium n-heptanesulfonate. Another study describes HPLC analysis of seven major crosslinking amino acids in elastin: desmosine, isodesmosine, allodesmosine, neodesmosine, aldosine, oxodesmosine, and cyclopentenosine.[659]

The sensitivity criteria usually required in amino acid analysis, calls for choice of derivatives with optimised physical characteristics that can be exploited for quantitation. OPA Fluorescence is sufficiently stable to yield good precision with a relative standard deviation of 0.8–7.3% depending on the use of relevant internal or external standards.[660] The presence of cyclodextrins in the mobile phase (a means of exploiting HPLC for the analysis of enantiomer mixtures) affects the fluorescence yield.[661] The protocol has been used for the analysis of tyrosine-O-sulfate (ion-pair variant with t-butylammonium phosphate in the mobile phase),[662] and in other amino acid areas,[663-665] one[665] describing a sample pretreatment procedure that allows proline to be included in the method (which is applicable only to primary amines) through chloramine-T/$NaBH_4$/60°C/11 min treatment that converts the imino acid into 4-aminobutan-1-ol.

Dabsylation of collagen hydrolysates after OPA-blocking of primary amines[666] is capable of extraordinary sensitivity, with hydroxy-L-proline being measureable at femtomole levels with its help.[667] Where radioactive-labelling is distributed between amino acids in physiological samples, the efficiency of HPLC separation with suitable detectors allows their assay in the form of dabsyl derivatives.[668]

7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole, or its 7-fluoro-analogue, has

been used increasingly recently, for derivatization of proline + hydroxyproline in mixtures after OPA blocking of primary amines,[664] or hydroxyproline alone,[669] and for homocysteine analysis.[670] A salutary warning has been published that the derivatization of cysteine by 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole is reversible in media containing reducing species at basic pH, undermining any quantitation protocol based on the fluorimetric assay using this reaction.[671]

N-Phenylthiocarboamoylation compares well with classical ion-exchange chromatographic analysis for the HPLC estimation of amino acids in plasma,[672] and of protein hydrolysates.[673] Using gas-phase acid hydrolysis, and the Waters Pico-Tag Workstation based on PTC-derivatization, the amino acid content of lysozyme as a typical protein was secured with a 22 min HPLC separation. L-Methionine sulfoxide assay in tissue extracts has been achieved through the sequence ion-exchange, Pico-Tag derivatization and HPLC after derivatization using diethoxymethylene malonate.[674] One-step amino acid derivatization by the new Waters AccQ-Tag Workstation has been described.[675] Traditional PTH analysis[676] and the similar DABTH assay employing HPLC[677] routinely deal with femtomole levels of analyte. Fluorogenic Edman reagents 7-N,N-dimethyl-aminosulfonyl-4-(2,1,3-benzoxadiazolyl)isothiocyanate and the 7-amino analogue yield amino acid derivatives showing $\lambda_{em}$ 505 nm for $\lambda_{excit}$ 385 nm.[678]

Several other derivatization protocols (some well-established, some new) have been reported, and there is perhaps more activity in this area than usual. N-Z-Amino acids,[679] N-acetylamino acid tetra-alkylammonium salts,[680] and amino acid pentafluorobenzyl esters[681] are familiar derivatives that have been studied further, the last-mentioned offering a suitable means of assay for tryptophan at low levels using electron capture detection and negative ion CI-MS. Novel fluorogenic reagents include N-quinolin-6-yl carbamic acid N'-hydroxysuccinimide ester[682] and 2-fluoro-4,5-diphenyloxazole and 2-chloro-4,5-di-(p-N,N-dimethylaminosulfonyl)phenyloxazole, the fluorescence and chemiluminescence from the latter reagent being detectable at 19–64 femtomole levels.[683] Known procedures for specific amino acids include the use of glyoxal (fluorescence generation with tryptophan),[684] and 3-bromopropylamine (for cysteine),[685] and 4,4'-dithiodipyridine (for post-column detection of homocysteine and other thiols).[686] 3'-Methylhistidine analysis has been accomplished through pre-column derivatization (reagent not stated in the abstract of this paper).[687]

The determination of the enantiomeric composition of amino acids in mixtures continues to stimulate the development of known methods, some based on chiral derivatizing agents, (+)-1-(fluoren-9-yl)ethyl chloroformate for amino acids,[688] or N-glycyl-L-(4-nitrophenyl)alanine methyl ester for N-Z-amino acids,[689] others employing chiral stationary phases (cellulose tris(3,5-dimethylphenyl)carbamate for N-protected amino acid esters (N-Z- is better than N-Boc or N-formyl; the L-enantiomer runs fastest),[690] and commercial phases for ligand-exchange HPLC resolution of non-derivatized amino acids (stereoisomers of 2,6-di-aminopimelic acid).[691] A review covers the HPLC resolution of amino acids.[692]

A number of topics in HPLC analysis of amino acids are briefly noted: hydroxylysine glycosides in collagen hydrolysates,[693] S-adenosyl-L-methionine

and its metabolites,[694] serotonin in insect brain tissue (amperometric detection),[695] tyrosine and isoquinoline alkaloids in papaver,[696] and carbidopa in clinical samples (electrochemical detection).[697] HPLC capacity factors have been correlated with Hansch hydrophobic parameters for N-dodecanoylamino acids.[698]

### 7.5 Fluorimetric Analysis

Much of the material under this heading is located elsewhere in this Section of this Chapter because analytical exploitation is the usual fate for fluorogenic derivatives of amino acids. The fluorescence generated in tryptophan through reaction with aldehydes in mildly acidic media (cf. glyoxal[684]) has been studied in some detail for methoxyacetaldehyde.[699] β-Carboline and 1-methoxymethyl-β-carboline are formed at pH 2.75 in aqueous $NaNO_2$, the intense fluorescence ($\lambda_{em}$ 450 nm at $\lambda_{excit}$ 253 nm) being detectable down to 10 picomole levels.

### 7.6 Other Analytical Methods

The growth area under this heading continues to be capillary zone electrophoresis (CZE), now routinely offering laser-based detection of thiohydantoins at sub-attomole levels,[700] and applicable to chiral separation of derivatized amino acids.[701]

Derivatization protocols for amino acids, and detection techniques, that are used for HPLC and other analytical purposes, are equally valid in the CZE area, as illustrated for laser-induced fluorescence (248 nm) for the detection of Fmoc amino acids (reaching $5 \times 10^{-10}$ M),[702] and for oxazoles formed by condensation of amino acids with the co-enzyme pyrroloquinolinequinone.[703] Micellar electrokinetic capillary chromatography is an important variant of CZE, used for PTH analysis,[704] and for chiral analysis employing bile salt micelles[705] or β-cyclodextrin (for charged analytes) or carboxymethylethyl-β-cyclodextrin (for neutral analytes).[706]

Absorption spectrophotometry at 440 nm allows simple estimation of the total amino acid content of a mixture reacted with benzoquinone (total protein at 350 nm).[707] Spectrophotometric estimation of phenylalanine in blood samples, on the basis of phenylpyruvate formed by oxidative deamination (phenylalanine dehydrogenase) compares favourably with results from fluorimetry, or other conventional amino acid analysis methods.[708] Asparagine in aqueous solutions develops a colour ($\lambda_{max}$ 340–350 nm) with ninhydrin that differs from that ($\lambda_{max}$ 405, 570 nm) for amino acids generally, leading to a simple colorimetric assay that compares favourably with HPLC methods for asparagine.[709] Glutamine colorimetry down to millimolar levels has been established.[710] The violet colour that develops between tyrosine methyl ester and iron(III) salts is the basis of a novel colorimetric assay applicable at 10 nanomolar levels.[711]

A carbon paste electrode impregnated with copper(II) cyclohexylbutyrate has an oxidation peak for copper(0) that is modified by the presence of amino acids in the solution.[712] The increased current taken to achieve the oxidation is proportional to the concentration of the total amino acids, which can thereby be estimated, down to $10^{-6}$ M levels.

### 7.7 Assays for Specific Amino Acids

Adding to the methods appropriate for particular amino acids discussed in preceding sections, papers cited here deal mainly with enzymatic methods linked to electrochemical measurements.

An amperometric electrode specific for L-alanine consists of a platinum surface that senses $H_2O_2$ produced in the presence of aqueous alanine by immobilized alanine aminotransferase and glutamate oxidase.[713] Alanine dehydrogenase and leucine dehydrogenase co-immobilized on chitosan, constitutes an HPLC post-column reactor that catalyses the degradation of alanine, leucine, valine and isoleucine into species amenable to fluorimetric assay.[714] Phenylalanine dehydrogenase, immobilized in a flow sensor, generates NADH from L-phenylalanine that encounters a nylon-immobilized bacterial enzyme capable of creating bioluminescence.[715]

L-Lysine biosensors have been described, one employing a lysine oxidase reactor combined with a fibre-optic $H_2O_2$ detector incorporating peroxidase and luminol, and capable of dealing with $10^{-6}$ M levels of analyte,[716] and another based on L-lysine decarboxylase combined with an optical transducer (a membrane carrying a lipophilic tartrate supporting an amine-sensitive dye-light source) for assaying the resulting cadaverine.[717]

Glutamate-sensing systems based on glutamic acid oxidase and other enzymes[718] or on glutamic acid oxidase alone[719] generating $H_2O_2$ at a Pt electrode in proportion to the concentration of glutamic acid. One of these studies describes ultra-miniaturisation of the sensor,[719] The extension of the amperometric exploitation of glutamic acid oxidase electrodes into glutamine, aspartic acid and aspartame sensing systems has been reviewed.[720] Glutaminase immobilized on an $NH_3$-sensing electrode constitutes an L-glutamine sensor,[721] while a broader range of analytes can be assayed by a device comprising an amino acid oxidase immobilized on aminated glass cloth and an $NH_3$-sensitive electrode.[722]

Non-enzymatic assays are involved in the remaining citations in this Section. A labelled L-leucine assay has been described, in which the amino acid is bound to its tRNA in the presence of either added radiolabelled tRNA-Leu and a deficiency of non-radioactive L-leucine, or in the presence of excess non-radioactive L-leucine to correct for other radiolabelled species.[723] This work extends a previously-disclosed method (Vol.25, p.78). A combined glutamine and α-ketoglutarate assay involves ion-exchange separation, o-phenylenediamine derivatization of the ketoglutarate, followed by conversion of the glutamine into ketoglutarate and its estimation as such through absorption spectrophotometry.[724]

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# 2
# Peptide Synthesis

BY DON T. ELMORE

## 1 Introduction

The format of this report is unchanged from its predecessor. There have been fewer reviews in total covering chemical synthesis including solid-phase peptide synthesis (SPPS) and the generation of peptide libraries[1-9], enzyme-catalysed peptide synthesis[10-12], peptide hormones [13,14], peptide mimetics[15-19], and a miscellany of more specialized topics[20-26]. In contrast to reviews, however, the number of research papers is greater than in previous reports.

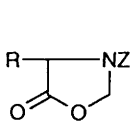
## 2 Methods

### 2.1 Amino-group Protection

–*N*-Z-*N*-methylamino acids can be prepared by hydrogenolysis of the oxazolidin-5-ones (**1**) with $Et_3SiH$ in the presence of $CF_3CO_2H$[27]. Z($NO_2$) groups can be removed with Zn dust in tetrahydrofuran/0.35 M phosphate buffer (pH 6, 1:2 or 1:3 v/v) at room temperature with stirring[28]. Hydrogenolytic removal of Z groups can be troublesome if carried out in MeOH. Mono- and di-methylation can occur as a result of oxidation of MeOH to HCHO with traces of adventitious $O_2$ followed by reductive methylation[29]. This side reaction can be avoided by rigorous exclusion of oxygen or by addition of 5% $H_2O$ or i-PrOH. *N*-α-Fmoc groups can be replaced by *N*-α-Z groups using KF/$Et_3N$ in the presence of *N*-Z-5-norbornene-2,3-dicarboximide[30] (**2**). It is proposed that the Fmoc group is removed by $F^-$ ion and that the liberated amine then reacts with (**2**). *N*-Fmoc groups can also be replaced by Boc groups using potassium in $CHONMe_2$ followed by $Et_3N$ then $(t\text{-}BuOCO)_2O$[31]. A one-pot method for removing Fmoc groups and coupling the liberated amine has been described[32]. $Bu_4NF$ is used with the novel variation of adding a thiol such as BzlSH in $CHONMe_2$ to react with the liberated dibenzofulvene. Reaction is complete in 1-2 min and the next amino acid derivative can be coupled *in situ*. One would like to be reassured that the excess of thiol does not interfere with the coupling step. Two methods of preparing *N*-Boc amino acids have been described[33,34]. $^{15}N$-Boc amino acids can be prepared from $^{14}N$-amino acids of the opposite chirality using $^{15}NHBoc_2$ as the isotopic source[35]. *N*-Boc groups on amide or carbamate N atoms are quite selectively removed by $Mg(ClO_4)_2$ in MeCN; Boc groups on amino N atoms are unaffected[36]. α-*N*-Boc groups are rapidly and quantitatively removed with $Me_3SiCl$/PhOH in $CH_2Cl_2$[37]. Removal of benzyl protecting groups and detachment of a peptide from a resin are respectively at least $10^5$ and 6000 times slower

than removal of Boc groups. It is postulated that the formation of complexes between PhOH and both $Me_3SiCl$ and the α-*N*-Boc derivative (Scheme 1) is followed by interaction between the complexes followed by a two-stage liberation of the amino derivative. A modified Boc group (**3**) has been described[38] but has not been deployed in peptide synthesis. Acidic deprotection is slower than the removal of Boc groups but can be accelerated by addition of $Ca^{2+}$ ions. A new reagent, *N*-Boc-(4-cyanophenyl)oxaziridine (**4**), permits electrophilic amination so that reaction with amino acid esters affords Boc-hydrazino esters[39]. α-*N*-Alloc groups can be removed by Pd catalysis using nitrogen or sulfur nucleophiles[40]. If $Et_2NH$ is the nucleophile, good yields are obtained provided an adequate excess is used. This is particularly important with *N*-Alloc derivatives of secondary amines in order to avoid the formation of *N*-allyl secondary amines. Protection of amino groups as Adpoc derivatives has not been widely used, but this may change following the development of reagents such as 0.1M $HCl/CF_3CH_2OH/CHCl_3$ (1:9:1) and 50% $HCO_2H/CF_3CH_2OH/CHCl_3$ (1:9:1) which can selectively remove Adpoc groups without affecting Boc groups or damaging Trp residues[41]. The 1-Adoc group has been known for a long time, but use of the isomeric 2-Adoc group has only recently been reported[42]. Just as 2-adamantyl esters are more stable than the 1-adamantyl isomers, so the 2-Adoc group is more resistant than the 1-Adoc group to acidolysis. It has been used in the synthesis of two fragments of a growth-inhibitory factor that resembles metallothionein. The synthesis of *NN*-bis-1-Adoc amino acids has been described[43] but awaits deployment in peptide synthesis.

The yellow *N*-3-(3,6-dioxo-2,4,5-trimethylcyclohexa-1,4-diene)-3,3-dimethylpropionyl group (**5**) (abbreviated to Q) is most unusual[44]. Although coupling reactions effected by DCCI are accompanied by extensive racemization, this does not happen if unsymmetrical anhydrides formed from isobutyl chloroformate in the presence of *N*-methylpiperidine are used. The *N*-Q group is indefinitely stable in $CF_3CO_2H$ and stable for up to 4 h in 20% piperidine in $CHONMe_2$. The latter observation was somewhat unexpected, since quinones are normally readily attacked by nucleophiles. The observed stability is attributed to the presence of substituents in the quinone ring. The Q group can be removed by hydrogenolysis but more interestingly by $Na_2S_2O_4$ in aqueous ether. A protecting group has been designed for the purification of peptides by affinity chromatography[45]. The [2-[(2-nitrophenyl)dithio]-1-phenylethoxy]carbonyl (NpSSPeoc) group (**6**) is electrophilic and derivatives can be immobilized on a thiol-containing support by an exchange reaction. The amino group of Asp or Glu can be protected using $CCl_3CH_2OCOCl$. After formation of the oxazolidone and reaction with $SOCl_2$ followed by coupling with an amino acid ester, the peptide derivative can be liberated by $Zn/AcOH/H_2O$ (Scheme 2)[46]. Another new protecting group (Dcm) generated from 5,5-dimethyl-2-(dimethylaminomethylene)cyclohexane-1,3-dione (**7**) (Scheme 3) is designed to preclude racemization via the oxazolone mechanism and none was detected following solution-coupling or SPPS using i-PrN=C=Ni-Pr/HOBt[47]. Unfortunately the Dcm derivatives of Pro and Sar were too unstable for peptide synthesis. The Dcm group can be removed using $N_2H_4$ or secondary amines in polar solvents. Leu-



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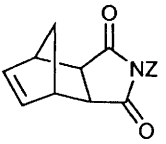

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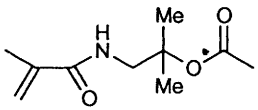

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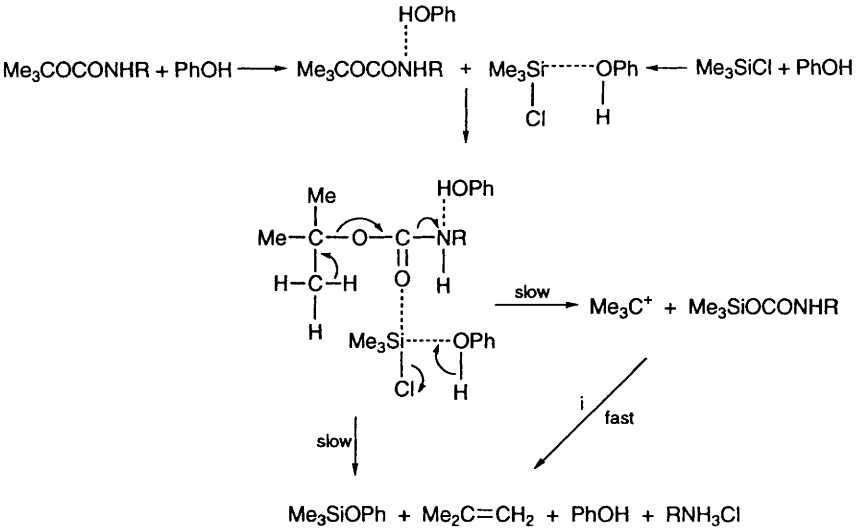
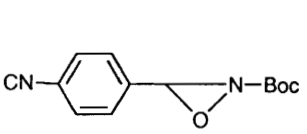



Reagent: i, $Me_3SiCl/PhOH$

**Scheme 1**

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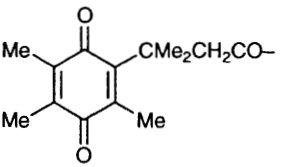


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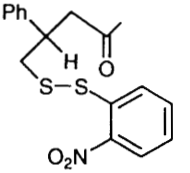


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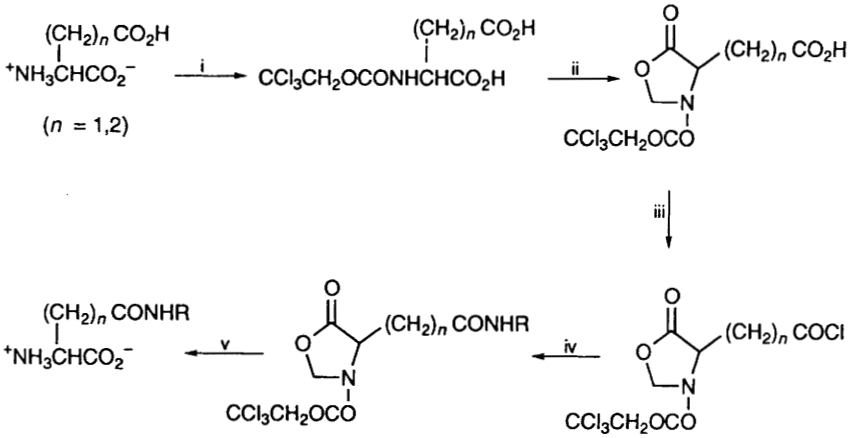



Reagents: i, $CCl_3CH_2OCOCl$, base; ii, $(CH_2O)_m$, TosOH; iii, $SOCl_2$; iv, $RNH_2$; v, Zn, $AcOH/H_2O$ (1:2)

**Scheme 2**

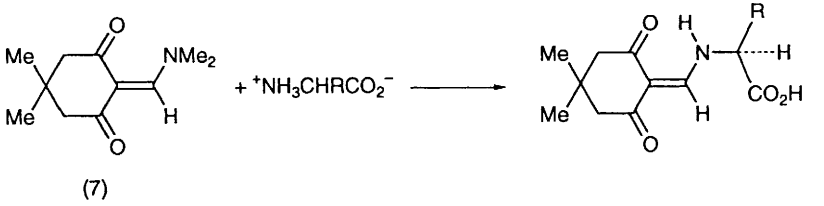
enkephalin and CCK8 were synthesized using the Dcm protecting group. Two methods for the synthesis of 3-nitro-2-pyridinesulfenyl chloride (NpysCl) have been worked out[48]. It is only moderately stable in solution and slowly forms the disulfide. The same byproduct is formed in the preparation of Npys amino acids, although its removal is straightforward. Finally, *N*-diphenylphosphinyltyrosine can be used in dipeptide synthesis without protection of the hydroxyl group using $Ph_2POCl$ as the coupling agent[49], but care was required to optimize the amount of $Ph_2POCl$ to avoid side reactions involving the hydroxyl group when a tetrapeptide ester was coupled.

## 2.2 Carboxyl-group Protection

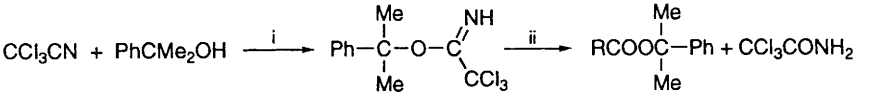
A simple synthesis of t-butyl esters of Z-amino acids involves reaction with t-BuBr in the presence of $BzlEt_3NCl$ and $K_2CO_3$ in $AcNMe_2$ at 55°C for 24 h[50]. Preparation of the ω-protected derivatives of Asp and Glu requires initial blocking of the α-carboxyl group by temporary formation of an oxazolid-5-one ring. The previously reported 2-(1-adamantyl)-isopropyl esters (Adp) have been used in peptide synthesis[51], and the Adp esters are more sensitive than t-Bu esters to acidolysis. Possibly more interesting is the report of the synthesis of 2-phenylisopropyl esters from the intermediate 2-phenylisopropyl trichloroacetimidate (Scheme 4)[52]. Deprotection with 2% $CF_3CO_2H/CH_2Cl_2$ does not affect *O*-t-Bu or Boc groups. *p*-Nitrobenzyl esters, like $Z(NO_2)$ derivatives, can be deprotected with Zn dust in tetrahydrofuran/phosphate buffer (pH 6)[28]. Phenacyl esters are cleaved by $Bu_4NF$ hydrate (3 equivalents) in the presence of BzlSH at room temperature in 75 s[53]. ω-Benzyl and ω-4-nitrobenzyl esters are stable under these conditions. Phenylhydrazide protecting groups can be removed readily with horseradish peroxidase or laccase (*Coriolus hirsius*) at pH 7 in presence of 1 mM $H_2O_2$ and aerial $O_2$ without affecting Met and Trp residues[54]. The 2-methoxy-5-nitrophenyl ester of Gly is photolysed by a laser pulse at 308 or 337 nm within 3μs[55]. Although this observation has not yet been applied to peptide synthesis, it is included here as a tempting morsel of information.

## 2.3 Side-chain Protection

The 2-(4-acetyl-2-nitrophenyl)-ethyl group (Ampe) has been recommended[56] for protecting the side chain of Asp. One synthesis (Scheme 5) involves an oxoborole complex in the protection of the α-carboxyl group during esterification. Deprotection is effected with 0.1M $Bu_4NF$. The trimethylsilyl ester (Tmse) group is an alternative orthogonal protecting group for Asp and its use was demonstrated in the synthesis of a cyclic peptide in which ring closure was effected between the ß-$CO_2H$ group of the *C*-terminal Asp and the *N*-terminal amino group while the peptide was still attached to the resin[57]. ε-*N*-Alloc-Lys-OH and its α-*N*-Fmoc derivative can be prepared on a large scale in a one-pot reaction[58]. If the Dnp group is used to protect the imidazole group of His, it is not stable during the aminolysis of ε-*N*-Fmoc from Lys; some dinitrophenylation of deprotected Lys side chains can occur[59]. Protection of the ε-amino group of Lys during SPPS with the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl

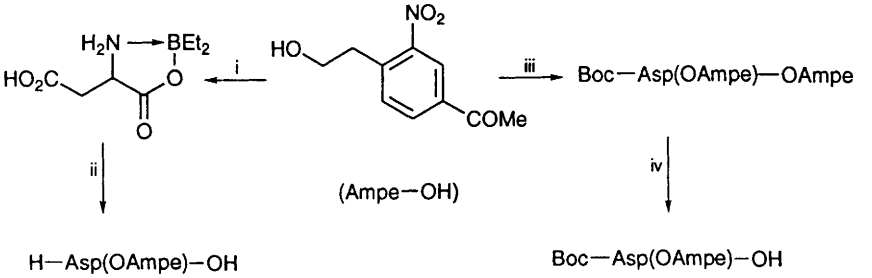



**Scheme 3**




Reagents: i, NaH; ii, $RCO_2H$ in $CH_2Cl_2/C_6H_{12}$

**Scheme 4**




Reagents: i, H–Asp–OH, $Et_3B$, tetrahydrofuran; ii, Ampe–OH;
iii, Boc–Asp–OH, DCCI, DMAP, $CH_2Cl_2$; iv, LiOH, $Me_2CO$, $H_2O$

**Scheme 5**

group (Dde) permitted its selective removal with 1.5% $N_2H_4$ in $CHONMe_2$ and elaboration of an ε-*N*-peptidyl chain prior to formation of a cyclic peptide, all while still attached to the resin[60]. The synthesis and use of α-*N*-Fmoc-ε-*N*-(6-nitroveratryl)oxycarbonyl-lysine in SPPS has been described[61]. A method for the selective protection by Boc of the α-amino group in methyl 2,3-diaminopropionate has been reported[62]. The synthesis of α-*N*-Boc and α-*N*-Fmoc derivatives of Arg, Asp, Cys, Glu, His, Lys, Ser, Thr and Tyr with either *O*-allyl or Alloc side-chain protection has been described[63]. These protecting groups can then be selectively removed using $Bu_3SnH$ with $PdCl_2(PPh_3)_2$ catalyst. Carpino's group has designed and used the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf) as an alternative to the Pmc group for protecting the side chain of Arg[64]. The Pbf group is more rapidly removed than Pmc in $CF_3CO_2H$/$H_2O$ (95:5). This could be useful, for example, in SPPS using Fmoc chemistry, especially in view of the report that removal of Pmc or Mtr groups from Arg residues in peptides containing Ser and/or Thr protected by *O*-t-Bu groups can be complicated by concomitant *O*-sulfation of the latter residues[65]. In addition, alkylation of Trp residues is diminished if Pbf rather than Pmc is used to protect Arg[66]. It is recommended that a combination of Boc protection of Trp and Pbf protection of Arg should be used and deprotection can be effected with a variety of acidolytic reagents and cation scavengers are not essential.

Surprisingly, in the SPPS of nafarelin, which contains Glu, His, Trp, Ser, Tyr and Arg, it is claimed that only His and Ser must have side-chain protection[67]. Novel silicon-based groups, $Me_2RSiCH_2CH_2$-, (R = Me, Ph), have been used to protect the hydroxyl group of Tyr[68]. Both groups are more stable than t-Bu and resistant to tertiary amines, HOBt, $R_3P$ and other nucleophiles. They are readily removed by $CF_3CO_2H$ in 5-20 min and cation scavengers are not necessary. Attempted removal of Acm groups from Cys residues in peptides with a high Ser/Thr content was found to be accompanied by some transfer of Acm to the latter residues[69]. Addition of glycerol as scavenger is recommended. The Tmob group has been used for protecting the side chain in ω-$SO_2NH_2$ analogues of peptides of Asn and Gln[70]. Thiol groups can be protected using the chromophoric reagent, $[(C_6H_7)Fe(CO)_3]^+BF_4^-$, in aqueous solution[71]. This lipophilic group is removed by $HBF_4/CHCl_3$ and the reagent is recoverable.

## 2.4 General Deprotection

Detailed comparison of the relative merits of 55% $CF_3CO_2H/CH_2Cl_2$ and 100% $CF_3CO_2H$ for Boc removal during SPPS has been carried out[72]. The first reagent gave purer products and it was shown that when incorporation of the second residue was low, this was due to insufficient swelling of the resin during Boc group removal. Acidolytic deprotection and cleavage of peptides containing acid-labile Tyr($SO_3H$) residues have been studied following SPPS[73]. Reagents containing 90% $CF_3CO_2H$ with scavengers such as m-cresol and 2-methylindole at 4°C were recommended. Formation of aminosuccinimide and isoAsp residues from peptides containing Asp can be suppressed by a stepwise deprotection using reductive acidolysis with $SiCl_4/CF_3CO_2H$ of ß-Tmob groups on Asp residues[74]. Lys and Tyr were protected by the Msz and Dpp groups respectively. Removal of

the latter was achieved with $F^-$ ion subsequent to the first stage of deprotection. Problems with $CF_3CO_2H$/PhSMe for side-chain deprotection induced another team to examine the use of 1M $HBF_4$ at 4°C for 90 min.[75]. This proved satisfactory especially since it was desired to retain protection for thiol groups. The latter were protected with t-$BuCONHCH_2$- (Tacm) which is stable to $HBF_4$. Disulfide bond formation was effected by reaction of the Tacm-peptide with $MeSiCl_3$ in presence of PhSOPh/PhSMe.

## 2.5 Peptide Bond Formation

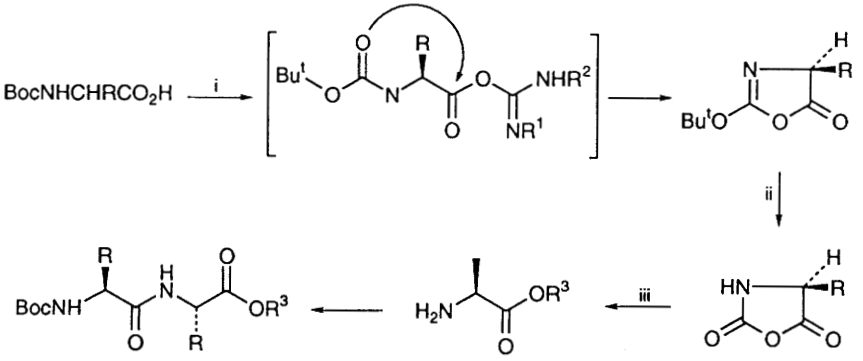
Although *NN*-bis-Boc amino acids cannot cyclize and undergo racemization via an oxazolone intermediate, the usual routes to peptides involving e.g. carbodiimides or acid anhydrides are inconveniently slow so that α-hydrogen exchange might have time to promote the alternative mechanism of racemization, especially since a second electron-attracting substituent on nitrogen labilizes the α-hydrogen atom. Bis-Boc amino acid fluorides, as briefly reported by Savrda and Wakselman last year and now further explored by Carpino's group[76] react sufficiently rapidly with negligible racemization. Fmoc-Aib-F is a useful reagent for introducing adjacent residues of Aib during SPPS[77]. Another problem has been reported in the attempted azide coupling of His derivatives with an unprotected imidazole ring[78]. The intermediate acyl azide underwent the Curtius rearrangement and the resulting isocyanate cyclized to give a dihydroimidazo [1,5-c]pyrimidine-5-one (**8**). Tos-*N*-Me-Aib-$N_3$ and the unsymmetrical anhydride, Tos-*N*-Me-Aib-$OCOCMe_3$ have been crystallized and their stuctures have been determined[79].

The effect of solvent on racemization in couplings mediated by a carbodiimide has been further studied[80]. Boc-Gly-L-Ala-OH was coupled to L-Leu-PAM-resin with or without HOBt in a range of solvents using i-PrN=C=Ni-Pr. After deprotection with 50% $CF_3CO_2H/CF_3SO_3H$ (9:1), the yield and degree of racemization were quantified by reversed-phase HPLC. Addition of HOBt always improved yield and diminished racemization. Results in $CHONMe_2$ were good, but protic solvents such as $CF_3CH_2OH$ and $CF_3CO_2H$ were much less satisfactory, although admixture with $CH_2Cl_2$ improved matters. Contrary to recent studies with BOP, a mixture of *N*-methylpyrrolidone and MeSOMe gave poor yields in carbodiimide-mediated peptide couplings. Benoiton has highlighted a further problem when carbodiimides are used to couple Boc amino acids[81]. When Boc-Xaa-OH was coupled with phenols in $CH_2Cl_2$, up to 25% of the product consisted of Boc-Xaa-Xaa-OAr. This side reaction was attributed to the formation of a 2-alkoxy-5(4)-oxazolone (Scheme 6) followed by fragmentation to the *N*-carboxyanhydride and then ring-opening in the presence of an alcohol giving the amino ester. The latter reacts with the *O*-acylisourea formed from the bulk of the Boc-Xaa-OH. This side reaction can be suppressed by the addition of 1 equivalent of *N*-methylmorpholine in syntheses effected by DCCI. No sooner is a cure apparently found for a problem and somebody rocks the boat. The addition of HOBt to minimize racemization in the coupling of Z-Pro-D-Leu-OH with 2,3-benzo-10-aza-1,4,7,13-tetraoxacyclopentadeca-2-ene (**9**) with DCCI actually promotes racemization[82].

The disproportionation of unsymmetrical anhydrides derived from *N*-protected amino acids and chloroformates is one possible explanation for occasional low yields. Anhydrides derived from Boc, Z and Fmoc amino acids and ethyl chloro-formate were kept for 24h and then analysed by HPLC[83]. Symmetrical anhydrides were formed with derivatives of Leu and Phe but not with Val or Ile. Polar solvents favoured disproportionation whereas anhydrides derived from bulky chloroformates were more stable. This work was extended[84] in a study of the generation of aryl esters of *N*-protected amino acids by reaction of the appropriate phenol with an unsymmetrical anhydride. The synthesis is catalysed by tertiary amines. The unsymmetrical anhydrides derived from *N*-alkoxycarbonylamino acids and isopropenyl chloroformate slowly form the corresponding 2-alkoxy-4-alkyl-5(4H)-oxazolone (**10**) with concomitant production of $CO_2$ and $MeCOMe$[85]. A polar solvent favours this process. Some symmetrical anhydride is usually formed as well. The oxazolones (**10**) had previously been shown to undergo aminolysis without racemization. *N*-Carboxyanhydrides have been recommended for coupling in SPPS[86], since reaction rates and extent of racemization are comparable to those observed with BOP and HBTU. Good yields of peptides containing adjacent Aib residues are obtained using the *N*-Fmoc carboxyanhydride derived from Aib[87]. *N*-Carboxyanhydrides have been used to synthesize peptide prodrugs from *C*-4-ß-aminoalkylcarbapenems[88].

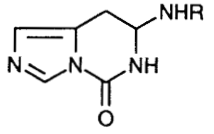
3-Dimethylphosphinothioyl-2(3H)-oxazolone (**11**), which is synthesized by the interaction of 2(3H)-oxazolone and $Me_2P(:S)Cl$ in the presence of $Et_3N$ in benzene at room temperature[89], approximates in reactivity to $(PhO)_2P(:O)N_3$ and $(MeO)_2P(:S)N_3$ but is free from side reactions associated with the azide group. No racemization was detected during the coupling of Z-Glu-Val-OH with H-Val-OMe in $CHONMe_2$ at 0°C and the slight racemization detected at room temperature could be suppressed by addition of HOBt. Good results are also claimed for $(PhO)_2P(:O)OPfp$[90].

A study of the extent of racemization during aminolysis of reactive esters of *N*-alkoxycarbonylamino acids by amino acid anions in aqueous solution revealed that good results could be obtained by using $Na_2CO_3$ as base with 50% excess of amino acid anion[91]. In most cases, the degree of racemization was 0.7%. An improved method of minimizing racemization involves the use of 1-hydroxy-7-azabenzotriazole (**12**; HOAt) as an additive during coupling reactions in place of HOBt[92]. The improved performance observed with HOAt is attributed to the presence of the weakly basic pyridine ring. The combined use of 2-trifluoroacetylthiopyridine and HOBt gives good yields of peptides with very little racemization[93]. The reaction of 1-hydroxy-6-(trifluoromethyl)benzotriazole with oxalyl chloride or phthaloyl chloride generates the benzotriazolyl oxalate and phthalate which are claimed to be excellent reagents for peptide coupling[94]. Similarly, 6-nitro-1-ß-naphthalene-sulfonyloxybenzotriazole gives good results with no detectable racemization on adding one amino acid[95]. Unfortunately, almost total racemization occurred during fragment coupling. A new saccharin derivative prepared by the interaction of the 3-chloro compound with pyridine-2-thiol gave moderate yields of protected dipeptides but no data were reported

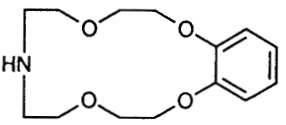



Reagents: i, $R^1 = C{=}NR^2$; ii, $H^+$; iii, $R^3OH$

**Scheme 6**




(8; R = Z, Boc or Z– Gly)

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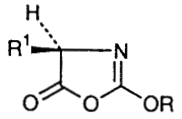


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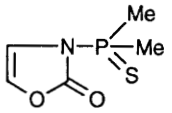


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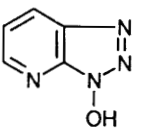


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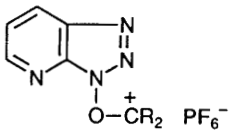



(13; R = pyrrolidino
or piperidino)

about possible racemization[96]. Blake's method of peptide bond formation involving reaction of a thiolester with $AgNO_3$ in the presence of *N*-hydroxysuccinimide and *N*-methylmorpholine has been applied to fragment condensation in which each fragment contained a protected Cys residue destined to be contributors to a disulfide bond[97]. The 4-MeBzl protecting groups were removed with HF in presence of cation scavengers then the disulfide bond was formed by oxidation with $K_3Fe(CN)_6$. Diphenylphosphinoyl chloride can be used for both amino group protection and peptide bond formation[49]. A comparative assessment[98] of a new reagent, pentafluorophenyl diphenylphosphinate, and several other reagents revealed that it was very good for forming cyclic peptides but was not satisfactory with Fmoc derivatives. Two other new reagents based on 1-hydroxy-7-azabenzotriazole (**13**) are also very good for synthesizing cyclic peptides[99]. The one-pot peptide synthesis involving Se-phenyl carboselenoates as key intermediates has been extended to include the use of unesterified amino acids or peptides as nucleophiles[100]. Kemp's thiol capture method of forming a peptide bond with a Cys residue as the carboxy component has been tested in the synthesis of 39- and 25-residue peptides[101]. This work demonstrates that the methodology allows fragment coupling to be achieved in high yield. Moreover, in the particular sequence synthesized, an Asp ß-$CO_2H$ group situated two residues before the key Cys residue is suitably stereochemically situated relative to the the transition state of the *O,N*-acyl transfer reaction to provide intramolecular general base catalysis in addition to the entropic activation due to the dibenzofuran template. Another method of intramolecular peptide bond formation has been devised[102] (Scheme 7) and uses tetrahydrophthalazine as the rigid template. Although a protected tripeptide is the most ambitious essay to date, further improvements and more enterprising goals are to be expected, perhaps by fine tuning of the template structure as Kemp has demonstrated. Hexafluoroacetone can be used simultaneously as a protecting and activating agent for peptide bond formation[103]. The reactive intermediate is a 2,2-bis-(trifluoromethyl)oxazolid-5-one. The method of generating a peptide derivative by photolysis of chromium aminocarbene complexes in the presence of amino acid esters has been extended to elongate peptides including those attached to a resin in SPPS[104]. A novel method of introducing a primary amide group at the *C*-terminus of a peptide uses $NH_2CH_2NH_2$[105]. Reactive esters or acyl chlorides react with $NH_2CH_2NH_2$ in dioxan in the presence of $Et_3N$. The reagent slowly releases $NH_3$ and by avoiding concentrated aqueous $NH_3$ protects against racemization.

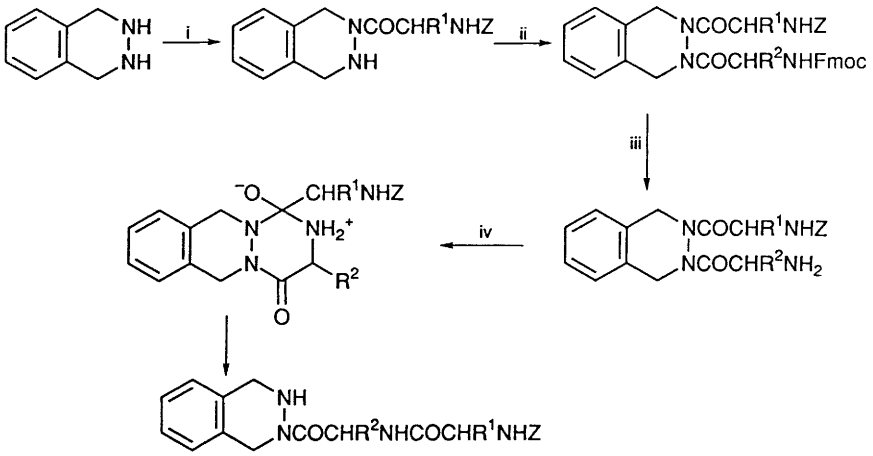
### 2.6 Disulfide Bond Formation

Disulfide bonds are formed from *S*-protected or unprotected cysteine by the action of MeSOMe/$CF_3CO_2H$ (1:9)[106]. The method was tested by synthesizing oxytocin and human calcitonin gene-related peptide. Alternatively, treatment of Cys(Acm) residues with $CF_3SO_3Ag$ followed by MeSOMe in aqueous HCl can be used[107]. *N*-Halosuccinimides convert cysteine, Cys(Acm), Cys (MeOBzl) or Cys(MeBzl) into cystine[108]. *N*-Iodosuccinimide is the mildest reagent and it can be used to generate cyclic disulfides while the open-chain peptides are still attached to the resin. Vasopressin, oxytocin, hGRH and apamin

were successfully synthesized. Dimethyl(methylthio)sulfonium tetrafluoroborate removes several types of thiol protecting groups from cysteine with concurrent disulfide formation[109]. It was found that the linear -Cys(SSMe)- peptide was sometimes formed, but this can be reduced and the resulting dithiol oxidized with $K_3Fe(CN)_6$. 2-Quinolylmethyl thioethers, which can be prepared by the interaction of 2-chloromethylquinoline with thiols in the presence of base, are deprotected and converted into disulfides by treatment with $FeCl_3$ or $CuCl_2$ in aqueous $CHONMe_2$ or EtOH[110]. Several cases of the synthesis of cyclopeptides containing one or two disulfide bonds have been reported. One example illustrates the use of orthogonal protecting groups to synthesize a peptide (Scheme 8) with a high affinity for $Ba^{2+}$ ions[111]. If disulfide formation involves the thiol group of penicillamine, the latter need not be protected[112]. Note that the amide group in the ring can have either Z or E configurations. When analogues of reduced endothelin containing two Pen and two Cys residues were aerially oxidized, the nature of the major product depended on the locations of the Pen residues[113]. Oxidation of reduced [$Pen^{1,11}$,$Nle^7$]ET-1 or [$Pen^{3,15}$, $Nle^7$]ET-1 gave mainly unsymmetrical disulfides which had the correct 1-15, 3-11 pairing. In contrast, reduced [$Pen^{1,15}$,$Nle^7$]ET-1 afforded the unnatural 1-11, 3-15 isomer because of the sterically unfavoured formation of the Pen symmetrical disulfide. The parallel and antiparallel bis-cystine peptides (**14, 15**) have been synthesized using Fm and Acm groups to protect pairs of thiol groups followed by stepwise deprotection and oxidation[114]. (The original paper should be consulted for details of the neat method of producing the isomeric protected heptapeptides). The synthesis of α-conotoxin S1 (**16**) involves the formation of a cyclic peptide containing two disulfide bonds[115]. Although random oxidation of the linear tetrathiol gave a moderate yield of the desired product, correct pairing was ensured by protecting $Cys^2$ and $Cys^7$ with Tmob and $Cys^3$ and $Cys^{13}$ with Acm and generating the disulfide moieties in a stepwise fashion. The disulfide groups could also be generated while the peptide was still attached to the resin after SPPS and this may well be a general method of choice. The most ambitious project[116] involved the total synthesis of human insulin using three orthogonal groups, Trt, Acm and t-Bu, to protect thiol groups during the generation of the three disulfide groups. [$Cys(t\text{-}Bu)^{6,11}$, $Cys(Acm)^7$, $Cys(Trt)^{20}$]A-chain(1-21) and [$Cys(Acm)^7$, $Cys(Trt)^{19}$] B-chain (1-30) were constructed. Removal of the Trt groups allowed the formation of the first disulfide bond by the thialysis method. Next, the Acm groups were removed and the second disulfide bond was formed by the action of $I_2$. Then the product was treated with $MeSiCl_3$in PhSOPh in order to form the last disulfide bond. The same team synthesized conotoxin M1 and an unnatural parallel dimer of hANP, both with two disulfide bonds. Finally, a peptide containing a diselenide group has been made by oxidation with $I_2$ or MeSOMe/$CF_3CO_2H$ of a pentapeptide containing a selenocysteine residue[117].

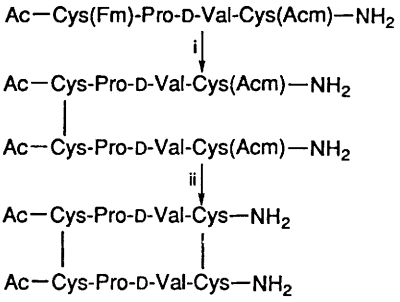
## 2.7 Solid Phase Peptide Synthesis

Several new supports for SPPS have been described[118-123]. Cotton is making a good showing[120,121], but perhaps the most intriguing support is bovine serum albumin[123]. A new synthesis of the PAL handle (**17**) involves reductive




Reagents: i, $ZNHCHR^1CO_2H$, BOP, DIEA, $CH_2Cl_2$, 30 min;
ii, $FmocNHCHR^2COCl$, DIEA, $CH_2Cl_2$;
iii, 40% aq. $HNMe_2$/THF (1:2 v/v), 1 h;
iv, 1.5% AcOH/THF

**Scheme 7**

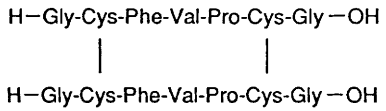



Reagents: i, piperidine/$CHONMe_2$ (1:1); ii, $I_2$/80% $CH_3CO_2H$ in $H_2O$

**Scheme 8**

amination of the corresponding 4-aldehyde with tritylamine as an ammonia equivalent[124]. The HMPB linker (**18**) has been designed[125] for Fmoc chemistry and the benzyl ester bond between the synthetic peptide and the linker is about 30-fold more acid labile than the Sheppard linker. This enables peptide derivatives to be detached from the support with side-chain protecting groups intact ready for fragment coupling in solution. A linker has been designed specifically to permit detachment of the peptide under neutral conditions[126]. The peptide is attached to an *N*-hydroxymethyl group and when the synthesis has been completed, the azide group is reduced to the amine with $Bu_3P$ and ring closure and peptide detachment ensues. The yields are good but not excellent and one wonders if the liberated HCHO leads to unwanted byproducts. A new safety catch linker[127], $N^3$-phenyloxycarbonyl-L-2,3-diaminopropionic acid, which is stable under neutral and acidic conditions, but undergoes activation at pH 10 leading to cyclization and release of the peptide acid or amide (Scheme 9), has been described. Other new linkers (**19**; R = polystyrene, R'= Fmoc or NHBoc; see page 121) have been designed[128] for the construction of peptide amides (R'= Fmoc) and peptide hydrazides (R'= NHBoc), the latter being intended for fragment coupling by the azide route. Linkers containing Si have been widely studied and a new one is reported[129] to provide peptide amides, particularly when Trp is *C*-terminal and prone to alkylation by carbocations (Scheme 10). A more specific linker contains cysteamine attached to the support through a disulfide group[130]. When cleaved reductively, the *C*-terminal cysteamine residue reacts through the thiol group to an activated protein carrier to form an immunogenic conjugate. A Co(III) ammine complex (**20**, see page 121) has been developed for attachment to polystyrene and use in SPPS. The product is detached reductively using e.g. dithiothreitol[131].

Although Boc and Fmoc share the top position for α-amino group protection in SPPS, the report that the rate of removal of the α-Z(4-MeO) group by $CH_3SO_3H$ in $CH_2Cl_2$ is accelerated by the addition of m-cresol[132] introduces another competitor, especially since pancreastatin and magainin I were successfully made by this route. An economical double-coupling procedure, in which reaction with a symmetrical anhydride, followed by a second coupling using the HOBt ester procedure, has been recommended[133]. For esterification of 4-hydroxymethylpolystyrene resin with Fmoc amino acids, the use of i-Pr-N=C=N-i-Pr in presence of pyridine in $CH_2Cl_2$/$CHONMe_2$ (9:1) or reaction with preformed Fmoc aminoacyl fluoride are reported to give high yields with negligible racemization[134]. An attempt has been made to predict quantitatively difficulties due to aggregation of peptide chains during SPPS[135]. 87 peptides were made and the volume of the resin was determined after each coupling step leading to the evaluation of an aggregation parameter, $<P_a>$ for each of the 20 coded amino acids. Coupling difficulties, if they occur, begin at the 5th residue and 6-9 *C*-terminal residues dominantly influence the character of the whole sequence. The table of $<P_a>$ values will be useful to incorporate into an algorithm to determine coupling procedures and times of reaction for use in SPPS hardware. Another group[136] has donned the hair shirt and synthesized a model antibody molecule which was extremely hydrophobic and had 70% ß-structure. Coupling

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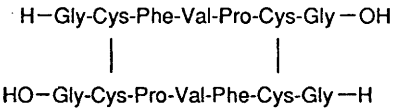


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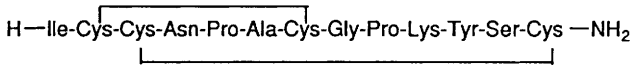


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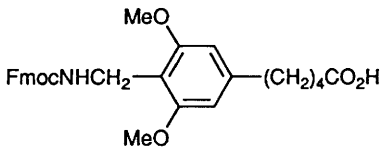


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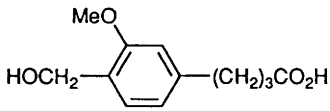


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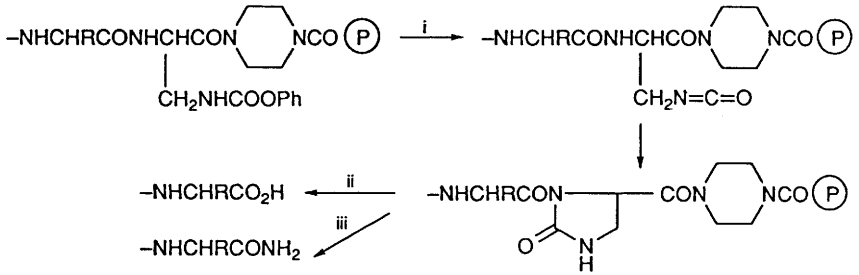
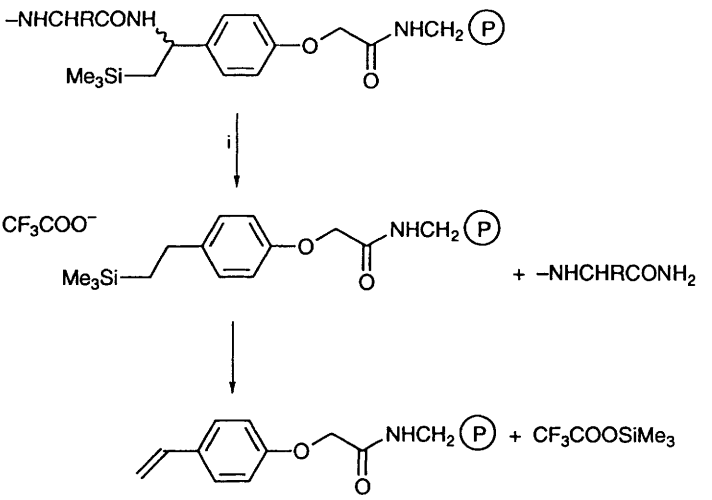


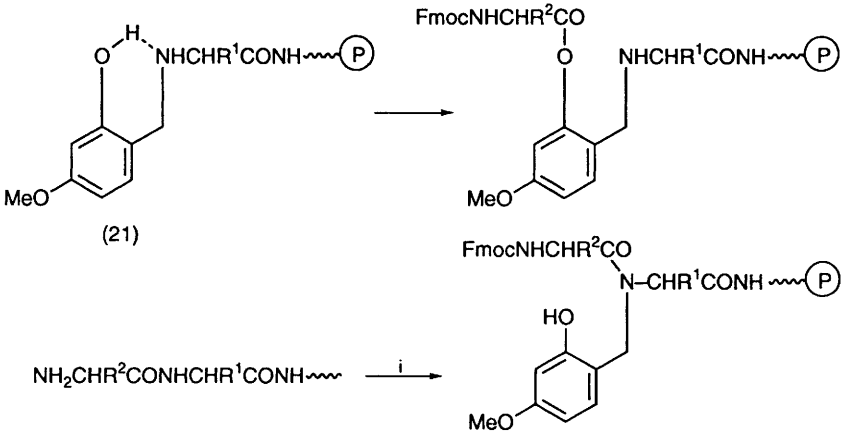

Reagents: i, pH 10; ii, $OH^-$; iii, $NH_3$

**Scheme 9**




Reagent: i, $CF_3CO_2H$

**Scheme 10**




Reagents: i, $CF_3COOH$ then piperidine

**Scheme 11**

difficulties were experienced throughout the whole synthesis, and there was little to choose between results when single, double or triple coupling stages were used. Fortunately, a new approach for overcoming the construction of difficult sequences has been proposed[137]. It was argued that the introduction of a suitable removable substituent on appropriate peptide bond nitrogen atoms would prevent the latter from acting as donors in hydrogen bond formation thus precluding the adoption of a secondary structure. An *N*-benzyl group seemed an obvious choice, but steric hindrance could interfere with the coupling of the next residue. Fortunately, it was found that an *ortho*-hydroxyl group in the benzene ring facilitated acylation by providing the initial site of acylation; intramolecular base-catalysed $O \rightarrow N$ acyl transfer completed the *N*-acylation step. One refinement remained; by incorporating a methoxy group in the benzene ring, the benzyl protecting group was rendered more sensitive to acidolysis. The required amino acid derivative (**21**) then undergoes the steps outlined in Scheme 11. By using the Ala derivative corresponding to (**21**) at position 7, the notorious ACP(65-74) sequence was assembled without difficulty.

The bromophenol blue method of monitoring coupling was used in a manual apparatus for the synthesis, using Fmoc/t-Bu protection, of two peptides containing >40 residues[138]. Coupling efficiency could be increased by raising the temperature in an ultrasonic bath. The tendency for Ala-rich peptides to form ß-sheets during SPPS on a polyacrylamide gel using Fmoc protection has been monitored by Fourier transform Raman spectroscopy during deprotection with piperidine[139]. Deprotection was essentially complete until the number of Ala residues was a multiple of 6 when 1/3 of the chains did not react. The Fmoc group favoured ß-sheet formation whereas Boc groups had no influence on the conformation of the pendant peptide. The well-known reactivity of mono esters of catechol, which stems from anchimeric assistance by the free hydroxyl group, has never been fully exploited and a recent paper[140] may change that. If a peptide is assembled on an oxime resin, then detached by interaction with the 2-phenacyloxyphenyl ester of an amino acid, the unreactive peptide ester can be converted into the much more reactive 2-hydroxyphenyl ester by removal of the phenacyl group using Zn in aqueous $CH_3CO_2H$. So far the method has been used to make sequential polypeptides, but could have a more important future in fragment coupling. Peptides with a *C*-terminal thioacid group, which are useful for fragment coupling and perhaps for enzyme-catalysed syntheses, can be prepared by treatment at room temperature of a peptide-oxime resin with $Me_3SiSSiMe_3/Bu_4N^+F^-$ followed by $NaHSO_4$[141]. There is a new method for synthesizing peptides containing *N*-methylamino acids by carrying out the *N*-methylation during SPPS[142]. With the introduction of more powerful coupling agents, it remains to be seen if this method will be preferred to the conventional synthesis from previously methylated amino acids. For the preparation of cyclic disulfide peptide amides, assembly of the open-chain derivative with Acm protection can be performed on an oxime resin. The Acm groups can be removed and oxidation effected, then the peptide can be detached as the amide using ammonium acetate[143]. Homodetic cyclic peptides can also be synthesized by effecting the cyclization step on the resin provided that the peptide contains an

amino acid such as Asp or Glu so that the peptide can be attached to the resin through the side-chain carboxyl group[144].

A number of papers describe the synthesis of peptide derivatives using solid-phase methodology. For example, by siting a Lys-Lys near the *C*-terminus, three identical chains can be constructed simultaneously, one on the α-amino group and two on the ε-amino groups[145]. A triple helical collagen model was produced by this method. *O*-Palmitoyl peptides are important in regulatory processes so their synthesis is of interest. There is a choice between using *O*-palmitoyl Ser/Thr in SPPS or *O*-acylating the assembled peptides while still on the support[146]. Peptides containing Gla residues can be prepared using Boc-Gla(OcHx)$_2$-OH as starting material[147]. Manual SPPS has been employed to synthesize a peptide which was phosphorylated on the resin[148]. An aminocarboxyphosphinic acid has been successfully incorporated into a peptide without protection of the P-OH group[149]. Removal of Fmoc groups by morpholine during the SPPS of *O*-glycopeptides is slow, incomplete and accompanied by byproducts[150]. Piperidine/CHONMe$_2$ gives better results. After assembly of a peptide by SPPS and liberation of the α-amino group, the latter can be converted into the isocyanate by reaction with bis-(2,4-dinitrophenyl)-carbonate and *N*-methylmorpholine. The isocyanate is allowed to react with an Fmoc amino acid hydrazide to give an azapeptide[151]. Coy's method for preparing ψ[CH$_2$NH] pseudopeptides by SPPS has been improved[152]. Reexamination of the use of the PAL linker for SPPS failed to detect a recently reported side reaction and it is concluded that the authors' methodology is sound[153].

There are several papers dealing with peptide detachment from supports. After assembly of thymosin $\alpha_1$ on MBHA resin, the product was detached and deprotected with Me$_3$SiBr/PhSMe/CF$_3$CO$_2$H starting at −10°C and rising to room temperature[154]. Purity was reported to be 99%. Peptides spanning the whole sequence of uteroglobin monomer were synthesized using the 4-hydroxymethyl-3-nitrobenzoyl linker and detached photolytically[155]. This last step caused Met to be oxidized to the sulfoxide. Schemes for the controlled stepwise release of peptides from a support have been devised[156-158] and part of one process is portrayed (Scheme 11). An automated allyl cleavage scheme on a continuous-flow synthesizer has been developed[159] for the preparation of head-to-tail cyclic peptides, branched peptides and multiple antigenic peptides. An insoluble Pd catalyst cannot be used and, after overcoming several problems, it was eventually found that Pd(PPh$_3$)$_4$ in CHCl$_3$ containing 5% CH$_3$CO$_2$H and 2.5% *N*-methylmorpholine was satisfactory. Peptides assembled on Pepsyn KB resin can be released as amides by NH$_3$ in tetrahydrofuran[160]. NH$_3$ in MeOH gave products that were contaminated by methyl esters. When peptides containing 5-aminopentanoic acid were detached from supports with HF in the presence of anisole, a Friedel-Crafts type of reaction occurred with the latter giving peptidyl-NH(CH$_2$)$_4$COC$_6$H$_4$OMe[161]. Peptides made by SPPS involving α-*N*-Fmoc protection and t-Bu groups on side chains can be sequenced by the Edman procedure[162]. The peptides are detached by the CF$_3$CO$_2$H used in the first cycle and subsequently embedded in a membrane. Results are satisfactory with peptides containing $\leqslant$20 residues. Sequencing has been accompanied by electro-

spray mass spectrometry[162,163]. Some home-made hardware has been described[164-166], one of which has a computer-controlled robot arm that delivers solvents and reagents to reaction vessels. There has been a further application of Blake's method of fragment coupling in the assembly of a 90-residue DNA-binding protein[167].

The remaining papers cited in this section[168-180] are concerned with multiple syntheses. Some papers describe[168-171] the assembly of relatively small libraries. Others describe libraries containing millions of peptides. Very small scale syntheses, often employing a single bead of resin support, are virtually obligatory. A few points deserve special mention. Assembly of a library can be achieved in a stepwise manner. In a search for peptides that inhibit trypsin[177], a library of hexapeptides was first assembled. The most active was then incorporated into a library of dodecapeptides. Two groups have developed methods for indexing peptides chemically by tagging the bead with an oligonucleotide[178] or with a carbonate ester[179], part of which can be detached photolytically and identified by electron-capture gas chromatography. The final reference[180] in this section describes a Pascal programme to convert linear amino-acid sequences into multiple small overlapping peptides, software which will be useful to those wishing to make candidate epitopic peptides with economy of effort.

## 2.8 Enzyme-mediated Synthesis and Semisynthesis

There is little to report on supports for immobilizing proteinases. Trypsin can be attached to polystyrene by first coupling the enzyme to azobis(4-cyanovaleric acid), then irradiating to generate free radicals in the presence of styrene[181]. α-Chymotrypsin, thermolysin and papain have been covalently attached to polyethylene glycol and used in the synthesis of enkephalins[182]. α-Chymotrypsin has been immobilized on a variety of insoluble supports and all were found to afford fairly good yields in peptide synthesis[183].

Considerably more effort has been deployed in studying the use of organic solvents for enzyme-catalysed synthesis. The crystal structure of Carlsberg subtilisin in anhydrous MeCN is almost identical to that in water. The catalytic triad is intact and only four water molecules are displaced by MeCN[184]. Another group propose that subtilisin and alcalase retain their conformation in EtOH but without definitive crystallographic proof[185]. Several papers are devoted to the use of alcalase in t-BuOH[186-189]. DL-Amino acids as their esters can be resolved[186], peptides with *C*-terminal amide groups are accessible using $NH_4Cl$ as the source of amide nitrogen[187], but there is no stereospecificity at the $S_1$' site in t-BuOH[188,189]. The synthesis of H-Gly-Phe-OH from Boc-Gly-OH and H-Phe-OMe using papain has been optimized[190] but some nonenzymic hydrolysis of the latter occurs. Papain was also used to synthesize H-Gly-Gly-Phe-$NH_2$ and enzyme activity was enhanced by incorporating polyols in the medium[191]. Z-Dipeptides can be esterified in 98% v/v EtOH using papain[192]. A suspension of thermolysin in hexane in presence of $Na_2SO_4.10H_2O$ catalyses the synthesis of *N*-protected di- and tri-peptide amides in good yield[193]. Carlsberg subtilisin somewhat surprisingly gives modest yields of dipeptide amide when H-L-Phe-OEt and an amino acid amide are the substrates in solvents where the second

substrate is only slightly soluble[194]. Indeed, solvent is unnecessary with the amino acid amide dispersed in H-Phe-OEt. Further, when both substrates were solid, reaction still occurred provided that the substrates formed a eutectic mixture or a small quantity of an organic adjuvant was added to disperse the substrates. Again, using Carlsberg subtilisin to catalyse reaction between Ac-Ser-OEt or Ac-Phe-OEt and n-PrOH, enantioselectivity is displayed towards the L-substrate in most solvents especially $CH_2Cl_2$, $CHCl_3$, MePh, $C_6H_6$ and $HCONMe_2$, but this is reversed in $C_5H_5N$, t-AmOH, t-BuOH and t-$BuNH_2$[195]. A thermodynamic model was derived which correctly predicted substrate specificity as a function of solvent/water partition coefficient of substrates and the substrate specificity of enzyme-catalysed hydrolysis of the esters in water. The use of $^2H_2O/H_2O$ as solvent helped to demonstrate that ACV-synthetase from *Cephalosporium acremonium* and *Streptomyces clavuligerus* catalyses both the synthesis of H-L-δ-(α-aminoadipoyl)-L-Cys-D-Val-OH and the epimerization of the Val residue[196]. Thus, use of [2-$^2$H]-valine as one of the substrates afforded the tripeptide with essentially complete loss of $^2H$ from the α-position of the incorporated Val. In contrast, synthesis of the tripeptide from unlabelled amino acids in (1:1) $^2H_2O/H_2O$ resulted in a product which was partially labelled with one $^2H$ atom in the Val residue. Finally, in connection with choice of solvent, the synthesis of Z-Phe-Phe-$NH_2$ from Z-Phe-OEt and H-Phe-$NH_2$ in the presence of lipase proceeded in $CHONMe_2$[197]. Optimal yields were obtained in 50% $CHONMe_2$ at 10°C.

There is little to report concerning the choice of protecting group in the acylating substrate. *N*-Malyl-L-Tyr-OEt has been synthesized in large quantities using aminopeptidase from *Staphylococcus chromogenes*[198]. This water-soluble substrate was then used to synthesize two peptide derivatives using α-chymotrypsin as catalyst. This could be a useful method for making water-soluble substrates for assaying enzymes such as pepsin, chymotrypsin and collagenase which have a marked predilection for substrates containing hydrophobic amino acids. This property usually precludes the use of substrate concentrations high enough to achieve $V_{max}$. A variety of protecting groups (X = For, Ac, Boc, Fmoc, Mal, Phac, Alloc and Z) were used in the synthesis of X-Phe-Leu-$NH_2$ catalysed by chymotrypsin in MeCN or EtOAc[199]. Excellent yields could be obtained and there was a good correlation between log(hydrophobicity), ovality, dipole moment and the global reaction rate. Highest velocities were found with less hydrophobic groups (Ac, For, Mal), ovality values close to unity and high dipole moments.

A substantial number of kinetically orientated studies have been described. In the hydrolysis of Ac-Tyr-OEt by α-chymotrypsin in $CH_2Cl_2$, no reaction occurred unless $NH_4HCO_3$ was present[200]. Complete reaction was then attained. One possible explanation proposed that the salt prevents bound water from being stripped from the enzyme in its native conformation. Several other salts and $HCONH_2$ were much less effective. This observation could be important in peptide syntheses catalysed by proteinases. In other kinetic studies with α-chymotrypsin, the high stereospecificity displayed towards Ala at the $S_1$' site in some polar solvents diminished as the water content was decreased[201]. Although

aqueous 1,4-butanediol is a satisfactory medium, the velocity decreased as the water content decreased, mainly due to an exponential increase in $K_m$[202]. Finally, the kinetics of reactions catalysed by immobilized α-chymotrypsin have been studied in detail taking into account the influence of intraparticular diffusion[203]. When trypsin was immobilized on porous glass, only 10% of the enzyme was active and amidase activity was affected more than esterase activity[204]. QSAR methodology has been applied to the chemoselectivity of chemically modified α-chymotrypsin[205]. Peptide synthesis has been studied at low temperatures including in frozen solutions. Using unprotected amino acids as amino components in reactions catalysed by α-chymotrypsin, higher yields were obtained when the medium was frozen[206,207] and it was proposed that freezing results in a higher concentration in a liquid phase which is in equilibrium with solid phase. The use of temperatures in the range −22°C to −35°C was found to suppress hydrolytic side reactions[208]. Kyotorphin was synthesized from H-Tyr-OEt and H-Arg-OH in >80% yield in frozen solution[209].

The thermodynamics of the synthesis of Boc-Phe-Gly-$NHC_6H_4X$ from Boc-Phe-OH and substituted Gly anilides in the presence of α-chymotrypsin were investigated[210]. The overall equilibrium constant is a function of three components, $K^{\ominus}_{con}$ for synthesis from the electrically neutral forms of the substrates, $K_{neu}$ for the equilibrium between neutral and ionized forms of substrates and $K_{pre}$ for the precipitated and soluble forms of the product. It is concluded that the last component provides the driving force for the overall reaction. This conclusion is strongly supported by a linear relationship between yield of product and the square root of its solubility. Stepanov's group[211] have derived equations for predicting the yield of product in pepsin-catalysed, thermodynamically controlled synthesis. This group have also found[212,213] that these reactions are complicated by the coprecipitation of pepsin and product so that the reaction can stop before thermodynamic equilibrium is attained. In contrast to the loss of enzyme activity by coprecipitation with product, an important and simple method of boosting enzyme activity in an organic solvent has been reported[214]. When solutions of various proteinases are freeze-dried in the presence of lyoprotectants such as Ac-Phe-$NH_2$, sorbitol or polyethylene glycol, the enzyme activity in organic solvent is 10–20 times that observed in the absence of ligand, although the activity of treated and untreated samples of otherwise identical enzyme were the same in aqueous solution. It is proposed that the added ligand or lyoprotectant alleviates reversible denaturation. Oxazol-5-ones serve as acyl donor substrates with α-chymotrypsin[215]. Since the amino component can be a peptide and peptidyl oxazolones are stereospecifically coupled, this could be a useful method for fragment coupling.

The next seven papers cited are chosen to reflect the range of enzymes used in peptides synthesis. Several genetically engineered subtilisin BPN' mutants have been examined for resistance to solvent denaturation[216]. The Met222Phe mutant has particularly favourable properties. A specific Glu endopeptidase from *Bacillus licheniformis* has been used in the kinetically controlled synthesis of Glu-X peptide bonds[217]. Specificity for the $S_1$' site is low except that X must not be Pro. This amino acid, however, is well catered for by a Pro-specific dipeptidyl-

carboxypeptidase from *Streptomyces sp.* Boc-Pro-Pro-OH and H-Pro-Pro-OH were coupled to give the tetraproline derivative[218]. The coupling of Z-Phe-OH and H-Met-OMe using the acid proteinase from *Cynara cardunculus L.* has been studied in biphasic systems[219]. Thrombin[220] and *Pseudomonas aeruginosa* elastase[221] have been used to synthesize dipeptide derivatives. Glycopeptides containing Asn(tri-*O*-acetyl-ß-GlcNAc) in the $S_2$ or $S_3$ (but not $S_1$) positions can be prepared using subtilisin BPN' to form a peptide bond with an amino acid or peptide amide[222]. Both α- and ß-glycosides of Ser derivatives can be similarly coupled using mutants of subtilisin BPN'. The carbohydrate substituents of the last reaction can be lengthened using, for example, UDP-Gal and galactosyl transferase. The way now seems open for the synthesis of quite complex glycopeptides. An alternative method for synthesizing *O*-ß-glycosyl derivatives of Ser used the 4-nitrophenylglycoside and either the ß-galactosidase of *E. coli* or *Aspergillus oryzae* or the ß-glucosidase from almonds[223].

Attention is now focused on particular target molecules. There are more proposals for the synthesis of 'Aspartame'[224-226]. Papain can catalyse the coupling of Z-Gly-OMe and α-hydroxy- or α-keto-amines to form peptide derivatives[227]. Unsymmetrical cystine peptides can be obtained by coupling amino acid esters to *N,N'*-$(Boc)_2$-cystine using immobilized papain in EtOAc[228]. Papain also provides a route to dehydropeptides[229]. Thus, Z-ΔGlu(OMe)-OMe and H-Leu-NHR can be coupled in moderate yield. With the same substrates, however, α-chymotrypsin affords the isopeptide. Carboxypeptidase Y has been used to effect the conversion of calcitonin-Leu into human calcitonin[230]. A more unusual method of preparing peptide amides involves coupling α-substituted derivatives of 2-nitrobenzylamine and a peptide using carboxypeptidase Y then photolysing the product[231]. The photolysis product is a 2-acylnitrosobenzene which can be trapped by $NaHSO_3$. Excellent yields are claimed. In a new synthesis of α-MSH, a protected *N*-terminal octapeptide and a protected *C*-terminal pentapeptide were assembled on a 4-nitrophenone oxime resin then detached and deprotected with HF. The octapeptide had Arg at the *C*-terminus so trypsin was used to effect the final coupling[232]. An analogue of human insulin with glutaminamide at the *C*-terminus of the B chain was synthesized from porcine insulin under trypsin catalysis[233]. A combination of genetic engineering and enzyme-catalysed synthesis was used in a novel route to substance P[234]. An artificial substance P precursor was produced as a ß-galactosidase (1-459) fusion protein containing nine copies of the decapeptide sequence: RLRRPKPQQF. This accumulated in *E. coli* as insoluble inclusion bodies which were easily isolated. The decapeptide sequences were detached by α-chymotrypsin, a process facilitated by the Arg residues at $S_1$' and $S_3$' which kinetically favour chymotrypsin cleavage. By incorporating H-Phe-Gly-OMe into the digest, transpeptidation occurred leading to a dodecapeptide. This was incubated with papain and H-Leu-Met-$NH_2$ giving RLRRPKPQQFFGLM-$NH_2$. Finally, hydrolysis with trypsin removed the *N*-terminal tripeptide giving substance P.

There is very little to report on the use of enzymes to remove protecting groups used in peptide synthesis. 2-(*N*-Morpholino)-ethyl esters can be hydrolysed with lipase N at pH 7.0 and 37°C[235]. Finally, there is a report on the use of

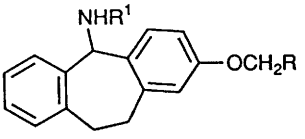
penicillin G acylase for removing *N*-phenylacetyl groups and the use of lipase M for hydrolysing heptyl esters[236].

### 2.9 Miscellaneous Reactions Related to Peptide Synthesis

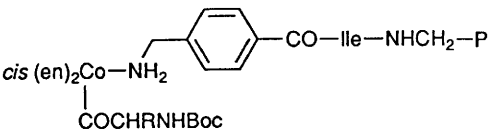
The side chains of Asp and Asn continue to enliven peptide synthesis. The kinetics and mechanism of formation of a succinimide ring from an Asn residue in the peptide: Ac-Gly-Asn-Gly-Gly-$NH_2$ over the pH range 5.5–10.4 at 37°C have been determined[237]. A multistep process (Scheme 12) is proposed. When a similar peptide of Asn was incubated in 0.1M phosphate buffer at pH 7.4 and 60°C, a mixture of the Asp and isoAsp peptides was formed[238]. The related peptide containing 5-methylasparagine also deamidated but additionally underwent main-chain cleavage. During attempts to prepare cyclic peptides by coupling the ß-$CO_2H$ group of an Asp residue and the ε-amino group of a Lys residue using either PyBOP or HBTU, the amide group of a *C*-terminal residue of Asp-$NH_2$, if present, was converted into -CN[239]. Attempted coupling of pyroglutamic acid to a derivative of Gln-Pro or Asn-Pro gave rise to the diketopiperazine derived from Glp-Gln or Glp-Asn[240]. Construction of the Glp-Gln (or Asn)-Pro-sequence is possible by coupling Glp-Gln(or Asn)-OH with a Pro derivative. Thr residues in peptides can be converted into alloThr without affecting the chirality at the α-carbon atom[241]. Treatment of Z-Xaa-Thr-OMe with $EtOCON^-SO_2N^+Et_3$ (Burgess reagent) causes cyclization to the *cis*-oxazoline with inversion at the ß-carbon atom. Mild acid treatment then reopens the ring to give an *O*-acylamine which then undergoes an O → N acyl shift on adjustment of the pH to 9.5 with $K_2CO_3$. Peptides containing free thiol groups tend to oxidize to disulfides on storage. Complete restoration of the reduced peptide can be achieved with $NaBH_4$ in pH 8–10 buffer during 30–60 min[242]. Excess $NaBH_4$ is destroyed by acidifying to pH 4. Ser and Thr residues in peptides can be converted into α-acetoxyglycine residues by $Pb(OAc)_4$[243]. The α-acetoxyglycine residues can then be treated with a tertiary amine, if desired, giving dehydroglycine residues.

## 3 Selected Examples of Peptide Syntheses

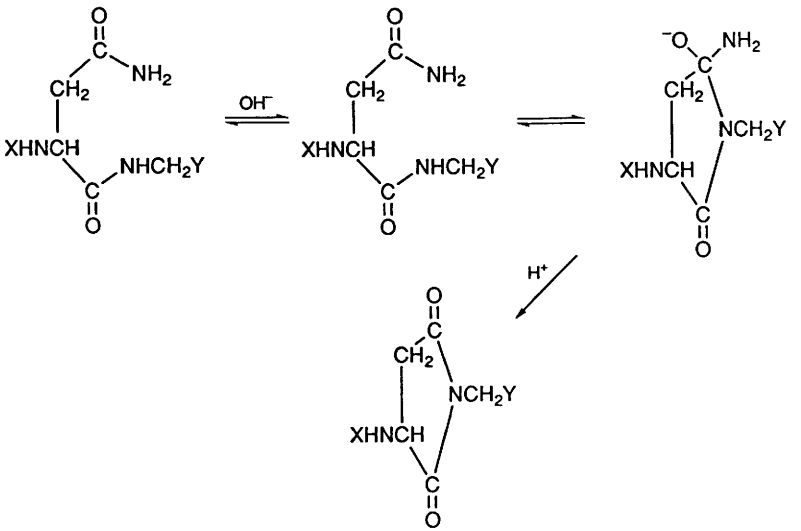
We are well past the point where entry in this section demands the synthesis of a protein of record length. The examples cited here have been chosen simply because they interested the Reporter and it is hoped that they will evoke a similar reaction in the reader. Charybdotoxin, a 37-residue peptide in the venom of the Israeli *Leiurus quinquestriatus* scorpion, has been assembled on PAM resin with Boc/Bzl protection[244]. The synthesis was somewhat complicated because of the need to make four disulfide bonds. Acm groups were not completely stable to HF and removal by Hg(II) was not complete or totally free from side reactions. The disulfide bonds were generated in aqueous solution in the presence of a mixture of oxidized and reduced glutathione. The enzyme Barnase has been constructed from four fragments which were made by SPPS[245]. Fragment condensation was effected by the Blake method from *C*-terminal Gly thioesters. $Gly^{52}$ and $Ala^{74}$




(19; R = polystyrene,
$R^1$ = Fmoc or NHBoc)




(20)




**Scheme 12**

were isotopically labelled with $^{13}C$. Insulin analogues containing $\psi[CH_2NH]$ pseudopeptide bonds in the *C*-terminal region of the B chain have been synthesized[246]. Cyclic peptides containing 29 amino acid residues and a disulfide bond have been synthesized that mimic the active sites and catalytic activities of α-chymotrypsin and trypsin[247]. The peptides have been given the trivial class name of pepzymes. Inhibitors of the native enzymes, $BzlSO_2Cl$, DFP, SBTI, BPTI and human $\alpha_1$-antitrypsin, but not TPCK, inhibit these model enzymes. There is obvious scope here for the synthesis of other pepzymes with specificities made to order. α-hANF and h-big endothelin have been made by SPPS using Fmoc protection and coupling by the symmetrical anhydride procedure[248]. Acm protection of thiol groups was preferred to *S*-Trt or *S*-t-Bu despite its sensitivity to the use of PhSMe as a carbocation scavenger. The synthesis of the 60-residue homeo domain of the Drosophila gene regulatory protein Antennapedia[249] is notable because assembly was effected in separate stages. Ten small fragments (4–10 residues) were made. These were assembled to give medium-sized fragments (17–20 residues) on an oxime resin. Final assembly was carried out in solution. A 14-residue peptide, GGHGYSPTSPSYGK, has been designed to contain a Cu(II)-binding domain (residues 1–3), a DNA-binding domain (residues 5–12) and a charged residue (K) to confer water solubility[250]. CD and NMR data indicated that the DNA-binding domain contains two ß-turns resulting in the two Tyr residues assuming a parallel orientation poised for intercalation into DNA. It was shown that the peptide effects oxidative cleavage of DNA after addition of sodium ascorbate and/or $H_2O_2$. Finally, a synthetic peptide containing 134 residues corresponding to the *N*-terminal half of the HIV-1 nucleoprotein, p24, has been assembled by SPPS[251]. Somewhat surprisingly, the side chains of Arg and His were protected by tosyl groups. Nevertheless, it was claimed that 100 mg of peptide with a purity of > 90% can be obtained in a month.

