Investigations of factors affecting binding affinity with peptidylglycine α-amidating monooxygenase (PAM)

A thesis submitted in fulfillment of the requirements for admission to the degree of

Doctor of Philosophy (Organic Chemistry)

By

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September 2007

THE AUSTRALIAN NATIONAL UNIVERSITY
RESEARCH SCHOOL OF CHEMISTRY
INSTITUTE OF ADVANCED STUDIES

Nothing in the world can take the place of persistence.
Talent will not;
nothing is more common than unsuccessful men with talent.
Genius will not;
unrewarded genius is almost a proverb.
Education will not;
the world is full of educated derelicts.
Persistence and determination alone is omnipotent.

Israel Regardie

Declaration

This is to declare that the work presented in this thesis represents original work that I carried out during my PhD candidature, with the exception of the following.

The crystal structure image of the oxidised peptidylglycine α-hydroxylating monooxygenase (PHM) catalytic core with bound substrate, shown on page 3, was generated from the protein data bank (PDB) file 1opm by Dr. Hideki Onagi.

The work described in Chapter Three of the Results and Discussion section has been published in a paper in the *Journal of the American Chemical Society*, **2004**, 126, 13306-13311 by B. J. W. Barratt, C. J. Easton, D. J. Henry, I. H. W. Li, L. Radom, J. S. Simpson. My contribution to the collaboration is clearly specified in Chapter Three.

The synthesis of (*S,E*)-5-[*N*-(*t*-butoxycarbonylamino)]-6-phenylhex-3-enoic acid was performed by Dr. Satish Chand.

To the best of my knowledge the work presented in this thesis does not contain material that has been submitted for a degree or diploma in any university or any other tertiary institution. Established results and methodologies published or written by another person have been acknowledged by citation of the original work throughout the text.

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Iris Hin Wah Li
September 2007

Acknowledgements

So here we are. What a journey it has been!

Today I am submitting my PhD thesis - this work would not have been possible had it not been for the contributions of many people.

Firstly, I would like to express my sincere gratitude to my supervisor, Professor Chris Easton for all his help, guidance, encouragement, patience and support throughout the course of my PhD. No one could have asked for a better PhD supervisor!

I would also like to thank the past and present members of the Easton research group. Thank you for all your support, encouragement, stimulating chemistry discussions and fun over the past three years. You've all made this whole PhD experience very enjoyable and memorable.

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To my wonderful friends, Tanmaya, Gape, Michael and especially Clifford, thank you for all your love and encouragement. Thank you for sharing many wonderful experiences with me and for sticking by me, especially during my low points. Thank you for your friendship.

To Candace, my best friend, words cannot express how grateful I am to have a friend like you. Thank you for all the fun, laughter and tears that we have shared. Thank

you for being there in my times of need and when I am up for a bit of fun. May we remain the best of friends.

Finally, I am forever indebted to my parents and my sister. Thank you for your constant love, encouragement, understanding and patience, without which many of my dreams and aspirations, including my PhD would not have been possible. Thank you.

Table of Contents

Abbreviations

Ac	acetyl
ACE	angiotensin-converting enzyme
Boc	*t*-butoxycarbonyl
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
^{t}Bu	*t*-butyl
^{n}BuLi	*n*-butyl lithium
Bz	benzoyl
ca.	circa (approximately)
calcd.	calculated
Cbz	carbobenzyloxy
CDI	1,1'-carbonyldiimidazole
m-CPBA	3-chloroperoxybenzoic acid
Cu_H	copper H (copper atom in PHM active site)
Cu_M	copper M (copper atom in PHM active site)
Cys	cysteine
δ	chemical shift
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
DIPA	diisopropylamine
DIPEA	*N,N'*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EDC	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
EI	electron impact
ESI	electrospray ionisation
et al.	*et alia*
EtOAc	ethyl acetate

EtOH	ethanol
GH	growth hormone
Gly	glycine
HCl	hydrochloric acid
His	histidine
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HWE	Horner-Wadsworth-Emmons reaction
Hz	hertz
IC_{50}	inhibitory concentration
J	coupling constant (Hz)
KF	potassium fluoride
K_I	inhibition constant
K_M	Michaelis-Menten constant
$K_{M,app}$	apparent dissociation constant
k_{rel}	relative rate
LDA	lithium diisopropylamide
lit.	literature
m/z	mass to charge ratio
Me	methyl
MeOH	methanol
Met	methionine
mp	melting point
MS	mass spectrometry
*N*BS	*N*-bromosuccinimide
NMR	nuclear magnetic resonance
PBA	(*E*)-4-phenyl-3-butenoic acid
PAL	peptidylamidoglycolate lyase
PAM	peptidylglycine α-amidating monooxygenase
Pd/C	palladium on carbon
Ph	phenyl
Phe	phenylalanine
PHM	peptidylglycine α-hydroxylating monooxygenase
ppm	parts per million

quant.	quantitative
RSE	radical stabilisation energy
t	*tert*
TBDMS	*t*-butyldimethylsilyl
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMS	trimethylsilyl
t_R	retention time (chromatography)
Tyr	tyrosine
v	volume
Val	valine
$V_{M,app}$	apparent maximum velocity
<	less than
>	greater than
$[\alpha]_D$	specific rotation

Abstract

In this thesis, the interactions of a number of classes of compounds with the enzyme peptidylglycine α-amidating monooxygenase (PAM) are investigated. The studies were conducted to establish the effects a range of modifications to natural PAM substrates have on binding affinity with the enzyme.

Incorporation of a hydrophobic or a copper binding functional group was found to preserve binding affinity with PAM, while extensions to the backbone of natural PAM substrates resulted in a loss in binding affinity.

Based on comparisons of the stability and the relative ease of formation of the α-carbon centred radical of an acylglycine, with that of the corresponding glycolic acid derivatives and γ-keto acids led to the latter two classes of compounds being identified as inhibitors of PAM. The glycolic acid derivatives displayed higher binding affinity with PAM than the analogous γ-keto acids.

The presence of the ester carbonyl group of glycolic acid derivatives was established to be important in preserving binding affinity with PAM. Unexpectedly, two of the β-oxa acids appeared to enhance the activity of the enzyme, which may be as a result of an activating allosteric effect.

α,β-Unsaturated γ-keto acids, which mimic the geometries of the glycyl NH and that of the α-carbon centred radical of natural PAM substrates were found to inhibit PAM activity.

Amino acid based analogues of (*E*)-4-phenyl-3-butenoic acid, an established PAM inactivator, displayed effective inhibition of the enzyme.

Analogues of natural PAM substrates that possess a thio ether substituent were found to inhibit PAM activity. The methionine containing dipeptides displayed greater

binding affinity with PAM than the corresponding leucine derivatives. In contrast, the methionyl glycolic acid derivative displayed poorer binding affinity than the corresponding leucyl glycolic acid.

Compounds possessing diamino substituents as copper binding functional groups did not display high binding affinity to PAM. Interestingly, one of the diamines behaved in a similar manner to the two β-oxa acids and appeared to act as an enzyme activator.

Chapter One

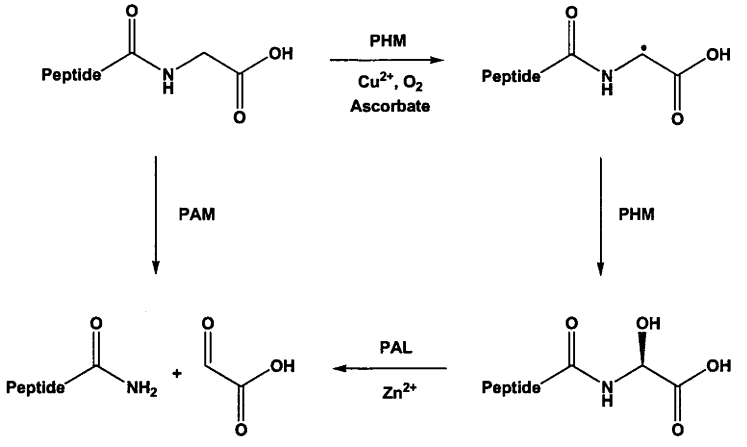
Introduction

1.1 Peptide Hormones

Peptide hormones are a class of peptides secreted directly into the bloodstream by the endocrine glands[1] and involved in a wide range of biological roles. Substance P, a member of the tachykinin neuropeptide family, has been shown to affect cardiovascular functions[2] as well as the transmission of pain.[3] The peptide hormone oxytocin is involved in the modulation of milk ejection and uterine contractions in mammals.[4] Vasopressin, a nonapeptide similar in structure to oxytocin, functions as an antidiuretic regulator.[5] The body's metabolism of calcium and phosphorus is regulated by the 32 amino acid polypeptide, calcitonin.[5] The neuropeptide bombesin along with calcitonin has been shown to induce the proliferation of prostate carcinoma cells by inhibiting cell apoptosis.[6] The fatty acid primary amide, oleamide (*cis*-9-octadecenamide) is involved in sleep induction and enhances the anti-proliferation of breast cancer cells by arachidonylethanolamide.[7]

1.2 Peptidylglycine α-Amidating Monooxygenase (PAM)

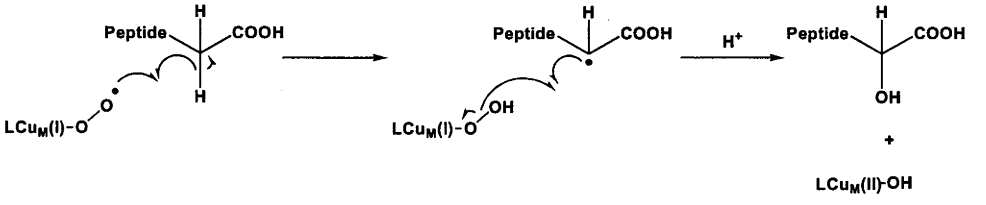
C-Terminal amidation is a ubiquitous post-translational modification of bioactive peptides, including peptide hormones. Over half of all bioactive peptides require amidation to achieve full biological activity.[8] The post-translational activation of the peptides is catalysed by peptidylglycine α-amidating monooxygenase (PAM) *via* the oxidative cleavage of the terminal glycine residue to give the corresponding bioactive *C*-terminal amides (Scheme 1). PAM is a bifunctional enzyme consisting of two catalytic subunits - peptidylglycine α-hydroxylating monooxygenase (PHM; EC 1.14.17.3) and peptidylamidoglycolate lyase (PAL; EC 4.3.2.5).



Scheme 1. Mechanism PAM catalysis

In the amidation process, PHM is responsible for the rate-limiting, catalytic hydroxylation of the glycine-extended precursor to give the corresponding α-hydroxyglycine intermediate. The activity of PHM is dependent on the presence of copper, molecular oxygen and ascorbate. PHM belongs to the same family of monooxygenases as dopamine β-monooxygenase (DβM), a norepinephrine synthesizing enzyme. DβM's activity is also dependent on molecular oxygen and ascorbate.

The mechanism of the PHM catalysed reaction of glycine extended precursors is outlined in Scheme 2.



Scheme 2. Reaction at the α-carbon of a substrate catalysed by PHM [8]

The initial step in the PHM mechanism involves the reduction of the two copper ions, Cu_H and Cu_M, from the cupric to cuprous states by two one electron transfers from two ascorbates.[8] Next, molecular oxygen is bound to Copper M (Cu_M) in the presence of a substrate to give a copper-peroxy radical. The radical species then

abstracts the pro-*(S)*-hydrogen from the α-carbon of the bound substrate's glycine, resulting in the formation of an α-carbon centred radical. This is followed by the radical cleavage of the oxygen to oxygen bond in the copper bound hydroperoxide species to give an α-hydroxyglycine as the product.[9]

According to a crystal structure of an oxidised PHM-substrate complex, a number of interactions take place between the substrate and the enzyme (Figure 1). Two interactions in particular are considered to be especially important in the binding of substrates to the enzyme active site. As observed in the crystal structure, the free carboxyl group of the substrate is anchored in a bidentate bridge by the guanidinium group of the R240 residue and also forms a hydrogen interaction with the hydroxyl group of the tyrosine residue Y318.[10] The other key interaction involves the hydrogen bonding of the glycyl NH of the substrate with the oxygen of the asparagine residue N316. In addition to the two interactions mentioned, the diiodotyrosine side chain of the bound substrate appears to occupy a hydrophobic pocket within the PHM active site.[8]

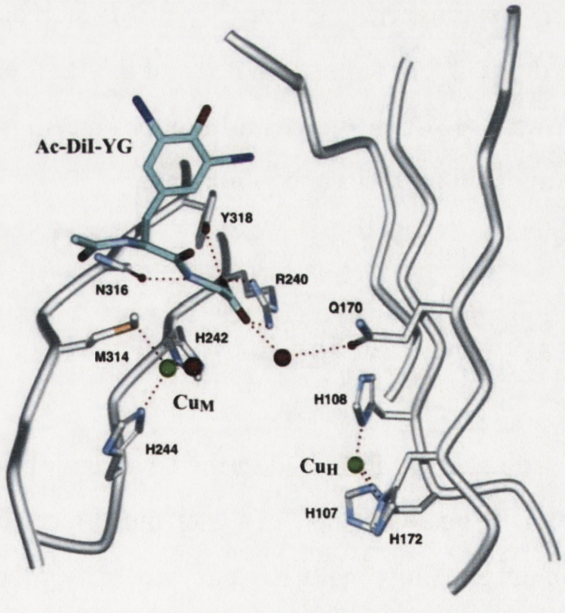
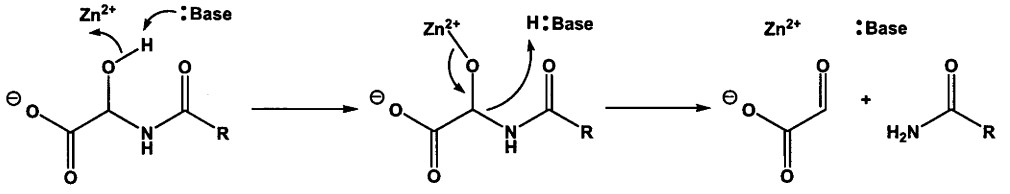


Figure 1. Crystal structure of oxidised PHM catalytic core with bound substrate (Ac-DiI-YG).[8,9] The image was generated from protein data bank (PDB) file 1opm.

The carbinol product from the PHM catalysed reaction is then processed by PAL, the second enzymatic functional subunit. The presence of zinc has been shown to be necessary for maintaining the catalytic activity of PAL.[11] However, its specific role in the PAL mechanism remains unknown. The mode of catalysis by PAL has been suggested to be similar to that of ureidoglycolate lyase, which converts ureidoglycolate into urea and glyoxylate.[12] The PAL mechanism has also been suggested to be closely related to that of alcohol dehydrogenase,[8] with the α-hydroxyglycine species being cleaved to the corresponding *C*-terminal amide and glyoxylate as products (Scheme 3).[8,13]



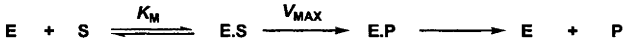
Scheme 3. Proposed reaction mechanism of PAL[8]

It has been established that the two enzyme components of PAM process their substrates in an asymmetric manner.[14] PHM stereoselectively produces *(S)*-configured α-hydroxyglycine intermediates and the PAL enzyme has been shown to be only active towards *(S)*-configured carbinol products in the conversion to give the corresponding amidated peptides and glyoxylate.

1.3 Quantification of interactions with PAM

The purpose of the research discussed in the Chapters of the Results and Discussion of this thesis was to quantify then evaluate the effects of modifications to PAM substrates on binding affinity with the enzyme. In order to establish the binding affinities of individual compounds with PAM, enzyme assays are carried out. A brief description of the parameters used to define enzyme-substrate and enzyme-inhibitor interactions is now provided.

The simplest equation to represent an enzyme **(E)** catalysed reaction of substrates **(S)** is referred to as the Michaelis-Menten equation (Scheme 4). E.S represents the enzyme-substrate complex formed in the reaction between an enzyme **(E)** and a substrate **(S)**. The enzyme-substrate complex is then converted into the enzyme-product complex **(E.P)**, followed by the dissociation of the complex to give the product **(P)**. The K_M, or the Michaelis-Menten constant, represents the substrate concentration required to produce a reaction rate that is half its maximal rate ($V_{max}/2$) or the dissociation constant of the enzyme-substrate complex.[15,16]



Scheme 4. Michaelis-Menten Equation

The two most common parameters use to describe the activity of an inhibitor with an enzyme are the K_I and IC_{50} values. The inhibition constant, K_I, represents the equilibrium constant of the dissociation of the enzyme-inhibitor complex. An IC_{50} denotes the concentration of inhibitor necessary to reduce the rate of the enzyme catalysed reaction by 50 percent.

In order to establish a K_I value, the activity of the enzyme catalysed reaction must be measured at a number of substrate concentrations, against a specific inhibitor concentration. The experiment is then repeated at various inhibitor concentrations. Based on the rate measurements collected, the values are interpreted in graphical form, which allows the extrapolation and determination of a K_I value.

On the other hand, fewer experimental measurements are required in the determination of an IC_{50} value. The IC_{50} value is established at one substrate concentration over a range of inhibitor concentrations.[17] Given the high cost of the PAM enzyme solution, the methodology used to establish IC_{50} values was used to establish the binding affinities of all the compounds researched as part of this thesis.

The relationship between an IC_{50} and a K_I value is described in the Cheng-Prusoff relationship (Equation 1).

$$IC_{50} = K_I\left(1+\frac{[S]}{K_M}\right)$$

Equation 1. Cheng-Prusoff equation[18]

The Cheng-Prusoff equation states that the IC_{50} value for any given competitive inhibitor is related to the K_I value of the inhibitor as a function of the substrate concentration [S] and the Michaelis constant K_M, of the substrate. A competitive inhibitor is defined as an inhibitor which prevents the substrate from binding to the enzyme active site.[16]

The Dixon plot of a competitive inhibitor is displayed in Figure 2. A Dixon plot is created from a series of data points generated at a number of substrate concentrations ($[S_A]$ and $[S_B]$). The negative K_I value is acknowledged as the point at which the two lines $[S_A]$ and $[S_B]$ intersect.[17]

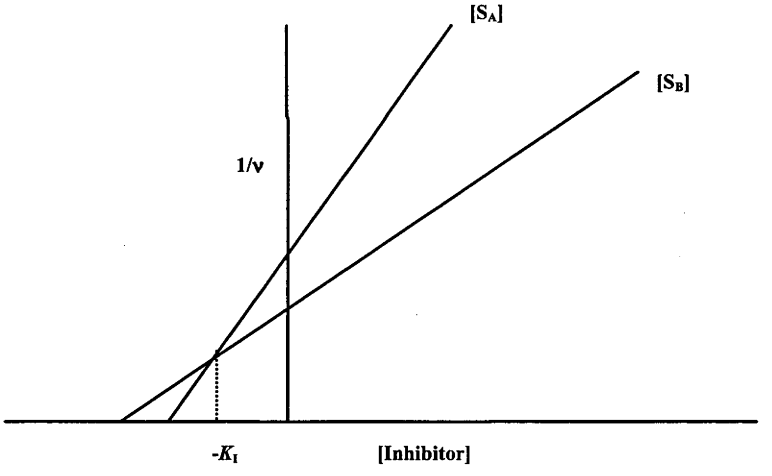


Figure 2. Dixon plot of a competitive inhibitor[17]

The Dixon plot may also be used to express how an IC_{50} value and a K_I value of a given inhibitor are related. By viewing the lines of the Dixon plot as a series of IC_{50} determinations, it can be seen that as the concentration of substrate decreases, the χ-intercept of the line approaches the K_I value, as illustrated in Figure 3.

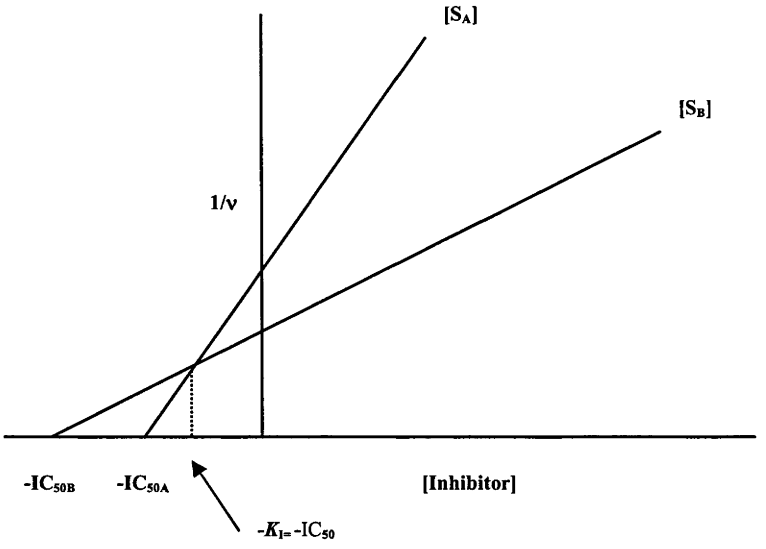


Figure 3. Dixon plot for K_I and IC_{50} value determinations[17]

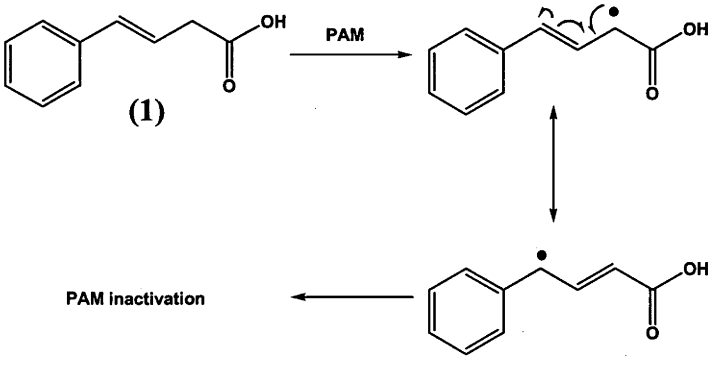
In the case of competitive inhibition, an IC_{50} value approximates the K_I value when the concentration of substrate used in the assay is lower than the K_M value.[17] The starting concentration of the substrate (*R*)-Tyr-(*S*)-Val-Gly employed in the enzyme assays conducted as part of the research for this thesis was 0.1 mM, with a $K_{M,app}$ value of 0.2 mM,[19] therefore, in this instance, the IC_{50} value should correlate reasonably well to the K_I value.

1.4 PAM inhibitors

PAM is a broad spectrum enzyme which catalyses the conversion of a large selection of prohormones to give their corresponding bioactive amide products.[20] The enzyme has been associated with the development of a large number of diverse pathological conditions, which are brought about by an overproduction of peptide hormones, including chronic inflammation or arthritis,[5] neuropathic pain,[21,22] cancer,[6] and asthma.[23] This has led to significant interest in the scientific community in developing pharmacological interventions of PAM's activity.[24]

A number of compounds which inhibit PAM activity have been identified including, *(E)*-4-phenyl-3-butenoic acid (PBA) **(1)**, which was one of the earliest established

potent inhibitors of PAM.[25,26] The proposed mechanism of inhibition by PBA is outlined in Scheme 5.[27]



Scheme 5. PBA mechanism of PAM inhibition

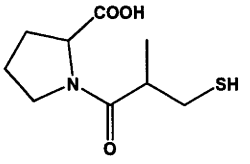
PBA has been established to be a mechanism based inhibitor of PAM, with a K_I value of 1 μM.[28] PAM activity is irreversibly inhibited by the formation of a delocalised radical which prohibits the normal oxidative cleavage process by the enzyme from being carried out.[27] Administration of PBA in rats was found to produce an anti-inflammatory effect.[29] PBA has also been shown to compromise the enzyme's activity in cardiac atrium, pituitary and brain tissue.[26]

Other proposed mechanism based inhibitors of PAM include *N*-formylamides, which are postulated to inactivate the enzyme *via* the formylation of a nucleophile in the enzyme active site.[30]

In the presence of benzylhydrazine, PHM activity in a rat α-amidating enzyme was lost in a time and concentration dependent manner.[31] Inorganic sulfites (SO_3^{2-}), which are common food additives, displayed potent inhibition of both PAM and the copper-dependent DβM. The sulfite mediated inactivation is suggested to proceed *via* modifications of amino acid residues in the enzyme active site by a sulfite radical ($SO_3^{-\bullet}$).[32]

A number of existing drug treatments for a range of pathological conditions have also been found to be effective in inhibiting PAM activity.

Captopril **(2)**, a sulfhydryl group containing compound, which has been established as an angiotensin-converting enzyme (ACE) inhibitor, also displays PHM inhibitory activity *in vitro* at around 100 μmol/L dosage.[33] While captopril **(2)** displayed PHM inhibition, nonsulfhydryl analogues such as enalprilat **(3)** did not inhibit the enzyme's activity. The results from the study suggest that the observed inhibition is the result of the sulfhydryl group of captopril **(2)** impeding on electron transfers between the copper ions in the enzyme active site.[33]



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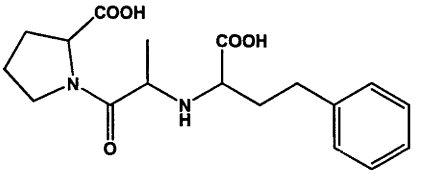
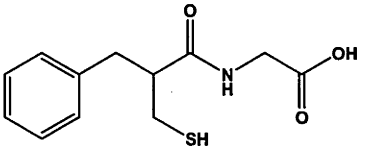


(3)

Similarly, thiorphan **(4)**, tiopronin **(5)** and related analogues have been shown to inhibit PAM activity.[34] Thiorphan **(4)** is administered as an antidiarrheal treatment,[35,36] while tiopronin **(5)** has been used to treat mercury and copper poisoning[37] as well as rheumatoid arthritis.[38] Theoretical modeling studies attributed the high binding affinity displayed by both thiorphan **(4)** and tiopronin **(5)** to PAM being a result of interactions between the sulfur of the thiol group with the enzyme-bound copper.



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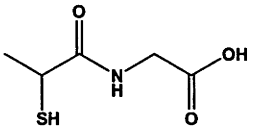
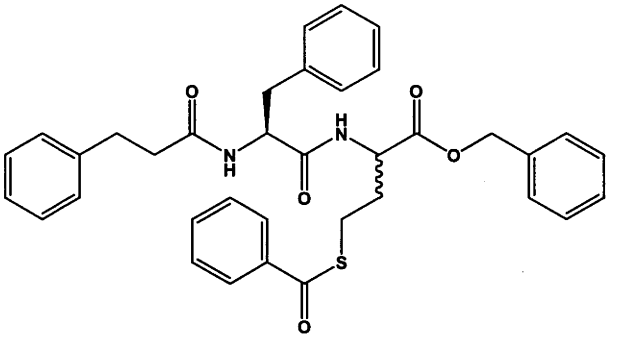


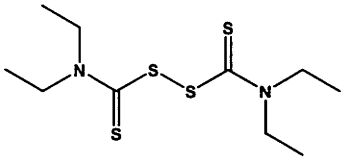
(5)

To date the most potent inhibitors of PHM are *N*-substituted homocysteine analogues, with reported IC_{50} values in the low nanomolar range.[5] The potent inhibition expressed by the homocysteine analogues is postulated to be a result of the co-ordination of the thiolate with the enzyme's copper.[5] Ester derivatives of the homocysteine compounds were subsequently found to inhibit the production of the peptide hormone substance P. One example, the hydrocinnamoylphenylalanyl benzyl ester **6**, displayed an IC_{50} value of 3 μM.[39]



(6)

Two other compounds which may also inhibit the peptide amidation process by chelating with the copper of the enzyme active site are disulfiram **(7)** and *N,N*-diethyldithiocarbamate **(8)**.[40]



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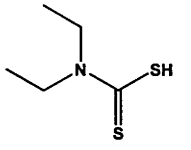
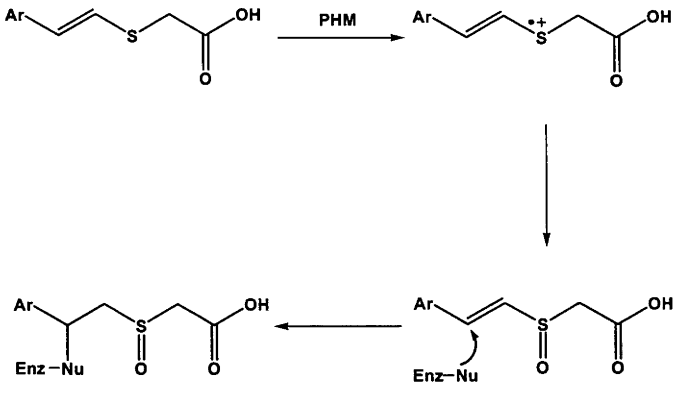


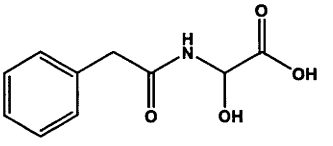
(8)

A series of unsaturated thio acetic acids has also been determined to inhibit the activity of PHM.[41] The compounds were investigated given the literature precedence of the conversion of sulfides to sulfoxides by PHM.[42] The sulfoxides generated were proposed to act as Michael acceptors, alkylating a nucleophilic residue in the active site of PHM, leading to enzyme inactivation. The proposed mechanism is outlined in Scheme 6.[41]



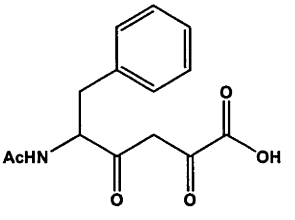
Scheme 6. Proposed PHM inhibition by unsaturated thio acetic acids[41]

Two classes of compounds have been identified to be specific towards the inhibition of PAL. The substrate analogue, phenylacetyl-α-hydroxyglycine **(9)** was determined to be a competitive inhibitor of PAL with a K_I value of 770 μM.[43]

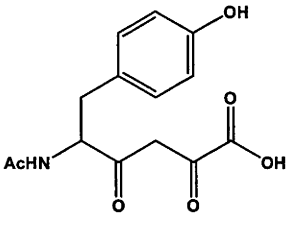


(9)

A later study identified that pyruvate-extended *N*-acylamino acid derivatives with hydrophobic side chains preferentially inhibited the activity of PAL over PHM.[44] This class of compounds was designed to mimic the geometry of the transition state of the substrate likely to be formed along the PAL catalytic pathway. *N*-Acetylphenylalanylpyruvate **(10)** and *N*-acetyltyrosylpyruvate **(11)** displayed potent inhibition of PAL, exhibiting K_I values of 0.24 μM and 0.52 μM, respectively.[44]



(10)



(11)

1.5 The stability and relative ease of formation of α-carbon centred amino acid radicals

In Chapter Three of this thesis, the use of an alternative approach to identify PAM inhibitors by evaluating the stability of α-carbon centred amino acid radicals and their relative ease of formation is discussed. A short review describing the characteristics of α-carbon centred amino acid radicals and the methods employed to quantify their stability and relative ease of formation is now provided.

α-Carbon centred amino acid radicals are readily formed in peptides, proteins and other amino acid derivatives. These radicals are much more stable compared to typical carbon radicals due to extensive radical stabilisation achieved by the captodative effect. The captodative effect enhances the stability of the radical through delocalisation of the unpaired spin density *via* a 'push-pull' mechanism initiated by the electron-donating (dative) action of the amino or amido substituent, and the electron withdrawing (capto) effect of the carboxyl group.[45]

Radical stabilisation energies (RSEs) are determined from a series of high level theoretical calculations that correspond to the difference in energy between the dissociation of the C-H bond in methane and the bond dissociation energy of the R-H bond in the compound of interest (Scheme 7).[46,47]

Scheme 7. Isodesmic reactions used to determine RSE values

In the context of this thesis, the RSE values are used to establish the relative stability of radicals. The more stable a radical is, the more positive the calculated RSE value will be.

All RSEs reported in this thesis correspond to RMP2/G3large//B3-LYP/6-31G(d) calculations at 0 K. The B3-LYP/6-31G(d) level of theory was used in the determination of geometries and zeropoint vibrational energies. Single point

calculations were carried out on the optimised structures at the RMP2 level with the 6-311+G(2df,p) and G3 large basis sets to obtain the improved relative energies values. The levels of theories mentioned have been established to be suitable for RSE calculations.[46]

The calculations for the RSE values discussed in Chapters Three and Six of this thesis were determined by Professor Leo Radom.[19] Calculations for the RSE values reported in Chapters Four and Five were conducted by Dr. Michelle Coote.

The rate at which an amino acid derivative undergoes radical α-bromination reflects the relative ease of formation of the corresponding α-carbon centred amino acid radical (k_{rel}). The radical bromination experiments were carried out according to a literature method.[48] A mixture of the substrate in the presence *N*-bromosuccinimide and carbon tetrachloride is heated at reflux and photolysed with a 300W sun lamp. Following the irradiation process, a small sample is obtained from the crude mixture and analysed by proton NMR to distinguish whether bromination had taken place.

The aim of the research discussed in this thesis was to investigate the effect a selection of modifications to PAM substrates have on binding affinity with the enzyme. The extent to which the modifications, which include atom substitutions and the incorporation of hydrophobic and copper coordinating substituents, affect binding with the enzyme was determined by comparing the binding affinities of the substrate analogues to that of the corresponding PAM substrates. The findings from the initial investigation of recognition features affecting binding affinity with PAM will now be described in Chapter Two of the Results and Discussion.

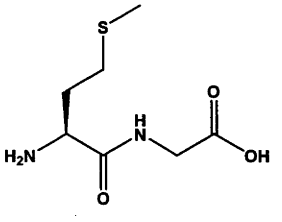
Chapter Two

Preliminary investigation of recognition features affecting binding affinity within the PAM enzyme active site

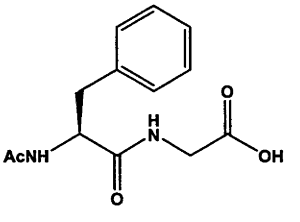
2.1 Introduction

Strong hydrogen bonds between the carboxyl functionality at the *C*-terminus of the substrate with guanidine and tyrosine residues of the active site of PAM are observed in the crystal structure of an enzyme-substrate complex.[49] These play a vital role towards maintaining binding affinity within the enzyme active site. For this reason, all of the compounds considered in this project possess a free carboxyl group.

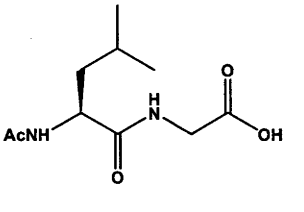
In order to maintain high binding affinity within the enzyme active site, the amino acid residues located at the penultimate position of peptide substrates should have either a hydrophobic or a sulfur containing side chain.[50] Peptides containing charged residues like aspartic acid or lysine displayed poor binding with PAM.[51] A methionine residue at the penultimate position resulted in inhibition of the amidation process as demonstrated with the methionylglycine **12**.[52] The dipeptides **13** and **14** containing phenylalanine and leucine, respectively, displayed high binding affinity.[7,43]



(12)

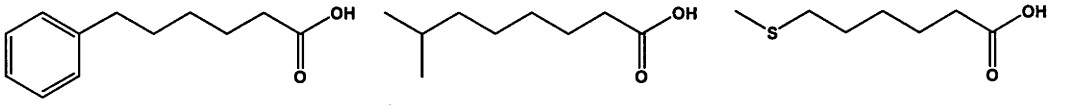


(13)



(14)

The simplified analogues **15**, **16** and **17** of the dipeptides **13**, **14** and **12** were studied, to explore the binding effects of only the carboxyl group and the side chains.



(15) **(16)** **(17)**

The visual comparisons shown below illustrate the geometric similarities between the two classes of compounds (Table 1).

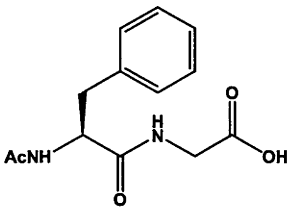
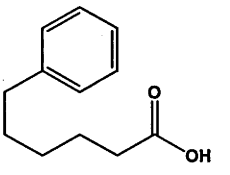
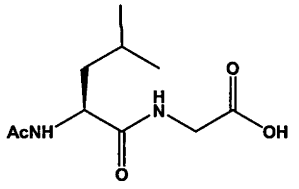
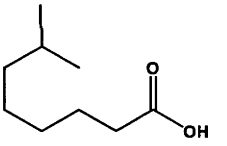
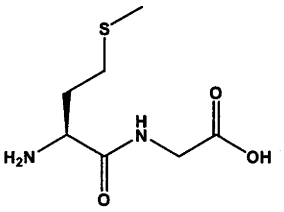
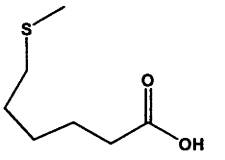
Dipeptides	**Acid analogues**
 **(13)**	 **(15)**
 **(14)**	 **(16)**
 **(12)**	 **(17)**

Table 1. The structures of the dipeptides **13**, **14** and **12** and the corresponding acid analogues **15**, **16** and **17**

Hydrogen bonding between the glycyl NH group of a substrate with an asparagine side chain in the enzyme active site has been established in a PHM-substrate crystal structure.[9] It was envisaged that the absence of such interactions would affect the binding affinity of the three acids **15**, **16** and **17** with PAM.

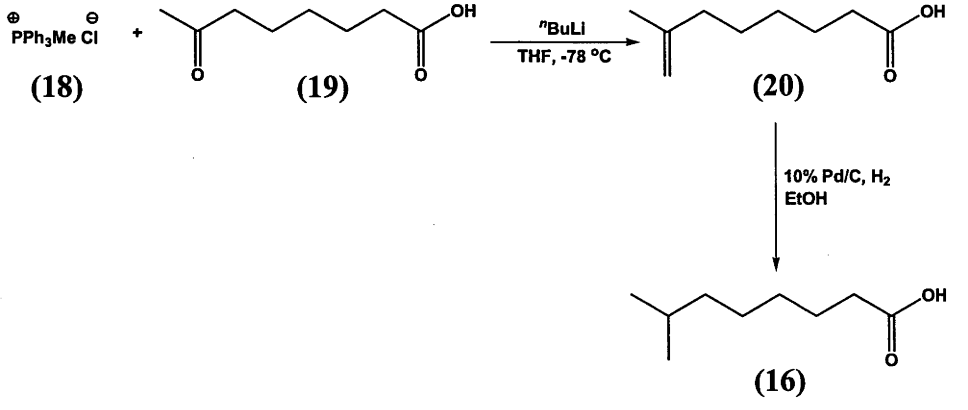
2.2 Syntheses of acids 15, 16 and 17

2.2.1 6-Phenylhexanoic acid (15)

The synthesis of 6-phenylhexanoic acid **(15)** was not necessary as the compound was available commercially from Sigma-Aldrich, Inc.

2.2.2 7-Methyloctanoic Acid (16)

The synthesis of 7-methyloctanoic acid **(16)** is depicted below (Scheme 8). The preparation proceeded according to the experimental conditions and methods described by Davidson and Schumacher.[53]



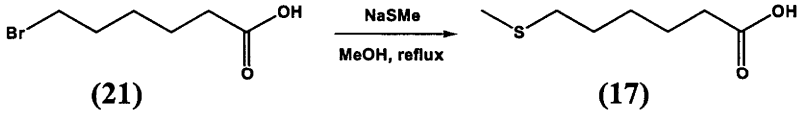
Scheme 8. Synthesis of 7-methyloctanoic acid **(16)**

Firstly, the transformation of the ketone group of 7-oxooctanoic acid **(19)** to an alkene *via* a Wittig reaction was carried out. Methyltriphenylphosphonium chloride **(18)** in tetrahydrofuran was treated with *n*-butyl lithium, followed by the addition of

commercially available 7-oxooctanoic acid **(19)**. The negative mode electrospray ionisation (ESI (-ve)) mass spectrum of the acid **20** displayed the deprotonated molecular ion at *m/z* 155 as the base peak. Hydrogenation of 7-methyloct-7-enoic acid **(20)** was carried out using hydrogen and 10% Pd/C in ethanol. Formation of 7-methyloctanoic acid **(16)** was confirmed by ^{1}H NMR spectroscopy and high resolution mass spectrometry. The ^{1}H NMR spectrum displayed resonances consistent with literature values,[53] and the ESI (-ve) mass spectrum presented the base peak at *m/z* 157 for the deprotonated molecular ion.

2.2.3 6-Methylthiohexanoic acid (17)

Commercially available 6-bromohexanoic acid **(21)** was converted to 6-methylthiohexanoic acid **(17)** in one step by treatment with sodium thiomethoxide (Scheme 9). The conversion was carried out following the procedure described by Knaus and co-workers.[54]



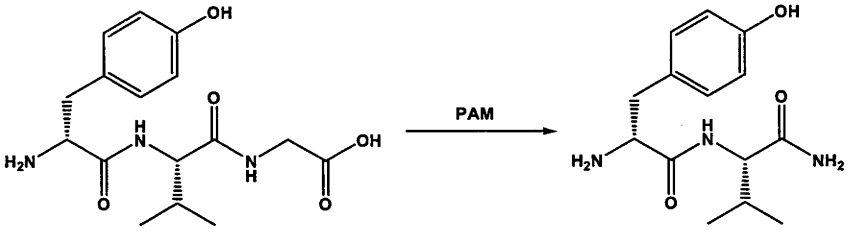
(21) **(17)**

Scheme 9. Synthesis of 6-methylthiohexanoic acid **(17)**

A mixture of 6-bromohexanoic acid **(21)** and sodium thiomethoxide in methanol was heated at reflux overnight. The ^{1}H NMR spectrum of the distilled product **17** displayed resonances identical to those recorded in the literature.[54] Further evidence for the successful conversion to the sulfide **17** was provided by the presence of a peak at *m/z* 162 due to the deprotonated molecular ion in the ESI (-ve) mass spectrum.

2.3 PAM binding affinity of acids 15, 16 and 17

For the purpose of the work within this thesis, the binding affinity of individual compounds with PAM is quantified by the determination of IC_{50} values. An IC_{50} value is defined as the concentration of an inhibitor that is necessary to reduce enzyme activity or turnover of substrate by fifty percent. The substrate subjected to the enzyme assays was the tripeptide **22** and the product from the substrate conversion by PAM was the amide **23**. The overall reaction is outlined in Scheme 10.



(22) **(23)**

Scheme 10. Conversion of substrate **22** to product **23** catalysed by PAM

IC_{50} values were determined by conducting enzyme assays at varying concentrations of the compounds **15**, **16** and **17**. The experiments carried out were in the form of serial dilution assays, and solutions were analysed directly by HPLC to determine the ratio of the substrate **22** to the product **23**. A Dixon plot is then generated from the inverse of the averaged substrate turnover values plotted against inhibitor concentration. The IC_{50} value is the value of $-x$ when $y = 0$ from the equation generated from the line of best fit in the Dixon plot. Although an IC_{50} value is only a preliminary indicator of inhibitor binding, a more thorough analysis of the mode of inhibition was not practical due to the expense of the enzyme.

A compiled table of the IC_{50} values of the acids **15**, **16** and **17** is provided in Table 2.

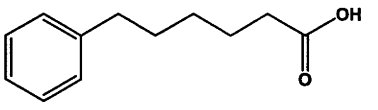
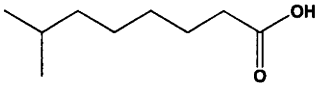
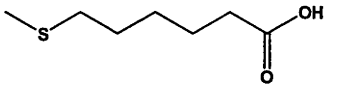
Compound	IC_{50} (mM)
 (15)	0.9
 (16)	4.4
 (17)	1.7

Table 2. IC_{50} values of the compounds **15**, **16** and **17**

The IC_{50} values show that compounds bearing only a free carboxyl group and either a hydrophobic portion or a sulfide group inhibit the enzyme at millimolar concentrations. It seems likely that the aromatic and aliphatic groups of the acids **15** and **16** reside in a hydrophobic pocket of the enzyme active site, while the sulfur of the sulfide **17** binds to the enzyme's copper.

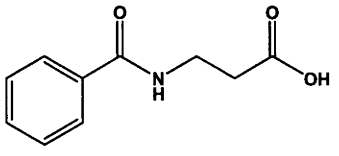
The compounds investigated in the remaining sections of this thesis were therefore chosen to incorporate either a hydrophobic moiety or a sulfur-containing group, which have been established to be beneficial in maintaining high binding affinity with the enzyme.

2.4 Effect on PAM binding affinity of substrates with backbone extension

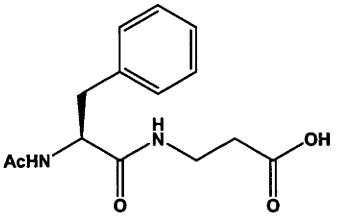
Compounds possessing only a free carboxyl group and a hydrophobic moiety have thus been shown to inhibit the catalytic activity of the PAM enzyme, while systems containing both the mentioned characteristics as well as a *C*-terminal glycine residue

displayed high binding affinity with PAM and are processed as substrates by the enzyme.[7,42,43]

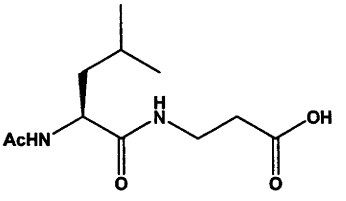
To study changes to binding affinity of compounds with an extra methylene between the peptidyl nitrogen and carboxyl group of a substrate glycine residue, the three compounds **24**, **25** and **26** all terminating with a β-alanine residue were investigated.



(24)

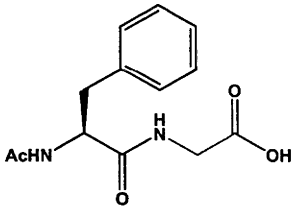


(25)

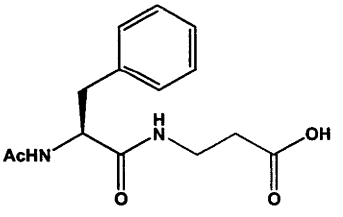


(26)

A visual comparison of the structures of the PAM substrate **13** and the corresponding β-alanyl dipeptide **25** is provided, with the key differentiation between the two classes of compounds highlighted.