## 4 Appendix: A List of Syntheses Reported in 1993

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.* |
|---|---|
| **4.1 Natural Peptides, Proteins and Partial Sequences** | |
| Adenylate cyclase activating polypeptide SPPS of 27 and 38 Residue peptides | 252 |
| Adrenergic peptides $ß_1$- and $ß_2$-adrenergic pentapeptides | 253 |
| Adrenodoxin Fragment of adrenodoxin precursor | 254 |
| Amyloid protein (chromosome 20) Peptides corresponding to normal and mutated alleles of gene probably linked to Creutzfeld-Jakob disease | 255 |

### 4.5 Glycopeptides

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# 3

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

BY CHRISTINE M. BLADON

## 1 Introduction

Material for this chapter, as in previous years, was obtained by scanning the major organic and bioorganic journals and Chem. Abs. Selects: Amino Acids, Peptides, and Proteins (up to issue 9, May 2nd 1994). Two changes have been made to the format of the chapter this year. Statine and the hydroxyethylene-type of isostere are now discussed under a separate heading (**5.2**) in section 5 rather than as part of the reviews on renin and HIV-1 protease inhibitors while conformational studies on peptidomimetics are now distributed over the entire chapter rather than being concentrated in a single section (formerly section 7). This latter change is the culmination of a gradual trend which has been occurring over recent years. Many publications on peptidomimetics are now multi-faceted with synthetic, conformational (NMR, X-ray) and, more recently, molecular modelling, studies being combined in a single article. It has become more appropriate therefore to discuss various aspects of a particular compound or series of compounds together rather than separately.

For those readers interested in numbers, approximately 60% of the papers in this Chapter originate from academic institutions, 30% from industrial organisations with the remaining 10% being joint academic/industrial publications.

## 2 Peptide-backbone Modifications

The use of peptidomimetics in drug design and development continues to be of intense interest and a number of reviews on this subject were published in 1993.[1-10] Topics covered included the general strategies involved in peptidomimetic design, computer-assisted design, and the synthetic application of these techniques. Useful data on the conformational consequences of introducing a peptidomimetic group into a sequence has been obtained from a detailed analysis of the solution and solid state structures of model dipeptides RCO-Xaa$\psi$Yaa-NHR′ ($\psi$ = amide bond surrogate, R, R′ = various alkyl).[11] The Spatola group continues to accumulate data on backbone-modified cyclic pentapeptides with general structure cyclo[-Gly-Pro$\psi$Gly-D-Phe-Pro-]. This year the group reported the solution conformation of the

ψ[$CH_2NH$] analogue[12] and the crystal structures of the ψ[$CH_2S$] and ψ[$CH_2SO$] analogues.[13]

### 2.1 ψ[CSNH]-Thioamide Analogues

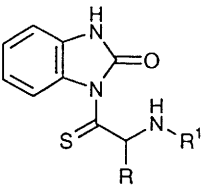
A novel thioacylating agent (1) has been developed which shows promise for the introduction of ψ[CSNH] linkages into peptides.[14] Aminolysis of (1) with amino acids or peptides (resin bound or in solution) gave good yields of thiodi- and tri-peptides. However low yields, eg 15% of thiothymopentin Arg-Lysψ[CSNH]Asp-Val-Tyr, were obtained from solid phase syntheses when the isostere was introduced into longer peptide sequences. The authors suggested that steric factors may be hindering the reaction between the thioacylating reagent and the growing resin-bound peptide. A conformational study of Ac-ψ[CSNH]Proψ[CSNH]Gly-NHMe has been carried out.[15] The reverse turn backbone conformation, stabilised by a 10-membered 1←4 hydrogen bond adopted by this compound in the solid state was also found to be the dominant structure in DMSO-$d_6$ solution.

### 2.2 ψ[NHCO]-Retro-inverso Analogues

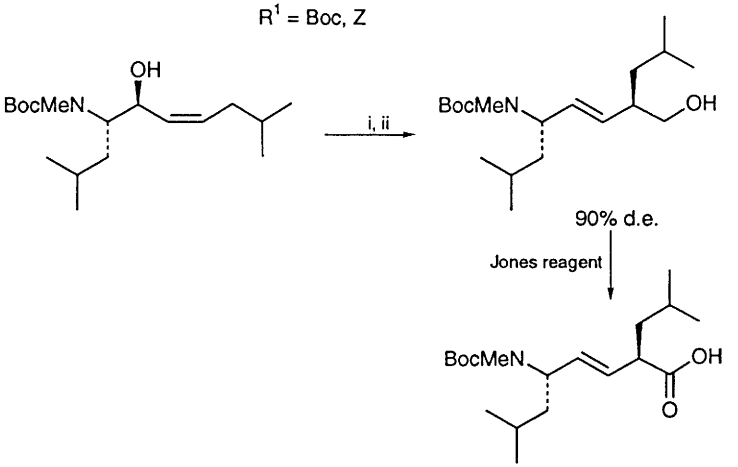
Three papers were published on the retro-inverso modifications this year. One was a review,[16] one described the synthesis of H-Gly-Hisψ[NHCO]Lys-OH,[17] an analogue of the growth factor glycyl-L-histidyl-L-lysine, and in the third, the pharmacological properties of a tritiated derivative of the CCK-B agonist Boc-Tyr($SO_3Na$)-gNle-mGly-Trp-(NMe)Nle-Asp-Phe-$NH_2$ were reported.[18] The growth factor analogue,[17] prepared by coupling Boc-Gly-gHis(Bom)-H with either TFA-$NH(CH_2)_4CH(CO_2Ph)CO_2H$ or BocNH-$(CH_2)_4$-meldrum's acid, was found to be highly resistant to degradation by peptidases.

### 2.3 ψ[$CH_2NH$]-Amino Methylene Analogues

Reductive amination of a Boc-amino aldehyde by the resin-bound peptide in the presence of $NaBH_3CN$ continues to be a popular method for the introduction of a ψ[$CH_2NH$] linkage. This technique was used in the synthesis of a series of C-terminal neurotensin hexapeptides[19] in which each amide bond was systematically replaced with the reduced amide isostere, in the preparation of insulin analogues with a ψ[$CH_2NH$] moiety in the COOH-terminal B chain domain,[20] in the synthesis of the somatostatin analogue cyclo[-Pheψ($CH_2N$)Pro-Phe-D-Trp-Lys-Thr-],[21] and in the development of δ-opioid[22] and gastrin-releasing peptide (GRP)[23] antagonists. The results of biological assays on the neurotensin hexapeptide (and also on a pentapeptide series *vide infra*), insulin, and somatostatin analogues were on the whole disappointing. More promising data was obtained on the opioid and GRP series. The analogues 4′-(hydroxy)-3-phenylpropanoyl]-His-Trp-Ala-Val-D-Ala-His-D-Proψ[$CH_2NH$]Phe-$NH_2$ and 1-naphthoyl-His-Trp-Ala-Val-D-Ala-His-D-Proψ[$CH_2NH$]Phe-$NH_2$ were found to be particularly potent GRP antagonists while H-Tyr-Ticψ[$CH_2NH$]Phe-Phe-OH displayed subnanomolar δ-opioid receptor affinity. This latter compound was shown to have unprecedented δ specificity ($K_i^{\mu}/K_i^{\delta} = 10{,}500$), was highly stable against enzymatic degradation, and showed no μ or κ antagonist proper-

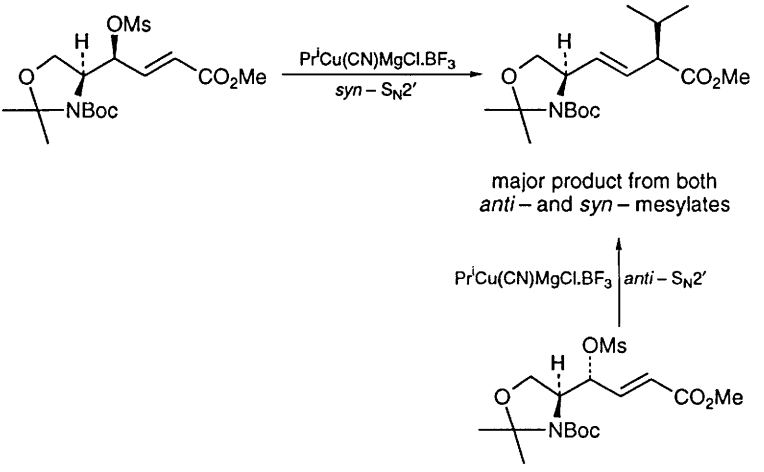


(1) R = amino acid side chains
$R^1$ = Boc, Z




Reagents: i, KH, $ICH_2SnBu_3$, 18–Crown–6; ii, BuLi

**Scheme 1**




**Scheme 2**

ties. Improvements to the *in situ* solid phase reductive amination procedure have been reported.[24] Solution phase reductive amination was used to generate the ψ[$CH_2NH$] bond in a series of neurotensin C-terminal pentapeptides[25] (*vide supra*), in a series of CCK-4 peptides,[26] and in cyclo[-Gly-Proψ[$CH_2NH$]Gly-D-Phe-Pro-].[12] The latter compound is one member of the cyclic pseudopentapeptides studied by the Spatola group - see comments at the beginning of this section. Two compounds in the CCK series, Boc-Trp-Lys(R)ψ[$CH_2NH$]Asp-Phe-$NH_2$ (R = 4-hydroxycinnamoyl, 2-tolylaminocarbonyl), showed high binding affinity and functional efficacy at CCK-A receptors.

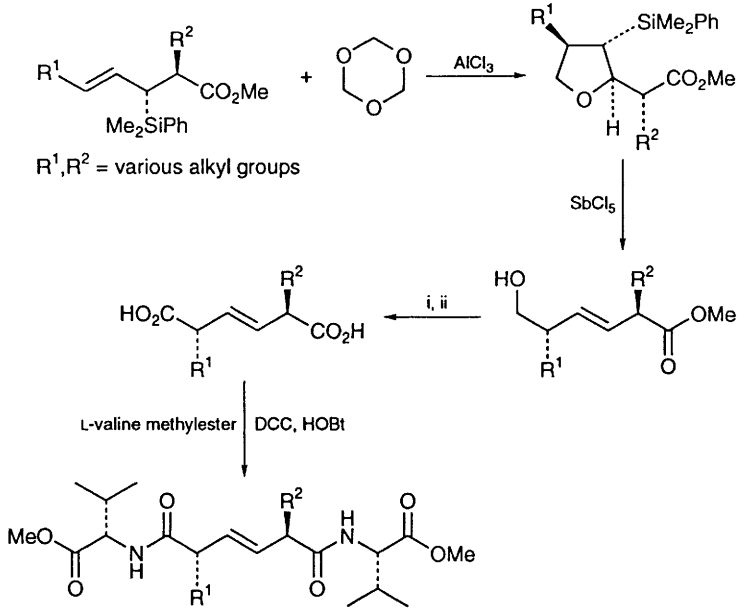
### 2.4 ψ[CH = CH]- and ψ[$CH_2CH_2$]- Ethylenic and Carba Analogues

Introduction, with high diastereoselectivity, of the alkyl group into the C-2 centre of *E*-alkene dipeptide isosteres was central to two of the synthetic routes published this year. The 2-(*R*)-chirality in BocMeLeuψ[E,CH = CH]Leu-OH was achieved *via* a stereoselective [2,3] Wittig rearrangement (Scheme 1)[27] while stereocontrol at the corresponding centre in a protected Serψ[*E*,CH = CH]Val isostere was effected, in part, by the use of bulky alkyl groups in the organocyanocuprate reagent (Scheme 2).[28] A third approach to the synthesis of optically pure [*E*,CH = CH] dipeptide mimetics involved *anti*-$S_E'$ addition of chiral (*E*)-crotylsilanes to the formaldehyde equivalent *s*-trioxane (Scheme 3).[29] This reaction resulted in the simultaneous introduction of a *trans* double bond and both amino acid side chains with high levels of diastereo- and enantio-selection. The isosteric units were subsequently incorporated into tetrapeptides.

Substance P analogues, [$Gly^9$ψ($CH_2CH_2$)$Xaa^{10}$]SP (Xaa = Gly, D-Leu, L-Leu),[30] with a carba bond between residues 9 and 10 and analogues of Boc-CCK-4, (Boc-Trp-Met-Asp-Phe-$NH_2$), in which each peptide bond was replaced by a *trans*-olefin bond[31] have been prepared for biological studies. The synthesis of both series of compounds involved incorporating racemic dipeptide mimetics into the sequence and, in the case of the substance P analogues, separating the resulting diastereoisomers by HPLC. Attempts to separate the diastereoisomeric pairs in the CCK-4 series failed and the biological studies were carried out largely on mixtures. The bis-phenylalanine mimetic (2), a component of the substance P endopeptidase inhibitor H-Arg-Pro-Lys-Pro-Gln-Gln-Pheψ[*E*,CH = CH]-$Gly(Bz)_2$-Gly-Leu-Met-$NH_2$, was prepared[32] *via* Julia olefination reaction of sulfone BocNHCH($CH_2Ph$)$CH_2SO_2Ph$ with aldehyde $(PhCH_2)_2CH(CHO)$-$CH_2OTBDMS$.

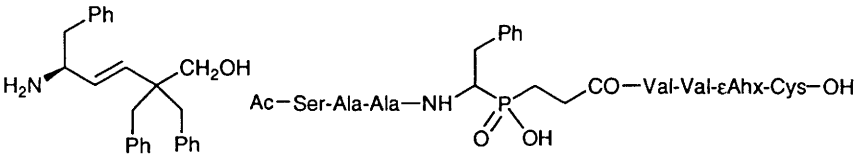
### 2.5 Phosphono-peptides

The design of novel phosphorous-based transition-state analogues of amide bond hydrolysis as well as new approaches to the synthesis of more well-known mimetics both feature in this year's crop of papers. The methylphosphinamide isostere falls into the first category and the key step in the synthesis of compounds containing this moiety was the coupling of a methylphosphinic chloride with an amino acid or peptide protected at the C-terminus (Scheme 4).[33] Phosphinic and phosphonic peptides continue to be synthetic targets and activity in this area has included the use of solid phase techniques [to

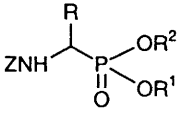



Reagents: i, Jones oxidation; ii, aq. $H^+$

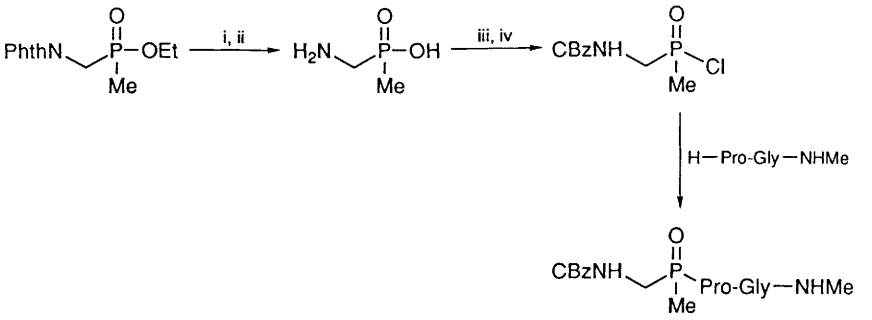
**Scheme 3**




(2) (3)

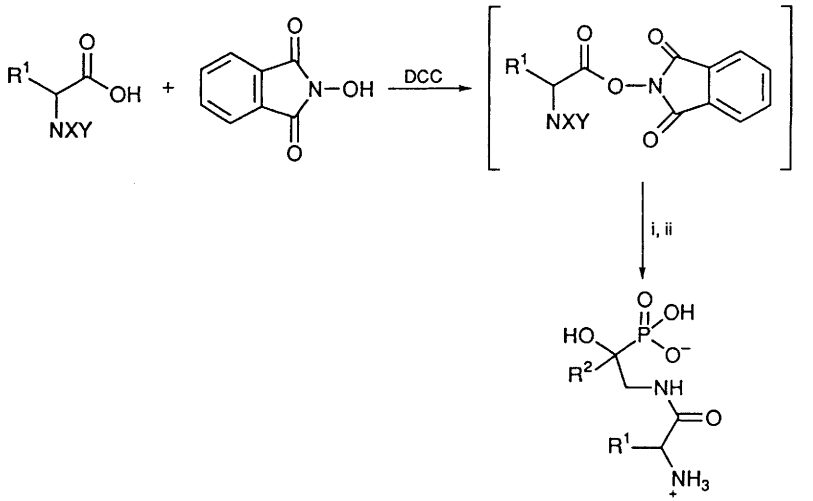


(4)a; R = $R^1$ = $CH_2Ph$, $R^2$ = various substituents
b; R = $(CH_2)_nNH_2$ ($n$ = 3–5), $R^1$ = $R^2$ = Ph

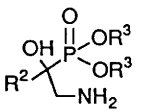



Reagents: i, 6NHCl; ii, propylene oxide; iii CBz–Cl; iv, $(COCl)_2$, DMF(cat).

**Scheme 4**




e.g. $R^1$ = D–Me, $R^2$ = *p* $FC_6H_4$,
$R^3$ = Et, XY = Phth

Reagents: i,  ; ii, deprotection

**Scheme 5**

give, for example, compound (3)],[34] the synthesis of *N*-protected phosphonate diesters (4a,4b),[35,36] and a new procedure for the preparation of phosphonopeptides bearing a free hydroxyl group (Scheme 5).[37] Synthesis of phosphonic analogues of historphin,[38] of dipeptides,[39] and of γ-glutamyl tripeptides and depsipeptides[40] have also been reported.

Analysis of the FAB mass spectra of several phosphonopeptides has provided some uesful fragmentation data.[41]

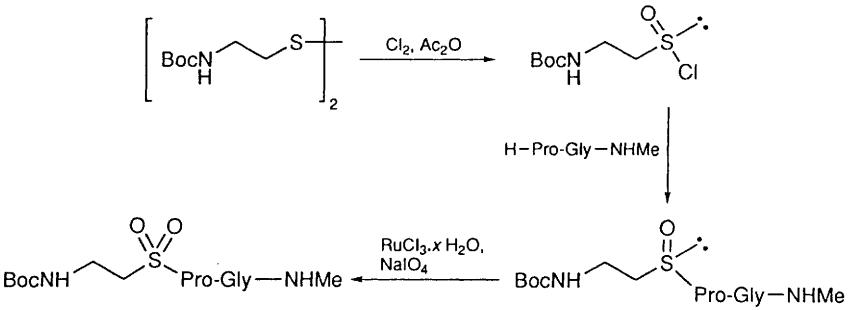
## 2.6 ψ[$SO_2$NH] Analogues

The peptides discussed in section **2.5** above containing the methylphosphinamide isostere were prepared by Liskamp and co-workers and this group have also synthesised analogous compounds with the sulfinamide and sulfonamide moieties (Scheme 6).[42] Both the phosphorous and sulfur containing peptides are analogues of the $Gly^{312}$-Pro-$Gly^{314}$ sequence in HIV gp120. Interest in the enzymes which control the γ-glutamyl cycle prompted the synthesis of the glutathione disulfide analogue [Glu-ψ($SO_2$NH)Cys-Gly]$_2$.[43]

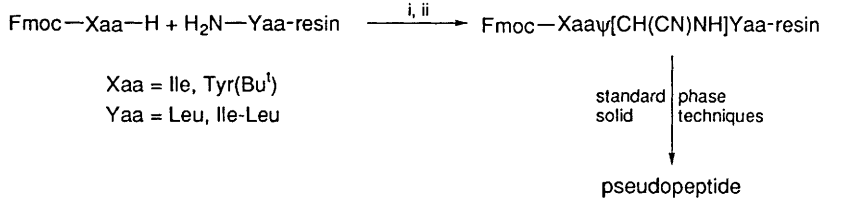
## 2.7 Miscellaneous Modifications

Herranz and co-workers have developed an easy method for the synthesis of ψ[CH(CN)NH]-pseudopeptides which is suitable for both solution[44] and solid phase strategies.[45] The procedure, which involves a modified Strecker synthesis to introduce the cyanomethyleneamino moiety, was applied to the preparation of neurotensin pseudohexapeptides H-Arg-Arg-Pro-Tyr-ψ[CH(CN)NH]Ile-Leu-OH and H-Arg-Arg-Pro-Tyr-Ileψ[CH(CN)NH]Leu-OH (Scheme 7). Azapeptides Ser-Glu-Val-azaLys-Met-Asp-Ala-Glu-Phe-Arg and Ser-Glu-Val-Lys-azaMet-Asp-Ala-Glu-Phe-Arg, required for a project associated with Alzheimer's disease, were prepared using the Fmoc-*tert*-butyl-polyamide technique.[46] The aza unit in these peptides was constructed by reaction of resin-bound *N*-terminal amino groups, activated as isocyanates, with the dipeptide synthons Fmoc-Val-NHNH$(CH_2)_4$NH-Boc and Fmoc-Lys(Boc)-NHNH$(CH_2)_2$SMe. Solid phase techniques were also used to prepare the thiomethylene-containing neurokinin antagonists β-Ala-Leuψ[$CH_2$S]Nle-Ser-Phe-Val and Ava-Pheψ[$CH_2$S]Phe-Abo-Leu-Trp.[47] Coupling on the resin to form the thioether bond involved nucleophilic substitution of the *N*-terminal bromide BrCH($^n$Bu or $CH_2$Ph)CO-peptide-resin with thiols FmocNHCH($^i$Bu or $CH_2$Ph)-$CH_2$SH.

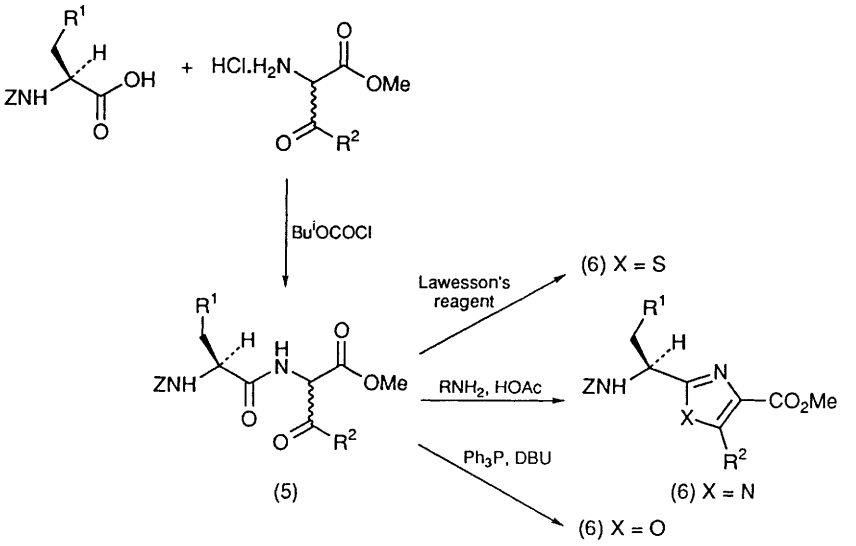
Progress has also been reported on the synthesis and application of heterocyclic dipeptide mimetics. In a versatile route to the azoles (Scheme 8)[48,49] the key intermediate (5) could be treated with either Lawesson's reagent to yield the thiazole (6, X = S), condensed with amine acetates to give the imidazole (6, X = N), or dehydrated to form the oxazole-dipeptide mimetic (6, X = O). Insertion of the azole mimetics ($R^1$ = 3-indolyl, $R^2$ = phenyl) at positions 7/8 of [$Pro^6$,D-$Trp^{7,9}$,$Phe^{11}$]$SP_{6-11}$ yielded analogues which were more potent antagonists than the parent hexapeptides.[50] Minor modifications to the procedure described last year for the synthesis of tetrazole dipeptide mimetics were necessary to obtain good yields of the bulky analogue Z-Trp($^{in}$Z)ψ[$CN_4$]Nle-OBzl.[51] Substitution of



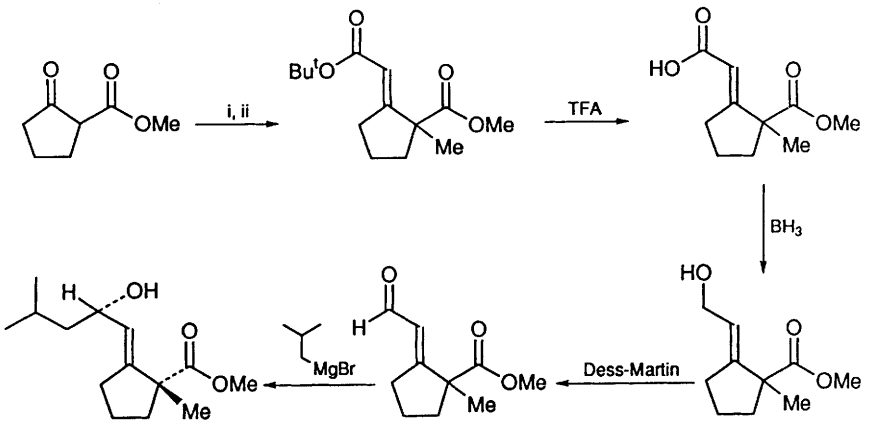

**Scheme 6**



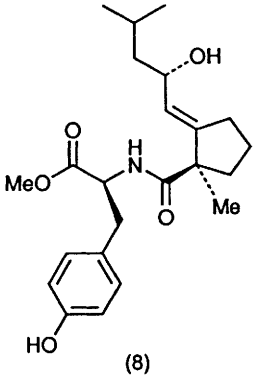

**Scheme 7**




**Scheme 8**



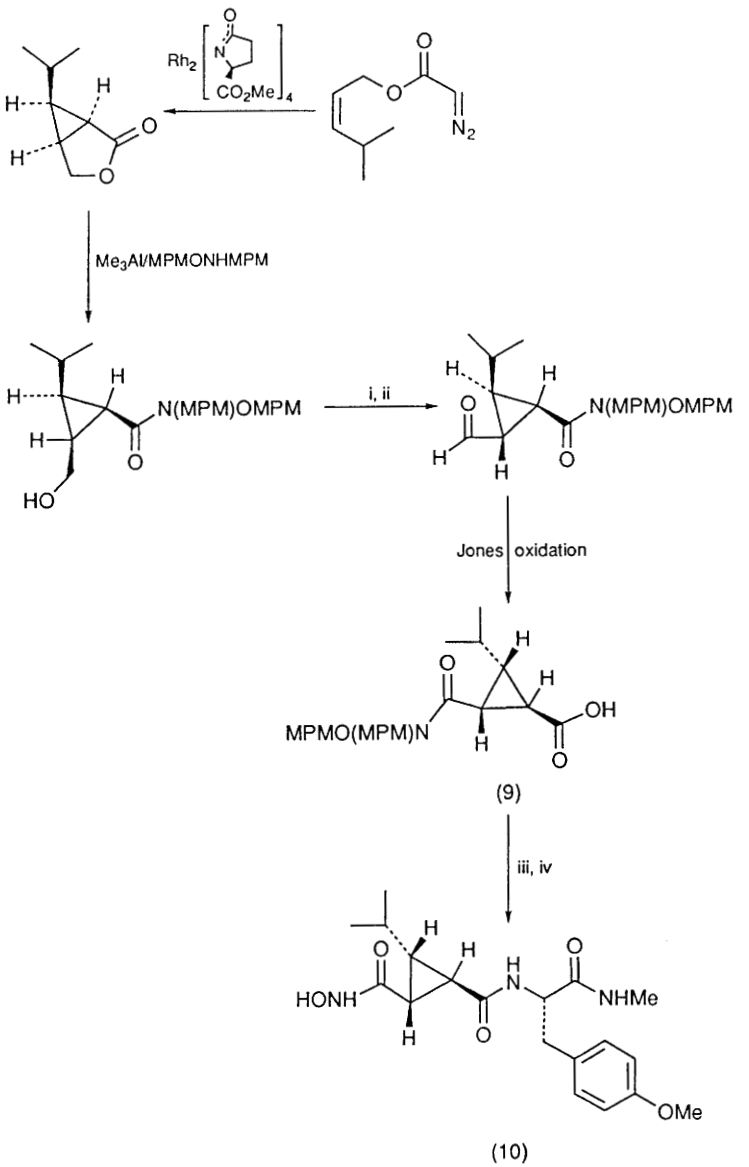

(7) (+ diastereoisomer with *R* - OH substituent)




(8)

Reagents: i, MeI; ii, Horner-Emmons

**Scheme 9**




Reagents: i, PCC; ii, $K_2CO_3$; iii, Tyr(Me)NHMe, EDC; iv, $MeSO_3H$/TFA

**Scheme 10**

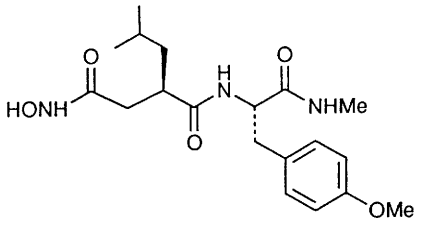
this *cis* amide bond mimetic for the Trp-(NMe)Nle moiety in the CCK-B receptor ligand Gly-Trp-(NMe)Nle-Asp-Phe-$NH_2$ caused a loss of activity.[52]

A rigid conformationally defined peptidomimetic of the *trans*-prolylamide bond has been prepared for studies with peptidyl prolyl isomerases (PPIases).[53] The mimetic (7) which possesses a *trans*-substituted alkene in place of the proline peptide bond was synthesised in 6 steps and 20% overall yield from methyl cyclopentanone-2-carboxylate (Scheme 9). Elaboration of this compound gave the Leu-Pro-Tyr tripeptidomimetic (8) which was found to be a potent inhibitor of PPIase activity of FKBP ($K_i$ of 8.6μM). Following their recent success in the synthesis of conformationally restricted renin inhibitors Martin *et al* have applied similar techniques to the design of collagenase inhibitors.[54] A 1,2,3,-trisubstituted cyclopropane dipeptide mimetic (9) similar to that used to great effect at the *N*-terminal/$P_3$ position of renin inhibitors, was prepared and coupled to tyrosine *N*-methyl amide, to yield, after removal of protecting groups, the conformationally restricted pseudopeptide (10, Scheme 10). This compound was found to be an inhibitor of collagenase ($IC_{50}$~50μM) although it was less potent than the flexible analogue (11, $IC_{50}$~1μM).

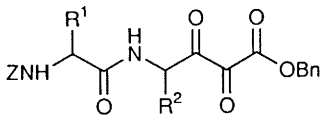
Other applications of recently developed synthetic processes include the preparation of a series of peptidyl tricarbonyl compounds with general structure (12),[55] several α-hydroxy ketomethylene dipeptide isosteres (13),[56] and *N*-terminally modified dermorphin and deltorphin-C analogues (compounds 14 and 15 respectively).[57] Several of the tricarbonyl compounds were found to be moderately potent inhibitors of serine proteases while the α-hydroxy dipeptide isosteres may find applications in the design of inhibitors for the renin-angiotensin system. Carbonylation of an enol triflate with α-amino acid esters has generated another series of dipeptide isosteres (Scheme 11).[58]

Lewis acid catalysed ring opening of a serine-derived *N*-Boc-aziridine with isoamyl alcohol yielded the ether-based dipeptide unit (16, Scheme 12).[59] Although the paper reported the preparation of several other *N*-Boc-aziridines the versatility of this route has yet to be asertained as no examples of the ring opening step were given apart from that leading to (16).

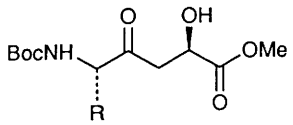
Three different approaches have been used to prepare peptides with *N*-alkylated residues. Preparation of $N^{\alpha}$-methyl substituted derivatives of LHRH agonists [D-Leu$^6$,Pro$^9$NHEt]LHRH (leuprolide), [D-Trp$^6$,Pro$^9$NHEt]LHRH (deslorelin) and [D-2-Nal$^6$]LHRH (nafarelin) used standard methods of solid phase techniques.[60,61] Most of the analogues were less potent agonists than the parent peptides but several, for example (NMe)Phe$^2$-leuprolide, (NMe)His$^2$-deslorelin, and (NMe)Arg$^8$-nafarelin, were antagonists. Substitutions of (NMe)-1-Nal$^3$, (NMe)Ser$^4$, or (NMe)Tyr$^5$ in leuprolide rendered the 3-4 peptide bond in these compounds completely stable to chymotrypsin. Standard solution techniques were used to incorporate a (NMe)Leu residue into the (n - 3) position of a series of oligoleucines with an alternating D,L-pattern of amino acids.[62] NMR data indicated that these *N*-methylated oligoleucines formed flawed $\beta^{4.4}$-helices in chloroform rather than the expected double-stranded helices of the type $\uparrow\downarrow\beta^{5.6}$ or $\uparrow\downarrow\beta^{7.2}$. The second method involved *C*-alkylation of sarcosine residues in cyclic tetrapeptides (Scheme 13)[63] while in the third method *N*-alkylation of the Ala$^2$



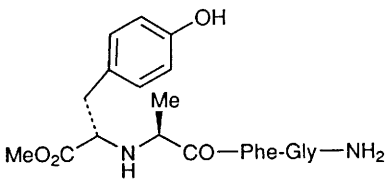
(11)



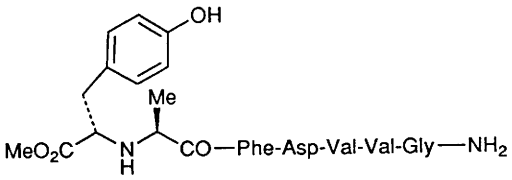
(12) $R^1$,$R^2$ = various alkyl



(13) R = side chain of Thr, Leu, Phe, Cha, Ala, Tyr



(14)



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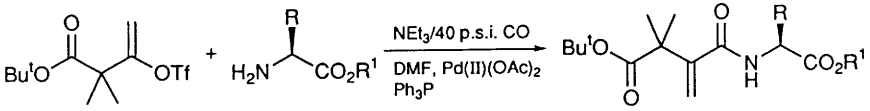
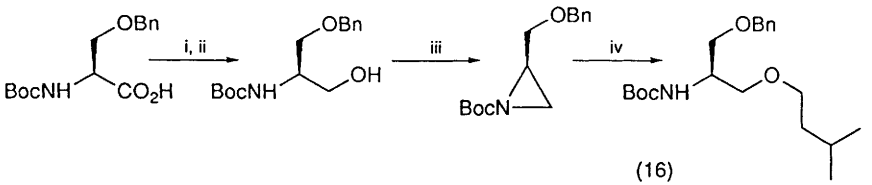


R = side chain of Phe, Tyr, Trp, Ile, Pro

**Scheme 11**

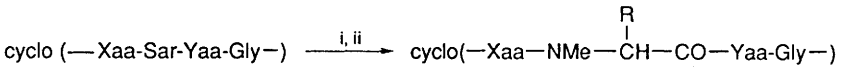



(16)

+ by product from C-2 attack

Reagents: i, $Bu^iOCOCl$, $NEt_3$; ii, $NaBH_4$; iii, $Ph_3P$, DEAD;
iv, $HOCH_2CH_2CH(CH_3)_2$, $BF_3.Et_2O$ or $Zn(OTf)_2$

**Scheme 12**

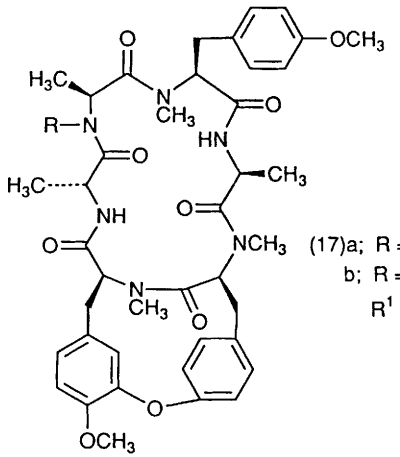



Xaa, Yaa = Leu, Sar; Val, Sar; D-Ala, MeLeu

R = D, $CH_3$, $CH_2Ph$, $CH_2OH$, $CH_2CH{=}CH_2$

Reagents: i, LDA/THF/LiBr/DMPU; ii, alkyl halide or $CH_2O$

**Scheme 13**




(17)a; R = H

b; R = $CH_2CH_2NR^1R^2$,
$R^1 = R^2 = CH_3$, $C_2H_5$, $Pr^i$, Bn, $-(CH_2)_4-$, $-(CH_2)_5-$

residue of the potent antitumor cyclic hexapeptide (17a) under phase-transfer conditions was found to give good yields of the [*N*-dialkylaminoethyl-Ala$^2$]-derivatives (17b).[64]

The general paper by Marraud *et al*[11] at the beginning of this section in which the conformational effects of amide bond modifications were discussed included some X-ray diffraction data on *N*-amino and *N*-hydroxy analogues of Bu$^t$CO-Pro-Gly-NH$^i$Pr and Bu$^t$CO-Pro-Gly-NHMe. A more extensive analysis of both the solid-state and solution-based conformations of these compounds has been carried out and reported in a separate paper.[65] This group have also investigated the conformational perturbations induced by an AzaPro residue by examining the crystal structures of Z-AzaPro-NH$^i$Pr, Z-AzaPro-L-Ala-NH$^i$Pr, and Boc-L-Ala-AzaPro-NH$^i$Pr.[66] Work by the Spatola group on backbone-modified cyclic pentapeptides continues (see comments at the beginning of this section) and X-ray diffraction data on two further members of the series, the sulfide cyclo[-Gly-Pro$\psi$($CH_2$S)Gly-D-Phe-Pro-] and the sulfoxide cyclo[-Gly-Pro$\psi$($CH_2$SO]Gly-D-Phe-Pro-] have been collected.[13] Variable temperature IR and $^1$H NMR spectroscopic techniques were used to assess the folding behaviour of several two- and three-residue depsipeptides.[67] Extrapolation from their data led the authors to predict that for tripeptides in a nonpolar environment a β-turn (fixed by a 10-membered ring amide-to-ester hydrogen bond) was generally enthalpically preferred over an isolated α-helical turn (13-membered ring amide-to-amide hydrogen bond).

Finally, the potential use of the peptoids (*N*-substituted oligoglycines) (18) in the development of pharmaceuticals has been noted.[68]

## 2.8 α,α-Dialkylated Glycine Analogues

The conformational preferences of peptides containing symmetrically α,α-disubstituted glycines such as Aib and those of compounds with other α-methylated amino acids, *eg* (αMe)Val, (αMe)Leu, (αMe)Phe, and Iva, have been discussed in several review articles.[69-72] The implications of these structural preferences in the design and construction of synthetic protein mimics has also been reviewed.[73] Structural data reported in the 1993 literature on other, similar, α,α-disubstituted glycine containing peptides is briefly outlined in Table 1.

A conformational study of the 20-residue peptaibols, saturnisporins SA II and SA IV, (19a,b) has been carried out on material isolated from natural sources.[90] NMR data indicated that these molecules adopted largely α-helical structures in methanol although there was some evidence of a bend, stabilised by two $3_{10}$ hydrogen bonds, in the Aib$^{10}$-Val$^{15}$ region. Acyclic peptide Boc-Cys(SBzl)-Val-Aib-Ala-Leu-Cys(SBzl)-NHMe was found to prefer a $3_{10}$-helical conformation in $CDCl_3$ whereas a β-hairpin structure was favoured in DMSO-$d_6$.[91] By linking the two cysteine residues *via* a disulfide bond to form (20) the β-hairpin type of structure could be observed in chloroform solution. Theoretical methods have been used to obtain free energy profiles for the α- to $3_{10}$-helical transitions of Ac-(Aib)$_{10}$-NHMe in various solvents.[92]

In a series of corticotropin releasing factor antagonists with N$^\alpha$- and C$^\alpha$-

**Table 1** Conformational studies on peptides containing α,α-dialkylamino acid residues

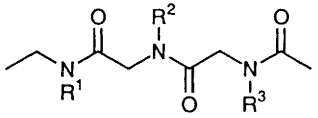
| *Peptide* | *Type of Structure* | *Reference* |
|---|---|---|
| Boc-(Ala-Aib)$_n$-OMe (n = 4,6,8) | crystal, solution | 74 |
| Boc-D-Phe-(Aib)$_4$-Gly-L-Leu-(Aib)$_2$-OMe | crystal | 75 |
| Ac-(Aib)$_2$-Nap-(Aib)$_2$-Phe-(Aib)$_2$NHMe | solution | 76 |
| Dnp-D- and L-Val-Aib-Gly-L-Leu-pNA | crystal | 77 |
| Boc-Val-Aib-X-Aib-Ala-Aib-Y-OMe (X,Y = Phe,Leu; Leu,Phe; Leu,Leu) | crystal | 78 |
| Z-L-Dap(*p*BrBz)-Aib-Aib-NHMe | crystal | 79 |
| Z-L-Dab(*p*BrBz)-Aib-Aib-NHMe | crystal | 79 |
| N$^{\alpha}$-acylated-L-Pro-Ac$_n$c-Gly-NH$_2$ (n = 3,5,6) | crystal | 80 |
| Z-(Aib-Hib)$_n$-Aib-OMe (n = 1,2) | crystal, solution | 81 |
| Boc-L-Ala-Hib-L-Ala-OMe | crystal, solution | 81 |
| (αMe)Phe]$_n$ (n = 1-5) | crystal<br>solution | 82<br>83 |
| Z-L-Pro-D-(αMe)Phe-OH | crystal | 84 |
| Z-L-(αMe)Phe-L-Ala-L-Ala-OMe | crystal | 85 |
| *p*CN-C$_6$H$_4$-NH-CO-L-Asp-L-(αMe)Phe-OMe | crystal | 86 |
| HCO-L-Met-L-Leu-D-(αMe)Phe-OMe | crystal | 86 |
| *p*BrBz-D-(αMe)Phe-(Aib)$_2$-D-(αMe)Phe-Aib-O$^t$Bu | crystal | 86 |
| Z-[D-(αMe)Val]$_n$-NH$^i$Pr (n = 2,3) | crystal, solution | 87 |
| Boc-D- and L-Iva-L-Pro-OBzl | crystal | 88 |
| Boc-Val-(*S*)-(αMe)Ser-OMe | crystal | 89 |

methylated alanine and leucines, one compound, [D-Phe$^{12}$,Nle$^{21,38}$,(αMe)eu$^{37}$] CRF$_{12-41}$ was found to be more potent and longer acting than the parent non-methylated analogue in two *in vivo* assays.[93] Structure-activity studies have also been reported on analogues of the chemoattractant HCO-Met-Leu-Phe-OH containing a C$^{\alpha}$-methylated residue at position 2.[94]

A new route is being explored for the synthesis of peptides containing symmetrically disubstituted residues.[95] In this approach the key step involves formation of an α,α-dichloroglycyl moiety which can, in turn, react with nucleophiles in a double elimination-addition reaction (Scheme 14). Synthesis of Aib-rich peptides using solid-phase techniques has also been investigated.[96] The best results, in terms of yield and quality of crude peptide was obtained using Fmoc-Aib-F for the introduction of the Aib residues. Solution phase methods were used to prepare the α,α-diphenylglycyl dipeptides Z-Dph-Xaa-OMe and Z-Xaa-Dph-OMe (Xaa = Ala, Leu, Val, Ile, Pro).[97] Chiral auxillary[98] and enzymic resolution techniques[99] both continue to be employed in the synthesis of optically pure, asymmetrically α,α-disubstituted amino acids.

## 3 Conformationally Restricted Cyclic and Bridged Analogues

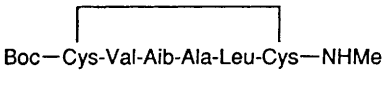
Residue substitutions to well established lactam or disulfide bridged analogues such as the cyclic somatostatin agonist cyclo[-Pro-Phe-D-Trp-Lys-Thr-



(18)

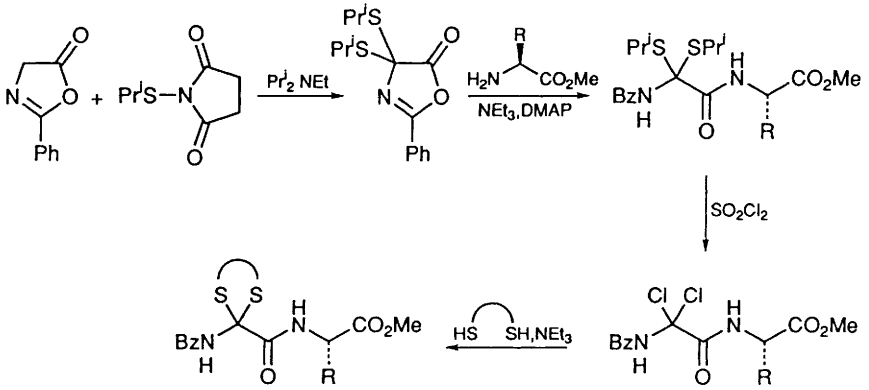
Ac–Aib-Ala-Aib-Ala-Aib-Xaa-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Iva-Gln-Gln-Phe—Ol

(19)a; SA II Xaa = Ala
b; SA IV Xaa = Aib



Boc—Cys-Val-Aib-Ala-Leu-Cys—NHMe

(20)




**Scheme 14**

Phe-] and the δ-selective opioid ligand H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE) are largely covered in section **6**. This section concentrates on reporting the synthesis, biological properties, and conformational analysis of new cyclic analogues of the peptide hormones.

### 3.1 Rings and Bridges formed *via* Amide Bonds

The design and synthesis of analogues with multiple lactam bonds has been a feature of a number of papers this year. The bicyclic LHRH analogues (21)[100] and (22)[101] were prepared by different groups who used as their lead compounds the monocyclic antagonists Ac-$\Delta^3$-Pro$^1$-D-*p*FPhe$^2$-D-Trp$^3$-cyclo(Asp$^4$-Tyr$^5$-D-2-Nal$^6$-Leu$^7$-Arg$^8$-Pro$^9$-Dpr$^{10}$)-$NH_2$ and Ac-D-*p*ClPhe$^1$-D-*p*ClPhe$^2$-D-Trp$^3$-Ser$^4$-cyclo(Glu$^5$-D-Arg$^6$-Leu$^7$-Lys$^8$)-Pro$^9$-D-Ala$^{10}$-$NH_2$ respectively. Although a direct comparison cannot be made between the two bicyclic compounds the overall impression given by the biological and structural data on these two molecules was that a bridge between the side chains of residues 5 and 8 was fully compatible with receptor geometry whereas an N- to C-terminal link was not. Two groups have reported results of receptor binding studies with conformationally constrained analogues of neuropeptide Y (NPY). Introduction of a γ-Glu$^2$-β-D-Dpr$^{27}$ lactam bridge into the truncated $Y_2$-ligand des-AA$^{7-24}$[Gly$^6$]NPY[102] and a γ-Glu$^2$-ε-Lys$^{30}$ bridge into [εAhx$^{5-24}$]NPY[103] gave compounds which exhibited higher selectivity for the $Y_2$ receptor subtype than the linear parents. Further cross-linking between residues 28 and 32 resulted in a $Y_2$-selective ligand (23) devoid of $Y_1$ affinity. Introducing an (i-i+3) lactam bridge into linear glucagon antagonists gave cyclic analogues (desHis$^1$)[Glu$^9$,Lys$^{12}$] glucagon amide and (desHis$^1$)[Glu$^9$,Lys$^{12}$,Asp$^{15}$]glucagon amide which showed biphasic binding curves.[104] The authors speculated that as these cyclic compounds appeared to differentiate between GR1 and GR2 receptor sites they may provide lead structures for the design of site-selective ligands.

The design of the $Y_2$-selective ligand (23) and also that of the cyclic octapeptide[105] Ac-D-Glu-Ser-Ala-Ala-Abu-Ala-Ala-Lys-$NH_2$ was partly the result of molecular modelling experiments. These experiments predicted that the Lys,Glu lactam bridge in (23) and the D-Glu,Lys linkage in the octapeptide would constrain, respectively, one- and two- turns of an α-helical conformation. CD spectroscopic data on the synthetic octapeptide indicated that, as predicted, a fixed structure with high α-helicity even in a buffer containing 6M guanidine hydrochloride.

Solution conformations of several cyclic endothelin and NK-2 antagonists have been reported. NMR data on the two endothelin antagonists cyclo(-D-Glu-L-Ala-D-allo-Ile-L-Leu-D-Trp-)[106] and cyclo(-D-Trp-D-Cys($SO_3^-Na^+$)-Pro-D-Val-Leu-)[107] indicated that both these molecules adopted backbone conformations in solution comprising of a type II β-turn and an inverse γ-turn. Each of the three NK-2 antagonists cyclo(-Gln-Trp-Phe-Gly-Leu-D- and L-Met-) and the bridged analogue (24) were found to exist in solution as multiple conformations in fast exchange on the NMR timescale.[108] However, backbone conformations for these three hexapeptides were proposed on the basis of molecular dynamics simulations.

Two of the NK-2 antagonists [cyclo(-Gln-Trp-Phe-Gly-Leu-Met-) and (24)] also cropped up in a SAR study of cyclic peptides derived from the C-terminal hexapeptide sequence of substance P.[109] The synthesis and biological properties of a series of hexapeptides based on the cyclic peptide T analogue cyclo(-Thr-Thr-Asn-Tyr-Thr-Asp-)[110] and of a cyclic octapeptide cyclo(-Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-)[111] corresponding to TNF-α-(59-66) have also been described.

### 3.2 Bridges formed by Disulfide Bonds

NMR data on the disulfide bridged peptides (25) indicated that a *cis*-amide bond was preferred in compounds with (*R*,*R*) stereochemistry while *trans*-geometry predominated in the diastereoisomeric (*R*,*S*)-peptides.[112-114]

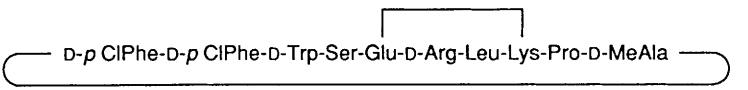
Structure-activity studies have been carried out on disulfide-containing cyclic analogues of angiotensin II,[115] bradykinin antagonists,[116] dynorphin A,[117] endothelin,[118] and vasoactive intestinal peptide.[119] Several of the angiotensin II analogues, for example, [Sar$^1$,Cys$^3$,Mpt$^5$]ANG II and the antiparallel dimer of [Sar$^1$,Cys$^{3,5}$]ANG II, and the cyclic bradykinin antagonists D-Arg-Arg-Cys-Pro-Gly-Cys-Ser-D-Tic-Oic-Arg and D-Arg-Arg-Cys-Pro-Gly-Phe-Cys-D-Tic-Oic-Arg bound with high affinity to their receptors. Retention of high activity by the dimeric angiotensin II analogue was rather unexpected. However conformational studies suggested that the side chains of the Tyr$^4$ residue of one monomer and those of the His$^6$ and Phe$^8$ residues of the other monomer unit could together present a conformation analogous to the three-dimensional arrangement of these critical side chains in a cyclic monomer. The dynorphin A analogues were conformationally constrained in the putative "address" segment of the peptide and several of these compounds exhibited unexpected apparent selectivities for the κ and μ opioid receptors of the central *vs* peripheral nervous system. These findings led the authors to propose, somewhat tentatively, that different subtypes of the κ and μ opioid receptors may exist in brain and peripheral tissues.

### 3.3 Miscellaneous Bridges and β-Turn Mimetics

The growing interest in β-turn mimetics was reflected this year by the publication of a *Tetrahedron* "symposium-in-print" on the topic of peptide secondary structure mimetics.[120] This issue of *Tetrahedron* included not only contributions on β-turn mimetics but also articles on strategies to stabilise β-strand conformations and template assisted approaches to the synthesis of helix bundles. The area of β-turn mimetics was also separately reviewed.[121,122]

A series of five papers by R.L. Johnson and co-workers on Pro-Leu-Gly-$NH_2$, a tripeptide which modulates dopamine receptor activity, has described the use of complex and rigid structures to investigate the hypothesis that the biologically active conformation of this peptide is a type II β-turn. Some of the tripeptide analogues incorporated different sized lactam rings (26) to vary the $\psi_2$ angle of a β-turn[123] while others (27, 28) used spirolactam[124,125] and bicyclic thiazolidine lactam[126] constraints to restrict the conformational freedom of the peptide backbone. The study culminated in the preparation of the highly rigid spiro bicyclic peptidomimetic (29).[127] This compound was found to enhance the


(21)

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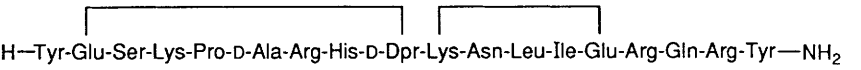


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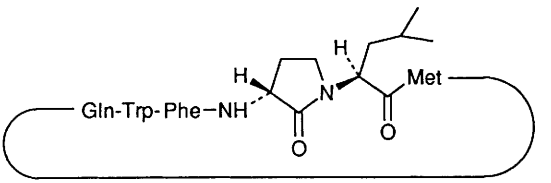


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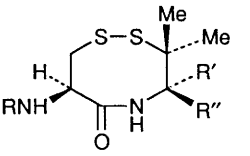
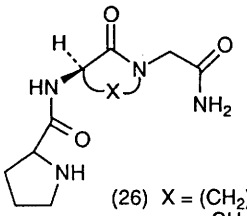
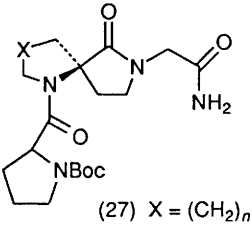


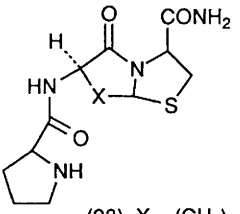

(25) R = $CF_3CO$ or Boc
R′, R″ = H, $CO_2Me$, or $CO_2Me$, H



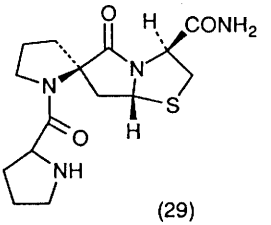

(26) X = $(CH_2)_n$, $n$ = 1–4; $CH_2CO$, $CH_2O$




(27) X = $(CH_2)_n$, $n$ = 1,2




(28) X = $(CH_2)_n$, $n$ = 1,2




(29)

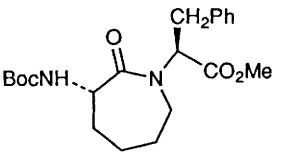
binding of a dopamine receptor agonist to dopamine receptors by 40% at $10^{-6}$M (*cf* 26% increase with Pro-Leu-Gly-$NH_2$). A similar type of design approach by other workers has resulted in the synthesis of several turn-inducing dipeptide mimetics including the 7-membered ring lactam-based building block (30),[128] the bicyclic γ-lactam (31),[129] the spiro-compound (32),[130] and the bicyclic mimetics (33,[131] 34,[132] 35,[133] and 36[134]).

A benzodiazepine scaffold (37)[135] and the large ring frameworks (38)[136] have been identified as structures which can reproduce both the geometrical constraints of a 4→1 β-turn as well as maintain the positions of the four side chains in their correct three-dimensional orientations. Encouraging biological results were obtained with the cyclic "decapeptide" (39)[137] which incorporates the benzodiazepine to mimic one of the two β-turns of gramicidin S [(Leu-D-Phe-Pro-Val-Orn)$_2$]. This analogue retained about 50% of the activity of gramicidin S in antibacterial assays. Macrocyclic-based enkephalin analogues (40) were prepared but the results of the biological assays on these compounds was rather less promising.[136] Only the 14-membered ring analogue (40, X = NH-C$(CH_3)_2$-CH=CH-$CH_2$) demonstrated any, albeit minimal, binding activity at the μ-receptor. Pharmacological data on the related 11-membered ring 25 β-turn mimetics (41) of enkephalin indicated that one of the diastereoisomers exhibited significant *in vivo* analgesic activity despite relatively weak *in vitro* binding activity.[138] Some evidence for a β-turn structure in the bioactive conformation of hypertrehalosemic hormone (an insect neurohormone) has been obtained.[139] Analogue (42) with the 11-membered ring bis-lactam mimetic was found to exhibit limited hyperglycemic activity while isomers with extended conformations showed no activity.

The only conformationally constrained mimetic of a γ-turn reported this year is the retro-amide $C_7$ compound (43, X = Boc, R = $CH_2$Ph).[140] This compound was elaborated to give, for example, an analogue (43, X = Ac-(NMe)Arg, R = H) of a linear peptide fibrinogen receptor antagonist.

An area which to some extent overlaps with β-turn mimetics is the design of compounds which will in effect constrain the side chain of a particular amino acid residue into a specific orientation. NMR studies established that in the substituted oxazepine (44) the 7-membered ring was in a chair conformation with the phthalimide moiety and the phenyl ring pseudo-equatorial, consistent with the *gauche* (-) conformation.[141] Another example was the use of a benzazepinone in the dermorphin analogue (45)[142] to fix the Phe side chain into the *trans*-conformation.

Several other papers describe the synthesis and/or use of conformationally restricted dipeptide units. The *N,N'* ethylene-bridged Phe-Met and Phe-Leu dipeptides (46, X = H) were prepared and used as the C-terminal residues of enkephalin analogues (46, X = H-Tyr-D-Ala-Gly-)[143-145] while the protected derivative of (2*R*,3*S*)-3-amino-2-piperidineacetic acid (47) has been prepared but not yet incorporated into peptide sequences.[146] Preliminary pharmacological tests on the enkephalin analogues indicated that these compounds had some affinity for the opiate receptors. The cyclic analogue (48) of Gly-Phg/Phg-Gly could be obtained *via* an intramolecular amidoalkylation reaction of MocNHCH(OMe)

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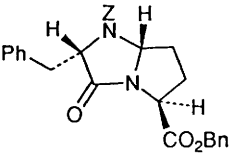


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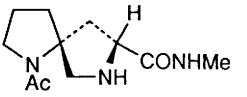


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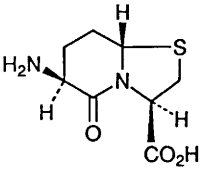


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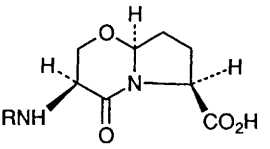
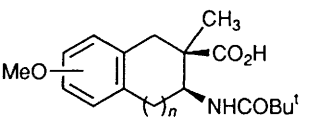
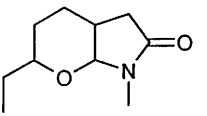



(34) R = Z or Boc
3 other diastereoisomers
also prepared




(35) $n$ = 0,1

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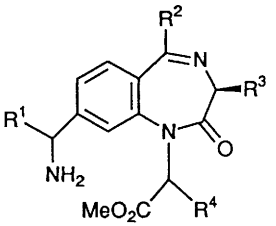


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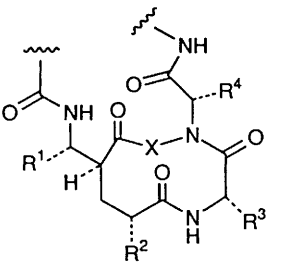


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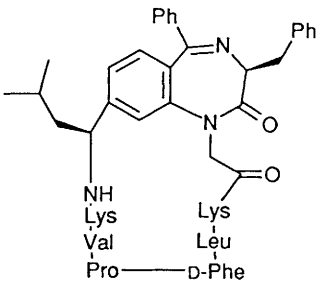


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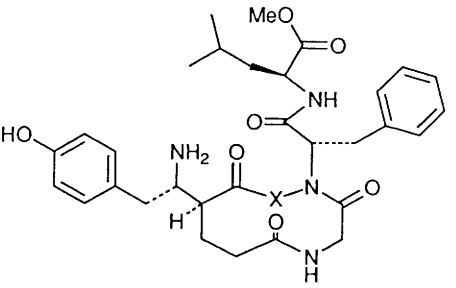
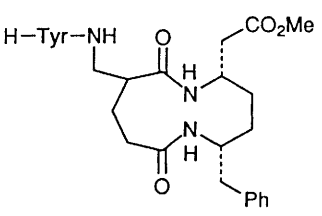



(40) X = NH; NH-C$(CH_3)_2$-$(CH_2)_n$, $n$ = 1,3; NH-C$(CH_3)_2$-CH =CH-$CH_2$

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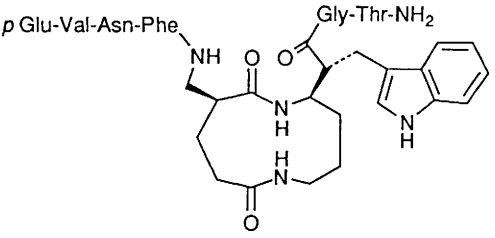


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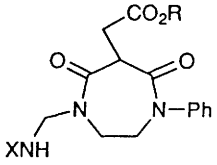


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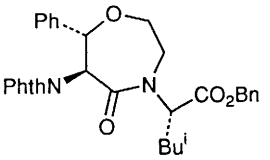
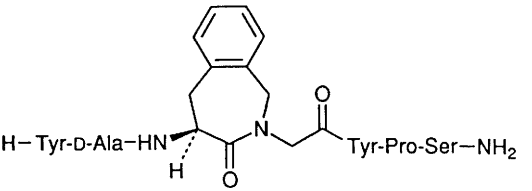



(44)

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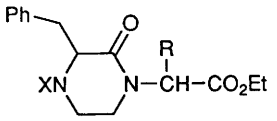
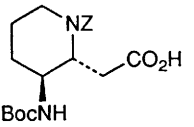



(46) R = $CH_2CH_2SCH_3$, $CH_2CH(CH_3)_2$

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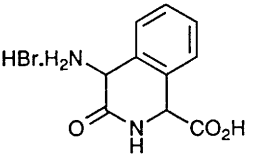


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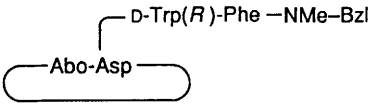
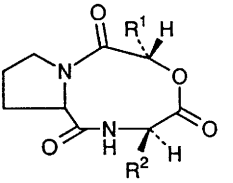
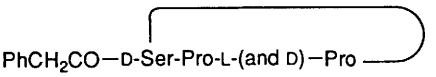


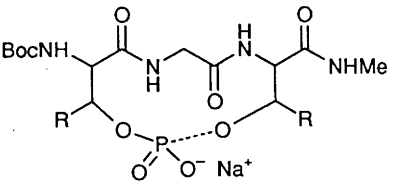

(49) R = H,$CH_3$,COR′ (R′ = various alkyl, alkoxy)




(50) $R^1$ = $CHMe_2$, Me
$R^2$ = $CH_2Ph$, Me




(51)




(52) R = H, Me

$CONHCH(Ph)CO_2Me$ (where Moc is methoxycarbonyl).[147] The corresponding reaction with $MocNHCH(Ph)CONHCH(OMe)CO_2Me$ gave a mixture of products.

The rationale behind the design of the partially cyclic tachykinin antagonists (49) was to elaborate the D-Trp-Phe dipeptide with substitutents which could increase affinity and stability.[148] Cyclo(-Abo-Asp-) and *N*-methylbenzylamine emerged as the best substituents for, respectively, the N- and C-termini of the D-Trp-Phe dipeptide. The pseudotetrapeptides were obtained by coupling the H-D-Trp-Phe-NMe-Bzl segment to the side chain carboxyl on the Abo-Asp ring and then acylating the resulting compound to introduce the various substitutents onto the indole nitrogen. One water-soluble compound [49, R = $CO(CH_2)_2CO_2H$] showed high analgesic potency in several *in vivo* assays both after intravenous or oral administration.

The synthesis and conformational studies of several cyclodepsipeptides have been reported. NMR data on the nine-membered ring peptide lactones (50)[149] and X-ray analysis of the 10-membered ring compounds (51)[150] indicated that both types of molecules adopted *cis-cis-trans* backbone conformation in which the lactone junction was *trans*. The cyclic phosphopeptides (52) were designed as models for an intramolecular phosphodiester cross-link in a protein.[151] These compounds were obtained by treating the linear peptides Boc-Ser(or Thr)-Gly-Ser(or Thr)-NHMe with a phosphitylating agent (4-chlorobenzyl dichlorophosphinite), oxidising the intermediate phosphite triester and then removing the 4-chlorobenzyl group by hydrogenolysis. Finally, two spacer units have been designed to induce other structural motifs in peptides. Phenoxathiin derivatives in (53) allowed two parallel peptide chains to adopt a conformation which resembles that found in a β-sheet.[152] In the cyclic peptide (54) the 2,7-disubstituted naphthalene moiety was used to span the intrachain distance between the two ends of a Ω-loop sequence from interleukin-1α and to maintain this loop in a conformation which mimicked that found in the protein.[153]

## 4 Dehydroamino Acid Analogues

Articles published in 1993 describing the conformational properties of dehydropeptides are summarised in Table 2. The experimental studies indicated that ΔPhe strongly induces a β-turn structure in small peptides. In longer peptides containing several ΔPhe residues the overlapping of consecutive β-turns results in a $3_{10}$-helical conformation. The conformational preferences of peptides containing an ΔAbu residue are not as clearly defined as those with ΔPhe and ΔLeu residues. Two groups reported structures for the Boc-Phe-ΔAbu-NHMe; one group found that this peptide adopted a β-turn conformation in the solid state[162] while the second group reported extended conformations for this peptide in $CDCl_3$ and DMSO-$d_6$ solutions.[161]

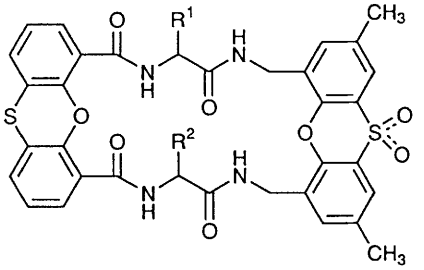
The usefulness of *N*-carboxy-α-dehydroamino acid anhydrides (ΔNCA) in the synthesis of dehydropeptides has been further explored. Experiments to optimise the conditions for coupling ΔNCA with both amine and carboxyl

**Table 2** Conformational studies on peptides containing dehydro amino acid residues

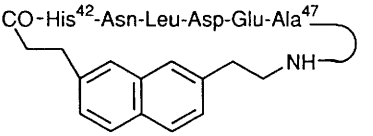
| *Peptide* | *Type of study* | *Result of study* | *Ref.* |
|---|---|---|---|
| Boc-Phe-ΔPhe-Val-Phe-ΔPhe-Val-OMe | Crystal structure | $3_{10}$-Helical conformation | 154 |
| Boc-Gly-$\Delta^{Z}$Phe-Leu-$\Delta^{Z}$Phe-Ala-NHMe | Crystal structure | $3_{10}$-Helical conformation | 155 |
| Boc-(Ala-$\Delta^{Z}$Phe-Aib)$_n$-OMe, n = 1-4 | Solution structure | $3_{10}$-Helical conformation for compounds n = 2-4 | 156 |
| Boc-Ala-ΔPhe-ΔPhe-NHMe | Crystal and solution structures | Incipient $3_{10}$-helical conformation | 157 |
| Ac-ΔPhe-Ala-ΔPhe-NHMe<br>Ac-ΔPhe-Val-ΔPhe-NHMe<br>Ac-ΔPhe-Gly-ΔPhe-Ala-OMe<br>Boc-Ala-ΔPhe-Gly-ΔPhe-Ala-OMe | Solution structures | $3_{10}$-Helical structures | 158 |
| HCO-Met-Leu-$\Delta^{Z}$Phe-Phe-OMe | Crystal structure | "Open-turn" conformation | 159 |
| HCO-Met-$\Delta^{Z}$Leu-Phe-OMe | Crystal and solution structures | β-Turn conformation | 160 |
| Boc-X-$\Delta^{Z}$- and $\Delta^{E}$Abu-NHMe (X = Phe,Ala,Val) | Solution structures | Extended conformations | 161 |
| Boc-Phe-ΔAbu-NHMe | Crystal structure | β-Turn conformation | 162 |
| Ac-Pro-$\Delta^{Z}$Xaa-NHMe (X = Phe,Val,Leu,Abu) | CD studies | Preference for β-turn conformations in apolar solvents, unordered structures in water | 163 |
| [poly-(ΔAbu)] | Molecular mechanics study | $3_{10}$-Helix conformation preferred in solid state | 164 |

components led to the preparation of several dehydrotetrapeptides[165] and ultimately to the synthesis of Fragment A of berninamycin A.[166] Papain has been found to catalyse the coupling of CBz-ΔGlu(OMe)-OMe with α-amino acid amides (H-AA-NHR, AA = neutral aliphatic or aromatic amino acid) to give reasonable yields (*ca* 50%) of CBz-ΔGlu(OMe)-AA-NHR.[167] Similar reactions with α-chymotrypsin A gave CBz-ΔGlu(AA-NHR)-OMe. Another method used to prepare dehydrodi- and tri-peptides involved perrhenate-catalysed decomposition of 2-azidocarboxylates and 2-(2-azidoacyl)amino acid esters with *N*-phthaloyl-protected amino acid chlorides.[168,169] Dehydroalanine-containing peptides were obtained by elimination of water from serine residues in a two-step procedure: acetylation and elimination of AcOH. The elimination reaction was effected by DBU or DBN in THF in the presence of large amounts of $LiClO_4$.[170]

A brief report last year on the synthesis of 1-azabicyclo[3.1.0]hex-2-ylidene dehydroamino acid derivatives (55), models for the azinomycin antitumor antibiotics, has now been followed by a full paper giving detailed experimental protocols for the formation of these compounds from D-arabinose and L-serine.[171] A key step in the synthesis of these compounds was the introduction of the pyrrolidine ring. This was accomplished by formation of the N-$C_8$ bond *via* an intramolecular Michael addition-elimination reaction sequence between the aziridine nitrogen and the β-bromo-dehydroamino acid ester (Scheme 15). Bromination of dehydroamino acids has been the subject of a separate comprehensive study.[172] Dehydroamino acid derivatives/synthons have been coupled to



(53) $R^1 = R^2 = CH(CH_3)_2$, $CH_2Ph$

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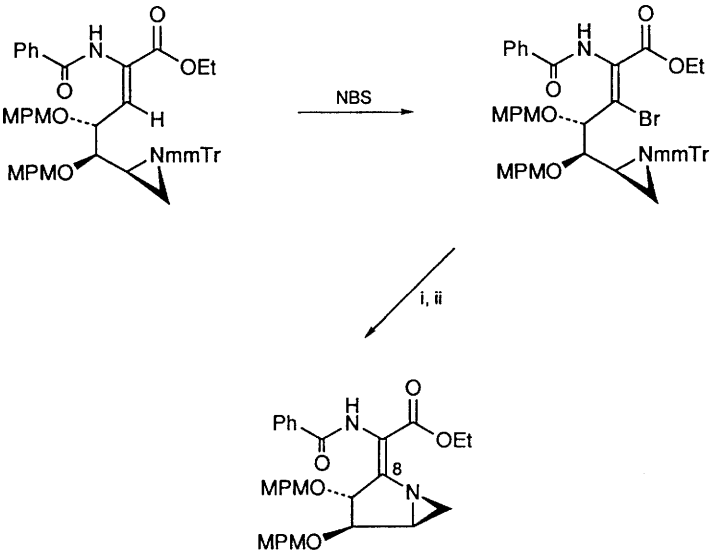



(55) *Z* – isomers also prepared

Reagents: i, trichloroacetic acid; ii, $NEt_3$

**Scheme 15**

3-amino-1,4-diphenylazetidin-2-one to give peptides (56) featuring a β-lactam moiety[173] and, in a cycloaddition reaction, to benzonitrilium *N*-phenylimide to give peptides (57) bearing a heterocyclic side chain.[174]

Other papers relevant to this section describe the synthesis and pharmacological properties of *N*-benzoyl-α,β-dehydrodipeptides (58),[175] the reaction of Ac-ΔPhe-L-Pro-OR (R = H, Me) with Ca(II) and Ni(II) ions,[176] and the catalytic deuteration and tritiation of dehydroproline[9]-buserelin.[177]

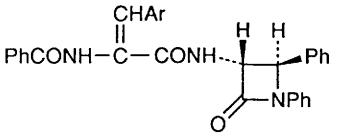
## 5 Enzyme Inhibitors

The growing interest in the synthesis of inhibitors, particularly for the aspartic protease HIV-1, has prompted some changes to the organisation of this section. ACE inhibitors are dealt with as in previous years. A new section (**5.2**) is included which summarises, in tabular form, the new synthetic routes to statine, its analogues, and hydroxyethylene and dihydroxyethylene dipeptide isosteres. Sections **5.3** on renin inhibitors and **5.4** on HIV-1 inhibitors concentrate on the design, synthesis, and biological activity of compounds based on the $P_4$ to $P_2'$ sequences of angiotensinogen and *gag*/*gag-pol* HIV polyproteins respectively.

### 5.1 Angiotensin Converting Enzyme (ACE) Inhibitors

The synthesis of enalapril maleate has been briefly reviewed.[178] A series of pseudopeptides (59) has been prepared containing 1,2-cyclomethylenedicarboxylic acids as the carboxyl terminal portion instead of the acylproline moiety common to many ACE inhibitors.[179] The most potent of the series was (59, n = 1, X = CONHOH, R = $CH_3$) with an $IC_{50}$ value of 7.0nM compared with the value of 3.0nM for captopril. Furthermore this compound was shown to be a highly selective and competitive ACE inhibitor. A rigid tricyclic dipeptide mimetic (60, R = H), designed to closely resemble the *anti*-orientation of the carboxy terminal His-Leu portion of angiotensin I, was synthesised.[180] Further elaboration of this mimetic gave [60, R = (*S*)-$CH(CO_2H)CH_2CH_2Ph$], a subnanomolar inhibitor of ACE while the mercaptoacetyl derivative (60, R = $COCH_2SH$) was found to be an inhibitor of both ACE and neutral endopeptidase 24.11 (NEP), a related metalloprotease, at low nanomolar concentrations.[181] The 13-membered ring thiol (61), designed as a dual ACE/NEP inhibitor, showed the desired *in vitro* profile with an ACE $IC_{50}$ of 12nM and an NEP $IC_{50}$ of 18nM.[182] This compound was also active after intravenous or oral administration, but the duration of action was less than 1h. The *N*-cyanoacetyl dipeptide (62, X = NH), reported last year to be a mechanism-based inhibitor of ACE, was also found to inactivate NEP.[183] The ketomethylene analogue (62, X = $CH_2$) only inactivated NEP.

Analogues of the cyclic peptide K-13 (63), a compound isolated from *Micromonospora halophytica*, have been prepared and tested for ACE inhibitory activity.[184] $BzCH_2CH(CO_2H)$-L-Ala-L-Tyr-OH was identified as the most potent ACE inhibitor ($IC_{50} = 7.9 \times 10^{-10}$mol/L) from the screening of several di- and tripeptides.[185] Useful applications of known compounds include the linkage of tri-,



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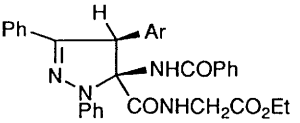

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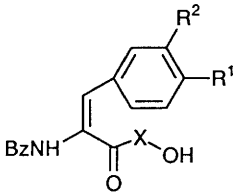
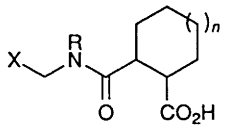
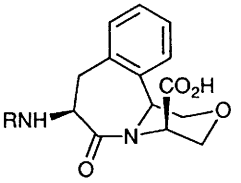


(58) $R^1$, $R^2$ = H or various alkoxy substituents
X = various amino acids (including β-amino acids)



(59) $n$ = 0, 1
X = $CO_2H$, CONHOH, $CH_2SH$
R = various alkyl substituents



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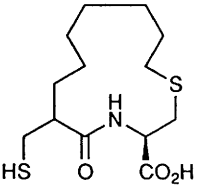

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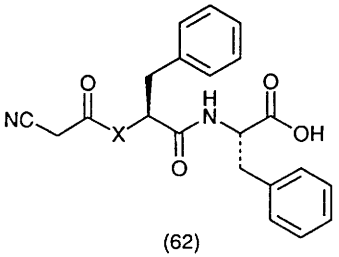
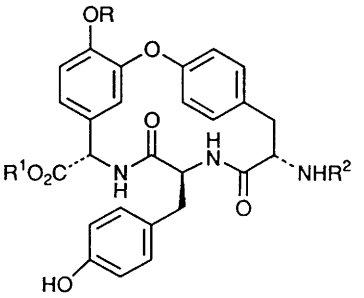
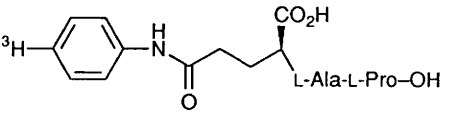


(62)



(63) K–13: R = $R^1$ = H, $R^2$ = Ac
analogues: R, $R^1$ = H or Me, $R^2$ = H or Ac



(64)

penta-, and nona-peptide ACE inhibitors to dextran polymers for therapeutic purposes[186] and the synthesis of tritium-labelled-RAC-X-65 (64).[187]

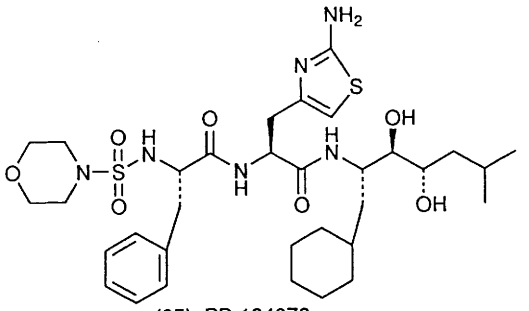
Models to predict the potency of molecules designed as ACE inhibitors have been developed using 3D-QSAR data on known inhibitors.[188,189]

## 5.2 Statine and Hydroxyethylene-type Dipeptide Isosteres

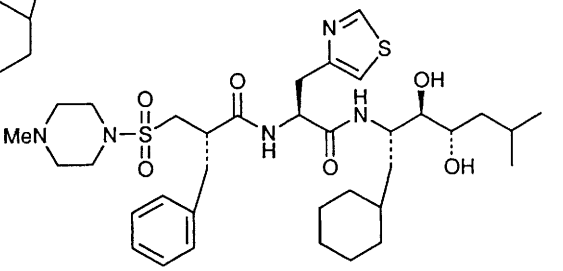
As indicated in the preamble this is a new section and consists entirely of a table. Table 3 summarises the synthetic routes to statine, its isomers and hydroxyethylene dipeptide isosteres and replaces the more detailed coverage given to these topics in previous years. The synthesis of dihydroxyethylene isosteres is referred to in the table purely for completeness and the preparation of these compounds is discussed in more detail in sections **5.3** and **5.4**.