(13)



(25)

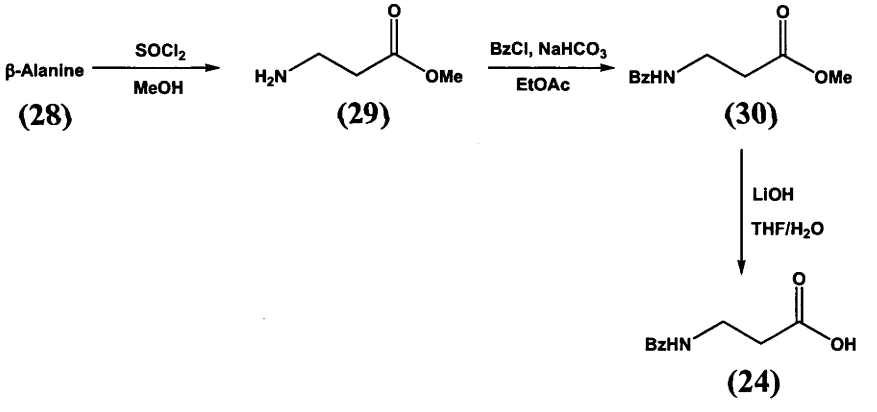
The β-alanine derivatives **25** and **26** of phenylalanine and leucine, respectively, were studied as the incorporation of a hydrophobic side chain in structures had been established to retain or enhance binding affinity in the PAM active site. In addition, the binding affinities of the phenylalanyl and leucyl dipeptides **13** and **14** terminating with a glycine residue had been reported.[7,43] This allows comparisons of the binding affinities of a dipeptide system terminating with β-alanine versus glycine to take place. Similarly the PAM binding affinity of *N*-benzoylglycine **(27)** had been established,[42] which allows for comparison with *N*-benzoyl-β-alanine **(24)**.

The binding affinity of the tripeptide Tyr-Val-β-Ala with an α-amidating enzyme has previously been reported.[51] No inhibition was observed at the highest concentration (2 mM) of the tripeptide examined, while the corresponding tripeptide terminating with glycine inhibited the enzyme activity at a concentration of 126 μM. As the enzyme from rats and employed in the study of the tripeptides mentioned above differs from the enzyme derived from frog skins and used in the current studies, direct comparisons of the results from the earlier work cannot be used to establish any conclusions or trends with the results from the present investigation.

2.5 Syntheses of β-alanine derivatives 24, 25 and 26

2.5.1 *N*-Benzoyl-β-alanine (24)

The preparation of the *N*-protected amino acid **24** is outlined in Scheme 11.



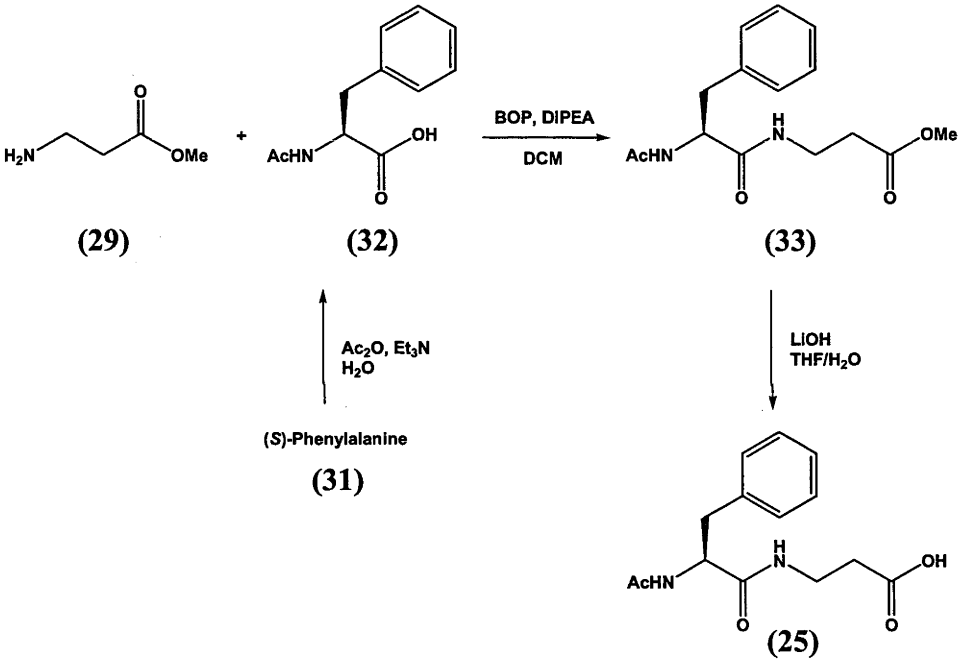
Scheme 11. Synthesis of *N*-benzoyl-β-alanine **(24)**

The conversion of β-alanine **28** to the methyl ester **29** was carried out by treatment with thionyl chloride and methanol. The melting point obtained for compound **29** is consistent with the reported value.[55] Benzoylation of the methyl ester **29** was conducted by treatment with benzoyl chloride and sodium bicarbonate to give the amide **30**. The product's ^{1}H NMR spectrum is consistent with data provided in literature.[56] The ESI positive ion (+ve) mass spectrum presented the base peak as

the sodiated molecular ion at *m/z* 230. Hydrolysis of the ester **30** was conducted in the presence of lithium hydroxide. Formation of the acid **24** was confirmed by ^{1}H NMR spectroscopy. The spectral characteristics obtained are consistent with reported information.[57] The high resolution ESI (-ve) mass spectrum presented the deprotonated molecular ion peak at *m/z* 192.0654.

2.5.2 (*S*)-*N*-Acetylphenylalanyl-β-alanine (25)

The synthesis of (*S*)-*N*-acetylphenylalanyl-β-alanine **(25)** is illustrated in Scheme 12.



Scheme 12. Synthesis of (*S*)-*N*-acetylphenylalanyl-β-alanine **(25)**

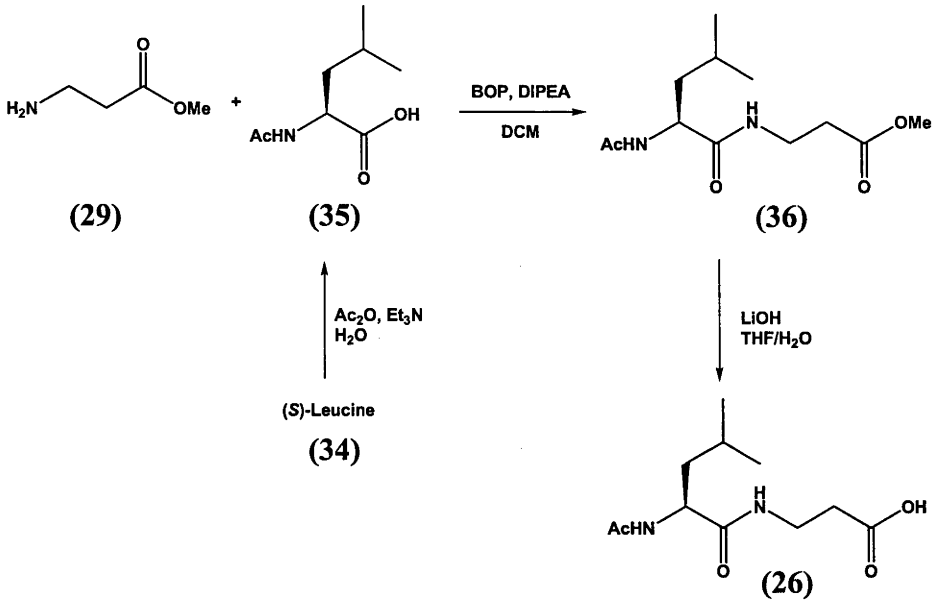
The conversion of the amino acid (*S*)-phenylalanine **(31)** to the protected derivative **32** was carried out by treatment with acetic anhydride. The ^{1}H NMR spectrum of the amide **32** displayed a singlet resonance at 1.78 ppm, corresponding to the three protons of the newly installed *N*-acyl group. The melting point of the acid **32** matches reported data.[58] The dipeptide **33** was synthesized by treating a mixture the methyl ester **29** and the *N*-acetylated acid **32** with the BOP reagent. The successful synthesis of the protected dipeptide **33** was confirmed by the display of a singlet

resonance at 3.64 ppm in the ^{1}H NMR spectrum corresponding to methyl ester protons. The deprotection reaction was achieved by treating the ester **33** with lithium hydroxide. Formation of the acid **25** was confirmed by the observation of a molecular ion peak in the high resolution ESI (+ve) mass spectrum at *m/z* 278.1269. The ^{1}H NMR spectrum displays characteristics consistent with those provided in the literature.[59]

Only a small sample of the dipeptide **25** was prepared, so its optical purity was not established. It is assumed to be homochiral with the stereochemistry illustrated, as the conditions selected for the synthesis of the dipeptide **33** from the *N*-acyl amino acid **32** and the methyl ester **29** have been established to result in negligible racemisation,[60] and the saponficiation converting the ester **33** to the acid **25** was also not expected to cause racemisation. However, even in the unlikely event of isomerisation, the IC_{50} value would only be reduced by at most a factor of two, unless the enantiomer showed peculiarly enhanced binding. An earlier example suggests this is unlikely to be the case. The study found that the dipeptide *(S)*-*N*-acetylphenylalanylglycine **(13)** exhibited binding affinity with PAM over two orders of magnitude greater than that of the corresponding dipeptide (*R*)-*N*-acetylphenylalanylglycine.[43] So, should isomerisation have taken place, the variation introduced would be likely to reduce the observed binding affinity by approximately a factor of two, and this is within the limit of accuracy of a preliminary determination of IC_{50} values.

2.5.3 (*S*)-*N*-Acetylleucyl-β-alanine (26)

The *N*-protected dipeptide **26** was prepared by following reactions depicted in Scheme 13.



Scheme 13. Synthesis of (*S*)-*N*-acetylleucyl-β-alanine **(26)**

Protection of the amino functional group of (*S*)-leucine **(34)** was carried out by treatment with acetic anhydride to give the acid **35**. The melting point of the acid **35** matches reported data.[61] The coupling reaction between the acid **35** and the methyl ester **29** was carried out using BOP. Formation of the product **36** was confirmed by ^{1}H NMR spectroscopy, which displayed resonances at 2.54 and between 3.45-3.57 ppm corresponding to the four methylene protons of the β-alanine residue. The ester **36** was deprotected to give the acid **26** *via* treatment with lithium hydroxide. The formation of the dipeptide **26** was confirmed by ^{1}H NMR spectroscopy and high resolution ESI (+ve) mass spectrometry, which displayed the peak of the sodiated molecular ion at *m/z* 267.1326.

As for the phenylalanine derivative **25** discussed above, the leucine derivative **26** is assumed to be optically pure, although this was neither established nor considered to be particularly important in the current context.

2.6 Binding studies of β-alanine derivatives 24, 25 and 26

The IC_{50} values of the β-alanine derivatives **24**, **25** and **26** with PAM were determined by treating samples containing various concentrations of the compounds with aliquots of enzyme in the presence the substrate *(R)*-tyrosyl-*(S)*-valylglycine **(22)**. The reaction mixtures were analysed by HPLC to quantify any changes in the ratio of the substrate **22** to amidated product *(R)*-tyrosyl-*(S)*-valinamide **(23)** to determine the activity displayed by the compound of interest with PAM. The concentrations of the β-alanine derivatives **24**, **25** and **26** ranged from 0 to 16 mM. It was deemed impractical to carry out enzyme assays at inhibitor concentrations greater than 16 mM due to limits on solubility. The binding affinities of the three β-alanine derivatives **24**, **25** and **26** and the corresponding glycine analogues **27**, **13** and **14** are presented in Table 3.

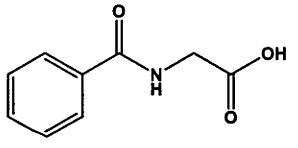
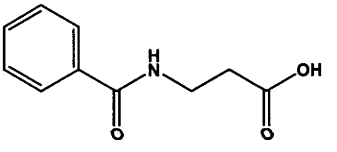
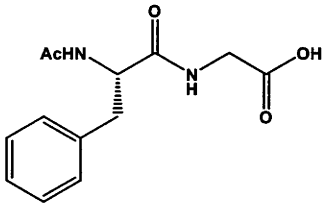
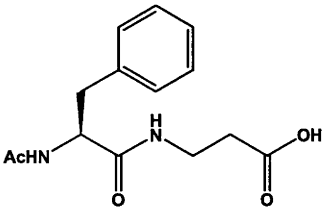
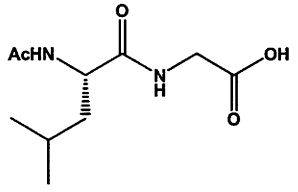
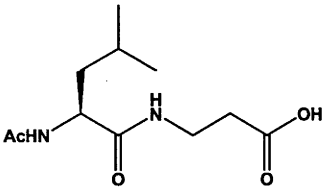
PAM substrate	K_M value	β-Alanine derivative	IC_{50} value
 **(27)**	**1.7**[42]	 **(24)**	**9.3**
 **(13)**	**0.008**[43]	 **(25)**	**>16**
 **(14)**	**0.096**[7]	 **(26)**	**>16**

Table 3. K_M values of PAM substrates **27**, **13**, **14** and IC_{50} values of corresponding β-alanine derivatives **24**, **25**, **26**

The protected β-alanine **24** inhibited PAM activity with an IC_{50} value of 9.3 mM. Both the derivatives of leucyl-β-alanine **26** and phenylalanyl-β-alanine **25** did not display any activity.

Extension of the methylene chain by one carbon between the peptidyl nitrogen functionality and the carboxyl group leads to a significant loss of binding affinity, as observed with the β-alanine derivatives **24**, **25** and **26** in contrast to the corresponding acylglycines **27**, **13** and **14**. Even in the presence of a hydrophobic phenyl or isopropyl substituent, which has been demonstrated above to enhance binding affinity with PAM, the derivatives of leucine **26** and phenylalanine **25** display poorer binding with PAM, at least two to three orders of magnitude less than that observed for the corresponding acylglycines **14** and **13**.

These results suggest the enzyme possesses quite tight geometric restrictions for the accommodation of compounds in the active site, limiting the degree of the modifications that can be made to PAM substrates before a loss of binding affinity results. Additionally, the IC_{50} values also signify the importance of the distance between key substrate recognition features like the free carboxyl group, the hydrophobic substituent and the amido functionality for maintaining high binding affinity with PAM.

Based on the results discussed in this Chapter, compounds designed as potential PAM inhibitors discussed in the upcoming Chapters exploit the characteristics that have been identified to preserve binding affinity with the enzyme. Such characteristics include a hydrophobic substituent like a phenyl or isopropyl group, or a copper-binding functional group, like a sulfide, and take into account the importance of the geometry of the *C*-terminal glycine.

Chapter Three

Inhibition of PAM by exploiting factors affecting the stability and ease of formation of glycyl radicals

3.1 Introduction

Radical stabilisation energies (RSEs) and the relative rates (k_{rel}s) at which compounds undergo bromination have been incorporated into a method to identify potential PAM inhibitors.[19] As discussed in Chapter One, RSE values are theoretical calculations of the stability of a radical relative to the methyl radical. Irradiation of compounds in the presence of a bromine source provides a practical method of quantifying the relative ease of formation of the corresponding intermediate radicals.[19] Previously, conventional bromination reactions were carried out with glycine derivatives using *N*-bromosuccinimide and the RSEs of the corresponding α-carbon centred radicals were used to determine the effect of substituents on the relative ease of radical formation and the stability of radicals, respectively.[48,62]

3.2 Impact of *N*-acyl substituents on glycyl radical stability and their relative ease of formation

The effects of different *N*-acyl substituents on the stability of α-carbon centred glycyl radicals and the relative ease of formation of the radicals were examined in an earlier study.[19] Compounds **39** and **40**, which possess electron withdrawing fluorines, were slower to undergo radical bromination than the corresponding non-halogenated analogues **37** and **38** (Table 4).

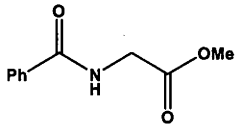
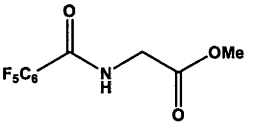
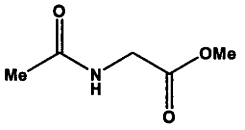
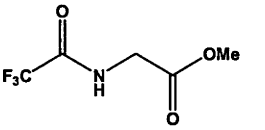
Compound	Relative rate of bromination (k_{rel})	Compound	Relative rate of bromination (k_{rel})
 (37)	1.0	 (39)	0.25
 (38)	1.2	 (40)	0.05

Table 4. Relative rates of bromination of amides **37**, **38**, **39** and **40**[48]

As depicted in Table 4, the benzamide **37** was established to be four times more reactive than the pentafluorobenzamide **39** in the conversion to the corresponding brominated products, while the acetamide **38** was observed to be twenty four times more reactive than the trifluoroacetamide **40**.[48]

For comparison, the RSE values of the radicals **41** and **42**, the intermediates in the bromination of the glycine derivatives **38** and **40**, were established.[19] The RSEs were not determined for the *N*-benzoyl and the corresponding *N*-pentafluorobenzoyl systems, as the aryl groups would significantly increase the complexity of the calculations.

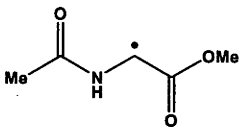
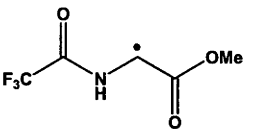
Radical	RSE (kJ mol^{-1})	Radical	RSE (kJ mol^{-1})
 (41)	79.1	 (42)	69.9

Table 5. RSE values of the radicals **41** and **42**[19]

As observed from the results presented in Table 5, the fluorines of the radical **42** decrease its RSE by 9.2 kJ mol^{-1}. It follows that the presence of electron withdrawing groups on the *N*-acyl substituents reduces the stability of glycyl radicals, as well as the relative ease of their formation.

This is reflected in the interactions of analogous systems with PAM (Table 6).

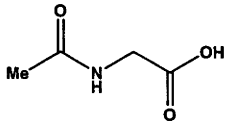
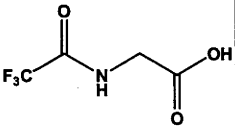
Compound	$V_{M,app}$ (μmol min^{-1} mg^{-1})	Compound	$V_{M,app}$ (μmol min^{-1} mg^{-1})
 (43)	6.4	 (44)	1.4

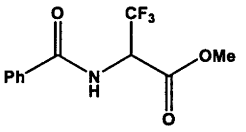
Table 6. Interactions of the glycine derivatives **43** and **44** with PAM[19]

The trifluoroacetamide **44** is turned over four- to five-fold more slowly than the acetamide **43**. Thus, the presence of fluorines reduces the stability of glycyl radicals, the rates of their formation in bromination experiments, as well as their rates of formation through catalysis by PAM. However the effect of the fluorines is insufficient to stop enzyme catalysis.

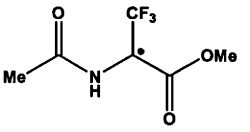
3.3 Effect of substituents at the α-carbon on the stability and relative ease of formation of the α-carbon centred radicals

Substitution at the α-carbon also affects the stability and relative ease of formation of α-carbon centred amino acid radicals, as well as the interactions of analogous compounds with PAM. A trifluoromethyl group at the α-carbon causes a more marked reduction in the stability and relative ease of formation of the radicals than the presence of the electron withdrawing groups on the *N*-trifluoroacetyl substituent. No detectable bromination was recorded for the trifluoroalanine methyl ester **45**, and

the stability of the trifluoroalanyl radical **46** was determined to be 39.2 kJ mol^{-1} lower than that of the corresponding glycyl radical **41**.[19,62]

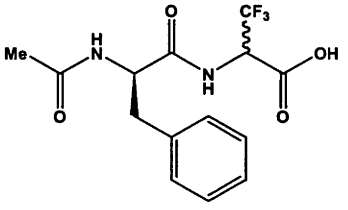


(45)

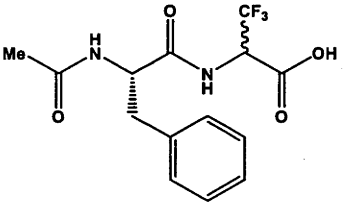


(46)

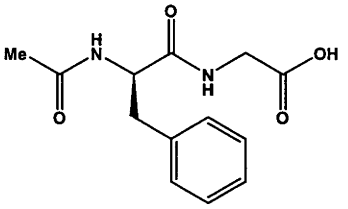
A study of the analogous compounds **47** and **48** found that neither was processed by PAM.[19] Thus the trifluoromethyl substituent at the α-carbon sufficiently reduces the stability of the amino acid radical to the extent that it prevents bromination from taking place, and the radical does not form with PAM, resulting in the inhibition of PAM catalysis. However, substitution at the α-carbon with a trifluoromethyl group prevents tight binding to the enzyme, as reflected in the IC_{50} values obtained. IC_{50} values of 5 mM and 5 mM were determined for the trifluoroalanine containing dipeptides **47** and **48**, compared with the K_M values of 1.3 and 0.0079 mM for binding of the corresponding glycine derivatives **49** and **13**.[43] Thus trifluoroalanine derivatives are only poor inhibitors of PAM.



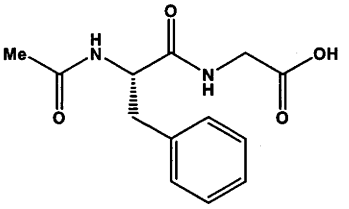
(47)



(48)



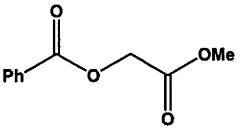
(49)



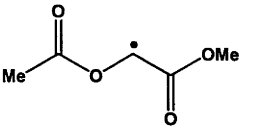
(13)

3.4 Inhibition of PAM with glycolic acid derivatives

In a similar manner, the effects on PAM activity brought about by the exchange of the *C*-terminal peptidyl amido group in PAM substrates with an ester were established.[19] As with the α-trifluoroalanyl system, subjecting the glycolic ester **50** to conventional bromination reaction conditions did not result in any reaction, while comparisons of the RSE values of the glycyl radical **41** versus the glycolyl radical **51** determined the latter to be 34.7 kJ mol^{-1} less stable.[19]



(50)



(51)

The reduction in radical stability may be explained by the change in the electro-chemical properties of the glycolic acid. Replacing the acetamido group with an acetoxy group results in an increase in the σ-electron withdrawing ability, while lowering the π-electron donating properties. Comparison of the RSEs of $MeCONHCH_2^{\bullet}$ (41.3 kJ mol^{-1}) and $MeCO_2CH_2^{\bullet}$ (17.11 kJ mol^{-1}) supports such an observation.[19]

The relative stability of the glycolyl and corresponding glycyl radicals and the relative ease of their formation correlate with the identification of glycolic acid derivatives as PAM inhibitors. The inhibition quotients of the glycolyl esters **55**, **56**, **57**, **58** and **59** are presented in Table 7, but none are processed by the enzyme.

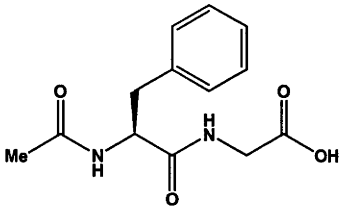
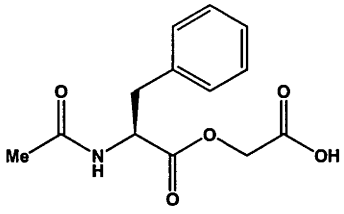
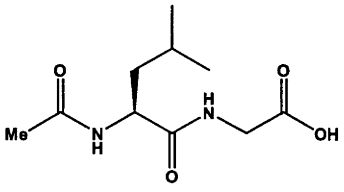
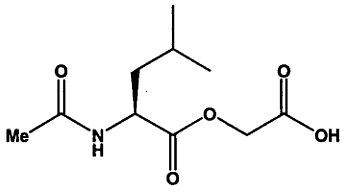
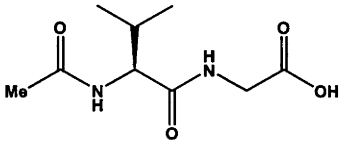
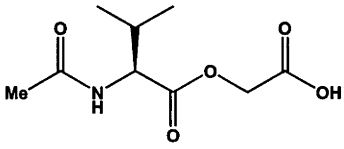
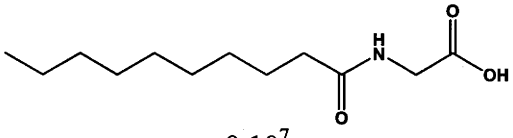
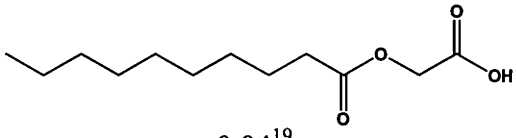
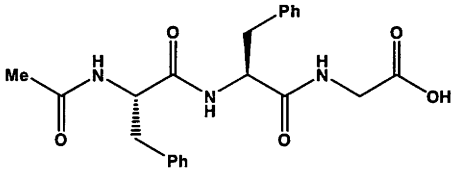
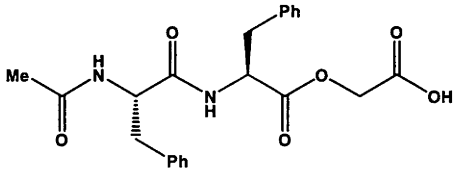
Acylglycine - K_M,app (mM)	Glycolic Acid - K_I or IC_{50} (mM)
	
0.0079[43]	0.045[43]
(13)	**(55)**
	
0.096[7]	0.060[43]
(14)	**(56)**
	
0.03[19]	0.5[19]
(52)	**(57)**
	
0.10[7]	0.04[19]
(53)	**(58)**
	
0.0012[43]	0.05[19]
(54)	**(59)**

Table 7. K_M, K_I and IC_{50} values of acylglycines **13**, **14**, **52**, **53** and **54** and glycolyl esters **55**, **56**, **57**, **58** and **59**[7,19,43]

It is noteworthy that the introduction of the ester functional group does not significantly compromise the binding affinity of the glycolic acid derivatives with PAM. The IC_{50} and K_I values of the glycolyl esters **55**, **56**, **57**, **58** and **59** presented in Table 7 all fall into the range of 0.04-0.5 mM, while the $K_{M,app}$ values of the corresponding acylglycine derivatives **13**, **14**, **52**, **53** and **54** vary between 0.001-0.1 mM.[7,19,43]

Therefore, replacement of glycine by glycolate prevents catalytic turnover by PAM. Furthermore, glycolic acid derivatives retain binding affinity with PAM, comparable to that of the corresponding acylglycine derivatives.

3.5 Amino acid based γ-keto acids as PAM inhibitors

In my contribution to this work,[19] γ-keto acids were explored as a potential class of PAM inhibitors. The γ-keto acid system is derived from replacing the NH of an acylglycine or the ether O of a glycolic acid with a methylene.

The interaction of one γ-keto acid, 3-tyrosylalanylpropionic acid with PAM had been investigated, however, no substrate or inhibition activity had been observed at the highest concentration of the γ-keto acid tested.[51]

The potential of γ-keto acids as PAM inhibitors was established by following the approach used to identify glycolic acid derivatives as inhibitors of the enzyme, that is by evaluating the differences in the stability of radicals and the relative ease at which they form in comparison to the corresponding acylglycines.

Substitution of the NH in an acylglycine with a CH_2 to give a γ-keto acid had similar effects on radical stability and relative ease of radical formation as the exchange to the ester of the glycolate, as observed from the results of RSE calculations and measurements of relative rates of bromination that are presented in Table 8.

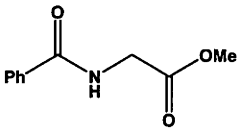
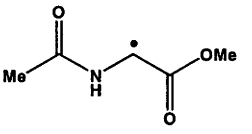
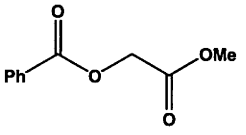
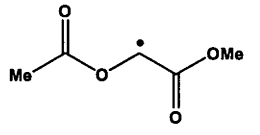
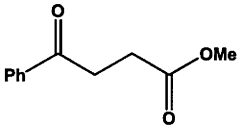
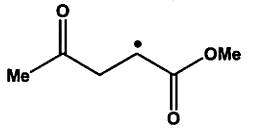
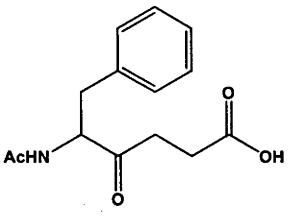
Compound	k_{rel}	Radical	RSE (kJ mol^{-1})
 (37)	1.0	 (41)	79.1
 (50)	<0.0005	 (51)	44.4
 (60)	<0.0005	 (61)	34.9

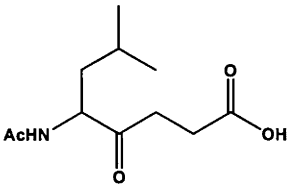
Table 8. Values of k_{rel} for bromination of the esters **37**, **50** and **60** and the RSE values for the corresponding radicals **41**, **51** and **61** [19]

The γ-keto ester radical **61** was established to be 44.2 kJ mol^{-1} less stable than the analogous glycyl radical **41** based on RSE calculations, and exposure of the ester **60** to conventional bromination conditions did not result in any reaction. With the analytical methods used in the latter experiment, this indicates that the k_{rel} for the glycolate **50** is <0.0005 relative to that of the acylglycine **37**.

The phenylalanyl and leucyl γ-keto acids **62** and **63** were therefore selected for investigation with PAM as the presence of a hydrophobic substituent had already been proven to increase binding affinity with the enzyme. In addition, the interactions with PAM of the analogous glycolyl esters **55** and **56** and acylglycines **13** and **14** had been reported,[7,43] allowing direct comparisons of their activities with the enzyme to be made.



(62)

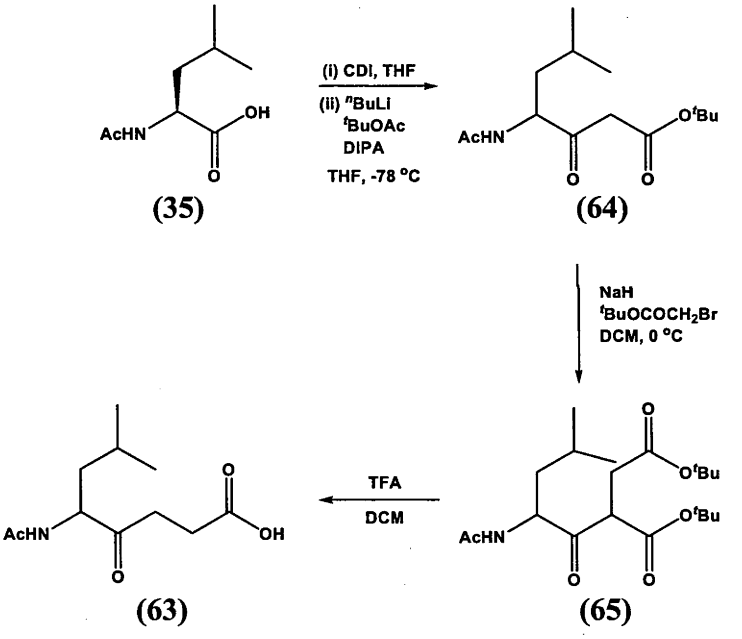


(63)

3.6 Syntheses of γ-keto acids 62 and 63

3.6.1 3-(*N*-Acetylleucyl)propionic acid (63)

The reactions used in the synthesis of the propionic acid **63** are shown in Scheme 14.



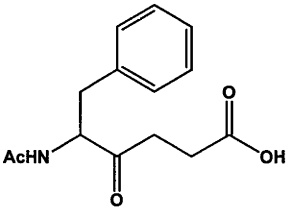
Scheme 14. Synthesis of 3-(*N*-acetylleucyl)propionic acid **(63)**

The acid **35** was synthesized following general methods previously described.[63] The successful synthesis of the acid **35** was confirmed by the presence of a singlet resonance corresponding to the acetyl protons at 1.98 ppm in the ^{1}H NMR spectrum. The synthesis of the malonate **64** was carried out in two steps. First, a solution of the acid **35** was treated with 1,1'-carbonyldiimidazole. The resulting mixture was added to a mixture of lithium diisopropylamide (LDA) generated *in situ*. *t*-Butyl acetate

was then added, affording malonate **64**. The ^{1}H NMR spectrum of the malonate **64** displayed resonances at 3.41 ppm and 3.51 ppm corresponding to the diastereotopic geminal malonate protons. A mixture of the malonate **64** in dichloromethane was treated with *t*-butyl bromoacetate in the presence of sodium hydride to give the diester **65** as a 1:1 mixture of diastereomers. Formation of the diester **65** was confirmed by the exhibition of the protonated molecular ion at *m/z* 386 as the base peak in the ESI (+ve) mass spectrum. Hydrolysis and decarboxylation of the diester **65** was carried out by treatment with trifluoroacetic acid in dichloromethane and heating the mixture to reflux, to yield the γ-keto acid **63**. The ^{1}H NMR spectrum of the keto acid **63** lacked any evidence of resonances corresponding to *t*-butyl ester groups, confirming the reaction was successful. The purity of the acid **63** was confirmed by elemental analysis. Although a chirally pure starting material was used in the synthesis of the γ-keto acid **63**, the final product was not optically active and is expected to exist as a racemic mixture. Racemisation is likely to have occurred as a result of the use of strong bases like lithium diisopropylamide and sodium hydride during the reaction sequence. However, the nature of the chiral centre in the acid **63** is expected to have little consequence on the inhibition of PAM, for similar reasons to those discussed earlier in relation to the dipeptide **25**.

3.6.2 3-(*N*-Acetylphenylalanyl)propionic acid (62)

3-(*N*-Acetylphenylalanyl)propionic acid **(62)** was generously prepared by Dr. Brendon Barratt,[19] employing procedures analogous to those outlined above for the synthesis of the corresponding leucine derivative **63**.



(62)

3.7 Binding affinity of γ-keto acids 63 and 62 with PAM

The IC_{50} values of the γ-keto acids **63** and **62** with PAM were determined following the protocol described above, under which there was no evidence that either compound was a substrate of the enzyme.

The binding affinities of the acylglycines **14** and **13**, glycolic acid derivatives **56** and **55** and γ-keto acids **63** and **62** are presented in Table 9.

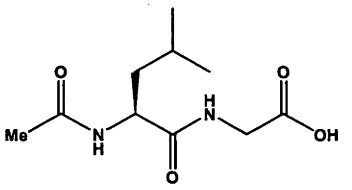
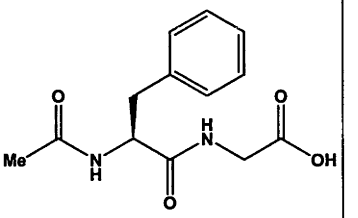
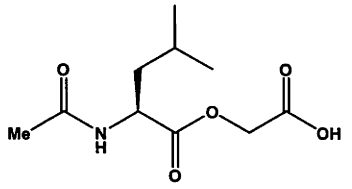
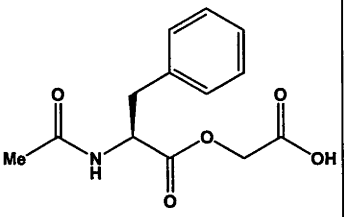
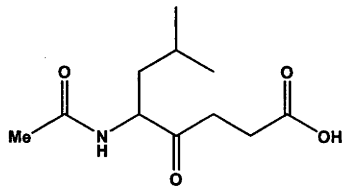
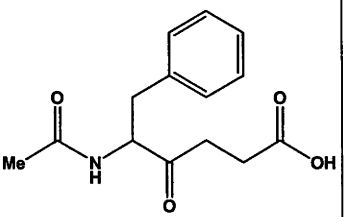
Compound	K_M, K_I or IC_{50} (mM)	Compound	K_M, K_I or IC_{50} (mM)
 **(14)**	**0.096**[7]	 **(13)**	**0.0079**[43]
 **(56)**	**0.060**[43]	 **(55)**	**0.045**[43]
 **(63)**	**6**[19]	 **(62)**	**3**[19]

Table 9. K_M values of acylglycines **14** and **13**, and K_I and IC_{50} values of glycolate esters **56** and **55** and γ-keto acids **63** and **62**[7,19,43]

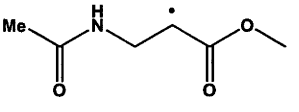
The γ-keto acids **63** and **62** inhibited PAM activity with IC_{50} values of 6 and 3 mM, respectively.

Although γ-keto acids inhibit the activity of PAM, they also exhibited the poorest binding, based on comparisons with the binding affinities of the corresponding acylglycines and glycolic acids. Replacing the NH of an acylglycine with a methylene resulted in a greater loss in binding affinity in comparison to substitution with an ether O to give a glycolic acid.

The greater binding affinity displayed by the glycolic acid derivatives may be attributed to hydrogen bonding interactions between the ether O and the amide of an asparagine residue in the enzyme active site. Hydrogen bonding interactions between the NH of an acylglycine and an asparagine residue in the active site have been documented in a substrate-enzyme crystal structure (Figure 1).[9] Such interactions are not possible with the methylene of γ-keto acids.

The reduced binding affinity exhibited by the γ-keto acids could also be related to their geometry. The NH of an acylglycine and the ether O of a glycolic acid are both classified as sp^2 hybridised adopting trigonal planar geometry. However, in the equivalent position of a γ-keto acid, the methylene is sp^3 hybridized and adopts tetrahedral geometry. In addition, both the acylglycines and the glycolic acids have free rotation around only the bonds of the α-carbon whilst there is free rotation around the bonds of the α- and β-carbons of a γ-keto acid.

The importance of geometric factors on binding affinity with PAM was already established with the β-alanine dipeptides **25** and **26** discussed in Chapter Two. The RSE value of the β-alanyl radical **66** (38.2 kJ mol^{-1}) had indicated that analogous compounds would not be turned over by the enzyme but the dipeptides **25** and **26** did not inhibit PAM activity, presumably because their geometry was unsuitable for strong binding.



(66)

In summary, glycolic acid derivatives and γ-keto acids were established to be inhibitors of PAM based on evaluations carried out comparing the stability and relative ease of formation of the corresponding α-carbon centred radicals in the two systems, relative to that of an acylglycine. The differences in binding affinities observed between the glycolic acid derivatives and the γ-keto acids most probably highlight the significance of a compound's geometry in effective binding with the enzyme.

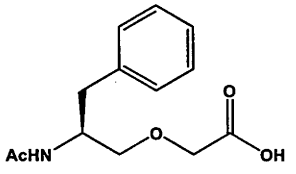
Chapter Four

Examination of the interactions of β-oxa acids with PAM

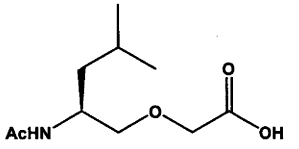
4.1 Introduction

Glycolic acid derivatives have been established to be PAM inhibitors displaying high binding affinity. To determine the effect of the absence of the ester carbonyl group in glycolic acid derivatives on binding affinity with the enzyme, the interactions of β-oxa acids were examined in the work described in this Chapter.

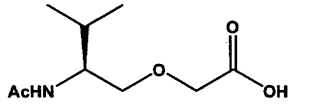
The phenylalanine, leucine and valine derived β-oxa acids **67**, **68** and **69** were selected for investigation as the binding affinities of the corresponding three esters **55**, **56** and **57** were available, as discussed in Chapter Three. This allows for direct comparisons to be made.



(67)



(68)

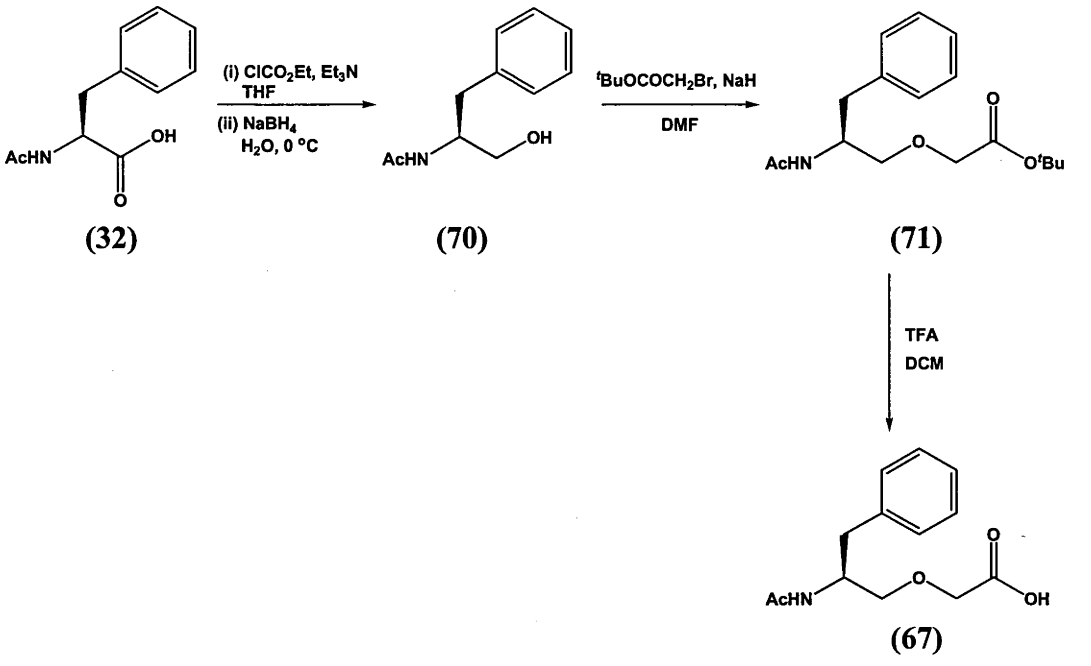


(69)

4.2 Syntheses of the β-oxa acids 67, 68 and 69

4.2.1 (*S*)-5-Acetamido-3-oxa-6-phenylhexanoic acid (67)

(*S*)-5-Acetamido-3-oxa-6-phenylhexanoic acid **(67)** was synthesized following the reaction sequence presented in Scheme 15.



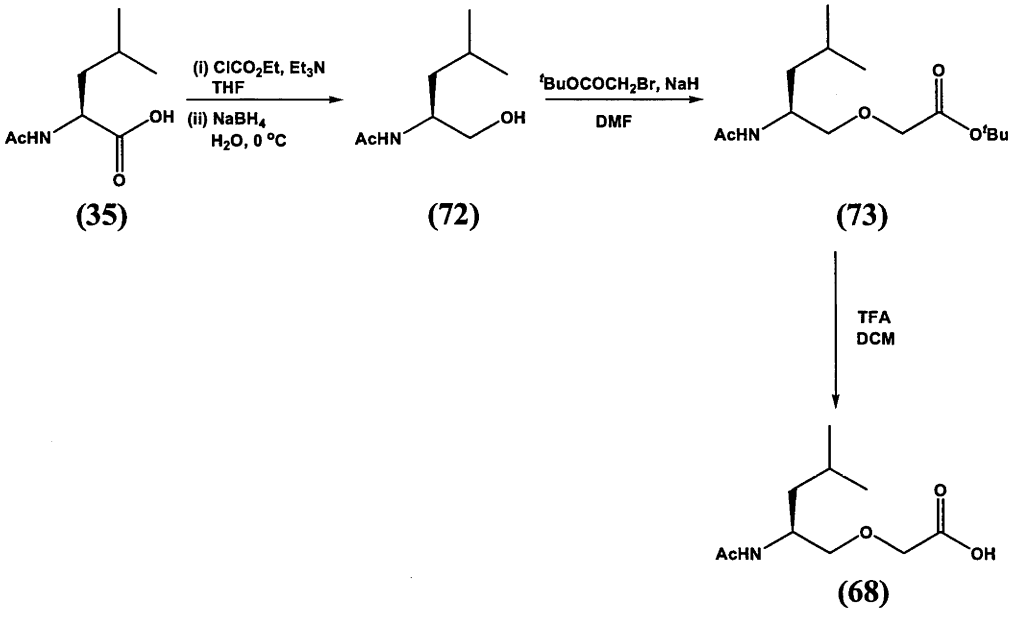
(32) (70) (71) (67)

Scheme 15. Synthesis of (*S*)-5-acetamido-3-oxa-6-phenylhexanoic acid **(67)**

The reduction of the carboxylic acid **32** to the alcohol **70** was carried out according to an established protocol.[64] The acid **32** was firstly treated with ethyl chloroformate and triethylamine to generate the corresponding acyl chloride, followed by the addition of sodium borohydride to complete the reduction process. The melting point of the alcohol **70** (99-100 °C) is consistent with the literature value for (*S*)-*N*-acetylphenylalaninol (100-102 °C).[65] The ester **71** was generated from treatment of a solution of the alcohol **70** with *t*-butyl bromoacetate. The high resolution ESI (+ve) mass spectrum of the ester **71** presented a peak for the sodiated molecular ion at *m/z* 330.1681. Further evidence for the successful synthesis was provided by the singlet resonance at 1.48 ppm observed in the ^{1}H NMR spectrum corresponding to the nine *t*-butyl ester protons. Deprotection of the ester **71** was carried out in the presence of trifluoroacetic acid to yield the acid **67** which was purified by high performance liquid chromatography. A peak corresponding to the deprotonated molecular ion was observed at *m/z* 250.1081 in the high resolution ESI (-ve) mass spectrum of the acid **67**.

4.2.2 (*S*)-5-Acetamido-7-methyl-3-oxaoctanoic acid (68)

(*S*)-5-Acetamido-7-methyl-3-oxaoctanoic acid **(68)** was prepared following the experimental protocol outlined above in the synthesis of the acid **67** (Scheme 16).