## 5.3 Renin Inhibitors

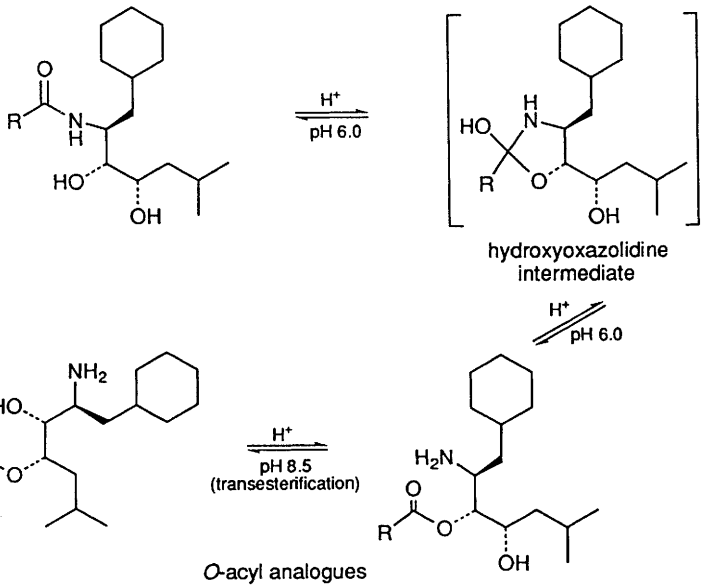
Last year two orally active and structurally very similar renin inhibitors were reported, PD 134672 (65) and A-72517 (66). Byproducts obtained by the Parke-Davis group during decomposition studies with their inhibitor were found to be more water soluble than the original material.[206] The new compounds were identified as *O*-acyl analogues and were probably the result of *N*- to *O*-acyl transfer reactions (Scheme 16). A similar type of reaction was noted by the Abbott group in a detailed account of the work which led to the discovery of A-72517.[207,208] Small quantities of the Abbott inhibitor were initially prepared using the D,L-form of the β-sulfonamidopropionic acid, the $P_3$ component. After coupling the racemic $P_3$ component to the $P_2$-$P_1'$ moiety the resulting diastereoisomers were separated by chromatography. A method of preparing optically pure β-sulfonamidopropionic acid has since been developed.[209] This route (Scheme 17) involves an enzyme resolution step and is suitable for the preparation of bulk quantities of the acid. The Abbott group have also synthesised a series of inhibitors (67) containing a 2,3-disubstituted cyclopropanecarboxamide at the $P_3$ position.[210] A similar synthetic strategy to that used for the original synthesis of A-72517 was employed to prepare these compounds, *i.e.* the racemic cyclopropanecarboxylic acids were coupled to the $P_2$-$P_1'$ components. In an interesting series of compounds by the Parke-Davis group the side chains of the $P_3$ residue were transferred and thereby directly attached to the ligand in the $P_1$ position.[211] The binding affinities of the tethered compounds (68c) lay between those of the parent $P_3$ phenylalanine derivative (68a, $IC_{50} = 0.2$nM) and the $P_3$ glycine analogue (68b, $IC_{50} = 82$nM). Several new syntheses have been devised of the dihydroxyethylene $P_1$-$P_1'$ dipeptide component in the various Parke-Davis and Abbott inhibitors mentioned above (Scheme 18, see also Table 3).[205] Each of the syntheses started from cheap readily available materials. The most intriguing route started from chlorobenzene. In this route the hydroxylactone (69) was obtained from chlorobenzene in three steps: microbial oxidation using *Pseudomonas putida* 39-D, protection of the resulting *cis*-cyclohexadiendiol as the acetonide, and finally ozonolysis. A further series of reactions converted the hydroxylactone into the *N*,*N*-dimethylhydrazone (70), the intermediate common to each of the three routes.



(65) PD 134672



(66) A–72517

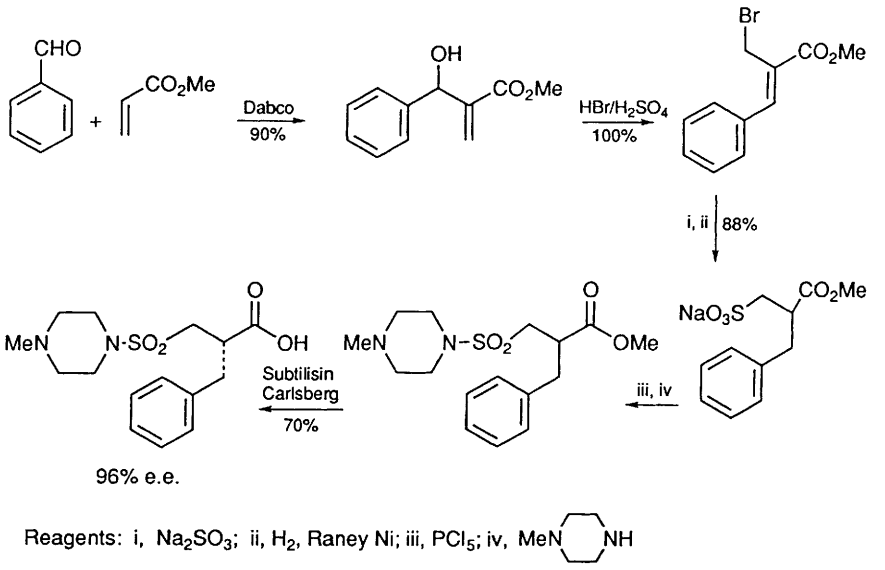



**Scheme 16**

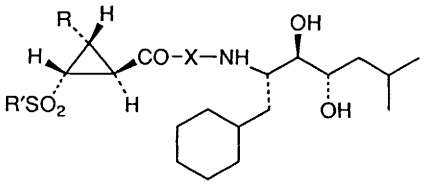
**Table 3** New synthetic routes to statine and related compounds

| *Compound* | *Comments on Synthesis* | *Note* | *Ref.* |
|---|---|---|---|
| *β-Hydroxy-γ-amino acids* | | | |
| Statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid] | – Stereoselective functionalisation of 2-oxazolone – use of chiral auxiliary; route also gave 2,2-dichloro and 2,2-difluoro analogues | a | 190 |
| | – Tetramic acid route – involves lipase-catalysed kinetic resolution step | b | 191 |
| | – D-Glucosamine starting material; chiral oxazolidinone as key intermediate | c | 192 |
| Statine isomer with (3*R*,4*S*) stereochemistry | – Stereoselective aldol route: use of iron acetal complex as chiral auxillary | | 193 |
| Valine analogue[(3*S*,4*S*)-4-amino-3-hydroxy-5-methylhexanoic acid] | – Stereoselective route *via* tetramic acids | a | 194 |
| AHPPA [(3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid] | – See statine comments | b,c | 191,192 |
| | – Chiral aziridine starting material | b | 195 |
| *α-Hydroxy-β-amino acids (norstatines)* | | | |
| (2*S*,3*R*)-3-Amino-2-hydroxy-4-phenylbutanoic acid | – See AHPPA comments | d | 195 |
| | – Ketomethylene analogue | | 196 |
| (2*S*,3*S*)-Isomer | – Stereoselective synthesis from *trans*-$PhCH_2CH=CHCO_2Bu^t$ | | 197 |
| Various isomers | – Use of papain to catalyse coupling of Z-Gly-OMe to amino group of bestatin-type isosteres – method applicable to acylation of α-keto-amines | | 198 |
| *Hydroxyethylene* | | | |
| BocChaψ[$CH(OH)CH_2$]Ala-OH | – Stereocontrolled synthesis (kilogram route) | e,f | 199 |
| ZChaψ[$CH(OH)CH_2$]Val | – Isolated in lactonised form (ring opened with amines) | f,g | 200 |
| BocPheψ[$CH(OH)CH_2$]Xaa-OH (Xaa = Gly, Ala, Phe) | – Stereoselective synthesis | f | 201 |
| BocPheψ[$CH(OH)CH_2$]Xaa (Xaa = Ala, Pro) | – Nonselective route to γ-lactone precursors – all diastereoisomers obtained | | 202 |
| Boc Trpψ[$CH(OH)CH_2$]ValOBu$^t$ | – Route also gave access to other Trp-Val isosteres *e.g.* BocTrp-$CH=C(Pr^i)$-$CO_2H$ | h | 203 |
| *Dihydroxyethylene* | | | |
| See **5.3** (renin inhibitors) | | | 204 |
| See **5.3** (renin inhibitors) and **5.4** (HIV-1 inhibitors) | | | 205 |

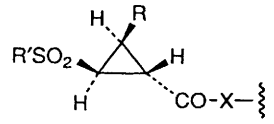
*Notes*: (a) isolated as *N*-Boc/OMe ester derivative; (b) isolated as *N*-Boc derivative; (c) no details of final steps given (conversion of known intermediate to statine/AHPPA); (d) isolated as *N*-Z/OMe ester derivative; (e) *O*-Tcboc form; (f) (2*R*,4*S*,5*S*)-isomer; (g) (2*S*,4*S*,5*S*)-isomer; (h) (2*R*/*S*,4*S*,5*S*)-isomer.



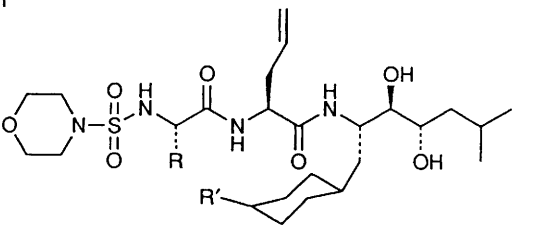

**Scheme 17**



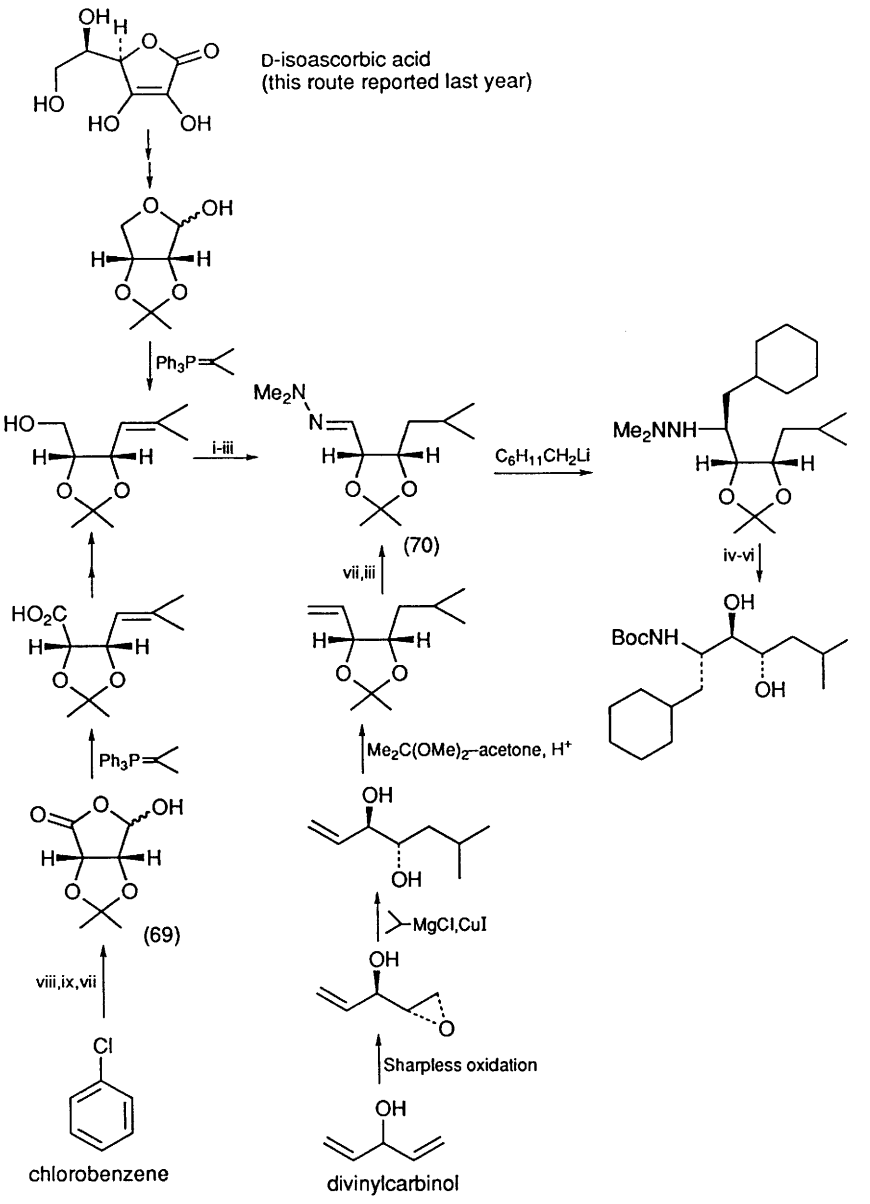

AND




(67) R = Ph, cyclohexyl
R′ = various alkyl
X = L-3-(4-thiazolyl)alanine, His, Nle, Leu

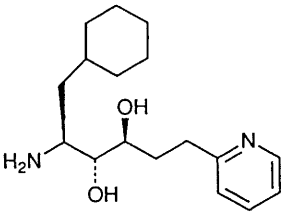



(68)a; R = $CH_2Ph$, R′ = H
b; R = H, R′ = H
c; R = H, R′ = phenyl, 1-naphthyl

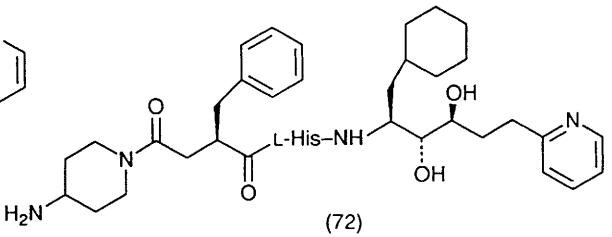



Reagents: i, $H_2$, Pd–C; ii, Swern oxidation; iii, $Me_2NNH_2$; iv, $H_2$, Raney Ni; v, HCl; vi, $Boc_2O$; vii, $O_3$; viii, Microbial oxidation; ix, $Me_2CH(OMe)_2$–acetone, $H^+$

**Scheme 18**




(71)

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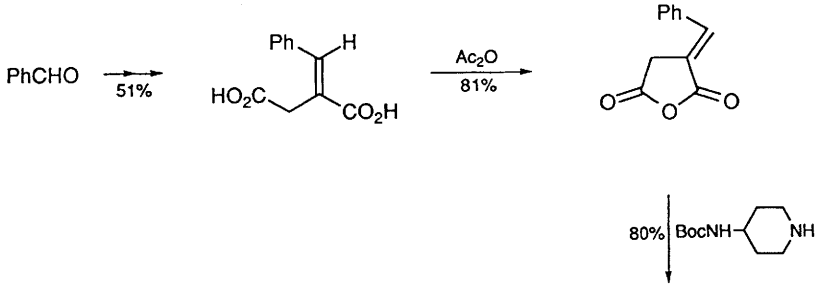
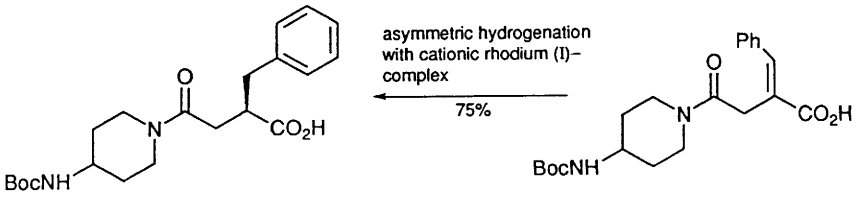
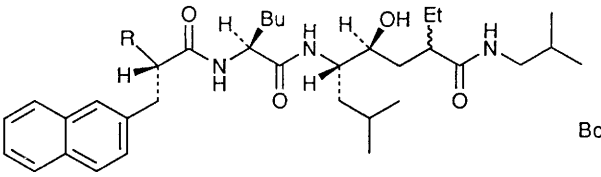


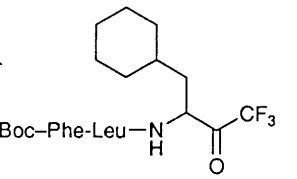




> 99.5% e.e.

**Scheme 19**



(73) R = various amino, sulfonyl and heterocyclic groups/derivatives

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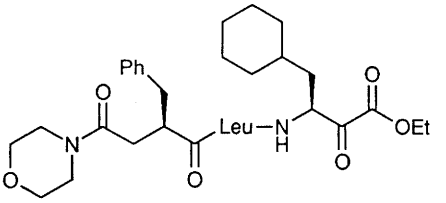


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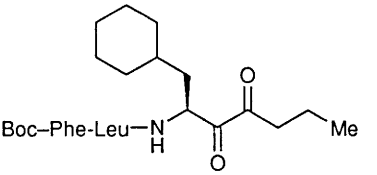
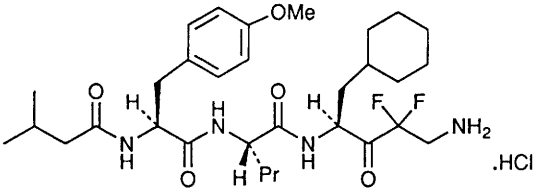
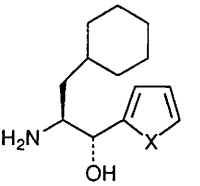


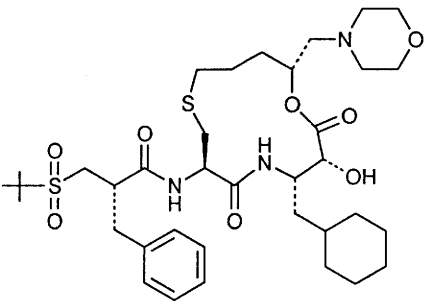

(76)




(77)




(78) X = O,S




(79)

The other dihydroxyethylene dipeptide isostere referenced in Table 3 was compound (71).[204] This compound, prepared from cyclohexylalaninal *via* iterative homologation (Dondoni reaction), was used as the $P_1$-$P_1'$ component in a series of renin inhibitors. The most promising member of that series (compound 72) inhibited human renin with $IC_{50} = 0.38nM$, did not affect other human aspartic proteases, and decreased mean arterial blood pressure in sodium-depleted rhesus monkeys.[212] A short and efficient large scale synthesis of the amino piperidinylsuccinic acid N-terminal moiety of inhibitors such as (72) has also been developed (Scheme 19).[213]

A sulfone derivative was found to be the best substituent for the N-terminal position of a homostatine series of inhibitors (73, R = $EtSO_2CH_2$, $IC_{50} = 0.5nM$).[214] The effect of incorporating activated ketone based groups into the $P_1$-$P_1'$ site has been investigated. The results of biological studies indicated that the trifluoromethylketones (74), α-keto esters (75), and α-diketones (76) were equipotent or more active than the corresponding alcohols.[215]

Five inhibitors containing the Phe-His-Sta-εAhx sequence,[216] an inhibitor (77) with a β-amino-α,α-difluoroketone functionality,[217] and transition-state mimetics (78) with C-terminal furan and thiophene moieties[218] have been prepared.

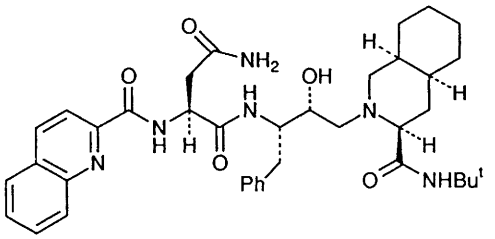
A group from Merck has continued to develop macrocyclic inhibitors and have synthesised the cysteine-containing compound (79).[219] This compound was a subnanomolar inhibitor ($IC_{50} = 0.35nM$) and showed good oral activity and duration of action.

Finally, a better understanding of the interaction between the inhibitor and active site may result from studies using molecular modelling techniques to analyse X-ray crystallographic data on inhibitor-enzyme complexes[220] and in interpreting structure-activity data on a series of $P_2$-$P_2'$ inhibitors with different $P_1$-$P_1'$ components.[221]

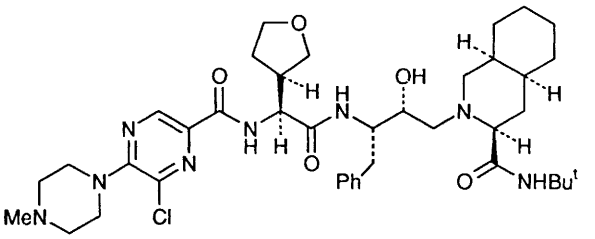
### 5.4 HIV-1 Protease Inhibitors

The majority of the articles in this section this year relate to the continuing efforts by researchers, largely based in the pharmaceutical industry, to improve the pharmacokinetic properties of a few lead compounds.

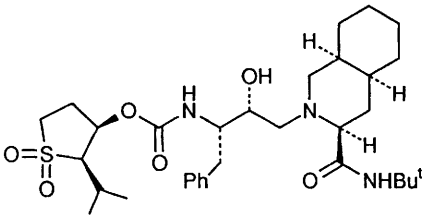
One inhibitor undergoing clinical evaluation is Ro 31-8959 (80) and several groups have reported the results of studies associated with this compound. A group at Merck found that replacing the asparagine $P_2$ residue of (80) with 3-tetrahydrofuranylglycine[222,223] or a cyclic sulfolane moiety[224] and/or the $P_3$ quinolinoyl ligand with pyrazine derivatives[225] resulted in an improvement in enzymic inhibitory as well as antiviral potencies. Of particular interest is compound (81) with (2*S*,3′*R*)-tetrahydrofuranylglycine at $P_2$ and a pyrazine derivative at $P_3$. This compound is one of the most potent inhibitors to date of HIV-1 ($IC_{50}$ value 0.07nM) and HIV-2 ($IC_{50}$ value 0.18nM) proteases. In (82) both the $P_2$ asparagine and $P_3$ quinoline moieties of Ro 31-8959 have been replaced by a sulfolane ligand and this analogue is an inhibitor of comparable *in vitro* antiviral potency to the parent compound (80) but of reduced molecular weight.[224] A practical route to obtaining multigram quantities of (2*R*,3*R*)-



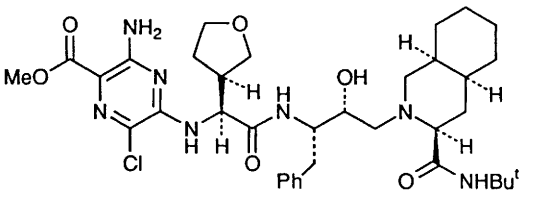
(80) Ro 31–8959



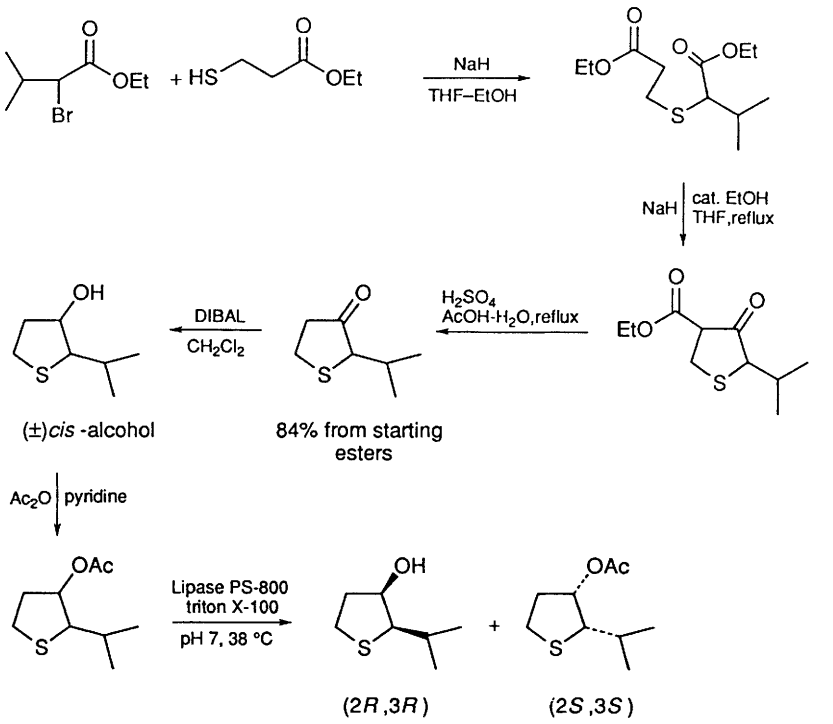
(81)



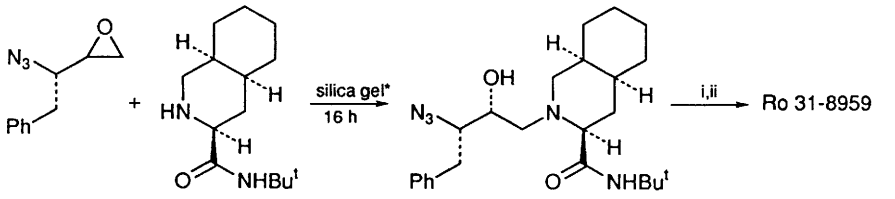
(82)



(83)



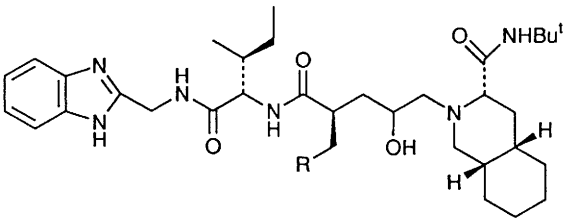

**Scheme 20**



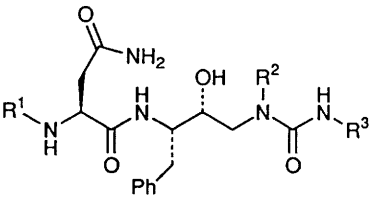

* Note: normal reaction conditions for preparation of azidoalcohol are reflux starting materials in 2-propanol for 12 h

Reagents: i, $H_2$, Pd-C; ii, *N*–(quinolinylcarbonyl)–L–asparagine, BOP

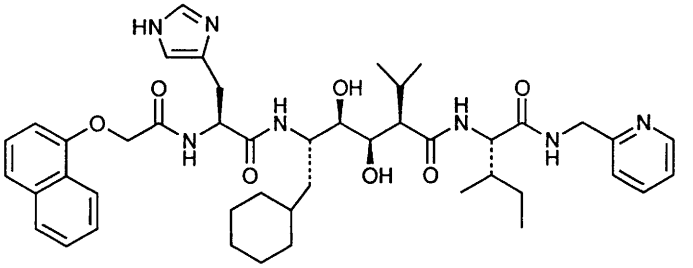
**Scheme 21**



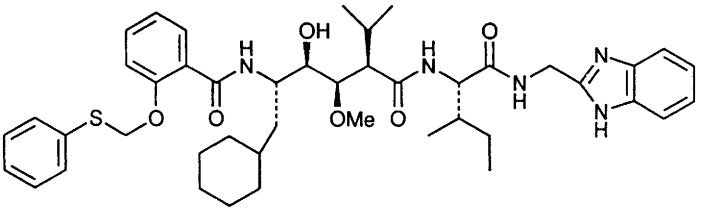
(84)



(85) $R^1$ = CBz, Qua; $R^2$, $R^3$ = various alkyl



(86) U–75, 875



(87)

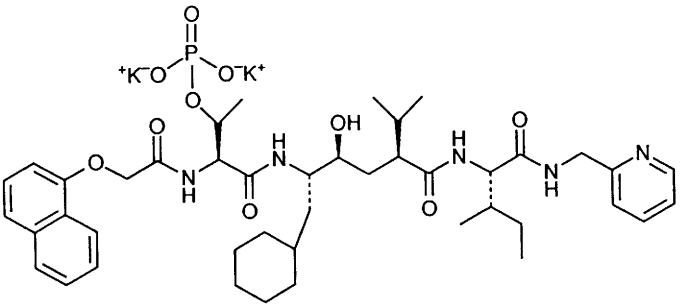
hydroxyisopropyltetrahydrothiophene, the precursor to the $P_2$ ligand of (82) has been developed (Scheme 20).[226] Another finding by the Merck group was that removing the amide carbonyl between the $P_2$ and $P_3$ residues resulted in a compound (83)[225] which exhibited an improvement in antiviral potency over Ro 31-8959 whilst retaining comparable enzyme inhibitory potency. In an interesting synthesis of the $P_1$-$P_1'$ hydroxyethylamide dipeptide component of Ro 31-8959 (Scheme 21) the azido oxirane and decahydroisoquinoline were adsorbed together on silica gel and after 16hrs the mixture was added to a silica column and eluted with an appropriate solvent to give a 72% yield of the azido alcohol.[227] Comparison of the NMR structure of Ro 31-8959 and the crystal structure of the inhibitor bound to HIV-1 protease indicated that, apart from the expected flexibility of part of the backbone, the conformational features obtained in solution were largely conserved on binding to the enzyme.[228]

The biological activity of two further compounds (84, R = Ph, $^{i}$Pr) has been compared with that of Ro 31-8959.[229] Although both these compounds were equipotent to Ro 31-8959 as enzyme inhibitors they were significantly less potent as antiviral agents. The most potent compound of a series of inhibitors (85) based on the hydroxyethylurea $P_1$-$P_1'$ isostere was structurally not unrelated to Ro 31-8959.[230] A quinoline moiety was found to be the preferred $P_3$ ligand while at the C-terminus a *t*-butyl group was identified as optimal. This compound (85, $R^1$ = Qua, $R^2$ = $^{i}$Bu, $R^3$ = $^{t}$Bu; $IC_{50}$ value 6nM) is currently being developed for clinical trials.

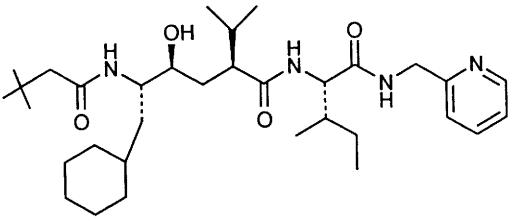
Modifications to the N and C-termini of U-75,875 (86) led to a compound (87)[231] which was a more effective antiviral agent than the parent inhibitor whilst a pro-drug (88) was obtained by replacing the histidine at $P_2$ in the related hydroxyethylene inhibitor with a phosphothreonine moiety.[232] Results of synthetic tailoring to N and/or C-termini of U-81,749E (89) have also been described.[233]

Studies with the $P_2$-$P_2'$ inhibitor (90, R = Ph) included replacing the $P_2$ *t*-butyl ligand with tetrahydrofuran/pyran moieties[223] and the synthesis of an analogue (91, R = $C_6H_{11}$) with an N-terminal ketone group rather than the carbamate.[234] Alternative methods to those described last year for the preparation of the hydroxyethylene Phe-Pro mimetic (92)[235] and JG-365 (93)[236] have been developed. A difluorostatone inhibitor (94) with a $K_i/IC_{50}$ of 1nM[237] and an enolic inhibitor (95) with a $Ki_{app}$ of 0.48μM[238] have also been prepared.

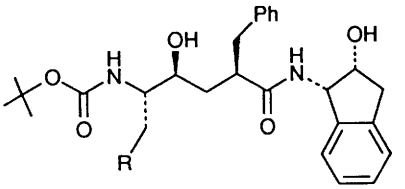
A full account of the structure-activity studies which led to the discovery of the $C_2$-symmetric inhibitor A-77003 (96) has been published.[239] Although A-77003 gave a good balance of activity and solubility in *in vitro* studies this compound showed no oral bioavailability in the rat. However clinical investigations of intravenous A-77003 for HIV infection are currently underway. Syntheses of the diamino diol (97) and diamino alcohol (98) core units of $C_2$-symmetric and pseudo-$C_2$-symmetric inhibitors have been reported by a number of groups. The diamino alcohol moiety could be obtained from D-(+)-arabitol,[240] a dihydroxyacetone[241] or 4-hydroxypyrrolidin-2-one[242] derivative while either D-(+)-mannitol[240,243] or (-)-2,3-*O*-isopropylidene-D-threitol[205] acted as starting materials for the synthesis of the diamino diol unit. Phosphorous[244]- and



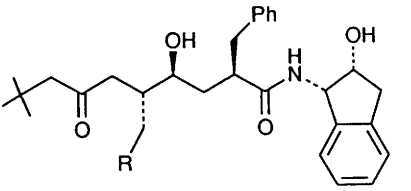
(88)



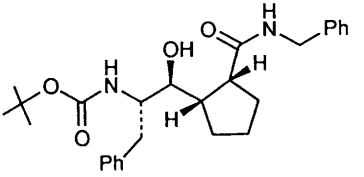
(89) U-81, 749E



(90)

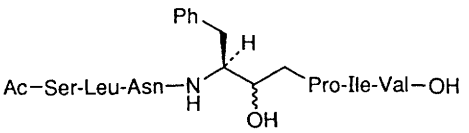


(91)

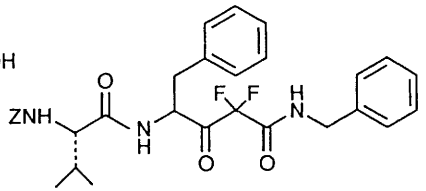


and *trans* –(2*S*, 3*R*, 4*R*, 5*S*) isomer

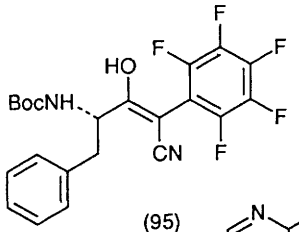
(92)



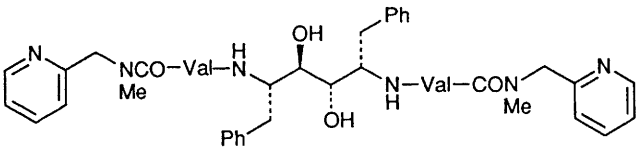

(93) JG–365



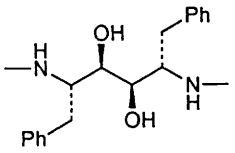

(94)



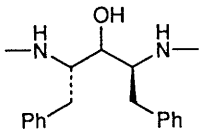

(95)



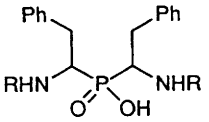

(96) A–77003



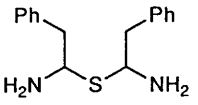

(97) diamino diol



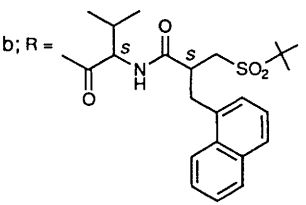

(98) diamino alcohol

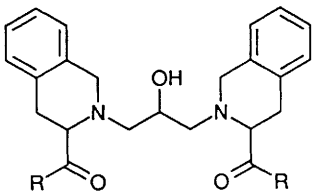



(99) a; R = H

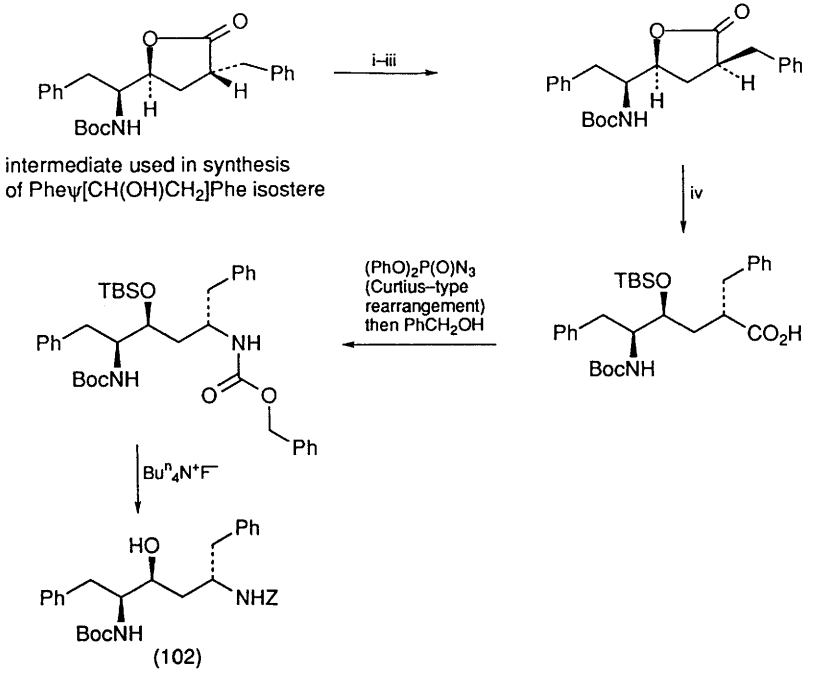



(100)




(101) R = NHBu$^t$, Val-NHBu$^t$

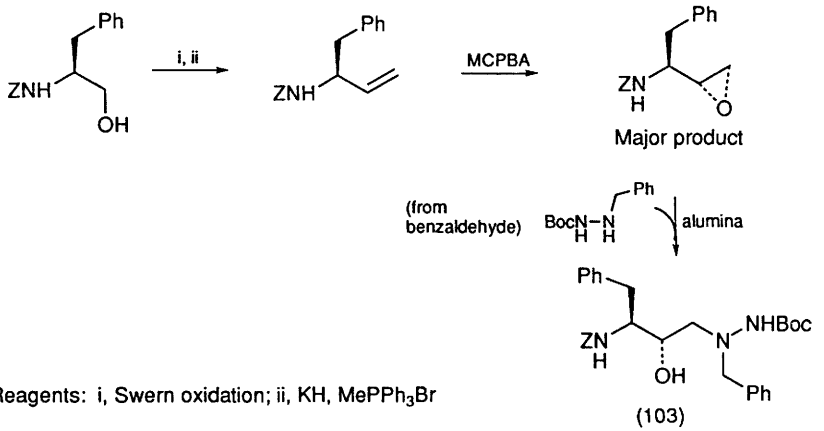



intermediate used in synthesis
of Phe$\psi$[CH(OH)$CH_2$]Phe isostere

(102)

Reagents: i, LDA, PhSeSePh; ii, MCPBA; iii, $H_2$, 10% Pd–C; iv LiOH, TBDMSCl, imidazole

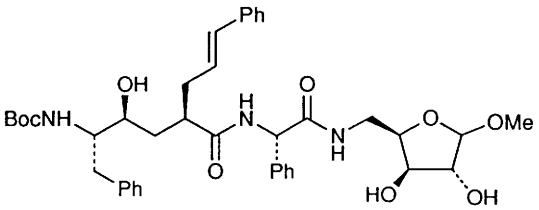
**Scheme 22**



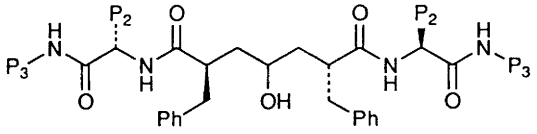

Reagents: i, Swern oxidation; ii, KH, $MePPh_3Br$

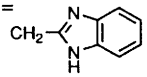
(103)

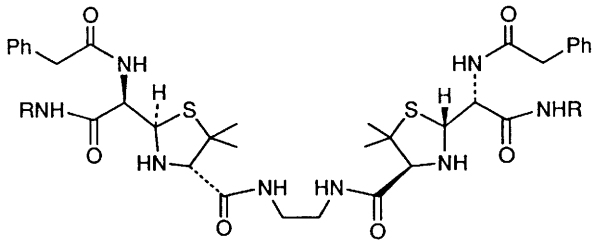
**Scheme 23**



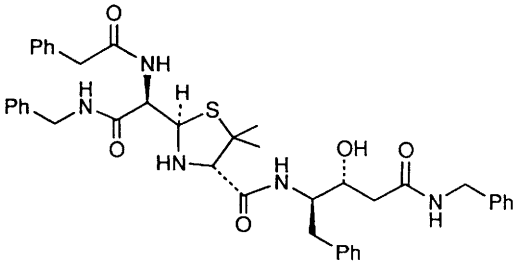
(104)



(105)a; $P_2 = CHMe_2$, $P_3 = CH[CH(Me)_2]CO_2Me$
b; $P_2 = CH(Me)Et$, $P_3 =$ 



(106)



(107)

sulfur[245]-containing pseudo-$C_2$-symmetric core units have also been prepared [compounds (99a) and (100) respectively]. The phosphinic acid-based derivative (*R,R*)-(99b) was found to be a potent inhibitor of HIV-1 protease ($IC_{50}$ value 0.5nM) but the bis-(CBz-valinyl)-sulfide, sulfoxide, and sulfone compounds were all inactive in *in vitro* assays. Also inactive in *in vitro* assays were the diamino alcohols (101),[246] compounds based on the C-terminus of the hydroxethylamide inhibitor Ro 31-8959 (80).

Groups from Merck and Abbott have both designed and synthesised novel dipeptide mimetics which are in effect hybrids of the hydroxyethylene- and symmetry-based core units. Both compounds [(102) from Merck[247] and (103) from Abbott[248]] were orthogonally protected at the two amino groups and both synthetic procedures (Schemes 22 and 23) possessed the necessary flexibility to allow the introduction of a range of ligands at the $P_1/P_1'$ positions. A series of glycopeptide mimetics based on the Phe$\psi$[CH(OH)$CH_2$]Phe isostere have been prepared.[249] The most potent inhibitor of this series (104, $ED_{50} = 0.17$nM) was shown to block the spread of HIV-1 in T-lymphoid cells.

Optimisation of side chains at the $P_2$ and $P_3$ positions of pseudo-symmetrical inhibitor (105a, $IC_{50} = 1.6\mu$M) gave a compound (105b) which was less peptidic in character and slightly more potent as an antiviral agent ($IC_{50} = 0.4\mu$M) but which was rather insoluble in a range of solvents.[250]

Glaxo researchers have reported results on the design, synthesis, and structure-activity studies of a series of penicillin-derived inhibitors. A crystal structure of the lead compound (106, R = Et), a $C_2$-symmetric inhibitor, complexed to HIV-1 revealed that the interaction between the inhibitor and the catalytic aspartates of the active site was rather poor.[251] Modification to this lead compound to improve the interaction with the binding site included varying the $P_2/P_2'$, $P_3/P_3'$ and linker groups[252,253] and replacing one of the penicillin units with, *inter alia*, statine-based isosteres.[254,255] The "monomeric" compound (107) showed similar levels of inhibitory and antiviral activity to the original dimeric lead compound (106, R = $CH_2Ph$) [$IC_{50} \sim 5$nM, $K_i \sim 0.1$nM, $ED_{50} \sim 5\mu$M].

Incorporation of a $\gamma$-turn mimetic into the $P_2$ to $P_1'$ positions of the substrate hexapeptide Ala-Asn-Tyr-Pro-Val-Val-$NH_2$ ($K_m$ in millimolar range) resulted in a constrained inhibitor (108a) with significantly improved binding affinity to HIV-1 protease ($K_i = 147\mu$M).[256] Furthermore, reduction of the amide bond in the $C_7$ mimetic of (108a) gave an inhibitor (108b) with a $K_i$ in the high nanomolar range ($K_i = 430$nM). Other substrate analogues prepared and reported to be inhibitors of HIV-1 protease include the phosphonamidate-containing compounds[257] Boc-Asn-Phe$\psi$[$PO_2$-N]Pro-Ile-$NH^iBu$ and Boc-Asn-D-Phe$\psi$ [P(OMe)O-N]Pro-Ile-$NH^iBu$ and the tripeptidyl epoxide (109).[258] N- and C-terminal peptides of varying lengths corresponding to the dimerisation interface of HIV-1 protease were found to be weak inhibitors of the enzyme.[259] Finally, design of new inhibitor candidates by using theoretical approaches have been the subject of several articles.[260-263]

## 5.5 Inhibitors of Other Proteases

*5.5.1 Serine Protease Inhibitors.* – Appropriately substituted cyclic peptides and enol lactone systems continue to be developed as mechanism-based inactivators of some serine proteases. Last year the cyclopeptides (110) with a phenylalanine residue at $P_1$ and chloro or bromo leaving groups (X = Cl, Br) were reported to be suicide substrates for α-chymotrypsin. Derivatives with lysine or arginine at $P_1$ and benzylic sulfonium leaving groups [X = $^+S(CH_3)_2$, $^+S(CH_3)C_6H_5$] have since been prepared and found to be selective inhibitors of bovine trypsin and human urokinase ($k_{inact}/K_I$ values in range $10^2$-$10^3 M^{-1}s^{-1}$).[264] Inhibition studies with the enol lactone valine mimics indicated that the α-alkyl-substituted derivatives (111) were much better inhibitors of human neutrophil elastase (HNE) ($k_a/K_i$ values ranging from 14,500 to 37,500$M^{-1}s^{-1}$) than the β-alkyl-substituted analogues (112).[265]

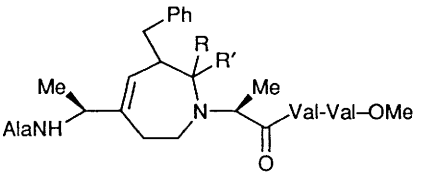
Dual inhibitors of serine proteases have been prepared by attaching known peptide-based inhibitors to the carboxy termini of a central terephthalic acid moiety.[266] Potent inhibition of both HNE ($K_i$ value of 15.9nM) and human cathepsin G ($K_i$ value of 2.2μM) was effected with compound (113) containing both Val-Pro-Val and Val-Pro-Phe tripeptide sequences. Replacing the $P_3$-$P_2$ components of a tripeptidyl trifluoromethyl ketone inhibitor of human leukocyte elastase (HLE) with a conformationally restricted lactam moiety gave compounds such as (114).[267] This bicyclic lactam derivative, although less potent than the original tripeptide inhibitor, exhibited good *in vitro* and *in vivo* inhibition of HLE.

The loss of inhibitory activity observed when prolineboronic acid-containing dipeptides are exposed to aqueous media has been explained in terms of cyclisation to give the boron analogue of a diketopiperazine (Scheme 24).[268] Proof of the equilibrium between the active and inactive compounds was obtained by using $^1H$ NMR to follow the course of the reaction and by isolating the cyclic material and establishing its structure.

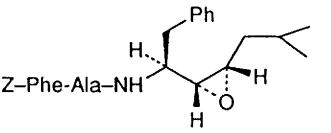
Other articles on serine proteases are listed in Table 4.

*5.5.2 Cysteine Protease Inhibitors.* – Several series of di- and tri-peptidyl derivatives have been prepared and tested for inhibitory activity against a range of cysteine proteases. These studies are briefly summarised in Table 5.

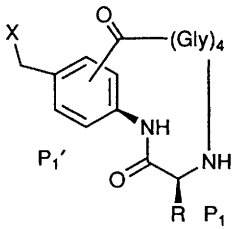
In the epoxysuccinyl series (117) the best inhibitor in terms of cathepsin B selectivity was (117, R = $NH^iBu$, $R^1$ = OH) (93-fold preference over papain).[279] Two factors appeared to be important for cathepsin B selectivity; a derivatised carboxylic acid group on the epoxide ring combined with a free C-terminal carboxylate on the dipeptidyl portion of the inhibitor. Selectivity for cathepsin B over trypsin-like serine proteinases was acheived in the leupeptin (Ac-Leu-Leu-Arg-H) series by incorporating a lysinal group at the C-terminus of the tripeptide or by altering the side chains at the $P_2$ and $P_3$ positions in the argininal compounds.[282] Ac-Leu-Val-Lys-H and Ac-Phe-Val-Arg-H were both 100-1000 times more potent inhibitors of cathepsin B ($IC_{50}$ values of 4nM and 39nM respectively) than of the serine proteases (trypsin, kallikrein, thrombin, and plasmin).




(108)a; R = R′ = O
b; R = R′ = H

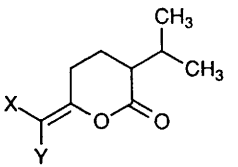



(109)

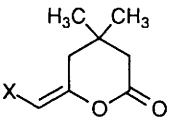



(110) R = $CH_2Ph$, $(CH_2)_4NH_2$, $(CH_2)_3NHC(=NH)NH_2$

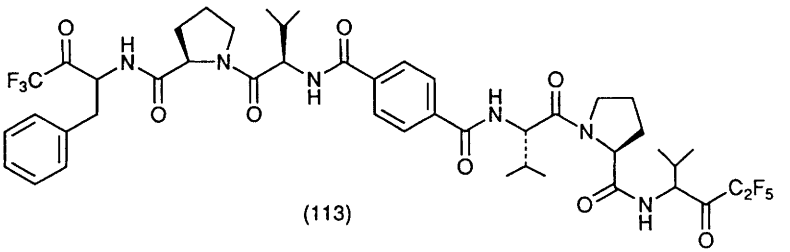
X = leaving group



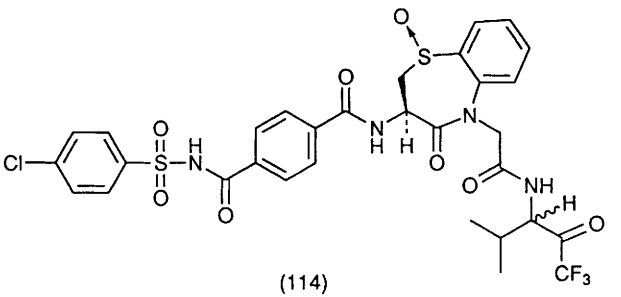

(111) X,Y = H,H; H,Br; Br,H




(112) X = H, Br




(113)




(114)

**Table 4** Serine protease inhibitors not discussed in the text

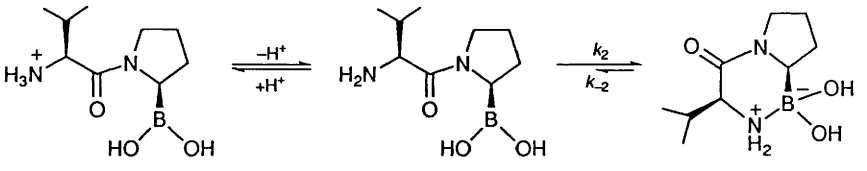
| *Enzyme* | *Reference* |
|---|---|
| *Trypsin* | |
| – Synthesis of semi-synthetic "backbone" variants of BPTI | 269 |
| *Kallikrein* | |
| – Structure-activity studies on phenylalanine anilide (115) | 270 |
| *Elastase* | |
| – QSAR study of porcine pancreatic elastase (PPE) substrates (Suc-X-Y-Ala-pNA) and inhibitors ($CF_3CO$-X-Y-Ala-pNA) (X,Y = various amino acids) | 271 |
| – Crystallographic and NMR investigation of cyclic thiolic compound (116) | 272 |
| *Thrombin* | |
| – SAR study on a series of di- and tri-peptide aldehydes based on Boc-D-Phe-Pro-Arg-H | 273 |
| – SAR study on a series of dipeptide arginine aldehydes based on desamino-Phe-Pro-Arg-H | 274 |
| – Synthesis and inhibition studies of D-Phe-Pro-NHCH[$(CH_2)_4$NHR] $CR^1(OH)CO_2Me$[R = $R^1$ = H; R = H, $R^1$ = OMe; R = $C(NH_2)$=NH, $R^1$ = H] | 275 |
| – Synthesis and inhibition study of Z-D-Phe-Pro-methoxypropylboroglycine | 276 |
| – Solution conformation of D-Phe-Pro-containing peptides | 277 |
| – Crystal structure of thrombin-cyclotheonamide A complex | 278 |

**Table 5** Di- and tri-peptidyl cysteine protease inhibitors

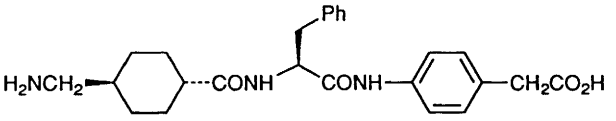
| *Compound* | *Enzymes inhibited* | *Reference* |
|---|---|---|
| *Epoxysuccinyl dipeptides | Papain, cathepsin B | 279 |
| Peptidyl diazomethanes | Papain, cathepsin B | 280 |
| *N*-Haloacetyl amino acid amides | Papain, cathepsin B | 281 |
| *Leupeptin (Ac-Leu-Leu-Arg-H) analogues | Cathepsin B, trypsin-like serine proteinases | 282 |
| Azapeptide halomethanes | Cathepsin B, calpain | 283,284 |
| Dipeptidyl and tripeptidyl α-keto esters, α-keto amides, and α-keto acids | Cathepsin B, calpain, and, to varying degrees, papain | 285 |
| *N*-Peptidyl-*O*-acyl hydroxamates | Cathepsins S, L, B and to a much lesser degree trypsin-like serine proteases | 286 |

* further details in text

The cyclopropenone-containing compounds (118) were designed as papain inhibitors by combining in a single molecule, a cyclopropenone reactive site and an enzyme recognition site.[287] Of the two structures, only the 1′*S*-isomer (118, R = OH, R′ = H) was found to be a potent inhibitor of papain ($IC_{50}$ = 0.054μM); the *R*-epimer was, by comparison, practically inactive ($IC_{50}$ = 32μM). Work




**Scheme 24**



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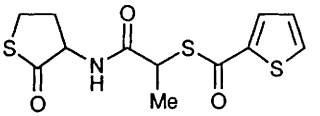
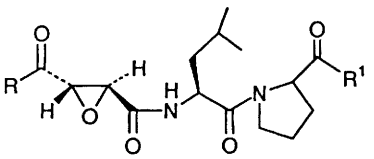
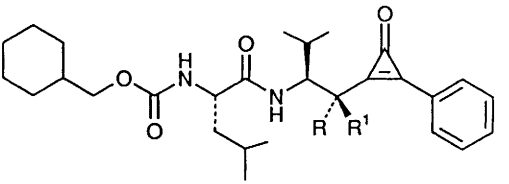


(116)



(117) R = OH, OEt, NHBu$^{i}$
R$^{1}$ = OBzl, OH



(118) R,R$^{1}$ = OH, H; H, OH

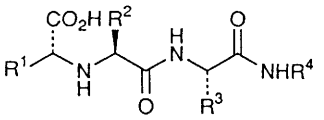
carried out by several groups has led to the conclusion that interleukin-1β converting enzyme is probably a cysteine protease.[288,289]

*5.5.3 Metalloprotease Inhibitors.* – Useful data on the requirements for potent inhibition of the matrix metalloproteases has emerged from structure-activity studies on *N*-carboxyalkyl dipeptides (119)[290] and β-mercapto carboxylic acid derivatives (120).[291] Nanomolar inhibition of collagenase was obtained by compounds in the latter series which featured *S,S* or *R,R* stereochemistry at the $P_1'$ and thiol bearing centres, an ester or small amide substituent at the $P_1$ position and an aromatic L-amino acid at the $P_2'$ position. One of the most significant findings in the *N*-carboxyalkyl dipeptide series was that compounds with a β-phenylethyl group at the $P_1'$ position were particularly potent inhibitors of stromelysin. One particular compound [119, $R^1 = CH_3$, $R^2 = CH_2CH_2Ph$, $R^3 = CH_2CH(CH_3)_2$, $R^4 = Ph$] was relatively selective for rabbit stromelysin with a $K_i = 6.5nM$ and the authors suggested that this inhibitor may prove useful in elucidating the role of the enzyme in cartilage degradation.

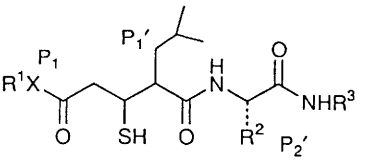
A multipeptidase inhibitor of the major neurotensin/neuromedin N (NT/NN) degrading enzymes has been developed.[292] Based on the C-terminal sequence common to NT and NN this compound (121) was a nanomolar *in vitro* inhibitor of three metalloendopeptidases and of leucine aminopeptidase ($IC_{50}$'s from 30 to 60nM). A range of pharmacological assays confirmed the protecting effects of the inhibitor on the metabolism of the two neuropeptides *in vitro*. An orally active neutral endopeptidase (NEP) inhibitor (122) has been synthesised which slows down the degradation of atrial natriuretic factor *in vitro* and produces a lowering of blood pressure in DOCA-salt hypertensive rats.[293] *In vitro* NEP inhibition was exhibited by the *gem*-cyclopentyl and *gem*-cyclohexyl substituted thiols (123).[294] These compounds, designed as hybrids of the thiorphan and candoxatrilat type of inhibitor, were prepared by a route employing formation of strained 2,2-spiro-β-lactones from β-hydroxy acids using triflic anhydride followed by *O*-alkyl cleavage with potassium thioacetate. Other studies on NEP included a structure-activity investigation with thiol inhibitors (124, 125) bearing a variety of substitutents[295] and the synthesis of isosteric analogues of thiorphan $HSCH_2CH(CH_2Ph)\psi CH_2CO_2H$ ($\psi$ = $COCH_2$, $CH_2NH$, CSNH, *E*,CH = CH].[296]

It is not yet clear whether endothelin converting enzyme (ECE), the enzyme which liberates endothelin from the prohormone big endothelin, is a metalloprotease or an aspartic protease. Compounds which have been evaluated for *in vitro* inhibition of ECE include several analogues of phosphoramidon, a metalloprotease inhibitor,[297,298] *N*-terminal cysteine di- and tri-peptides,[298] and several series of statine- and dihydroxyethylene-based inhibitors.[299] Nanomolar inhibition of ECE was exhibited by several of the statine and dihydroxyethylene compounds while $IC_{50}$ values of ~1-10μM were obtained for some of the phosphoramidon analogues.

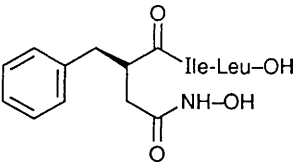
Finally, several Schiff base derivatives, (RCH = NNHCS-L-Phe-OMe; R = 2-pyridyl, 1-oxo-2-pyridyl, 1-oxo-2-quinolyl) of α-*N*-thiocarbazoyl-L-phenylalanine methyl ester were found to be competitive inhibitors of thermolysin.[300]



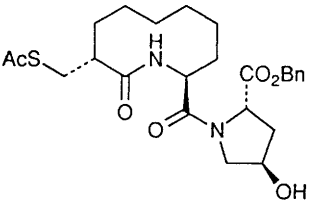
(119) $R^1$–$R^4$ = various alkyl, aryl groups



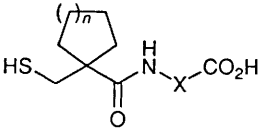
(120) $R^1X$ = RO, RNH, piperazinyl
RCOCHRNH (R = H or alkyl)
$R^2$ = $ArCH_2$, (3-indolyl) $CH_2$,
($PhCH_2O$) CHMe
$R^3$ = H, Me



(121)

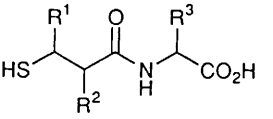


(122) CGS 25155

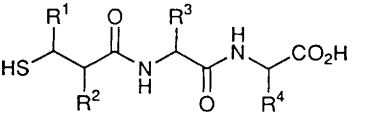


(123) X = *m*-cyclohexane,
(*S*)-$CH_2Ph$

*n* = 1,2



(124)



(125) $R^1$–$R^4$ = various alkyl, aryl groups

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

### 6.1 Peptides with 'Opioid Characteristics'

The solution structures of several δ-selective opioid peptides has been examined using NMR techniques and/or energy calculations. The cyclic portion of H-Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE) was well-defined in both DMSO and water with a γ-turn at the $Gly^3$ residue and torsion angles close to those of an α-helix for the backbone of the $Phe^4$ residue.[301,302] Low energy conformations obtained from independent energy calculations were in good agreement with the NMR data and one conformation in particular was very similar to the proposed receptor bound conformation of DPDPE. Solution structures of (*S*,*S*)- and (*S*,*R*)-β$MePhe^4$-DPDPE resembled those of DPDPE whereas the conformations adopted by analogues with an *R*-configuration at the $C^\alpha$ atom of residue 4 were somewhat different. This observation was in line with the high biological potencies displayed by the former three compounds but not by the latter two. The orientation of the phenyl ring of $Phe^4$ was identified as being the crucial difference between the low energy conformations of other high and low affinity analogues of DPDPE.[303]

Two groups have investigated the conformation of deltorphin A (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-$NH_2$) in DMSO-$d_6$ solution. A quantitative NMR analysis pointed to the existence of a conformational equilibrium between extended and folded structures.[304] while a possible bioactive conformation for the N-terminal tripeptide moiety was identified from a combination of NMR data and energy calculations.[305] Molecular dynamics simulations of the three naturally occurring deltorphins (deltorphins A, B, and C) have been carried out.[306] (Deltorphin B and C = H-Tyr-D-Ala-Phe-Xaa-Val-Val-Gly-$NH_2$, Xaa = Glu for deltorphin B and Asp for deltorphin C).

Structure-activity studies on the δ-selective ligands have emphasised that the conformational requirements at the opioid receptors are very exacting. Deltorphin C analogues containing the conformationally restricted bicyclic amino acids 2-aminoindan-2-carboxylic acid (Aic) and 2-aminotetralin-2-carboxylic acid (Atc) at position 3 exhibited higher δ-affinities than the natural peptide.[307] One of the compounds [(*R* or *S*)$Atc^3$]deltorphin C was also four-fold more selective for δ-receptors than deltorphin C. At the other extreme [NMe-$Phe^3$]deltorphin $C^{307}$ and [des$Gly^3$]BUBU [Tyr-D-Ser(O$^t$Bu)-Phe-Leu-Thr(O$^t$Bu)],[308] for example, were practically inactive. Incorporation of a disulfide bridge into deltorphin C did not affect its high affinity for the δ-receptor but this analogue, H-Tyr-D-Cys-Phe-Asp-Cys-Val-Gly-$NH_2$, showed low δ-receptor selectivity as a result of a large increase in its affinity for μ-receptors.[309] An interesting observation emerged from a study with the somatostatin-derived opioid peptide D-Tca-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ (Tca = cyclic tryptophan analogue). This compound proved to be a weak δ- and μ-agonist in receptor binding assays but in antinociceptive assays it was as potent as DPDPE.[310]

A comprehensive mass spectral analysis of DPDPE has been reported.[311]

Conformational and/or biological data on several μ-selective peptides has been analysed. The bioactive conformation of morphiceptin (Tyr-Pro-Phe-Pro-$NH_2$) at the μ-receptor was estimated as a result of studying the solution structures of a family of analogues with different biological potencies.[312] The main charateristics of this model are a requirement of a *cis* amide bond linking residues 1 and 2 and *trans* conformations ($\chi_1 \sim 180$) for the Tyr and Phe side chains. NMR results and theoretical calculations on two other μ-selective opioids, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol and Tyr-D-Ala-Gly-(NMe)Phe-Met(O)-ol, pointed towards folded low energy conformations[313] characterised by a type II β-turn around $Gly^3$-(NMe)$Phe^4$. Analysis of receptor binding data on dermorphin (H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-$NH_2$) and several analogues revealed that the interaction with brain μ-receptors may be more complex than a "simple reversible bimolecular equilibrium".[314] The ring portion of a cyclic dermorphin tetrapeptide H-Tyr-Lys-Phe-Asp-$NH_2$ was found to adopt a well-defined conformation in DMSO-$d_6$ but the exocyclic $Tyr^1$ and $Phe^3$ side chains were more flexible.[315] No biological data was reported for this compound.

Two conformationally constrained analogues of dynorphin A have been prepared.[316] Both compounds, [*cis*- and *trans*-$ACCA^{2-3}$]Dyn $A_{1-13}$-$NH_2$ were selective for κ-receptors. This selectivity was largely due to a significant decrease in affinity for the μ-receptor ($K_i$ κ/μ/δ = 1/13/210 and 1/21/103 for the *cis* and *trans* compounds respectively). Substitution of $Tyr^1$ in dynorphin $A_{1-11}$-$NH_2$ with Ac-Tyr, D-Tyr, Phe- or *p*BrPhe also led to analogues that were quite potent at the κ-opioid receptor.[317] Dynorphin A derivatives containing a *S*-(3-nitro-2-pyridinesulfenyl)cysteine residue at position 8 were found to be highly potent and specific to κ receptors and bound through disulfide bond formation.[318] Analysis of NMR data on dynorphin A bound to dodecylphosphocholine micelles indicated that the $Gly^3$ to $Arg^9$ residues adopted an α-helical conformation.[319] The synthesis of, and limited biological data on, several dynorphin B analogues has been reported.[320,321]

A number of opioid peptides have been coupled, *via* their C-termini, to oligosaccharides. Leu-Enkephalin[322] and DPDPE[323] were conjugated to β-cyclodextrins while Met-enkephalin[324] was linked to various D-glycopyranose moieties. Dimeric enkephalin structures have also been prepared.[325]

## 6.2 Cholecystokinin Analogues

A group from Organon Laboratories has published a useful overview of the approaches adopted by themselves and other workers in the development of peptidomimetics with CCK-A agonist activity.[326] This work used CCK-8 as a lead compound. Other structure-activity studies on CCK fragments are summarised in Table 6. Several articles of a more miscellaneous nature are also included in the Table.

A series of papers by Horwell's group at Parke-Davis UK has reported the results of systematically modifying various components of the CCK-B dipeptoid antagonist CI-988 (126). In addition to detailing the structure-activity studies which led to the development of CI-988[340-342] the group has also investigated the biological consequences of introducing a dehydro residue,[343] a conformational

**Table 6** Studies on CCK fragments

| *CCK fragment* | *Compounds synthesised and evaluated* | *Reference* |
|---|---|---|
| | *Structure-activity studies* | |
| $CCK_2$ (32-33) | – $ArSO_2$-Asp-$NH(CH_2)_2R$; Ar,R = various aryl groups | 327 |
| $CCK_4$ (30-33) | – Series of tetrapeptides Boc-Trp-Xaa-Asp-Yaa; Xaa, Yaa = natural and unnatural residues | 328 |
| $CCK_7$ (27-33) | – Analogues of Boc-Tyr($SO_3H$)-Nle-Gly-(L- or D)-Trp-Nle-Asp-$O(CH_2)_2Ph$ with $CH_2CH_2$ linkage in place of ester bond at C-terminus | 329 |
| | – Analogues of BocTyr($SO_3H$)-Nle-Gly-(L- or D)-Trp-Nle-Asp-$NH(CH_2)_2Ph$ with various groups replacing C-terminal phenethyl moiety | 330 |
| | – Analogues of $HO_2C(CH_2)_2CO$-Tyr($SO_3H$)-Met-Gly-Trp-Met-Asp-$NH(CH_2)_2Ph$ | 331 |
| | – Analogues of Boc-$CCK_7$ with Met and Phe residues replaced by neopentylglycine and 2,4,6-trimethylphenyl-alanine respectively (synthesis only, no biological data) | 332 |
| $CCK_8$ (26-33) | – $CCK_8$ with N-terminal modifications and substitutions at $Gly^{29}$ | 333 |
| | – [Phe(*p*-$CH_2SO_3Na$)$^{27}$,Nle$^{28,31}$,Nal$^{30}$]$CCK_{26-33}$ | 334 |
| CCK-33 | – [Phe(*p*-$CH_2SO_3Na$)$^{27}$,Nle$^{7,28,31}$,Nal$^{30}$]CCK-33 | 334 |
| | *Miscellaneous Studies* | |
| | – CD study of CCK-peptides of varying length ($CCK_{26-33}$ up to $CCK_{19-33}$) | 335 |
| | – Synthesis of CCK-58 analogues | 336 |
| | – Synthesis and application to receptor studies of [$^3$H]BzBz-Orn ($COCH_2CH_3$)-$CCK_{26-33}$ | 337 |
| | – Solution conformation of [Thr$^{28}$,Nle$^{31}$]$CCK_{25-33}$ | 338 |
| | – N-terminal lipophilic derivatives of [Thr$^{28}$,Nle$^{31}$]$CCK_{25-33}$ | 339 |

constraint,[344-346] or a stereochemical modification[347,348] into dipeptoids related to(126). Inverting the configuration of the α-Me tryptophan and/or substituted phenethylamine groups in (126) caused a significant decrease in CCK-B binding affinity but had little effect on CCK-A receptor binding affinity.[347] The combined effect of inverting both chiral centres in (127a) ($IC_{50}$CCK-B = 0.15nM; $IC_{50}$CCK-A = 25.5nM), a less selective CCK-B ligand than (126), was a compound (127b) ($IC_{50}$CCK-B = 260nM; $IC_{50}$CCK-A = 2.8nM) that was selective for the CCK-A receptor.[348] Other examples of "reversed" receptor selectivity in pairs of compounds which differed only in the stereochemistry of the chiral centres included the diastereoisomers (127c and 127d)[348] with a (1*R*,2*R*)-*trans*-2-methylcyclohexyl group at the N-terminus and the enantiomers (128)[343] containing a α,β-dehydrotryptophan residue. Combining the strategies of stereochemical inversions of the tryptophan and substituted phenethylamine centres with an N-terminal (1*S*,2*S*)-*trans*-2-methylcyclohexyl group resulted in a ligand (127e, $IC_{50}$CCK-A = 3.9nM; $IC_{50}$CCK-B = 4.2nM)[348] which had mixed, nanomolar affinity for both CCK-A and CCK-B receptor subtypes. Racemic

α,α-disubstituted tryptophan derivatives such as [129, R = OH, R′ = $(CH_2)_2$Ph; R = H, R′ = $(CH_2)_4$NHCONH(*o*ClPhe] were found to be CCK-A selective.[349]

A tritiated derivative of a compound closely related to (127a) has also been prepared[350] and decomposition studies on (126) and several analogues have been carried out.[351]

Macrocyclic analogues (130) of CI-988 have been prepared by a group at the USA laboratories of Parke-Davis.[352] Although molecular modelling studies indicated that the 11 to 14-membered ring analogues (130, n = 2-5) overlapped the X-ray structure of CI-988 at a number of key points none of these four macrocycles showed any affinity for the CCK-B receptor.

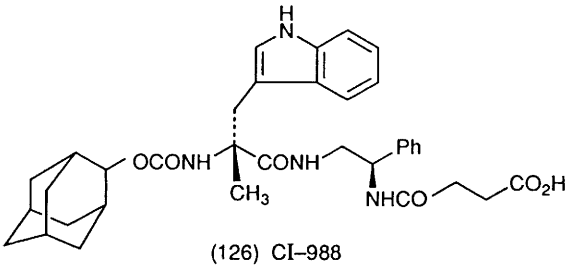
Roques' group has investigated the structure-activity relationships of compound (131) and derivatives.[353] Most of the compounds synthesised and evaluated proved to be selective for the CCK-B receptor and the most potent ($K_i$ = 6.1nM, $K_i$ CCK-A/$K_i$ CCK-B = 174) analogue had an (*R*)-configuration at the α-carbon atom and a *p*-chloro substitutent on the benzene ring.

### 6.3 Angiotensin Analogues

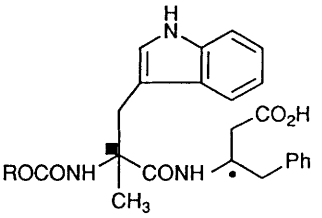
Replacement of $Pro^7$ with its four- or six-membered ring homologues azetidine (Aze) or pipecolic acid (Pip) in [$Sar^1$]ANG II (agonist), [$Sar^1$,$Ile^8$]ANG II (Sarilesin, type I antagonist), and [$Sar^1$,Tyr(OMe)$^4$]ANG II (Sarmesin, type II antagonist) resulted in analogues which retained high activity, whereas similar modifications to ANG II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) resulted in substantially lower levels of activity.[354] Further analysis of the biological data on the antagonist (Sarilesin, Sarmesin) analogues indicated that Pip rather than Aze at position 7 gave higher levels of type I antagonist activity while the reverse substitution pattern gave higher levels of type II antagonist activity. The authors concluded that subtle changes in the ring properties of the secondary amino group at position 7 (Aza = planar, Pro = envelope, Pip = boat/chair) influenced the conformations necessary for receptor recognition and expression of agonist or antagonist activity. The conformational and biological consequences of introducing a methyl group into the imidazole ring of $His^6$ in [$Sar^1$]ANG II was also examined.[355] The chemical shifts in the NMR spectrum of the His $C_2$ and $C_4$ protons and the Sar *N*-methyl protons in the major isomer of [$Sar^1$,His(π-Me)$^6$]ANG II were found to be markedly different from those in [$Sar^1$,His(τ-Me)$^6$]ANG II and [$Sar^1$]ANG II. This suggested that methylation of the π-nitrogen of the imidazole ring had a greater perturbation effect on the overall conformation of the molecule than methylation of the σ-nitrogen. This perturbation effect was mirrored by the biological activities of the two analogues; [$Sar^1$,His(σ-Me)$^6$]ANG II maintained significant levels of biological activity whereas [$Sar^1$,His(π-Me)$^6$]ANG II was essentially devoid of biological activity.

### 6.4 Oxytocin and Vasopressin Analogues

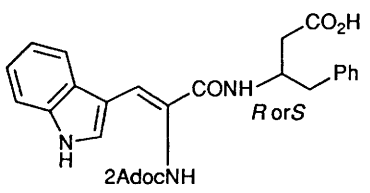
Structure-activity studies on oxytocin and vasopressin continue. Residues 3 to 8 of the uterotonic antagonist [Pmp$^1$,D-$Trp^2$,$Arg^8$]oxytocin ($pA_2$ = 7.77) were systematically replaced by the corresponding D-amino acids.[356] A surprise finding was that replacement of $Cys^6$ with D-Cys led to an analogue ($pA_2$ = 8.29)



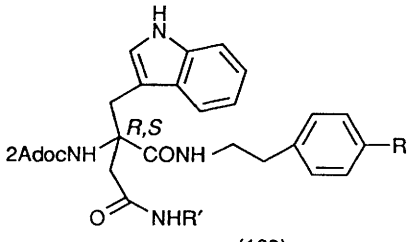

(126) CI–988



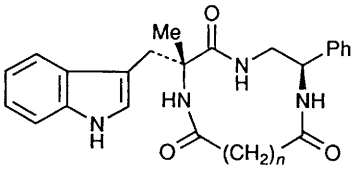

| R | ■ | • |
|---|---|---|
| (127)a; 2–adamantyl | *R* | *S* |
| b; 2– adamantyl | *S* | *R* |
| c; (1*R*, 2*R*)–*trans*–2–methylcyclohexyl | *R* | *S* |
| d; (1*R*, 2*R*)–*trans*–2–methylcyclohexyl | *S* | *R* |
| e; (1*S*, 2*S*)–*trans*–2–methylcyclohexyl | *R* | *S* |



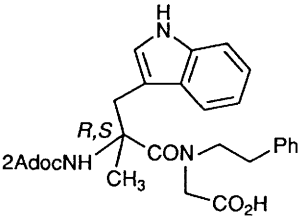

(128)




(129)




(130) $n$ = 2–5




(131)

which was more than three times as potent as the parent antagonist. Other modifications to the ring portion of the molecule gave compounds which were weaker antagonists than the parent peptides or were inactive. Substitutions in the tail sequence were well tolerated and the D-Pro$^7$ and D-Arg$^8$ analogues were equipotent to the parent antagonist. Several series of arginine-vasopressin analogues have been prepared with modifications at, in particular, position 2.[357-360] One compound, [D-Phe(2,4,6-triMe)$^2$,D-Har$^8$]vasopressin, was reported to be a potent *in vitro* and *in vivo* uterotonic inhibitor ($pA_2$ values equal to 8.1. and 7.5 respectively) while another, [Cpp$^1$,Thi$^2$,Val$^4$]arginine-vasopressin, showed selectivity in antiuterotonic, antipressor, and anti-antidiuretic effects.

Two sulfydryl-reactive probes for receptor studies, deamino[Dab($N^\delta$-*N*-maleoyl-β-alanine)$^4$]arginine-vasopressin and deamino[Lys($N^\varepsilon$-*N*-maleoyl-β-alanine)$^8$]vasopressin,[361] and a parallel dimer of deamino-oxytocin[362] have also been prepared. Rather disappointingly neither of the maleoyl peptides formed covalent bonds to the sulfydryl groups in or close to the hormone binding site of the $V_2$ receptor. The deamino-oxytocin dimer showed approximately 0.1% to 2% of the activity of the monomer as well as prolonged action.

Kinetic and thermodynamic data on the interconversion between the *cis* and *trans* conformations of oxytocin and arginine vasopressin have been obtained from NMR experiments.[363] The rate of rotation about the Cys$^6$-Pro$^7$ imide bond was found to be significantly faster in methanolic than in aqueous solution for both compounds. These results were consistent with the proposed interconversion mechanism which involves a less polar, twisted Cys$^6$-Pro$^7$, imide bond in the transition state. The solution conformations of oxytocin (protonated and unprotonated forms)[364] and triglycyl-lysylvasopressin[365] have been studied by NMR spectroscopy.

## 6.5 Luteinising Hormone-releasing Hormone (LHRH) Analogues

Several $N^\alpha$methyl analogues of LHRH have been prepared and these are discussed in section **2.7**. Formylating or acetylating the free amino groups of the $A_2$pr and $A_2$bu moieties of [Ac-D-2-Nal$^1$,D-*p*ClPhe$^2$,D-3-Pal$^3$,D-Lys($A_2$pr or $A_2$bu)$^6$,D-Ala$^{10}$]LHRH gave compounds which produced 100% inhibition of ovulation in cycling rats in doses of 0.75-1.5μg/rat.[366] The most potent analogue of a series of antagonists with histidine at position 8 was Ac-D-Qal-D-*p*ClPhe-D-3-Pal-Ser-*c*PzACAla-D-Lys(Pic)-Leu-His-Pro-D-Ala-$NH_2$. This compound showed 33% antiovulatory activity (AOA) at 0.25μg and neglible levels of histamine release at an $ED_{50}$ of 308μg/ml.[367] Antiovulatory activity data on [Ac-D-Xaa$^1$,D-*p*ClPhe$^2$,D-3-Pal$^3$,*c*PzACAla$^5$,D-Lys(Pic)$^6$,Ilys$^8$,D-Ala$^{10}$]LHRH [Xaa = *O*-phenyltyrosine (Opt), 3-(2-dibenzofuranyl)alanine (Dbf)] indicated that the compound with the more rigid Ac-D-Dbf in position 1 was twice as potent as the analogue with the less rigid Ac-D-Opt substitutent in this position (82% and 38% AOA respectively at 0.5μg).[368] The parent compound [Xaa = 3-(2-naphthyl) alanine (2-Nal)] was more potent than both analogues with 90% AOA activity at 0.5μg.

Energy calculations on LHRH and a series of analogues found that the most rigid part of the molecule was the central tetrapeptide Tyr$^5$-Gly$^6$-Leu$^7$-Arg$^8$

and that the antagonists differed from the agonists in the common geometrical shape of their fragments 5-8.[369,370] In a multigram solid phase synthesis of the LHRH agonist nafarelin (pGlu-His-Trp-Ser-Tyr-D-Nal-Leu-Arg-Pro-Gly-$NH_2$) using $N^{\alpha}$-Boc protected amino acids and minimal side chain protection the quality of the crude product was significantly improved by using temporary protection for the serine residue.[371] Solution and solid phase methods of preparing [D-Trp$^6$]LHRH using urethane-protected α-amino and *N*-carboxy-anhydrides have been compared[372] and several antagonists, [Ac-D-Nal$^1$,D-*p*ClPhe$^2$,D-Pal$^3$,D-Pal(*N*R)$^6$,Lys($^i$Pr)$^8$,D-Ala$^{10}$]LHRH (R = Me, $CH_2Ph$, $^n$Bu, $^i$Pr) with an *N*-alkylated pyridylalanine residue at position 6 have been prepared using solid phase techniques.[373]

### 6.6 Tachykinin Analogues

Multiple synthesis techniques have been used to prepare large numbers of substance P analogues. A set of naturally occurring substance P analogues, eight *N*-terminal truncated analogues, several structural variants, and 512 stereo-isomers with all possible combinations of L- and D-amino acids at 9 of the 11 positions (except Gly$^9$ and Met$^{11}$) were prepared using multipin methods.[374-377] A single D-amino acid replacement set of substance P was prepared on cotton discs.[378] Results of receptor binding experiments with the analogues largely confirmed previous findings. Briefly, a significant reduction in binding affinity to NK-1 receptors was observed in compounds with D-amino acid substitutents in the *C*-terminal residues (Gln$^6$-Leu$^{10}$) whereas reasonable levels of activity were retained upon either loss of or chirality changes to the *N*-terminal residues (Arg$^1$-Gln$^5$) of substance P.

A study[379-381] investigating the effect of replacing Met$^{11}$ in [Orn$^6$]$SP_{6-11}$ with dialkyl esters of glutamic or aspartic acid is complementary to similar work reported last year on the hexapeptide analogues containing secondary and tertiary amides of glutamic acid at position 11. The nature of the alkyl groups in [Orn$^6$,Glu/Asp(OR)-OR′]$SP_{6-11}$ (132) was found to be a determining factor for activity. For example compounds, (132, R = R′ = $^t$Bu, Bz), with bulky lipophilic C-termini were potent antagonists in NK-1 receptor while the bis-methyl glutaminyl analogue was a weak agonist. Each of these compounds showed weak antagonist activity in NK-2 and NK-3 receptor subtypes.