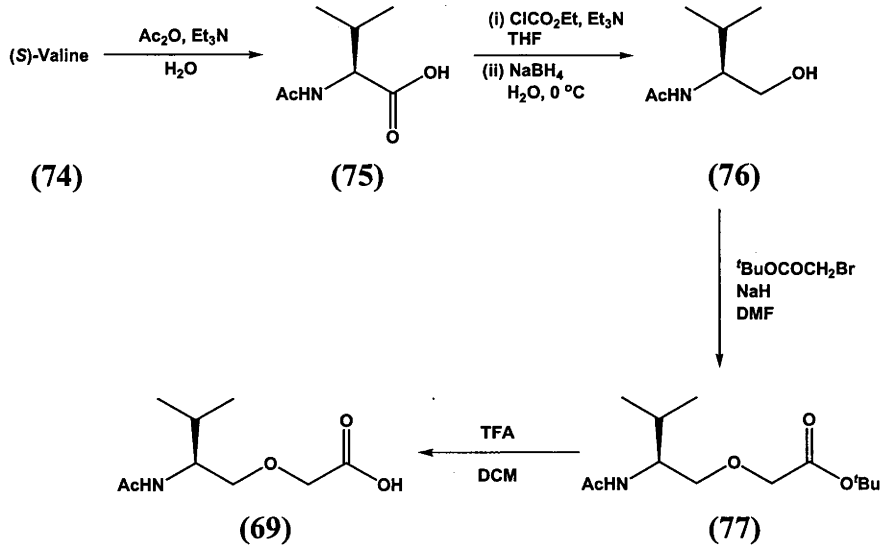


Scheme 16. Synthesis of (*S*)-5-acetamido-7-methyl-3-oxaoctanoic acid **(68)**

Evidence for the successful preparation of the alcohol **72** was provided by the display of the peak of a molecular ion at *m/z* 160.1334 in the high resolution ESI (+ve) mass spectrum. The ^{1}H NMR spectrum of the ester **73** displayed a singlet resonance at 1.45 ppm corresponding to the nine protons of the *t*-butyl group. Deprotection of the ester **73** gave the acid **68**, which was purified by reverse phase high performance liquid chromatography. The characteristic singlet peak corresponding to the protons of the *t*-butyl ester substituent was not observed in the ^{1}H NMR spectrum of the acid **68**, confirming the deprotection reaction was successful.

4.2.3 (*S*)-5-Acetamido-6-methyl-3-oxaheptanoic acid (69)

The acid **69** was prepared according to the general method used in the synthesis of the acid **67** described above (Scheme 17).



Scheme 17. Synthesis of (*S*)-5-acetamido-6-methyl-3-oxaheptanoic acid **(69)**

The acid **75** was generated through the acetylation of (*S*)-valine **(74)**. The experimentally obtained melting point of the protected valine **75** is consistent with literature data.[66] The ESI (+ve) mass spectrum of the alcohol **76** displayed a peak corresponding to the protonated molecular ion at *m/z* 146, confirming the reaction was successful. The high resolution ESI (+ve) mass spectrum of the ester **77** presented a peak for the sodiated molecular ion at *m/z* 282.1669. Formation of the acid **69** was confirmed by a peak at *m/z* 202.1076 in the high resolution ESI (-ve) mass spectrum corresponding to the deprotonated molecular ion.

The melting points recorded for the alcohols **76** and **70** correspond closely to those reported in literature, which indicates that isomerisation up to this point in the synthetic sequences had not taken place. The β–oxa acids **67**, **68** and **69** and the corresponding ester precursors are all assumed to exist as single enantiomers, given the literature precedence of compounds prepared under similar experimental conditions being established to be homochiral.[67] However, no optical rotation

measurements were carried out on the three β–oxa acids **67**, **68** and **69** as they were prepared only in small quantities and further analysis was not considered to be warranted.

4.3 Interactions of the β-oxa acids 67, 68 and 69 with PAM

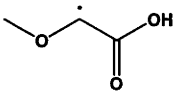
The PAM binding affinities of the three β–oxa acids **67**, **68** and **69** were investigated following the general procedure described earlier. No interaction was observed between PAM and the phenylalanyl β-oxa acid **67** up to highest concentration (16 mM) tested. The lack of activity observed must relate to the absence of the ester carbonyl functionality. The β-oxa acid **67** displayed binding over two orders of magnitude less strong than that observed for the equivalent glycolic acid derivative **55**.

Interestingly and unexpectedly, as the concentration of the β–oxa acids **68** and **69** in the enzyme assay samples increased, the percentage of substrate **22** turned over also increased. At concentrations of 1.0, 4.0 and 8.0 mM of the leucyl β–oxa acid **68**, the yields of the amidated product **23** were greater by factors of 1.5, 1.6 and 2.4 respectively, in relation to the sample with no β–oxa acid **68** present. A more marked effect was observed from the results of the assays with the β–oxa acid **69**. At concentrations of 1.0, 4.0 and 8.0 mM of the valyl β–oxa acid **69**, the yields of the amidated product **23** were greater by factors of 1.2, 2.5 and 4.1 respectively, relative to the sample with no β–oxa acid **69** present.

The enhanced formation of the amidated product **23** is likely to be a result of an activating allosteric effect[68] imposed on the enzyme by the two β-oxa acids **68** and **69**. These compounds probably induce modifications to the enzyme's structure and as a result the substrate **22** is more readily accommodated.

It is not clear whether the β-oxa acids **68** and **69** are processed by the enzyme or are simply allosteric activators. An RSE value of 67.6 kJ mol^{-1} was established for the radical **78**, which is similar in magnitude to the RSE value of the *N*-trifluoroacetyl

radical **42** (RSE value 69.9 kJ mol^{-1}). The close proximity of the two values led to the assumption that the β-oxa acids **67**, **68** and **69** could be processed as PAM substrates, as the *N*-trifluoroacetyl substituted compound **44** was turned over by the enzyme. However, HPLC analysis of the enzyme assay mixtures showed no evidence of the reaction of the β-oxa acids **68** and **69**, although this was not examined in detail since the main focus of this investigation is the effect of compound modifications on binding affinity.



(78)

Nevertheless, the activating effect of the β-oxa acids **68** and **69** may be useful in developing therapies associated with abnormally low hormone levels. A number of pathological conditions have been associated with depleted levels of peptide hormones,[69] including stunted bone growth which has been linked with a deficiency in the growth hormone (GH),[70] while a shortage in the peptide hormone calcitonin has been associated with the pathogenesis of osteoporosis.[71] There is continual interest in the pharmaceutical industry in developing drug therapies to treat certain disease states by replenishing hormone levels.[72,73]

In summary, the poor binding displayed by the phenylalanyl β-oxa acid **67** in comparison with the analogous glycolic acid derivative **55** highlights the importance of the ester carbonyl group in preserving binding affinity with the enzyme. However, this disparity in binding affinity was not observed with the other two β-oxa acids. The β-oxa acids **68** and **69** appeared to act as activators of the enzyme by enhancing the release of the amidated product **23**. The two acids may be useful leads towards the development of therapeutic agents in treating conditions linked with hormone deficiencies.

Chapter Five

α,β-Unsaturated γ-keto acids as PAM inhibitors

5.1 Introduction

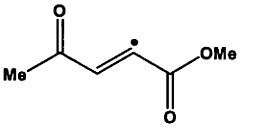
The results from the binding studies presented in Chapters Two and Three indicate that a compound's geometry plays a major role in determining its binding affinity with PAM. A compound which can closely adopt the conformation desired by the enzyme for binding displays overall greater affinity. Glycolic acid derivatives were established to be PAM inhibitors, displaying similar binding affinities to the enzyme as the analogous acylglycines.

α,β-Unsaturated γ-keto acids were next selected for investigation to examine the effects on binding affinity with the enzyme of compounds which mimic the geometries of the amide NH and that of the α-carbon centred radical of natural PAM substrates.

Like the NH of an acylglycine or the ether O of a glycolic acid derivative, the olefinic carbon in the equivalent position of an α,β-unsaturated γ-keto acid is sp^2 hybridised. Both acylglycines and glycolic acid derivatives display good binding affinity to PAM, presumably because the geometric configurations of the compounds closely mimic the configuration desired by the enzyme for tight binding.

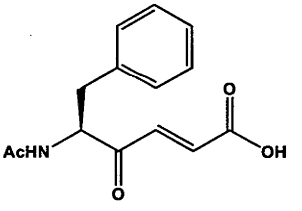
The geometry of the olefinic carbon positioned adjacent to the carboxyl group of an α,β-unsaturated γ-keto acid is also sp^2 hybridised. It mimics the geometry of the radical that normally forms at the α-carbon in systems processed as substrates by the enzyme. Given the geometric similarities between the α,β-unsaturated γ-keto acid and the acylglycine systems, it was anticipated that derivatives of the former would display tight binding towards PAM.

An RSE value of -29.5 kJ mol^{-1} was calculated for the acrylate radical **79**. It is by 108.6 kJ mol^{-1} less stable than the corresponding acylglycine radical **41**, and hence such radicals are unlikely to form through catalysis by PAM, indicating that α,β-unsaturated γ-keto acids are not likely to be substrates even if they bind to the enzyme.

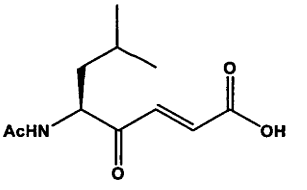


(79)

The phenyl and isopropyl substituted α,β-unsaturated γ-keto acids **80** and **81** were chosen for examination as the interactions of the analogous glycolic acid derivatives **55** and **56** and acylglycines **13** and **14** had already been studied, as discussed above, thus allowing direct comparisons of the interactions with PAM between the different classes of compounds.



(80)



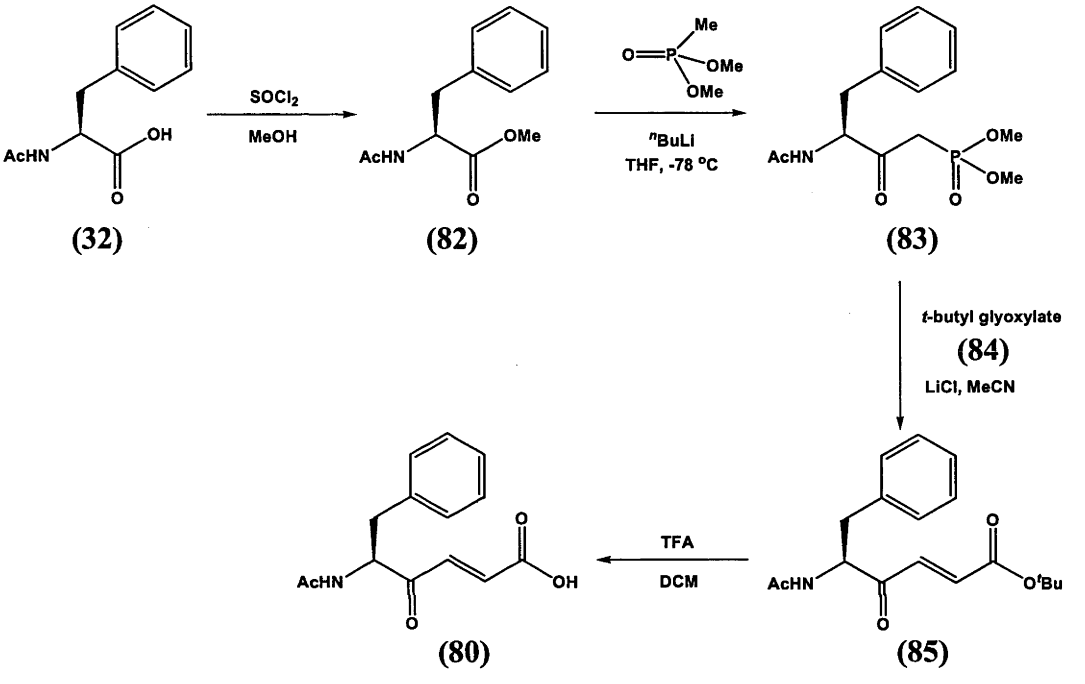
(81)

The binding affinity of the acrylic acid **80** with PAM has previously been established and a K_I value of 90 μM was determined.[74] However, as the enzyme assay parameters employed in the earlier investigation differ from those of the current study, direct comparisons of the literature results cannot be used to derive any correlations with the findings from the present work.

5.2 Syntheses of the α,β-unsaturated γ-keto acids 80 and 81

5.2.1 (*S,E*)-5-Acetamido-4-oxo-6-phenylhex-2-enoic acid (80)

The preparation of the acrylic acid **80** proceeded according to the method described by Luthman and co-workers (Scheme 18).[75]



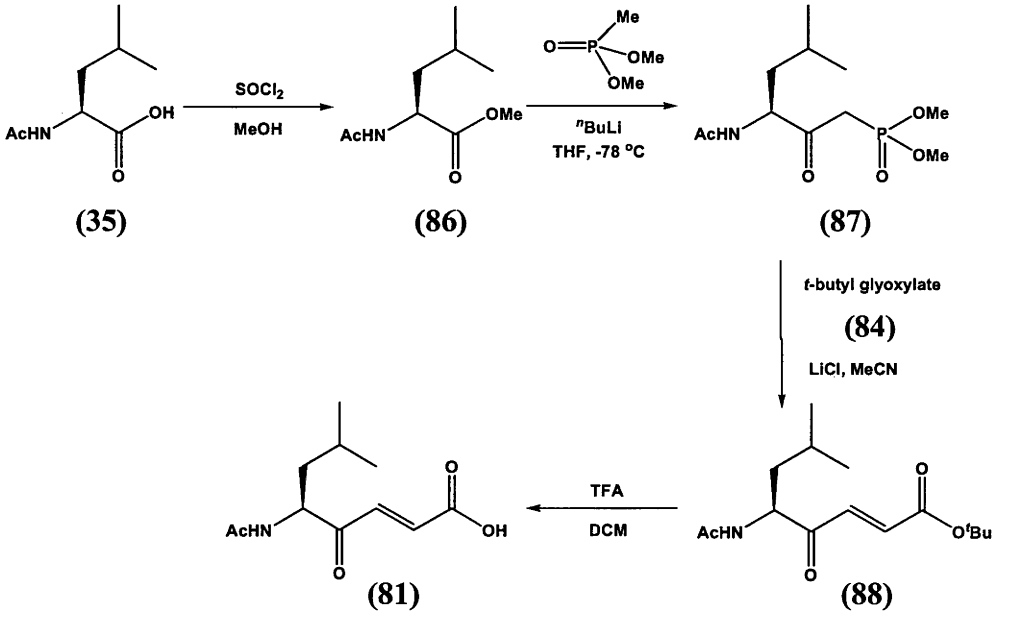
Scheme 18. Synthesis of (*S,E*)-5-acetamido-4-oxo-6-phenylhex-2-enoic acid **(80)**

A methanolic solution of the acid **32** was treated with thionyl chloride to give the methyl ester **82**. The ^{1}H NMR spectral data acquired for the methyl ester **82** is consistent with literature information.[76] The phosphonate ester **83** was prepared following the reported synthetic protocol.[77] The ester **82** was added to a mixture of dimethyl methylphosphonate and *n*-butyl lithium and stirred for 1 hour. The ESI (+ve) mass spectrum of the ester **83** presented a peak at *m/z* 336 corresponding to the sodiated molecular ion. *t*-Butyl glyoxylate **84** was generated in two steps from *(S)*-tartaric acid, following an established method.[75] The Horner-Wadsworth Emmons (HWE) reaction between the phosphonate ester **83** and the glyoxylate **84** was carried out according to a reported procedure.[75] The ^{1}H NMR spectrum of the ester **85** displayed two sets of resonances at 6.70 and 7.02 ppm corresponding to the olefinic

protons. Further evidence supporting the successful synthesis of the ester **85** was observed in the ESI (+ve) mass spectrum in which the peak for the molecular ion was displayed at *m/z* 317. Removal of the *t*-butyl group from the ester **85** proceeded *via* treatment with trifluoroacetic acid to give the acid **80**. The ^{1}H NMR spectral information obtained for the acid **80** is consistent with data reported.[74]

5.2.2 (*S*,*E*)-5-Acetamido-7-methyl-4-oxooct-2-enoic acid (81)

(*S*,*E*)-5-Acetamido-7-methyl-4-oxooct-2-enoic acid **(81)** was synthesized following the protocol exploited in the preparation of the acrylic acid **80** (Scheme 19).



Scheme 19. Synthesis of (*S*,*E*)-5-acetamido-7-methyl-4-oxooct-2-enoic acid **(81)**

The ^{1}H NMR spectral information obtained for the methyl ester **86** is consistent with previously reported data.[78] Formation of the ester **87** was verified by the display of the protonated molecular ion at *m/z* 280 as the base peak in the ESI (+ve) mass spectrum. The ^{1}H NMR spectrum of the ester **88** presented resonances at 6.75 and 7.07 ppm corresponding to the two olefinic protons. The ^{1}H NMR spectrum of the acid **81** did not display any evidence of the singlet resonance corresponding to the

t-butyl group protons of the ester **88**. In addition, the ESI (-ve) mass spectrum of the acid **81** displayed a peak for the deprotonated molecular ion at *m/z* 226.

The acrylic acid **80** was prepared following a literature method used to synthesize the identical compound. The compound reported in the literature was established to be homochiral.[74] It is therefore assumed that the acrylic acid **80** is also homochiral.

In addition, the leucine derivative **81** is assumed to exist as a single enantiomer, as it was prepared under similar experimental conditions to the acid **80**. However, no optical rotation measurements were conducted on the acrylic acids **80** and **81** as their syntheses were performed only on a small scale.

5.3 PAM binding studies with the α,β-unsaturated γ-keto acids 80 and 81

The binding affinities of the α,β-unsaturated γ-keto acids **80** and **81** were determined as per the usual protocol, and the results together with those of the corresponding acylglycines **13** and **14** and glycolic acid derivatives **55** and **56** are presented in Table 10.

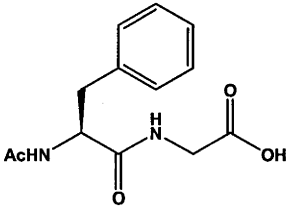
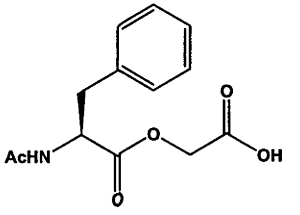
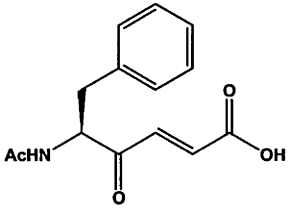
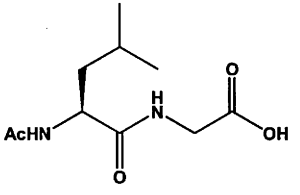
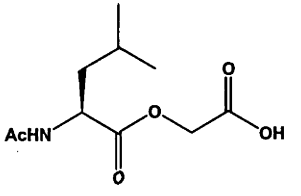
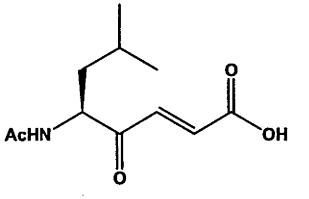
Acylglycines	K_M (mM)	Glycolic acid derivatives	K_I/IC_{50} (mM)	α,β-Unsaturated γ-keto acids	IC_{50} (mM)
 (13)	0.0079[43]	 (55)	0.045[43]	 (80)	0.0007
 (14)	0.096[7]	 (56)	0.060[43]	 (81)	0.642

Table 10. K_M values of the acylglycines **13** and **14** and the K_I/IC_{50} values of the corresponding glycolic acid derivatives **55** and **56** and α,β-unsaturated γ-keto acids **80** and **81**

The α,β-unsaturated γ-keto acids **80** and **81** inhibited PAM activity with IC_{50} values of 0.0007 and 0.642 mM, respectively. Although the binding affinity of the acrylic acid **80** had previously been reported, the enzyme assay conditions under which the binding values were determined differ from those used in the current binding studies, which prevents correlations of the results from the reported work with those collected in this study.

The acrylic acid **80** displayed binding affinity one to two orders of magnitude higher than that observed with the corresponding acylglycine **13** and glycolic acid derivative **55**. The enhanced binding effect could be associated with the geometry of the olefinic carbon adjacent to the carboxyl group in the acrylic acid **80**, which resembles the geometry of the α-carbon centred radical formed from the corresponding substrate **13** during PAM catalysis.

Alternatively, the greater binding affinity displayed by the acid **80** might not simply be as a result of competitive inhibition. In the literature report of its interactions with PAM, the acid **80** was established to be a time-dependent inactivator of the enzyme.[14] The mechanism of inactivation was suggested to proceed *via* epoxide intermediates.[42]

In any event the enhanced binding affinity cannot be generalised to other α,β-unsaturated γ-keto acids, as the leucine derivative **81** exhibited binding affinity around one order of magnitude lower than that observed for the corresponding acylglycine **14** and glycolic acid derivative **56**.

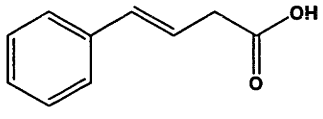
Given the complexity of factors contributing towards the binding affinity of the α,β-unsaturated γ-keto acids **80** and **81** with PAM, and the poor binding observed with the leucine derived acrylic acid **81**, no further investigations with α,β-unsaturated γ-keto acids were conducted since more promising results were obtained with an alternative class of compounds, as discussed in the next Chapter.

Chapter Six

Examination of amino acid based PBA analogues as mechanism based PAM inhibitors

6.1 Introduction

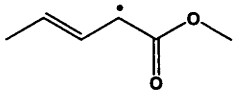
As discussed in the Introduction of this thesis, (*E*)-4-phenyl-3-butenoic acid (PBA) **(1)** has been established to be a suicide substrate of PAM, with a determined K_I value of 0.001 mM.[28]



(1)

The suggested mechanism of inhibition presented in Scheme 5 proceeds through hydrogen atom transfer from the α-carbon to give a delocalised radical resulting in termination of the enzyme's activity.[27]

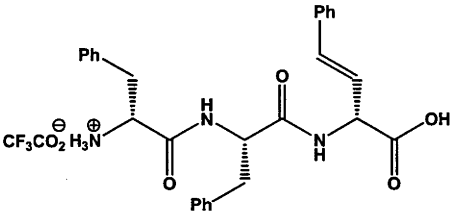
Theoretical calculations established an RSE value of 100.2 kJ mol^{-1} for the propenyl radical.



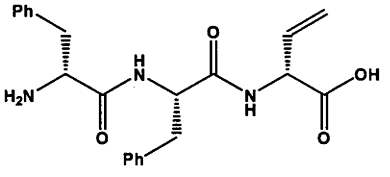
(89)

The propenyl radical **89** is by 21.1 kJ mol^{-1} more stable than the corresponding acetamido radical **41** and hence is likely to form. This observation supports the mechanism of PAM inhibition by PBA **(1)** discussed above.

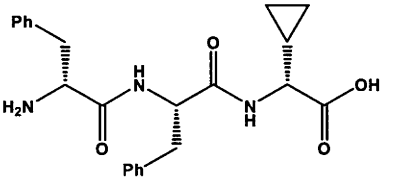
The interactions of a number of compounds designed to exploit the PBA **(1)** mechanism of enzyme inhibition have also been reported. An IC_{50} value of 0.4 mM was recorded for the tripeptide **90**,[27] while the tripeptides **91** and **92** were both established to inhibit PAM activity, with determined K_I values of 0.020 and > 5 mM, respectively.[79]



(90)



(91)

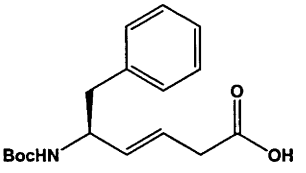


(92)

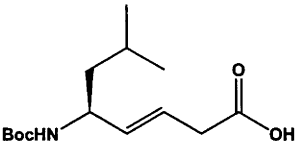
Although the presence of a hydrophobic substituent has been established to retain binding affinity with PAM, in the instance of the tripeptide **90**, the position of the phenyl substituent near the carboxyl group hindered tight binding. This is in contrast to the styryl group of PBA **(1)**, which is suggested to adopt a conformation and occupy a position normally reserved for a substrate of PAM, thus preserving binding affinity with the enzyme.[27]

The tripeptide **92** was found to be a poor inhibitor of the enzyme. The cyclopropyl substituent may have been too sterically demanding to be tolerated by the enzyme active site.[79]

To explore the effect on enzyme inhibition of amino acid based PBA analogues, the *trans*-β,γ-unsaturated acids **93** and **94** were selected for investigation.



(93)



(94)

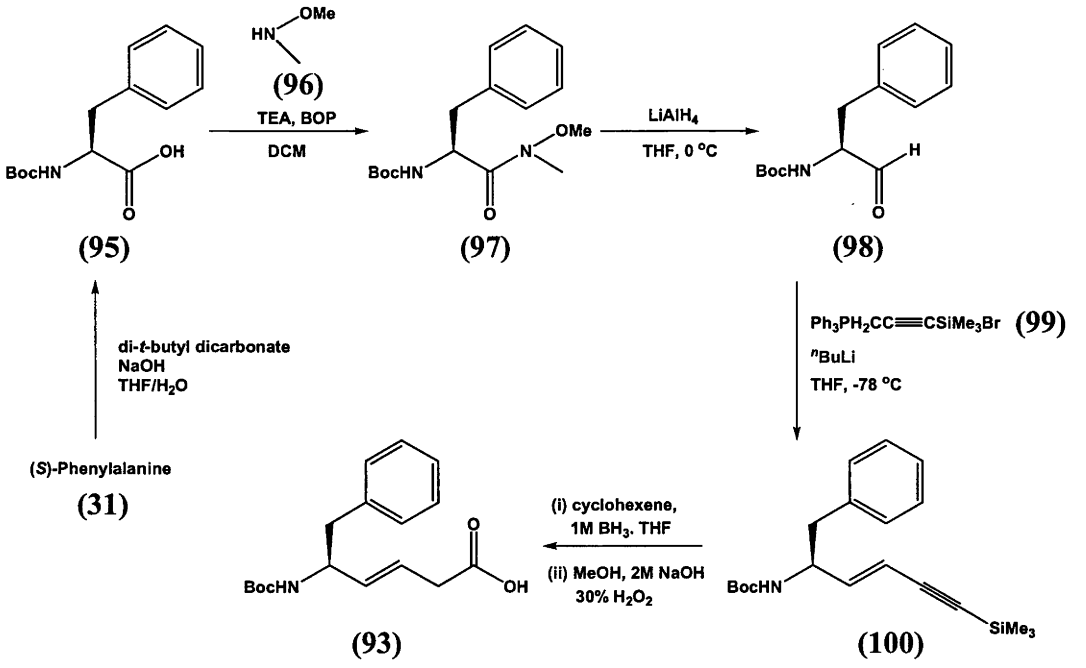
The basis of the design of the *trans*-β,γ-unsaturated acids shown above was to combine two features which are now recognised to retain binding affinity and inhibit the activity of PAM. The incorporation of hydrophobic substituents, like a phenyl or isopropyl group, has been shown to preserve binding affinity, while the PBA **(1)** system has been proven to effectively inhibit enzyme activity.

In addition to the reasons described above, justifying the selection of the acids **93** and **94** for investigation, the inhibition constants of the corresponding glycolic acid derivatives **55** and **56** are discussed above, thus allowing comparisons of the inhibitory effects on PAM by the two classes of compounds.

6.2 Syntheses of the *trans*-β,γ-unsaturated acids 93 and 94

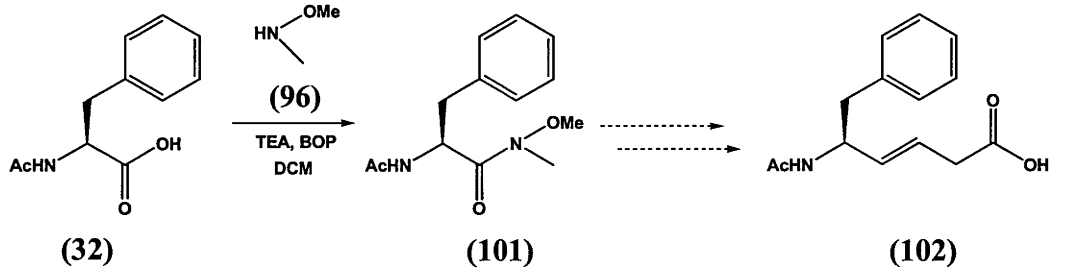
6.2.1 Attempted preparations of the *trans*-β,γ-unsaturated acids 102 and 109

The preparation of the *N*-(*t*-butoxycarbonyl)-substituted *trans*-β,γ-unsaturated acid **93** has previously been reported.[80] The compound **93** was prepared as an intermediate in the synthesis of renin inhibitors. The activity of the unsaturated acid **93** with PAM was not examined in the literature report. The acid **93** was prepared following the reaction sequence illustrated in Scheme 20.



Scheme 20. Synthesis of (*S*,*E*)-5-[*N*-(*t*-butoxycarbonylamino)]-6-phenylhex-3-enoic acid **(93)**

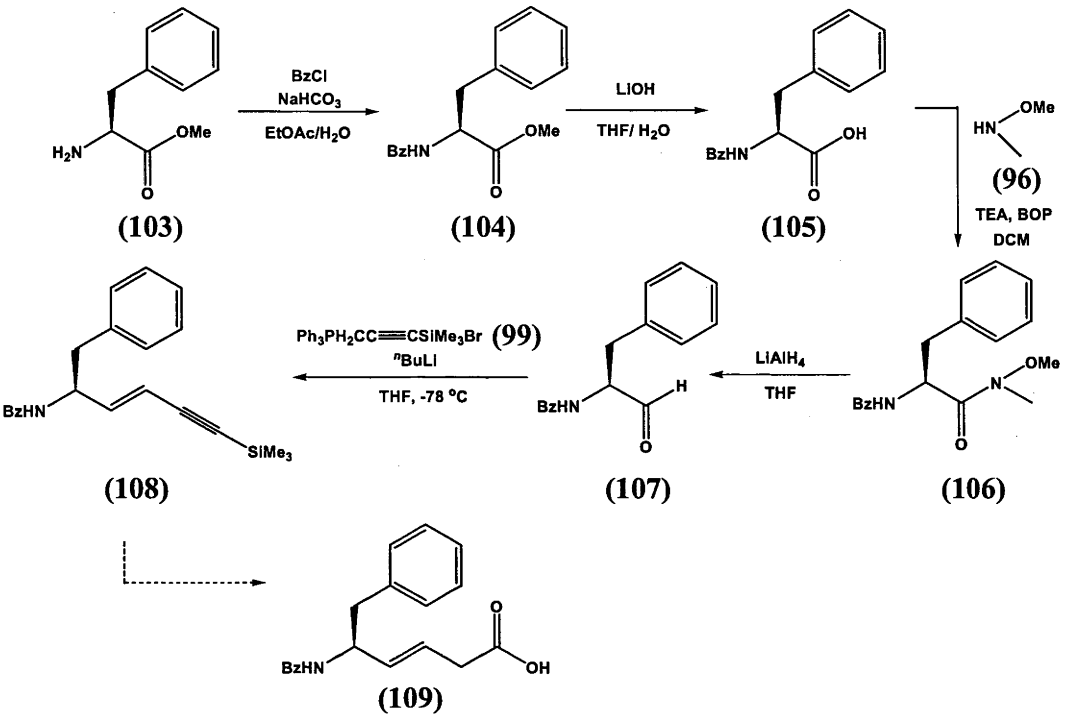
An attempt was made to prepare the corresponding *N*-acetylated unsaturated acid **102** following the reaction scheme outlined above, as the analogous acylglycine **13** and glycolic acid derivative **55** were prepared as the *N*-acetylated compounds. The reaction sequence towards the acid **102** is outlined in Scheme 21.



Scheme 21. Towards the synthesis of the *N*-acetylated acid **102**

The Weinreb amide **101** was prepared from a coupling reaction of the acid **32** with the amine **96** using BOP. The ^{1}H NMR spectrum of the amide **101** displayed resonances at 3.17 and 3.67 ppm, corresponding to the protons of the *N*-methylamide and *N*-methoxy functionalities, respectively. However, due to the low yields of

around 30 percent obtained for the amide **101**, an attempt was then made to prepare the *N*-benzoylated unsaturated acid **109** following the same experimental protocol. It was anticipated that the benzoyl derivatives would be easier to handle. The synthetic pathway attempted is depicted in Scheme 22.



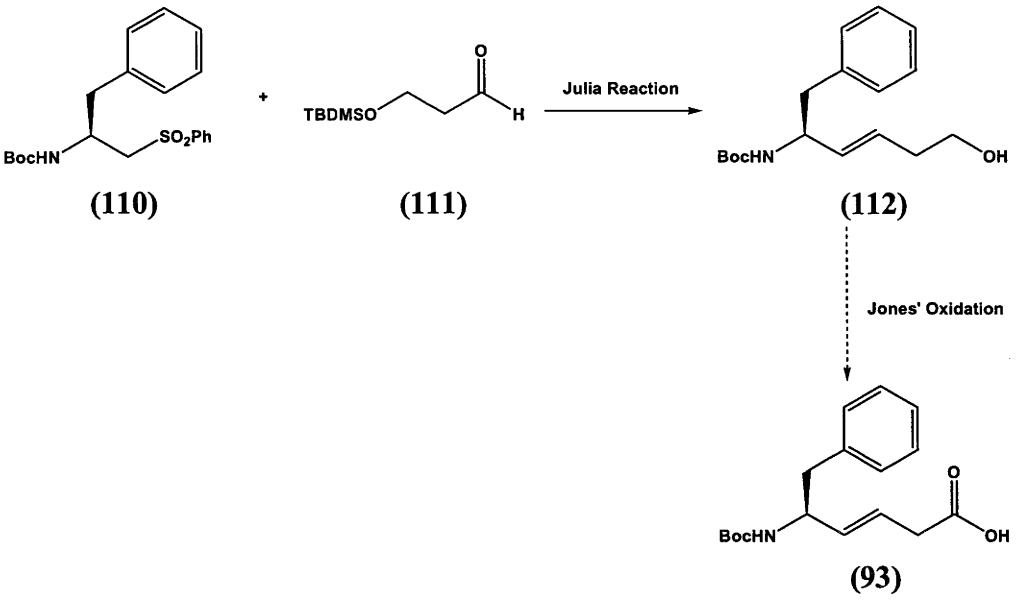
Scheme 22. Towards the synthesis of the *N*-benzoylated acid **109**

The amine **103** was converted to the amide **104** in a Schotten-Baumann reaction.[81,82] Formation of the amide **104** was confirmed by the presence of resonances between 7.10-7.73 ppm in the ^{1}H NMR spectrum. Deprotection of the ester **104** with lithium hydroxide gave the acid **105**. The melting point obtained for the acid **105** is consistent with reported data.[83] Next, the BOP coupling reaction between the acid **105** and *N,O*-dimethylhydroxylamine **(96)** was carried out. The ^{1}H NMR spectrum of the Weinreb amide **106** displayed singlet resonances at 3.21 and 3.74 ppm, which correspond to the protons of the *N*-methylamide and *N*-methoxy functionalities, respectively. The aldehyde **107** was generated through the reduction of the amide **106** with lithium aluminum hydride following established procedures.[84] The ^{1}H NMR spectrum of the aldehyde **107** displayed peaks consistent with the literature.[85] The phosphonium bromide salt **99** was synthesized from triphenylphosphine and

3-(trimethylsilyl)propargyl bromide. The recorded melting point of the bromide salt **99** is consistent with literature values.[86] Next, the Wittig reaction between the aldehyde **107** and the phosphonium salt **99** in the presence of *n*-butyl lithium was attempted. The trimethylsilyl intermediate **108** was isolated as a 2.5:1 mixture with the *Z*-isomer. A peak for the molecular ion was observed at *m/z* 347.1709 in the electron impact (EI) mass spectrum of the intermediate **108**. The mixture was then subjected to a hydroboration reaction, in the presence of a mixture of cyclohexene and 1 M borane-tetrahydrofuran complex.

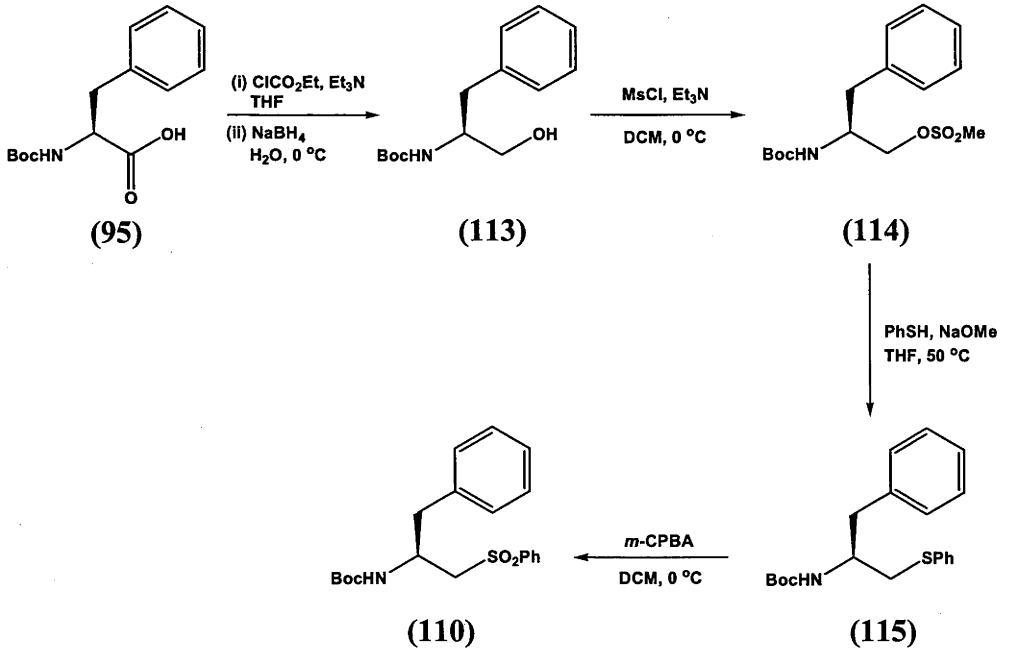
The hydroboration reaction was carried out on 70 milligrams of a 2.5:1 mixture of the intermediate **108** and the *Z*-isomer. The ^{1}H NMR spectrum of the crude product mixture indicated the presence of the unreacted starting material **108** in addition to a large amount of impurities. The presence of the impurities made the recovery of the starting material from the reaction mixture difficult. A number of factors may have contributed towards the failed attempt to prepare the acid **109**, including the small scale on which the reaction was carried out and the sensitivity of the reagents in the reaction to water.

In addition, the reactions to give the aldehyde **107** and the amide **106** were low yielding and the two compounds were prepared in less than 50 percent isolated yields, which adversely affected the subsequent synthesis of the intermediate **108** and the acid **109**. Given the unfavourable elements in the syntheses of the *N*-acetylated and *N*-benzoylated *trans*-β,γ-unsaturated acids **102** and **109**, a decision was made to focus on the preparation of the *N*-(*t*-butoxycarbonyl)-substituted analogue **93**, as its synthesis had previously been reported, as discussed above. In addition to the synthetic sequence towards the acid **93** outlined in Scheme 20, the acid had also been prepared *via* an alternative pathway.[87] The key step in the reported alternative synthetic sequence to give the acid **93** is outlined in Scheme 23.



Scheme 23. Julia reaction of the sulfone **110** and the aldehyde **111** to give the alcohol **112**

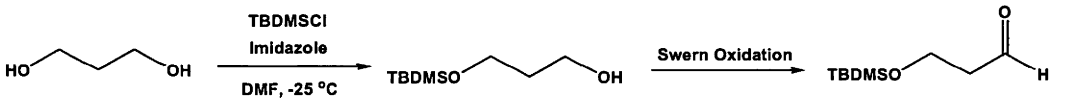
The synthesis of the key intermediate sulfone **110** from the acid **95** is depicted in Scheme 24.



Scheme 24. Synthesis of (*S*)-2-[*N*-*t*-butoxycarbonylamino)]-3-phenyl-1-(phenylsulfonyl)propane **(110)**

The acid **95** was reduced to the alcohol **113** following the established experimental protocol.[64] The ^{1}H NMR spectrum of the alcohol **113** displayed two sets of resonances located between 3.52-3.58 and 3.65-3.69 ppm, associated with the two protons located on the carbon adjacent to the hydroxyl functionality. Next, the newly furnished alcohol **113** was stirred in a mixture of triethylamine and methansulfonyl chloride at 0 °C to yield the mesylate **114**. The ^{1}H NMR data obtained for the mesylate **114** is consistent with reported data.[88] The mesylate **114** was then converted to the sulfide **115** following a literature method.[64] The ESI (+ve) mass spectrum of the sulfide **115** displayed a peak for the protonated molecular ion at *m/z* 344. The sulfide **115** was then oxidised to the corresponding sulfone **110** through the use of 3-chloroperoxybenzoic acid. The melting point recorded for the sulfone **110** is consistent with the reported value.[64]

The monosilylated aldehyde **111** was prepared from the diol **116** following literature procedures.[89] Oxidation of the monosilylated diol **117** under Swern conditions gave the aldehyde **111** (Scheme 25).



(116) **(117)** **(111)**

Scheme 25. Synthesis of 3-[(*t*-butyldimethylsilyl)oxy]propanal **(111)**

The Julia reaction to couple the sulfone **110** with the aldehyde **111** was attempted according to the literature procedure presented in Scheme 23.[89]

The ^{1}H NMR spectrum of the crude product mixture indicated the presence of a large amount of impurities. The spectrum also only indicated the presence of a negligible amount of the alcohol **112**, as signified by the magnitude of the resonances observed in the region of the spectrum where the signals of the olefinic protons of the Julia reaction product were expected, relative to the other signals. In addition, the Julia reaction was only performed on a small scale of 100 milligrams of the starting materials **110** and aldehyde **111**, which would have made the isolation of 20 milligrams of the acid **93** necessary for conducting enzyme assays and compound

characterisation unfeasible. Given the undesirable outcomes in the synthesis of the acid **93** *via* the synthetic route described above, an attempt was then made to prepare the acid **93** following the original literature synthesis[80] of the same compound depicted earlier in Scheme 20.

6.2.2 (*S*,*E*)-5-[*N*-(*t*-Butoxycarbonylamino)]-6-phenylhex-3-enoic acid (93)

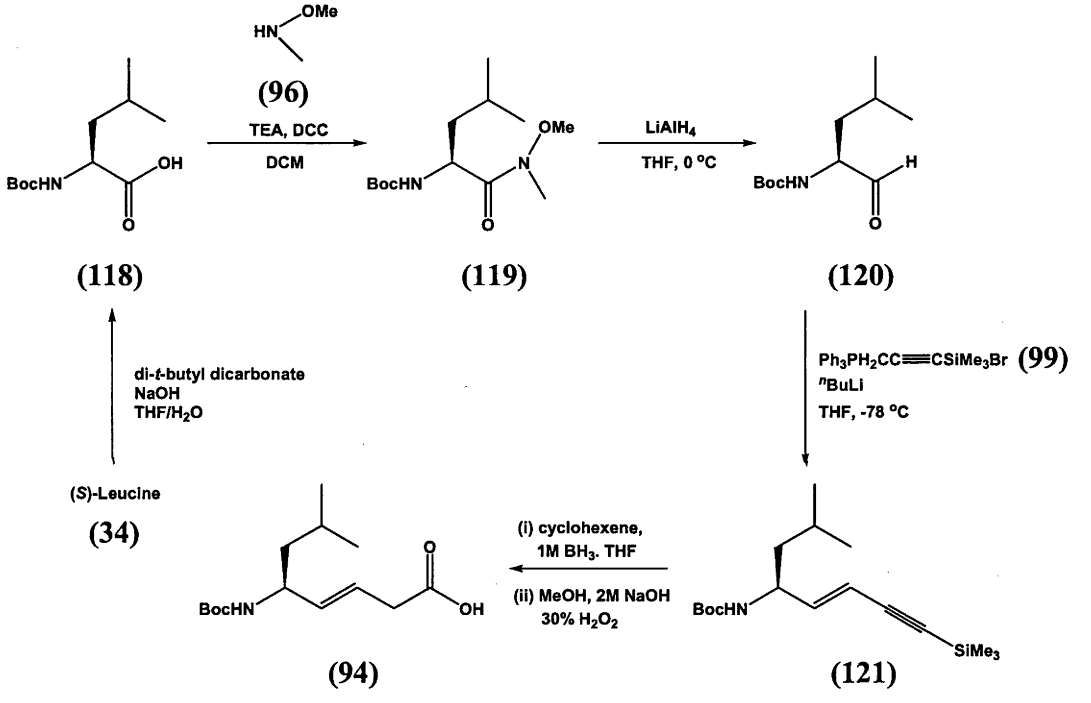
Finally, the *N*-*t*-butoxycarbonyl-protected *trans*-β,γ-unsaturated acid **93** was successfully synthesized following the experimental protocol described by Kaltenbronn *et al.*[80] (Scheme 20).

The acid **95** was prepared from treating a solution of (*S*)-phenylalanine **(31)** with di-*t*-butyl dicarbonate. A peak corresponding to the deprotonated molecular ion was observed at *m/z* 264 in the ESI (-ve) mass spectrum of the acid **95**. Next, the Weinreb amide **97** was synthesized through a BOP coupling reaction between the acid **95** and *N,O*-dimethylhydroxylamine **(96)**. The ^{1}H NMR spectrum of the amide **97** displayed characteristic resonances that correspond well with reported information.[84] Reduction of the amide **97** with lithium aluminium hydride yielded the aldehyde **98**. The ^{1}H NMR spectral information obtained for the aldehyde **98** is consistent with literature data.[90] The aldehyde **98** was added to a mixture of the phosphonium bromide salt **99** and *n*-butyl lithium at –78 °C to give the intermediate **100**. The ^{1}H NMR spectrum of the trimethylsilyl intermediate **100** displayed resonances consistent with literature records.[91] Hydroboration of the intermediate **100** was carried out according to the procedure described by Kaltenbronn *et al.*[80] The deprotonated molecular ion was displayed as a peak at *m/z* 304.1554 in the high resolution ESI (-ve) mass spectrum of the acid **93**.