Conformational analysis of several C-terminal hexapeptide analogues of substance P have been carried out. NMR data on pGlu$^6$-Phe-Phe-Pro-Leu-Met$^{11}$-$NH_2$ (NK-1 ligand) and the D-Pro$^9$ analogue (NK-2 ligand) indicated that both compounds, but in particular the latter, tended towards folded conformations in $CDCl_3$ whereas in TFE/$H_2O$ (70:30) the structures were largely unordered.[382] The main isomer of the NK-3 ligand (pGlu$^6$,(NMe)Phe$^8$,Aib$^9$]$SP_{6-11}$ detected from a study in DMSO-$d_6$/water (90:10) contained a *cis* Phe-(NMe)Phe peptide bond and a type VI β-turn spanning residues 6-9 followed by a helical segment extending to the C-terminus.[383] Substance P methyl ester (NK-1 selective agonist) was found to exist as a conformational equlibrium between unordered and helical structures in TFE/$H_2O$ with high TFE content.[384] The helical region in the more ordered structures existed between Pro$^4$ and Gly$^9$. In each of these

studies the authors discussed the conformation(s) adopted by the peptides in the light of their (known) biological activities.

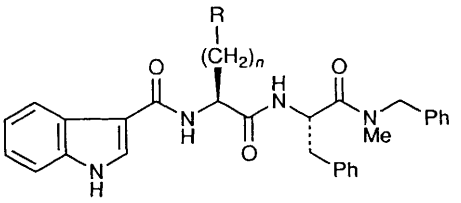
Structure-activity studies on scyliorhinin I modified in positions 3, 6, 7, and 8 concluded that Phe$^6$ was essential for biological activity in the GPI test and that replacing Tyr$^7$ with Phe or D-*p*FPhe gave compounds with significantly enhanced activity.[385] The most potent member of the series was [Abu$^8$]Scyl (26 times more active than Scyl) and the CD spectrum of this compound (in 75% aqueous TFE or 15mM SDS) indicated the presence of a significant degree of ordered structure.

The synthesis of fluoresceinyl-neurokinin-A as a probe for NK-2 receptors,[386] the preparation of dimeric neurokinin B analogues for studies with NK-1 receptor,[387] and an investigation into the degradation of substance P[388] have also been described. Peptides with tachykinin-like activity have been isolated from the brain of the dogfish *Scyliorhinus canicula*[389] and from the ventral nerve cords of the echiuroid worm *Urechis unicinctus*.[390] The structure of a macrocyclic heptapeptide lactone, isolated from *Streptomyces violaceoniger* and identified as a tachykinin antagonist from a screening program, has been determined.[391]

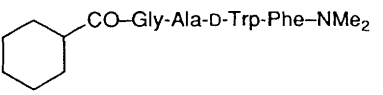
Three groups have reported progress on the development of low molecular weight peptide tachykinin antagonists. Molecular modelling techniques aided the design of the structural mimetics (133)[392] of the lead compound Ac-Thr-D-Trp(CHO)-Phe-NMeBzl. The development of this lead tripeptide was reported last year. In the branched tripeptides (133a, 133b) modelling studies indicated that the indole nucleus and the two benzene rings in the Phe-(NMe)Phe structure adopted the same spatial relationship as in the lead compound. The new compounds were found to potently inhibit $^3$H-substance P binding to guinea pig lung membranes in the $10^{-8}$M range (*cf* $IC_{50}$ of lead compound = $5.8 \times 10^{-9}$M). Furthermore, in the lysine series, dipeptides having either nothing or a simple acyl group at the ε-amino group also exhibited potent activity [activity of (133c) was 91% of (133b)]. A deletion-optimisation strategy based on the heptapeptide lead structure PhCO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-$NH_2$ led to potent and selective neurokinin NK-2 antagonists (134, $pK_B = 9.3$) and (135, $pK_B = 7.9$) of substantially reduced molecular size.[393] In the third approach a weak inhibitor (136, R = NHEt, R′ = H; $IC_{50} = 3.8\mu M$) of substance P binding to human NK-1 receptor was optimised to give [136, R = NHAc, R′ = 3,5-$(CF_3)_2$; $IC_{50} =$ 1.6nM].[394] The lead compound in this study was identified from screening the Merck sample collection.

## 6.7 Somatostatin Analogues

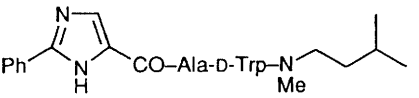
Several modifications have been made to the potent somatostatin hexapeptide cyclo[-Pro$^6$-Phe$^7$-D-Trp$^8$-Lys$^9$-Thr$^{10}$-Phe$^{11}$-] (137) with the aim of reducing the conformational flexibility of the molecule. The modifications included replacing Pro$^6$ with conformationally constrained mimetics (1- and 2-aminocyclopentane carboxylic acid),[395] replacing the Phe$^{11}$-Pro$^6$ segment with the retro-inverso gPhe$^{11}$-mAla$^6$ moiety[395] or the *cyclo*-cystine unit,[396] and introducing methylated residues into the sequence.[397] Conformational and biological data on the various analogues have provided further evidence to support the proposed model for the receptor-bound conformation of the somatostatin hexapeptide. For



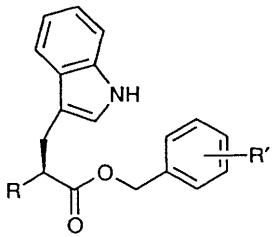
(133)a; R = $COThrNH_2$, $n$ = 1
b; R = NHThrAc, $n$ = 4
c; R = $NH_2.HCl$, $n$ = 4



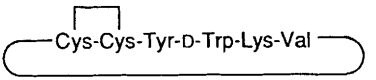

(134)



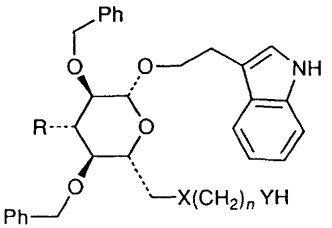

(135)



(136)




(138)



(139)a; R = $OCH_2Ph$; X,Y = O,NH; NH, O; $n$ = 4–6
b; R = H; X,Y = O,NH; NH, O; $n$ = 5,6

example, the importance for bioactivity of a *cis* amide bond at the bridging region was strengthened by results with compounds featuring conformational constraints in this segment. Of the constrained compounds only the *cyclo*-cystine analogue (138) and the retro-inverso analogue cyclo[-*S*-mAla-Phe-D-Trp-Lys-Thr-gPhe-] containing an *S*-mAla component showed both high potency in biological assays and were found to adopt a *cis* (11-6) amide bond in conformational studies. The view that *trans* conformations for the side chain groups of D-Trp$^{8}$ and Phe$^{11}$ in (137) are important for receptor binding was strengthened by the finding that the corresponding β-methylated derivatives, which are constrained to prefer the *trans* rotamers, retained binding potency.

Full details of the design, synthesis, and biological properties of the non-peptide peptidomimetics (139) of somatostatin have been reported.[398] These compounds bind to the somatostatin receptor in a dose-dependent manner with affinity in the μM range. The fact that the glucose scaffold of these molecules cannot participate in hydrogen bonding confirms the importance of the side chain functionality rather than the peptide backbone in somatostatin receptor binding and activation.

Glycated analogues of the Sandoz somatostatin drug octreotide have been prepared.[399]

## 6.8 Bradykinin Analogues

NMR conformational analysis of several bradykinin analogues has been carried out. The very active antagonists [D-Arg$^{0}$,Hyp$^{3}$,Thi$^{5}$,D-Cpg$^{7}$,Cpg$^{8}$]-BK and [D-Arg$^{0}$,Hyp$^{3}$,D-Cpg$^{7}$,Cpg$^{8}$]-BK were found to adopt turn-like structures between residues 2 and 5 in methanol/water (80/20).[400] However no turn-like structure was detected in the NMR spectra of the less potent antagonist [D-Arg$^{0}$,Hyp$^{3}$,Cpg$^{7,8}$]-BK. In a similar study, by the same group, on a bradykinin agonist-antagonist pair (the agonist D-Arg$^{0}$-Arg$^{1}$-Pro$^{2}$-Hyp$^{3}$-Gly$^{4}$-Thi$^{5}$-Ser$^{6}$-Pro$^{7}$-Thi$^{8}$-Arg$^{9}$ and the corresponding antagonist with a D-Phe in position 7), a type I β-turn was found between residues 6 and 9 in the agonist, and some possibly indication of a turn-like structure between residues 2 and 5 was detected in the antagonist.[401] No evidence of a β-turn in the C-terminal region of the antagonist was detected. Somewhat in contrast to these findings a third study investigating the structure of C-terminal fragments Ser$^{6}$-D-Xaa$^{7}$-Oic$^{8}$-Arg$^{9}$ [Xaa = Phe, Tic, Hyp(*trans*-propyl)] of three further bradykinin antagonists reported that each of the tetrapeptides adopted a β-turn as the primary conformation in water.[402] Thus there appears to be two schools of thought. One hypothesis is that a turn-like structure between residues 2 and 5 could be important for antagonist activity while the second view held is that bradykinin antagonists adopt a C-terminal β-turn conformation when bound to the receptor.

## 6.9 Miscellaneous Examples

This year coverage of peptides coming under the heading "miscellaneous examples" is dealt with in the form of a table (Table 7). Systematic substitutions, particularly with D-amino acids and multiple synthesis techniques have now been applied to a number of peptides. In endothelin, for example, a D-amino acid scan

**Table 7** Miscellaneous examples

| *1. Structure-activity studies* | *Reference* |
|---|---|
| *Corticotropin-releasing factor* | |
| – Single-point D-amino acid substitution of oCRF | 403 |
| *Endothelin* | |
| – Substitution of $Asp^{18}$ and $Ile^{19}$ of endothelin-I | 404 |
| – Single and multiple D-amino acid replacements in C-terminal hexapeptide ligand Ac-D-$Phe^{16}$-Orn-Asp-Ile-Ile-$Trp^{21}$-OH | 405 |
| – Systematic replacement of each residue of Ac-D-$Phe^{16}$-Orn-Asp-Ile-Ile-$Trp^{21}$-OH with 50 amino acid substitutes | 406 |
| – Studies centred around position 16 of Ac-D-$Phe^{16}$-Leu-Asp-Ile-Ile-$Trp^{21}$-OH | 407,408 |
| *Gastrin* | |
| – Lipophilic derivatives of tetragastrin | 409 |
| *Neuropeptide Y* | |
| – Single point D-amino acid substitutions | 410 |
| – Amino acid replacements in segment 27 to 32 of [1-4-εAhx-25-36]NPY | 411 |
| *Neurotensin* | |
| – N-and C-terminal deletions and Ala scan | 412 |
| – $N^{\alpha}$RCO-$Lys^{9}$-Pro-Tyr-Ile-$Leu^{13}$ (R = lipophilic group) | 413 |
| – Di-, tri-, and tetra-peptides fragments of C-terminal (9-13) sequence | 414 |
| *Thyrotropin-releasing hormone* | |
| – Heteroaromatic replacements for *N*-terminal pGlu | 415 |
| *Tuftsin* | |
| – [$Hyp^{3}$]-Tuftsin and O-glucosylated derivatives | 416 |
| *2. Conformational studies* | |
| *Bombesin* | |
| – CD studies with C-terminal (7-14) fragment | 417 |
| *Growth hormone-releasing factor* | |
| – CD and 2D NMR analysis of $Leu^{27}$hGRF(1-32)$NH_2$ and its deamidation products | 418 |
| *Melanostatin* | |
| – $^{13}C$,$^{15}N$-REDOR NMR analysis of the isotopically enriched tripeptide | 419 |
| *Muramyl dipeptide* | |
| – NMR and molecular dynamics study of carbocyclic analogues | 420,421 |
| *Neuropeptide Y* | |
| – CD studies of $N^{\alpha}$-acyl-$NPY_{15\text{-}36}$ | 422 |
| *Parathyroid hormone* | |
| – Simulated annealing analysis of fragment (1-34) | 423 |
| *3. Miscellaneous Studies* | |
| *Synthesis of* | |
| – Biotinylated derivatives of [β-$Ala^{1}$,$Lys^{17}$]-$ACTH_{1\text{-}17}$-NH-$(CH_2)_4$-$NH_2$ for receptor studies | 424 |
| – Tritiated derivative of a growth hormone-releasing peptide | 425 |
| – Lipophilic and fluorescent derivatives of muramyl dipeptide | 426 |

of the C-terminal hexapeptides revealed that D-stereochemistry at position 16 afforded a large increase in binding affinity and antagonist activity at both $ET_A$ and $ET_B$ receptors. SAR studies centered around the D-Phe$^{16}$ position have led to the potent functional $ET_A/ET_B$ antagonists Ac-D-Dip$^{16}$-Xaa-Asp-Ile-Ile-Trp$^{21}$ (Xaa = Leu, Glu).

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# 4
# Cyclic, Modified and Conjugated Peptides

BY JOHN S. DAVIES

## 1 Introduction

This year's 'mission statement' on the coverage in this Chapter, is really a repeat of the last three years of reporting under this heading. The Chapter has become an annual sanctuary for about 200 papers, mostly on naturally occurring cyclic and modified peptides, not taken care of in Chapter 3. The latter however is still the place to access cyclic analogues of biologically active domains and peptidomimetic analogues. However an expanding theme in this Chapter is the coverage of phosphorylated, glycosylated and lipophilic peptides.

The availability of CA selects[1] on Amino Acids, Peptides and Proteins (up to Issue 12, 1994 have been covered) was again the core source of the 1993 references, but many of the mainstream Journals were also scanned manually. The patent literature was not covered, neither have publications emanating from conferences, the latter being left to await their appearance in refereed Journals. About three quarters of the papers represented work carried out in academia.

Many of the naturally-occurring peptides highlighted in this Chapter often become templates in peptidomimetic design. The complex stages involved and current perspectives on progress towards commercial pharmacophores have been reviewed[2-4], and have also been the subject of a Tetrahedron Symposium in Print[5]. Within the latter the hierarchical approach to peptidomimetic design has been highlighted[6]. The marine environment is proving to be a rich source of bioactive peptides, as reflected in a review of bioactive sponge peptides[7], and in a review[8] on marine metabolites in general it is stated that cyclic hepta- and octapeptides constitute the largest group of metabolites from *Lissoclinum* tunicates.

## 2 Cyclic Peptides

### 2.1 General Considerations

The key step to an efficient synthesis of a cyclic peptide is the final step of ring closure. The newly developed HOAt (1) has recently been introduced as an improved substitute for HOBt and the prowess[9] of two of its derivatives (2) and (3) in the cyclisation of the linear GnRH-derived decapeptide, H-Nal-D-Cpa-D-Pal-Gln-Tyr-D-Arg-Leu-Arg-Pro-Lys(Ac)-OH has been monitored. Both reagents gave 100% cyclisation in 30 min, when compared to TBTU, TOPPipU and DPPA which gave yields of 60, 10 and 12% respectively. Development and optimisation of the on-resin method of cyclisation continues. Head to tail

cyclisation, after initial anchoring of Glu or Asp side chains to *p*-alkoxybenzyl or tris(alkoxy)benzylamide supports, using allyl group protection for the α-carboxyl group has been examined[10] for a number of options for the synthesis of cyclo-(-Ala$^1$-Ala$^2$-Arg$^3$-D-Phe$^4$-Pro$^5$-Glu$^6$-Asp$^7$-Asn$^8$-Tyr$^9$-Glu$^{10}$). Starting the synthesis either with Glu$^{10}$, Asp$^7$, Asn$^8$ or Glu$^6$ attached to the resin, all strategies gave the desired cyclic product, but the Asn$^8$ strategy gave the highest yield (71%) and the Glu$^{10}$ option gave the greatest number of by-products. BOP and PyBOP were used in the cyclisation step. Problems associated with the removal of the allyl group using an insoluble palladium catalyst have been overcome in the development of protocol for continuous flow systems[11]. The allyl ester also showed improvement over a phenacyl ester in the four-dimensional orthogonal protection scheme[12] used in the head to tail cyclisation leading to the calcitonin analogue (4) using the oxime resin method for cyclisation. The oxime resin has also been the anchor for an on-resin assembly of a disulfide bond as demonstrated[18] for the salmon calcitonin (1-10) fragment in Scheme 1.

Most of the structures derived for the molecules reviewed annually in this Chapter rely heavily on 'state of the art', high field NMR, X-ray and mass spectrometric technology. Allied to these in conformational work are molecular dynamics simulations. A mini review[14] has appeared on the methodologies which has assisted the simulation work, and molecular dynamics simulations have been carried out[15] to determine the flexibility of a series of analogues derived from the epitope loop in the snake toxin, erabutoxin.

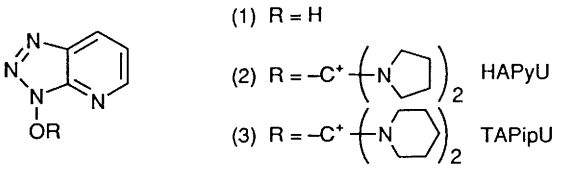
### 2.2 Naturally Occurring Dioxopiperazines (Cyclic Dipeptides)

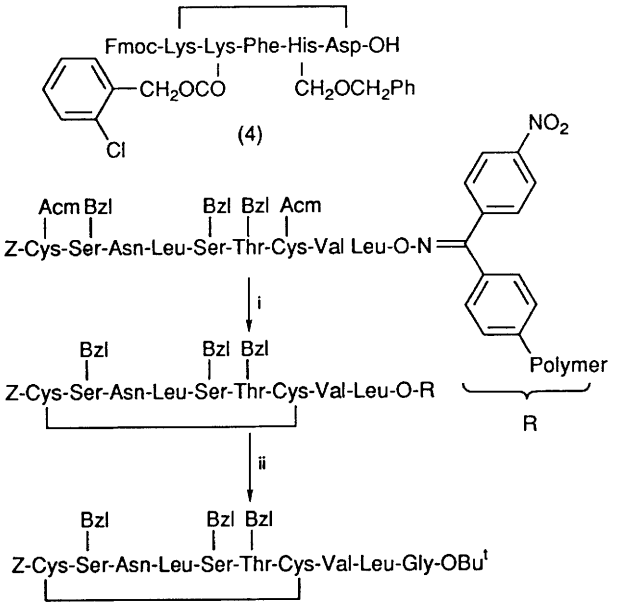
The only report seen under this category was the characterisation[16] of three novel polychlorinated cyclodipeptides designated dysamide A, B and C and a known compound 2,3-dihydrodysamide C from the sponge Dysidea. Lack of access to the Chinese journal concerned precluded a report of their structures.

### 2.3 Other Dioxopiperazines

Interest in cyclo[-(*S*)-His-(*S*)-Phe-] as an asymmetric catalyst has been reviewed[17] in the general context of asymmetric cyanohydrin synthesis, while the more detailed mechanistic studies[18,19] continue within the same group. Variable pH NMR studies[18] and molecular mechanics calculations indicate that the interaction between the dioxopiperazine and HCN is covalent rather than ionic. This, together with evidence[19] from the catalytic oxidation of benzaldehyde to benzoic acid, suggests the catalytic action is best explained by intermediate (5). An improved synthesis[20] of cyclo[-(*S*)-His-(*S*)-Phe-] has been reported using the protected His precursor, Z-Phe-His(Bzl)-OMe. Catalytic transfer hydrogenation removed the Z group to give spontaneous cyclisation to the dioxopiperazine which was followed by hydrogenolysis of the imidazolyl-benzyl protection.

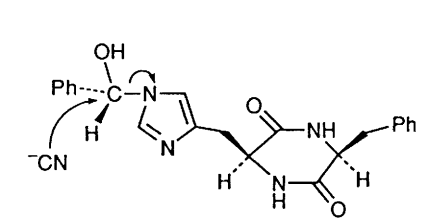
Quantitative yields of dioxopiperazine (6) have been obtained[21] by reacting the corresponding N-substituted alanine precursor with di-t-butyl dicarbonate in the presence of triethylamine and dimethylaminopyridine (DMAP). Variation in the substituent R on the D-Trp residue in cyclic dipeptide analogues (7) has been shown[22] to modulate water solubility and transport properties of these tachykinin



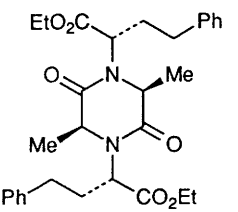




Reagents: i, $I_2$/DMF; ii, H-Gly-OBu$^t$

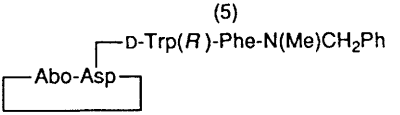
**Scheme 1**



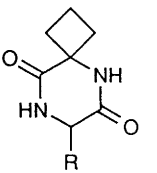

(5)




(6)




(7) Abo = 2-azabicyclo[2,2,2] octane-3(*S*)-carboxylic acid




(8)

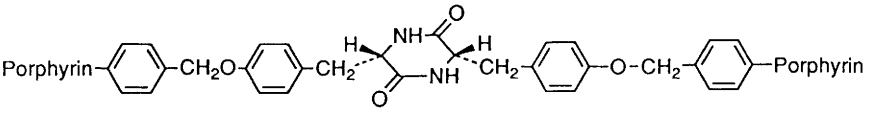
antagonists, with R = $COCH_2CH_2COOH$ showing prolonged reaction time in the mouse hot plate test. Previous reports on dioxopiperazines containing a spiro atom shared with a cyclopentane ring have now been supplemented[23] by the synthesis of a series (8) based on the cyclobutane ring. However their inhibition qualities in the chick embryotoxicity screening test was generally lower than their cyclopentane analogues. *Cis* and *trans* forms of the dioxopiperazine based on the condensation of the fluorescent chromophore, β-(1′-naphthyl)-L-Ala and the heavy atom perturber *p*-bromo-L-Phe have been synthesised[24] to study spin-forbidden excitation transfer and heavy-atom induced intersystem crossing. Subtle factors arising from the intervening cyclodipeptide have been introduced to explain some of the effects. The dioxopiperazine ring has also been used[25] as a bridging unit between porphyrin units as shown in (9). Both the D,L- and L,L-forms of the dioxopiperazine were synthesised, and the $^1H$ NMR, UV and CD spectra showed that the porphyrins in the L,L-molecule interacted more strongly than those in the D,L-isomer. The tendency for a resin-bound peptide linker derivative to cyclise to a dioxopiperazine, has been developed[26] for release of peptide molecules from polymer beads, while the dioxopiperazine (10) has been identified[27] as an unwanted side reaction during the synthesis of thyroliberin (Glp-His-Pro-$NH_2$). Formation of the dioxopiperazine (11) has also been shown[28] to be the cause of spontaneous degradation of δ-opioid antagonists, H-Tyr-Tic-Phe-OH and H-Tyr-Tic-Phe-Phe-$NH_2$ in DMSO solution (Tic = tetrahydroisoquinoline-3-carboxylic acid).

Reversed phase HPLC capacity factors, roughly correlated with hydrophobic parameters such as partition coefficients in octanol-water, have been measured[29] for a series of diastereoisomeric dioxopiperazines. For a pair of diastereoisomers of cyclo(-L-X-L-Phe-) and cyclo(-L-X-D-Phe-), $k'_{LL}$ was found to be larger than $k'_{DL}$, while in the case of cyclo(-D-Ala-L-Trp-) and cyclo (-L-Ala-L-Trp), $k'_{LL}$ is smaller than $k_{DL}$ especially in highly aqueous solutions.

## 2.4 Cyclotripeptides and Cyclotetrapeptides

There appear to be no reports on work on cyclotripeptides reported in 1993.

However there was activity on cyclotetrapeptides, which included the discovery[30] of cyclo(-Pro-Tyr-Pro-Val-) a strong inhibitor of mushroom tyrosinase ($I_{C50}$ = 1.5 mM), in the bacterium *Lactobacillus helveticus*. Cyclo(-Pro-Ala-D-Phe-Leu) has been modelled (Quanta-Charmm) and synthesised[31] in order to investigate complexing properties with Boc-amino acids. Total correlation spectroscopy and nOe spectra showed that the molecule existed as one dominant conformer 1, and three minor ones 2a, 2b and 2c in the ratio of 92:6:1:1, but this ratio could be changed to 48:40:6:6 on complexing with Boc-hydroxyamino acids. This suggested that the minor conformers bound more strongly with the amino acid derivatives and a possible complex between 2a and Boc-D-Ser is depicted in (12). Synthesis was achieved by producing Boc-Pro-Ala-D-Phe-Leu-$NHNH_2$ *via* a Kaiser oxime gel, and then treating it with $NaNO_2$/HCl for 2 days under high dilution conditions. In a continuation of the success achieved with a specific

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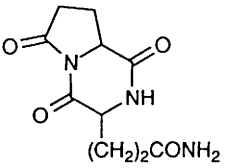


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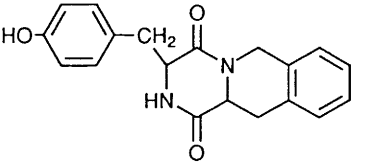


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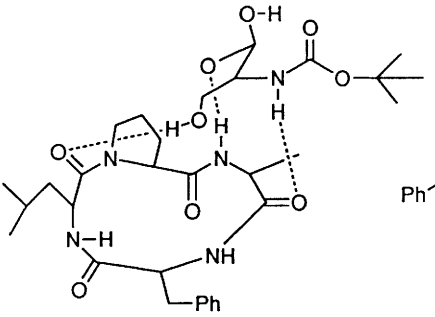
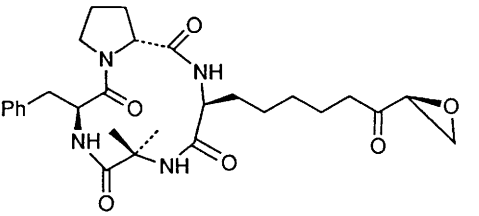
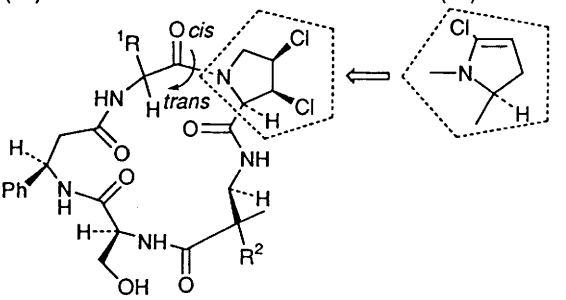


(12)



(13)




(14) Asterin, $R^1$ = Et, $R^2$ = H (*c.f.* cyclochlorotine $R^1$ = $CH_2OH$)
(15) Astin A, $R^1$ = $CH_2OH$, $R^2$ = H, acyl-Pro bond *cis*
(16) Astin B, $R^1$ = Et, $R^2$ = OH, acyl-Pro bond *cis*
(17) Astin C, $R^1$ = Et, $R^2$ = H
(18) Astin D, $R^1$ = Et, $R^2$ = H acyl-Pro bond *cis* } Dichloroproline replaced by
(19) Astin E, $R^1$ = Et, $R^2$ = OH acyl-Pro bond *cis* } $\Delta^4$ Pro(Cl)

C-alkylation reaction in cyclosporin A, the Seebach group have investigated[32] the use of lithium enolates to generate insertion positions in sarcosine residues in the cyclic tetrapeptides cyclo(-Leu-Sar-Sar-Gly-), cyclo(-Val-Sar-Sar-Gly) and cyclo (-MeLeu-Gly-D-Ala-Sar-). Multiple deprotonation with LDA in THF/LiBr/DMPU and addition of highly reactive electrophiles ($CF_3CO_2D$, MeI, $CH_2O$, $CH_2=CHCH_2Br$, $PhCH_2Br$) produced cyclic tetrapeptides with diastereoselectively introduced substituents. The sarcosine enolates were only produced adjacent to another N-methyl amino acid. Pentafluorophenyl esters of linear precursors were used to synthesise the original cyclic sarcosine peptides.

Solution phase synthesis[33], using cyclisation with DCC under high dilution has provided crystals of cyclo(-β-Ala-L-Pro-β-Ala-L-Val) for an X-ray determination. All peptide bonds were found to be *trans*, but the conformation derived from NMR data and from molecular dynamics simulations is quite different to the solid state, reminiscent of cyclo(-β-Ala-L-Pro-β-Ala-L-Pro-), in that it contains two $\gamma^i$ turns around Pro and Val. Problems with the lability of the epoxy ketone moiety in the synthesis of chlamydocin (13) have been overcome in a new stereospecific synthesis[34] *via* free radical homologation from a 2-amino-5-iodopentanoic acid containing cyclic tetrapeptide intermediate using a chirally pure form of the epoxyenone $CH_2=CHCO—CH=O=CH—SiMe_3$. Specific fragmentation patterns and a reduction phenomenon have been discussed[35] in the FAB mass spectra of chlamydocin analogues.

Molecular modelling using a GENMOL programme[36] has been used to predict the best cyclisation precursor for small peptides. Cyclo-(-$Phe_4$-) was used as a model with Boc groups as substituents on the main-chain nitrogen atoms. Cyclisation 'yield' increased if there is a preferred conformation having terminal functions close together as induced by the Boc substituents. Molecular mechanics calculations (CHARMM programme), and other physical methods applied to the diastereoisomers cyclo(L-Pro-)$_4$, cyclo(L-Pro-D-Pro)$_2$ and cyclo[-(L-Pro)$_2$-(D-Pro)$_2$-], revealed[37] that most of the *cis*/*trans* transitions are hindered by energy barriers $>30$ kcal/mol. In cyclo(L-Pro-D-Pro)$_2$ four *cis*/*trans* isomerisations could be identified as *ctct*→*cttt*→*tttt*→*tctt*→*tctc*.

## 2.5 Cyclopentapeptides

The plant *Aster tataricus* (Compositae) has proved to be a rich source of cyclopentapeptides. Asterin (14), an analogue of cyclochlorotine has been identified[38] and shown to have hepatotoxic activities. Another research group have also found antitumour activity in the astins A, B and C which they have identified[39] as the compounds (15)-(17). Detailed NMR analysis confirmed that the acyl-substituted prolyl bond had the *cis* conformation. In the structures deduced[40] for astins D (18) and E (19) the dichloroproline ring has been replaced by a $\Delta^4$-chloroproline residue, with the other parts of the cyclic backbone remaining the same.

The naturally-occurring cyclopentapeptide endothelin A receptor antagonists have been subjected to further structure-activity investigations. Cyclo(-D-Trp-D-Cys(SOO(-)$_3$)$Na^+$)-Pro-D-Val-Leu) has been shown[41] to be a potent antagonist. Its synthesis was carried out according to Scheme 2 (page 244), and

conformational analysis confirmed a type II β-turn involving the residues D-Val-Leu-D-Trp-D-Cys($SO_3^-Na^+$) and a γ-turn centred around D-Val-Pro-D-Cys($SO_3^-Na^+$). Similar types of conformational profile with β- and γ-turns have been identified[42] in the solution conformation of the selective endothelin antagonists BE18257B, cyclo(D-Glu-Ala-D-aIle-L-Leu-D-Trp-), and BQ123, cyclo(-D-Asp-Pro-D-Val-Leu-D-Trp). The proposition has been made that these antagonists derive their activity from their ability to mimic the C-terminus of the endothelins. In the synthesis[43] of the endothelin antagonist cyclo(-D-Trp(CHO)-D-Cys($SO_3^-Na^+$)-Pro-Thg-Leu-) where Thg is D-2-thienylglycine, the solubility of Fmoc-cysteic acid in DMF has been enhanced by using its tetrabutylammonium salt. In the series of cyclic analogues (20)-(25) prepared, four were synthesised *via* the azide method for cyclisation while the other two were obtained by EDC or DCC/HOBt couplings. All the analogues inhibited [$^{125}$I]-ET-1 binding to receptor $ET_A$ *ca.* 1000-fold more potently than to $ET_B$.

The role of proline in defining β-turn conformations has been studied[44] using 20 NMR and restrained molecular dynamics simulations on cyclopenta- and cyclohexapeptides containing L- and D-prolyl residues. The *cis* conformations of cyclo(-Pro-Ala-Ala-Pro-Ala-Ala-), cyclo(-Arg-Gly-Asp-Phe-Pro-Gly-), cyclo(-Arg-Gly-Asp-Phe-Pro-Ala-), cyclo(-Pro-Ala-Ala-Ala-Ala-) and cyclo (-Pro-Ala-Pro-Ala-Ala) form uncommon bVI turns similar to those found in proteins. This work bears particular relevance to the challenge of incorporating the -Arg-Gly-Asp-bioactive domain into a constraint that would mimic its conformation in the natural glycoprotein. The potent fibrinogen receptor antagonist[45] Ac-Pen-Arg-Gly-Asp-Cys-OH represents the many current attempts at constraining the RGD domain. Even with this constraint, NMR studies and molecular modelling indicate the molecule exchanges between a number of conformers which comprise type II′ or type V β-turns. The Arg-Gly-Asp-portion is flexible due to amide-plane rotations of the Arg-Gly and Gly-Asp peptide bonds. Substitution of Pen for another analogue containing Cys at that position has given the molecule better conformational rigidity.

Biosynthetic studies[46] based on $^{13}C$ and $^{2}H$-labelled, serine, alanine and 2,3-diaminopropionic acid, fed to *Streptomyces capreolus A250*, have shown that the 2,3-diaminopropionate (Dap) moiety of the antibiotic capreomycin is derived from serine *via* the dehydroalanyl pathway. Since the 2,3-diaminopropionic acid can also be incorporated it is presumed that the pathway involves a prepeptide assembly modification of serine.

## 2.6 Cyclohexapeptides

Since cyclisation is often the limiting step in the synthesis of cyclic peptides, comparison of the various coupling reagents contributes useful information. The merits of diphenylphosphoryl azide (DPPA), TBTU/HOBt and HBTU/HOBt/DIEA in the key cyclisation step for a series of cyclic peptides can be seen from the results summarised in Table 1[47]. Cyclisation by TBTU proved the best and took place so rapidly that no kinetic studies could be performed. Using the HOAt derivative (3) it has been possible to cyclise[9] the all L-form of H-Val-Arg-Lys(Ac)-Ala-Val-Tyr-OH in 55% yield.

**Table 1** Cyclicisation of cyclohexapeptides

| *Cyclic Product* | *Method* | *Time* | *% yield* |
|---|---|---|---|
| cyclo[-Tyr(Bzl)-Asp(OBzl)-Phe-Phe-Ser($Bu^t$)-D-Ala-] | TBTU/HOBt | 2 hr | 100 |
| | $DPPA/NaHCO_3$ | 17.50 hr | 100 |
| cyclo[-Asp(OBu$^t$)-Phe-Phe-Ser($Bu^t$)-D-Ala-] | TBTU/HOBt | 20 mins | 100 |
| | $DPPA/NaHCO_3$ | 1 day | 50 |
| cyclo[-Tyr-Asp(OBu$^t$)-Phe-Phe-Ser($Bu^t$)-D-Ala-] | TBTU/HOBt | 20 min | 100 |
| | $DPPA/NaHCO_3$ | 1 day | 100 |
| cyclo[-Tyr(Bzl)-Asp(OBzl)-Phe-Phe-Thr($Bu^t$)-D-Ala] | TBTU/HOBt | 4 hr | 74 |
| | HBTU | 4 hr | 60 |
| cyclo[-Tyr-Asp(OBu$^t$)-Phe-Phe-Thr($Bu^t$)-D-Ala] | DPPA | 25 hr | 100 |

All amino acid components of the antifungal echinocandins (26) have been stereospecifically synthesised[48] and incorporated into a linear precursor *via* a novel coupling method (Scheme 3). The final link at 'a' in the cycle was achieved in 39% yield using diphenylphosphorylazide. In contrast to other cyclic peptide analogues, up to this time cyclohexapeptides containing the Arg-Gly-Asp domain have not been seen to interact strongly with fibrinogen receptors. However the situation has changed with the design and synthesis[49] of cyclo(-Phe-Asp-Gly-Arg-Gly-Phe-) (27), cyclo(-D-Ser-Asp-Gly-Arg-Gly-Phe-) (28), and cyclo(-D-Ser-Asp-Gly-Arg-D-Phe-) (29). In the ELISA GPIIb/IIIa-fibrinogen receptor assay, (29) with its two D-amino acids bound to the receptor with an $IC_{50}$ value of 0.016 lM, with all three completely displacing fibrinogen. DPPA was used in the final cyclisation step in each case, but in (29) there seemed to be a post cyclisation tendency for aspartimide formation catalysed by piperidine, when the side-chain 9-fluorenyl methyl ester was removed. DPPA was also the reagent of choice[50] in the cyclisation of a series of peptide T analogues. In all the examples chosen the cyclic forms showed higher biological activity than their linear counterparts. Cyclo(-Thr-Hse-Asn-Tyr-Thr-Asp-) showed the highest affinity to the CD4 receptor of monocytes, and was highly resistant to degradation by plasma or brain enzymes.

The potent cyclic peptide analogue cyclo($Pro^6$-$Phe^7$-D-$Trp^8$-$Lys^9$-$Thr^{10}$-$Phe^{11}$) of somatostatin, has been subjected to further attempts to reduce its conformational flexibility. Through the synthesis[51] of an α-$MeVal^{10}$ analogue, constraint was built into the main chain, but the resultant flat conformation led to loss of binding affinity. Analogues with β-methyl substituents in the side-chain, *e.g.* β-MeTrp at position 8 and another analogue with β-MePhe at position 11, did not lose their high binding potency. The structural role of the $Phe^{11}$-$Pro^6$ bridging region has also been investigated[52] through the synthesis of the retro-inverso $mAla^6$-$gPhe^{11}$ analogue and five analogues with 2-aminocyclopentane carboxylic acids (2-$Ac_5c$) and 1-aminocyclopentane carboxylic acid (1-$Ac_5c$) as proline mimetics. In these analogues the βII'-turn characteristics are maintained but the 'bridging region's' conformation was changed. Only a *trans*

amide was seen for Phe[11]-Pro[6] in the (R)-mAla retro-inverso analogue, and the five $Ac_5c$ analogues, while it showed *cis-trans* isomerisation in the (*S*)-mAla form. Only the latter showed high binding affinity. Confirmation of the significance of the Phe[11]-Pro[6] bond to activity has also been obtained[53] from the low receptor affinity of cyclo(-Phe$\psi$[$CH_2N$]Pro-Phe-D-Trp-Lys-Thr-). For the latter, NMR studies confirmed the presence of a β-turn, and a δ-turn over the reduced peptide moiety. Constraining the Phe[11]-Pro[6] to *cis* geometry has also been attempted[54] by replacement of this dipeptide moiety with a cystine unit. Both (-Cys-Phe-D-Trp-Lys-Thr-Cys), and cyclo(-Cys-Tyr-D-Trp-Lys-Val-Cys-) retained the activity of the parent somatostatin analogue, thus supporting the evidence for the need for a *cis* Phe[11]-Pro[6] bond. Solution phase cyclisation using the azide method on a linear hydrazide precursors released from oxime solid phase gel was the method chosen[55] to synthesise cyclo[-Pro-Gly-Glu(OBzl)-Pro-Phe-Leu-]. NMR techniques used on a $CDCl_3$ solution of the cyclic peptide revealed the presence of three conformers, two dominant (Ia and Ib) that exchanged chemically with each other and a third Ic (4%) that exchanged exclusively with Ib. The cyclic peptide was designed to show chiral discrimination in its interaction with amino acid derivatives. Only Ia interacted strongly with Boc- and Fmoc-amino acids in $CDCl_3$, the interaction being localised at the Phe-Leu domain of the cyclic peptide *via* their NH groups. Differences in interaction between the D- and L-enantiomers of Boc-Trp and Fmoc-Trp were also observed and explained in terms of a 3-point docking relationship discriminating between the enantiomers. The minor conformational isomer present has been shown[56] to possess an unusual *cis* Phe peptide bond while both the Pro bonds were *trans*.

The potent antitumour cyclohexapeptide RA-VII (30) now undergoing clinical trials, and its congener deoxybouvardin (31) have been synthesised[57] using the key Ullmann reaction in Scheme 4 as the starting point. For (30) the diphenyl ether unit was then coupled to Boc-D-Ala-Ala-MeTyr(Me)-Ala-OH and the final macrocyclisation carried out with DPPA. The desmethyl analogue (32) was synthesised to study the conformational features at the ring junctions, and it is concluded that the tetrapeptide housed within the 18-membered ring induces the 14-membered cycloisodityrosine to adopt a disfavoured *cis* secondary or tertiary amide. In contrast to previous views the present work suggests a reversal in the previously accepted functional roles of the rings, and it is now believed that it is the tetrapeptide ring that potentiates the activity and alters the conformation of the cycloisodityrosine. When substituents were varied[58] in the Tyr[3] and Tyr[6] rings, the changes were only effective within the Tyr[3] ring. Thus (33) with no substituent at the $R^4$ position was nearly as active as RA-VII (30). Effect of substituents at residue 2 have been explored[59] *via* the formation of (34) by thioacetylation of a serine precursor. The thiol analogue showed antitumour properties, but was less toxic. Amide nitrogen in position 2 has been alkylated[60] under phase-transfer conditions to form derivatives such as (35) with improved solubilities. RA-XII (36) isolated from *Rubia cordifolia* appears[61] to undergo a fast exchange process in the presence of rat 80S ribosomes. Line broadening in the NMR spectra during binding correlate with conformational change (*cis*/*trans* at the N-methylated amide bond between Ala[2]-Tyr[3]).

Boc-D-Trp(CHO)-D-Cys(Acm)-Pro-D-Val-Leu—PAM—Resin

↓ i, ii

Cyclo(—D-Trp(CHO)-D-Cys(Acm)-Pro-D-Val-Leu—)

↓ iii

Cyclo(—D-Trp(CHO)-D-Cys-Pro-D-Val-Leu—) (Cys 1–2)

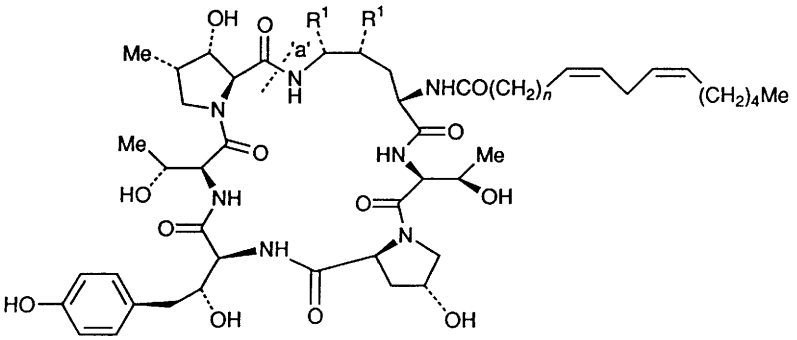
↓ iv

Cyclo(—D-Trp-Cys-($SO_3^-Na^+$)-Pro-D-Val-Leu —)

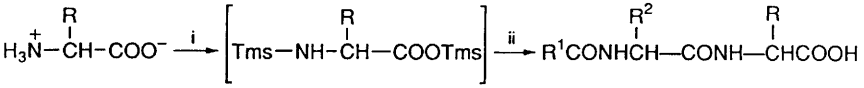
Reagents: i, HF/anisole; ii, EDC/HOBt; iii, $I_2$/80% HOAc; iv, $HCO_3H$

**Scheme 2**

(20) Cyclo(—D-Trp-D-Cys($SO_3^-Na^+$)-Cys($SO_3^-Na^+$)-D-Val-Leu—)
(21) Cyclo(—D-Trp-D-Cys($SO_3^-Na^+$)-Pro-D-aIle-Leu—)
(22) Cyclo(—D-Trp-D-Cys($SO_3^-Na^+$)-Glu-D-Val-Leu—)
(23) Cyclo(—D-Trp-D-Cys($SO_3H$)-Lys-D-Val-Leu—)
(24) Cyclo(—D-Trp-D-Cys($SO_3^-Na^+$)-Pro-D-Val-Nle—)
(25) Cyclo(—D-Trp-D-Cys($SO_3^-Na^+$)-Pro-D-Thg-Leu—)

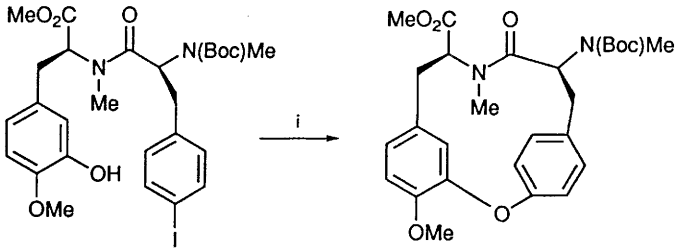



(26) echinocandin C($R^1$ = OH) and D($R^1$ = H)



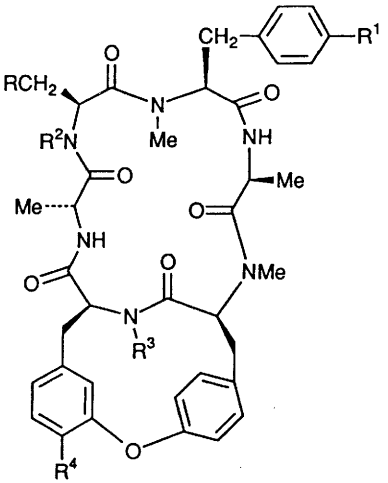

Reagents: i, 1-Tms-imidazole; ii, $R^1CONHCH(R^2)COSPyridyl$

**Scheme 3**




Reagent: i, MeCu in collidine at 130°C

**Scheme 4**




(30) RA VII, R = H, $R^1$ = OMe, $R^2$ = H, $R^3$ = Me, $R^4$ = OMe
(31) deoxybouvardin, R = H, $R^1$ = OMe, $R^2$ = H, $R^3$ = Me, $R^4$ = OH
(32) $N^{29}$-desmethyl RA VII, R = H, $R^1$ = OMe, $R^2$ = H, $R^3$ = H, $R^4$ = OMe
(33) R = H, $R^1$ = OMe, $R^2$ = H, $R^3$ = Me, $R^4$ = H
(34) [$Cys^2$]-RA VII, R = SH, $R^1$ =OMe, $R^2$ = H, $R^3$ = Me, $R^4$ = OMe
(35) R = H, $R^1$ = OMe, $R^2$ = *e.g.* $CH_2CH_2NMe_2$, $R^3$ = Me, $R^4$ = OMe
(36) R = H, $R^1$ = OH, $R^2$ = H, $R^3$ = Me, $R^4$ = *O*-glucoside

Contained in a mini-review[62] of conformational mobility in cyclic oligopeptides is a specific nOe constrained distance geometry conformational search of the isomeric cyclo(-Pro-Arg-Gly-Asp-Gly-D-Pro-) and cyclo(-Pro-Arg-Gly-Asp-D-Pro-Gly-). A narrowly defined backbone conformation was proposed for one but the other's conformation remained ambiguous, possibly due to a physical difference in internal mobility. $^{1}H$ and $^{13}C$ NMR studies[63] on cyclo(-Pro-Pro-Gly-)$_2$ in $CDCl_3$ and $d_6$DMSO has revealed that in the former solvent there is conformational homogeneity with a single *cis* Pro-Pro bond. The Gly-Pro-Pro-Gly segment in the β-bend has a *trans* Pro-Pro bond. In $d_6$DMSO there is conformational heterogeneity with two main conformers in the ratio 80:20, the major one having two *cis* peptide bonds, at Gly-Pro and Pro-Pro, while the minor component has a single Pro-Pro *cis* bond included in the β-bend. Neurokinin-2 (NK-2) antagonisitic cyclic peptides cyclo(Gln-Trp-Phe-Gly-Leu-X) where X is varied between Met, and D-Met and the bridged analogue (37) have been studied[64] using NMR techniques. The two Met analogues were known to be active in the hamster trachea assay, but (37) was inactive as agonist and antagonist. From the NMR results and restrained molecular dynamics simulations, the active peptides showed a $\gamma^{i}$-turn at Phe and βI′ or βII turns with Gly and Leu at the corners. The competing stereochemical effects of a disulfide ring as in (38) against the Aib residue only in a linear analogue Boc-Cys(Bzl)-Val-Aib-Ala-Leu-Cys(Bzl)-NHMe have been analysed[65]. A single Aib residue without the ring appears to stabilise a helix in polar solvent, while the disulfide bridge locks the peptide in a β-hairpin conformation. MeAib and NMeαAc$_5$c highly hindered residues in the Xaa position of cyclo(-Xaa-Phe-D-Trp-Lys(Boc)-Thr(Bu$^{t}$)-Phe-) seem to cause[66] a special strain on the Xaa-Phe bond, sufficient for the homodetic peptide to cleave at this bond when treated with TFA to remove the protecting groups. Line broadening is a feature also seen in the NMR spectra and believed to be due to this diminished amide character of the Xaa-Phe link. If the cyclohexapeptide backbone is still too troublesome a skeleton, why not consider moving to the cyclopentanoperhydrophenanthrene (steroid) skeleton which has been modelled as a peptidomimetic[67] cyclohexapeptide ring and used successfully for attachment of RGD side-chains!

## 2.7 Cycloheptapeptides and Cyclooctapeptides

The cycloheptapeptide seems to be a favourite backbone of the marine sponges. Table 2 summarises the diversity of structures isolated and characterised. Hymenamides A and B show[68] the presence of a type IIβ-turn in their conformation and are similar to evolidine. In the analogues hymenamides C-E one proline participates in a *cis* Xaa-Pro bond[69] and in the two novel nairaiamides A and B[73] the Ile-Pro amides are *cis*. It is also reported[73] that the nairaiamides could well be the same as the axinastatins.

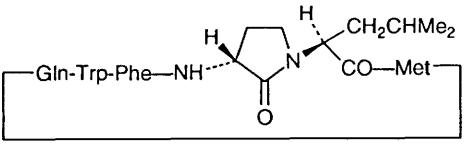
X-ray structural studies[74] on two crystalline forms of the antibiotic ternatin cyclo(-βOH-D-Leu-D-Ile-MeAla-MeLeu-Leu-MeAla-D-MeAla) (39) have relieved the frustration of a decade of study of its conformational structure. The current work was assisted by a new direct method phasing algorithm, called minimum principle, as well as the availability of two crystalline forms, one with

**Table 2** Cycloheptapeptides isolated and characterised

| *Cycloheptapeptide* | *Name* | *Source* | *Ref.* |
|---|---|---|---|
| cyclo(-Pro-Pro-Val-Pro-Phe-Trp-Arg-) | hymenamide A | Hymeniacidon Sponge | 68 |
| cyclo(-Pro-Pro-Asn-Phe-Val-Glu-Phe-) | " B | " | 68 |
| cyclo(-Trp-Pro-Phe-Gly-Pro-Glu-Leu) | " C | " | 69 |
| cyclo(-Ile-Pro-Tyr-Asp-Pro-Leu-Ala-) | " D | " | 69 |
| cyclo(-Phe-Pro-Thr-Thr-Pro-Tyr-Phe-) | " E | " | 69 |
| cyclo(-Pro-Ile-Pro-Ile-Phe-Pro-Tyr-) | phakellistatin 1 | *Phakellia costata* | 70 |
| cyclo(-Tyr-Pro-Phe-Pro-Ile-Ile-Pro-) | phakellistatin 2 | *Phakellia carteri* | 71 |
| cyclo(-Trp-Val-Pro-Leu-Thr-Pro-Leu-) | axinastatin 4 | *Axinella cf.carteri* | 72 |
| cyclo-(Ile-Ile-Pro-Val-Thr(-$CMe_2CH=CH_2$)-Ile-Pro-) | nairaiamide A | *Lissoclinum bistratum* | 73 |
| cyclo-(-Ile-Ile-Pro-Ile-Thr(-$CMe_2CH=CH_2$)-Ile-Pro-) | nairaiamide B | *Lissoclinum bistratum* | 73 |

minimum principle, as well as the availability of two crystalline forms, one with 2 mols of peptide to one of dioxane, the other 2 mols of peptide to one of water. The conformation of (39) is characterised by (i) a *cis* peptide bond between MeAla[3] and MeLeu[4], (ii) a type II β-bend involving residues Leu[5]-MeAla[6]-D-MeAla[7]-βOH-D-Leu[1] and (iii) a third intramolecular H-bond, between the hydroxyl group of βOH-D-Leu and the D-Ile[2] CO. In the on-resin synthesis[75] of cyclic peptide amide (42), summarised in Scheme 5, the orthogonal protecting group used on the α-COOH group of the aspartyl residue was trimethylsilylethyl(TMSE) (as in 40) which was deblocked by the fluoride anion of $Bu_4NF$. A cycloheptapeptide bearing a disulfide bridge has been synthesised[76]. The peptide, Boc-Cys-Val-Pro-Pro-Phe-Phe-Cys-Ome was able to complex $Ca^{2+}$, and the structure of the complex in acetonitrile showed two transannular H-bonds giving two ring structures of the $C_{10}$ and $C_{14}$ type. In one peptide unit the Pro-Pro bond is *cis* while all the others are *trans*.

Calcium complexes of cyclo(-Ala-Leu-Pro-Gly-)$_2$ have also been determined[77] by X-ray crystallographic techniques. In both the crystalline forms analysed, the cyclic octapeptide has 2-fold symmetry in the form of a slightly elongated bowl, and may be described as incomplete encapsulation, very similar to antanamide complexes. Complexation takes place without large conformational change in the peptide, with the interior substantially hydrophilic and the external surface of the bowl largely hydrophobic. NMR and molecular dynamics simulations have been applied[78] to synthesised hymenistatin 1, cyclo(-Pro-Pro-Tyr-Val-Pro-Leu-Ile-Ile-), originally isolated from the Hymeniacidon sponge. The dominant conformation contains a βVIa turn about Ile[8]-Tyr[3] and includes a Pro[1]-Pro[2] *cis* peptide, with a βI or βII turn around Val[4]-Ile[7] depending on whether the solvent is $CHCl_3$ or DMSO respectively. The linear precursor was synthesised using the Boc/Merrifield strategy on a resin and cyclised in the solution phase between Pro[2] and Tyr[3] using the azide method, or between Leu[6]

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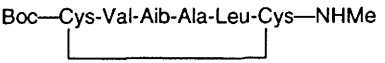


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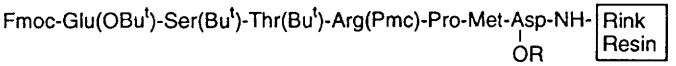
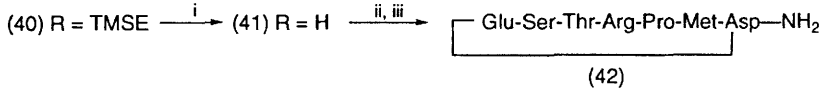
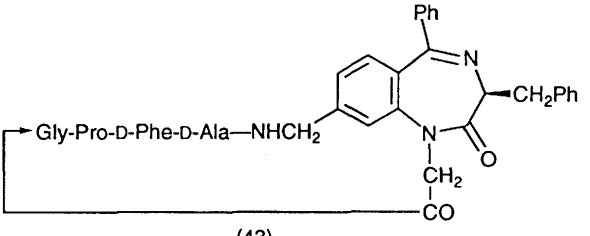



(42)

Reagents: i, $Bu_4N^+F^-$; ii, piperidine; iii, BOP

**Scheme 5**

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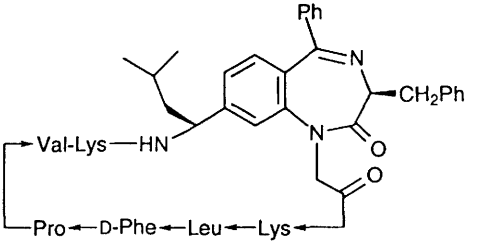



(44)

and Ile[7] using EDC/HOBt. Solution phase synthesis[79] and cyclisation using a pentafluorophenyl ester was the strategy used for the synthesis of cyclo(-Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-) which corresponds to the binding domain of human tumour necrosis factor, TNF-α-(59-66). When tested for induction of interleukin-1(IL-1) production at low concentrations, the IL-1 levels induced were similar to that of recombinant TNF-α thus suggesting that the fragment TNF-α-(59-66) may represent certain binding domains which elicit IL-1 production. Two kinds of $C_2$-symmetry conformations have been elucidated[80] for cyclo(-Phe-Pro-Gly-Pro)$_2$ in $CDCl_3$ solution. One form is an all *trans* conformation and the other with amide bonds in *cis-trans-trans-trans* combinations. However when either $Cs^+$ ions or D- or L-forms of H-Phe-OMe.HCl were added the resulting complexes reverted to a single all *trans* conformation of the peptide backbone. A comparison has also been made with cyclo[-(Phe-Pro)$_2$-(Gly-Pro)$_2$], synthesised *via* a DCC/HONSu activation step in the formation of the ring. The $^{13}$C-NMR spectra of complexes of both cyclooctapeptides, with D-H-PheOMe.HCl and L-H-PheOMe.HCl displayed separate resonances for each carbon atom in the Phe esters. The ability of cyclo(-Phe-Pro-Gly-Pro-)$_2$ to distinguish between the D and L-enantiomer is superior to cyclo[-(Phe-Pro)$_2$-(Gly-Pro)$_2$]. A benzodiazepine β-turn mimetic has been incorporated[81] as a 4-residue mimic into the model compound (43). The macrocyclisation step was carried out using azide coupling and the NMR study showed the backbone conformation to be similar to the all peptide analogue.

## 2.8 Cyclodecapeptides

The benzodiazepine β-turn mimetic discussed in the last sub-section has also been introduced[82] into the gramicidin S sequence to give (44), and on taking into account that it is lysine based (rather than ornithine) its biological activity confirms that the β-turn mimetic is a satisfactory insert to make. The biosynthesis of gramicidin S (45) involves linking a Leu residue to the GS synthetase as the first step, followed by formation of a pentapeptide which then cyclodimerises. To mimic such a strategy using an unprotected ornithyl side-chain, pentapeptide active esters based on N-hydroxysuccinimide have been synthesised[83]. Amongst the five pentapeptide hydroxysuccinimide esters carrying, Val, Orn, Leu, D-Phe or Pro as C-terminal only the 'natural' sequence H-D-Phe-Pro-Val-Orn-Leu-ONSu gave semi GS(cyclic monomer) and GS(cyclic dimer) in yields of 15 and 38% respectively. The result therefore confirms the existence of 'sequence' features that promote cyclisation. These features also seem to affect[84] the cyclisation yields when a series of ornithinyl protected pentapeptides (46–50) were cyclised to GS derivatives as recorded in Table 3, using water soluble carbodiimide and HOBt coupling agents. The CD spectrum of (46) was significantly different from pentapeptides (47)-(50), so it may have secondary structure less disposed for monomer formation. In order to clarify an earlier report in the 1960's that higher acylated analogues of the ornithinyl groups in GS were biologically active, while the lower analogues n < 7 were inactive, a new series of acylated derivatives [Orn($COC_nH_{2n+1}$)$^{2,2'}$]GS have been synthesised[85] with n = O-11,13,15 and 17. However when assayed for antimicrobial activity these

**Table 3** Cyclodimerisation of pentapeptides to gramiciidin S (GS) derivatives

| | *(Z)semi GS* : *(di-Z)GS* |
|---|---|
| (46) H-Val-Orn(Z)-Leu-D-Phe-Pro-OH | 10 : 90 |
| (47) H-Orn(Z)-Leu-D-Phe-Pro-Val-OH | 56 : 44 |
| (48) H-Leu-D-Phe-Pro-Val-Orn(Z)-OH | 97 : 3 |
| (49) H-D-Phe-Pro-Val-Orn(Z)-Leu-OH | 65 : 35 |
| (50) H-Pro-Val-Orn(Z)-Leu-D-Phe-OH | 58 : 42 |

new acylated analogues were all inactive. It is suggested that the 1960 samples were contaminated with unmodified GS.

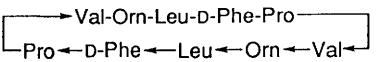
Assembly[86] of the linear precursor on a resin containing an acid labile linker [4-(4-hydroxymethyl-3-methoxyphenoxy)butanoyl-] using Fmoc chemistry, released (1% TFA) sufficient protected linear precursor to yield in turn the cyclic peptides:-

cyclo(-Ala-Val-Phe-Gln-D-Phe-Pro-Asp-Asn-Gly-Tyr-)
cyclo(-Ala-Leu-Phe-Gln-D-Phe-Pro-Asp-Asn-Gly-Tyr-)
cyclo(-Ala-Ile-Phe-Gln-D-Phe-Pro-Asp-Asn-Gly-Tyr-)

Cyclisation was carried out using the BOP reagent. The same acid labile linker approach was utilised[87] for the synthesis of decapeptide precursors for a cyclisation to give the following cyclodecapeptides based on the sequences surrounding $Tyr^{416}$ the autophosphorylation site of $pp60^{c\text{-}src}$, the retroviral protein tyrosine kinase. The cyclodecapeptides synthesised for testing as inhibitors represented the D-Phe-Pro in different environments:-cyclo(-D-Phe-Pro-Asn-Glu-Tyr-Ala-Ala-Arg-Gln-Gly-)

cyclo(-D-Phe-Pro-Asp-Asn-Glu-Tyr-Ala-Ala-Arg-Gln-)
cyclo(-D-Phe-Pro-Glu-Asp-Asn-Glu-Tyr-Ala-Ala-Arg-)
cyclo(-D-Phe-Pro-Ile-Glu-Asp-Asn-Glu-Tyr-Ala-Ala-)

These showed marked differences in $K_i$, $K_m$ and $V_{max}$ values so the position of the D-Phe-Pro can markedly affect recognition and rate of phosphorylation. Two cyclic decapeptides (51) and (52), with sequences related to the virus glycoprotein V3 domain sequences of the MN and IIIB variants of HIV-1 have been synthesised[88], using Fmoc chemistry followed by cyclisation with DPPA. 2D NMR and distance geometry modelling techniques indicated that the highly conserved Gly-Pro-Gly-Arg- of the loop tip was in a conventional β-turn less than 50% of the time. Immune responses elicited in rabbits immunised with (51) and (52) showed antipeptide responses could be achieved, but the lack of a strong preference for a β-turn in the peptides may explain poor reproducibility of the neutralising antibody response. Astute orthogonal protection of side-chain cysteine thiols have led to the synthesis[89] of parallel (53) and antiparallel (54) dimers. The ensemble of conformations were different in each case, (53) giving a mainly folded conformation while (54) gave an extended structure.

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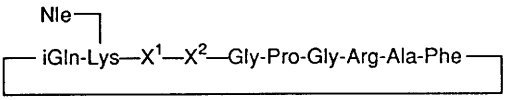
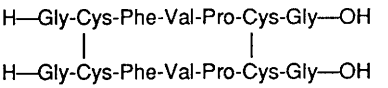



(51) $X^1$ = His, $X^2$ = Ile, MN Serotype L697, 250
(52) $X^1$ = Gln, $X^2$ = Arg, III B Serotype L700, 064

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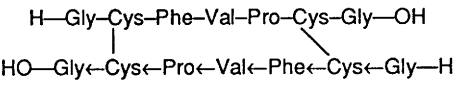


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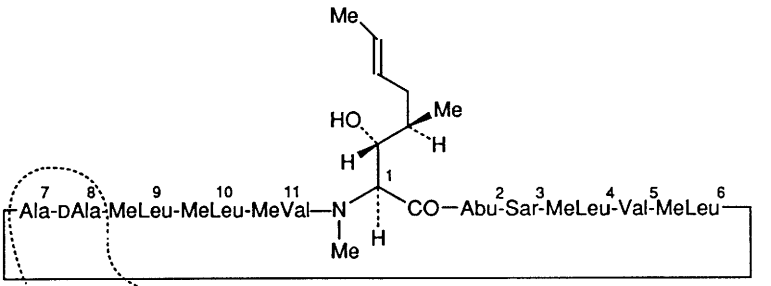


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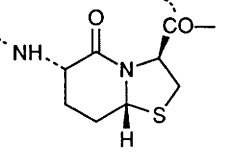


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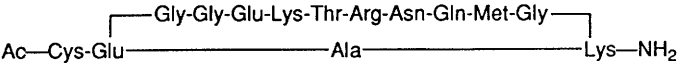



(57)

## 2.9 Higher Cyclic Peptides

This section is again this year dominated with work on the immunosuppressant cyclosporin A (55). The serendipitous modification[90] of one sarcosine residue ($Sar^3$) in cyclosporin A by firstly converting the molecule to its hexalithio derivative using LDA, and then reacting the Li-enolate unit with electrophiles, gives rise to $R_e/S_i$ selectivity of 7:1 in some cases at position 3. Useful labelled derivatives can be obtained as well as [$MeAla^3$]-cyclosporin A, which was also synthesised conventionally for comparison. [$D\text{-}Cys^8$]-Cyclosporin A has been made[91] by first acetylating [$D\text{-}Ser^8$]-cyclosporin A to a diacetate, followed by treatment with Lawesson's reagent which converted amide bonds in the 4 and 7 positions to thioamides. After chromatographic separation, hydrolysis of the diacetates, treatment with tosyl chloride followed by acid, [$D\text{-}Cys^8$]-cyclosporin was obtained *via* intramolecular sulfur transfer. When cyclosporin A is bound to its receptor cyclophilin the backbone of the cyclosporin is turned inside out and all elements of secondary structure in the unbound state are absent. To avoid structural alterations which might interfere with binding, a β-turn constraint (56) has been inserted[92] to replace $Ala^7$-$D\text{-}Ala^8$ which is not involved in binding with cyclophilin. The affinity of the more constrained analogue for cyclophilin was shown to be 3 x that of cyclosporin itself. Using[93] molecular modelling packages INSIGHT II on a Silicon Graphics workstation and SYBYL on an Evans and Sutherland machine, it is claimed, with a certain degree of caution, that the conformation of some cyclosporin A fragments in the cyclophilin-bound form, pre-exist in the free crystalline form and in aqueous solution. Solvent influences on the conformation have also been studied by FT-IR[94], and from partition coefficient determinations and molecular dynamics simulations[95]. In apolar solvents cyclosporin is internally H-bonded, while the H-bonding groups are exposed in polar solvents.

Two additional orthogonal strategies utilising the allyl ester and 1-(4,4-dimethyl-2,6-dicyclohex-1-ylidine)ethyl (Dde) for the Lys residue were required[96] for an on-resin cyclisation synthesis of (57). Linear development of the peptide chain was effected using Fmoc/$Bu^t$ chemistry. This approach can conveniently incorporate a cyclic region into a peptide at any point. On-resin synthesis of disulfide bridges has also been shown[97] with the synthesis of a bicyclic tridecapeptide α-conotoxin SI (58) on tris(alkoxy)benzylamide PAL resin using the Fmoc for N-protection. Side chain protection of Cys was carried out *via* suitable combinations of S-2,4,6-trimethoxybenzyl(Tmob) and Acm. The strategy worked best if the smaller disulfide loop was closed before the larger loop. Using three orthogonal thio-protecting groups, Trt, Acm and $Bu^t$, the three disulfide bonds of human insulin have been efficiently constructed[98] by stepwise disulfide formation. In an attempt to produce antagonists to the anaphylatoxic peptide $C_{3a}$, a potent inflammatory mediator, 26 cyclic disulfide bridged $C_{3a}$ analogues have been synthesised in a multiple peptide synthetic approach[99] using Acm and Trt for Cys side-chain protection, and $I_2$ for ring closure. Some of the cyclic peptides showed $C_{3a}$ antagonist properties. Cyclisation *via* the -S-S-bond has been possible[100] from Cys(Acm) using $MeSiCl_3/Ph_2SO$ over a period of only 10 mins, and this approach has found application in constraining the RGDSPASS sequence, to

give constrained molecules still recognised by the fibrinogen receptor. Cyclic peptides (59)-(61) are examples of peptides showing inhibition of platelet aggregation using this RGD domain.

### 2.10 Peptides Containing Thiazole Type Rings

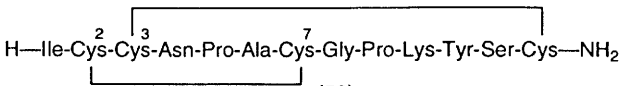
The conformation of patellamide A (62) from the ascidian *Lissoclinum patella* has undergone[101] X-ray crystallographic analysis. The shape of the molecule has been found to be a rectangular saddle-shaped form wrapped around water and methanol solvents, and similar to the related ascidiacyclamide. The biosynthesis of the thiopeptide antibiotic thiostrepton has been investigated[102] in cultures of *Streptomyces azures* and *S.laurentii*, and the origins of all components have been established. Serine was incorporated intact into the thiazoline and thiazole rings as well as into the dehydroalanine, alanine and tetrahydropyridine moieties. The quinaldic acid residue seems to arise from (*S*)-tryptophan and a methyl group donated from methionine, a process which occurs prior to incorporation into the peptide chain. A similar study[103] of the modified peptide antibiotic nosiheptide in *Streptomyces actuosus*, confirmed that the dehydroalanine and butyrine moieties were formed by *anti*-elimination of water from Ser and Thr, while cysteine proved to be the source of the thiazole rings with loss of the *pro*-3*R* hydrogens in the oxidation step. Again the indolic acid was derived from tryptophan.

### 2.11 Cyclodepsipeptides

This year again, this sub-section takes the Chapter prize for the greatest number of novel structures elucidated from nature's rich sources. Again the most modern technology in high field NMR and mass spectrometry have contributed greatly to the detective work.

*Streptomyces violaceoniger* has been found to be a source of a novel tachykinin antagonist WS9326A (63)[104]. The spectroscopic evidence which helped to clinch the structure was carried out on the triacetyl derivative. WS9326A inhibits the binding of [$^3$H]-substance P to a guinea pig lung membrane preparation with an $IC_{50}$ value of $3.6 \times 10^{-6}$M. The blue-green algae *Microcystis aeruginosa*, the most deleterious of freshwater bloom, yet again proved a rich source of depsipeptides. Microcystilide A (64), has been shown[105] to be similar to dolastatin 13 a peptide found in *Dolabella auricularia*. Micropeptins A and B from the same algae have also been shown[106] to be related to dolastatin 13 and given the structures (65) and (66). Both cyclodepsipeptides are potent inhibitors of plasmin and trypsin, at the $IC_{50}$ values of 0.026/0.071 μg/mL level, but did not inhibit thrombin, chymotrypsin and elastase. Further toxic fractions from these toxic cyanobacteria have yielded[107] another four depsipeptide moieties aeruginopeptins 95A (67), 95B (68), 228A (69) and 228B (70). Again the resemblance to dolastatin 13 can readily be seen in the structures.

The siderophore alterobactin A from an open ocean bacterium *Alteromonas luteoviolacea* has been found[108] to have structure (71) and is capable of forming a 1:1 complex with $Fe^{3+}$ ions. The sacoglassan mollusk *Elysia rufescens* and its food source a green alga, *Bryopsis* has been reported[109] to be a source of

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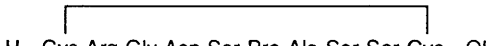
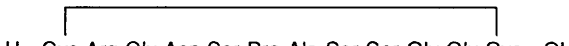



(59)

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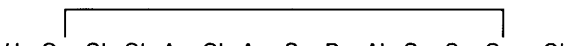


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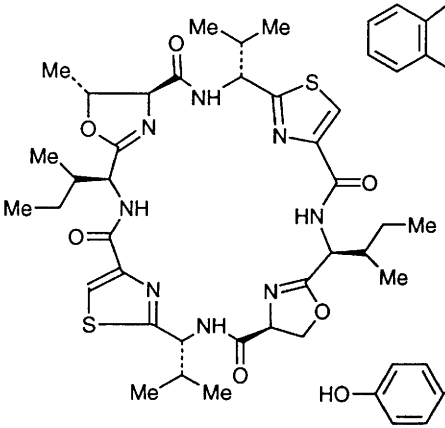

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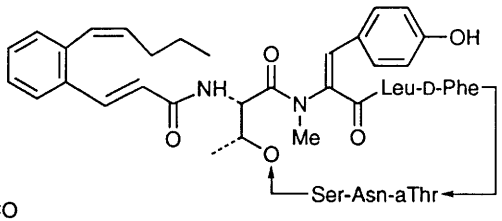


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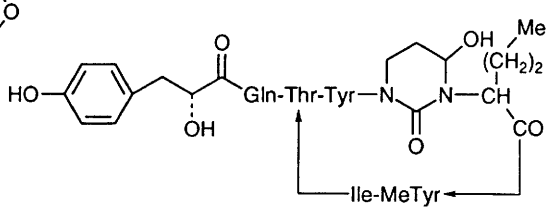


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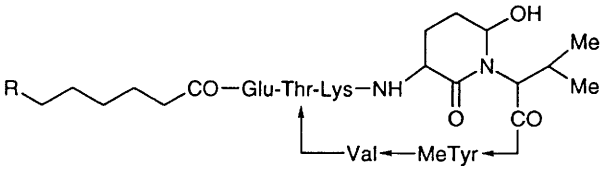


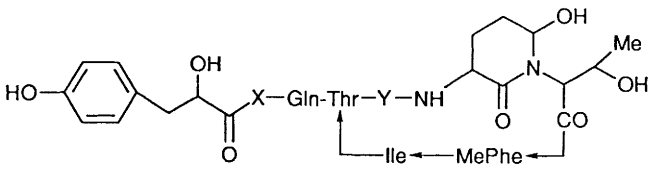

(65) R = Et
(66) R = H

kahalalide F whose structure has been elucidated by high field NMR technology as (72). Majusculamide C (73) a metabolite of the blue-green alga *Lyngbya majuscula* has been isolated[110] from the sponge *Ptilocaulis trachys*. Structure (73) contains a novel β-amino acid, 2-methyl-3-aminopentanoic acid (MAP) which was characterised as being in the 2*S*, 3*R* form in the cyclic depsipeptide.

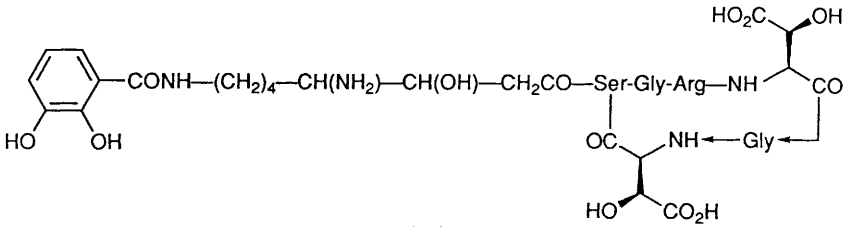
The beauveriolides I (74) and II (75), metabolites of the entomopathogenic fungi Beauveria Sp. have been known since 1978 but the detailed structures have only now been refined[111] using more modern techniques. The host selective toxin phomalide has been shown[112] to have structure (76) when isolated from the phytopathogenic fungus *Phoma lingam*.

Some of the complicated depsipeptide structures continually offer great synthetic challenges, especially as the formation of a depside bond demands higher activation than its amide counterpart, and the field of HO group protection has not been so enriched in options as its $NH_2$ counterpart in peptide synthesis. Such a synthetic challenge has been described[113] for the synthesis of the potent antitumour antibiotic (-)-sandramycin (77), whose structure has a two-fold axis of symmetry. The synthetic strategy involved a late stage introduction of the quinolyl moiety, so that simple analogues could be made accessible. The final macrocyclisation step was achieved in 90% yield *via* the secondary amide site using DPPA/10 eq $NaHCO_3$ over 48 hrs. The depside links were made using DCC/DMAP, and the comment is made that *increasing* amounts of DMAP was found to *suppress* epimerisation. The latter needs explanation. Dolastatin D (78) from the sea hare *Dolobella auricularia* has been identified[114] as a cyclic depsipeptide while dolastatin C is an acyclic depsipeptide. The synthesis of both report the development of novel usage of t-butyldimethylsilyl derivatives for the hydroxy components. These silyl derivatives could be readily deprotected by fluoride ion. Scheme 6 summarises the stepwise construction of dolastatin D. The final cyclisation results confirmed BOP/$NaHCO_3$ as the most efficient reagent. The depside link in aureobasidin A (79) was made[115] using DCC/4-pyrrolidino pyridine, while the macrocyclisation step was best achieved by coupling between Pro and aIle using bromotrispyrrolidinophosphonium hexafluorophosphate (PyBroP) under high dilution. Key stages in the synthesis[116] of [D-Lac$^6$]destruxin E (80) involved coupling of Boc-Ile-MeVal-MeAla-β-AlaOMe with (*R*)-MeCH(OH)CO-Pro-OBu$^t$ to make the depside link, followed by cyclisation with either DEPC (21% yield) or DPPA/HOBt/DMAP (30% yield). A stereoselective synthesis[117] of the polyketide-derived unit ($C_1$-$C_8$) has provided the key link in the synthesis[118] of jaspamide (81) and geodiamolide-D (82) by linking the unit to H-Ala-Me(BrTrp)-NH-CH[PhOTBS]$CH_2CO_2Et$ and H-Gly-Me[3-I-Tyr(TBS)]-AlaOMe, respectively. Final formation of the depside link required drastic conditions (DCC/DMAP under reflux in chloroform) giving a cyclisation yield of 7% for geodiamolide and 22% for jaspamide. Virginiamycin $S_1$ ($VS_1$) analogues such as (83) and [Ala$^5$,Gly$^6$]-$VS_1$ have been prepared[119] by partial synthesis from the 1-4 sequence prepared from degradation of the native antibiotic. The MePhe epimerised during the synthesis so diastereoisomers had to be separated on preparative TLC.