The acid **93** was prepared on a larger scale to that used with the *N*-benzoylated and *N*-acetylated acids **109** and **102**, which facilitated the isolation of sufficient quantities. The intermediate compounds in the synthetic sequence of the acid **93** were also generally synthesized in higher yields than the intermediates prepared in the syntheses of the acids **109** and **102**.

6.2.3 (*S*,*E*)-5-[*N*-(*t*-Butoxycarbonylamino)]-7-methyloct-3-enoic acid (94)

The acid **94** was prepared following the literature protocol used to synthesize the same compound.[92] In the reported synthesis of the *t*-butoxycarbonyl-protected acid **94**, the acid was coupled with a dipeptide and the inhibitory activity of the resultant product with renin was investigated. The interaction of the acid **94** with PAM was not studied in that investigation. The literature synthesis of the acid **94** proceeded in a similar manner to the reported synthesis of the phenylalanine derivative **93** and is depicted in Scheme 26.[80]



Scheme 26. Synthesis of (*S*,*E*)-5-[*N*-(*t*-butoxycarbonylamino)]-7-methyloct-3-enoic acid **(94)**

The DCC coupling reaction between the acid **118** and the amine **96** gave the Weinreb amide **119**. The ^{1}H NMR spectrum of the amide **119** displayed resonances which correspond well to data reported in literature.[93] Conversion of the amide **119** to the aldehyde **120** was carried out following the previously established experimental protocol.[94] The ESI (+ve) mass spectrum of the aldehyde **120** exhibited a peak corresponding to the protonated molecular ion at *m/z* 216. The Wittig reaction between the aldehyde **120** and the phosphonium salt **99** was carried

out according to the procedure described by Johnson.[92] The high resolution ESI (+ve) mass spectrum of the intermediate **121** presented a peak at *m/z* 332.2016 for the sodiated molecular ion. Hydroboration of the trimethylsilyl intermediate **121** to the acid **94** was carried out according to literature protocol.[80] The ^{1}H NMR spectrum obtained for the acid **94** displayed resonances consistent with reported data.[92] Further evidence for the successful synthesis of the acid **94** was provided by the display of a peak at *m/z* 270 for the deprotonated molecular ion in the ESI (-ve) mass spectrum.

The acid **93** is reported to be homochiral.[80,89] Since the synthesis of the leucyl derivative **94** was carried out under similar experimental conditions to those used to prepare the acid **93**, coupled with the measured optical rotation value of -20.6°, it is also assumed to be a single enantiomer.

6.3 Interactions of the unsaturated acids 93 and 94 with PAM

The IC_{50} values of the two *trans*-β,γ-unsaturated acids **93** and **94** with PAM were determined according to the method described in Chapter Two, and together with those of the corresponding glycolic acid derivatives **55** and **56**, and the K_M values of the analogous acylglycines **13** and **14** are provided in Table 11.

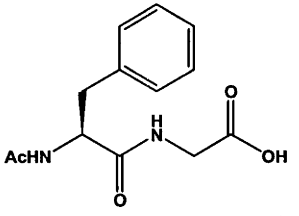
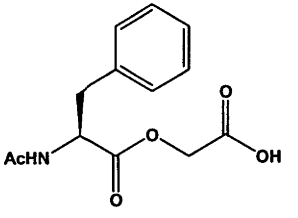
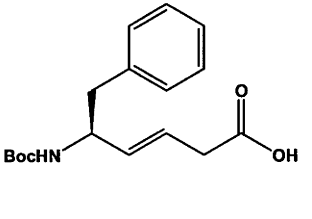
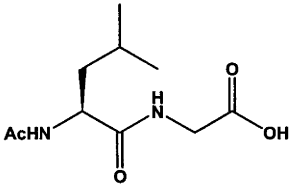
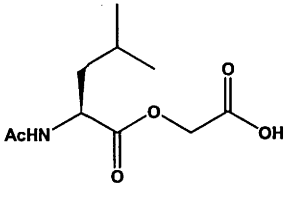
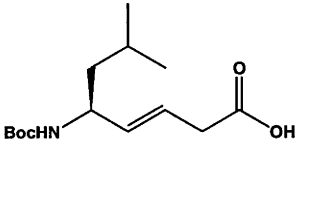
Acylglycines	K_M (mM)	Glycolic acid derivatives	K_I/IC_{50} (mM)	*trans*-β,γ-Unsaturated Acids	IC_{50} (mM)
 (13)	0.0079[43]	 (55)	0.045[43]	 (93)	0.0026
 (14)	0.096[7]	 (56)	0.060[43]	 (94)	0.0015

Table 11. IC_{50} values of the acids **93** and **94** and the corresponding glycolic acid derivatives **55** and **56**, and the K_M values of the acylglycines **13** and **14**

An IC_{50} value of 0.0026 mM was determined for the phenylalanyl *trans*-β,γ-unsaturated acid **93**. The corresponding leucyl derivative **94** displayed an IC_{50} value of 0.0015 mM.

The acids **93** and **94** are more effective at inhibiting PAM activity than the corresponding glycolic acid derivatives **55** and **56**, by over an order of magnitude.

As discussed in Chapter One, with respect to compounds which undergo competitive inhibition, an IC_{50} value provides a good preliminary approximation of the K_I value. However, inhibition of PAM by the olefinic acids **93** and **94** is likely to proceed in a similar manner to that proposed for (*E*)-4-phenyl-3-butenoic acid (PBA) **(1)**, which has been established as an irreversible inactivator of the enzyme,[26] and thus the IC_{50} values of the acids **93** and **94** do not compare so directly with the K_I value obtained for PBA **(1)** to establish relative potency. Nevertheless, the IC_{50} values obtained for the two *trans*-β,γ-unsaturated acid **93** and **94** indicate that the compounds are potent inhibitors of PAM.

In order to determine the mode of PAM inhibition displayed by the *trans*-β,γ-unsaturated acids **93** and **94**, the K_I values of the two compounds will need to be determined. It is however extremely costly to establish this type of inhibition as a large amount of enzyme is required. An individual assay will cost several thousands of dollars to perform, therefore, K_I determinations of the acids **93** and **94** were not carried out.

Given the effective inhibition of PAM displayed by the olefinic acids **93** and **94**, derivatives of the *trans*-β,γ-unsaturated acids may be useful in the development of therapeutic agents to treat disease states associated with the over production of peptide hormones. Incorporation of a *trans*-β,γ-unsaturated acid into peptidic systems which resemble specific peptide prohormones would give compounds that could interfere with the catalysis of the particular prohormones by the enzyme. The peptide analogues of the *trans*-β,γ-unsaturated acids are expected to display potent inhibition of the catalysis of the corresponding peptide prohormones by PAM.

To summarise, the combination of a hydrophobic substituent which has been shown to preserve binding affinity with PAM, incorporated in a *trans*-β,γ-unsaturated acid, produced two compounds **93** and **94** that displayed effective enzyme inhibition. The *trans*-β,γ-unsaturated acids **93** and **94** are by far the most effective PAM inhibitors of all the compounds studied thus far as part of the research work conducted for this thesis.

Chapter Seven

Evaluation of the effect of methionine based compounds on PAM binding affinity in comparison with the analogous leucine derivatives

7.1 Introduction

In Chapter Two, the interaction of 6-methylthiohexanoic acid **(17)** with PAM was investigated. The acid **17** was identified as a PAM inhibitor and the incorporation of a sulfur-containing group was established to preserve binding affinity with the enzyme. The high binding affinity displayed by the sulfide **17** may be a result of the sulfur coordinating with a copper of the enzyme.

The availability of the copper is necessary for maintaining normal PAM activity.[10,95,96] Coordination to the copper renders it unavailable, and consequently PAM activity is inhibited.

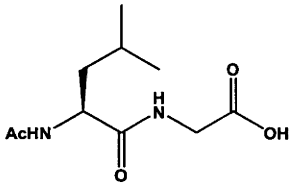
The incorporation of a copper binding substituent enhances binding affinity with PAM and at the same time inhibits the activity of the enzyme.

The interactions of a number of inhibitors designed to exploit PAM's dependence on the presence of copper have been documented. All of the compounds possess a substituent aimed at binding with the enzyme's copper. Examples of the inhibitors include the therapeutic agents captopril **(2)**, thiorphan **(4)** and tiopronin **(5)** discussed in Section 1.4.

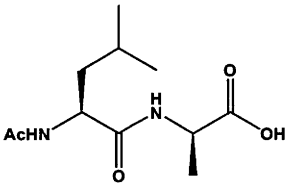
In addition, the tripeptides Gly-Cys-Gly and Gly-Met-Gly were found to be effective at inhibiting PAM activity, with established K_I values of 0.0031 and 0.023 mM, respectively.[51] As with the other sulfur containing compounds discussed above, the

mechanism of PAM inhibition by the tripeptides is likely to involve binding with the enzyme's copper.

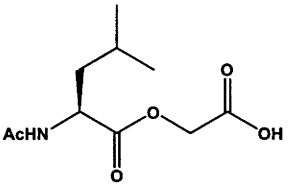
To establish the mode of binding of methionine containing compounds to the enzyme, and to determine whether the binding affinity of the copper coordinating compounds is affected by the same factors as the corresponding analogues derived from non-copper binding amino acids, the interactions with PAM of the leucine derivatives **14**, **122** and **56** and the corresponding methionine analogues **123**, **124** and **125** were studied.



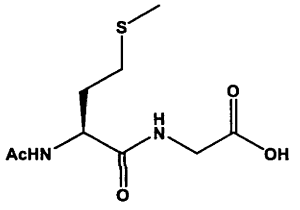
(14)



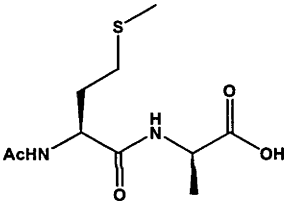
(122)



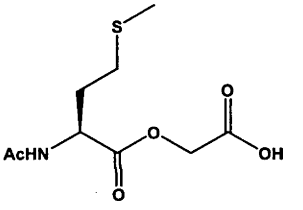
(56)



(123)



(124)



(125)

The incorporation of a hydrophobic moiety has been established to retain high binding affinity with PAM, as discussed in Chapter Two. The binding affinities of the dipeptide **14** and the glycolic acid derivative **56** with PAM have previously been reported.[7,43] The two compounds displayed similar binding affinities with the enzyme, presumably due to the similarities in the geometric configurations adopted by the compounds, which are desired by the enzyme for tight binding.

It was envisaged that the dipeptide **122** would exhibit poor binding affinity, as substituents at the α-carbon have been established to disrupt binding with PAM, as observed with the trifluoroalanine containing dipeptides **47** and **48** discussed in Chapter Three. To contrast the binding affinity of the leucyl dipeptide **122** with the corresponding methionyl derivative, the dipeptide **124** was selected for examination.

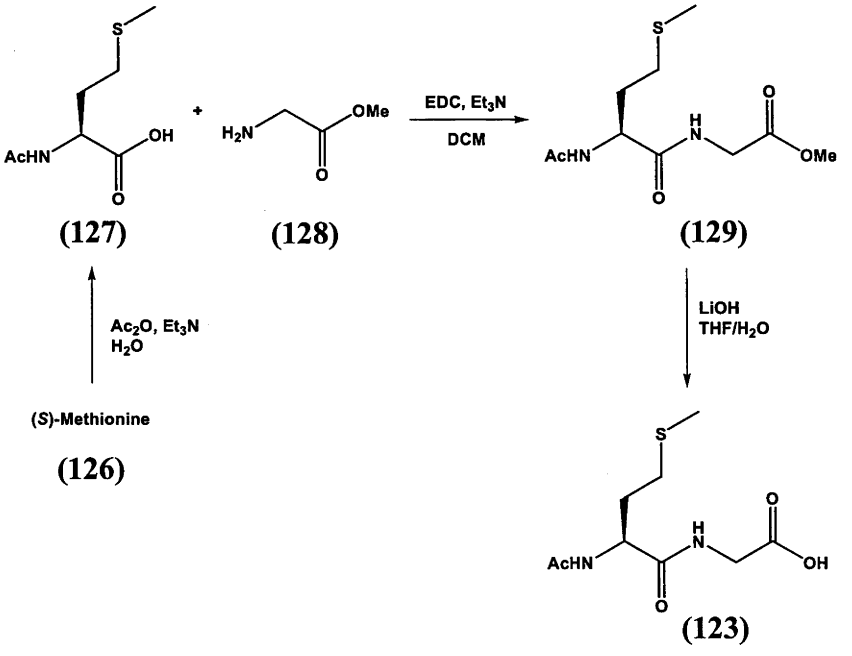
As mentioned in Chapter Two, the methionylglycine **12** has been established as an inhibitor of PAM.[52] However, as binding affinity of the dipeptide **12** with the enzyme was not determined in the earlier work, the interaction of the dipeptide **123** with PAM was re-examined as part of the work for this Chapter.

The acid **125** encompasses both a glycolic acid functionality as well as a thio ether side chain from methionine, which have both been established to preserve binding affinity with PAM. It was anticipated that the two features might act in a complementary fashion, thus the acid **125** would display particularly high binding affinity with the enzyme.

7.2 Syntheses of the methionine based compounds 123, 124 and 125

7.2.1 (*S*)-*N*-Acetylmethionylglycine (123)

The dipeptide **123** was prepared according to the reaction sequence outlined in Scheme 27.

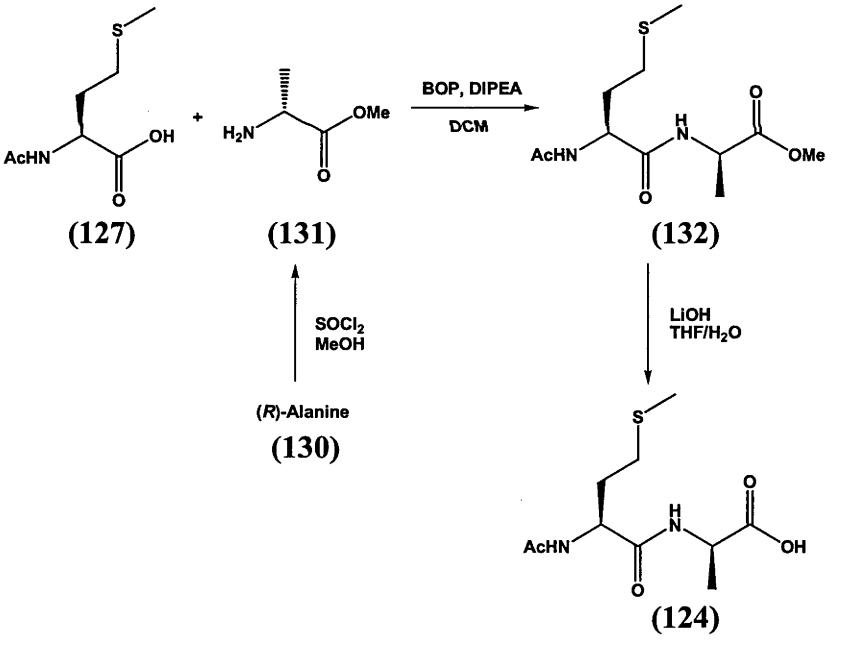


Scheme 27. Synthesis of (*S*)-*N*-acetylmethionylglycine **(123)**

The acid **127** was prepared from (*S*)-methionine **126,** using acetic anhydride. The resonances in the ^{1}H NMR spectrum of the acid **127** are consistent with those reported in the literature.[97] A mixture of the ester **128** and the acid **127** in dichloromethane was treated with triethylamine followed by the addition of 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide (EDC) hydrochloride and the resulting mixture was stirred overnight. Formation of the ester **129** was verified by the exhibition of the peak for the sodiated molecular ion at *m/z* 285 in the ESI (+ve) mass spectrum. Saponification of the ester **129** with lithium hydroxide yielded the acid **123**. Microanalysis of the dipeptide **123** confirmed the purity of the compound.

7.2.2 (*S,R*)-*N*-Acetylmethionylalanine (124)

The synthetic pathway employed in the preparation of the dipeptide **124** is outlined in Scheme 28.



Scheme 28. Synthesis of (*S,R*)-*N*-acetylmethionylalanine **(124)**

The acid **127** was prepared as per above. The methyl ester **131** was successfully prepared from treating a methanolic solution of the amino acid **130** with thionyl chloride. Formation of the methyl ester **131** was confirmed by the singlet resonance

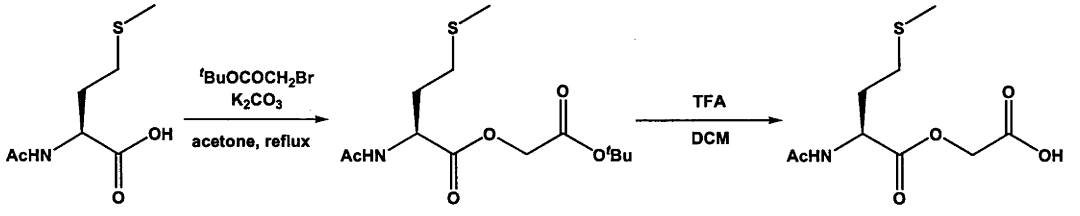
observed in the ^{1}H NMR spectrum at 3.81 ppm, which corresponds to the three protons of the methyl ester group. The coupling reaction between the acid **127** and the methyl ester **131** was carried out using BOP. A peak corresponding to the molecular ion was observed at *m/z* 276.1145 in the high resolution EI mass spectrum of the ester **132**. Saponification of the ester **132** using lithium hydroxide gave the acid **124**. The ^{1}H NMR spectrum obtained for the acid **124** matches the reported data.[98] The high resolution EI mass spectrum displayed a peak corresponding to the molecular ion at *m/z* 262.0987, confirming the formation of the acid **124**.

Only one set of resonances was observed in the ^{1}H NMR spectrum of the dipeptide **124**, which confirmed the absence of diastereomers. This, together with the optical rotation value of -12.0° recorded for the dipeptide **124** is the basis of the assumption that it is present as a single stereoisomer.

An optical rotation value of -3.8° was recorded for the dipeptide **123**, it is therefore also assumed to be homochiral.

7.2.3 (*S*)-O^{α}-(*N*-Acetylmethionyl)glycolic acid (125)

The glycolic acid derivative **125** was synthesized as illustrated in Scheme 29.



(127) **(133)** **(125)**

Scheme 29. Synthesis of (*S*)-O^{α}-(*N*-acetylmethionyl)glycolic acid **(125)**

The acid **127** was prepared following the experimental protocol described earlier in this Chapter. A mixture of the acid **127** and *t*-butyl bromoacetate was heated at reflux and stirred overnight to give the ester **133**. The ESI (+ve) mass spectrum of

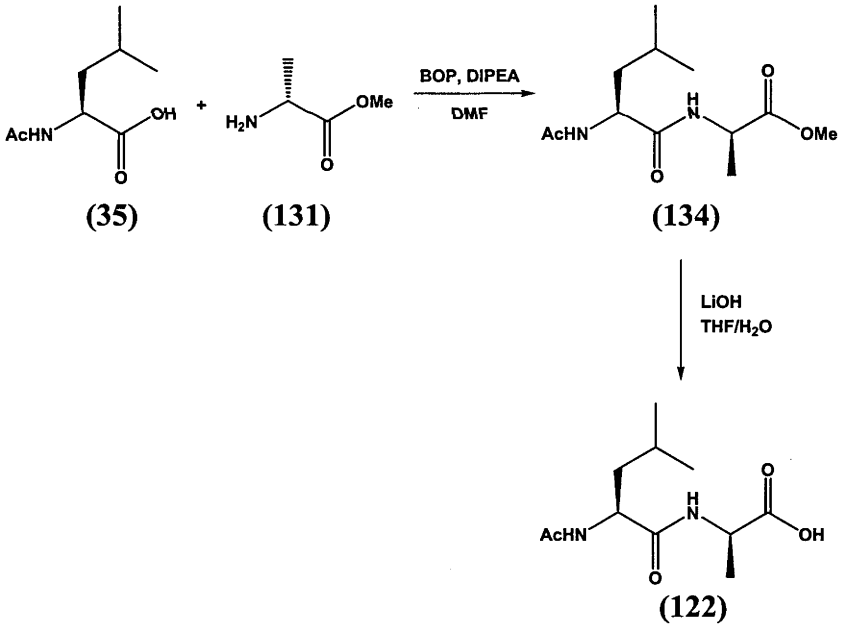
the ester **133** displayed the sodiated molecular ion at *m/z* 328 as the base peak. The deprotection of the ester **133** was carried out with trifluoroacetic acid. The singlet resonance corresponding to the *t*-butyl protons of the ester **133** was not present in the ^{1}H NMR spectrum of the acid **125**, indicating the reaction was successful.

An optical rotation of –63.8° was obtained for the acid **125**; it is therefore assumed to be present as a single enantiomer.

7.3 Synthesis of the leucylalanine dipeptide 122

7.3.1 (*S,R*)-*N*-Acetylleucylalanine (122)

(*S,R*)-*N*-Acetylleucylalanine **(122)** was synthesized following the experimental protocol used to prepare the dipeptide **124** (Scheme 30).



Scheme 30. Synthesis of (*S,R*)-*N*-acetylleucylalanine **(122)**

The acid **35** was prepared following the experimental procedures described in Chapter Two and the methyl ester **131** was prepared according to the protocol described earlier in this Chapter. The coupling reaction between the amine **131** and

the acid **35** was carried out using BOP. The high resolution ESI (+ve) mass spectrum of the ester **134** exhibited a peak at *m/z* 281.1469 corresponding to the sodiated molecular ion. Saponification of the ester **134** was carried out in the presence of lithium hydroxide. The ESI (+ve) mass spectrum of the acid **122** presented the sodiated molecular ion at *m/z* 267 as the base peak.

Only one set of resonances was observed in the ^{1}H NMR spectrum of the dipeptide **122**, indicating the absence of diastereomers. Furthermore, an optical rotation value of –44.8° was recorded for the acid **122**, leading to the assumption that it is homochiral.

7.4 PAM binding affinities of compounds 123, 124, 125 and 122

The binding affinities of compounds **123**, **124**, **125** and **122** with PAM were established according to the protocol described in Chapter Two. The results from these binding studies, together with the IC_{50} values of the acids **17** and **16**, the K_M value of the dipeptide **14** and the K_I value of the leucylglycolic acid **56** are presented in Table 12.