It is claimed[120] that the cyclisation of $PhCH_2CO$-D-Ser-Pro-Pro-ONp and




(67) X = Thr, Y = Tyr, (68) X = Thr, Y = tetrahydrotyrosine
(69) X = —, Y = Tyr, (70) X = —, Y = tetrahydrotyrosine

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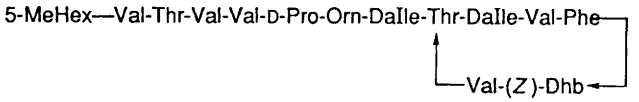


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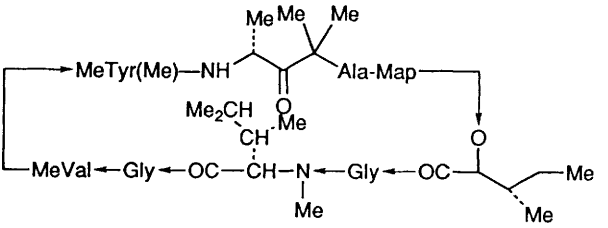


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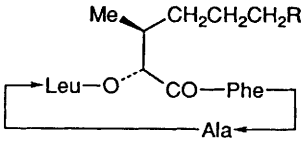
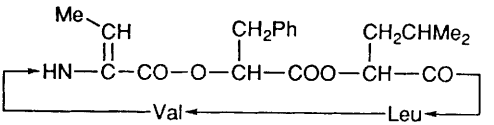
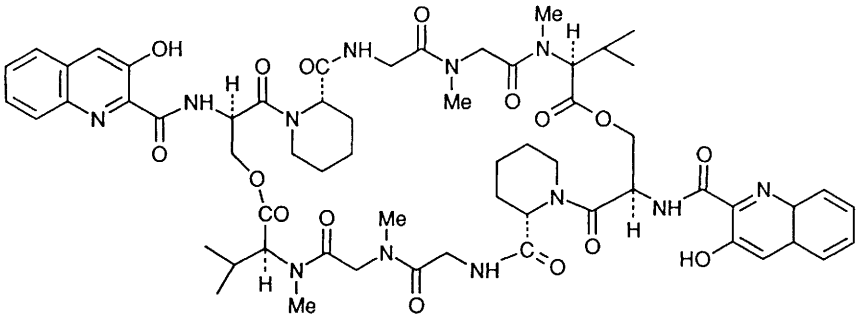



(74) R = Me
(75) R = Pr




(76)



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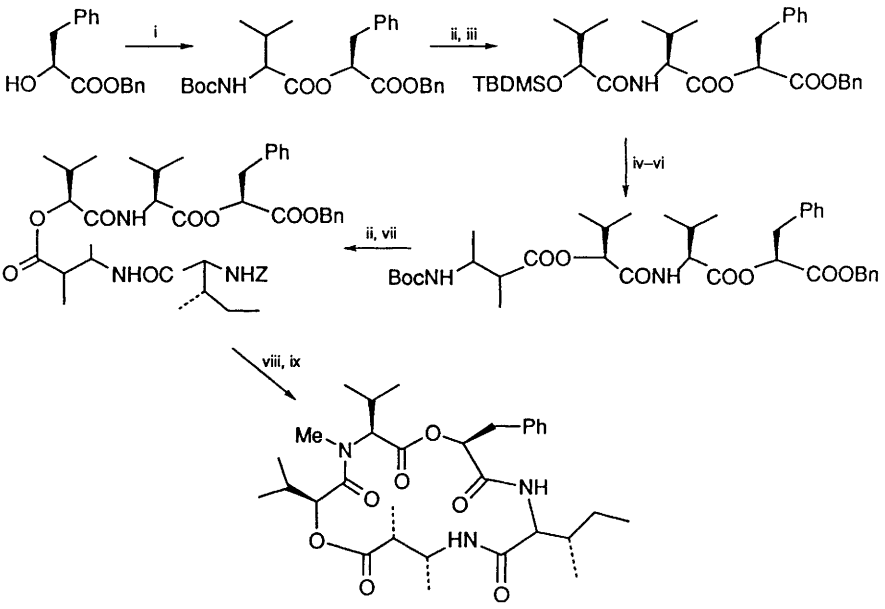
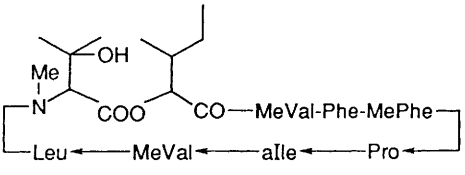


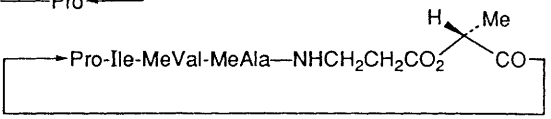
(78)

Reagents: i, Boc-Val, DCC, DMAP;
ii, TFA; iii, TBDMS-(*S*)-PhLac, DEPC(diethylphosphorocyanidate); iv, NaH/MeI;
v, HF/$H_2O$; vi, Boc-(2*R*, 3*R*)-3-amino-2-methylbutanoic acid;
vii, *Z*-Ile, DEPC; viii, $H_2$/Pd/C;
ix, BOP-Cl/$Et_3N$ 13% yield, HOSu/DCC 41% yield,
DPPA/$Et_3N$ 47% yield, BOP/$NaHCO_3$ 66% yield

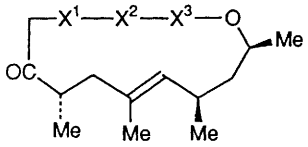
**Scheme 6**



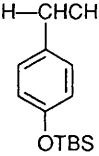

(79)



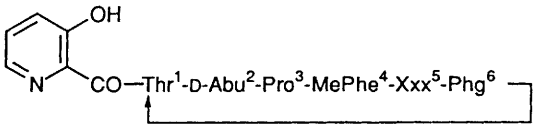

(80)




(81) $X^1$ = Ala, $X^2$ = Me(BrTrp), $X^3$ = NH—CHCH$_2$CO

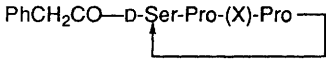



(82) $X^1$ = Gly, $X^2$ = Me[3-I-Tyr(TBS)], $X^3$ = Ala

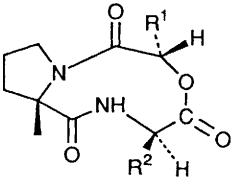



Xxx = Ala, Asp, Asn and Lys

(83)




(84) X = L or X = D




(85) $R^1$ = CHMe$_2$, Me; $R^2$ = CH$_2$Ph, Me

$PhCH_2CO$-D-Ser-Pro-D-Pro-ONp using DBU to form compounds (84) represents the smallest cyclodepsipeptide models synthesised using such a strategy. X-ray analyses of (84) showed that both rings adopted a *cis-cis-trans* conformation with the lactone junction *trans*. Another 'small' cyclodepsipeptide ring[121], was formed by incorporation of a retroisomeric sequence into the ergot peptide sequence. The 'normal' ergot peptide sequence cyclises *via* the aza-cyclol intermediate but in the retroisomeric form this is not stable and collapses to (85), which has a *cis-cis-trans* conformation. The asymmetric synthesis of the non-peptidic C(28)-C(47) subunit of the azinothricin antibiotic A83586C has been reported[122].

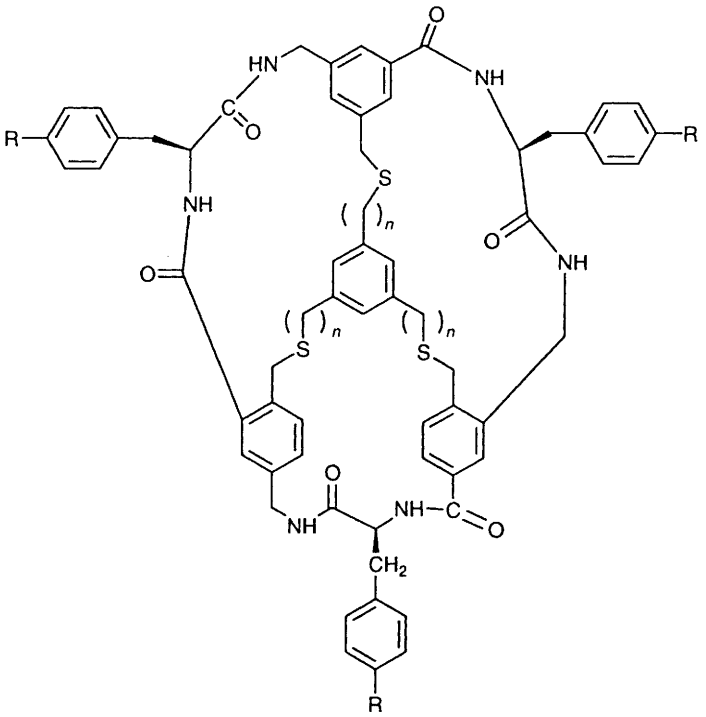
An unusual formation of thermostable gels occurs[123] when cyclodepsipeptides cyclo($X\text{-}X^1\text{-}OCH_2CH_2CO)_n$ with $X = X^1 = Leu$, $n = 1$ and $X = X^1 = Val$, $n = 2$ are added to the solvents $CH_2Cl_2$, MeCN $Et_2O$, EtOAc or acetone. As little as 10 mg can gelate up to 1 g of solvent. The effect is dependent on having the side chains of Leu and Val present. Low energy tandem mass spectrometry of valinomycin has been studied[124]. Different ionisation conditions do not seem to affect the M + H ions, but the M + Na ions are affected by the ionisation methods chosen. FAB-Mass spectrometry[125] has identified novel cyclic depsipeptides involving Ser and Thr residues during the production of elcatonin.

## 2.12 Cyclic Peptides Containing 'Other' Non-protein Ring Components

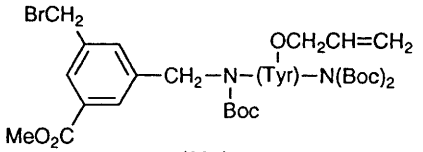
The highly enantioselective, $C_3$-symmetry host molecule (86) has been synthesised[126] in a more direct manner than previously published. Key to the strategy was the formation of (88a), and the final macrolactamisation step was achieved *via* pentafluorophenyl ester activation in the presence of i-Pr2NEt. Binding experiments[127] have shown that analogue (87) is capable of binding tightly and selectively a variety of donor/acceptor substrates. Both Boc-amino acids or $MeO_2C$-Val-$OBu^t$ bind enantioselectively with a preference for the L-form. Binding with carbohydrates is not such a clear-cut system. Increasing the flexibility of the synthetic receptor as in model (88)[128] resulted in a decrease in binding of the otherwise selectively-bound 'guests'.

Templates such as (89) with incorporated turn-inducing mimics have been synthesised[129] in the solution phase using DCC/HOBt coupling methods. The final cyclisation between Gly and Lys was achieved using bis(phenyloxy)-phosphoryl azide $(PhO)_2PON_3$. Macrocyclic analogues such as (90) of the potent and selective CCK-B antagonist CI-988 have been prepared and evaluated[130]. It is worth noting the results in Table 4 of the macrocyclisation at point (a) in (90), using DPPA/$NaHCO_3$ at high dilution.

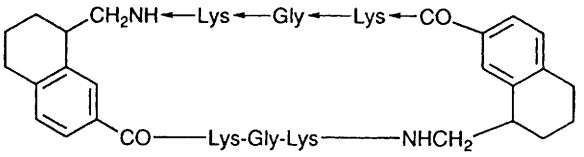
The lantibiotics, characterised by the presence of monosulfide analogues of cystine in their structure, have been studied in terms of their structure, synthesis and biosynthesis. NMR, followed by distance geometry and restrained molecular mechanics calculations on duramycins B (91) and C (92) unambiguously assigned the three thioether bridges and a lysinoalanine ring. The overall conformation is seen as U-shaped, and this clamplike conformation is stabilised by the thioether bridges and β-strands at the termini. Duramycins (B) and (C) differ mainly in the relative mobilities of the A, C and D rings. A direct cyclisation[132] on a Kaiser



(86) R = $OCH_2CH{=}CH_2$, $n$ = 0
(87) R = H, $n$ =0
(88) R = H, $n$ = 1



(88a)



(89)

**Table 4** Macrocyclisation at point (a) to form the peptide (90)

| *Structure* | *Monomer (90) yields %* | *Dimer %* |
|---|---|---|
| $n=2$ | 26 | 27 |
| $n=3$ | 53 | 24 |
| $n=4$ | 65 | 0 |
| $n=5$ | 61 | $\approx 6$ |

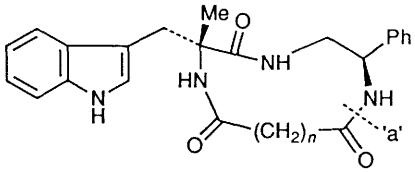
oxime resin has yielded an orthogonally protected lanthionine amino acid unit as depicted in Scheme 7. The biosynthesis of the lantibiotics have been 'modelled'[133] *via* the synthesis of H-Ala-DAla-Pro-Gly-Cys(SBu$^t$)-Ala-OBzl which spontaneously cyclised in a biomimetic fashion to (2*S*,6*R*)-lanthionine (93).

A total synthesis[134] of cyclotheonamide A, a potent inhibitor of thrombin and plasmin, has been designed to allow efficient synthesis of analogues. The ring closure at point (a) from a protected linear precursor as a pentafluorophenyl ester was carried out in 52% yield. The formation of the α-ketoamide was from a precursor α-hydroxy amide *via* a Dess-Martin oxidation. A total synthesis of (94) is also described[135] in a paper whose main emphasis is on understanding the molecular basis of inhibition of human α-thrombin by (94). In the synthetic part cyclisation was achieved at position (c) using BOP-Cl/DMAP for coupling of the linear precursor, which had been synthesised by a convergent [2+3] fragment-condensation. In the main work in the paper X-ray studies revealed details of the 'molecular recognition' of cyclotheonamide within the active site of α-thrombin. Formation of a H-bonded 2-strand anti-parallel β-sheet with Ser$^{214}$-Gly$^{216}$ and the α-keto amide of cyclotheonamide serving as a transition state analogue are primarily responsible for the binding.

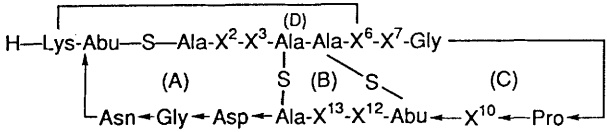
Four water soluble cyclic peptides, the lyciumins A-D(95)-(98), have been discovered[136] in the root bark of *Lycium chinense Mill*, used as an oriental crude drug, as an antifebrile, tonic and an antihypertensive. The structures contain the novel C-N linkage between the N of Trp and the $C_\alpha$ of Gly. A 2,7-disubstituted naphthalene spacer unit has been inserted[137] to constrain residues present in the exposed Ω loop of interleukin 1α, and provide the mimetic (99) whose conformation has been shown to mimic sequence 41-48 in interleukin-1α. Activation of the C-terminal Ala residue in the linear precursor with DPPA gave (99) in 42% yield. Tentative structures (100) and (101) have been ascribed[138] to alkaloids from the flowers of *Sphaeranthus indicus*, and it has been reported[139] that there is significant assemblage of water in crystals of cyclo(-D-Val-MeArg-GlyAsp-*m*-$NHCH_2C_6H_4CO$-).

## 3 Modified and Conjugated Peptides

As in last year's Report, this section, which is expanding annually, concentrates on peptides which usually have non-peptidic conjugates attached to their side chains.

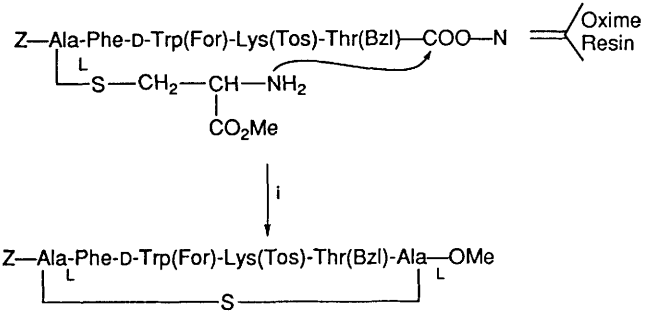


(90) $n$ = 2, 3, 4 or 5



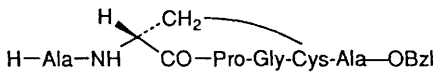
(91) $X^2$ = Arg, $X^3$ = Gln, $X^6$ = Ala linked to $Lys^{19}$ as lysinoalanine
$X^7$ = Phe, $X^{10}$ = Leu, $X^{12}$ = Phe, $X^{13}$ = Val.

(92) $X^2$ = Ala, $X^3$ = Asn, $X^6$ = Ala, $X^7$ = Tyr, $X^{10}$ = Leu, $X^{12}$ = Trp

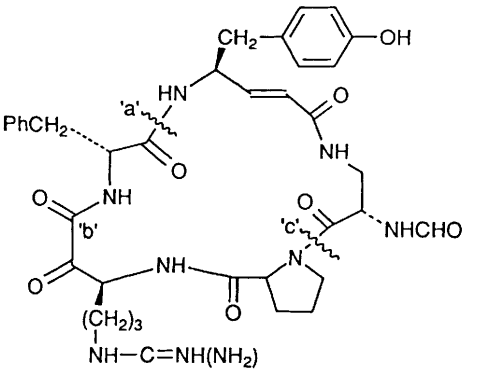


Reagent: i, 10 mol. eq. AcOH

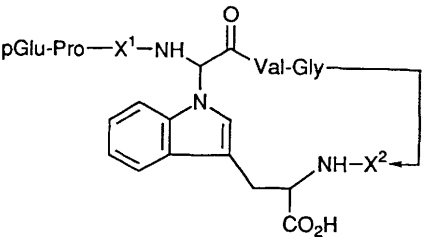
**Scheme 7**



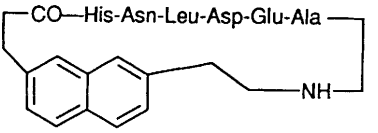
(93)



(94)



(95) Lycium A, $X^1$ = Tyr, $X^2$ = Ser
(96) Lycium B, $X^1$ = Trp, $X^2$ = Ser
(97) Lycium C, $X^1$ = Tyr, $X^2$ = Ser
(98) Lycium D, $X^1$ = Tyr, $X^2$ = Ile



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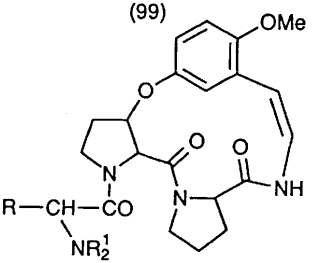


(100) R = $CH_2Ph$, $R^1$ = Me
(101) R = Me, $R^1$ = H

### 3.1 Phosphopeptides

The two general methods for introducing the phosphate group into the side-chains of Tyr, Ser and Thr, (i) the building block approach and (ii) post-assembly approach, seem to still have their supporters. For the Fmoc/$Bu^t$ solid phase strategies[140] the use of -Tyr-[$PO(OCH_2CH_2SiMe_3)_2$], formed from i-$Pr_2N$-$P(O\text{-}CH_2CH_2SiMe_3)_2$ is useful, as it can form -Tyr($PO_3H_2$)-during the last stage treatment of the protected peptides with TFA. Reactions summarised in Scheme 8 have provided[141] good yields of phosphoserine and phosphothreonine synthons which are compatible with a number of peptide synthetic strategies. Introduction of $(CH_2=CH\text{-}CH_2O)_2P(O)\text{-}CH_2CH_2Br$ into the Schollkopf bislactim ether method for synthesising chiral amino acid derivatives has given gram quantities of Fmoc-Abu-[$PO(OCH_2CH=CH_2)_2$]-OH for use in solid phase synthesis[142]. The ester released in the synthesis was hydrolysed by chymotrypsin to give D and L forms of the derivative. In a re-examination[143] of the use of Boc-Tyr($PO_3Bzl)_2$-OH in solid phase synthesis, it was discovered that one of the benzyl groups was completely removed even after two consecutive cycles of Boc-deprotection by 50%/TFA/$CH_2Cl_2$. However the other benzyl group seems to remain intact throughout a synthesis of 35 cycles. So the loss of one benzyl does not preclude the synthesis of pure phosphopeptides. Another efficient use of the same phosphate protecting group as Boc-Thr[$PO(OBzl)_2$]-OH, was reported[144] in its use in the making of H-Cys-Ile-Val-Arg-Lys-Arg-Thr(P)-Leu-Arg-Arg-Leu-Leu-OH, Cys[EGFRPP(649-659)]. A slightly different phosphate protection using Boc-Thr($PO_3Ph_2$)-OH, has been developed[145] for the synthesis of phosphopeptides involved in $Ca^{2+}$ binding. The phosphate protection is removed by Pt-mediated hydrogenolysis and in the series of three peptides synthesised – H-Thr(P)-Glu-Glu-NHMe.TFA, H-Thr(P)-Thr(P)-Glu-Glu-NHMe.TFA and H-Thr(P)-Thr(P)-Thr(P)-Glu-Glu-NHMe.TFA – only the latter with three phosphate residues was able to bind to calcium phosphate. Fmoc-Tyr[$PO(OMe)_2$]-OH has been utilised[146] in a continuous flow solid phase synthesis of the O-phosphotyrosine analogue of human angiotensin II. The methylphosphate groups were removed at the same time as cleavage of the peptide from the solid support using 1M trimethylsilyl bromide/thioanisole in TFA. The product itself had the same potency as native angiotensin II. The Fmoc technique was also used[147] to study whether phosphorylation had any effect on cell binding of an RGD-containing hexapeptide H-Pro-Arg-Gly-Asp-Thr(O-phosphoryl)-Tyr-OH. Phosphorylated peptide inhibited binding of R1 cells to bone sialoprotein 10 times less efficiently than its non-phosphorylated peptide.

Single serine and threonine residues required for phosphorylations were left unprotected in the small scale manual multiple peptide synthesis[148] of 5 monophosphorylated isomers of H-Ala-Ser-Thr-Thr-Val-Ser-Lys-Thr-Glu-OH from the C-terminal region of rhodopsin. Post assembly introduction of the phosphate group was made by phosphitylation/oxidation according to published procedures. Phosphitylation of free serine and threonine on peptides assembled on solid phase has been carried out[149] with $(4\text{-}ClC_6H_4CH_2O)2PN(CHMe_2)_2$ as demonstrated for H-Leu-Ser-Asp-Gln-Glu-Lys-Arg-Lys-Gln-Ile-Ser(R)-Val-Arg-Gly-Leu-OH. Ser(R) in this case comprises the regulatory site ($Ser^{14}$) of phos-

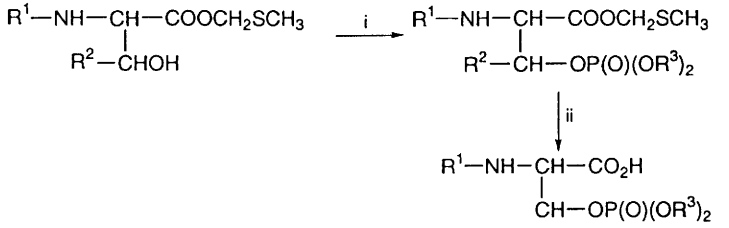
phorylase b. The phosphitylated peptide could then be converted to the phosphorothioyl Ser [R = P(S)(OH)$_2$] by treatment with phenylacetyl sulfide, or to the phosphopeptide with Ser (R = $PO_3H_2$) using peracid. Synthesis[150] of phosphorylated tripeptides representing poisoned acetylcholinesterase have been carried out by post assembly phosphitylation with $Pr_2NP(OMe)_2$ followed by oxidation with m-chloroperbenzoic acid to give tripeptides such as Z-Glu(OMe)-Ser[P(O)(OMe)$_2$]-AlaOEt. Conversion to the Ser[P(S)(OMe)] analogue was achieved by $P_4S_{10}$.

Although there is evidence for the presence of a phosphodiester linkage between Ser and Thr in *Azobacter vinelandii* flavodoxin, it has not been widely studied. The challenge to synthesise[151] such a bridge has been successful with the formation of bridged phosphopeptide (102). The linear precursor was synthesised *via* the mixed anhydride procedure and then phosphitylised by 4-Cl-$C_6H_4$-$CH_2OPCl_2$, oxidised by m-chloroperoxybenzoic acid to (103) before hydrogenolysis to (102). Synthetic phosphopeptide models have been used[152] to study cooperativity during multiple phosphorylations catalysed by rhodopsin kinase. Dodecapeptides containing notionally Ser$^{353}$ from rhodopsin's C-terminus sequence were phosphitylated with di-t-butyl-N,N-diethylphosphoramidite and oxidised by m-chloroperoxybenzoic acid. The phosphopeptides had a greater affinity for the kinase compared with their non-phosphorylated analogues. Useful synthons as stable analogues of O-phosphotyrosine have been prepared[153] with Fmoc protection (104) and inserted into hexapeptides such as H-Gly-Pmp($XPO_3H_2$)-Val-Pro-Met-Leu-OH, important in mitogenic cellular signal transduction. Low m.w. linear and cyclic phosphopeptides have been studied[154] by FAB and tandem MS to verify position of phosphate group. Low abundance fragment ions can yield unequivocal information about the positions of the phosphate moieties.

### 3.2 Glycopeptide Antibiotics

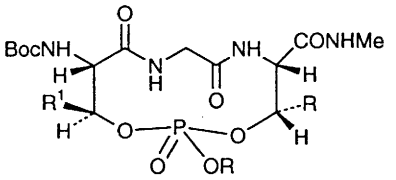
A glance at the structure (105) re-iterates once again the synthetic challenges associated with a total synthesis of vancomycin. Once again, reports have appeared on the construction of vital parts, but the total synthesis still appears elusive. Derivatives (106) have been made[155] *via* DCC-mediated condensation reactions to test the relative potencies of antibiotics that act stoichiometrically *vs* catalytically against a common target. The derivatives bind to $N^{\alpha}$,$N^{\omega}$-diacetyl-Lys-D-Ala-D-Ala which demonstrated substrate turnover and specificity. The 12-membered cyclic fragment (107) has been synthesised[156] from its linear tripeptide precursor by an efficient oxidative biaryl coupling at position (a) with $VOF_3$ in 64% yield, but the unnatural biphenyl rotamer was obtained, so it had to be atropisomerised to the natural unit by overcoming a 21 kcal mol$^{-1}$ rotational barrier. The $C_{\alpha}$-stereochemistry of the arylglycine residue in (107) is pivotal[157] to determining the atropdiastereoselection as it seems to interact with the neighbouring ortho-substituent in the aromatic ring.

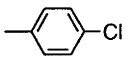
Displacement of bromine on a benzoquinone by phenoxide (see later for K-13) has also been extended[158] to 2,6-dibromobenzoquinone for the synthesis of the C, D and E ring segments (108) of vancomycin. Mild and suitable for chiral

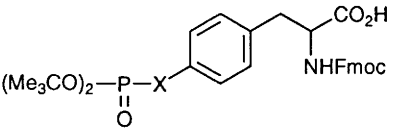


Reagents: i, Cl $P(O)(OR^3)_2$/pyridine; ii, $MgBr_2/Et_2O$ where $R^3$ = Ph or $CH_2CCl_3$

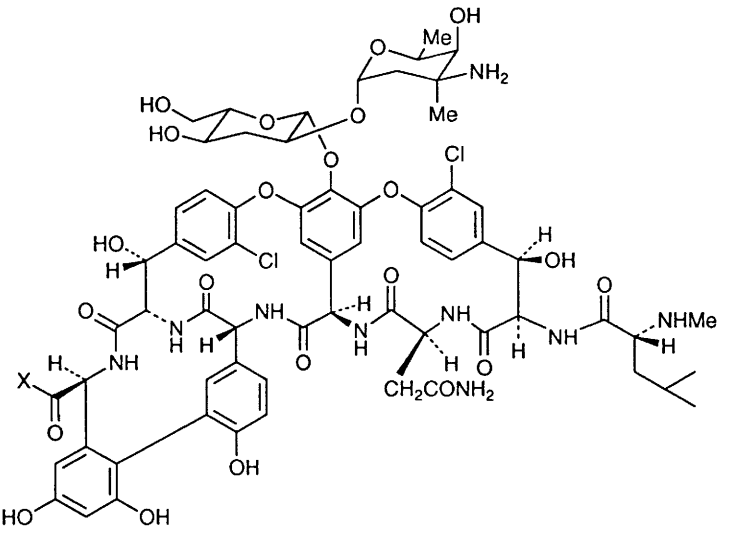
**Scheme 8**



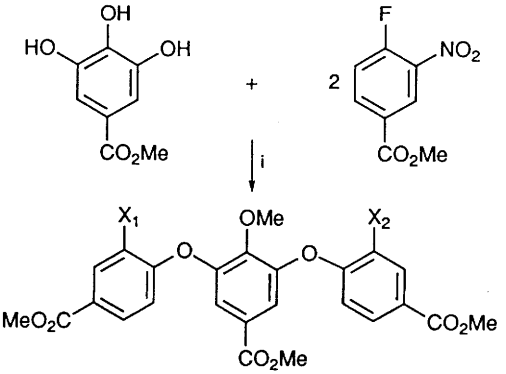
(102) R = Na
(103) R = $CH_2$ 



(104) X = CHF or CH(OH)
*p* -(phosphonomethyl) Phe (Pmp)

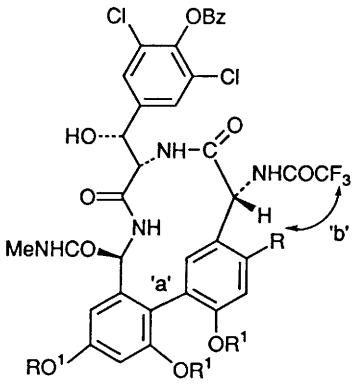


(105) X = H
(106) X = PrNH, 2-(imidazol-4-yl)ethylamino, or $NH_2(CH_2)_3NH$

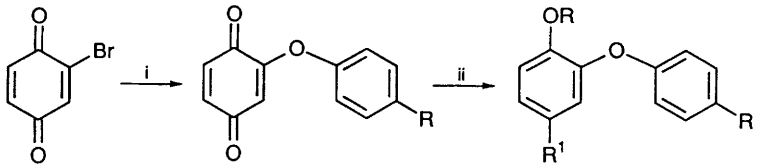



Reagent: i, $K_2CO_3$/DMF

**Scheme 9**




(107) R = $R^1$ = H




Reagents: i, KF/DMF at 90°C; ii, sodium dithionite
R = (*S*)-$CH_2$(NHBoc)$CO_2$Bn

**Scheme 10**

amino acid groups is the verdict[159] on a facile $S_NAr$ approach to rings C, D and E suitable for ristocetin, vancomycin and other antibiotics. The essentials are summarised in Scheme 9. Using model compounds to represent the parent skeletons of the C,D and D,E diphenyl ether rings, linear precursors have been cyclised[160] using the Ullmann reaction of MeCu at 130°C. Closure of ring D,E was faster than C,D and gave a higher yield. $^1$H NMR spectroscopy has provided[161] evidence of dimers existing in aq. solutions of eremomycin, A82846B, vancomycin and eremomycin-ψ. The sugar functionalities are believed to contribute to the dimerisation in addition to their increasing aqueous solubility, and the Cl in ring 2 may act as a stabilising influence.

A new route to the isodityrosine fragment has provided[162] a key intermediate in the making of K-13. Scheme 10 summarises the key features, which relies on displacement of a bromoquinone by a phenolate.

### 3.3 Glycopeptides

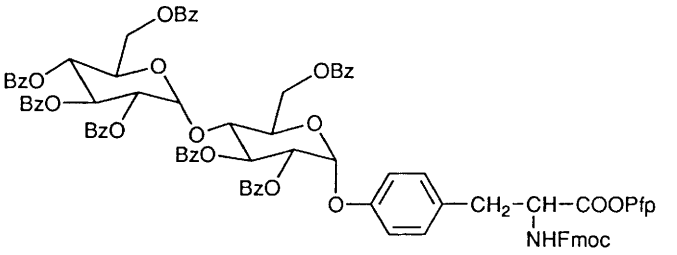
In last year's Report under this category, it was expedient to categorise the published work into sub-sections, under the sub-headings, *O*-glycopeptides, *N*-glycopeptides and *C*-glycopeptides. The first two remain by far the most productive sections, but the last category this year has to encompass a greater variety of conjugates, so 'miscellaneous' is a more accurate sub-title to use. Glycopeptide synthesis remains a major interest and challenge and has generated two reviews[163,164] on the subject. Enzymic syntheses[165] of both N- and O-linked glycopeptides have been explored, and it has been shown that subtilisin enzymes can accommodate as substrates both N-and O-glycopeptide fragments for coupling to other peptide fragments, *i.e.* peptide bond formation is catalysed by the enzyme. It has also been shown[166] that β-D-galactosidase can take on board a protected serine dipeptide ester attached to galactose and catalyse the addition of a second galactosyl unit, *i.e.* carbohydrate links can be enzymatically formed.

*3.3.1 O-Glycopeptides.* – The combination of Fmoc N-protection, and pentafluorophenylester(Pfp) activation of O-glycosylated amino acid residues have worked satisfactorily in the building block approach to glycopeptide synthesis on the solid phase. Hence the need for a number of these derivatives as the building units, as exemplified by tyrosine derivatives[167] such as (109), formed by silver triflate-promoted reactions with glycosyl bromides. Variations in the structures included Fmoc-Tyr($Ac_4$β-DGlc)-OPfp, Fmoc-Tyr-($Ac_4$αD-Glc(1→4)-$Ac_3$β-D-Glc)-OPfp, and Fmoc-Tyr($Bz_4$-α-D-Glc(→)-$Bz_3$α-D-Glc)-OPfp. The incorporation of the unit (110) has in a similar way led[168] to O-mannosylated threonine tripeptides. During the synthesis[169] of O-mannosylated hexapeptides, the use of building blocks such as (110) was compared with assembling the hexapeptides first without protection of side chain OH groups followed by glycosylation of the completed peptide using mannosyl bromide and silver triflate. The building block method gave a 12.5% overall yield while the latter post-assembly approach gave 5% overall yield. Cyclised hexapeptides from blood clotting factor IX have been synthesised[170] by incorporating building blocks Fmoc-Ser(β-D-Glc)-OPfp or Fmoc-Ser(α-D-Xyl(1→3)-α-D-Xyl(1-3)-β-D-

Glc)-OPfp into -Cys(Acm)-Glu-Ser(OR)-Asn-Pro-Cys(Acm)- and further conversion to a cyclic disulfide analogue using $TFA/Tl(OCOCF_3)_3$. Fear of β-elimination of the side-chain O-glycoside during treatment of Fmoc derivatives with piperidine has led to a move towards the weaker base morpholine. However, as part of the synthesis[171] of a fragment (111) of the HIV envelope glycoprotein $gp^{120}$, it was found that cleavage of the Fmoc group in the later residues added was very slow with morpholine and produced several by-products. When repeated using 20% piperidine all was well. By using the Kaiser nitrobenzophenone-oxime resin the final C-terminal residue in a peptide can be inserted[172] as a glycosylated serine as shown in Scheme 11.

Quite complex glycopeptides from the carbohydrate-protein linkage of most mammalian proteoglycans have been synthesised[173] by condensing a tetrasaccharide trichloroacetimidate with a protected L-serylglycine, followed by peptide chain elongation. The O-glycopeptide link in (112), where X′ can be Ser or Thr and X = Ala or Val, was formed[174] *via* reaction with thioether (113). The resulting O-GlcNAc, after exchanging of $CCl_3CH_2$-OCO-with the acetyl group, could be elongated further by enzymic galactosylation using β-1,4-galactosylation transferase. [D-$Ala^2$,$Met^5$]-Enkephalin has been linked[175] through its C-terminal carboxyl group to the 6-OH group of D-glycopyranose moieties, by first coupling Boc-Phe-Met-OH to the sugar by active ester or DCC coupling, and then building up the peptide chain. Most derivates were full agonists in GPI (μ-receptor) and MVD (δ-receptors) tests. $N^2$-(N-acetyl-6-O-stearoyl-α-D-muramoyl)-L-Ala-isoGln (114) (R = H) when synthesised[176] by a published procedure, and compared to commercial material was found to be the benzyl ether ($R = CH_2Ph$). Hydrogenation with $H_2$/Pd/C for 52 hrs yielded (114) R = H. Acid- and base-labile O-glycopeptides carrying the key structural element of the tumour associated $T_N$-antigen (GalNAc α→Ser/Thr) have been formed[177] through deprotection of their heptyl esters with lipase M. Similarly hydrophilic 2-(N-morpholino)ethyl esters of peptides and glycopeptides are hydrolysed[178] by lipases in water/acetone at pH 7. Cleavage of the α-bond between mannose (or galactose) and serine (or threonine) in the presence of α-mannosidase and α-galactosidase has been studied[179] using model glycopeptides.

It is reported[180] that glycosylation of the hexapeptide Ac-Phe-Phe-D- Trp-Lys-Thr(R)-Phe-$NH_2$ (R = H) to its derivative (R = αGalNHAc) dramatically alters the ensemble average conformations, according to ROESY NMR experiments on both molecules, and by comparison with a cyclic analogue, which has a similar conformation to the glycosylated form. α- or β-O-D-Glucosylated forms of the hydroxy proline analogue [L-$Hyp^3$]-tuftsin have been formed[181] by reacting the Boc(Glcα + β)Hyp-OH with H-Arg($NO_2$)-OBzl *via* the mixed anhydride procedure, the N-terminal derivative having been formed *via* the trichloroacetimidate procedure. The dipeptide derivative was further acylated with Z-Thr-Lys(Z)-OH. After deprotection, the capacity of the analogues to evoke release of interleukin I from mouse peritoneal macrophages, and to stimulate cytokinine release was measured. [(Glcβ)$Hyp^3$]-tuftsin was amongst the most potent at $5 \times 10^{-8}$M, but all analogues showed tuftsin-like activity.

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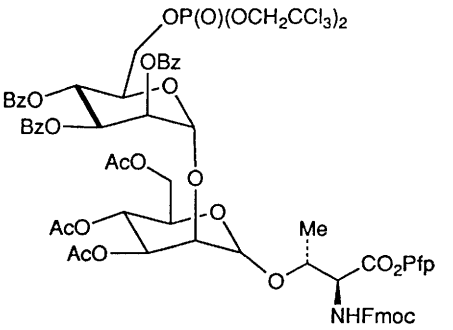


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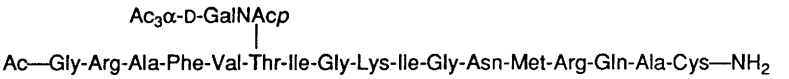
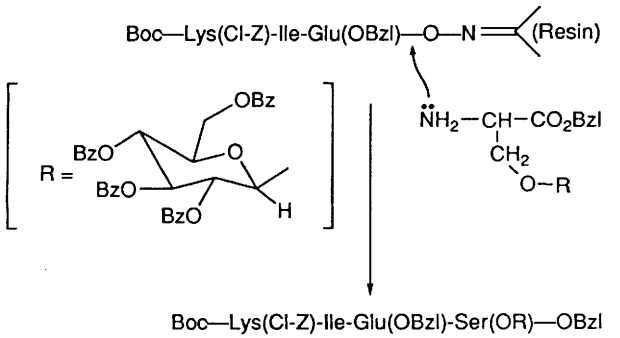



(111)

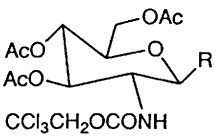



**Scheme 11**

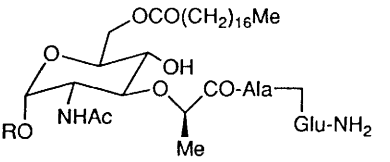
*3.3.2 N-Glycopeptides.* – Again the Fmoc/OPfp option has become popular in this field, as exemplified[182] by the formation of glycosylated Fmoc-asparagine-pentafluorophenyl esters, by conversion initially to the side-chain acid chloride N-Fmoc-Asp(Cl)-OPfp, which then acylated per-O-acetylated glycosylamines. The application of the building block containing a maltose moiety has been demonstrated for the synthesis of the glycosylated D-Ala[1]-peptide T amide analogue (115). Silyl protection of the carbohydrate moiety, *e.g.* as per-O-(trimethylylsilylated)-1-amino-1-deoxy-D-glucitol, followed by selective reaction[183] with FmocAsp(Cl)-OPfp is also an option which was used to modify the D-Ala[1]-peptide T amide. Fmoc-Derivatives such as (116), where $R = CH_2Ph,Bu^t$, $R^1 = OAc$, NHAc and $R^2 = Ac$, β-D-2-deoxy-2-acetamido-3,4,6-tri-O-acetyl glucopyranosyl have been obtained[184] by reaction of glycosyl azides with Fmoc-Asp-OR in the presence of $Et_3P$. Optimised procedures[185] have been developed for the acylation of a β-glycosylamine with a partially protected peptide. The amine was derived from any reducing sugar using the Kochetkov reaction, and then joined to the side-chain of N-protected aspartyl peptides using HBTU as the best reagent for the purpose. The N-glycopeptide link has been used[186] for grafting Leu-enkephalin on to unmodified cyclodextrin as in (117). The link was made by reaction of Fmoc-[Leu]-enkephalin with 6-amino-6-deoxy cyclomaltoheptaose in the presence of DCC/HOBt. NMR studies showed that the free and 'grafted' peptide exhibit similar conformations. A comprehensive CD, molecular dynamics and NMR analysis[187] of eight linear N-glycopeptides has confirmed the X-ray analysis which showed a nearly *trans* orientation of the CH and H hydrogens of the 1-N-acetyl- and 1-N-(β-aspartoyl)-2-acetamido-2-deoxy-β-D-glucopyranosylamine. In general the CD results confirmed that N-glycosylation has a significant effect on the conformation of the peptide resulting in a decrease in type I β-turn content and an increase in type II β-turn to about 20%.

*3.3.3 Miscellaneous Glycopeptides.* – The biological importance of naturally occurring glycopeptides has stimulated the interest in modified structures for pharmaceutical purposes. Already known for its higher stability, the S-glycosyl peptide link has now been incorporated[188] *via* intermediate (118) into cyclo[-D-Pro-Phe-Ala-Cys(β-D-galactopyranosyl)-Phe-Phe-]. Boc-derivative (118) was synthesised by treating Boc-Cys-OH with acetobromogalactose in the presence of sodium hydride. Once inserted into the linear precursor H-Phe-D-Pro-Phe-Ala-Cys(β-D-galactopyranosyl)-Phe-OH, the final cyclisation was achieved using DPPA/$NaHCO_3$. Compared with cyclo(D-Pro-Phe-Ala-Ser-Phe-Phe) the S-glycosylated analogue gave the same β-turn characteristics, so it should be possible to design glycopeptides without affecting the conformation of the parent non-glycosylated pharmacophore. A series of glycopeptide mimetics based on the hydroxyethylene Phe-Phe isostere have been synthesised[189] as inhibitors of HIV-1 protease. Compound (119) proved to be the most potent ($ED_{50}$ = 0.17 nM).

Disaccharide glycosyl isothiocyanates, useful for glycopeptide synthesis, have been made[190] *via* the corresponding glycosylamines, and a route to pseudo-glycopeptides[191] *via* lithiated anions derived from N-substituted (diethylβ-keto-




(112) R = Z-X[1]-X-OBu[t]
(113) R = SEt

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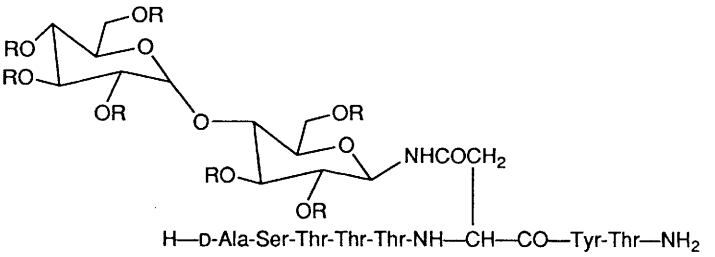
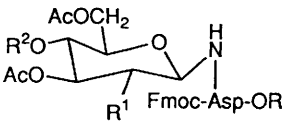
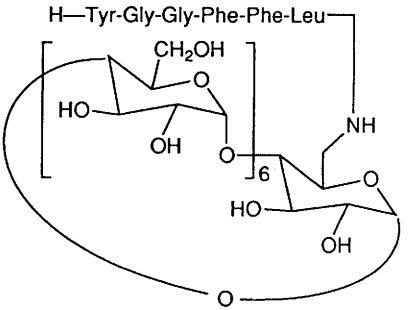


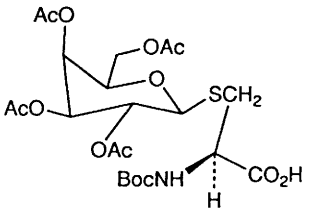

(115)



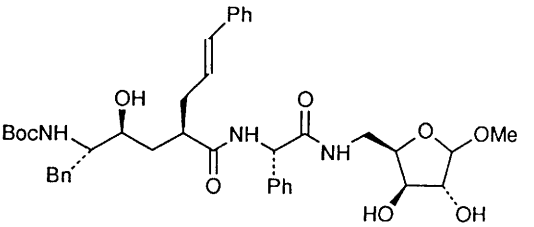

(116)



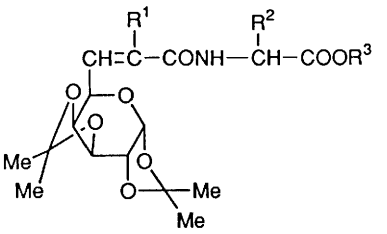

(117)



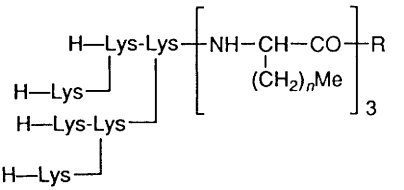

(118)



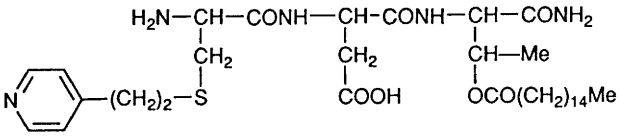

(119)



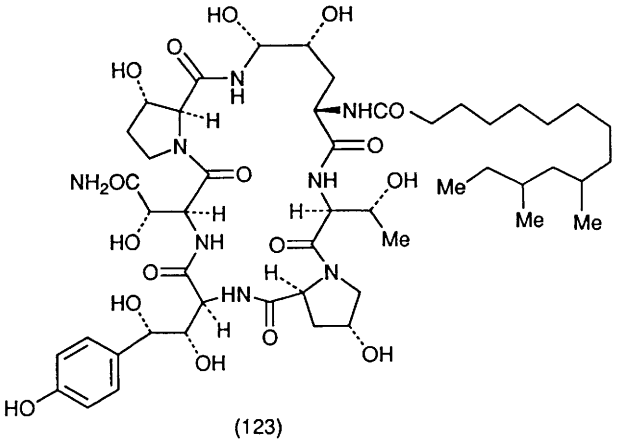
(120)



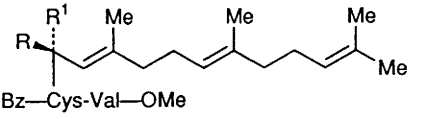
(121)



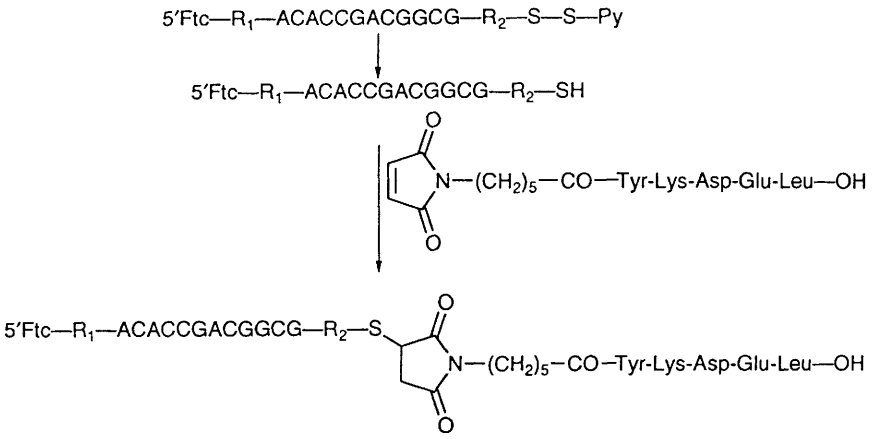
(122)



(123)



(124)


5'Ftc—R1—ACACCGACGGCG—R2—S—S—Py
5'Ftc—R1—ACACCGACGGCG—R2—SH
N—(CH2)5—CO—Tyr-Lys-Asp-Glu-Leu—OH
5'Ftc—R1—ACACCGACGGCG—R2—S
N—(CH2)5—CO—Tyr-Lys-Asp-Glu-Leu—OH

**Scheme 12**

phosphono)-α-amino acid derivatives and sugar aldehydes gives rise to structures such as (120).

### 3.4 Lipopeptides

Lipidic amino acids attached to a polylysine system to enhance lipophilicity and membrane binding effects has been shown[192] to be an excellent means of enhancing antigenicity. In the combined adjuvant and carrier system (121) different spacer-(lipidic amino acid)$_n$ systems, *e.g.* (121) n = 9, 13, R = $NH_2$, Ala-OH, Pro-OH have been synthesised using the Boc procedure in the solid phase. To keep up with the synthetic approaches to post-translational, glycosylation, phosphorylation or sulfation successful attempts have been made[193] to synthesise O-palmitoylated peptide (122) by either palmitoylation of the resin-bound peptide or incorporation of Fmoc-Thr(palmitoyl)-OH using the solid phase strategy. The latter approach was hampered by an O→N shift during Fmoc removal and the bulkiness of the palmitoyl side-chain reduced the coupling efficiency of Fmoc-Asp(OBu$^t$)-OH. Pneumocandin $B_0$I (123) can be selectively oxidised/reduced at the homotyrosine residue[194], but removal of the phenolic hydroxyl gave a > 140-fold loss of activity in 1,3-β-glucan synthetase inhibition and loss of antifungal activity. Even inversion of the C-4 homotyrosine hydroxyl causes a 70-fold decrease in potency. In order to elucidate the stereochemistry of protein farnesylation, deuterium-labelled farnesylated peptides (124) R = H, $R^1$ = D; R = D, $R^1$ = H have been reported[195]. Clear differences in the signals of the two pro-chiral hydrogens of C-1 are seen in the 500 MHz NMR spectra. Fluorescence microscopy and FACS analysis have been used to analyse[196] fluorescence at the surface of cells after incubation with a fluorescein-labelled lipopeptide. Thus using tripalmitoyl-S-glycerylcysteinyl ($Pam_3Cys$) peptides containing spin labels or fluorescent markers it could be shown that sequestering of the fatty acid chains of the lipopeptide within the membrane is an early step in the interaction.

### 3.5 Oligonucleotide Peptide Conjugate

A synthesis has been reported[197] of a conjugate containing a KDEL signal sequence, which is known to be the C-terminal sequence of proteins retained in the endoplasmic reticulum after being newly biosynthesised. Steps in the synthetic sequence for producing the conjugate are summarised in Scheme 12.

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# 5
# Current Trends in Protein Research

BY JENNIFER A. LITTLECHILD

## 1 Introduction

The field of protein research is now so great that it is impossible to cover all of the literature appearing in 1993 on this subject. The approach has been to pick some of the highlights appearing in this year. Some areas have been covered that were not addressed in the Amino acids, Peptides and Proteins Vol. 25. Since this is only the second year that proteins have been included in recent specialist reports the earlier references for many of the topics discussed below cannot be included.

## 2 Water and Proteins

Proteins have water inside them and around the outside. Protein crystals prepared for X-ray crystallography typically contain 50% water, and this accounts for their fragile nature. In fact X-ray diffraction of crystals was the first method to reveal the exact position of the water molecules within the protein as revealed by peaks of electron density. Neutron scattering studies showed the water hydrogen positions and the earlier X-ray diffraction studies, the water oxygen positions. It was observed that between one half and two ordered water molecules were found per residue in protein crystal structures. This is equivalent to about 200 water molecules for a typical protein. A careful study of buried water (not contacting bulk solvent) was made in seven homologous structures of serine proteases[1]. The buried waters form hydrogen bonds with the protein and appear to be an integral part of it. This was also observed by Kossiakoff[2,3] in neutron scattering studies of trypsin. The buried waters seem to be highly conserved whereas the water on the surface of the protein is not so conserved. It appears that deep grooves in the protein surface are formed by protein/water interactions. A study by Kuhn *et al.*[4] has looked at 56 high resolution crystal structures and concludes that ordered water is three times more likely to be in surface grooves of the protein, than elsewhere on the surface. The most accurate complete pictures of hydration in protein crystals come from very high resolution studies such as that described in 1993 for Crambin at 0.83Å resolution[5]. Pitt *et al.*[6], have reported updated software called AQUARIUS2 to predict probabilities for various possible first shell hydration sites. The prediction is based on the empirical analysis of water sites in 16 crystal structures with a resolution of $\leqslant 1.7$Å. By analysis of protein crystal structures polar amino acid side chains are found to interact more with water molecules than non polar amino acid side

chains. This means that ordered water molecules generally make hydrogen bonds with polar atoms of the protein. Two recent papers have analysed the distribution of solvent molecules around polar[7] residues and apolar residues[8].

Many critics have said that protein structure observed in the crystalline state is not the same as in solution. Clearly the crystal traps one conformation of the protein, but now that a complete structural elucidation is possible by NMR, comparisons can be made. This has convinced the sceptics that solution structure by NMR is, by and large, the same as that observed by X-ray methods. Ideally to monitor the dynamic structure of an enzyme it is better if the structure can be compared by both methods. NMR is limited to solving structures of ~20,000 daltons, however for larger proteins once the structure has been determined by X-ray methods this can be used to help interpret the NMR spectra. Comparisons of this type have been made with a mutant protein structure of the enzyme phosphoglycerate kinase[9].

The position of bound water for several proteins has previously been located by NMR techniques. Bovine pancreatic trypsin inhibitor contains four internal water molecules which have been detected by X-ray crystallography and NMR in solution[10]. In 1993 this was also examined by studying FK506 binding protein[11]. In all cases the water structure was in the same positions as that found by X-ray methods. It has also been possible to look at the surface water in some proteins by NMR[12]. These waters appear to be in rapid exchange with bulk water and have resonance times below 500 pico seconds.

In addition to X-ray and NMR methods the problem has also been tackled by studying theoretical methods[13]. In 1993 Brunne *et al.* made a comparison of experimental residence times of water molecules solvating the bovine pancreatic trypsin inhibitor with theoretical model calculations[14]. They find that their simulation agrees well with the experiment and that the average residence times for surface waters are less than 100 pico seconds. The average simulated residence times of water molecules near backbone and side chains of the protein are 39 pico seconds and 24 pico seconds respectively. Residence times near charged side chain atoms are shorter than those for other polar and non-polar atoms. These residence times are what one might estimate from diffusion.

Another molecular dynamics simulation of water around a protein was published by Komeji *et al*[15]. They examined the problem by analysing a trajectory of molecular dynamics simulation of the Trp holorepressor protein. The calculated self diffusion coefficient indicated that the solvent within 10Å of the protein had lower mobility. Examination of the solvent diffusion of different atoms around different kinds of residues showed no general trend. They found that the solvent mobility was not affected by the kind of atoms or residue solvated. Distribution analysis around the proteins revealed two peaks of water oxygen: a sharp one at 2.8Å around polar and charged atoms and a broad one at ~3.4Å around apolar atoms. The former was stabilised by water-protein hydrogen bonds and the latter was stabilised by water-water hydrogen bonds suggesting the existence of a hydrophobic shell. An analysis of protein atom-water radial distribution functions confirmed these shell structures around polar or charged atoms and apolar ones. Steinbach and Brooks[16] have used molecular

dynamics to study protein hydration in myoglobin. The results suggest that the protein is fully hydrated by about 350 water molecules which is consistent with experiments results.

Clearly both internal and surface water is important for maintaining protein structure. This issue has to be addressed when using enzymes in biotransformation reactions. Chemists like to carry out reactions in organic solvents. Proteins stable in some solvents such as the lipase enzymes will only be stable if some water is still present in the reaction mixture. The layer of surface water is clearly important to maintain the structure of the protein and internal water is important to compensate for poor steric fit of side chains and of substrates of binding sites. An interesting study reported in 1993 by the group of Klibanov[17] reported the crystal structure of the protein subtilisin in pure acetonitrile. The protein crystals were crosslinked with glutaraldehyde. The subtilisin structure was barely altered by the change in solvent, and a similar number of preferred hydration sites were occupied by water. About half of them were in the same place as in the crystal structure analysed in aqueous solvents. It is important to address the role of water in protein structure and activity, during modelling studies and the analysis of the catalytic mechanisms. A review discussing water in proteins[18] appeared in the new journal "Structure" launched in 1993.

The contribution of water to the folding and stability of proteins is discussed in a later section.

## 3 Protein Folds

The increasing number of protein structures now available in the Brookhaven data bank from both X-ray and NMR analysis reinforces the idea that proteins can be classified into distinctive folds. The two main secondary structures in proteins are the α helix and β pleated sheet. This allows proteins to be divided into four main classes: 1) mainly α, 2) mainly β, 3) alternating α/β, and 4) α + β. Two papers published in 1993 demonstrated the now well accepted idea that the structure of a protein is better conserved than its amino acid sequence[19,20]. As proteins evolve, amino acid residues mutate and if these occur in the core of the protein they can cause shifts in secondary structure orientation in order to optimise residue packing. There can also be insertions and deletions in the sequence which occur mainly in loops connecting secondary structures[21,22]. Benner and Cohen have produced empirical and structural models for insertions and deletions in the divergent evolution of proteins. The probability of a gap (deletion) was found to increase with evolutionary distance. The method of dynamic programming algorithms has been developed to make multiple structural alignments of proteins[23,24]. Several other approaches have been used to compare proteins[25-27]. These include the use of Monte Carlo based simulated annealing by Godzik *et al.*[26] to better optimise overlap between global contact maps. Artymiuk *et al.*[28] and Grindley *et al.*[29] have developed a method of compensation called POSSUM, where the secondary structure elements form the

nodes of a graph, whilst the edges represent distances and angles between secondary structures. Other approaches to this problem were published in 1993 from Bachar *et al.*[30], Orengo and Taylor[31] and Luo *et al*[32]. A relatively successful method has been used for alignment and searching for common protein folds using a data base of structural templates[33].

The structures in the Brookhaven data bank have been clustered into groups by Holm *et al.*[34] and Orengo *et al*[35]. The latter group have applied their method (SSAP) to the June 1993 release of the database and found ~100 fold families. Figure 1 shows the major fold groups observed in the four classes mentioned earlier. The $\alpha + \beta$ class is most diverse, having many complex folds which cannot easily be clustered into groups[36]. A recent paper by Chothia[37] suggests that the upper limit for the number of protein folds would be 1,000. A recent review by Prof. T. Blundell[38] discusses current protein fold comparison, classifications and recognition algorithms.

## 4 New Protein Folds

A group of peptides called trefoil peptides are found in the gastrointestinal tract. They stabilise the mucus layer and may effect healing of peptic ulcers, inflammatory bowel disease and other diseases of the intestine. The first three dimensional structure of a member of this family was described by Gajhede *et al.*[39] in 1993. This was of the porcine pancreatic spasmolytic polypeptide (PSP) which contains two trefoil domains. The domains comprise 39 amino acid residues, including six cysteine residues which form disulfide bridges. The trefoil domain is compact and is composed of a central short anti-parallel $\beta$ sheet with one short helix above and one below. The two domains are related by two-fold symmetry and each domain contains a cleft which could accommodate a polysaccharide chain. The primary structure of PSP showing the disulfide bridges is shown in Figure 2. The compact structure of the trefoil peptide explains its resistance to degradation by proteolytic enzymes and its survival in the gastric tract. The trefoil fold represents a new motif, distinct from the folds found in all other known protein structures.

In 1993 several unusual protein structures have been described. Often large proteins are folded into several domains but some have recently been reported to have unusual shapes. The pancreatic ribonuclease inhibitor resembles a horseshoe. The inner side is a seventeen stranded parallel $\beta$ sheet and the outer side a sixteen $\alpha$ helical structure[40]. This protein has leucine amino acid rich repeats in the sequence and is shown in Figure 3. These leucine rich repeats are found in a number of proteins involved in protein-protein interactions. The ribonuclease inhibitor is the first protein 3D structure solved with these repeats. The inhibitor inhibits ribonucleases and is thought to regulate RNA turnover and might have a function in the inhibition of angiogenesis. The ribonuclease inhibitor has 30 cysteine residues which in the active protein are all free. They however all form disulfide bonds in the absence of reducing agent and the inhibitor is inactivated.

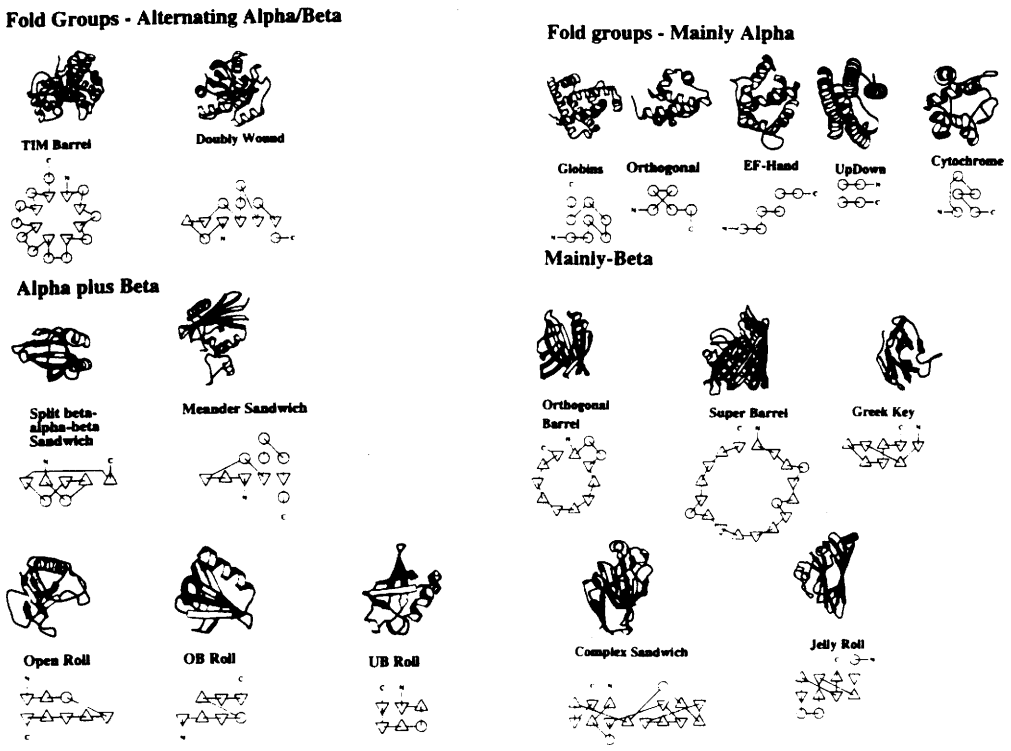


**Figure 1.** Schematic TOPS diagrams to show some of the major fold groups observed in the mainly α, mainly β, alternating α/β and α + β classes of protein (reproduced from reference 35 by permission of Oxford University Press).

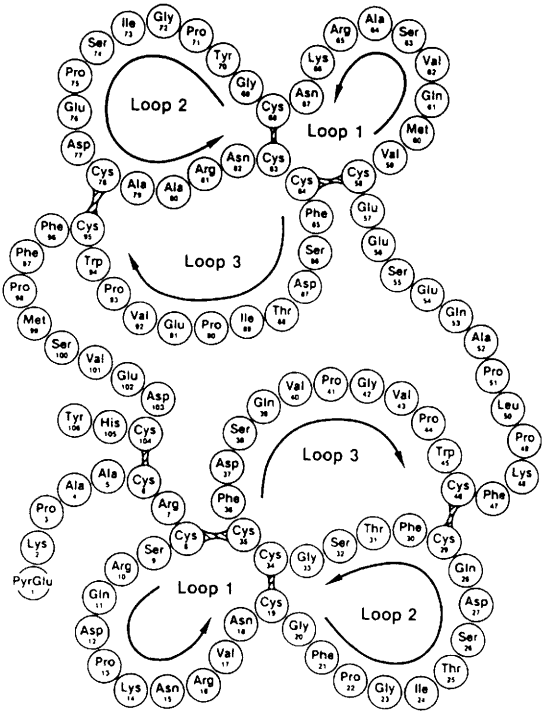


**Figure 2.** Primary structure of porcine pancreatic spasmolytic polypeptide, PSP, showing the two characteristic trefoil domains (reproduced with permission from reference 39).

The pectate lyases[41] have been found to have an unusual structure in the form of a parallel β helix motif. These are secreted enzymes which digest the buried pectate component in plant cell walls. The protein chain coils into a right handed super helix. In each turn of the β helix there are three strands of β sheet. Each strand hydrogen bonds to the equivalent strands in neighbouring turns to produce three parallel β sheets. Two of these sheets pack into a β-sandwich of distinct architecture. The side chain atoms form linear stacks that include asparagine ladders, serine stacks, aliphatic stacks, and ringed residue stacks. Figure 4a shows a schematic diagram of the pectate lyase PelC. Figure 4b shows the asparagine ladder of PelC. The side chains are orientated so that the maximum number of hydrogen bonds are formed. With exception of the first and last asparagine of the ladder, each asparagine forms five hydrogen bonds. The asparagine ladder and stack of serine residues are important to stabilise β turns of this unusual type.

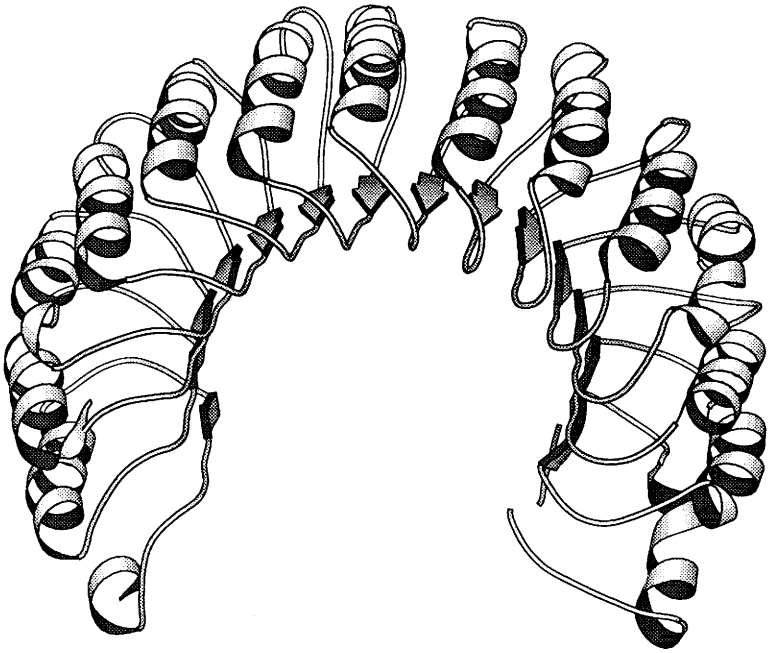

**Figure 3.** The structure of the porcine ribonuclease inhibitor [modified from reference 40 using MOLSCRIPT (Kraulis, reference 40a)].

## 5 Protein Folding and Protein Stability

How a protein folds into its three dimensional structure has received much attention in the last few years. Earlier studies and many described below concentrate on monitoring folding pathways *in vitro*. It is clear that these pathways are often not those followed *in vivo* where one finds pre-existing proteins called chaperones which assist correct folding. Some of these hydrolyse ATP and act by minimising the formation of misfolded structures. Discussion of the molecular chaperones was covered last year. Several reviews and collected articles have appeared in 1993 on this topic[42-45]. A detailed account of the folding of the small protein barnase in the presence of chaperone has been reported by Gray and Fersht[46].

Several techniques have improved our understanding of folding pathways. NMR techniques now allow the determination of the three dimensional structure of proteins but they also allow us to look at the structure denatured states of proteins and intermediates in the folding pathways. NMR data has recently been reported for the urea-unfolded forms of bacteriophage 434 repressor[47,48] and FK 506 binding protein[49]. These experiments have used two different methods. The

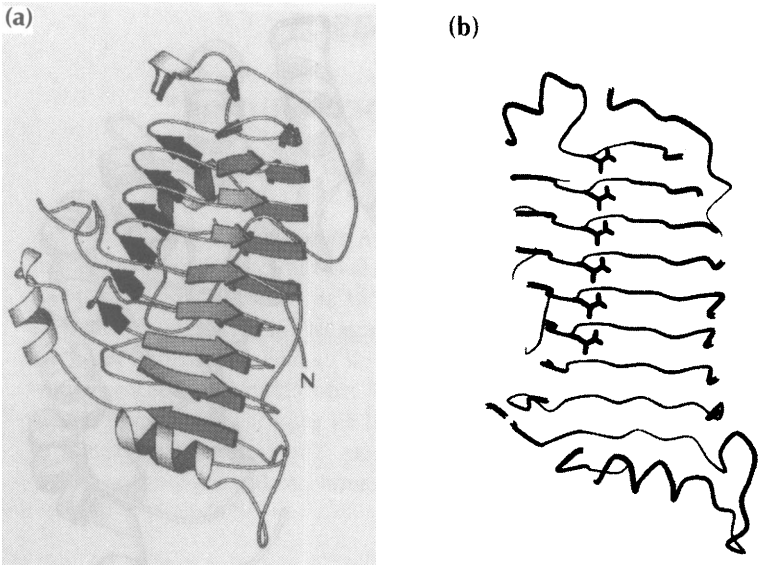


**Figure 4.** (a) Schematic figure of Pel C. Arrows represent β structure and coils α-helices. (b) The asparagine ladder in pectate lyase Pel C. The Cα chain is in grey and all side chain atoms including hydrogens are in black (reproduced with permission from reference 41).

first involved a complete assignment of the spectrum of the native state using $^{15}N$ labelled protein. At 7M urea the unfolded protein spectrum was obtained and at 4.2M urea the native and the unfolded protein were present in equal quantities. Using the spectrum at 4.2M urea the amide $^{15}N$ and $^{1}H$ assignments of the native protein were transferred to the unfolded form by exchange of heteronuclear longitudinal two-spin order in a 2D difference [$^{15}N$, $^{1}H$] correlation spectroscopy (COSY) experiment. In the second step using the 7M urea spectrum the sequence-specific assignments of the amide $^{15}N$ and $^{1}H$ resonances were extended to the complete $^{1}H$ spin systems of the individual amino acids. The work on FK 506 binding protein was carried out using a direct assignment approach with the protein labelled uniformly with $^{15}N$ and $^{13}C$ in 6.3M urea where it is fully denatured. The structure and dynamics of the acid-denatured molten globule state of α lactalbumin has been studied by 2D NMR[50]. Structural determination in denatured proteins will intrinsically be limited by conformational polymorphism.

Another approach to this problem has been to carry out NMR studies with polypeptide fragments from folded proteins with or without addition of structure inducing solvents. This has been used by Waltho *et al.*[51] with secondary structure formation by peptides corresponding to the G and H helices of myoglobin, and by Sancho *et al.*[52] with an N-terminal fragment of the protein barnase.

Many proteins have stable partially folded states which are referred to as 'molten globules'. The structural energies of α lactalbumin, cytochrome c, apomyoglobin and the $T_4$ lysozyme are reviewed as a basis to analyse the high stability of certain molten globule states and the low probability of other potential folding intermediates[53]. Spectroscopic methods such as absorbance, fluorescence and circular dichroism can be used to detect events that happen in the first few minutes of protein folding[54-57]. Khorazanizadeh *et al.*[56] used a unique tryptophan residue engineered into ubiquitin as a fluorescent probe for equilibrium and kinetic folding studies. Some proteins have a unique tryptophan residue as the case studied with the all β sheet protein interleukin 1β[58].

Two papers in 1993 report studies of protein denaturation using computer simulations[59,60]. Camacho and Thirumalai[59] use Monte-Carlo simulations for their studies which result in the proposed three distinct stages of folding which are random collapse, parallel folding pathways and the existence of a late transition state. Daggett and Levitt[60] describe the unfolding transition of bovine pancreatic trypsin inhibitor from the native to molten globule states.

Hydrogen exchange rates have been used to study protein folding. This method takes advantage of the high pH dependence of exchange and the very slow exchange of some NHs in native proteins. The slowest exchanging amides in native bovine pancreatic trypsin inhibitor are proposed to form the folding core of the protein[61]. A study relating to the rate of hydrogen exchange of amide protons in barnase and a more stable mutant containing a disulfide bond suggest that the relationship between hydrogen exchange behaviour in the native state and folding pathways is not a straightforward one[62].

Far UV circular dichroism has recently been used in a stopped flow system to study folding kinetics[63-65]. This has been used as probe for studying secondary structure in proteins, especially for α helical structures which show a strong maximum dichroism at 222 nm. Significant amounts of secondary structure is formed very rapidly in the first 2-4 milliseconds of the experiments which is much faster that the formation of the native site. Mann and Matthews[65] use this technique to study the structure and stability of an early folding intermediate of *Escherichia coli* Trp aporepressor.

A novel method using electrospray ionisation mass spectrometry in combination with hydrogen exchange labelling has been used to study the folding of hen lysozyme[66]. It has been found by Buck *et al.*[67] that a partially folded state of hen lysozyme can be obtained in trifluoroethanol which is a good mimic of the major transient intermediate in the folding pathway of this protein. A recent review of the kinetics and equilibrium folding intermediates of apomyoglobin has been published by Barrick and Baldwin[68].

Small angle scattering, experiments (SAXS) can be used to monitor transitions between the folded and denatured states of proteins. Several groups have used this approach in the last two years. Sosnick *et al.*[69] have used SAXS and Fourier transform infrared spectroscopy to study denatured ribonuclease. Kataoka *et al.*[70] have used SAXS to analyse the denatured states of cytochrome c. This study examined the salt-mediated conversion of the moderately expanded acid denatured form of cytochrome c to a molten globule. Flanagan *et al.*[71] have

monitored the changes in radius of gyration ensuing from single point mutations in a polypeptide. The results supported earlier ideas from Shortle[72] on a mutant form of *Staphylococcus* nuclease which suggested that apparent changes in the stability of a protein in response to a mutation more accurately reflected changes in the free energy difference between denatured and folded states. Mutations could affect the energies of the folded state, the denatured state or both. A paper by Calmettes *et al.*[73] has attempted to make a representation of the scattering of denatured phosphoglycerate kinase in 4M guanidinium chloride. They use a statistical model of the denatured protein which is represented by a 'string of beads'. The model has good agreement with experimental data and has the potential to accommodate atomic information.

Calorimetry is being used to study mutational effects of protein energies. Free energies are much easier to rationalise than entropies, enthalpies and heat capacities. There is little or no correlation between enthalpy and free energy changes for processes involving proteins. This was evident from the studies of Kimura *et al.*[74] who monitored the effect of thermal unfolding of ribonuclease (RNase) H1 from *Escherichia coli* resulting from the replacement of various residues with the corresponding residues from RNase H of the thermophilic bacteria, *Thermus thermophilus*. Similar results were obtained by Varadarajan *et al.*[75] using isothermal titration calorimetry to look at heat capacity changes for the protein-peptide interactions in the ribonuclease S system. Tanaka *et al.*[76] have studied the thermal unfolding of *Staphylococcal* nuclease and several mutants of this enzyme by differential scanning calorimetry. A triple mutant enzyme caused slight stabilisation amounting to 0.3 kcal $mol^{-1}$ but a huge decrease in enthalpy of 51 kcal $mol^{-1}$ and in entropy of 161 kcal $mol^{-1}$.

An interesting study of Liang *et al.*[77] has been reported where the genetic fusion of subunits of a dimer protein (gene V) substantially enhanced its stability and rate of folding. Herning *et al.*[78] have made a detailed study of the role of proline residues in human lysozyme stability combining scanning calorimetry with X-ray studies of mutants. Differential scanning calorimetry has been used to study the thermal unfolding of mutant forms of T4 lysozyme[79]. This study resulted in a difficulty in accounting for the changes in thermodynamics produced by a single amino acid mutation in the protein studied.

The last two or three years have provided papers which show that hydrogen bonds play a large part in protein stability[80,81]. Privalov and Makhatadze[82-83] examined the contribution of hydration to protein folding thermodynamics by looking at the entropy and Gibbs energy of hydration. Berndt *et al.*[84] have genetically modified bovine pancreatic trypsin inhibitor to eliminate one of the four internally bound water molecules. This lead to a small decrease in stability and in the free energy of unfolding.

Studies of the importance of hydrophobicity to protein stability were discussed briefly in Vol. 25 of this series. The contribution of the hydrophobic effect is discussed in an article by Pace[85]. Spolar *et al.*[86] have liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of non-polar and polar surface water. The consequences of hydrophobic replacements in the case of T4 lysozyme have

recently been studied by Eriksson *et al.*[87] Kim *et al.* have reported stability changes and new hydrophobic surfaces by cervice forming mutations of bovine pancreatic trypsin inhibitor[88]. Apart from the cervices the mutants caused few changes observable by X-ray crystallography but all were destabilising.

Some of the experiments above have been carried out with genetically engineered proteins. Clearly the ability to introduce amino acid changes which do not alter the structure and can act as reporter groups offers a lot of potential. The specific addition of tryptophan residues can be used as reporter groups to monitor fluorescent changes whereas chemical activity of cysteine residues can also be used. This latter approach has been studied using the enzyme phosphoglycerate kinase[89] to characterise intermediates in the folding pathway. Martensson and Jonsson[90] have used the introduction of genetically engineered cysteines into the protein carbonic anhydrase II to analyse the solvent exposure of different regions of the folding intermediate of this protein. Disulfide bonds in proteins can be used as a probe to monitor protein folding. This approach has been reviewed by Creighton[91] who has carried out much of this work. In 1993 he published a paper using mutants of bovine trypsin inhibitor to analyse the role that different disulfide bridges play on the folding of this protein[92]. A detailed study of the folding pathway of the small protein barnase has been carried out by Clarke and Fersht[93]. They have used structurally non-essential disulfide bonds to probe protein folding. In this case disulfide bond formation is not required for protein folding and so one can perform a kinetic analysis of the disulfide and corresponding dithiol proteins. When the engineered disulfide bond is located in a region of the protein that folds early in the kinetic pathway, it results in the stabilisation of the kinetic intermediate. Whereas if it is located in a region that does not fold until the end of the folding reaction, it stabilises the protein late in the reaction.

Double mutant cycles can be used to detect the interaction between two amino acid residues during protein folding[94]. The method consists of localising two residues which are interacting in the folded protein and mutating them both independently and simultaneously. The differences in free energy of unfolding are measured with respect to the wild type protein. This method has been used for more complicated interactions involving several residues[95]. This has revealed the existence of cooperative interactions in certain regions of the kinetic intermediate of a protein. Application of physical organic chemistry has been used to study the effect that point mutations could have on the folding pathway of a protein[96]. This used the Hammon postulate that states that if two consecutive states in a reaction are close in their energy content then interconversion requires a small reorganisation of the structure. The results emphasise the importance of engineering small conservative mutations that will not destabilise the structure of the protein. A detailed analysis of the folding reaction of barnase studied by protein engineering and quenched-flow NMR methods results in very similar pictures for the structure of the folding intermediates of this protein[97].

These many protein folding studies indicate that the kinetic intermediate of the protein is a weakened hydrophobic core probably with some trapped water molecules. It appears that the majority of the secondary structure of the protein is

present. Some small subdomains and long loops are unfolded and many tertiary interactions are broken. It is still unclear how the kinetic intermediates are similar to 'molten globules'. A review of the 'molten globule' state has been recently written by Haynie and Freire[98]. Kinetic intermediates show cooperativity in the regions of the protein that are still folded and for many regions the population of the intermediate is quite homogeneous, whereas 'molten globules' show little cooperativity and many non-native conformations exist.

It is clear that despite all these studies we are only beginning to understand protein folding and protein stability.

## 6 New Protein Structures

### 6.1 Elongation Factors

The year 1993 has given us information on two elongation factor proteins both crystallised from thermophilic sources. The first is the elongation factor EF-Tu from *Thermus aquaticus*[99]. This is a GTP binding protein that is crucial for protein biosynthesis. In the GTP form of the protein it binds tightly to aminoacyl tRNA which interacts with the ribosome. During this interaction GTP is hydrolysed and EF-Tu-GDP is released. The crystal structure of the Tu complexed to the GTP analogue GDPNP has been determined to 2.5Å resolution and when compared to the earlier published structure of the enzyme from *Escherichia coli* EF-Tu-GDP[100] shows large conformational changes. This shows that proteins are dynamic structures and emphasises the importance of determining their structures with different ligands bound. The large conformational change that occurs (a rotation of 90.8° of domain 1 relative to domains 2 and 3) exposes the tRNA binding site. The structure of the EF-Tu from *Thermus thermophilus* has also been described to 1.45Å resolution and in this case complexed with the analogue GppNHp[101]. This structure also shows major changes to the *Escherichia coli* EF-Tu-GDP structure. The hole between domains 1 and 3 found in the inactive EF-Tu-GDP complex disappears. The changes observed between the two structures leads to differences in atomic position of up to 42Å. The structure of EF-Tu-GppNHp is shown in Figure 5.

### 6.2 Protein–Nucleic Acid Complexes

Several important structures have been reported in 1993 where a protein is complexed with a nucleic acid fragment which contains its specific binding side.

*6.2.1 Transcription Factors.* – The first of these is the oestrogen receptor complexed with its DNA binding domain[102,103]. The oestrogen receptor regulates gene expression by binding to DNA response elements associated with their target genes. The receptor protein has two domains, (see Trends in Protein Research, Vol. 25). These are made of a strongly conserved DNA binding domain containing two zinc binding motifs and an N-terminal hormone binding domain. The protein acts as a dimer with each monomer recognising a six base pair half site on the DNA. The protein makes base specific contacts with the

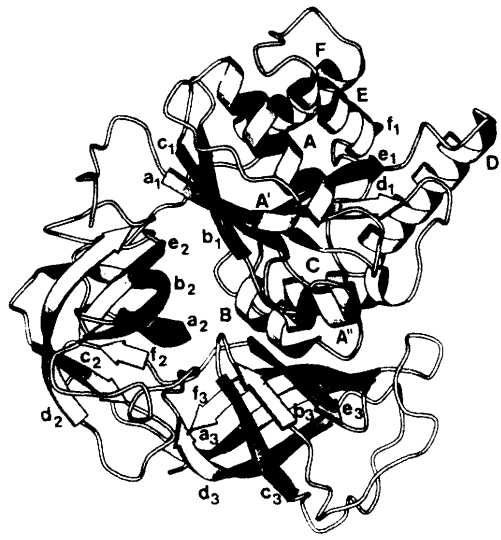


**Figure 5.** The conformation of EF-Tu when complexed with GppNHp. Domain 1 is at the top, domain 2 on the left and domain 3 at the bottom. The helices and the β strands are labelled with capital and small letters respectively. The subscripts denote the domain to which the β strands belong (reproduced with permission from reference 99).

DNA, consisting of a network of hydrogen bonds to the base pairs and to ordered water molecules that are in turn hydrogen bonded to base pairs. The two papers show how the oestrogen receptor domain recognises its own half sequence rather than that of the related glucocorticoid receptor which differs by only two base pairs.

Another transcription factor DNA-binding domain structure is described for the GATA-1 factor which is involved with the generation of the erythroid lineage. This structure has been solved by NMR techniques[104]. The protein fragment of GATA-1 in this study consists of a single zinc binding site and a C-terminal tail, which constitutes the minimal requirement for specific binding. The zinc is co-ordinated by four cysteines. This is a class IV zinc-dependent binding protein and this is the first tertiary structural data for a molecule of this class. The protein core is composed of two irregular antiparallel β sheets and an α helix with a long loop leading into the extended C-terminal tail. The DNA oligonucleotide is B form with the central eight base pairs which make up the target site making contact with the protein. The loop and connecting two sheets of the protein interact with the major groove of the DNA and the C-terminal tail wraps around in the minor groove. There is some structural similarity with the core of the GATA-1 binding protein and the N-terminal zinc module of the glucocorticoid receptor, however the mode of DNA binding is very different. This demonstrates

the subtle changes that can alter the specificity of molecular recognition between proteins and DNA.

The eukaryotic transcription factor HNF-3γ has been co-crystallised with its DNA binding region[105]. HNF or hepatocyte nuclear factor protein was first identified as an activator of liver specific gene expression in the rat. The monomer structure of HNF forms a compact α/β structure consisting of three α helices and three β sheets. The molecule binds to B-DNA through both the DNA backbone and direct water-mediated major and minor groove base contacts. In the N-terminal half, three helices form a compact structure that presents the third helix to the major groove. The remainder of the protein includes twisted, antiparallel β-structure and random coil that interacts with the minor groove. The helical region is similar to the 'helix-turn-helix' proteins. The factor is also structurally similar to the unrelated DNA-packaging histone H5 globular domain, and N-terminal DNA binding protein BirA, the *Escherichia coli* repressor of the biotin operon. The structure of HNF-3γ DNA complex is shown in Figure 6.

Another fascinating structure is that of the GCN4, basic region leucine zipper/DNA, ATF/CREB-site complex[106]. The gene regulating protein GCN4 binds to a seven base pair consensus DNA sequence from which it induces amino acid biosynthesis in yeast. The C-terminal half of the basic region leucine zipper motif of GCN4 forms a parallel coiled-coil of α helices with a core of interdigitating hydrophobic amino acid residues. The N-terminal basic region forms a continuation of each C-terminal α helix accommodated in the major groove of its half site on opposite sides of the DNA double helix. This structure is compared to a similar complex structure described for GCN4 bound to AP-1 site with a DNA sequence ATGACTCAT[107]. The ATF/CREB site in the latest complex is ATGACGTCAT. The protein structure is similar in both complexes

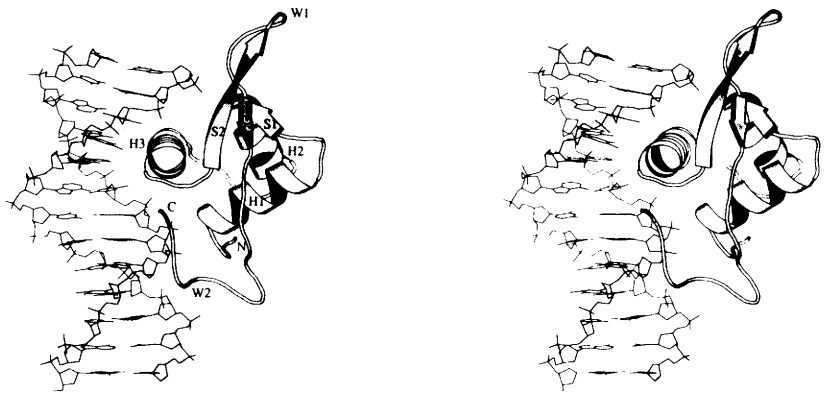


**Figure 6.** Structure of the HNF-3γ Transcription factor–DNA complex. The DNA is drawn as a stick figure (reproduced with permission from reference 105).

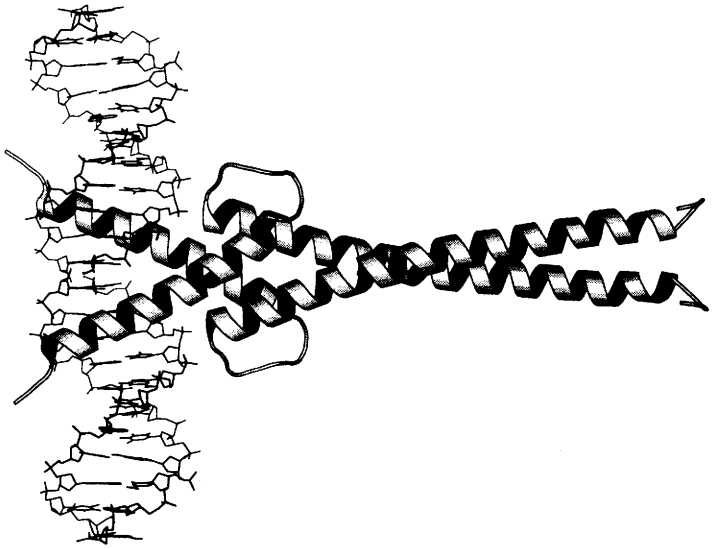

**Figure 7.** A schematic view of the Max protein homodimer and its interaction with DNA (reproduced with permission from reference 108).

and to that described for the unbound protein. The DNA conformation however differs between the two complexes. The DNA with the additional G-C base pair is bent symmetrically by 20° at the major groove toward the leucine zipper. The flexibility of the DNA allows the same protein contact to occur in both protein/DNA complexes.

An important Max protein has been co-crystallised with its DNA complex[108]. This is required for malignant transformation by Myc oncoprotein. The HLH transcription factors are important in regulating metabolism, cell differentiation and development in many eukaryotic cells. Two amphipathic helices are responsible for dimerisation of the protein while a conserved b region is required for binding DNA by HLH dimers. The DNA binding domain of Max (b/HLH/2) forms a symmetric homodimer and is shown in Figure 7. Each monomer consists of two α-helical segments separated by a loop. The N terminal helix includes the basic and first helix whereas the second helix comprises the second helix of HLH and the leucine zipper. The two basic regions extend from the four-helix bundle and enter the major groove of DNA similar to that observed in the yeast transcriptional activator GCN4. The two zipper regions are also similar to GCN4. The B DNA duplex in the crystal structure is straight.

The Trp repressor from *Escherichia coli* and its operator half site structure have been solved by X-ray methods[109,110]. This protein represses the transcription of genes related to tryptophan biosynthesis by binding to a promoter region

on the DNA. L-tryptophan is required as a co-repressor. This system has been known for many years and a knowledge of the protein DNA interaction is important to understand how this type of repressor carries out its role of controlling gene expression. The repressor protein is a stable twofold symmetrical protein with each subunit containing a helix turn helix DNA binding motif and these interact with successive major grooves of the DNA. A previously solved trp repressor DNA complex contains one repressor dimer bound to a DNA duplex containing two operator 'half site' sequences. An alternative DNA duplex was found to bind two repressor dimers per DNA duplex with high cooperativity. The biological significance of the two modes of binding is uncertain. In the new structure each repressor binds to the central half-site of the DNA duplex and to a half-site formed at the junction of the DNA duplexes. The binding of the different complexes are shown in Figure 8 parts (i) and (ii). The tandem binding in the latest study might explain the binding of repressor to the imperfect palindromic repeats found at eight base pair intervals in the natural trp operator sequences.

6.2.2 *TATA-box Binding Polypeptide.* – Two papers describe the very interesting TATA-box binding polypeptide complexed with the TATA box DNA. The importance of this structure is for initiation of gene transcription by RNA polymerase. The first X-ray structure reports the complex of binding polypeptide from *A. thaliana* with the TATA box element of the adenovirus major late promoter[111]. The protein is a 10 stranded antiparallel β sheet which folds into a saddle like structure with four α helices on its upper surface. The complex shows that the protein undergoes a modest conformational change on DNA binding allowing it to follow the minor groove of the distorted DNA duplex. The conformation of the DNA is grossly distorted. Binding of the protein to DNA has provided clues to the mechanism of the first step in transcription. The second structure is that of the yeast polypeptide with the TATA box of CYC1 promoter[112]. This structure shows very similar features to the first complex described above.

*6.2.3 HIV Reverse Transcriptase Complex.* – The structure of reverse transcriptase from HIV has been solved co-crystallised with a DNA template primer/Fab complex[113]. Information on the binding of this protein to DNA is important for the design of inhibitors of the enzyme. In retroviruses such as HIV, reverse transcriptase is the enzyme required to copy the single-stranded viral RNA into duplex DNA for integration into host cell chromosomes. The general structure of the HIV-1 reverse transcriptase enzyme is similar to that previously reported for the reverse transcriptase-nevirapine complex[114]. However the folding of the four individual polymerase subdomains, named finger, palm, thumb and connection, is similar in p66 and p51 (see Trends in Protein Research, Vol. 25) but the spatial arrangements of the subdomains is very different. The DNA template primer is A form near the polymerase active site and B form near the RNase H site. The

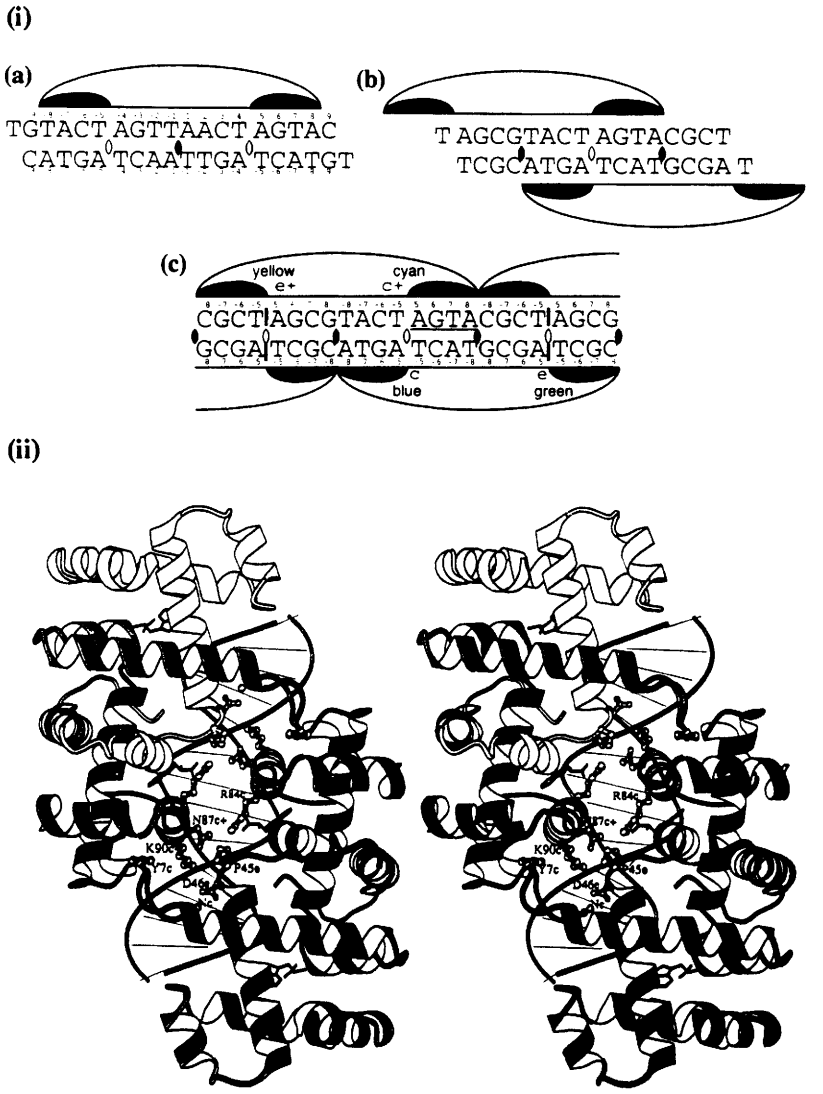


**Figure 8.** (i) Schematic of the structure of the trpR complex (ITRO). The 19-mer DNA duplex sequence, and the repressor duplex with the two symmetrical reading heads, are represented as block slivers. The filled and open dyad symbols represent repressor dimer and tandem two fold axes, respectively (reproduced with permission from references 109,110). (ii) The two repressor molecules bound to the central site viewed down the tandem dyad axis.

3'-hydroxyl of the primer terminus is close to the catalytically essential three aspartic acid residues of the p66 palm and is in a position for nucleophilic attack on the α phosphate of an incoming nucleoside triphosphate. The α helix of the p66 thumb makes contact with the sugar-phosphate backbone of the primer strand. The adjacent anti-parallel helix α1 makes contact with the sugar-phosphate backbone of the template strand. It is hoped that AIDS therapies will be enhanced by a fuller understanding of drug inhibition and resistance resulting from these studies.

*6.2.4 Restriction Enzymes.* – The type II restriction endonuclease *Eco*RV has been studied bound to its cognate and non-cognate DNA fragments[115]. Two type II restriction endonucleases that cleave double-stranded DNA at defined regions have been studied in detail. These are *Eco*RV and *Eco*RI which are both active as dimers and catalyse a similar reaction. The *Eco*RI appears to have little sequence or structural homology to *Eco*RV. The structure of the *Eco*RV monomer and the dimer complexed with DNA is shown in Figure 9. In the *Eco*RV endonuclease cognate DNA complex the enzyme has the same structure as the free protein. This appears to be the same also in the non-cognate DNA complex. Each protein monomer is made up from a small domain that is involved in dimerisation linked in a flexible fashion to a large domain that carries all the residues for DNA binding and recognition. The orientation of the two domains of the protein is different in the free enzyme from that found in the protein/DNA complexes. In the cognate DNA complex there is a kink of 50° at the centre TA (recognition sequence GATATC) with a compression of the major groove. The contacts between the protein and the DNA are by two short loops. This does not resemble any other known DNA-recognition motifs. One of these loops is disordered in the free enzyme and non-cognate complex. An arrangement of two aspartic acid residues close to the reactive phosphodiester group probably provides ligands to the essential cofactor magnesium. This arrangement, together with a lysine and a β strand are also seen in the otherwise unrelated *Eco*RI endonuclease. This demonstrates that even functionally related enzymes can carry out a similar reaction with a completely different protein motif except for the conservation described above.

*6.2.5 Klenow Fragment.* – The Klenow fragment from *Escherichia coli* DNA polymerase has been crystallised with its DNA fragment[116]. The Klenow fragment is the C terminal portion of the polymerase enzyme which has the polymerase activity and the 3'-5' exonuclease activity. The Klenow fragment when complexed to DNA has an area which becomes more ordered and a movement of 12 Å in this region produces a cleft where the DNA is bound. The contacts between the protein and duplex DNA are made exclusively through protein interactions with the DNA phosphate. This complex has produced new ideas regarding the mechanism of the polymerase enzyme. It suggests that the primer strand approaches the polymerase active site from the direction of the exonuclease domain and that duplex DNA product might bend to enter the polymerase site.

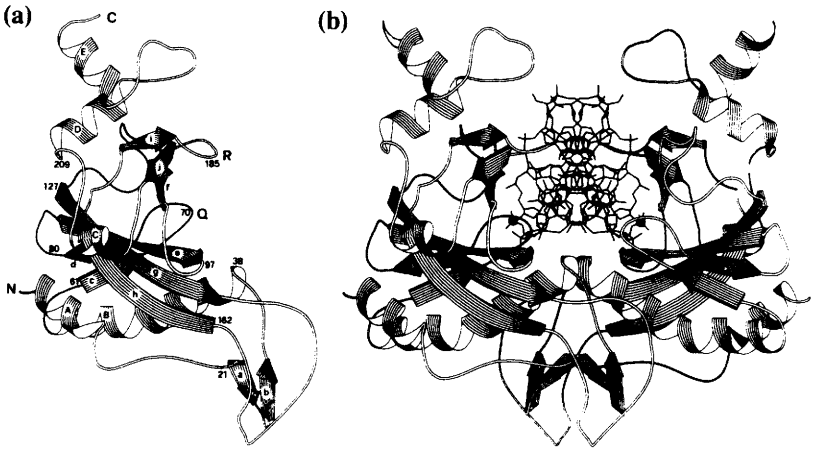


**Figure 9.** Ribbon diagrams showing the monomeric structure of a) *Eco*RV and b) the dimer with bound DNA (viewed down the axis of the DNA). Q and R mark the location of the two loops involved in DNA recognition (reproduced with permission from reference 115).

*6.2.6 Methyltransferase.* – DNA methyltransferase structure has been elucidated by Cheng *et al*[117]. This enzyme catalyses the transfer of a methyl group from S-adenosyl-L-methionine to adenine or cytosine residues of DNA. The function of this methylation in prokaryotic systems is in the restriction-modification system to recognise foreign DNA. In eukaryotic cells its role is thought to be in gene regulation. The enzyme has a large and a small domain separated by a hinge region as shown in Figure 10. The cofactor binds to the large domain close to the DNA binding cleft and has a structure different to that in the metJ repressor but similar to a NAD-binding motif. It is postulated that a loop with a Cys residue crucial for activity moves closer to the cofactor when DNA is bound. To verify this we must wait for the structure determination of the enzyme complexed with DNA.

## 6.3 Receptor Protein Complex

An important structure has been reported by Banner *et al.*,[118,119] where the tumour necrosis factor-β (TNFB) has been co-crystallised with a 55KD receptor (SSR), soluble fragment complex. TNF-β is one of the two factors secreted by the lymphocytes that can mediate inflammatory responses and induce necrosis of tumours. The receptor part of this complex is made up of a striking cysteine repeat motif, arranged end to end with little overlap. This very elongated protein has disulfide bonds arranged like rings on a slightly twisted ladder. Three SSR molecules are found with one TNF-β trimer, with each of the receptors interacting with two subunits of the ligand trimer. The receptor molecule binds to the bottom of the ligand trimer in a shallow groove. The three receptor molecules

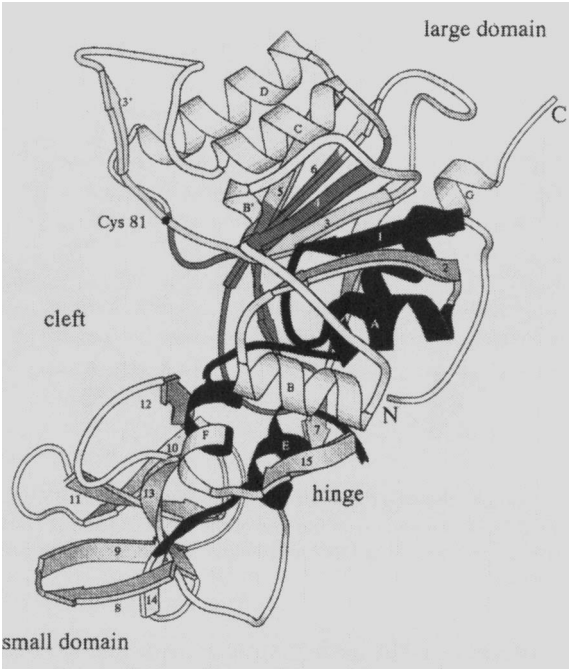


**Figure 10.** Ribbon showing the domain organisation of *H. haemolyticus* DNA methyltransferase (reproduced with permission from reference 117).

in the complex do not appear to interact with each other. Although the overall structure of the ligand does not change on receptor binding, two significant changes do occur on surface loops. These studies illustrate the unusual way proteins can be made to form elongated structures using disulfide bonds and the subtle yet specific interaction of TNF-β with such a structure that can in turn mediate changes in the cytoplasmic region of the receptor involved in signal transduction.

### 6.4 Visual Protein

Recoverin[120] is a calcium binding protein that mediates the activation of retinal rod guanylate cyclase, which in turn decreases the photosensitivity of cGMP phosphodiesterase and switches cGMP gated channels to high affinity. This cascade restores the dark state following visual excitation. Recoverin belongs to a family of proteins which include calmodulin, troponin C and visinin from retinal cones. The protein has a myristoyl moiety covalently attached to the N-terminus. Calcium and myristoylation are believed to regulate in a coordinated manner where calcium induces myristoylated but not unmyristoylated recoverin to bind to lipid bilayer membranes. Recoverin folds compactly into two domains separated by a narrow cleft, each domain contains a 29 residue helix-loop-helix

motif. Recoverin has an additional helix at the C and N termini when compared to calmodulin. The calcium coordination is extremely similar in the two structures.

## 6.5 Muscle Proteins

*6.5.1 Calmodulin.* – The complex of calmodulin with a peptide complex has been reported[121]. This study suggests that the role of calcium is to organise and stabilise domain structure and so restrict the available conformational states of the protein to those most favourable to target recognition and activation. A number of mutant proteins of calmodulin have been constructed by genetic engineering which have deletions in the central helix. A structural study has been carried out on one of these mutants[122] (DES-Glu84). Comparisons of the mutant structure to the native calmodulin supports the idea that the central helix functions as a flexible tether as shown in Figure 11. In this way the calmodulin can regulate in a calcium dependent manner the activities of numerous cellular

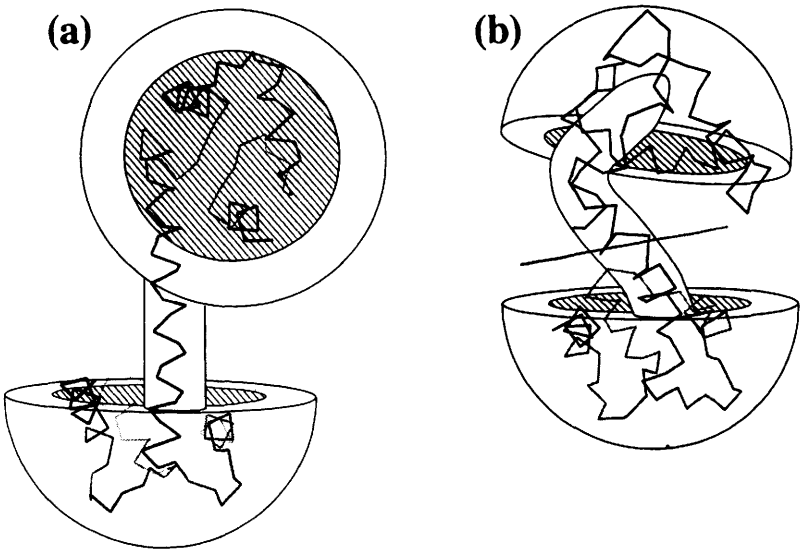


**Figure 11.** Diagrams showing the relative positions of lobe 1,2; linker; and lobe 3,4 of calmodulin. The hatched portion of the surface represents the hydrophobic patch which is on the opposite side and approximately 20Å from the two calcium-binding loops on the other surface. a) The crystal structure of the native calmodulin. The central linker region of the central helix is represented by a rod. b) The crystal structure of the des-Glu 84 calmodulin. The axis of approximate twofold rotation relating lobe 1,2 and lobe 3,4 is indicated. The central helix is bent by 95° (reproduced with permission from reference 122).

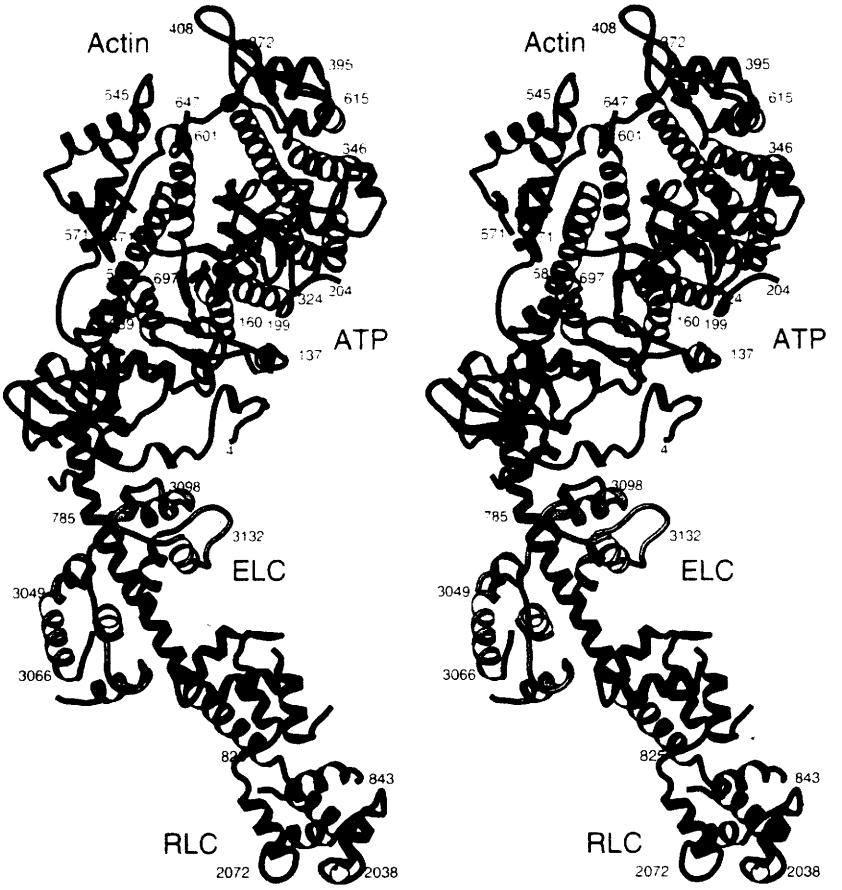


**Figure 12.** Ribbon diagram showing the myosin S1 heavy chain. Residue numbering from the regulatory light chain begins at 2001 and for the essential light chain at 3001 (reproduced with permission from reference 125).

enzymes. The mutant enzyme is still able to activate skeletal muscle myosin light chain kinase, NAD kinase and calcineurin (protein phosphatase).

*6.5.2 Myosin.* – Vital information has recently been obtained by the elucidation of the structure of the muscle protein, myosin subfragment 1 by Rayment *et al*[123,124]. Myosin subfragment-1 (S1) forms the crossbridges between the myosin thick filament and actin thin filaments. These filaments slide over one another during muscle contraction. A new crystal form of S1 which diffracted to 2.8 Å resolution was obtained after reductive methylation of most of S1's 102 lysine residues. The methylated S1 remains active as an actin-stimulated ATPase. The

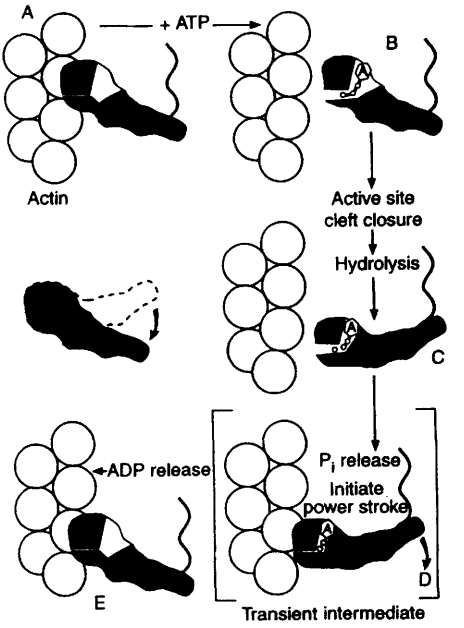


**Figure 13.** Schematic representation of the sequential changes in the actin cleft and the ATP-binding pocket of the whale-shaped myosin head as a plausible framework for progression of the contractile cycle. Protomers of F-actin are shown as spheres. The narrow cleft that splits the 50kDa segment of the myosin heavy-chain sequence into two domains is represented as a horizontal gap perpendicular to the filament axis. This representation of the nucleotide-bound state and the associated conformational changes relative to the X-ray structure of myosin is conceptual in nature (reprinted with permission from reference 124. © 1993 American Association for the Advancement of Science).

S1 molecule is a highly asymmetrical molecule as shown in Figure 12. The nucleotide binding pocket is located at the end of a seven-stranded mostly parallel β sheet. The topology of the GXXGXGX nucleotide binding motif is the same as that found in Ras protein and adenylate kinase. The X-ray structure of myosin S1 has been fitted into electron microscopy reconstructions of native ATP-free (rigor) complexes where actin is saturated with attached myosin heads. The schematic representation of the sequential changes in the actin cleft and the ATP-binding pocket of the 'whale-shaped' myosin head, as proposed by Rayment *et al.*,[124] is shown in Figure 13. The discussion of the structure of myosin S1 and its function as a molecular motor has been the subject of an article in Current Opinion in Structural Biology[125]. This work has significantly increased our understanding of actin-myosin interactions in muscle contraction.

*6.5.3 Actin/Gelsolin.* – The crystal structure of the actin/gelsolin segment 1 complex has been reported by McLaughlin *et al*[126]. Gelsolin is a member of a family of proteins that regulate actin filament organisation through their ability to fragment them. On calcium activation, gelsolin randomly severs an F-actin filament and remains tightly bound to the newly created (+) end. Certain *polyphosphoinositides* can dissociate this gelsolin cap and thus allow filament growth. The structure of actin remains virtually unchanged on binding to segment 1.

*6.5.4 Other Actin Complexes.* – Actin is also found in non-muscle cells where the assembly and disassembly of actin to filamentous actin (F-actin) is believed to be a key feature of cell structure and mobility. Several actin binding proteins are known including DNase, profilin and thymosin β4. The structure of the actin/profilin structure has been reported by Schutt *et al*[127]. Profilin is thought to have a role in the assembly of actin filaments. Although profilin and actin form a 1:1 complex, in solution crystals of the complex show a tendency to assemble further into filamentous-like structures.

## 6.6 Kinases

Several kinase structures have been reported in 1993. These enzymes catalyse the transfer of a phosphate from a nucleotide (typically ATP) to another substrate. The catalysis which is usually rapid follows a direct, in line transfer mechanism and requires the binding of both MgATP and a substrate. In several kinases substrate binding is accompanied by significant structural changes in the protein. The structure of two forms of the c-AMP-dependent protein kinase (cAPK) have been reported recently. The first structure is the structure of the enzyme complexed with a peptide inhibitor[128]. This represents an open conformation of the enzyme. The N-terminal of the pig enzyme has a myristylate moiety which has allowed the previously unresolved N-terminal region of the protein to be seen. On addition of MgATP the ternary complex is formed which results in a closed form of the enzyme. The major difference between the open and closed conformation is the rotation by 15° of a small lobe relative to the large lobe which results in the opening up of the active site cleft. Crystal structures of the catalytic domain of recombinant mouse cAPK as either binary or ternary complexes both adopt a closed conformation[129]. The crystal structure at 1.6 Å resolution of the thermophilic phosphoglycerate kinase enzyme was reported from the bacterium *Bacillus stearothermophilus*[130]. There are now 5 structures available for this kinase enzyme and all of them represent the 'open' form of the enzyme. If the *B. stearothermophilus* structure is superimposed on the structure of the enzyme from horse or yeast the main difference is that the domains are 4.2° closer together. This still makes the substrate binding sites for ADP and 3-phosphoglycerate which have been determined crystallographically too far away from each other for phosphoryl transfer. The structure of the *B. stearothermophilus* phosphoglycerate kinase and bound ADP is shown in Figure 14. The structure of glycerol kinase has been reported by Hurley *et al.*[131] complexed with phosphocarrier protein $III^{Glc}$. The phosphoenolypyruvate glycose phospho-

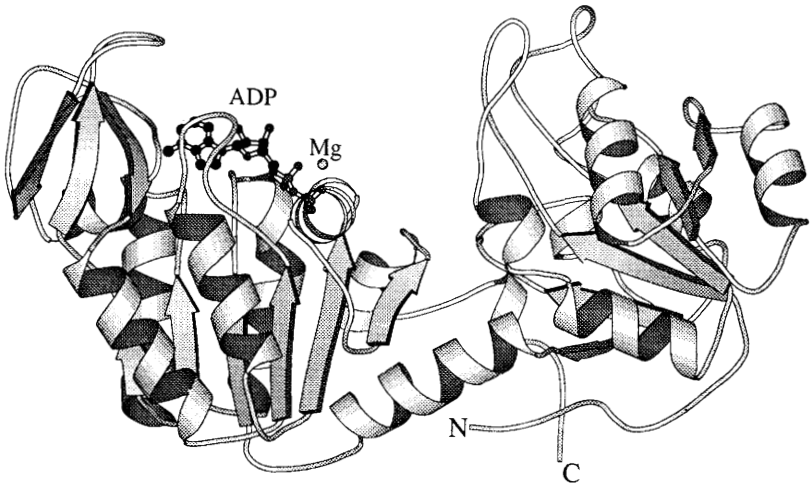


**Figure 14.** Ribbon diagram showing the structure of *B. stearothermophilus* phosphoglycerate kinase (PGK). The nucleotide substrate atoms are shown in 'ball-and-stick' representation.

transferase system (PTS) catalyses the transport and phosphorylation of a number of simple sugars. $III^{Glc}$ is a PTS protein specific for the uptake and phosphorylation of simple sugars. Glycerol kinase (GK) has two large domains with a deep narrow cleft between them. Glycerol binds at the bottom of the cleft and ATP above it. The only structural change of GK on ADP binding is the rotation of a specific arginine residue to interact with the pyrophosphate moiety of ADP. The first eleven residues of one molecule of $III^{Glc}$ bind to a GK monomer in extended conformation. This binding site is more than 30 Å from the catalytic site of GK, ruling out a direct short range effect. It is concluded that $III^{Glc}$ might destabilise one or more of the conformations of GK in the reaction pathway.

### 6.7 Proteases

Human immunodeficiency virus (HIV) protease has been the subject of many studies since it is essential to the viral life cycle and is responsible for catalysing the production of individual structural proteins and enzymes from polyprotein precursors. It is for this reason that inhibitors of this enzyme have received considerable interest in the treatment of AIDS. The different types of HIV have different proteases and some inhibitors are active against both types whilst others are specific for the protease from HIV-1 and vice versa. In 1993 Tong *et al.*[132] described the crystal structure of HIV-2 protease in a complex with a reduced amide inhibitor which is also active against HIV-1 protease. Figure 15 shows the hydrogen bonding interactions between the inhibitor and the HIV-2 protease dimer. Another complex between HIV-2 protease and two transition

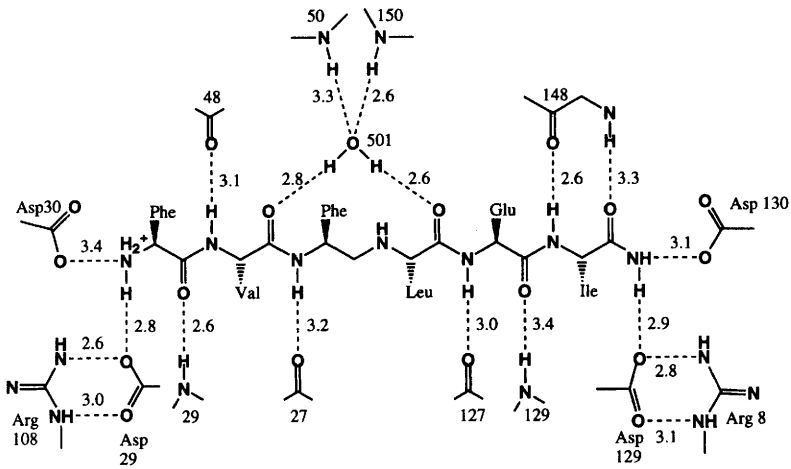


**Figure 15.** The hydrogen bonding interactions between the BI-LA-398 inhibitor and the HIV-2 protease dimer (reproduced with permission from reference 132).

state analog inhibitors has been described[133]. A comparison of the complex with HIV-1 protease has allowed an explanation of the 30 fold smaller, $K_i$ observed for this latter protease.

The structures of human cathepsin D in its native and inhibited form have been described by Baldwin *et al*[134]. Cathepsin D is an aspartic lysosomal protease thought to have an important role in protein catabolism, antigen processing, degenerative disease and breast cancer progression. Two features distinguish cathepsin from other members of the pepsin family. These are that cathepsin D is found as a two chain form in the mature protein and second it contains a phosphorylated, N-linked oligosaccharide that targets the protein into lysosomes *via* mannose-6-phosphate receptors. Correlations can be made between the $K_i$ values observed with cathepsin D for pepstatin and those slightly higher for rhizopuspepsin-pepstatin complex. The substrate binding cleft in cathepsin D is wider than in human renin. This information will help determine the best strategies for fine tuning the specificity of cathepsin D inhibitors. Metcalf and Fusek[135] have shown the schematic representation of cathepsin D and pepstatin A represented in Figure 16.

The structure of a glutamic acid specific protease[136] has been determined which uses a novel histidine triad in substrate binding. This is classed as a small bacterial serine protease which has a high specificity for cleavage next to glutamic acid residues. The structure is similar to *Streptomyces griseus* protease A and B and broadly similar to other serine proteases. Binding of the peptide analogue Boc-Ala-Ala-Pro-Glu-p-nitroanilide to the enzyme shows the substrate carboxylate to be bound to the histidine side chain and two serines. A mechanism for charge compensation involving a novel histidine triad as shown in Figure 17.

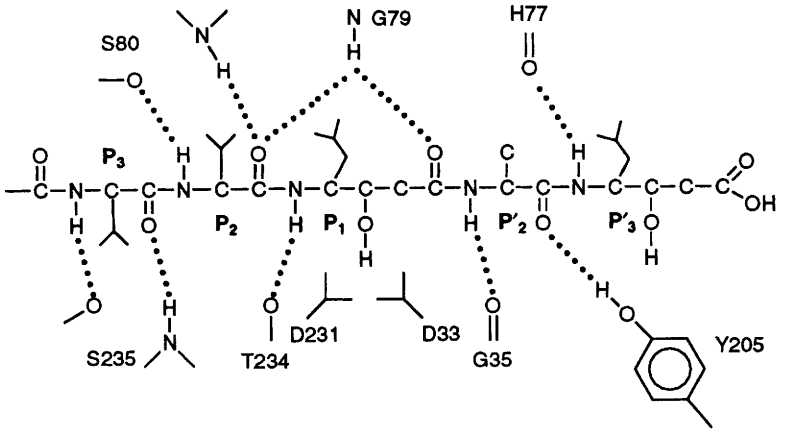


**Figure 16.** Schematic representation of cathepsin D hydrogen bond interactions (dashed lines) with pepstatin A (reproduced from reference 135 by permission of Oxford University Press).

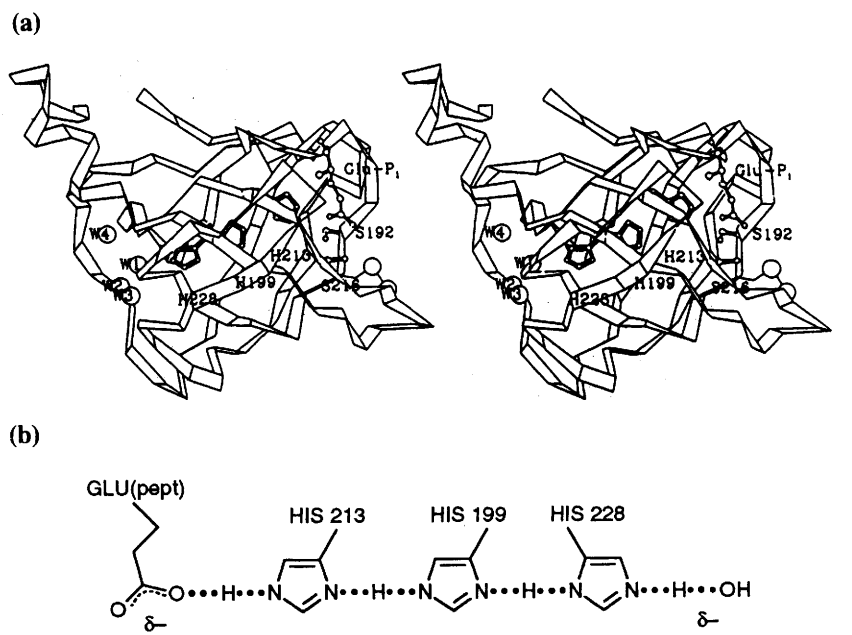


**Figure 17.** a) Ribbon diagram of the second β barrel of glutamic acid specific protease, with residues of the histidine triad, substrate and active site serine labelled. Solvent molecules (WI-4) which might be involved in linking the microdipole of the C-terminal helix to the histidine triad are also shown. b) Schematic representation of the histidine triad charge relay system (reproduced with permission from reference 136).

The structure of an alkaline protease has been described from *Pseudomonas aeruginosa*[137]. This is a secreted zinc-dependent protease thought to be involved in virulence. The N-terminal domain of the protein contains the zinc ion and is the proteolytic domain and includes the active site consensus sequence HEXXH-XUGUXH (X is an arbitrary residue, U is a bulky hydrophobic residue, H histidine, E, Glutamic Acid, G, Glycine). This has also been observed with other proteases in this group including astacin (see Amino Acids, Peptides and Proteins, Vol. 25). The terminal domain contains the repeat motif GGXGXDXUX (as above, D, aspartic acid) required for binding of calcium ions. The calcium binding motif is thought to be important in secretion of the protein and in its folding. The C terminal domain is a β sandwich containing 21 β strands and eight calcium ions.

The structure of adamalysin II, a zinc endopeptidase from the snake venom of the eastern diamondback rattlesnake, *Crotalus adamanteus* has been reported by Gomis-Rüth *et al*[138]. This is the first structure of a snake venom metalloproteinase. Adamalysin II is a 24 kD enzyme which has a shallow active site cleft separating the main calcium binding domain from an irregularly folded subdomain. The zinc binding motif is identical to that in astacin. The zinc ion is co-ordinated by 3 histidines and a water molecule acting as a catalytic base in a near tetrahedral co-ordination. It is suggested that the snake venom metalloproteinase, adamalysin, the astacins, the matrix metalloproteinases/mammalian collagenases and also the large bacterial proteases be grouped into a superfamily with distinct differences from the thermolysin family.

## 6.8 Lipases

Two further papers on lipases have appeared in 1993. A more general discussion on lipase and related enzymes was covered in Trends in Protein Research, Amino Acids, Peptides and Proteins, Volume 25. The first paper describes the structure of the lipase from *Candida rugosa*[139]. This is a single domain molecule with an α/β hydrolase fold. It is similar in overall structural features to the *Geotrichum candidum* lipase. The loops near the catalytic site however have a conformation that differs between the two lipases. The 'flap' region that covers the catalytic site in the *Geotrichum candidum* lipase move up to 25Å to produce a large depression exposing the active site Ser 109 and a hydrophobic surface around it. Another large difference is in a loop which extends towards the centre of the protein in *Candida rugosa* lipase whereas in *Geotrichum candidum* lipase this loop extends in the opposite direction and partially occludes the active site. The *Candida rugosa* lipase is thought to represent the active 'open' form of the enzyme. The second new lipase structure is that of human pancreatic lipase co-crystallised with a small proteic activator, co-lipase which is required to anchor the lipase at the lipid-water interface in the presence of bile salts. When a complex is formed between the lipase and procolipase in the absence of micelles, a helix forms a 'closed' lid over the catalytic triad of the lipase. The addition of micelles causes the helix to unwind and the lid to have an 'open' conformation[140]. These conformational changes alter the environment of the catalytic triad, allowing the active site serine to

become totally accessible to the solvent. A putative phosphatidylcholine molecule has been modelled into the active site.

## 6.9 β-Lactamases

β-lactamases catalyse the hydrolysis of β lactam rings, reducing antibiotics such as penicillins and cephalosporins to inactive acids. The enzymes work *via* a serine bound acyl intermediate The best studied enzymes are class A β lactamases or penicillinases[141,142]. Lobkovsky *et al.*[143] have studied the structure of cephalosporinase from *Enterobacter cloacae*. The β lactam binding site lies between the two domains of the protein and is generally more open than in class A β-lactamases. The class C cephalosporinase and class A penicillinase are shown in Figure 18. The 4 residues implicated in β lactam binding coincide (Ser 64, Lys 67, Lys 315 and Tyr 150 in class C). In cephalosporinase there is no equivalent of Glu 166 thought to be responsible for hydrolytic deacylation of the acyl intermediate in the penicillinases. Clavulanic acid resistance of cephalosporinases results from a difference in position of an arginine residue that is thought to bind clavulanic acid in class A enzymes.

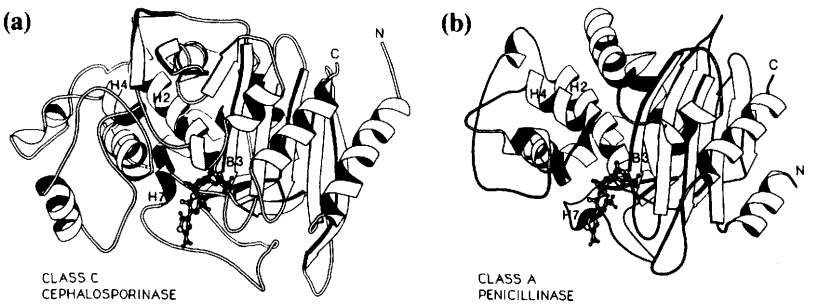


**Figure 18.** Cartoon diagrams showing a) the class C cephalosporinase and b) the class A penicillinase of *Bacillus licheniformis* (reproduced with permission from reference 143).

## 6.10 Other Enzymes

The structure of the enzyme dialkylglycine decarboxylase (DGD) has been determined[144] because of its unusual ability to catalyse rapidly both decarboxylation and transamination. This enzyme is a pyridoxal-5-phosphate enzyme. The enzyme forms a tetramer which is made up of a dimer of dimers. Each DGD monomer is made up of two domains and an N-terminal segment. The active site is located at the monomer-monomer interface. Both the reactions appear to be catalysed by a single active site.

The structure of lipoxygenase has been determined from soyabean[145]. This is a non-haem iron containing dioxygenase that acts on arachidonic acid, using molecular oxygen to form hydroperoxides. The reaction leads to the formation of

leukotrienes and lipoxins which are compounds important for the regulation of cellular response in inflammation and immunity. It is a two domain protein with an N-terminal domain that is an eight stranded antiparallel β-barrel with a jelly-roll topology and larger C terminal domain that is a helical bundle. The iron binding site is located in the larger domain and is co-ordinated to four ligands (3 histidines, C-terminal $COO^-$) in a distorted octahedron with two adjacent unoccupied positions. A similar co-ordination is seen in iron superoxide dismutase.

The structure of the aldehyde reductase from pig has been determined from two crystal forms[146]. The enzyme is a major member of a family of NADPH dependent oxidoreductases that catalyse the reduction of a wide range of aldehydes to their corresponding alcohols. The enzyme is a β barrel structure. At the N terminus two short antiparallel β-strands connected by a tight turn close off the bottom of the barrel. The enzyme is related to aldose reductase which catalyses the reduction of carbonyl groups to alcohols. This latter enzyme has been linked to a number of diabetic disorders. The aldose reductase from human has been crystallised with an inhibitor zopolrestat (3,4-dihydro-4-oxo-3-{[5-(trifluoromethyl)-2-benzothiazolyl]methyl}-1-phthalazineacetic acid)[147]. In the complex the inhibitor almost fills the pocket at the C terminal end of the barrel. Specific residues important for inhibitor recognition are two tryptophans, a phenylalanine and a leucine residue. Comparison of the ternary zopolrestat complex with the holoenzyme reveals a hinged flap motion which acts to close the active site pocket upon inhibitor binding. The structure of aldose reductase with the zopolrestat and NADPH bound is shown in Figure 19.

The structure of a protein, DsbA which is required for disulfide bond formation of proteins in *Escherichia coli* has been determined by Martin *et al*[148]. The fold of this protein is divided into two domains one of which is very similar

**Figure 19.** Diagram of aldose reductase viewed along the axis of the β barrel. The inhibitor zopolrestat and the cofactor NADPH are shown as stick models. The inhibitor lies in the active site pocket at the C-terminal end of the barrel (reproduced with permission from reference 147).

to that found in thioredoxin. The protein contains a sequence of four residues (-Cys-Pro-His-Cys) similar to that found in the active site of the thioredoxin super-family of oxidoreductases. Although the structures are similar there is only a 11% sequence homology.

### 6.11 Enzymes Acting on Carbohydrates

The structure of several enzymes acting on carbohydrates have recently been solved.

*6.11.1 Endocellulase.* – One of these is the endocellulase E2[149]. The enzyme has two domains; the C-terminal domain controls enzyme binding to insoluble cellulose while the N terminal domain carries out the catalysis. The structure described is that of the catalytic domain. The active site residues include three tryptophans and several polar amino acids which are found in other protein carbohydrate binding sites. The structure consists of eight β-strands and eight major and two minor α helices that form an unusual α/β barrel structure.

*6.11.2 Endochitinase.* – The structure of endochitinase has been determined from barley[150]. This enzyme is used by the plant to protect itself from fungal attack by hydrolysing the chitin polymer internally and releasing polysaccharides. The protein is made up of 10 α helices and has 3 disulfide bonds. An elongated cleft runs the length of the molecule and is thought to be the substrate binding and catalytic centre.

*6.11.3 Endoglucanase.* – The catalytic core of the endoglucanase V enzyme (EGV) structure has been reported by Davies *et al*[151]. This protein contains a six-stranded β-barrel in which the strands are both parallel and anti-parallel as shown in Figure 20. This fold has only previously been observed in barwin, a plant defence protein. The overall folds of the cellulose structure reported so far are different although their catalytic sites are similar. This suggests that these enzymes have arisen by convergent evolution. The active site of EGV is similar in shape to that of both hen white lysozyme and T4 lysozyme. For lysozyme the reaction proceeds with retention of the configuration around C, while for EGV it proceeds with inversion. In lysozyme the water acting as a nucleophile is able to attack only after the product has departed. The EGV-cellobiose complex, however, shows a bound water in a position suitable for nucleophilic attack without the need for release of product.

*6.11.4 Glucanase.* – The β-Glucanase enzyme structure[152] has been determined from hybrid protein constructed from two *Bacillus* species to improve the thermostability of the protein. The structure shows no similarity with any of the well known cellulases, lysozymes or amylases. The enzyme comprises a sandwich of two antiparallel seven stranded β-sheets organised as a jelly roll β barrel topology. The active site is located on the concave face and the convex face has a calcium ion and disulfide bridge. One side of the active site is lined with acid residues and the other with aromatic residues. A glutamic acid residue, modified

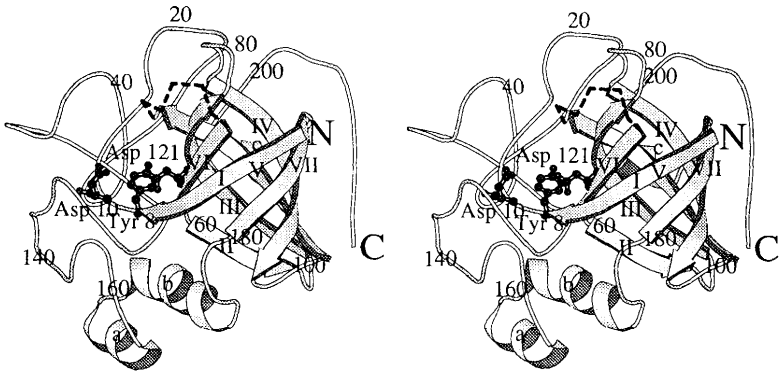


**Figure 20.** Diagram showing the overall topology of the endoglucanase V molecule. The flexible loop region only seen in substrate-inhibitor complexes is shown dashed. The catalytic aspartic acid residues and the tyrosine, lying at the floor of the binding site, are shown in ball and stick representation (reproduced with permission from reference 151).

when the inhibitor (314) expoxybutyl β-D-cellobioside is bound, is thought to be responsible for nucleophilic attack on the substrate.

*6.11.5 Glucose Oxidase.* – The crystal structure of glucose oxidase from *Aspergillus niger* has been solved to 2.3Å resolution[153]. This enzyme catalyses the oxidation of β-D-glucose by molecular oxygen to gluconic acid and hydrogen peroxide *via* δ-gluconolactone. The reaction can be divided into the oxidation of the substrate and the corresponding reduction of the enzyme. The glucose oxidase enzyme has two bound FAD molecules on each dimer. The enzyme shows a similarity with other FAD binding enzymes (glutathione reductase, p-hydroxybenzoate hydroxylase and cholesterol oxidase). A 20 residue lid covers the entrance to the active site. The first domain of the protein binds the FAD and the second the substrate. The second domain has a deep pocket where the substrate binds with the flavin ring of the FAD at the bottom and to one side.

*6.11.6 Lectins.* – S-Lac lectins bind lactose and are thought to be involved in modulating cell-cell and cell-matrix interactions. The structure of the S-Lac lectin L-14-II human/lactose complex has been determined[154]. The protein is composed of all β sheets. The β sheet fold is very similar to that found in plant lectins concanavalin A and pea lectin with which the S-Lac shows no significant sequence homology. The binding of the lactose moiety is seen in detail as shown in Figure 21. All of the residues interacting with the carbohydrate are conserved in all S-Lac lectins.

The lectin from pea has been crystallised with a trimannoside complex[155]. Unlike concanavalin A, pea lectin requires an α-1,6-linked fucose moiety for high affinity binding. The crystal structure only provides ordered density for a single

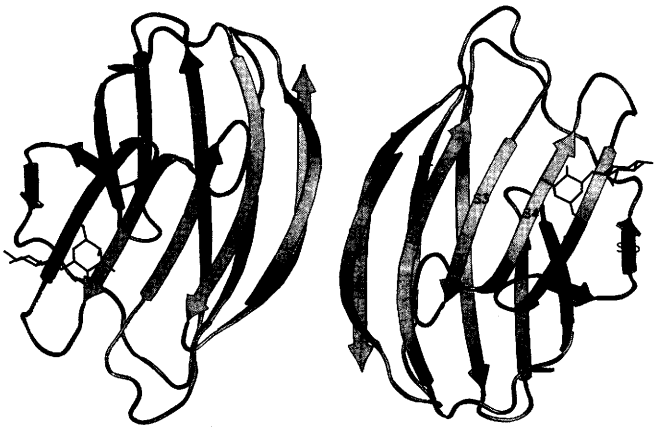

**Figure 21.** Ribbon diagram of the human L-14-II lactose complex. The lactose moiety in each monomer is shown in stick representation. The β-strands of the five and six stranded β sheets are labelled F1 to F5 and S1 to S6a/S6b respectively (reproduced with permission from reference 154).

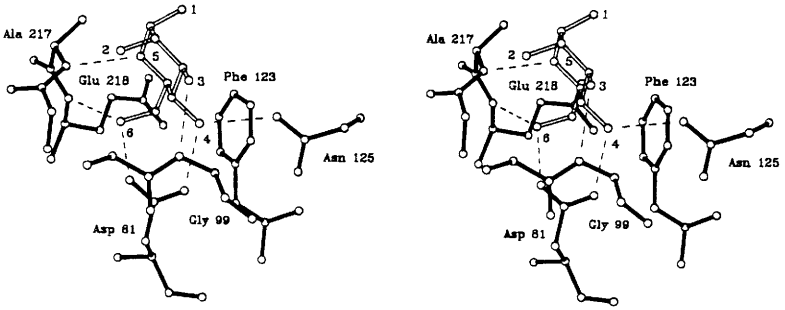


**Figure 22.** The pea lectin saccharide binding site. The filled bonds are for the protein, open bonds for the mannose residue. The sugar oxygen atoms are labelled and the hydrogen bonds are dashed lines (reproduced with permission from reference 155).

mannose residue in each of the saccharide binding pockets. That the trisaccharide binds through a single, terminal mannose suggests a direct role for fucose binding in high affinity oligosaccharide complexes. The terminal mannose residue forms six hydrogen bonds with the protein. A comparison of the lectin with and without saccharide bound indicates that a loop is displaced towards the carbohydrate binding site, maximising two of the maximum six hydrogen bonds formed with the ligand as shown in Figure 22.

*6.11.7 Aldolase.* – The structure of class II aldolase has been described by Dreyer *et al*[156]. This is the zinc containing L-fuculose-1-phosphate aldolase (FuA) that

reversibly cleaves L-fuculose-1-phosphate to dihydroxyacetone phosphate and L-lactaldehyde. The interesting result here is that the structure of this aldolase bears no resemblance to the structure of the more commonly studied TIM-barrel-containing class I Schiff base aldolases. The enzyme is a homotetramer in which each subunit is a central nine stranded anti-parallel β sheet with two parallel helices on one side and three parallel helices on the other. The zinc ion, which acts as an electron sink for the activation of the bound sugar molecule, is bound by five ligands from four side chains giving a distorted tetrahedral co-ordination sphere (as shown in Figure 23). Three of the ligands are the Nε atoms of histidines and the others the Oε1 and Oε2 atoms of a glutamic acid residue.

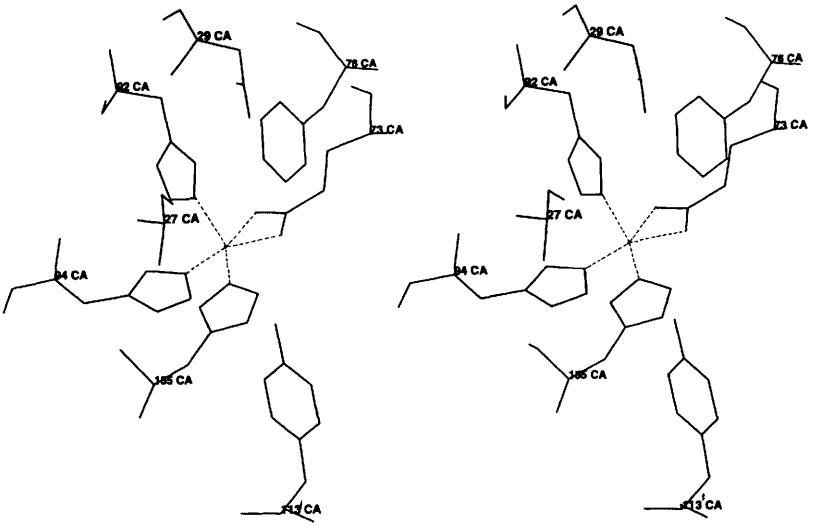


**Figure 23.** The environment of the zinc ion in the active site of fuculose-1-phosphate aldolase. Zinc co-ordinates to the Nε-atoms of histidines 92, 94 and 155 and to the Oε1 and Oε2 atoms of glutamic acid 73 (reproduced with permission from reference 156).

## 6.12 Parasitic Enzymes

The structure of enzymes from parasites such as *Trypanosoma brucei* are of considerable interest since detailed comparison of their structures with the equivalent human enzyme can be used as a target for drug design. The structure of glyceraldehyde phosphate dehydrogenase (GADPH) has been determined from the Trypanosome sleeping sickness parasite[157]. The largest differences observed between the *T. brucei* glycosomal GAPDH and the human enzyme mainly involve residues on the surface of the protein where two unique insertions are found in the parasitic enzyme. Amino acid differences occur in the NAD-binding region along the entire length of the cofactor. Appropriate modifications of the adenine ring might provide opportunities for the design of selective

inhibitors. The parasitic enzyme structure was solved by using Laue diffraction methods and comparison with the human GAPDH.

Another enzyme from the trypanosome has been studied by the group of Wierenga[158]. This is the glycolytic enzyme triosephosphate (TIM) isomerase which forms the well studied TIM barrel. The enzyme had been previously solved in another space group in 1991[159]. The new structure shows the nature of a shift in conformation of a loop which moves from an 'open' to 'closed' substrate bound state. Differences in the states between TIM from chicken and trypanosome have raised the question as to how much the crystalline conditions affect the ligand binding properties and conformation. These studies will help to determine whether differences between TIM from trypanosomes and chicken can be used for drug design purposes or not. The TIM structure has also recently been reported from *Escherichia coli*[160]. A related study has used an artificially constructed monomeric TIM enzyme[161] to study the normal dimeric enzyme. The study reveals that dimerisation is important for positioning the catalytic residues properly.

A variant surface glycoprotein (VSG) structure has been determined from the trypanosome[162]. This is an important protein since evasion of the immune response is accomplished by switching to expression of one or more antigenically distinct VSGs. Structural differences between this protein and other VSG structures are made to understand their antigenic variation. It is concluded that this is achieved mainly by sequence variation and not gross structural determination. The core of the VSG is comprised of two antiparallel α helices with other elements of secondary structure mounted on this scaffold, as shown in Figure 24.

### 6.13 Immunoglobulins

Determining the structure of an intact immunoglobulin molecule has been difficult due to the flexibility of the Fc unit resulting in disordered crystals. By deleting the hinge region of a human immunoglobin G1 the group of Edmundson[163] have been able to obtain the structure of this protein to 3.2Å resolution. The absence of the hinge stops the immunoglobulins function to fix complement but it is able to show binding affinity for the Fc receptor of human monocytes and for the protein A of *Staphylococcus aureus*. This immunoglobulin differs from the usual structures of Fab and Fc fragments. One of the differences is the angle adopted by the 'elbow' joint in the Fab. The active site between $V_L$ and $V_H$ is a large irregular cavity and is continuous with a tunnel that extends into the solvent space between the V and C domain pairs. The presence of a large cavity is unusual amongst known Fabs and such a tunnel has not been seen elsewhere.

The crystal structure has been reported of the Fab fragment TE33 complexed with the 15 residue cholera toxin peptide[164]. The peptide binds to the antibody as shown in Figure 25. A β turn formed in the peptide fits into a pocket lined with aromatic residues. This is a feature of other peptide antibody complexes. Another Fab complex has been solved recently. This is Fab-digoxin complex[165]. Digoxin inhibits the $Na^+$, $K^+$ - ATPase and is used to treat congestive heart failure. It is toxic and anti-digoxin antibodies are used both to

monitor serum levels of the drug and treat digoxin intoxication. Digoxin which is a cardenolide type steroid with an α,β-unsaturated lactone ring attached at C17 and three β (1-4)-D-glycoside linked digitoxases attached at O3. The molecule binds to the Fab combining site with its long axis approximately parallel to the pseudo twofold axis relating the $V_L$ and $V_H$ domains. The lactone ring is buried and the digitoxases are exposed to solvent. There are no H-bonds or salt links formed between the digitoxin and the Fab which is unusual. Here the specificity is clearly the result of only non-polar interactions. Only small changes are observed between the structure of the complexed and uncomplexed antibody.

A detailed study has been carried out on the analysis of the free and bound conformations of the anti-haemoglobin antibody and its heptapeptide complex[166]. A previous study[167] had looked at the structures of Fab in its free form and in two crystal forms in complex with peptide. This produced structural evidence for an induced fit as a mechanism for antibody-antigen recognition. The

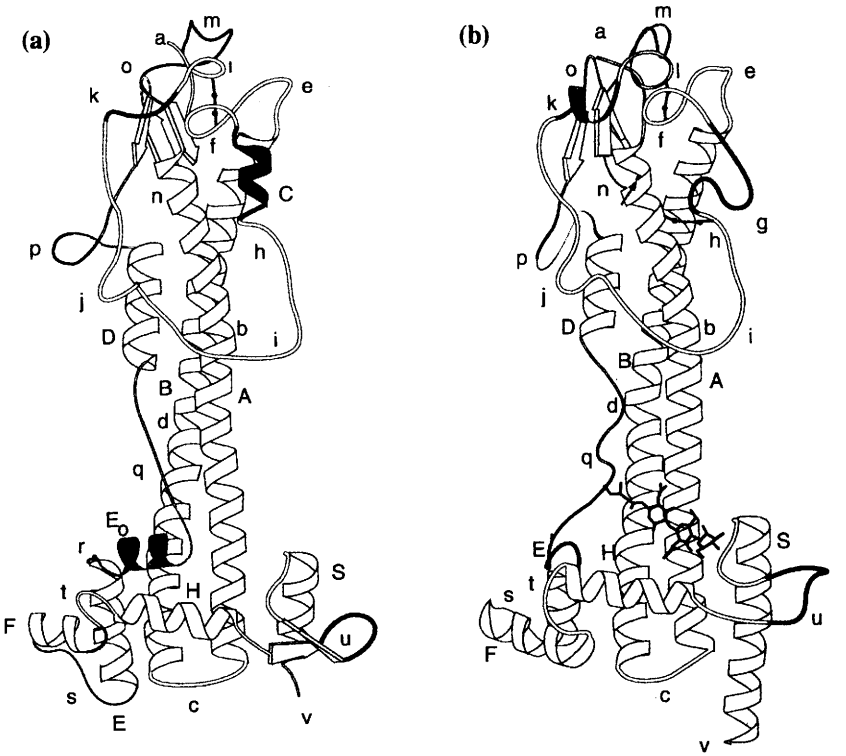


**Figure 24.** Ribbon drawings of a) ILTat 1.24 and b) MITat 1.2 variant surface glycoproteins from *Trypanosoma brucei*. Solid regions represent structural differences between the two molecules (reproduced with permission from reference 162).

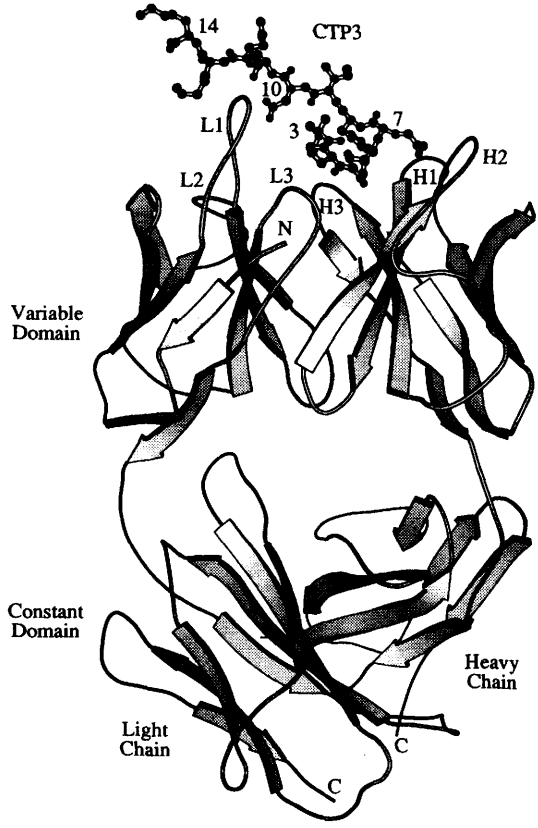


**Figure 25.** Overall view of the crystal structure of the Fab part of the monoclonal antibody T22 in complex with the 15 residue cholera toxin peptide 3 (CTP3) (reproduced with permission from reference 164).

H3 loop of the variable region of the Fab heavy chain has two different well defined conformations. One for the unligated Fab and one for all the crystal forms of the Fab complex. The hydrogen bonding pattern is different and differences up to 4Å for some of the main-chain atoms are discovered. The peptide has an extended conformation followed by a type 1β turn.

Another important Fab/peptide antigen complex is that formed between the HIV neutralising antibody 50.1 and the V3 loop peptide antigen[168]. The principle neutralising determinant of HIV-1 is located in the third hypervariable region (V3) of the envelope glycoprotein. This study has furthered our understanding of the nature of HIV-1 neutralisation. Some residues of the peptide in the complex have an extended structure and others form a type II β-turn. Peptide binding primarily involves hydrophobic interactions. A salt bridge between a

lysine residue of the peptide and two asparagine residues on the Fab also have a role in antigen recognition.

The anti-HPr antibody Jel42, Fab (Mouse)/histidine containing phospho-carrier protein HPr complex has been used to evaluate mutagenesis, for epitope mapping[169]. The epitope determined by X-ray structural analysis is similar to that predicted by site-directed mutagenesis experiments. The structures of an anti-progesterose Fab and its complex with progesterone[170] have been determined to investigate the structural basis of steroid binding. The relatively flat surface of the steroid rings are inserted into a narrow hydrophobic binding slot with specificity enforced by hydrogen bond interactions. A specific tryptophan residue from the Fab heavy chain shows two different conformations representing an 'open' and 'closed' form of the antibody combining site. This residue is thought to cover the hydrophobic binding site in aqueous conditions which then opens to allow binding of the hydrophobic ligand.

Some antibodies bind metal chelate haptens and they have an interest for medical imaging and therapy. An X-ray study has been carried out with two Fab/hapten complexes with different metals in the chelate[171]. The binding of metal-L-benzyl-EDTA-haptens with a murine monoclonal antibody where the metal is varied show a high specificity for an indium chelate with affinity decreasing by as much as $10^4$ for other metals. In the indium complex a histidine residue from the H3 loop of the heavy chain provides a seventh co-ordination of the indium atom chelated with the EDTA group. The histidine co-ordination is not observed in an iron complex.

A study of anti-lysozyme antibody D11.15 Fv/lysozyme complex has been carried out[172] to understand why a monoclonal antibody raised against hen-white lysozyme also binds to several other avian lysozymes with different affinities.

Structures of chimaeric mouse/human antibodies have been reported[173]. Mouse antibodies are easily produced by hybridoma technology. Their use in humans is restricted since the non-antigenic binding regions are sufficiently 'foreign' to raise an immune response. A large amount of work is now being carried out to make chimaeric antibodies to try to overcome this problem.

### 6.14 Chaperone–Peptide Complex

The structure of a PapD chaperone/peptide complex from *Escherichia coli* has been determined by Kuehn *et al.*;[174] PapD is a periplasmic chaperone which is involved in the assembly of the proteins that form the P pilus in *Escherichia coli*. PapD binds each of the pilus subunit types as they are translocated across the cytoplasmic membrane and escorts them in native-like conformations through the periplasm to outer membrane assembly sites. The structure of PapD is similar to that of an immunoglobulin fold. The peptide that is complexed with PapD binds in an extended conformation with its C terminus anchored to the protein. Significant hydrophobic interactions of the peptide are made with PapD with the alternating hydrophilic residues pointing away from the protein. The structure of the two domains of PapD are similar in the peptide complex to those of native PapD however the angle between them is more acute due to a 13° hinge bending motion.

## 7 Protein Engineering

Work in this area has continued to be carried out at an ever expanding rate. Knowing the structure of a protein is only a start. Site-directed mutagenesis has to be carried out to study its mechanism and its properties. Only several aspects of engineering that have been reported in 1993 will be covered here.

### 7.1 Antibody Engineering

The first is the topic of human antibody engineering. As explained in the previous section if antibodies are to be used in human therapy they will be engineered to avoid any unnecessary immune response. The framework of the antibody variable regions is a β sheet framework connected by loops (complementarity - determining loops - CDRs) that are responsible for interacting with the antigen. Five of the six loops adopt a limited number of conformations. The antibody framework residues affecting the conformation of the CDRs was discussed in papers by Foote and Winter[175] and Saul and Poljak[176]. Despite many crystal structures, some of which are discussed in the previous section, the mechanisms of antigen binding are only starting to be understood. A review published in 1993[177] by Mariuzza and Poljak discusses the current view of antigen-antibody binding.

Earlier work has been carried out to design antibodies for human therapy. Chimaeric antibodies have been made between rodent variable domains linked to human constant domains but these are still immunogenic. Another route has been to transplant CDRs from a non human antibody onto a human framework. These antibodies are 90% human and are less immunogenic[178].

*7.1.1 Phage Antibodies.* – Phage antibodies are now used to harness the power of engineering these human antibody variable region genes in *Escherichia coli.* This was first described by McCafferty *et al.* in 1990[179]. In this technique antibody V genes are cloned into the capsid genes of filamentous bacteriophages and displayed as functional proteins on the surface of the phage particles. The phage genome is a single copy of single stranded DNA and selections based on the functional properties of the antibody result in the selection of the gene encoding those properties. Phage can be selected by any method partitioning binders from non-binders. A recent technique has employed antigen coated onto plastic[180]. This report describes the isolation of highly specific antibodies to thyroglobulin, tumor necrosis factor, carcino embryonic antigen, epithelial mucin, CD4 and IgG anti-idiotype from an unimmunised human V gene repetoire.

Dimeric and bispecific antibodies have been recently reported. The group of Greg Winter have described small bispecific antibody fragments called Diabodies[181]. Several single antibody domains and subdomains have been described with binding affinity. A 61 residue peptide called a minibody has been described[182] which is a derivative of the variable region of the heavy domain of the antibody. This chelates metals by virtue of histidine residues in CDRs 1 and 2 (CDR 3 is lacking). A complementarity-determining region synthetic peptide has

been described that acts as a mini-antibody and neutralises human HIV-I virus in vitro[183]

The phage display technique used for antibodies has also been used for protein engineering of peptides and protein domains. Luzzago *et al.*[184] have reported the epitope mapping of human H ferritin using a phage library of cysteine-constrained peptides. Felici *et al.*[185] have attempted to map a monoclonal antibody to *Bordeteilla pertussis* toxin however no clear consensus sequence could be identified from the selected phage. With no three dimensional structure of the toxin it was difficult to determine which residues comprised the epitope. McLafferty *et al.*[186] have used bacteriophage to display disulfide-constrained micro-proteins. This method has also been used for investigation of receptor-ligand interaction[187]. This reports the selection of phage that bound the S-protein of ribonuclease (20 amino acids cleaved from N-terminal end of ribonuclease) using an unconstrained hexapeptide library. The selected peptide did not resemble the S-peptide of ribonuclease which is the first 15 residues that can bind to S1 and restore its function yet competed effectively for binding.

Kay *et al.*[188] have used a phage library to display random 38 amino acid peptides as a source of novel targets with affinity to selected lengths. When constructing complete libraries the display of longer 38 amino acid peptides reduces the number of independent clones required for complete coverage of hexapeptide sequences.

Recently a number of whole proteins have been displayed on phage. This includes trypsin in a study by Corey *et al*[189]. In this study the potential of studying protein-protein interactions was realised by the co-purification of another protein, ecotin (serine protease inhibitor) with the trypsin molecules displayed on the phage. Pannekoek *et al.*[190] have demonstrated the expression of a functional human plasminogen activator inhibitor 1 (PAI-1) on phages. A library of mutant PAI-1 molecules were then constructed using error-prone PCR mutagenesis.

A novel technique has been introduced to combine peptide and protein domain display to examine protease substrate specificity[191]. This phage technology has also been used for vaccine development. Willis *et al.*[192] showed that phage displaying different sequences from a protein of the malarial parasite fused to gVIII were strongly immunogenic in mice. Minenkowa *et al.*[193] have used a peptide from HIV-1 gag protein, p17 as a fusion to gVIII and raised antibodies in rabbits that react with the p17 protein.

It appears that displaying the peptides and proteins on the phage in these experiments does not affect their activity. This technique is proving to be invaluable for protein engineering. It should be possible to display active enzymes and modify substrate specificity by these techniques in the future.

## 8 Summary

An attempt has been made to cover some of the current trends in protein research reported in 1993. Topics such as the importance of water in protein

structure and function have been discussed. Several new protein folds have been discovered and a classification of protein structures can be made into those that are mainly α, mainly β, alternating α/β and α+β classes. We are beginning to understand how proteins fold and what stabilises their structures. 1993 has brought a wealth of new protein structures and we can now have a greater insight into the principles of molecular recognition which govern all biological processes. Advances in genetic manipulation techniques should help in understanding recognition processes, enzyme catalysis and the design of new and novel protein structures.

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# 6
# β-Lactam Chemistry

BY CHRISTOPHER J. SCHOFIELD and NICHOLAS J. WESTWOOD

## 1 Introduction

This review attempts to cover the β-lactam literature likely to be of interest to chemists published in 1992 and 1993. The continued high level of interest in the chemistry and biochemistry of the β-lactams is indicated by the large number of publications relevant to this review. Many of these relate to structure activity relationships of antibacterial β-lactams and are listed in the Appendix. A growing number of reports reflect the development of β-lactams for new therapeutic uses and these are reviewed in Section 10. The deletion of the word antibiotic from the title of this review reflects the growing interest in these new applications, as well as the use of β-lactams as synthetic intermediates. The format and section headings used by our predecessors have been generally retained.

As an antibiotic family the β-lactams have proved remarkably resilient and are possibly the most successful therapeutic agents of the 20$^{th}$ century. Several reviews have highlighted the threat that microbial resistance presents to the continued use of β-lactams and other antibiotics. How long the predominance of β-lactams in the antibiotic market will extend into the next century is likely to depend upon the ingenuity of medicinal chemists and biochemists in meeting the challenge of resistance.

Three books on β-lactams have been published. 'The Organic Chemistry of β-Lactams' (Ed. G. I. Georg) is concerned with the synthesis of β-lactams and their use as precursors for α and β-amino acids and natural products.[1] The emphasis is on modern β-lactam chemistry and it is an excellent text for anyone interested in the synthesis of β-lactams. Chapters on protecting groups in β-lactam chemistry, the introduction and transformation of functional groups in β-lactam chemistry, strategies for the synthesis of bicyclic β-lactams, the β-lactam synthon method, novel methods for the construction of β-lactams and the stereocontrolled ketene-imine cyclisation are included. 'The Chemistry of β-Lactams' (Ed. M. I. Page) is a more general text and contains recent reviews concerning the mode of action and resistance to the β-lactam antibiotics.[2] It includes chapters on the biosynthesis of β-lactams, chemical structure activity relationships, biological structure activity relationships, the mechanism of reactions of β-lactams, mode of action of β-lactam antibiotics, the mechanism of action and inhibition of β-lactamases, the carbacephems, non β-lactam mimics of β-lactam antibiotics and classical β-lactams. The proceedings of the '50 Years of Penicillin Application' conference, held in honour of Prof. E. P. Abraham, have also been published in book format.[3]

Some general reviews on antibacterial resistance have been published and

will be of interest to β-lactam chemists.[4-9] Other reviews have concentrated on the resistance threat to the β-lactam antibiotics.[10-16]. A review draws attention to the threat posed by metallo β-lactamases (to which there are currently no effective inhibitors) to β-lactam antibiotics.[17] Nine reviews on the molecular biology of β-lactam biosynthesis have been published[18-26] and on several of the enzymes involved in the penicillin/cephalosporin and clavulanic acid biosynthetic pathways.[27-30] Review articles have also been published on the use of NMR spectroscopy in the study of the metabolism of β-lactams,[31] the synthesis of non-classical β-lactams,[32] the tribactams,[33] the use of computational chemistry to provide insights into β-lactam antibiotics and their receptors,[34] dual action and pro-drug β-lactams,[35] strategies for the design of new β-lactams,[36] the synthesis and biological activity of fluorinated penicillins and other β-lactams,[37] historical perspectives,[38] penicillin and cephalosporin acylases,[39] the use of penicillin acylase in organic synthesis,[40] the industrial transformations of penicillins and cephalosporins,[41] and Cefprozil.[42] Other reviews are referred to in the specific sections below.

## 2 New Natural Products

The structure of valclavam has been revised from **(1)** to **(2)** on the basis of NMR and mass spectrometric studies.[43] Three herbicidal peptide antibiotics **(3a, 3b, 3c)** related to tabtoxin **(4)** have been isolated from a fermentation broth. **(3a)** was active on minimal media, but its activity was reversed by the addition of glutamine, indicating that like tabtoxin **(4)** its mode of action is via inhibition of glutamine synthetase.[44]

## 3 Biosynthesis

### 3.1 Penicillin and Cephalosporin Biosynthesis

The unravelling of the molecular biology of the penicillin and cephalosporin biosynthesis pathway continues, with particular recent emphasis on the factors regulating the flux through the pathway, including studies on the effects of the carbon and nitrogen sources.[45-48] A detailed discussion of these studies is beyond the scope of this review and the reader is referred to the reviews in the area. A pulsed field electrophoresis study has allowed mapping of the penicillin gene cluster of *Aspergillus nidulans* to chromosome VI.[49] Nine chemical mutants of *Penicillium chrysogenum* which demonstrated reduced levels of penicillin production have been generated and characterised.[50]

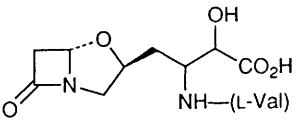
*3.1.1 ACV (5) Biosynthesis.* – Two reviews on ACV synthetase have been published.[51,52] Studies on the effect of dissolved oxygen on the production of ACV synthetase in *Streptomyces clavuligerus*[53] and on the regulation of its activity by carbon sources and their metabolites have been published.[54] A theoretical analysis suggests that ACV synthetase is the rate limiting enzyme in

penicillin and cephalosporin biosynthesis.[55,56] An interesting study on the subcellular compartmentation of the early stages of penicillin biosynthesis in *P. chrysogenum*, using [$^{14}$C]-labelled amino acids, has resulted in the proposal that ACV synthetase is located in or on the surface of the vacuolar membrane and that its substrates are drawn from within the vacuole.[57] Improved procedures for the purification of ACV synthetase from *Cephalosporium acremonium* and *S. clavuligerus*[58-60] and conditions for the *in vitro* stabilisation of ACV synthetase isolated from *S. clavuligerus* have been described.[61] The availability of *in vitro* ACV synthetase activity has enabled preliminary characterisation and mechanistic studies to be initiated. ACV synthetases from both *C. acremonium* and *S. clavuligerus* have been shown to contain phosphopantothenic acid, substantiating its characterisation as a member of the multienzyme peptide synthetase family.[58,60] Incubation of [2-$^{2}$H]-valine with purified ACV synthetase from *C. acremonium* and *S. clavuligerus* produced ACV **(5)** with complete loss of deuterium from the valinyl $C_2$-position and incubations with fully protiated valine in deuterium oxide produced ACV **(5)** with significant incorporation of deuterium at the valinyl $C_2$ position (Scheme 1).[62] These experiments were interpreted as confirming that ACV synthetase catalyses both the formation of the peptide bonds of ACV **(5)** and the epimerisation of the valinyl residue. Studies on the exchange of the valine oxygen during ACV **(5)** biosynthesis, using [$^{18}$O]-valine have revealed that *in vitro* the exchange of a single valine carboxylate oxygen occurs, indicating that formation of a valinyl acyl enzyme intermediate is not freely reversible (Scheme 1).[63,64]

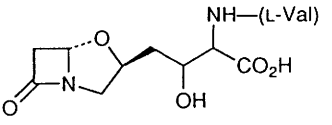
*S. clavuligerus* has been shown to contain an NADPH disulfide reductase with a broad substrate specificity, related to thioredoxin, which catalyses the reduction of ACV disulfide to its thiol form **(5)**.[65] An *in vitro* coupled enzyme system for the production of penicillins from ACV disulfide using the disulfide reductase and isopenicillin N synthase (IPNS) was described. The gene for the disulfide reductase has been cloned.[66]

The gene encoding for lysine 6-aminotransferase which is involved in the biosynthesis of L-α-aminoadipic acid has been shown to be located in the cluster of cephamycin biosynthesis genes in *Nocardia lactamdurans*.[67] Hybridisation experiments, using both cephamycin producing and non producing strains, suggested that this gene is a 'genuine' β-lactam biosynthetic gene.[67]

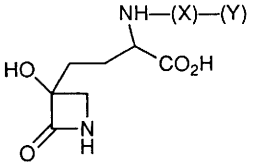
*3.1.2 Isopenicillin N Synthase.* – High level expression in *E. coli* of the genes for various IPNS isozymes has been achieved[68,69] and studies on the factors affecting the transcription of the IPNS genes from *A. nidulans*[70] and *S. clavuligerus*[71] have been reported. Expression of the IPNS gene is apparently regulated by (at least) two independent mechanisms, one mediating carbon regulation and one mediating pH regulation.[72] The results of a broad screening effort to examine the potential of IPNS for the synthesis of new penicillins from substrate analogues has been described.[73] The results complement previous substrate analogue studies. It was found that conversion of the D-valinyl residue of ACV **(5)** to an aromatic amino acid or to a residue containing strongly electron withdrawing groups resulted in the production of inhibitors. A full report, including a



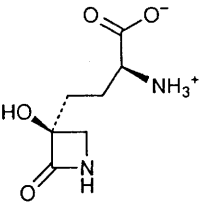

(1)



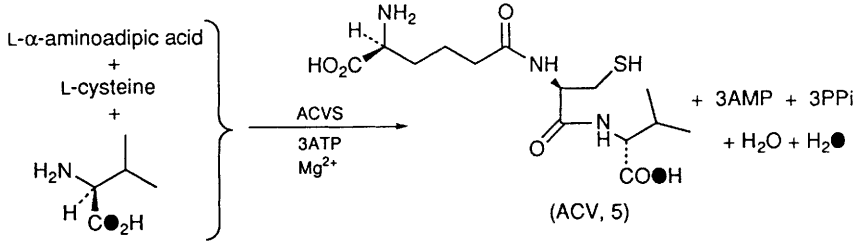

(2)




(3a) X = L-Ala, Y = L-Val
(3b) X = Y = L-Ala
(3c) X = L-Ala, Y = —

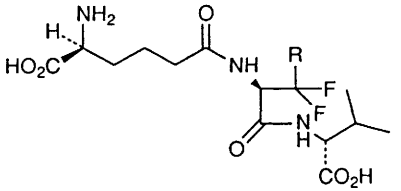



(4)

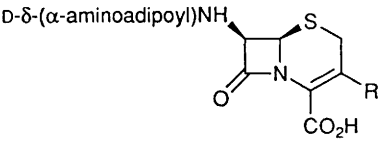



(ACV, 5)

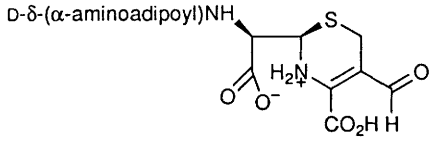
**Scheme 1**




(6) R = $CH_2SH$
(7) R = COSH




(8) R = $CH_2OAc$, Cephalosporin C
(9) R = $CH_3$, DAOC
(10) R = $CH_2OH$, DAC
(11) R = CHO
(13) R = $C(^2H)_3$




(12)

mechanistic interpretation, has appeared on the conversion by IPNS of an ACV **(5)** analogue **(6)** containing a difluorohomocysteinyl residue in place of cysteine, to a thiocarboxcylic acid **(7)**.[74] The availability of recombinant IPNS has encouraged biophysical studies by several groups. A variety of spectroscopic techniques including X-ray absorption,[75,76] NMR,[77] Mossbauer,[78] and spin echo spectroscopy[79] have been utilised to study the active site of IPNS. These studies are all consistent with the ligation of the ferrous iron of IPNS by two or three histidine residues. NMR studies[77] indicate that an aspartyl residue is located in close proximity to the active site iron. X-ray absorption[75,76] and other studies[78] indicate that the thiol of the substrate ligates to the active site iron, as has been previously proposed.[74]

*3.1.3 Cephalosporin Biosynthesis.* – The cloning and expression of the acetyl CoA:deacetylcephalosporin C (DAC) acetyl transferase gene which encodes for the final enzyme involved in cephalosporin C **(8)** biosynthesis has been reported.[80-82] The genes for the cephalosporin 7α-hydroxylase[83] (from *S. lividans*) and cephalosporin acetyl esterases (from *C. acremonium*[84] and *Bacillus subtilus*[85]) have also been cloned and expressed. The results of an ambitious experiment in which the production of cephalosporins in *P. chrysogenum* was engineered have been described.[86] Thus, *P. chrysogenum* was transformed with DNA containing a hybrid DAOC synthase gene and a hybrid isopenicillin N epimerase gene. The transformed *P. chrysogenum* was shown to produce DAOC **(9)** along with penicillin V. The latter was produced at lower levels than that observed for the untransformed *P. chrysogenum*, which (in common with other fungi) does not produce cephalosporins. The genes encoding for isopenicillin N epimerase and DAOC synthase from *N. lactamdurans* have been sequenced and expressed in *S. lividans*.[87] Several mechanistic studies have been carried out on DAOC/DAC synthase from *C. acremonium*, which has been expressed in *E. coli*.[88,89] DAC **(10)** itself has been shown to be a substrate for the enzyme being converted to the aldehyde **(11)**, which is highly unstable and undergoes rapid hydrolysis to the cephalosporinate **(12)**.[90] This is of interest because **(12)** has been previously isolated from fermentation broths of a *C. acremonium* mutant, believed to be blocked at the DAC acetyl transferase step. Furthermore it suggests that the evolutionary significance of the acetyl transferase may have been to preserve the cephem nucleus by modification of DAC **(10)** by acetylation, since cephalosporin C **(8)** is not a substrate for DAOC/DAC synthase. Incubation of a mixture of DAOC **(9)** and [3-$^2H_3$]-DAOC **(13)** with DAOC/DAC synthase led to the observation that the former was preferentially processed by the enzyme, consistent with a mechanistic proposals in which cleavage of the C-H bond of the exocyclic methyl group is an irreversible event.[91] The conversion of the unnatural substrate, exomethylene cephalosporin C **(14)**, by DAOC/DAC synthase has been studied using [4-$^2$H]-exomethylenecephalosporin C **(15)**.[92,93] Incubation of **(15)** led to the isolation of DAC **(10)**, the aldehyde **(11)** and the epoxide **(16)**. Reincubation of isolated **(16)** with DAOC/DAC synthase led to the production of an aldehyde analogous to **(12)**, but derived by ring opening of **(11)** with $NH_3$. Further evidence implied that DAC **(10)** was not an intermediate in this

process.[94,95] $^{18}O$ was incorporated into the products **(10)** and **(16)**, from both $^{18}O_2$ and $H_2{}^{18}O$, although the level of incorporation into the epoxide **(16)** from $^{18}O_2$ was higher than that observed for DAC **(10)**.[94,95]

*3.1.4 Acyl Coenzyme A: Isopenicillin N Amidohydrolase Acyltransferase (AT).* – Several studies have confirmed that a single gene product is responsible for the conversion of isopenicillin N to penicillin G. The enzyme is α,β-hetereodimeric and the intact gene product does not display activity.[96,97] A study has investigated the requirement for interaction in the production of the subunits of AT.[98] Another study which characterised the post-translationally modified protein using electrospray ionisation mass spectrometry provided evidence that the partial irreversible acylation of the α-subunit occurs *in vivo* by acylation with exogenously added substituted acetic acids.[99] It was also demonstrated that incubation of the enzyme with appropriate acyl coenzyme A derivatives led to reversible (by the addition of 6-aminopenicillanic acid) acylation of the β-subunit only.[99] Expression in *P. chrysogenum* of a mutant AT lacking a putative targeting sequence for microbody proteins led to the observation that whilst the mutant enzyme was active *in vitro*, the transformed *P. chrysogenum* did not produce penicillins. The mutant enzyme was also found to be located in vacuoles and the cytoplasm, rather than in microbodies as normally observed.[100] The isolation of acylcoenzyme A synthases (ACS) from *P. chrysogenum* and *A. nidulans* has been reported.[101] The ACS from the former is apparently an $\alpha_2$ homodimer and was shown to catalyse the synthesis of acyl coenzyme A derivatives of a number of acids ($C_2$ to $C_8$ and some aromatic acids) including some of commercial importance. The ACSs could be coupled to AT from *P. chrysogenum* to produce acylated penicillins from carboxylic acids and 6-aminopenicillanic acid.

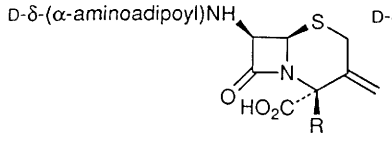
*3.1.5 Penicillin Acylases.* – Interest in the optimisation of conditions for the deacylation of fermented penicillins remains high, but full coverage is beyond the scope of this review and the reader is referred to the reviews in the area. This interest has, however, stimulated studies on the postranslational processing pathway and on the mechanism of penicillin acylases. The α and β-subunits of the *E. coli* acylase have been expressed separately and shown to be inactive, whereas activity was obtained when the genes for the two subunits were put into an *E. coli* host on separate plasmids.[102] The processing pathway has also been probed using site directed mutagenesis on *E. coli*[103] and *Kluyvera citrophilia*[104] acylases, which have also been used to investigate a putative penicillin binding site on the acylase.[105,106] The use of penicillin acylases has been described for the resolution of racemic mixtures by the hydrolysis of N-phenylacetyl derivatives of β-amino acids,[107] N-phenylacetylated 1-aminoethylphosphonic and 1-aminoethylphosphonous acids,[108] N-phenylacetylated γ-aminobutyric acid derivatives,[109] phenylacetate esters of secondary alcohols[110] and of methyl esters of phenylacetate derivatives in the preparation of compounds useful as penicillin side chains.[111] Penicillin acylase has also been used for the deprotection of N-phenylacetyl-L-Asp-L-Phe-OMe, in an aspartame synthesis,[112] and for the N-acylation of $C_7$-

aminocephalosporins[113-115] and amino acids.[116] In the latter case it was reported that L-amino acids with non branched side chains were the best substrates.

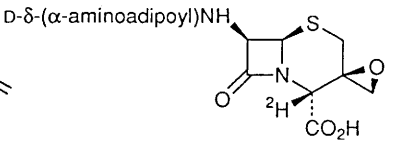
*3.1.6 Cephalosporin Acylases.* – The route for the preparation of 7-aminocephalosporanic acid from cephalosporin C **(8)** by oxidation with a D-amino acid oxidase, followed by decarboxylation and deacylation of the resultant glutaryl-7-aminocephalosporanic acid (GL-7-ACA) continues to receive attention. A kinetic study on the overall process has been reported[117] and D-amino acid oxidases from several sources have been characterised.[118-120] The GL-7-ACA acylases from *Bacillus laterosporous*[121] and *Pseudomonas* strains[122-124] have been cloned and expressed in *E. coli*. One of the *Pseudomonas* cephalosporin C acylases, which was cloned and expressed in *E. coli*, reportedly catalyses the removal of the D-α-aminoadipoyl side chain from cephalosporin C **(8)**.[124]

### 3.2 Clavam Biosynthesis

The past two years have seen significant advances in the understanding of the clavam biosynthetic pathway (Scheme 2). Two isozymes of clavaminic acid synthase (CAS), a key enzyme in clavam biosynthesis, from *S. clavuligerus* have now been cloned[125,126] and one of them expressed in *E. coli*.[126] Hybridisation and mapping studies have shown that the gene clusters for the clavulanic acid **(17)** and cephamycin biosynthesis pathways in three *Streptomyces* species are chromosomally adjacent. Thus, in *Streptomyces spp.* there appears to be a 'super cluster' of genes involved in the biosynthesis and protection of β-lactam antibiotics.[127] Substrate analogue studies on CAS have led to the proposal that it has an unprecedented trifunctional role in the biosynthesis of a secondary metabolite. Thus, incubation of the amino substrate **(18)** with CAS led to the production of the alkene **(19)**, as the major product, together with a low yield of proclavaminic acid **(20)**.[128] However, incubation of the guanidino compound **(21)** with CAS led to the production of alcohol **(22)** in an efficient fashion. Partly on the basis of these observations it was proposed that an amidino hydrolase catalyses the hydrolysis of guanidino alcohol **(22)** to proclavaminic acid **(20)**. The conversion of **(21)** to **(22)** was shown to occur with retention of configuration at the $C_3$ position of the side chain using stereospecifically labelled substrates **(23)**.[129,130] The oxygen of the hydroxyl group of **(22)** was shown to be derived in part from $O_2$ and in part from $H_2O$.[128] The ability of CAS to accommodate substrate analogues was further demonstrated by the conversion of the γ-lactam analogue **(24)** of proclavaminic acid **(20)** to the γ-lactam analogues **(25)** and **(26)** of dihydroclavaminic acid **(27)** and clavaminic acid **(28)** respectively.[131] A diastereoselective synthesis of the dihydroclavaminic acid analogue **(25)** by bicylisation of aldehyde **(29)**, was also reported.[131] CAS has been shown to catalyse the hydroxylation of N-acetyl arginine to give **(30)** and the hydroxylation and desaturation of N-acetyl ornithine to give alkene **(31)** and alcohol **(32)**.[132] The former transformation is useful as an assay for CAS. Evidence that supported the supposition that **(21)** was indeed an intermediate in clavulanic acid **(17)** biosynthesis came with the observation that arginine is a later intermediate than ornithine in the pathway.[133] Furthermore, **(21)** was subsequently isolated along with **(33)**




(14) R = H
(15) R = $^2$H

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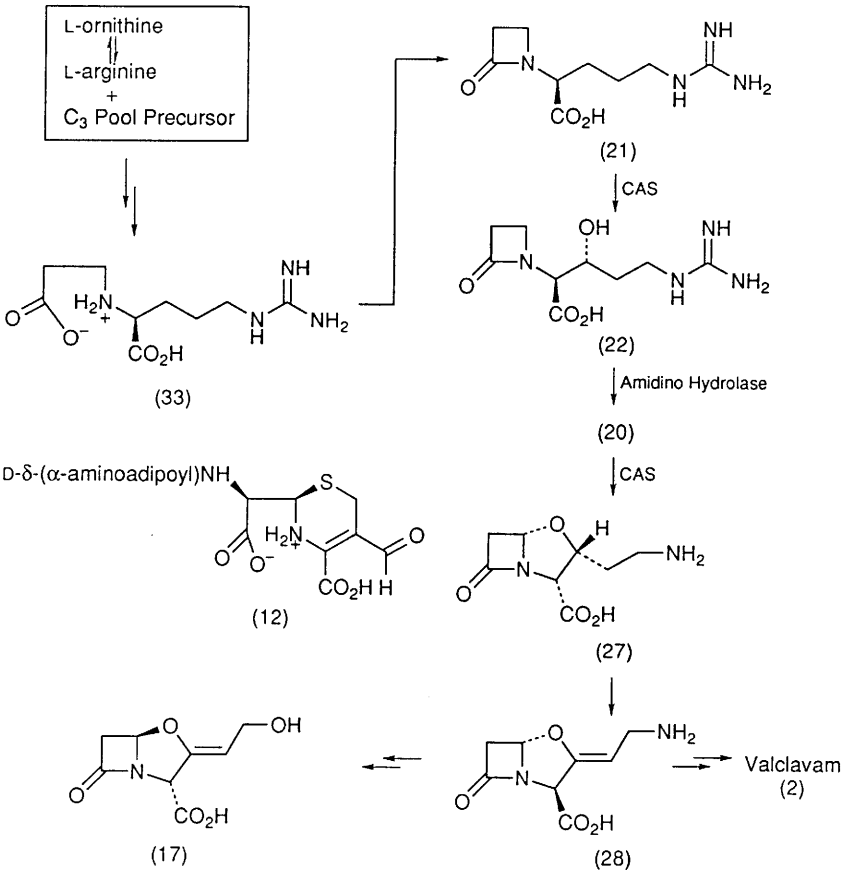



**Scheme 2**

from *S. clavuligerus*.[134,135] The processes by which arginine is converted to **(33)** and by which clavaminic acid **(28)** is converted to clavulanic acid **(17)** remain to be elucidated in the clavulanic acid **(17)** pathway. Biosynthetic studies on other clavams have now been initiated and these have resulted in the revision of the structure of valclavam from **(1)** to **(2)**.[43] A degradation product **(34)** of valclavam **(2)** was found to be identical to the antibiotic Tü1718B, which had previously been assigned another structure **(35)**.[43] Evidence is beginning to accumulate that the early stages of the pathway are common to the biosynthesis of all clavams. Labelling studies demonstrated that the biosynthesis of clavam-2-carboxylic acid **(36)**[136] and valclavam **(2)**[137,138] both proceed via proclavaminic acid **(20)**. Furthermore, both CAS and proclavaminic acid amidino hydrolase have been isolated from *S. antibioticus*,[139] which produces valclavam **(2)**. An outline biosynthetic pathway to all clavams has been proposed (Scheme 2).[139]

### 3.3 Carbapenem Biosynthesis

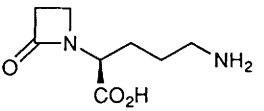
N-(β-Ketocaproyl)homoserine lactone **(37)** has been shown to act as a 'molecular control' signal for the expression of genes controlling carbapenem biosynthesis.[140,141] A number of the analogues were capable of restoring carbapenem biosynthesis to mutants lacking the ability to biosynthesise **(37)**.[142] Some of the genes involved in thienamycin biosynthesis have been cloned.[143]

### 3.4 Tabtoxin Biosynthesis

Investigations into the biosynthesis of tabtoxin β-lactam **(4)**, the agent which causes wildfire disease in tobacco plants, using blocked mutants of *Pseudomonas syringae*, led to the isolation of the amino acid **(38)**.[144] **(38)** was shown to be produced by tabtoxin **(4)** producing strains but not by toxin deficient strains and was therefore proposed as a putative intermediate in the biosynthesis of tabtoxin **(4)**. The stereochemistry of **(38)** was established by subsequent synthesis, in which the key step was $S_H2'$ coupling of allylic stannane **(39)** to protected β-iodoalanine to give **(40)**.[145] A summary, which includes some previously unpublished work, on tabtoxin **(4)** biosynthesis has been published.[146] Three studies on the molecular biology of tabtoxin **(4)** biosynthesis have been reported[147-149] and should pave the way for the detailed elucidation of the biosynthetic pathway. In particular it will be of interest to examine the mechanism of the formation of the β-lactam ring, since IPNS remains the only β-lactam forming enzyme to be characterised. Resistance to tabtoxin **(4)** is mediated by an acyltransferase which acylates tabtoxin **(4)**. The gene for the acyltransferase has been introduced into tobacco cells, and the resultant transgenic plants shown to be resistant to tabtoxin treatment and to bacterial attack by *P. syringae*.[150]

## 4 Penicillins and Cephalosporins

(2,3)-α-Methylenepenams **(41)** have been synthesised [via DBU mediated cyclisation of bromide **(42)** to cyclopropane **(43)**]. In contrast to the (2,3)-β-methylenepenams **(44)**, **(41)** was shown to be biologically active, consistent with



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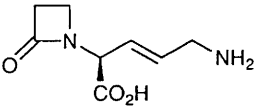

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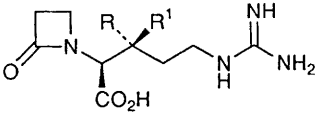
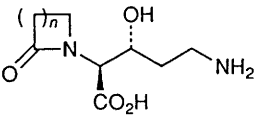
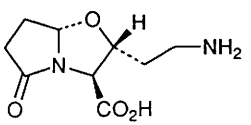


(23a) R = H, $R^1 = {}^2H$
(23b) R = $^2H$, $R^1$ = H



(20) *n* = 1
(24) *n* = 2



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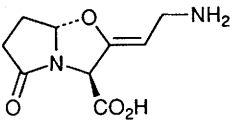

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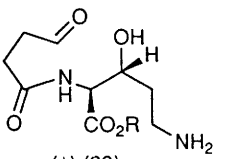

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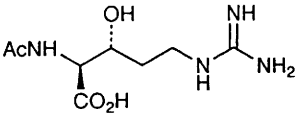

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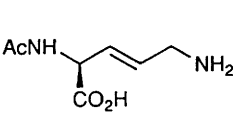

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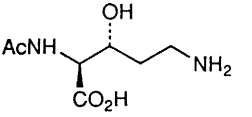

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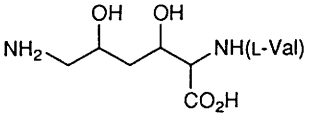

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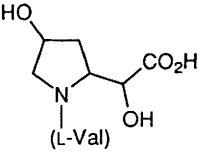

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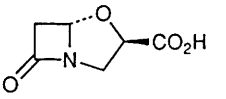

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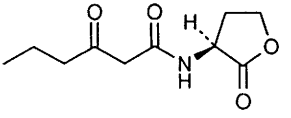

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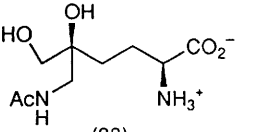

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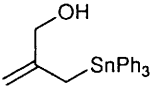

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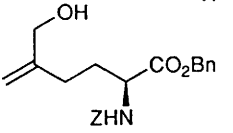
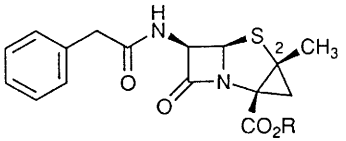
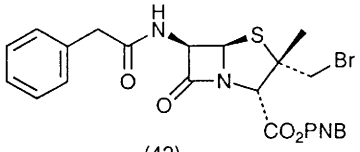


(40)

proposals that the open conformation of penicillins is the active form.[151] The absolute stereochemsitry at $C_3$ of **(41)** is opposite to that found in the naturally occurring penicillins and modelling suggests that the carboxyl group of **(41)** occupies a similar position to the carboxyl group in the cephalosporins. The spectrum of biological activity of **(41)** was consistent with this proposal.[151] Reaction of thiazoline **(45)** (derived from valine) with chromium-alkoxycarbene complexes **(46)** under photolytic conditions provides a route to penams **(47)**.[152] 6-Allenylpenicillins have been prepared by stereospecific addition of acetylide anions to 6-oxopenams **(48)** to give propargylic alcohols **(49)**.[153] $S_N2'$ displacement of the subsequently formed triflates **(50)** with cuprates and copper (I) halides proceeded efficiently to give haloallenes **(51)** and **(52)**. Metal halogen exchange of **(51)** and **(52)** followed by subsequent protonation or acylation can be stereoselectively achieved.[153] Penicillin and cephalosporin amides can be readily synthesised by reaction between their 2-pyridyl thiolester derivatives and a suitable N-silylamine.[154] Reaction of 6-diazopenicillanates **(53)** with ozone gives 6-oxopenicillanates **(48)**.[155] Dimethyldioxirane is a useful reagent for the diastereoselective oxidation of penicillanates and penams not containing a $C_6$-acylamino functionality. Generally the least hindered sulfoxide is formed.[156] Penams such as **(54)**, **(55)**, **(56)** and **(57)** have been synthesised by dirhodium tetraacetate mediated cyclisation of the corresponding diazo azetidin-2-ones **(58)**, **(59)** and **(60)**. In the case of **(54)** the rearranged vinyl thioether **(61)** was also isolated.[157] Allenes of type **(62)** (derived from penicillins) have been elegantly exploited in the synthesis of bicylic β-lactams. The first synthesis of $C_6$-unsubstituted exomethylene penam **(63)** has been reported[158] utilising reductive cyclisation ($BiCl_3$/Zn) of allene **(64)**. **(63)** was also converted to the thioether **(65)**. Michael additions (promoted by copper (I) chloride) of vinyl tin reagent **(66)** to the allene **(67)** provided a short route to vinyl cephems **(68)**.[159] Addition of lithium chloride to allene **(69)**, prepared from triflate **(70)** by triethylamine treatment, resulted in cyclisation to give cephem **(71)**. External nucleophiles can also be introduced as exemplified by reaction of **(72)** with lithium bromide and sodium 1-methyl-2-mercaptotetrazolate to give **(73)**.[160] Furthermore, the 1,4-conjugate addition of organocuprates to allene **(72)** followed by cyclisation leads to cephalosporins with carbon substituents at $C_3$.[161] The study of a potentially biomimetic process for DAOC/DAC synthase has demonstrated the conversion of thiol **(74)** to cephams **(75)** and **(76)**, by metals [Fe(III) and Mn(III)] capable of performing a single electron oxidation.[162,163] The addition of lithium chloride to the $Mn(OAc)_3$ oxidation mixture gave the further oxidised products **(77)**, **(78)** and **(79)**.[163] [2+2] Reaction of 2-methylthio-3,6-dihydropyrimidines **(80)** with aryloxyketenes gave the 1-aza-$\Delta^2$-cephems **(81)**.[164] Similarly, reaction of thiazines **(82)** with phenoxyketene gave the cephams **(83)**.[165] Full details of the approach to $C_3$-substituted cephems using the addition-elimination reaction of organocuprates to 3-trifloxycephems **(84)** have now appeared.[166] Organocuprates derived from Grignard reagents and a copper(I) bromide dimethylsulfide complex were the most effective and did not cause $\Delta^2$ / $\Delta^3$-double bond isomerisation.[166] $C_3$-Fluorosulfonyloxycephems **(85)** are cost effective alternatives to triflates for the organocuprate addition-elimination reactions.[167] $C_2$-Exo-methylenecephems **(86)**



(41) R = H
(43) R = PNB = *p*-nitrobenzyl



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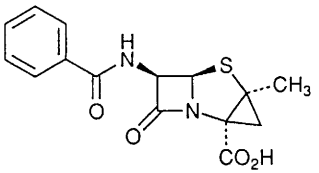
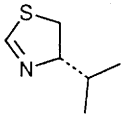


(44)



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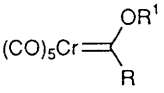

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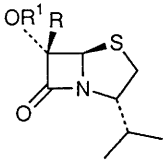
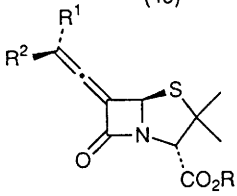
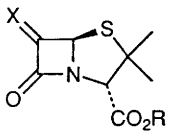


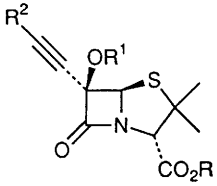
(47)



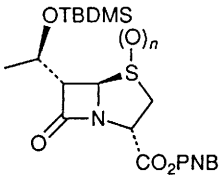
(51) $R^1$ = Br, I; $R^2$ = H, Me, $CH_2OTBS$, $CH(OEt)_2$, $CO_2Et$, CH(Ph)OTBS
(52) $R^1$ = Me, $CH_2OTBS$, $CH(OEt)_2$, $CO_2Et$, CH(Ph)OTBS; $R^2$ = Br, I



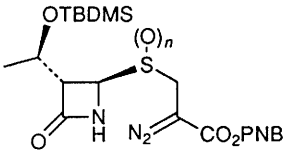
(48) X = O
(53) $X = N_2$



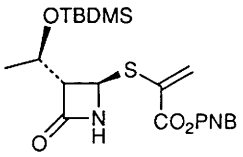
(49) $R^1$ = H
(50) $R^1$ = Tf



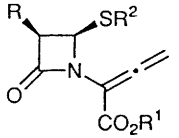
(54) *n* = 0
(55) *n* = 1, β-sulfoxide
(56) *n* = 1, α-sulfoxide
(57) *n* = 2



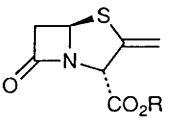
(58) *n* = 0
(59) *n* = 1, mixture of diastereomers
(60) *n* = 2



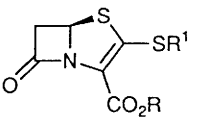
(61)



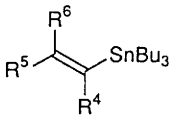
(62) R = H, $R^2 = SO_2Ph$
(64) R = H, $R^2$ = H
(67) R = $NHCOR^1$, $R^2 = SO_2Ar$



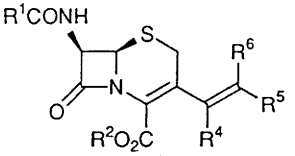
(63)



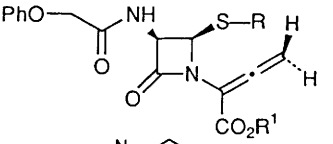
(65)

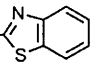


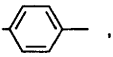
(66)

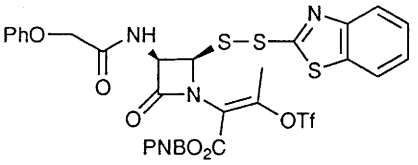


(68)

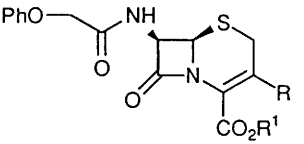


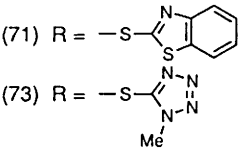
(69) R =  , $R^1$ = PNB

(72) R =  , $R^1 = CH(Ph)_2$



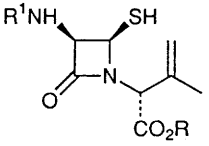
(70)





(71) R = 

(73) R = 



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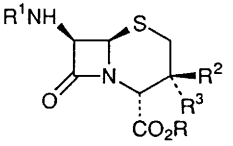
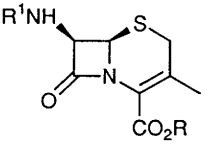


(75) $R^2$ = H, $R^3$ = Me
(76) $R^2$ = Me, $R^3$ = H
(77) $R^2$ = Me, $R^3$ = Cl
(78) $R^2$ = Cl, $R^3$ = Me



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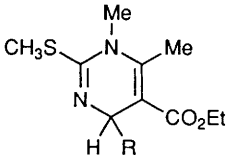

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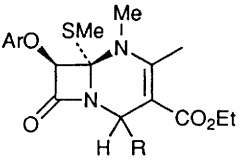
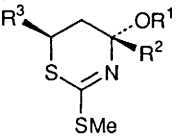


(81)



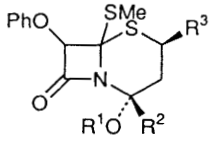
(82)

can be synthesised by treatment of diene **(87)** with sodium benzenesulfinate, followed by reaction with DBU or ethyldiisopropylamine.[168] The corresponding sulfoxides **(88)** or sulfones **(89)** can be synthesised by Mannich reaction of **(90)** and **(91)** respectively with dimethyl (methylene)imonium salts.[169] Reaction of dibromide **(92)** with triethylamine or pyridine gave a mixture of $C_2$-bromocephem **(93)** and cephem **(94)**.[170] Attempts at nucleophilic displacement of the bromide of **(93)** led to quantitative recovery of the sulfone **(94)**. Synthesis of the desired $C_2$ substituted sulfones was achieved by reaction of dibromide **(95)** with nucleophiles (ROH, $RCO_2K$) to give **(96)**, followed by *meta*-chloroperbenzoic acid oxidation to the sulfone.

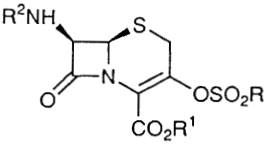
The cephalosporin triflate **(97)** reacts with acetylenes and alkenes in the presence of ethyldiisopropylamine to give 2,3-fused cyclobutenes, *e.g.* reaction of **(97)** with methyl propargylate gave a mixture of **(98)** and **(99)** and reaction with hept-1-ene gave **(100)**.[171] Reaction of cephalosporin $C_3$, phosphorous ylide **(101)** with keto-aldehydes (OHC-COR') led to mixtures of the olefins of type **(102)** and tricyclic alcohols **(103)**. The formation of **(103)** probably occurs via the aldol product **(104)**.[172] In an attempt to develop an intramolecular method for the replacement of the sulfur of cephems with a carbon to afford carbacephems, it was envisaged that oxime **(105)** may undergo radical rearrangement to give the oxime **(106)**, via **(107)**.[173] In fact, oxime **(105)** readily undergoes base catalysed (pH 10 phosphate buffer) rearrangement to **(108)**, presumably via **(109)**. **(108)** was converted to **(110)** using sodium hydroxide in $CH_3CN$:pH10 buffer 4:1. Tosylhydrazone **(111)** undergoes a similar rearrangement to give heterocycle **(112)**.[173] Reaction of $C_3$-halocephem **(113)** with samarium diiodide provides an excellent route to $C_3$-exomethylencephams **(114)** with the natural $C_4$ configuration.[174] The *R*-sulfoxides of cephems **(115)** can be produced diastereoselectively by oxidation of cephems with monochlorourethane.[175] Mixed anhydrides of carboxylic and sulfonic acids are useful reagents for the acylation of the $C_7$-amino group of cephalosporins.[176] The problem of isomerisation of the cephem alkene position from $D^3$ to $D^2$ during acylation of the $C_3$ alcohol can be avoided by carrying out the reaction without protecting the $C_4$ carboxyl group.[177,178]

## 5 Clavulanic Acid, Oxapenams and Oxapenems

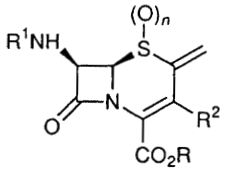
Efforts to develop an efficient synthesis of clavam derivatives via a route in which the key step is the [2+2] cycloaddition of isocyanates to glycals have continued.[179] Thus, α-D-galactopyranose derivative **(116)** was subjected to glycolytic cleavage to give dialdehyde **(117)**, which was transformed, by standard methodology, into **(118)**. Fluoride induced cyclisation of **(118)** gave the clavam **(119)**.[179] Using related methodology the ester **(120)** was prepared.[180] Attempts at fluoride induced cyclisation, however, met with failure. Treatment of **(120)** with *tert* -butylammonium fluoride, led to the production of **(121)** and fragment **(122)**, presumably both formed following deprotonation at $C_3$, whereas reaction of **(120)** with CsF led to the production of enamine **(123)**, possibly via the desired clavam **(124)**, which is believed to be unstable under the reaction conditons,



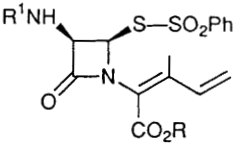
(83)



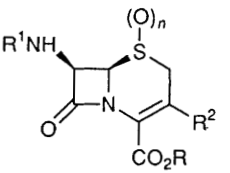
(84) R = $CF_3$, $R^1$ = $CHPh_2$, $R^2$ = $COCH_2OPh$
(85) R = F, $R^1$ = $CHPh_2$, $R^2$ = $COCH_2OPh$, COOPh
(97) R = $CF_3$, $R^1$ = PMB, $R^2$ = $PhOCH_2CO$



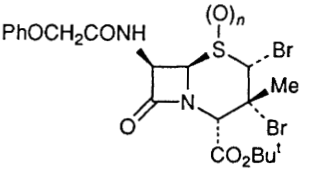
(86) *n* = 0, $R^2$ = $CH_3$
(88) *n* = 1
(89) *n* = 2



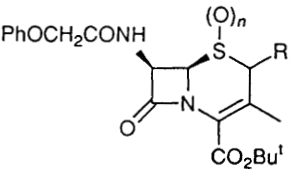
(87)



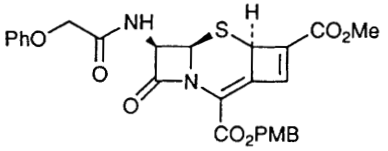
(90) *n* = 1
(91) *n* = 2



(92) *n* = 2
(95) *n* = 0



(93) *n* = 2, R = Br
(94) *n* = 2, R = H
(96) *n* = 0, R = OMe, OEt, OBn, OAc



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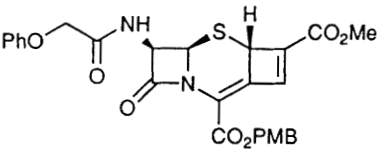
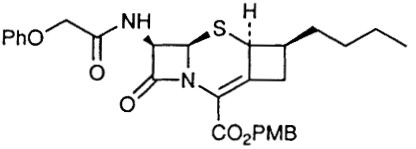


(99)



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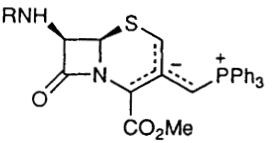

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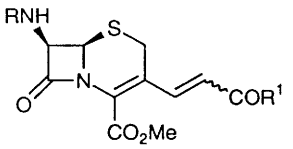

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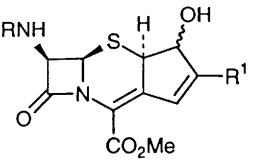

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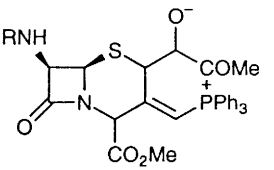
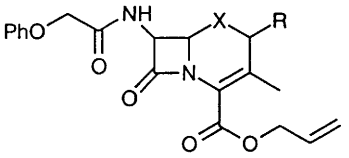
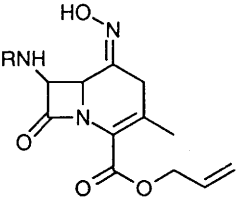


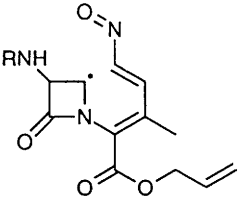
(104)



(105) R = CH=NOH, X = $SO_2$



(106)



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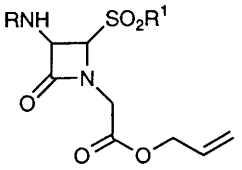
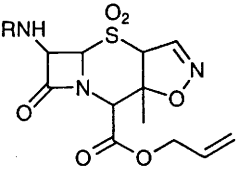


(108) $R^1$ =



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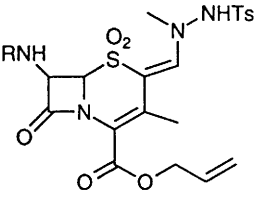
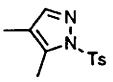
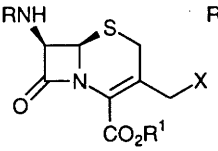


(111)

(110) $R^1$ =

(112) $R^1$ =





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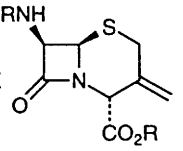

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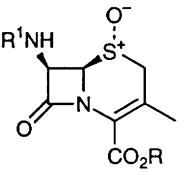

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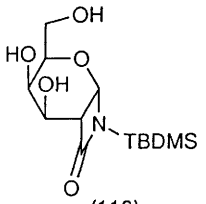


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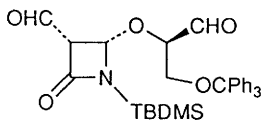
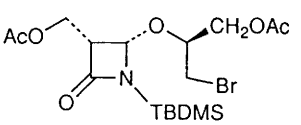
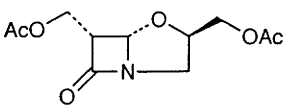


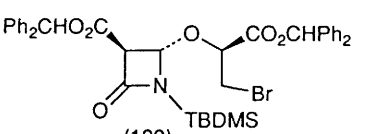

(117)



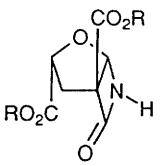

(118)



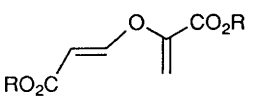

(119)



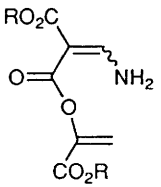

(120)



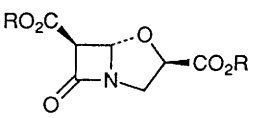

(121)



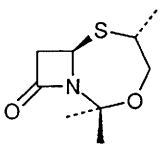

(122) R = $CHPh_2$



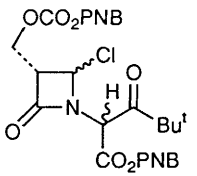

(123)



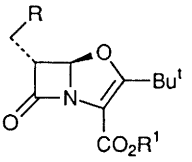

(124)



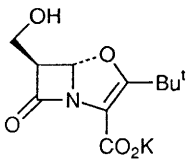

(125)



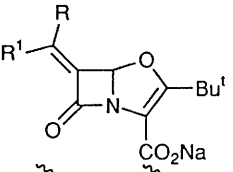

(126)

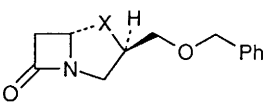



(127) R = $OCO_2PNB$
$R^1$ = PNB
(128) R = OH, $R^1$ = K

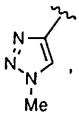


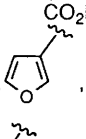

(129)



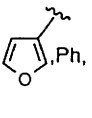



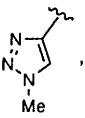

(132) X = O or $CH_2$

(130) R = 

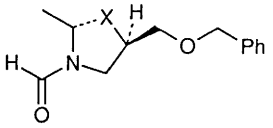
, Ph, 

, Me; $R^1$ = H

(131) R = H, $R^1$ = 

, Ph, 

, Me

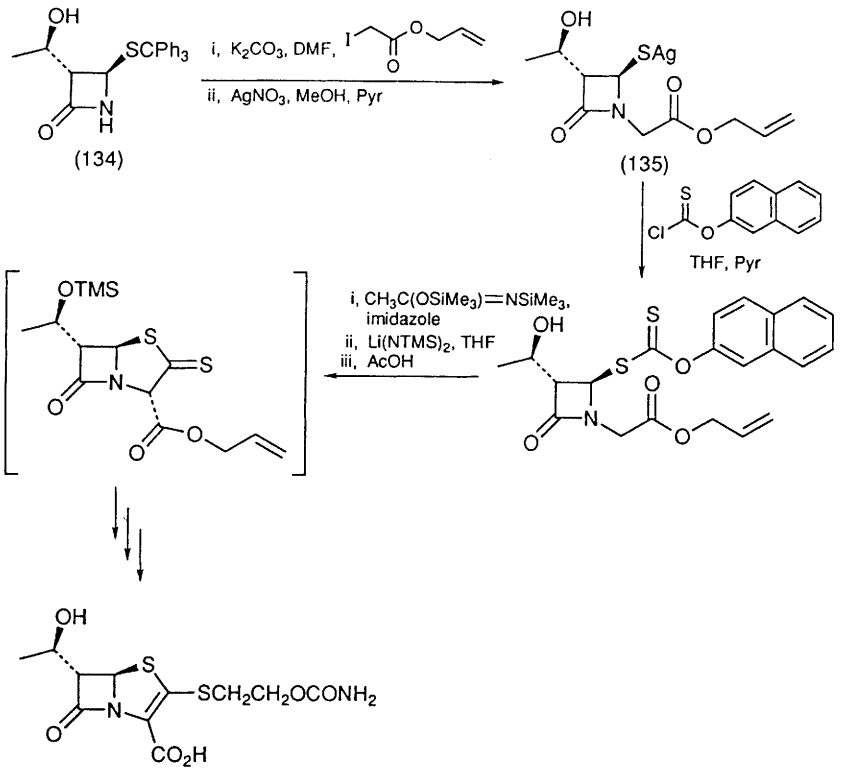



(133) X = O or $CH_2$

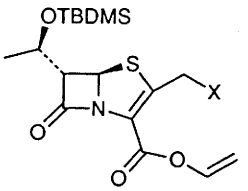
undergoing facile β-elimination.[180] The synthesis and biological activity of $C_2$-*tert*-butyloxapenemcarboxylic acids, which are much more stable than anticipated, have been described.[181] The synthesis of both enantiomers of the $C_6$-hydroxymethylene oxapenems has been reported.[182] The known intermediate **(125)** was converted to chloride **(126)** in 12 steps. Treatment with KO$^t$Bu effected cyclisation to the desired oxapenem **(127)** (34%), which was deprotected to give **(128)**. The enantiomer **(129)** of **(128)** was synthesised via closely related methodology.[182] Surprisingly, *both* enantiomers were more active as inhibitors of β-lactamase from *Staphylococus aureus* than clavulanic acid **(17)**. Several $C_6$-substituted oxapenems have been subsequently reported,[183,184] including the $C_6$-methylene oxapenems **(130)** and **(131)**. These compounds were however unstable with respect to hydrolysis.[183] Oxa- and carba-penams **(132)** undergo an abnormal Pd catalysed reductive cleavage between $C_6$ and $C_7$ to give formamides **(133)**.[185]

## 6 Penems

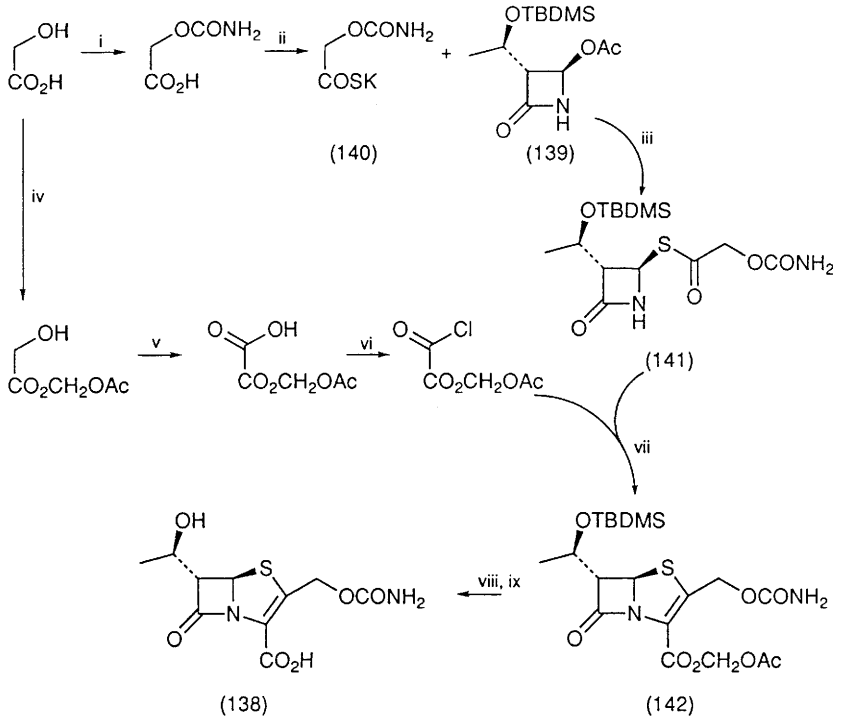
The full details of a practical (*i.e.* multikilo scale) and optimised (chromatography of intermediates is not required) eight step synthesis of penems from azetidin-2-one **(134)**, via the crystalline silver salt **(135)** have been published (Scheme 3).[186] The efficient synthesis of $C_2$-halogenomethyl penems **(136)** has been achieved by treatment of mesylate **(137)** with the appropriate calcium halogenide in DMSO.[187] The method was not applicable to the synthesis of $C_2$-fluoromethyl penems. A synthesis of Ritipenem **(138)** from glycolic acid and azetidin-2-one **(139)** has been described.[188] The key steps were the reaction of the potassium salt **(140)** with **(139)** to give **(141)** followed by 'one pot' acylation of the azetidin-2-one nitrogen and carbonyl-carbonyl coupling to give **(142)**. Subsequent deprotection of the silyl and ester protecting groups, the latter using enzymatic hydrolysis, gave the penem **(138)** (Scheme 4).[188] The first synthesis of $C_6$-spirocyclic penems has been reported.[189] Treatment of *E*-furylmethylene penem **(143)** with diazomethane gave a mixture of pyrozolines **(144)** and **(145)**. Thermolysis of the major diastereomer **(145)** gave the desired spirocycle **(146)**. Similarly, treatment of *Z*-furylmethylene penem **(147)** gave pyrazoline **(148)**, but the adduct **(149)**, arising from additional reaction of diazomethane with the endo-alkene was also isolated. Thermolysis of the mixture of **(148)** and **(149)** gave **(150)** and **(151)**. The esters **(146)**, **(150)** and **(151)** were deprotected to give the corresponding sodium salts, which all showed reduced activity compared with heterocyclic substituted $C_6$-methylene penems. The parent penem **(152)** was also reacted with diazomethane to give pyrazolines **(153)**. Thermolysis of the major (but not the minor) diastereomer gave the fused cyclopropane **(154)**.[189] Studies aimed at the synthesis of a penem isothiazolinone **(155)**, whilst unsucessful, have resulted in the synthesis of the first penem antibiotic containing a S-N bond at $C_2$.[190] Thus, treatment of thioxopenam **(156)** with Hunig's base and hydroxylamine-O-sulfonic acid led to the formation of the sulfeneamide **(157)**, which failed to undergo cyclisation to the desired isothiazolinone. The deprotected




**Scheme 3**

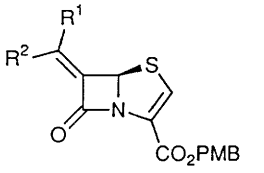



(136) X = halogen
(137) X = OMs



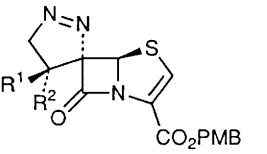

Reagents; i: CSI, 85%; ii: ClCOOEt, $CH_2Cl_2$, $H_2S$ then KOMe, 77%; iii: dioxane, 40 °C, 50%; iv: $CH_3COOCH_2Br$, $CH_3CN$, 50 °C, 85%; v: $CrO_3/H_2SO_4$, $CH_3COCH_3$, r.t., 79%; vi: $(COCl)_2$, $Et_2O$, r.t., 92%; vii: $Et_3N$, toluene, 0 °C, then $P(OEt)_3$, reflux, 71%; viii: $Bu^n{}_4NF$, $CH_3COOH$, THF, r.t., 76%; ix: PLE, phosphate buffer, pH 7, quantitative

**Scheme 4**

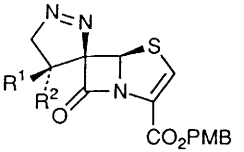



(143) $R^1$ = H, $R^2$ = 

(147) $R^1$ =  , $R^2$ = H

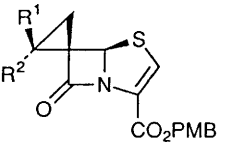



(144) $R^1$ = H, $R^2$ = 



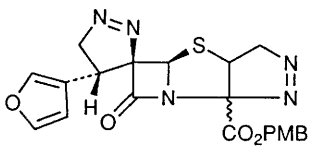
(145) $R^1$ =  , $R^2$ = H

(148) $R^1$ = H, $R^2$ = 

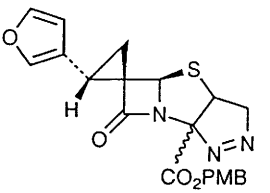


(146) $R^1$ =  , $R^2$ = H

(150) $R^1$ = H, $R^2$ = 



(149)



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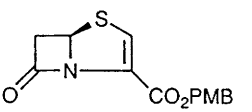
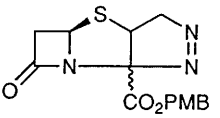
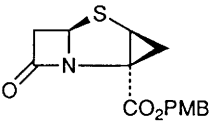


(152)



(153)



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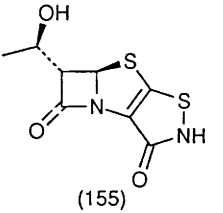

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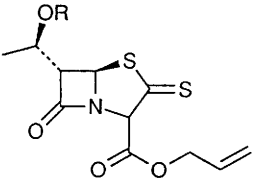
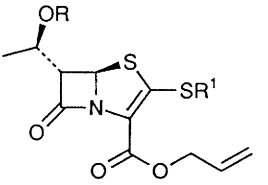
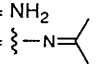
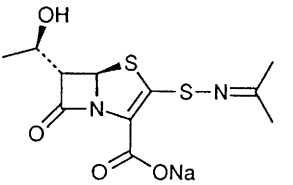


(156) R = TBDMS
(159) R = H



(157) R = TBDMS, $R^1$ = $NH_2$
(158) R = H, $R^1$ = $NH_2$
(160) R = H, $R^1$ = 



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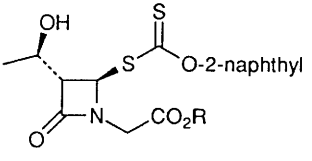

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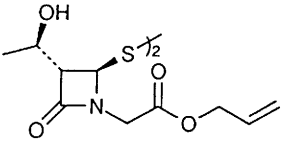


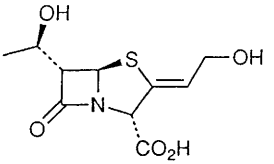
(163)

sulfeneamide **(158)** was also prepared by treatment of **(159)** with hydroxylamine-O-sulfonic acid. **(158)** was reacted with acetone in the presence of catalytic PPTS to give the sulfenimine **(160)**, which was deprotected to give the unstable crude sodium salt **(161)**.[190] The synthesis of **(162)** and **(163)**, which are intermediates for the synthesis of penems, from $C_6$-aminopenicillanic acid has been reported.[191] A concise synthesis of $C_6$-hydroxyethylthiaclavulanic acid **(164)** from the penem nucleus **(165)** has been reported.[192] Oxidation of **(165)** with $MnO_2$ gave aldehyde **(166)**, which was converted to the epoxide diastereomers **(167)** by treatment with diazomethane. 'One pot' rearrangement and deprotection (40% unoptimised) was achieved using $Pd/C/H_2/NaHCO_3$ to give the sodium salt of **(164)**. Alkylphosphonous acid diesters $MeP(OR)_2$ have been reported to be superior (higher yields and shorter reaction times) reagents to the classically used phosphites for the ring closure of thioesters or thiocarbonates to give penems.[193]

## 7 Carbapenems, Carbapenams, Carbacephems and Related Systems

### 7.1 Carbapenems and Carbapenams

A new approach to the carbapenams utilises the Lewis acid ($TiCl_4$) mediated intramolecular cyclisation of the β-lactam nitrogen of **(168)** onto a keto group, followed by dehydration to give **(169)**.[194] Reaction of iminoketone **(170)** with benzyloxyacetylchloride in the presence of triethylamine gave exclusively the *cis*-β-lactams **(171)**. Cyclisation of the latter with lithium hexamethyldisilylazide gave either the *cis*- **(172)** or *trans*-**(173)** carbapenams, depending upon the reaction conditons, which may be further utilised in the synthesis of carbapenems.[195] A new route involving photochemical ring contraction of pyrazolidinones to give β-lactams has been developed e.g. ring contraction of **(174)** (prepared from δ-valerolactone) gave the *cis*-β-lactam **(175)**.[196-198] The carbapenem precursor **(176)** was prepared via an extension of this methodology. Synthesis of **(176)** and related compounds which are also intermediates for the synthesis of 1β-methylcarbapenems continues to attract attention. **(176)** has been synthesied by a new route in which the key step was a [2+2] reaction of $ClSO_2NCO$ with **(177)** (prepared from 3*R*-hydroxybutyric acid) to give **(178)** (after N-deprotection).[199] Benzylic cleavage, followed by oxidation to ketone **(179)**, Baeyer-Villiger reaction (MCPBA in AcOH) and protecting group manipulation gave **(176)**. Reaction of the Reformatsky reagent from bromide **(180)** with **(176)** gave **(181)**, which was treated sequentially with $Zn(BH_4)_2$ then $NaIO_4$/cat. $OsO_4$ to give the desired carbapenem intermediate **(182)** in a stereocontrolled manner.[200] Regioselective ring opening of aziridines **(183)** or **(184)** with $AlMe_3$ gave alcohols **(185)** and **(186)** respectively. **(185)** and **(186)** were both converted to azetidin-2-one **(187)**, a 1β-methylcarbapenem precursor.[201] The 1β-methyl analogue **(188)** of PS-5 has been synthesised.[202] In an analogous route to that previously reported for the synthesis of PS-5, methyl pyroglutamate was used as the starting material for the synthesis of the carbapenem **(189)**.[203] A route to carbapenems employing a radical cyclisation to form the five membered ring has been explored.[204] Treatment of azetidin-2-ones **(190)** and **(191)** with tributyltin



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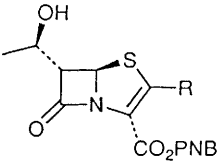
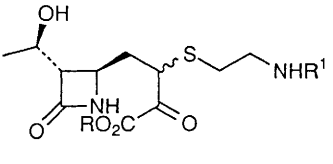
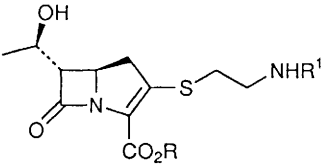


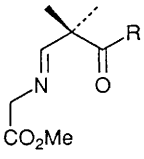
(165) R = $CH_2OH$
(166) R = CHO
(167) R =



(168) R = *p*-nitrobenzyl
$R^1$ = *p*-nitrobenzyloxycarbonyl



(169) R = *p*-nitrobenzyl
$R^1$ = *p*-nitrobenzyloxycarbonyl



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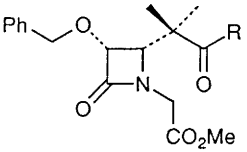
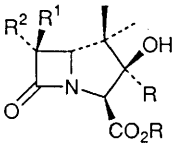
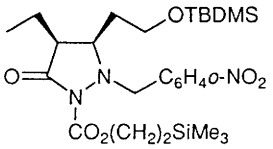


(171)



(172) $R^1$ = H, $R^2$ = $OCH_2Ph$
(173) $R^1$ = $OCH_2Ph$, $R^2$ = H



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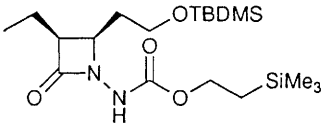

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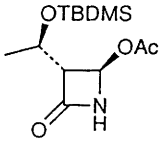

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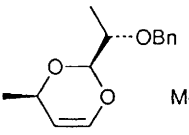

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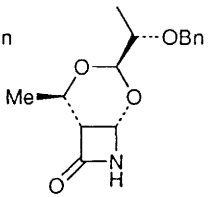

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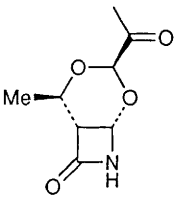

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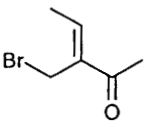


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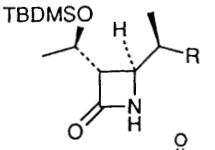
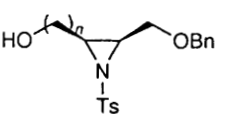
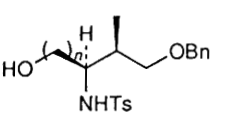



(181) R = 

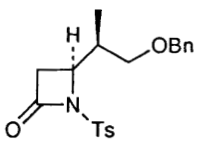
(182) R = $CO_2H$




(183) $n$ = 2
(184) $n$ = 1




(185) $n$ = 2
(186) $n$ = 1

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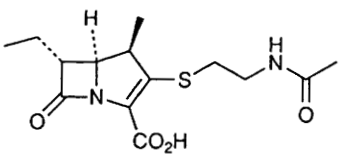


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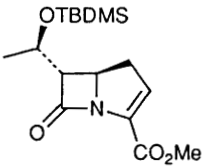


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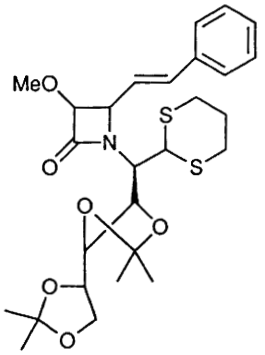
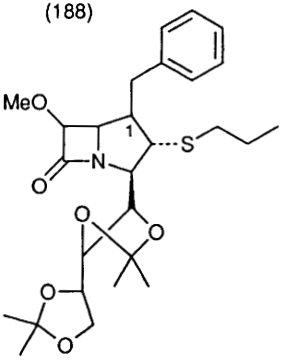
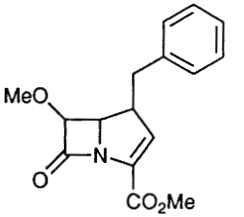


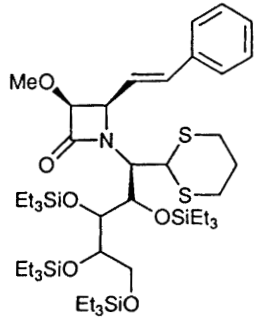

(190) 3α, 4α
(191) 3β, 4β




(192) 1β, 5α, 6α
(193) 1α, 5β, 6β




(194) 1β, 5α, 6α
(195) 1α, 5β, 6β

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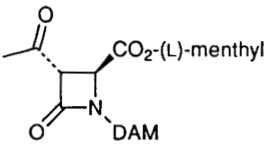


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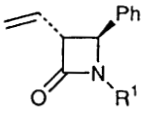


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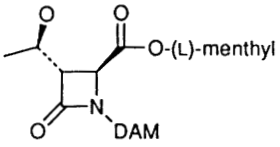


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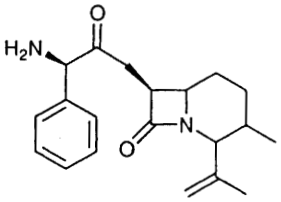



Loracarbef
(200)

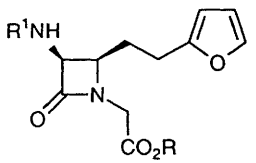
hydride and catalytic AIBN led to the carbapenams **(192)** and **(193)** respectively. The attempted conversion of **(192)** or **(193)** into the desired carbapenams **(194)** or **(195)** respectively was unsuccessful. Exchange of the isopropylidene protecting groups of **(190)** for silyl protection gave **(196)**, which underwent radical cyclisation and further manipulation (desilylation, followed by a two stage oxidation process) to give the carbapenem **(194)**.[204]

The highly selective reduction of $C_3$-acetyl azetidin-2-ones **(197)** to the carbapenem precursor **(198)** has been carried out using N-benzylaminoethoxyborane in the presence of boron trifluoride etherate.[205]

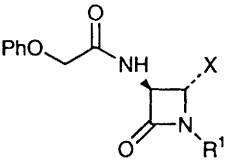
Several methods for the conversion of the $C_3$ vinyl group of azetidin-2-one **(199)** into a hydroxyethyl side chain with a view to preparing intermediates for the synthesis of thienamycin have been investigated.[206] Oxymercuration followed by demercuration was found to be low yielding so an elaborate route employing epoxidation, regioselective ring opening with bromide followed by tributyltin-hydride reduction was employed. *p*-Nitrobenzyl esters of 1β-methylcarbapenems can be efficiently hydrolysed (relative to catalytic hydrogenation) by treatment with zinc dust in THF/phosphate buffer.[207]

### 7.2 Carbacephems

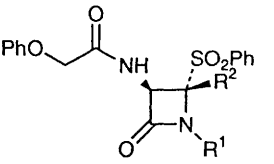
Spearheaded by efforts directed towards an efficient synthesis of Loracarbef **(200)**, interest in the carbacephems appears to be rising. Full details of early approaches proceeding via racemic **(201)** and the use of penicillin acylase to effect enantioselective acylation of **(201)** or acid **(202)** to give **(203)** and **(204)** respectively have been reported.[208] New routes to **(200)** have also been developed, several of which involve the use of penicillins as starting materials. The major challenge is the asymmetric construction of a requisite *cis*-azetidin-2-one intermediate. Thus, acetoxyazetidin-2-one **(205)**, readily available from penicillin V, was converted to the sulfonyl derivative **(206)** by treatment with sodium phenylsulfinate. N-Silylation of **(206)** to give **(207)**, followed by deprotonation with $^nBuLi$ and highly selective β-alkylation with a variety of electrophiles gave **(208)**, which was subsequently desilylated to give **(209)**.[209] Removal of the sulfone group gave predominantly the desired *cis*-azetidin-2-one **(210)**. **(211)** was oxidised using $KMnO_4$ to give **(212)** which was converted to **(200)**.[209] The $C_4$-allyl azetidin-2-one **(213)** was alkylated with *tert*-butyl bromoacetate (using Triton B) to give **(214)**. Epoxidation of **(214)** followed by cyclisation using $LiN(TMS)_2$ gave **(215)**, which was converted to the cephem **(216)** via the iodide **(217)** ($Ph_3P$, $I_2$, pyridine then DBU).[210] The synthesis of **(218)** from sodium erythorbate **(219)** has been reported.[211] Conversion of **(219)** to the unstable epoxy aldehyde **(220)** followed by condensation with *tert*-butyl glycinate under anhydrous conditions gave imine **(221)**. [2+2] reaction of **(221)** with phthalimidoacetyl chloride and triethylamine gave **(222)**, which was deoxygenated to give **(223)**. Hydrogenation of **(223)** gave **(224)** which was converted to Loracarbef **(200)**. A drawback in the latter stages of this methodology is that the phthalimido group was not suitable for the requisite Dieckman cyclisation and had to be exchanged.[211] An alternative strategy for the synthesis of carbacephems employed aldol reaction of the anion of **(225)** with succinic acid semialdehyde methyl ester to give racemic **(226)** as a



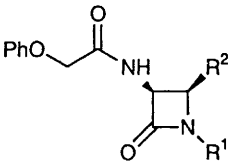
(201) R = Me, $R^1$ = H
(202) R = $R^1$ = H
(203) R = Me, $R^1$ = $COCH_2Ph$
(204) R = H, $R^1$ = $COCH_2Ph$



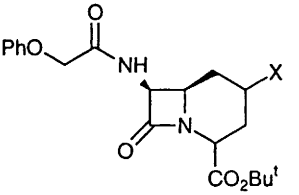
(205) $R^1$ = H, X = OAc
(206) $R^1$ = H, X = $SO_2Ph$
(207) $R^1$ = $SiBu^tMe_2$, X = $SO_2Ph$



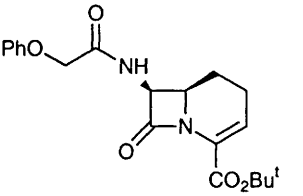
(208) $R^1$ = $SiBu^tMe_2$
(209) $R^1$ = H



(210) $R^1$ = H
(211) $R^1$ = H, $R^2$ = $CH_2CH_2CH{=}CH_2$
(212) $R^1$ = H, $R^2$ = $CH_2CH_2CO_2H$
(213) $R^1$ = H, $R^2$ = $CH_2CH{=}CH_2$
(214) $R^1$ = $CH_2CO_2Bu^t$, $R^2$ = $CH_2CH{=}CH_2$



(215) X = OH
(217) X = I



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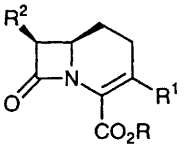

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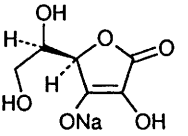
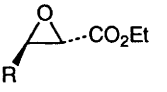
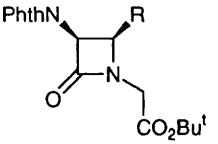
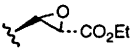
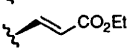
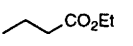


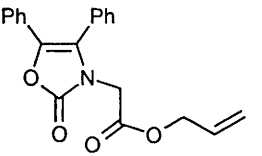
(219)



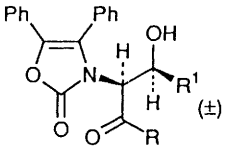
(220) R = CHO
(221) R = $CH{=}NCH_2CO_2Bu^t$

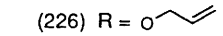


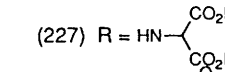
(222) R = 
(223) R = 
(224) R = 

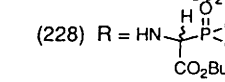


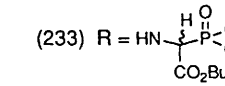
(225)

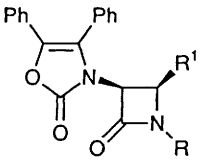


(226) R =  , $R^1 = CH_2CH_2CO_2Me$

(227) R =  , $R^1 = CH_2CH_2CO_2Me$

(228) R =  , $R^1 = CH_2CH_2CO_2Me$

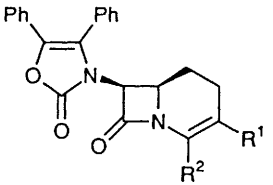
(233) R =  , $R^1 = CH_2CH_2CH{=}CH_2$



(229) R =  , $R^1 = CH_2CH_2CO_2Me$

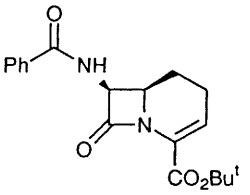
(230) R =  , $R^1 = CH_2CH_2CO_2Me$

(234) R =  , $R^1 = CH_2CH_2CH{=}CH_2$



(231) $R^1 = OH$, $R^2 = CO_2Et$

(232) $R^1 = OMe$, $R^2 = CO_2Bu^t$



(235)

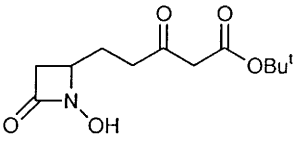
single diastereomer.[212] **(226)** was then reacted with diethylaminomalonate or *tert*-butylaminodiethylphosphonoacetate to give **(227)** and **(228)** respectively. Subsequent cyclisation gave the β-lactams **(229)** and **(230)** with the desired *cis*-stereochemistry. Ring closure reactions of **(229)** and **(230)**, in an attempt to prepare carbacephems **(231)** and **(232)**, proved unsuccessful. The β-hydroxy amino acid derivative **(233)** was prepared in an analogous manner and converted to **(234)**. Ozonolysis and treatment with base effected cyclisation, and cleavage of the side chain protection gave the carbacephem ester **(235)**. A remarkable three reaction 'one pot' protocol was employed in the synthesis of a carbacephem precursor.[213,214] Optimally, reaction of ketone **(236)** with trityl azide, N,N-diisopropylethylamine and trimethylsilylazide effected not only the anticipated diazo transfer but also reduction of the N-O bond and introduction of an azido group at $C_3$ to give **(237)** in 79% yield. An $S_N2'$ reaction (Scheme 5) was proposed in order to rationalise the latter two transformations.

The synthesis of 1,2-disubstituted carbacephems has been reported.[215] Acetonide **(238)** was prepared by [2+2] reaction of **(239)** and azidoacetyl chloride. Subsequent manipulation gave the bicylic intermediate **(240)**, which was converted to **(241)**. Reaction of **(241)** with the azaenolate derived from acetonitrile gave exclusive ring opening of the urethane activated δ-lactam ring to give **(242)**, which underwent *in situ* Wittig reaction to give the desired carbacephem **(243)**.[215]

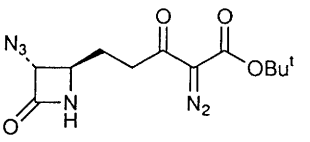
The preparation of 4-hydroxy-2-oxo carbacepham **(244)** with a $C_7$-acylamido side chain has been reported using silica gel induced cyclisation of **(245)**.[216] Improved synthetic routes to intermediates such as **(245)** have been developed. Thus, treatment of oxime **(246)** derived from ketone **(247)**, with $TiCl_3$ in acetone/aq.NaOAc at pH5 effected both hydrolysis of the oxime functionality and reduction of the N-hydroxy β-lactam to give **(248)**, which was cyclised using silica gel.[217] $\Delta^{1,6}$-Carbacephems have been synthesised via treatment of the 4,4-diphenylthioazetidin-2-one **(249)** with $Bu_3SnH$ and catalytic AIBN to give **(250)** in excellent yield.[218] Subsequent oxidation and thermal elimination gave **(251)** which was deprotected to give the desired carbacephem **(252)**. The syntheis of $C_3$-phosphonocarbacephems **(253)** has been described.[219] The key step involved an Arbusov reaction of the bromide **(254)**. The first synthesis of a $C_7$-methoxycarbacephem **(255)** has been reported.[220] Thus, **(256)** and **(257)** were synthesised by alkylation of lactam enolate **(258)** with ethyl iodide or acetaldehyde respectively (in the case of **(257)** diastereomerically pure material was obtained by oxidation followed by reduction with L-Selectride). Conversion of **(256)** and **(257)** to **(259)** and **(260)** using precedented methodology, followed by diazo transfer and rhodium carbenoid cyclisation gave **(261)** and **(262)** respectively. **(261)** was converted to the desired PS-5 analogue **(255)**.[220]

## 8 Azetidin-2-ones

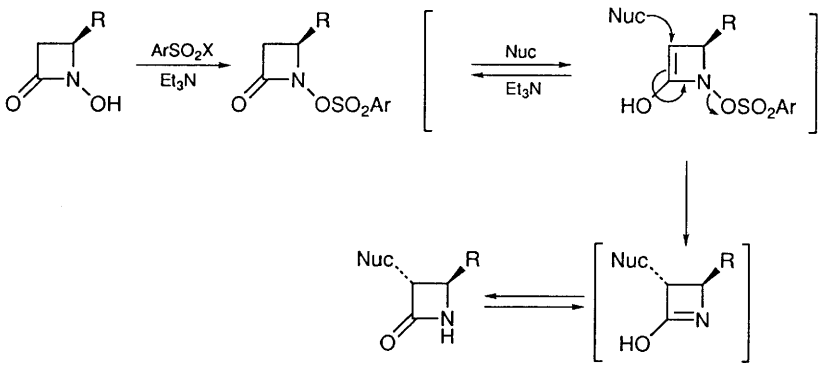
Emphasis is given to new methods for the synthesis of azetidin-2-ones. Further examples of existing methodologies are briefly described.



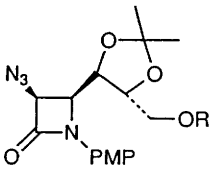
(236)



(237)



**Scheme 5**



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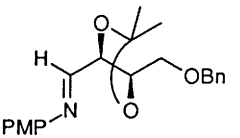

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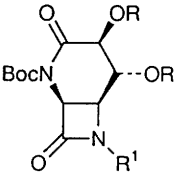
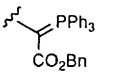
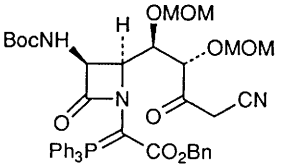


(240) R = MOM, $R^1$ = PMP

(241) R = MOM, $R^1$ = 



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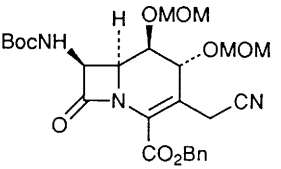

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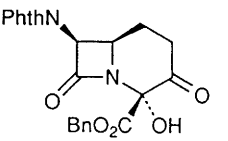

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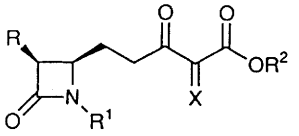
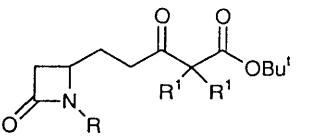


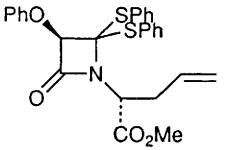
(245) R = NPhth, $R^1$ = H, $R^2$ = Bn, X = O

(±)(246) R = H, $R^1$ = OH, $R^2$ = $OBu^t$, X = NOH(mixture of isomers)



(247) R = OBn, $R^1$ = H

(248) R = H, $R^1$ = OH



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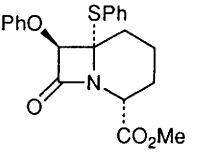

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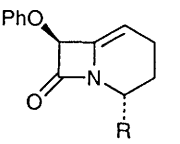
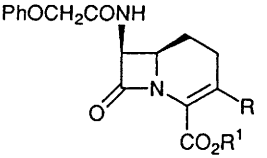


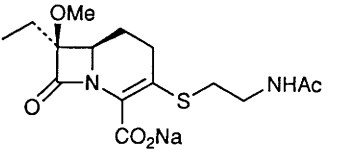
(251) R = $CO_2Me$

(252) R = $CO_2H$

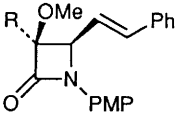


(253) R = $PO_3R^2_2$, $R^2$ = Me, Et, $Bu^n$

(254) R = Br, $R^1$ = $CO_2CHPh_2$

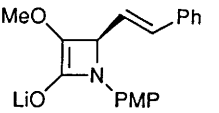


(255)



(256) R = Et

(257) R = 



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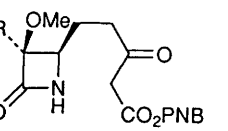
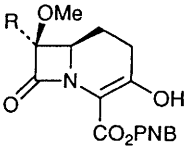


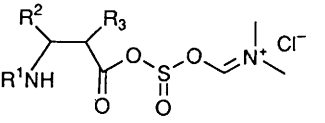
(259) R = Et

(260) R = 



(261) R = Et

(262) R = 



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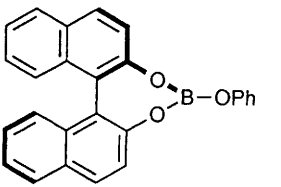
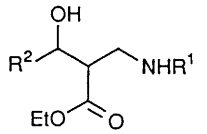


(264)

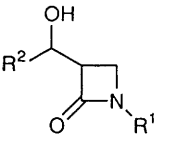
## 8.1 Reactions in which One Bond is Formed

*8.1.1 1,2-Bond Forming Reactions.* – N-[[(Chlorosulfinyl)oxy]methylene]-N-methylmethanaminium] chloride has been introduced for the cyclisation of β-amino acids to azetidin-2-ones via intermediate **(263)**.[221] The asymmetric aldol reaction of chiral imines with silyl ketene acetals mediated by boron reagent **(264)** or its enantiomer afforded β-amino esters with high diastereoselectivity. The esters were cyclised (PhMgBr) to *cis*-azetidin-2-ones.[222-224] Acidic clay montmorillonite has been found to catalyse the addition of silyl ketene acetals to imines to give β-amino esters.[225] Further examples of the cyclisation of β-amino acids using $PPh_3$-$(pyS)_2$ have been reported,[225-228] as has the cyclisation of β-amino acids using 1-methyl-2-chloro-pyridinium iodide.[229-231] **(265)** was converted to **(266)** by refluxing in methanol.[232] The addition of $Sn[N(TMS)_2]_2$ to β-amino esters **(267)** resulted in high yields of **(268)** provided substituents at $C_3$ and on nitrogen were not sterically demanding.[233] Azoacetates **(269)** formed by the reaction of the appropriate hydrazone with lead tetraacetate, were cyclised to **(270)** using a range of bases in acetone or an alcohol. When an alcohol was used as solvent, **(271)** was also isolated from the reaction mixture [formed by a rearrangement following deacetylation of **(269)**] and was subsequently cyclised to give the 1,2-diazetidin-3-one **(272)** in moderate yield.[234] The use of dicyclohexylcarbodiimide (DCC)[235-237] and Grignard reagents[238-243] for β-lactam ring closure reactions has continued. Treatment of isoxazolidine **(273)** with *tert*-butyl lithium gave a mixture of **(274)** and **(275)**. **(274)** was converted to **(275)** by treatment with methyl magnesium iodide.[238] Hydrogenolytic cleavage of $C_2$ ester substituted nitrones followed by ethyl magnesium bromide induced cyclisation gave azetidin-2-ones.[239] β-Amino ester **(276)**, prepared by diastereoselective alkylation of enolate **(277)** and nitrile reduction, was converted to **(278)** using methyl magnesium bromide.[240,241] **(279)** was prepared (following protecting group manipulation) by *tert*-butyl magnesium chloride induced cyclisation of the homotyrosine derivative **(280)**.[242] Attempts to cyclise **(281)** to give **(282)** using a variety of Grignard reagents failed due to the fragmentation of the thiazolidine ring. The use of triisobutylaluminium enabled a 30% yield of **(282)** to be attained.[243] LDA has also been used to promote β-amino ester cyclisations.[244]

*8.1.2 3,4-Bond Forming Reactions.* – Photocyclisation reactions of acrylylureas **(283)** bearing two chiral (1-phenylethyl)amino groups gave a diastereomeric mixture of **(284)** (major) and **(285)**.[245] Photocyclisation of chiral crystals of achiral N,N-diisopropylarylglyoxylamide **(286)**[246] and achiral monothiomide **(287)**[247] gave azetidin-2-ones **(288)** (> 90% e.e.) and **(289)** (40% e.e.) respectively. $SO_2$ extrusion from **(290)** gave a mixture of **(291)** and **(292)**.[248] Continuing studies into the rhodium (II) catalysed cyclisation of α-diazo amides **(293)** have demonstrated that for a series of ligands in the $Rh_2L_4$ catalyst, acetate ligands give the highest ratio of β-**(294)** to γ-lactam **(295)**.[249] Enantioselective CH bond insertions using substrate **(296)** and chiral ligands gave predominantly β-lactams with moderate e.e. provided substituent R in **(296)** was a strong electron withdrawing group (otherwise γ-lactam formation dominated).[249] Treatment of



(265) $R^1$ = $Pr^n$, $Pr^i$, $Bu^n$, $Bu^i$, $Bu^t$, c-$C_6H_{11}$, $PhCH_2$
$R^2$ = H, Me



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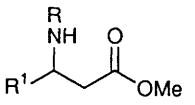
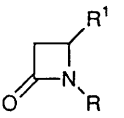


(267) R = Bn, PMP,



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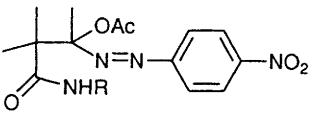
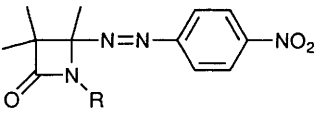


(269)



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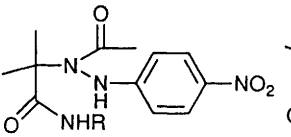

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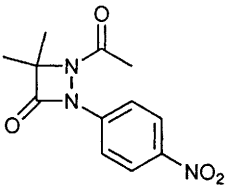

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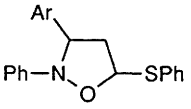

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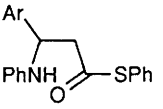

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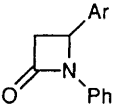

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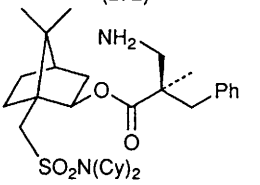

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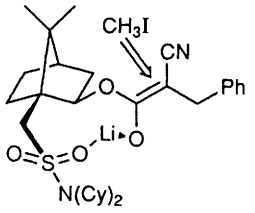

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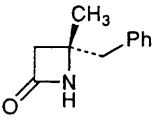

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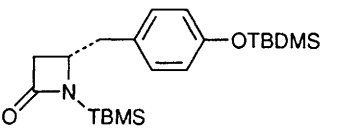

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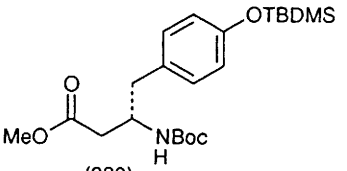

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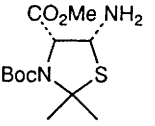

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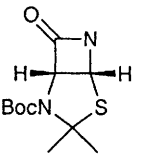

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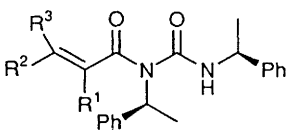

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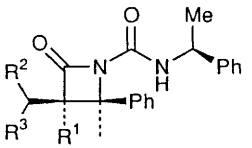
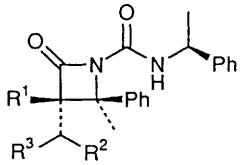
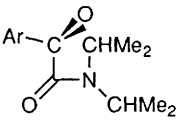


(284) $R^2 = R^3 = H$ or Me
$R^2$ = Me, Et, $Pr^n$, $Pr^i$, $R^3 = H$



(285) $R^2 = R^3 = H$ or Me
$R^2$ = Me, Et, $Pr^n$, $Pr^i$, $R^3 = H$



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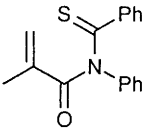

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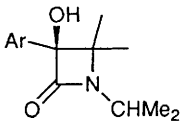

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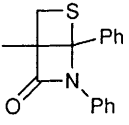

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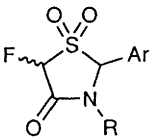

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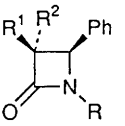
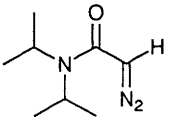


(291) $R^1 = F$, $R^2 = H$
(292) $R^2 = H$, $R^1 = F$



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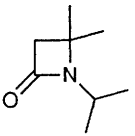

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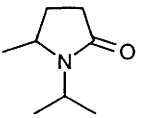

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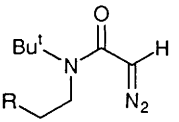

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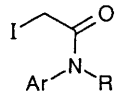
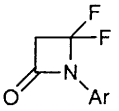


(297) R = H
(299) R = $CF_2^-$



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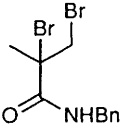

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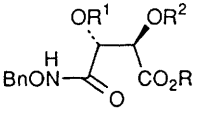
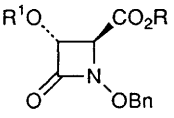
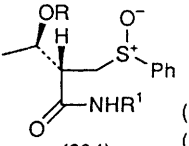


(301) $R^2 = H$, $R^1$ = Bn, Me
(302) $R^2$ = Ts, $R^1$ = Bn, Me



(303) $R^1$ = Bn, Me



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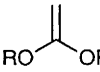
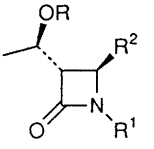


(305) R = Me, $R^1 = SiMe_2Bu^t$
(308) R = $PhCH_2$, $R^1 = SiMe_3$



(306) $R^2$ = SPh
(307) $R^2$ = SOPh
(309) $R^2 = CH_2CO_2Bn$

α-haloacetamides **(297)** with chlorodifluoromethane and base gave 4,4-difluoro-azetidin-2-ones **(298)** via **(299)**.[250]

*8.1.3 1,4-Bond Forming Reactions.* – Dibromide **(300)** was cyclised in optimum yield with potassium carbonate in acetone at reflux.[251] Analogous conversion of **(301)** to the tosylate **(302)** and treatment with base gave azetidin-2-one **(303)**. This two step process gave higher yields than the standard Mitsunobu or mesylation/potassium carbonate in acetone conditions.[252] Mitsunobu methodology has been successfully used by several groups.[253-255] Silicon induced Pummerer-type reaction of sulfoxide **(304)** in the presence of **(305)** and $ZnI_2$ gave predominantly **(306)**. Oxidation of **(306)** to **(307)** followed by $ZnI_2$ catalysed reaction with **(308)** gave **(309)** in high yield.[256] Treatment of **(310)** and **(311)** with MeI and silver perchlorate followed by cyclisation with potassium carbonate gave **(312)** and **(313)** respectively.[257] Stereoselective azide mediated ring expansion of cyclopropanone **(314)** in the presence of 15-crown-5 gave **(315)** via **(316)** in high yield.[258]

*8.1.4 2,3-Bond Forming Reactions.* – No new examples of this class of reaction were found.

## 8.2 Reactions in which Two Bonds are Formed

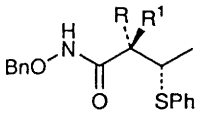
### 8.2.1 [3 + 1] Additions

Palladium catalysed carbonylation of vinyl aziridine **(317)** in the presence of excess $PPh_3$ gave **(318)** in moderate yield. The stereoselectivity reportedly results from isomerisation of initially formed **(319)** to **(320)** which may be stabilised by internal coordination between the nitrogen of the sulfonamide and palladium.[259] Reaction of haloacetanilides ($XCH_2$ CONHAr) with diethyl malonate anion gave **(321)** in moderate yield.[260]

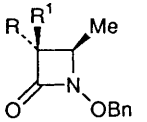
### 8.2.2 [2 + 2] Additions

*8.2.2.1 1,2 and 3,4-Bond Formation.* – A multistep route to **(322)**, an intermediate in the synthesis of Taxol, starting from reaction of acid chloride **(323)** with the appropriate imine, has been developed.[261] The reaction of ketenes with imines derived from 2-oxoalkanoic esters gave predominantly *cis*-azetidin-2-one **(324)**. Decarboxylation of **(324)** gave **(325)**.[262] Reaction of diketene with a chiral Schiff base was promoted by imidazole to give a diastereomeric mixture of *trans* azetidin-2-ones **(197)** and **(326)** in a 2:1 ratio in 90% yield. The predominance of *trans*-azetidin-2-ones was rationalised by imidazole mediated epimerisation of the initially formed *cis*-azetidin-2-ones.[263]

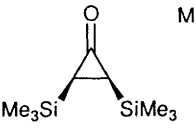
Microwave-induced organic reaction enhancement chemistry techniques have been applied to the synthesis of enantiopure $C_3$-hydroxyazetidin-2-ones,[264,265] as well as $C_3$-vinyl azetidin-2-ones.[266,206] The application of chiral imines to the Staudinger [2 + 2] reaction has continued with Schiff bases derived from threonine esters,[267] galactose,[268] N-Boc amino aldehydes,[269,270] methyl (*R*)



(310) R = Me, $R^1$ = H
(311) R = H, $R^1$ = Me



(312) R = Me, $R^1$ = H
(313) R = H, $R^1$ = Me



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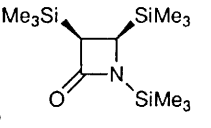

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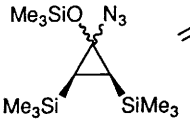

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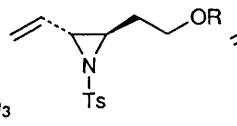

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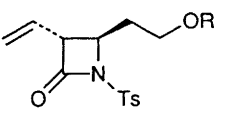

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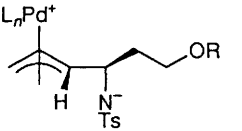

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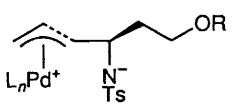

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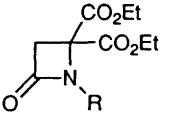

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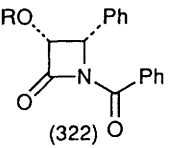

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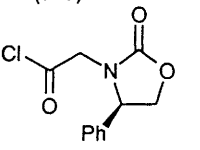

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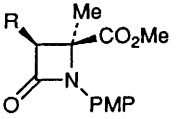
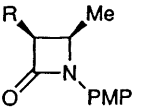


(324) R = Me, Et, $Pr^i$, $PhCH_2$, MeO, $PhCH_2O$



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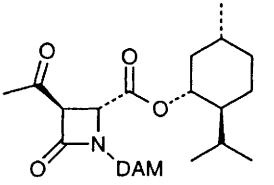

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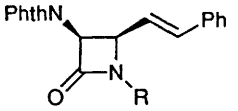

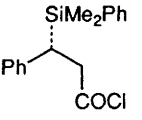

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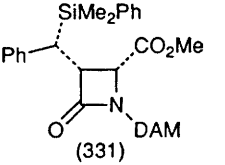

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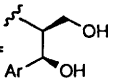
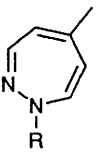
(327) R =



(328) R =

(329) R = H



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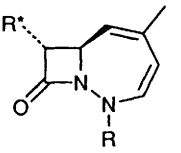

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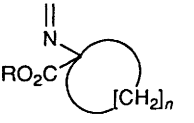


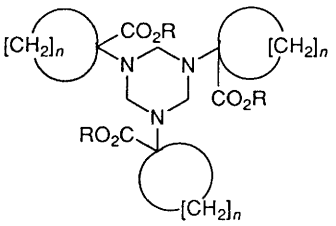
(334)

or (*S*)- mandelate[271] and glyoxalate esters.[272,273] The use of Schiff bases derived from optically active 2-amino-1-aryl-propane-1,3-diols has been reported for the chiral synthesis of azetidin-2-ones.[274] A method for the removal of this chiral auxillary from (**327**) has been developed using Jones oxidation to give (**328**) followed by treatment with wet $SiO_2$ or catalytic sodium methoxide in methanol/ THF to give (**329**).[274] The use of chiral acid chloride (**330**) enabled (**331**) to be synthesised.[273]

Reaction of diazepine (**332**) with the ketene generated from (**323**) gave (**333**). A review of the chemistry carried out on these derivatives has been reported.[275] Imine (**334**) (n = 4,5) [which exists predominantly as trimeric species (**335**)] reacted with phthaloylglycyl chloride, $BF_3.Et_2O$ and pyridine to give azetidin-2-ones (**336**).[276] The ketene (**337**) derived from (**338**) reacted with several imines to give (**339**) in >99% diastereomeric excess. Resolution of (**338**) followed by analogous reactions were carried out to give chiral material.[277] Saturated *cis*- and *trans*-3,1- (**340**) and 1,3-benzoxazines (**341**) reacted with chloroacetyl chlorides to give the corresponding azetidin-2-ones.[278] Palladium catalysed carbonylation of allyl diethyl phosphate in the presence of imines, triphenylphosphine and Hunig's base under CO pressure gave predominantly *cis*-(**342**) if the imine functionality was conjugated with a carbonyl group or *trans*-(**343**) if not.[279]

A series of spiro fused β-lactam oxadiazolines (**344**) have been prepared from imine (**345**). Their thermodynamic stabilities with regards to decomposition to $C_4$-ylidenes (**346**) were investigated [280,281] Several other reactions of (**346**) were explored.[282,283] A comparison of the chemistry of (**344**) with β-lactam thiadiazoline and triazoline systems (**347**) has been reported.[284] Schiff base (**348**) reacted with *in situ* generated chloroketene to yield *trans*-azetidin-2-one (**349**).[285] *In situ* generation of (**350**) from (**351**) and N-benzoylalanine followed by reaction with (**352**) and triethylamine gave (**353**) in good yield.[286] Addition of triethylamine to a solution of acetoxyacetyl chloride and imine (**354**) gave predominantly racemic *cis*-(**355**). In the initial absence of triethylamine *trans*-(**356**) was formed via acyliminium salt (**357**). (**357**) was trapped with isopropanol to give crystalline (**358**).[287] Reaction of imines with silyl ketene acetals in the presence of KF/18-crown-6 under microwave irradiation gave a mixture of *trans*-(major) and *cis*-azetidin-2-ones.[288] Two computational studies on the Staudinger reaction have been published.[289,290]

(**359**) has been developed as a chiral imine for stereocontrolled synthesis of azetidin-2-ones using the ester-enolate imine route.[226] Reaction of titanium enolates of $C_3$-sulfenyl esters with (**359**) gave *trans*-azetidin-2-one (**360**) in good yield. (**359**) reacted with zinc enolate (**361**) to give predominantly *cis*-(**362**), the reversal in diastereoselectivity being attributed to the different coordination characteristics of the enolate metals.[226] Analogous reaction of (**359**) with titanium, lithium and zinc enolates of *tert*-butyl-α-alkyl acetates[231] and titanium enolates of α-haloacetates[291] have also been carried out to give $C_{3,4}$-dialkyl substituted and $C_3$-haloazetidin-2-ones respectively. In contrast, lithium enolates of *tert*-butyl chloroacetate were found to react with (**359**) to give aziridine (2*R*,3*S*) (**363**) exclusively, while the zinc enolate gave aziridine (2*S*,3*R*) (**364**) exclusively.[291]



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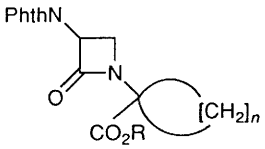

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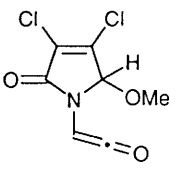

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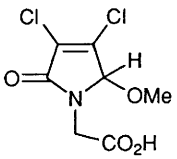

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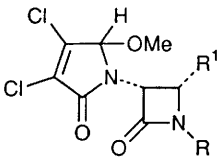

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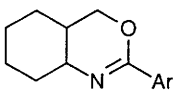

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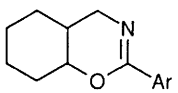

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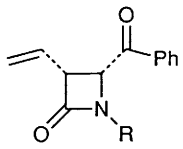

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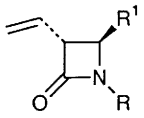

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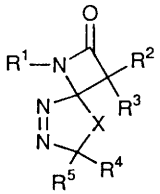
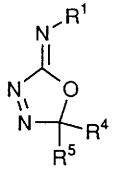


(344) X = O
(347) X = NR or S



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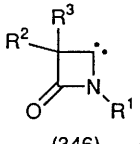

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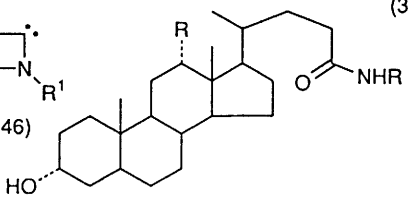
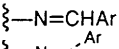
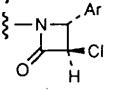
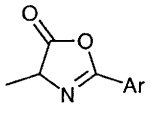


(348) R = 
(349) R = 



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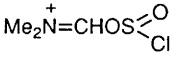

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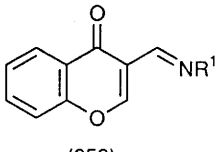

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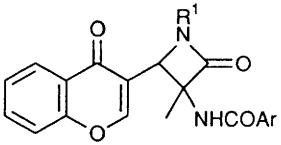

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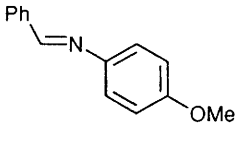

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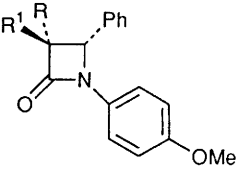


(355) R = OAc, $R^1$ = H
(356) R = H, $R^1$ = OAc

Detailed studies on the use of zinc mediated ester enolate-imine reactions have been carried out including their use for the enantioselective synthesis of $C_3$-aminoazetidin-2-ones using either chiral esters[292] or chiral imines.[292-294] Use of bis-silyl protected glycine esters (**365**) have enabled the free $C_3$-aminoazetidin-2-ones to be obtained.[293,294] Reaction of titanium enolates of 2-pyridyl thioester with (**366**) gave predominantly (**367**).[295,296]

Chiral ester (**368**) was converted to (**369**) in high yield and enantiomeric excess. (**369**) was converted to norstatine and several of its analogues.[297] Lithium enolates derived from esters containing Oppolzers chiral auxillary gave *cis* azetidin-2-ones with high e.e.[298] Treatment of (**370**) with an amine and reaction of the resulting imine with lithium ester enolates gave *trans*-(**371**).[299] A computational study on the reaction of α-hydroxyl ester lithium enolates with imines has indicated that the energetically more stable *Z*-enolate reacts with the imine through a boat like transition state.[300]

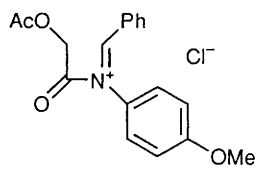
*8.2.2.2 1,4- and 2,3-Bond Formation.* – The reaction of trichloroacetyl isocyanate with pyranoid and furanoid glycals proceeds in both cases to give a mixture of [2+2] and [4+2] cycloadducts.[301] β-Lactam formation occurs in both cases exclusively *anti* to the $C_3$ carbon substituent. Reaction of racemic allene (**372**) with chlorosulfonyl isocyanate gave after deprotection (**373**) and (**374**) in a 7:1 ratio. Partial resolution of allene (**375**) followed by conversion to optically active (**372**) enabled the synthesis of optically active (**373**) with enantiomeric excess in the region of 50%.[302] (**376**), prepared from the corresponding enamine and acylisocyanate (ArCONCO), was converted to a pyrimidinone by treatment with ammonium acetate in acetic acid or methanol.[303] *Ab initio* studies on the alkene-isocyanate cycloaddition reaction indicated that a concerted suprafacial mechanism is favoured.[304]

## 8.3 Chemistry of Azetidin-2-ones

*8.3.1 $N_1$ Chemistry.* – Deprotection of N-hydroxyazetidin-2-ones using $Mn(OAc)_3/Cu(OAc)_2$ has been reported.[305] Electrodic cleavage of the N-S bond in N-tosylazetidinones gave N-unsubstituted derivatives in high yield.[306] Anomalous behaviour on attempted cerium (IV) ammonium nitrate (CAN) deprotection of (**377**) gave a range of products depending on the work up procedure. When R = H in (**377**), stable spirocycle quinone aminal (**378**) was isolated.[307] (**379**) gave the expected N-unsubstituted azetidin-2-one on treatment with CAN, whereas the diastereomer (**380**) gave (**381**) (X = pthalimido).[308]

*8.3.2 $C_2$ and $C_{2'}$ Chemistry.* – Treatment of (**382**) with $P_2S_5$ gave (**383**).[309] The Wittig reaction of azetidin-2-ones with stabilised ylides has been reported.[310]

*8.3.3 $C_3$ and $C_{3'}$ Chemistry.* – Attempts to cleave (**384**) to oxoazetidin-2-one (**385**) by ozonolysis proved unsuccessful. (**385**) was synthesised from (**386**) in three steps including a Baeyer-Villiger rearrangement of (**387**) followed by treatment with $NH_3$ in methanol.[311] Reduction of (**387**) gave a mixture of



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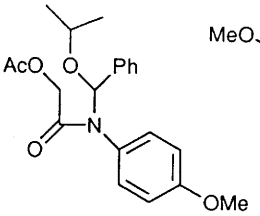

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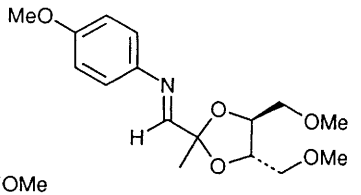

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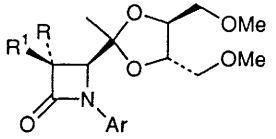
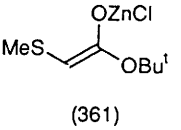


(360) R = SPh, SBu$^t$, SMe; R$^1$ = H



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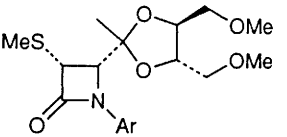

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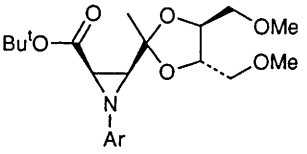

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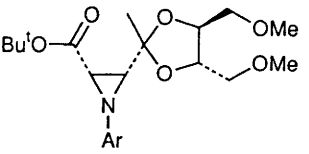

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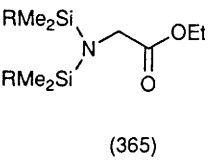

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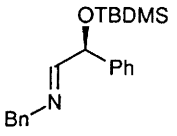

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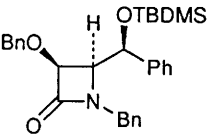

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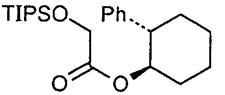

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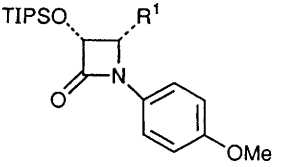

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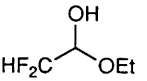

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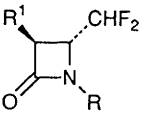

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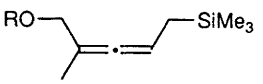
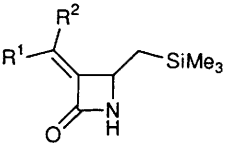
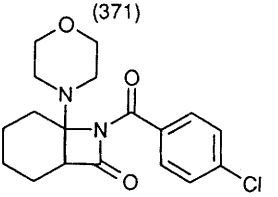


(372) R = TBDMS
(375) R = Ac



(373) R$^1$ = Me, R$^2$ = CH$_2$OH
(374) R$^1$ = CH$_2$OH, R$^2$ = Me



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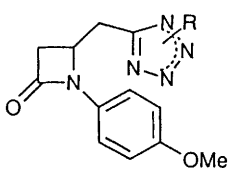

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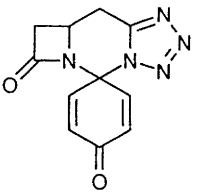

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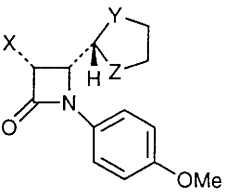
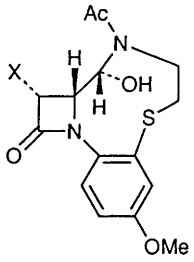


(379) Y = S, Z = NAc
(380) Y = NAc, Z = S



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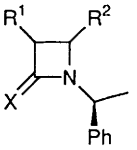
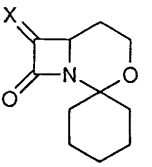
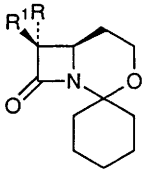


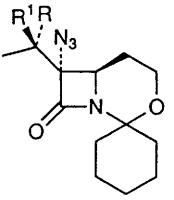
(382) X = O
(383) X = S



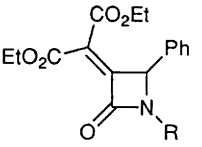
(384) X = $CHCH_3$
(385) X = O



(386) R = COMe, $R^1$ = H
(387) R = $N_3$, $R^1$ = COMe



(388) R = OH, $R^1$ = H
(389) R = H, $R^1$ = OH



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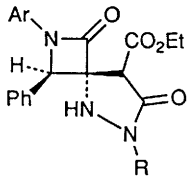

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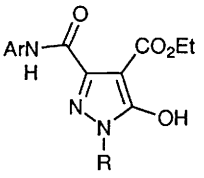

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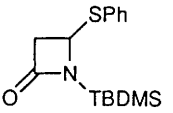

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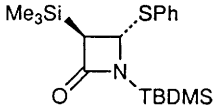

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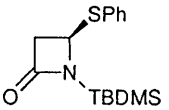

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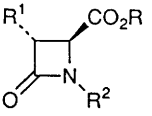


(396) R = H, $R^1$ = H, $R^2$ = TBDMS
(397) R = $R^2$, $R^1$ = Me, Et, $CH_2CH{=}CH_2$, Bn, $R^2$ = H

diastereomers **(388)** and **(389)** in an 8:1 ratio.[312] A detailed study on the addition of O-, S- and N-nucleophiles to the exocyclic double bond of **(390)** has been reported. The products from N-nucleophile addition were found to be unstable, in particular addition of hydrazines gave the spiroazetidin-2-one **(391)** which rearranged to **(392)**. The exocyclic double bond of **(390)** can also participate in cyclopropanation and Diels-Alder reactions.[313]

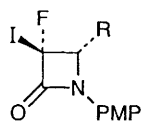
Kinetic resolution of racemic **(393)** by treatment with a homochiral lithium amide base gave **(394)** and **(395)** after quenching with TMSCl.[314] $C_3$ Alkylation of **(396)** followed by ester formation and N-deprotection gave **(397)** in high yields. (2*S*,3*R*)-$C_3$-Alkyl-L-aspartic acids were prepared from **(397)**.[315] **(398)** reacted with a variety of olefins under photolytic conditions to give **(399)** with retention of configuration at $C_3$.[316] Reduction of **(399)** using $Bu_3SnH$ gave **(400)**.[316] **(401)** was shown to undergo a stereoselective sulfilimine rearrangement on treatment with o-(mesitylenesulfonyl)hydroxylamine and $P(OEt)_3$ followed by sodium bicarbonate and $PhCH_2OCOCl$ to give **(402)** in 79% yield as part of a ten step synthesis of (-)-tabtoxin **(4)**.[317] Peterson olefination of **(403)** using LDA and cyclohexanone followed by epoxidation with MCPBA gave **(404)** which rearranged on treatment with $BF_3.Et_2O$ to give γ-lactam **(405)**.[318] **(406)** reacts with $HBF_4.Et_2O$ to give **(407)** which can be converted in low yield to **(408)**, a precursor of (±) thienamycin and (±)-β-hydroxy aspartic acid derivatives.[319] Dehydration of **(408)** gave a mixture of **(409)** and **(410)**. Analogously **(411)** was converted to **(412)** and subsequently to a mixture of **(409)** and **(410)**.[319] The conversion of **(413)** to **(414)** in 80% yield using this methodology has also been reported.[320] In an extension of existing methodology,[213,214] **(415)** was converted to **(416)** using a range of nucleophiles in good yield and diastereoselectivity.[321]

*8.3.4 $C_4$ and $C_{4'}$ Chemistry.* – Several reports on $C_4$ functionalisation of $C_4$-acetoxy-azetidin-2-ones, assumed to occur via the reactive acylimine **(417)**,[322] have been reported.[322-326] Trapping of **(417)** with a variety of oxygen[322,323], sulfur[322,209] and carbon[324,325,326] nucleophiles has been reported. $Pd(OAc)_2$ catalysed reaction of **(418)** with **(419)** gave predominantly **(420)**.[323] Asymmetric alkylation of **(417)** using tin enolate **(421)** ($R^1 = H$) gave **(422)**.[324] Diastereoselective $C_4$ alkylation of **(423)** and **(424)** using **(421)** ($R = Et$, $R^1 = H$) gave predominantly **(425)** and **(426)** respectively.[325] Copper (I) salts of carboxylates, thiocarboxylates and copper (I) enolates were found to displace the $C_4$-sulfur substituent of **(427)** with retention of configuration.[327]

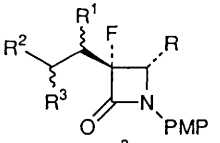
**(428)** reacted with a series of silylated N-, O-, S- and P-nucleophiles in the presence of a catalytic amount of $ZnI_2$ to give $C_4$-heterofunction substituted azetidin-2-ones.[328] Analogous reactions of **(428)** with O-silylated ketene acetals and silyl enol ethers,[329] as well as with tributyltin alkoxides with catalytic TMSOTf[330] have been reported.

Anodic oxidation of **(429)** in the presence of alcohols ($R^1OH$) gave **(430)** in good yield.[331] Treatment of **(431)** with $OsO_4$ and $NaIO_4$ gave **(432)**, a cephalosporin intermediate.[332] Hydrogenation ($R_3 = OR$) and hydroboration ($R_3 = H$) of **(433)** occurred with poor selectivity.[333]

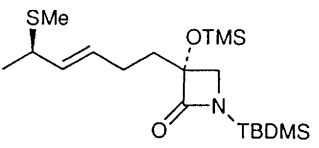
Osmylation of **(434)** in the presence of dihydroquinidine *p*-chlorobenzoate,



(398)



(399) $R^3$ = I
(400) $R^3$ = H



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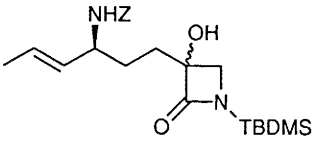

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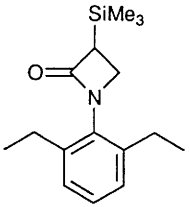
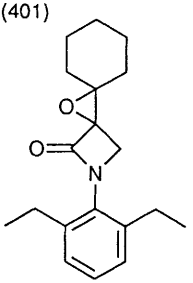
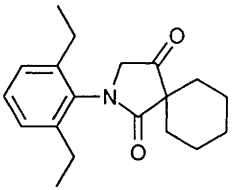


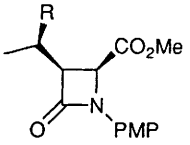
(403)



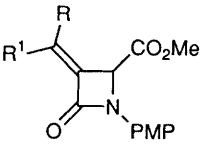
(404)



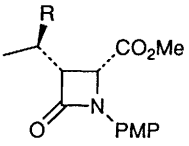
(405)



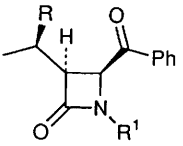
(406) R = $SiMe_2Ph$
(407) R = $SiMe_2F$
(408) R = OH



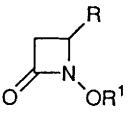
(409) R = H, $R^1$ = Me
(410) R = Me, $R^1$ = H



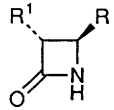
(411) R = $SiMe_2Ph$
(412) R = $SiMe_2F$



(413) R = $SiMe_2Ph$, $R^1$ = PMP
(414) R = OH, $R^1$ = PMP

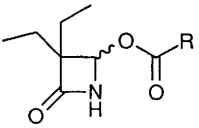


(415) $R^1$ = Ts, $PO(OPh)_2$



(416) $R^1$ = I, Br, Cl, OAc, SPh

(417)



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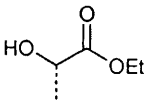

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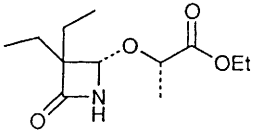

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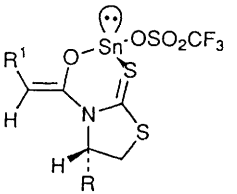

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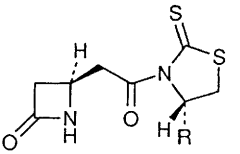

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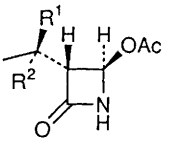
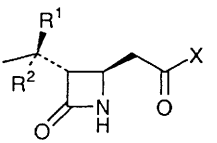
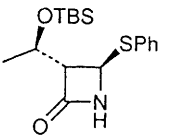


(423) $R^1 = OSiMe_2Bu^t$, $R^2 = H$
(424) $R^1 = H$, $R^2 = OSiMe_2Bu^t$



(425) $R^1 = OSiMe_2Bu^t$, $R^2 = H$
(426) $R^1 = H$, $R^2 = OSiMe_2Bu^t$



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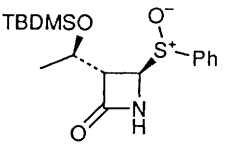
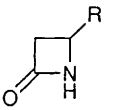
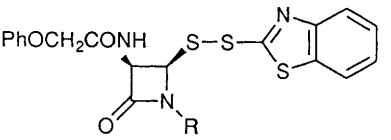


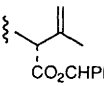
(428)



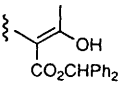
(429) $R = SiMe_3$
(430) $R = OR^1$

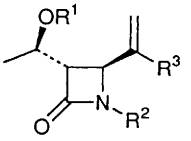


(431) R =



(432) R =





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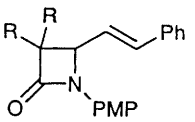

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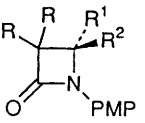
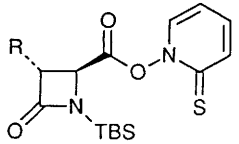
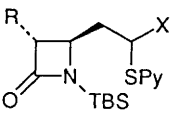
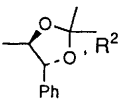


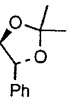


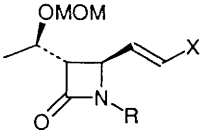
(437)



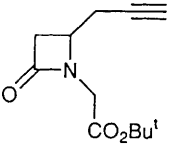
(438)

(435) $R^1 =$  $R^2 = H$

(436) $R^1 = H$, $R^2 =$ 



(439) X = $CO_2Me$, $SO_2Ph$, $NO_2$
R = 3,4-dimethoxybenzyl



(440)

$K_3F(CN)_6$ and $K_2CO_3$ in water:$^t$BuOH 1:1 followed by treatment with $(MeO)_2CMe_2$ and p-TSA gave a mixture of **(435)** and **(436)** in high e.e.[334] Barton decarboxylation of thiopyrimidylhydroxamate **(437)** followed by trapping of the radical with a Michael acceptor gave **(438)** which was converted to carbapenem precursor **(439)**.[335] Treatment of **(440)** with $Bu_3SnH$ led to a mixture of bicycle **(441)** and the monocycle **(442)** in 19 and 56% yield respectively.[336]

*8.3.5 Ring Opening and Rearrangement Reactions of Azetidin-2-ones.* – **(443)** was hydrolysed to **(444)** on heating with aqueous TFA.[337] **(445)** was converted to alkenamides **(446)** as a mixture of stereoisomers via palladium catalysed cross-coupling with aryl iodides[338] or organomercurials.[339] Interestingly, **(447)** reacted with PhI to give **(448)** as an 82:18 E:Z mixture, reportedly formed as a result of palladium migration.[338] Opening of **(449)** with trimethylsulfoxonium ylide and **(450)** with a sulfone ylide gave ylide **(451)** or sulfone **(452)** respectively.[340] Reaction of **(453)** with Boc-L-alanine-N-hydroxysuccinimide ester resulted in formation of lactone **(454)** which was further converted to a peptidyl difluoro ketone inhibitor of ICE.[341] Cleavage of **(455)** with $Me_3SiCl$ in MeOH gave **(456)**.[342]

Sodium borohydride reduction of **(457)**, prepared by reaction of the appropriate β-lactam imine with ozone, provided a stereoselective method of preparing vinyl ethers via decomposition of **(458)**.[343] $SnCl_2.2H_2O$ mediated $C_3$-$C_4$ bond cleavage of **(459)** followed by a cationic rearrangement gave **(460)**.[344] N-$C_2$ cleavage of N-unsubstituted azetidin-2-ones by a pyrazolylborate zinc hydroxide complex has been reported.[345] Methanolysis of **(461)** gave predominantly **(462)**, which was converted to an α-alkyl-α-amino acid via **(463)**.[346]

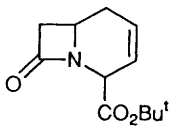
Amination of **(464)** in liquid ammonia followed by ring expansion gave **(465)**, a precursor to a series of alkaloids.[347,348] Thermal rearrangement of **(466)** gave γ-lactam **(467)**.[349] Treatment of **(468)** with excess sodium methoxide in refluxing methanol gave bicycle **(469)** via lactone **(470)**.[350] Rearrangement of *trans*-**(471)** and *cis*-**(472)** to the γ-lactams **(473)** and **(474)** respectively was carried out using TMSCl in refluxing MeOH.[351] β-Lactam derivatives of 1-benzoazepines **(475)**, synthesised by alkene-isocyanate methodology[352] have been ring opened using TMSCN to give β-cyanoamides **(476)** which were cyclised to iminopyrrolidinones **(477)** using $AlCl_3$.[353] A one pot conversion of **(475)** to **(477)** also proved possible.[353]

## 8.4 Further Uses of Azetidin-2-ones

With the increased interest in the synthesis of taxol analogues, a number of examples in which modified taxol side chains have been introduced using ring opening of activated azetidin-2-ones have been reported.[261,354-363] Analogous methodology has been used in the preparation of a brevifoliol analogue.[364]

# 9 Major Structural Variants

Phosphonimidates **(478)** were synthesised and shown to be time dependent inactivators of a class C β-lactamase. Evidence was obtained supporting for-



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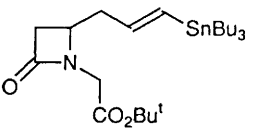

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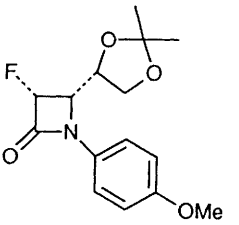

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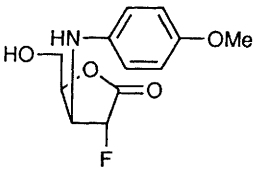

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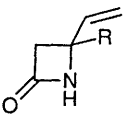
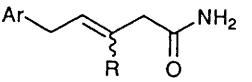


(445) R = H, Me



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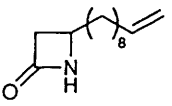

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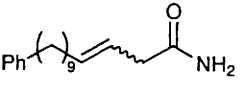

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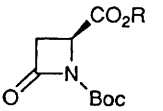
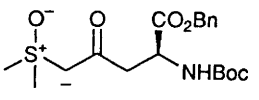


(449) R = Bn
(450) R = Bu$^t$



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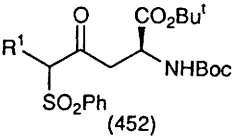

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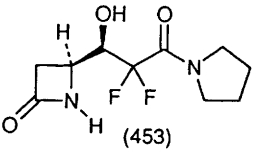

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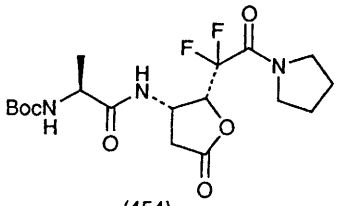

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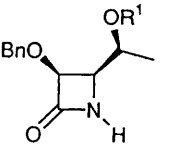

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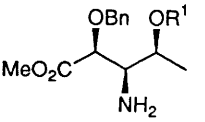

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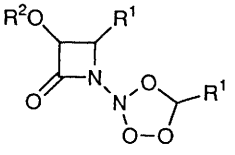

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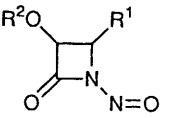

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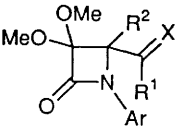
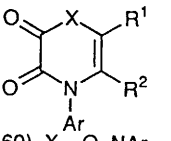
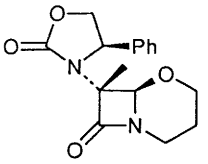


(459) X = O, NAr



(460) X = O, NAr



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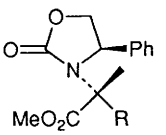
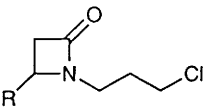


(462) R = CHO
(463) R = $CH_2OH$



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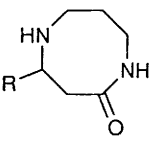

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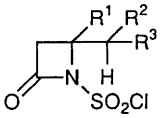
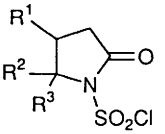
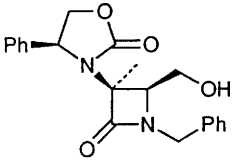


(466a) $R^1$ = Ph, $R^2$ = $R^3$ = Me
(466b) $R^1$ = H, $R^2$ = Ph, $R^3$ = Me
(466c) $R^1$ = Et, $R^2$ = Ph, $R^3$ = H



(467a) $R^1$ = Ph, $R^2$ = $R^3$ = Me
(467b) $R^1$ = H, $R^2$ = Ph, $R^3$ = Me
(467c) $R^1$ = Et, $R^2$ = Ph, $R^3$ = H



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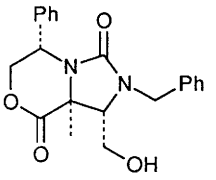
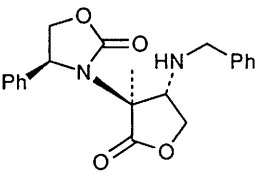


(469)



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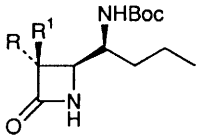
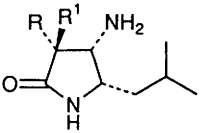
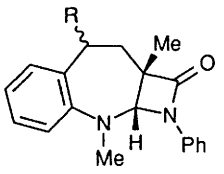


(471) R = Me, Et,  , $R^1$ = H
(472) R = H, $R^1$ = Me, Et, 



(473) R = Me, Et,  , $R^1$ = H
(474) R = H, $R^1$ = Me, Et, 



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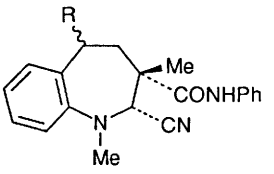

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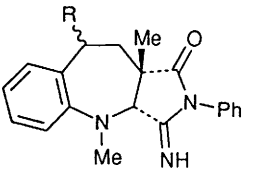

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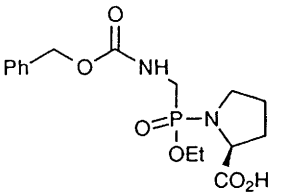

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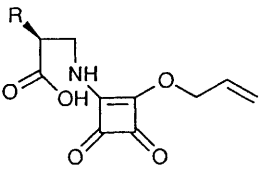

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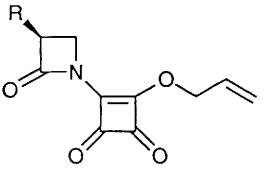

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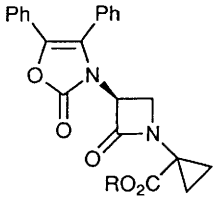

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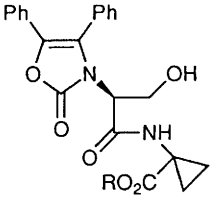

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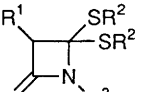

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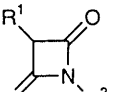

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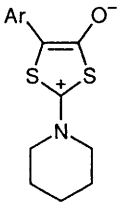

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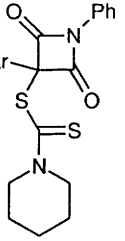

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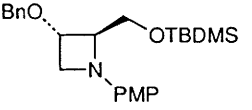

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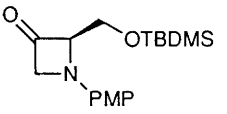

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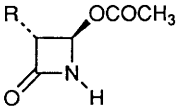
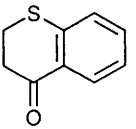


(489) R = 

(495) R = $NHCOCH_2OPh$



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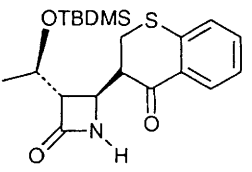

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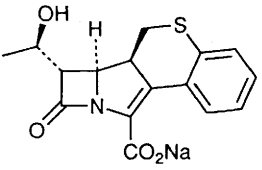
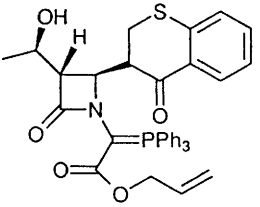


(492)



(493)

mation of a 1:1 covalent enzyme-inhibitor complex in which the prolyl residue of **(478)** was expelled by an enzyme bound nucleophile (probably the active site serine).[365] DCC cyclisation of **(479)** gave the N-(hydroxydioxocyclobutenyl)-β-lactam **(480)** in low yield (24%). **(480)** exhibited no useful antibacterial activity.[237] The 1-aminocyclopropane-1-carboxylic acid containing β-lactam **(481)** was prepared from **(482)** using Mitsunobu methodology. This route was not successful for the synthesis of more functionalised β-lactams.[254]

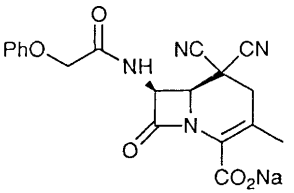
A new synthesis of azetidin-2,4-diones has been reported.[366] **(483)** was synthesised from the corresponding imine and acid chloride. Reaction of **(483)** with N-bromo succinimide gave **(484)** in high yield.[366] Reaction of **(485)** with phenyl isocyanate gave **(486)**.[367] Azetidine **(487)**, prepared in several steps from diethyl-L-tartrate, was converted by $Pd(OH)_2$ mediated debenzylation and Swern oxidation to azetidin-3-one **(488)**,[368] a precursor to polyoximic acid.[369]

Several tetracyclic carbapenems have been synthesised using related methodology and were shown to exhibit significant antibacterial activity.[370] In one case, reaction of azetidin-2-one **(489)** with **(490)** (using lithium hexamethyldisilylazide[371] or TMS triflate/$Et_3N$) gave **(491)** which was subsequently manipulated using standard carbapenem methodology to the tetracycle **(492)** via **(493)**.[370] Synthesis of the antibacterially active and highly reactive ($\nu_{max}$ β-lactam carbonyl = 1770 $cm^{-1}$) 1,1-dicyanocephem **(494)** has been described.[372] Reaction of acetoxyazetidin-2-one **(495)** with malononitrile derivative **(496)** gave *trans*-**(497)**. Ozonolysis of **(497)** followed by N-acylation gave **(498)** which underwent ring closure on treatment with triethylphosphite to give **(499)**. Epimerisation of **(499)** at $C_7$ and conversion to **(494)** was achieved using precedented procedures.[372]

A synthetic route to several isocephem analogues (all derivatives tested showed no useful antibacterial activity) has been developed.[373] The key step was reaction of enolate **(500)** with carbon disulfide.[373] A range of isocephems **(501)** with intercalated double bonds between the cephem ring and a leaving group have also been synthesised.[374]

Isopenam **(502)** and isocephems **(503)** and **(504)** displayed antibacterial activity.[375] The key step in the synthesis of the isocephems **(503)** and **(504)** was the Pummerer rearrangement of sulfoxides **(505)** and **(506)** respectively. **(504)** displayed potent carcinostatic activity.[375] The synthesis of 2-isocephems **(507)** and 2-oxa-isocephem **(508)** has been reported.[376] Anhydro-2-azacephams **(509)** were synthesised by intramolecular cyclisation (DCC) of sulfonamides **(510)**, readily prepared from penicillins.[377]

Interest in activated γ-lactam analogues of β-lactams continues.[378,379,382] The first synthesis of thienamycin-like γ-lactam analogues was achieved by the diastereoselective coupling of tin enolate **(421)** ($R = CHMe_2$) with γ-lactam **(511)** to give **(512)**. **(512)** was converted to the β-keto ester **(513)** by treatment first with imidazole followed by a decarboxylative Claisen-type reaction. Desilylation of **(513)** followed by diazotisation and rhodium catalysed ring closure gave the bicycle **(514)**. Subsequent manipulation gave the desired analogues **(515)**. None of these analogues were active as antibacterials.[378,379] An analysis of the activation energies required for the methanolysis of various β- and γ-lactams suggested that γ-lactams bearing electron donating groups at $C_2$, such as **(516)** or **(517)**,



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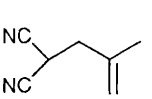

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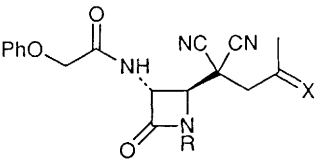
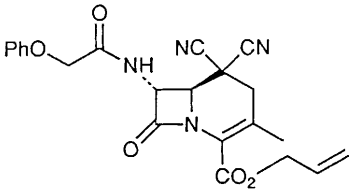


(497) R = H, X = $CH_2$
(498) R = $COCO_2$  , X = O



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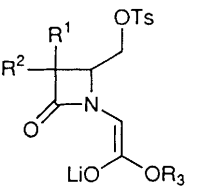
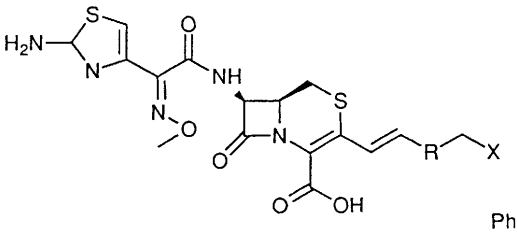
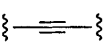
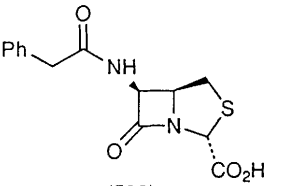


(500)



(501) R =  ,  ,  , , Y = S, O



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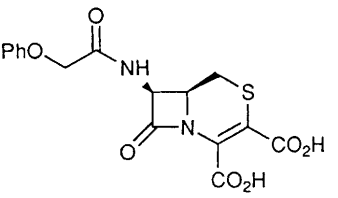

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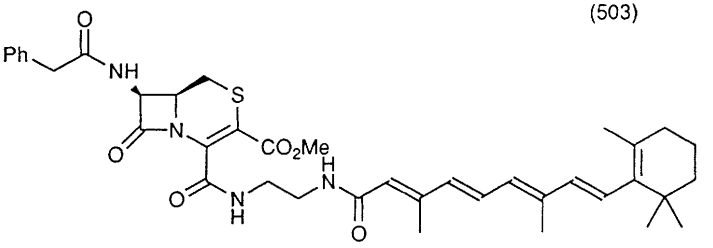

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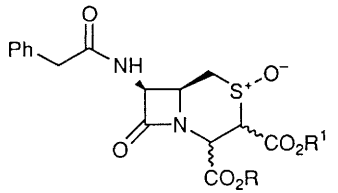
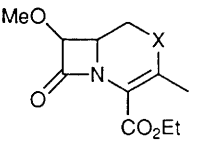
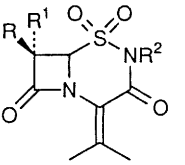


(505) R = Bn
(506) R = H, $R^1$ = Me



(507) X = S
(508) X = O



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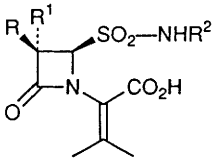

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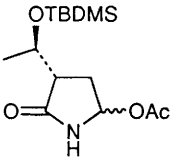

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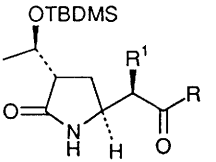
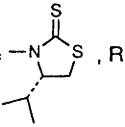
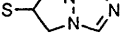
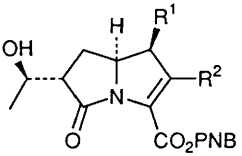
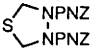
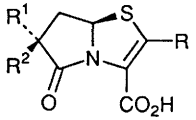


(512) R =  , $R^1$ = H, $S(CH_2)_2\overset{+}{N}H_3$, 

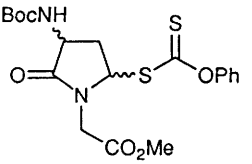


(513) R = $CH_2CO_2PNB$, $R^1$ = H

(514) $R^2$ = OH, $R^1$ = H
(515) $R^2$ = SPh, $SCH_2CH_2NHPNZ$,  , $R^1$ = H



(516) R = $SR^3$, $R^1$ = $R^2$ = H
(517) R = OMe, $R^1$ = $R^2$ = H
(520) R = SEt, $R^1$ = H, $R^2$ = $NHCOCH_2Ph$
(521) R = SEt, $R^1$ = $NHCOCH_2Ph$, $R^2$ = H



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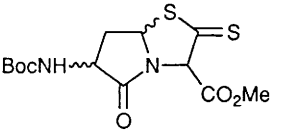

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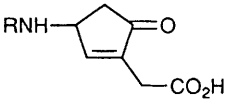
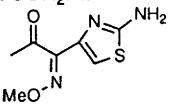
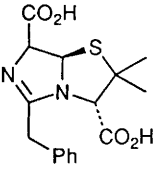


(522) R = $COCH_2Ph$

(523) R = 



(524)

may be more reactive as acylating agents, than those bearing $C_2$-electron withdrawing groups (which were previously supposed to increase activity).[380] **(516)** ($R^3$ = Et) was synthesised as a mixture of diastereomers in racemic form, by a route in which the key step was cyclisation of **(518)** using lithium hexamethyldisilylazide to give **(519)**. Subsequent manipulation of **(519)** gave a mixture of *cis*-**(520)** and *trans*-**(521)** diastereomers, which displayed weak antibacterial activity.[380] 2-cyclopenten-1-ones **(522)** and **(523)** were designed and synthesised as potential alkylating inhibitors of penicillin binding proteins.[381] None were active and the lack of activity was attributed to the inability of **(522)** and **(523)** to form stabilising hydrogen bonds with the enzymes.[381] A ring switching reaction was used for the synthesis of some γ-lactam analogues of the penams and penems.[382] Based on the knowledge that $C_6$-acylamino penicillins undergo decomposition by attack of the carbonyl oxygen on the β-lactam to give imidazoline derivatives **(524)** via oxazolidines **(525)**, $C_6$-methylenecarboxy penicillins **(526)** were reacted with trimethylsilyliodide to give γ-lactams **(527)**. In an extension of this methodology, the penem **(528)** was treated with zinc in THF/1M $KH_2PO_4$ which effected deprotection and ring expansion to give γ-lactam **(529)**. None of the subsequently deprotected γ-lactams were active as antibacterials.[382]

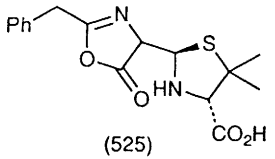
The bicyclic thiazoline carbapenem analogues **(530)**, **(531)** and **(532)** were synthesised and shown to be devoid of antibacterial or β-lactamase inhibitory activity.[383] Elaboration of thiazolidinone **(533)** using the Woodward-Wittig route led to **(530)** and **(531)**. The acetamido side chain was introduced by first converting **(533)** to bicycle **(534)** (anisaldehyde dimethyl acetal, boron trifluoride etherate). **(534)** was subsequently subjected to azide transfer using LDA and tosylazide followed by quenching with acetic acid to give **(535)**. **(535)** was converted to **(532)** in several steps.[383]

*E*-**(536)**- or *Z*-**(537)**-Alkylidene-β-sultams were synthesised in a stereochemically defined manner from $C_4$-hydroxyalkylated-β-sultams **(538)** via sulfonation of the hydroxy group and elimination.[384] Peterson reaction of $C_4$-silylated-β-sultams **(539)** gave mixtures of *E*- and *Z*-alkylidene β-sultams.[384] Reaction of the β-sultam anion with aromatic nitriles gave the enamino-β-sultam **(540)**.[385] N-Acylation of **(540)** gave sultams of type **(541)**, some of which were weak inhibitors of β-lactamases.[385] None of the sultams prepared displayed antibacterial activity.[384,385]

$C_3$-Isopropylidene, **(542)** and **(543)**, and $C_3$-isopropyl, **(544)** and **(545)**, substituted N-protected azetidin-2-ones are the first examples of monocyclic β-lactams with neutral substituents to show *in vitro* antibacterial activity.[386,387] Treatment of **(546)** with benzylamine and a Pd(II) catalyst gave a mixture of **(547)** and **(548)**. Refluxing this mixture in THF gave a complex mixture from which unchanged **(548)** and γ-lactam **(549)** were isolated.[387]

## 10 Mechanistic Studies, Mode of Action, Degradation and New Applications

The scope of this section encompasses structural studies, molecular modelling, degradative studies, some enzymatic studies, general mechanistic studies and



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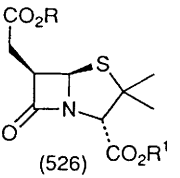

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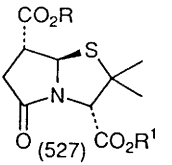

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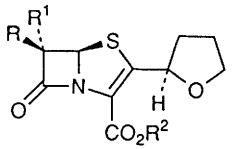
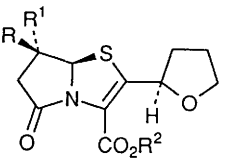
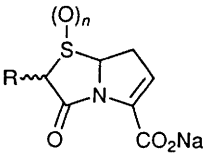


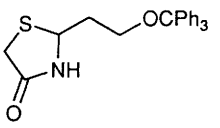
(528) R = $CH_2CO_2TCE$, $R^1$ = H, $R^2$ = 



(529) R = H, $R^1$ = $CO_2H$, $R^2$ = 



(530) *n* = 0, R = H
(531) *n* = 1, R = H
(532) *n* = 0, R = $NHCOCH_2Ph$



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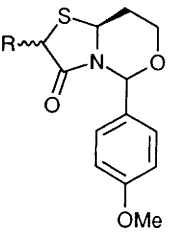
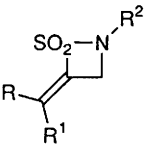
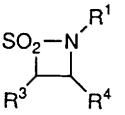


(534) R = H
(535) R = $N_3$



(536) R = H, $R^1$ = Ph, Me, Ar, $R^2$ = H
(537) R = Ph, Me, Ar, $R^1$ = H, $R^2$ = H
(540) R = Ar, $R^1$ = $NH_2$, $R^2$ = TBDMS, $C_6H_{11}$
(541) R = Ar, $R^1$ = $NHCOR^3$, $R^2$ = TBDMS, $C_6H_{11}$



(538) $R^1$ = TBDMS, Me, $R^3$ = $CH(OH)R^5$
$R^5$ = Ph, Me, Ar, $R^4$ = H
(539) $R^1$ = TBDMS, Me, $CH_2$– 
$R^3$ = $SiMe_3$, $R^4$ = H, Ph

new applications of β-lactams. Only structure activity, kinetic and mutagenesis studies, which have provided new mechanistic insights relevant to β-lactam chemistry, are covered.

Previous $^{13}C$ cross polarisation magic angle spinning (CP/MAS) NMR studies on frozen solutions of penicillins have been extended to a very detailed examination of the temperature dependent changes in the structure of the potassium salt of penicillin V **(550)**.[388] Using a combination of variable temperature (180-390K) CP/MAS NMR analysis and X-ray crystallographic analyses (at 293 and 373K) it was shown that **(550)** not only displays localised dynamic properties but also undergoes a phase transition at 356K. X-ray studies on penicillin V benzyl ester sulfoxide,[389] a $C_{7\alpha}$-methoxycephem,[390] a $C_6$-hydroxyethylpenem [391] and a further study on potassium penicillin V[392] have also been reported.

Theoretical calculations on cleavage of β-lactam rings are becoming a popular practise for molecular modellers, but the validity of some of the calculations using MINDO/3 and MNDO have been called into question.[393] The AM1 method is apparently the most suitable for studying β-lactam cleavage. MNDO-PM3 calculations have been used to model nucleophilic attack on β-lactams. Results have indicated that penams and especially cephems are sensitive for two major reasons: a lower energy of formation of the tetrahedral intermediates and lower energy barriers to reach the final products (relative to amide hydrolysis).[394] Other calculations predict breakdown of the tetrahedral intermediates is unlikely to be rate limiting.[395] MNDO-PM3 calculations have also been used to compare (and predict) the reactivity of $\Delta^2$- and $\Delta^3$-cephems.[396] The results predict that the latter will be more reactive to nucleophilic attack. Whilst not encompassing β-lactam measurements, a study reporting measurements of the core ionisation energies of three strained lactams and their comparison with model lactams and amides is of interest.[397] The effect of ring strain on the pyramidality of the lactam nitrogen was examined for six tricyclic β-lactams using MOPAC with AM1 and PM3 calculation methods and based on comparisons with crystallographic data. Novel tricylic carbacephem and carbapenem nuclei were proposed as transition state analogies for peptide bond hydrolysis.[398] Calculations have predicted that the reaction of $C_3$-aza-β-lactams with nucleophiles (including active site serine hydroxyls) will result in the formation of hydrolytically stable carbamates.[399] The structures of the products resulting from cycloaddition reactions of cephems were studied using n.O.e experiments and force field calculations.[400]

Studies of the degradation pathways of penems,[401] 7-(oxyiminoacyl)-cephalosporins[402] and Loracarbef **(200)**[403] have been carried out. Kinetic studies on penem degradation have also been reported.[404] The polymerisation of cephalosporins with neutral side chains in aqueous solution proceeds via ring opening of the β-lactam with an amine derived from hydrolysis of the β-lactam ring of another cepahalosporin. For cephalosporins with a $C_7$-amino group, polymerisation occurs in basic solution both via the mechanism described above and by attack of the β-lactam with the $C_7$-amino group. In acidic solution only the former process was observed.[405] The rate of polymerisation of cephalo-

sporins bearing various $C_3$ and $C_7$ substituents was examined and found to be proportional to the electron withdrawing power of the $C_7$ group.[406] A study on the complexation of penicillin V with cobalt (II) in methanolic solution led to the isolation of two complexes with molar ratios of 1:1 and 2:1.[407] An EPR study on the reaction of penicillins with hydrogen peroxide indicated the production of an aminoxyl radical.[408] It was suggested that the formation of aminoxyl radicals may occur during the *in vivo* destruction of penicillins by hydrogen peroxide.[408]

A study of the chelating properties of several derivatised catechols, which were models for the side chains of known β-lactam antibiotics, indicated that the most potent antibiotics are those bearing catechols with the highest iron chelating ability and the lowest $pK_a$ values.[409,410]

A study of keto-enol tautomerisation of $C_4$-ketocephems has shown enolisation confers hydrolytic stability and solubility, without necessarily destroying inhibitory activity of human leukocyte elastase.[410,411]

The base catalysed hydrolysis of the penem BRL 42715 **(551)** a potent serine β-lactamase inhibitor, resulted in a novel rearrangement to give **(552)**, presumably via **(553)**.[412] **(552)** was also shown to be produced upon incubation of **(551)** with a β-lactamase and although it was not proven that the enzyme inhibitor complex involved a dihydrothiazepeine, by analogy with studies on the inhibition of β-lactamases by 6β-halogenopenicillanic acids,[413] this would seem likely.[412] The β-lactamase catalysed hydrolysis of Sch 34343 **(554)** gives the penemoic acid **(555)**, which is further hydrolysed to **(556)**.[414] Surprisingly, the conversion of **(555)** to **(556)** was shown to be catalytic and β-lactamase mediated.[414] Utilising X-ray crystallographic data, reassessment of the mechanism of β-lactamase inhibition by clavulanic acid **(17)** (page 337) has been carried out and a key role for the Arg-244 of the β-lactamase in the inhibition process has been proposed.[415]

The applications of β-lactams for uses other than as antibacterials or β-lactamase inhibitors has been pioneered by their use as human leukocyte elastase (HLE) inhibitors.[416-430] A number of structure activity relationships on cephalosporins,[416] cephems,[417] azetidinones,[418-420] penems,[421] and penams[422,423] have been reported. The synthesis of HLE inhibitors has prompted a study of the functionalisation of the dihydrothiazine ring of the cephems at the $C_2$ and $C_4$ positions.[424]

Sulfenamides **(557)** and **(558)** have been shown to be HLE inhibitors[425] and details have now been reported on the synthesis and biological testing of the novel bicyclic β-lactams **(559)**, **(560)** and **(561)** and the sulfenamides **(562)** and **(563)**.[425] The synthesis of all the analogues was achieved from monocyclic azetidin-2-ones.[425] Reaction of enamine **(564)** with hydrogen sulfide and trifluoroacetic acid gave the ene thiol **(565)** which was cyclised using N-chlorosuccinimide and ethyldiisopropylamine to give the sulfenamide **(566)** (plus 5% of the isomeric alkene). Alkene isomerisation of **(566)** (*hv*, $CCl_4$) followed by desilylation gave **(559)**. Hydrolysis of **(564)** (R = allyl) followed by diazo transfer and rhodium (II) carbenoid insertion gave bicycle **(567)**, which was converted by ester hydrolysis, decarboxylation and Wittig reaction to **(560)**. Cyclisation (DCC) of thiol **(568)**

gave the thiolactone **(569)**, which after Wittig reaction and desilylation gave **(561)**.[425]

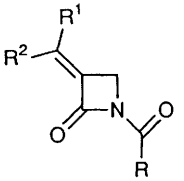
Studies on the mechanism of inhibition of HLE by two 7α-methoxycephem inhibitors **(570)** and **(571)** have demonstrated that following serine acylation, the $C_3$'-leaving group was released.[426] The proposed intermediate **(572)** may then either be hydrolysed or undergo further modification, proabably via Michael addition of a histidine imidazole onto the $C_3$'-methylene position. Support for this mechanism was provided by an electrospray ionisation mass spectrometric (ESI MS) investigation on the inhibition of porcine pancreatic elastase (PPE) by **(571)** and **(573)**.[427] In the case of **(573)**, mass shifts (compared to uninhibited PPE) which indicated the loss of HCl from **(573)** were also observed.[427] An ESI MS study on the inhibition of HLE by azetidin-2-one **(574)** indicated that HLE does not catalyse the elimination of *p*-nitrophenylsulfinate as previously suggested and consequently it is not necessary to follow a 'double hit' mechanism (i.e. covalent cross linking of the active site serine and another residue) to form a stable acyl enzyme complex.[428] The products resulting from both nonenzymatic and HLE mediated cleavage of the β-lactam of cephem sulfone inhibitors of HLE have been characterised.[429,430] It appears that the enzymatic process(es) lead to expulsion of a C-3' leaving group (if present) whereas methanolysis does not.

Several β-lactams, including monobactams, $C_6$-aminopenicillanic acid and $C_7$-aminocephalosporanic acid were recognised as bearing structural similarities to γ-aminobutyric acid (GABA) and to substrates and inhibitors of GABA transferase.[431] Examples of these classes of β-lactams were found to be competitive inhibitors of GABA transferase and this observation has led to the proposal that GABA is bound at the active site of the enzyme with its amino and carboxylate groups in a *syn* orientation.[431]

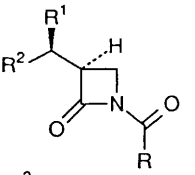
Three penicillin derivatives have been synthesised and tested as inhibitors of the interaction of the HIV envelope protein gp 120 with the cellular receptor CD4.[432] No inhibition was observed. Screening for inhibitors of HIV protease led to the identification of a crude sample of the penicillin dimer **(575)** as having inhibitory activity.[433] Purification of **(575)** demonstrated that it was not the active component but that the $C_2$-symmetric diester **(576)** was a potent inhibitor.[433] Subsequently, detailed SAR studies have been carried out[434-436] and the binding modes of a number of active compounds investigated by NMR, crystallography and molecular modelling.[437,438]

The synthesis of several types of cephalosporin prodrugs **(577)** (all with the antitumour agent attached to the cephalosporin at the C-3' methylene) that are intended to be activated by antibody directed β-lactamases has been reported by several groups.[439-442] In several cases the unmasking of the active drug from the prodrug by β-lactamase was demonstrated and preliminary results indicate that this approach is a promising line of treatment.[440,442]

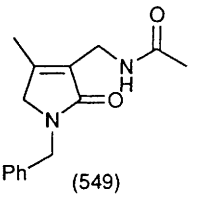
Acknowledgements: We thank Gareth Pritchard, Peter Roach and Mark Wood for proof reading this manuscript and our colleagues for their contributions to our efforts in the area of β-lactam chemistry.



(542) $R^1$ = Me, $R^2$ = $CH_2Y$, R = alkyl or O-alkyl
Y = H or electron withdrawing group
(543) $R^1$ = $CH_2Y$, $R^2$ = Me, R = alkyl or O-alkyl
Y = electron withdrawing group
(546) R = COMe, $R^1$ = Me, $R^2$ = $CH_2OAc$
(547) R = COMe, $R^1$ = Me, $R^2$ = $CH_2NHCH_2Ph$
(548) R = COMe, $R^1$ = $CH_2NHCH_2Ph$, $R^2$ = Me



(544) $R^1$ = Me, $R^2$ = $CH_2Y$, R = alkyl or O-alkyl
(545) $R^1$ = $CH_2Y$, $R^2$ = Me, R = alkyl or O-alkyl
Y = electron withdrawing group



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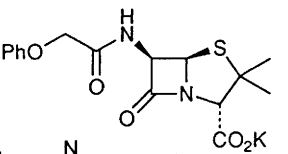

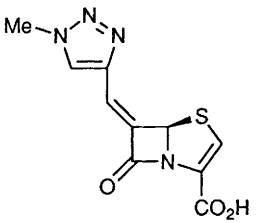

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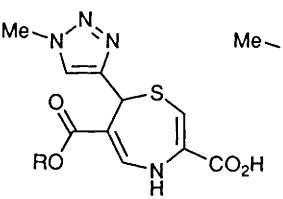

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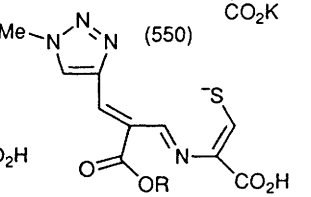

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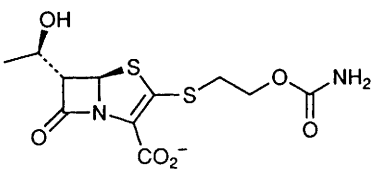

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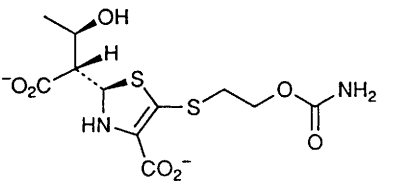

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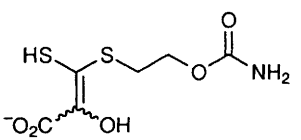

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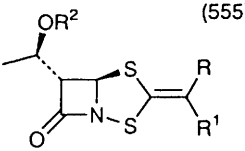
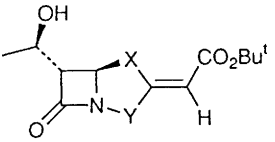
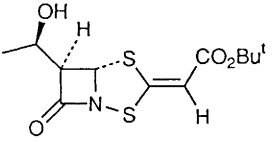


(557) R = $CO_2Bu^t$, $R^1$ = H, $R^2$ = H
(558) R = H, $R^1$ = $CO_2Bu^t$, $R^2$ = H
(562) R = $CO_2Bu^t$, $R^1$ = H, $R^2$ = $CO_2Bn$



(559) X = $CH_2$, Y = S
(560) X = $CH_2$, Y = $CH_2$
(561) X = S, Y = $CH_2$



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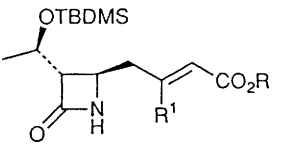
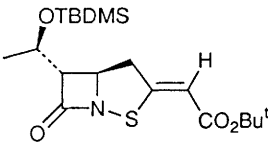


(564) $R^1$ = 
(565) $R^1$ = SH



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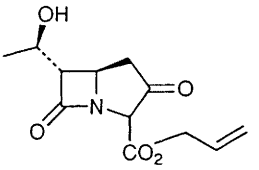

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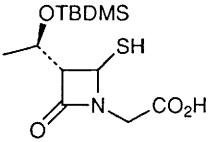

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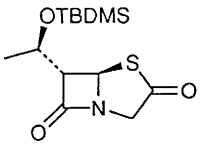

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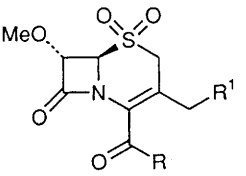
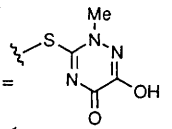
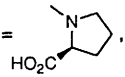
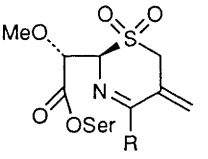


(570) R =  , $R^1$ = 

(571) R =  , $R^1$ = OAc



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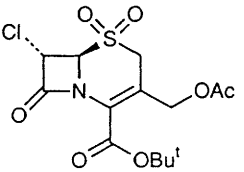

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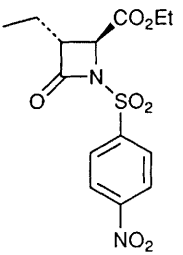

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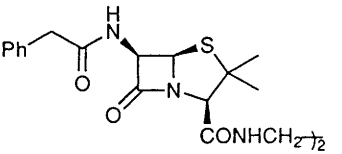

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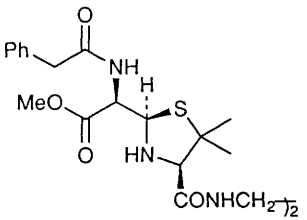

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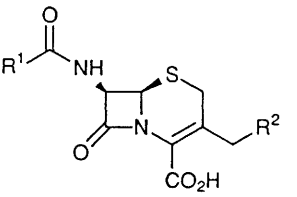

(577) $R^2$ = cytotoxic agent

## Appendix

**Penicillins and Cephalosporins**

1. Synthesis and structure-activity relationships of cephalosporins with C-3' catechol- containing residues.[443]
2. Synthesis and antibacterial activity of new ureido-β-lactam derivatives having dialkyloxyphosphoryl-2-oxo-imidazolidinyl moieties.[444]
3. Stereoselective synthesis of BRL 56173, a bicyclic acrylic penicillin highly stable to β-lactamases.[445]
4. (6*R*)-6-(Substituted methyl) penicillanic acid sulfones: new potent β-lactamase inhibitors.[446]
5. Transformation of 7-(4-hydroxyphenylacetamido)cephalosporanic acid into a new cephalosporin antibiotic.[447]
6. Enzymatic dimerisation of penicillin X.[448]
7. Incorporation of pyrrole residues into penicillin structures by azomethine bridge formation.[449]
8. Synthesis and antibacterial activity of some C-3-lactonyl substituted cephalosporins.[450]
9. Synthesis and biological activity of C-3' ortho-dihydroxyphthalimido cephalosporins.[451]
10. Structure-activity relationship study on cephalosporins with mechanism-based-descriptors.[452]
11. Synthesis and biological activity of 3-(dioxopiperazinylcarboxamido)-pyridinium-methylcephalosporins.[453]
12. Synthesis and biological activity of 3-(diaminotriazinyl) and 3-(aminothiazolyl)-pyridiniummethyl cephalosporins.[454]
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