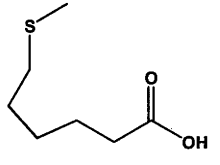
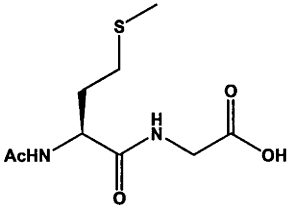
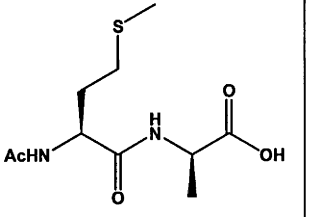
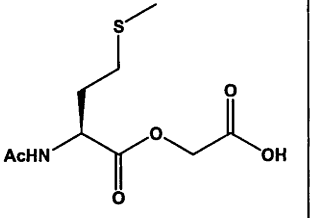
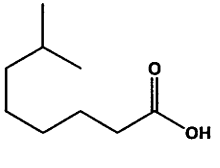
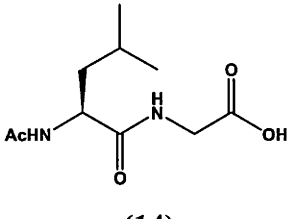
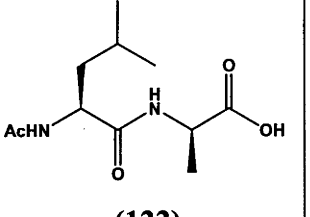
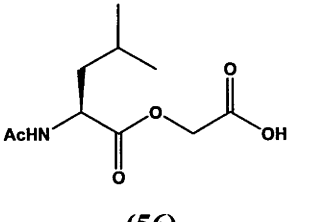
Acids	IC_{50} (mM)	Acylglycines	K_M/IC_{50} (mM)	Acylalanines	IC_{50} (mM)	Glycolic acid derivatives	K_I/IC_{50} (mM)
 (17)	1.7	 (123)	0.03	 (124)	0.2	 (125)	0.9
 (16)	4.4	 (14)	0.096[7]	 (122)	> 4	 (56)	0.060[43]

Table 12. IC_{50} values of compounds **17, 16, 123, 124, 122** and **125**, K_M value of the dipeptide **14** and K_I value of the glycolic acid derivative **56**

The dipeptide **123** inhibited PAM activity with an IC_{50} value of 0.03 mM. The dipeptide **123** displayed similar binding affinity to PAM as the analogous leucyl dipeptide **14**. The corresponding thio ether **17** also displayed similar binding affinity to the enzyme as its acid analogue **16**.

The similarities in binding affinities observed with the acylglycines **123** and **14**, and the acids **17** and **16** suggest that the mode of binding of compounds with a copper coordinating group to PAM may be similar to that of the corresponding compounds which lack the copper binding feature but have a hydrophobic group.

An IC_{50} value of 0.2 mM was recorded for the dipeptide **124**, while the corresponding leucyl dipeptide **122** did not display any activity. The α-alkylated dipeptides **124** and **122** displayed poorer binding affinity to PAM than the corresponding unsubstituted dipeptides **123** and **14**, respectively. This was the expected outcome, as substituents at the α-carbon of glycine-based substrates have been determined to disrupt binding with the enzyme.[99]

However, the methionyl dipeptide **124** displayed binding affinity that was greater by over an order of magnitude than that observed with the leucyl derivative **122**. This result is inconsistent with the similarities in binding affinities observed with the acylglycines and the acids discussed above. The disparity in binding between the dipeptides **124** and **122** indicates that the binding affinity of a compound with a copper coordinating substituent is not always affected to the same extent as the corresponding compound possessing a hydrophobic group, in the presence of another factor which has been established to disrupt binding.

The binding study results for the α-alkylated dipeptides **124** and **122** also suggest that the mode of binding to PAM of the methionine-containing compounds might differ in some ways to that of the analogous compounds derived from non copper-binding amino acids. The key interaction between the methionine containing compounds with PAM is suggested to be through coordination with the copper in the active site.

The glycolic acid derivative **125** was expected to exhibit high binding affinity with PAM as it incorporates two features which have been established to maintain high binding affinity with the enzyme. It was proposed that the glycolic acid functionality, together with the thio ether ligand could act in a synergistic manner to give a compound which would display tight binding to the enzyme. Unfortunately this was not the case.

The methionylglycolic acid derivative **125** displayed binding affinity that was by over an order of magnitude poorer than that observed with the corresponding acylglycine **123**. In contrast, the leucine derivative **56** displayed similar binding affinity to PAM as the analogous acylglycine **14**.

The reduced binding affinity displayed by the acid **125** is likely to be associated with the arrangement of the compound's functional groups with respect to the key recognition points in the active site, which may be preventing tight binding.

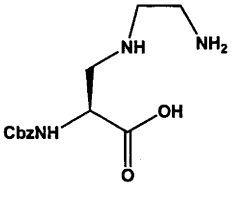
In summary, the methionyl dipeptides **123** and **124** displayed higher binding affinity with PAM that the analogous leucyl derivatives **14** and **122**, respectively, which highlights the significance of the thio ether functional group for maintaining binding affinity with the enzyme. In the case of the glycolic acid derivative **125**, the poor binding affinity displayed could be due to the arrangement of its functional groups within the active site, which may not be ideal for tight binding with PAM. Given the overall positive binding study results obtained for the compounds possessing a copper coordinating functional group examined as part of the research conducted for this Chapter, compounds discussed in the next Chapter of this thesis also possess a copper-binding substituent.

Chapter Eight

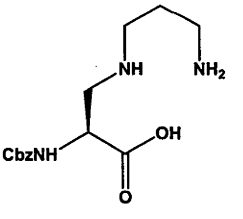
Investigation of the effect of 1,2-diaminoethane and 1,3-diaminopropane substituted amino acids on interactions with PAM

8.1 Introduction

The incorporation of a copper coordinating substituent has been established to preserve binding affinity with PAM as observed with the sulfur containing compounds discussed in Chapter Seven. In order to determine the effect of amino acid derivatives bearing 1,2-diaminoethane and 1,3-diaminopropane groups, which have been well established to form complexes with copper,[100-104] the interactions of the two diamines **135** and **136** with the enzyme were explored.



(135)



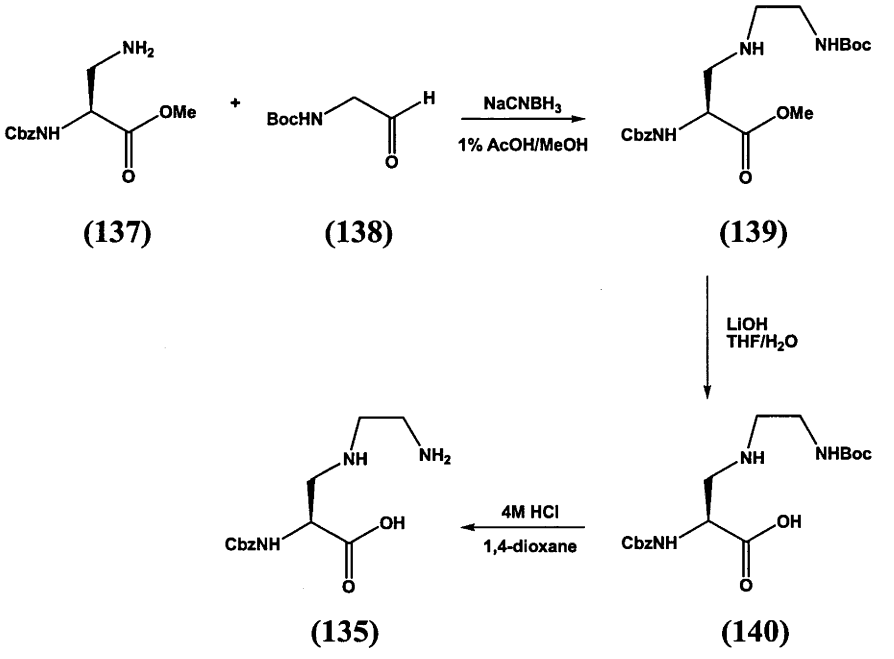
(136)

It was envisaged that the 1,2-diaminoethane moiety of the diamine **135** and the 1,3-diaminopropane ligand of the diamine **136** would coordinate with the copper in the PAM active site, thus inhibiting the enzyme's activity.

8.2 Syntheses of the diamines 135 and 136

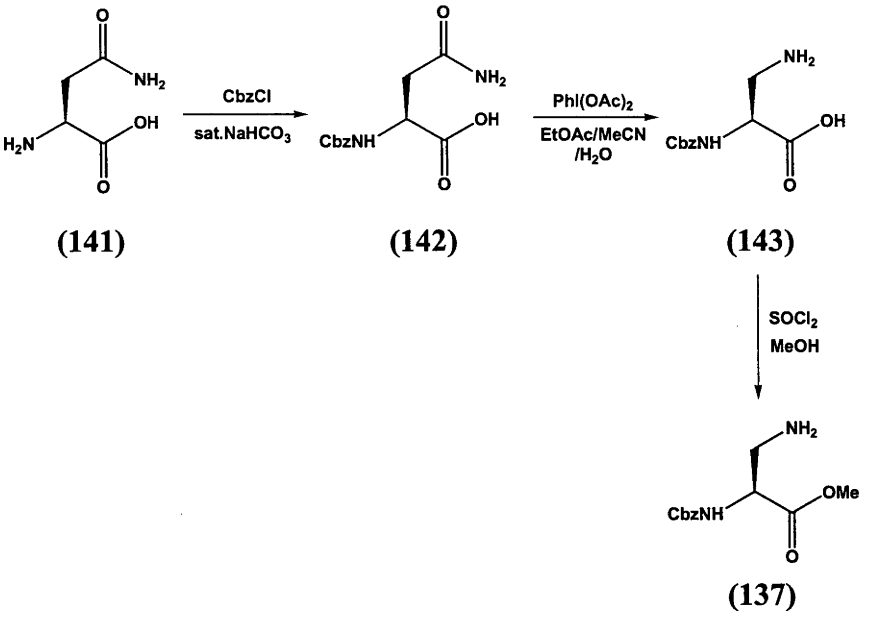
8.2.1 (*S*)-N^2-Benzyloxycarbonyl-2,6-diamino-4-azahexanoic acid (135)

The key steps in the synthesis of the azahexanoic acid **135** are outlined in Scheme 31.



Scheme 31. Synthesis of (*S*)-N^2-benzyloxycarbonyl-2,6-diamino-4-azahexanoic acid **(135)**

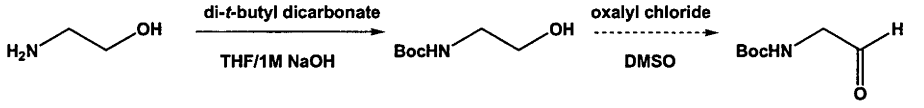
The ester **139** was prepared following the reported synthesis of the same compound.[105] Preparation of the first of the two key components in the synthesis of the ester **139** is depicted in Scheme 32.



Scheme 32. Synthesis of methyl (*S*)-N^2-benzyloxycarbonyl-2,3-diaminopropionate **(137)**

A mixture of the amino acid **141** in a sodium bicarbonate solution was treated with benzyl chloroformate. A multiplet resonance corresponding to the five aromatic protons was observed between 7.14-7.36 ppm in the ^{1}H NMR spectrum of the acid **142**. The conversion of the acid **142** to the amine **143** proceeded through treatment with diacetoxyiodobenzene. A peak corresponding to the protonated molecular ion was observed at *m/z* 239 in the ESI (+ve) mass spectrum of the amine **143**. Treatment of a methanolic solution of the amine **143** with thionyl chloride gave the ester **137**. The melting point obtained for the ester **137** is consistent with the reported data.[105]

Next, the second key component in the synthesis of the ester **139**, the aldehyde **138**, was prepared. An initial attempt was made to synthesize the aldehyde **138** from the aminoethanol **144** (Scheme 33).

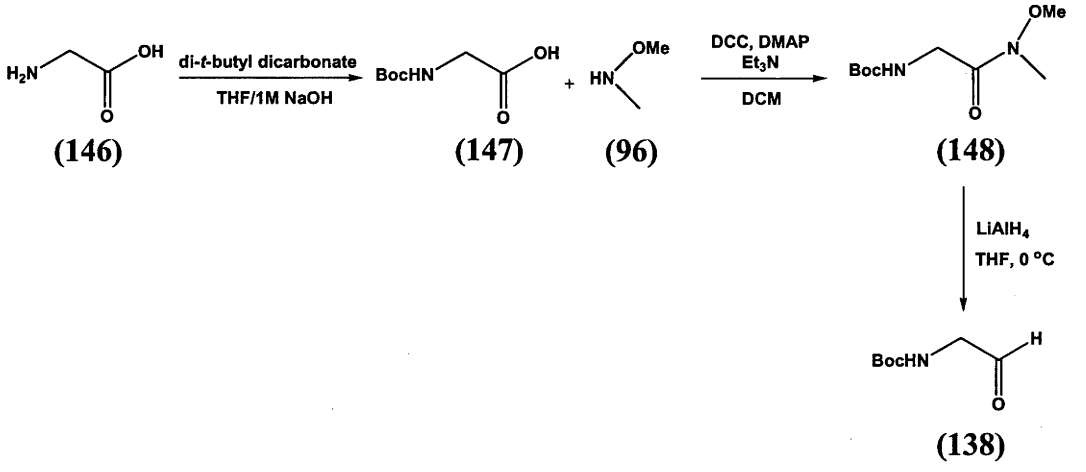


(144) **(145)** **(138)**

Scheme 33. Attempted synthesis of the aldehyde **138** from the alcohol **144**

The amine **144** was treated with di-*t*-butyl dicarbonate to give the carbamate **145**. The ^{1}H NMR spectrum of the carbamate **145** displayed resonances consistent with the literature data.[106] Attempts were made to oxidise the alcohol **145** under Swern oxidation conditions. The ^{1}H NMR spectra of the crude product mixtures did not display the characteristic singlet resonance corresponding to the aldehyde proton, indicating the oxidation reactions were not successful. Given that several unsuccessful attempts were made to prepare the glycinal **138** *via* the synthetic pathway described above, an alternative route for the synthesis of the glycinal **138** was identified and tried.

The aldehyde **138** was successfully prepared from glycine **146** as illustrated in Scheme 34.



(146) **(147)** **(96)** **(148)** **(138)**

Scheme 34. Synthesis of *N*-(*t*-butoxycarbonyl)glycinal **(138)**

The carbamate **147** was successfully prepared from the amine **146** following treatment with di-*t*-butyl dicarbonate. The ^{1}H NMR spectral data obtained for the

acid **147** is consistent with the literature records.[107] The Weinreb amide **148** was generated from a coupling reaction between the acid **147** and the amine **96** using *N,N'*-dicyclohexylcarbodiimide. The ^{1}H NMR spectrum of the amide **148** displayed two singlet resonances at 3.20 and 3.71 ppm, corresponding to the *N*-methyl and *N*-methoxy protons, respectively. Following the established experimental protocol, reduction of the Weinreb amide **148** with lithium aluminium hydride gave the aldehyde **138**.[94] The ^{1}H NMR spectrum of the aldehyde **138** displayed resonances consistent with the literature data.[94]

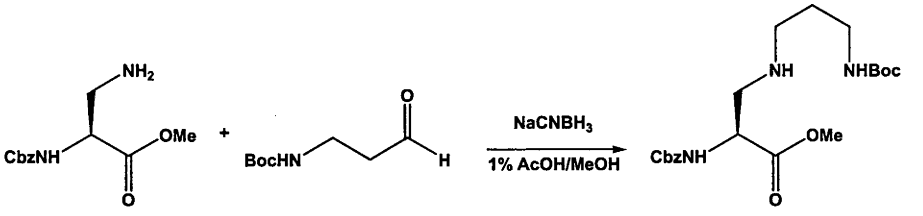
Next, an attempt was made to prepare the azahexanoic acid **135** following the method outlined earlier in Scheme 31.

A mixture of the glycinal **138** and the methyl ester **137** in a solution of 1% acetic acid and methanol was treated with sodium cyanoborohydride. The ESI (+ve) mass spectrum of the ester **139** presented a peak for the protonated molecular ion at *m/z* 396. Deprotection of the methyl ester **139** with lithium hydroxide yielded the acid **140**. The ^{1}H NMR spectrum of the acid **140** did not display the singlet resonance corresponding to the protons of the methyl ester functionality, confirming the reaction was successful. The ESI (-ve) mass spectrum of the acid **140** displayed a peak at *m/z* 380 corresponding to the deprotonated molecular ion. Removal of the *t*-butoxycarbonyl group from the amide **140** was achieved *via* treatment with 4M hydrochloric acid in 1,4–dioxane to yield the diamine **135**. The ^{1}H NMR spectrum of the diamine **135** did not display any evidence of the singlet resonance corresponding to the protons of a *t*-butoxycarbonyl group. Further evidence for the successful synthesis of the diamine **135** was provided by the high resolution ESI (+ve) mass spectrum which presented a peak for the protonated molecular ion at *m/z* 282.1462.

8.2.2 (*S*)-N^2-Benzyloxycarbonyl-2,7-diamino-4-azaheptanoic acid (136)

The synthesis of the azaheptanoic acid **136** initially proceeded following the protocol used in the preparation of the azahexanoic acid **135**. As with the diamine **135**, the

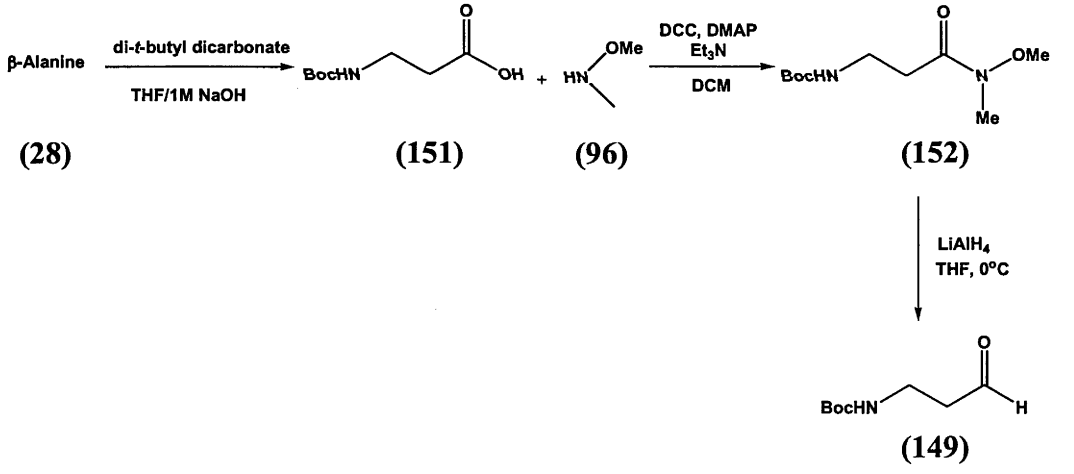
crucial step in the synthesis of the diamine **136**, was the reductive alkylation reaction between the ester **137** and the aldehyde **149** to give the azalysinate **150** (Scheme 35).



(137) **(149)** **(150)**

Scheme 35. Synthesis of the azalysinate **150** from the ester **137** and the aldehyde **149**

The β-alaninal **149** was synthesized following the experimental protocol employed in the preparation of the glycinal **138** from glycine **146** expressed above (Scheme 36).



(28) **(151)** **(96)** **(152)**

(149)

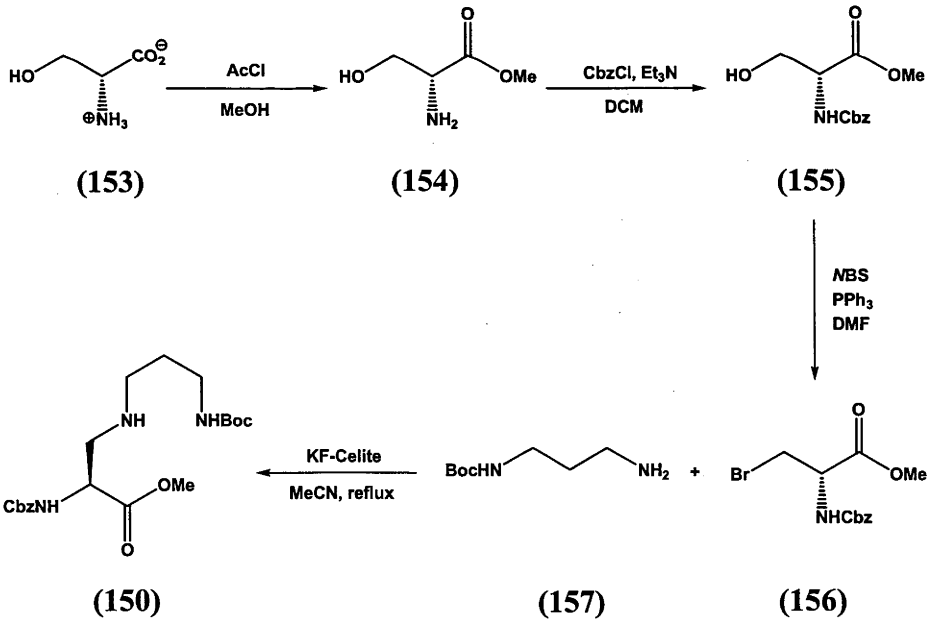
Scheme 36. Synthesis of *N*-(*t*-butoxycarbonyl)-β-alaninal **(149)**

The carbamate **151** was prepared from β-alanine **(28)** with di-*t*-butyl dicarbonate. A peak corresponding to the sodiated molecular ion was observed at *m/z* 212 in the ESI (+ve) mass spectrum of the carbamate **151**. The acid **151** was coupled with the amine **96** using *N,N'*-dicyclohexylcarbodiimide to give the Weinreb amide **152**. The ESI (+ve) mass spectrum of the amide **152** presented a peak at *m/z* 255

corresponding to the sodiated molecular ion. The amide **152** was reduced to give the aldehyde **149** using lithium aluminium hydride. The ^{1}H NMR spectrum of the aldehyde **149** displayed resonances that correspond with the literature data.[108]

The reductive alkylation reaction between the methyl ester **137** and the aldehyde **149** was carried out according to the experimental protocol utilised in the synthesis of the ester **139**, as outlined earlier in Scheme 35.

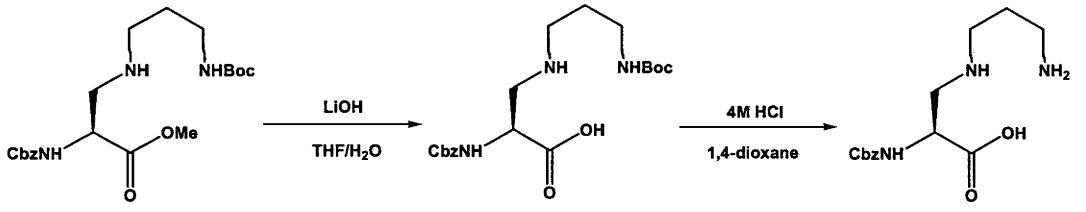
A peak for the sodiated molecular ion was observed at *m/z* 422 in the ESI (+ve) mass spectrum of the ester **150**, confirming its successful synthesis. However, the ^{1}H NMR spectrum of the product mixture indicated the presence of a large amount of impurities and the crude yield of the reaction was less than 10 percent, which was equivalent to 15 milligrams; this made the isolation of the ester **150** and the subsequent reactions to synthesize the diamine **136** impractical. Consequently, an attempt was made to prepare the azalysinate **150** following the reported procedure for its synthesis (Scheme 37).[109]



Scheme 37. Synthesis of methyl-(*S*)-N^2-benzyloxycarbonyl-N^7-(*t*-butoxycarbonyl)-2,7-diamino-4-azaheptanoate **(150)**

The ester **154** was obtained by heating to reflux a mixture of (*S*)-serine **(153)** in methanol with acetyl chloride. The ^{1}H NMR spectrum of the ester **154** displayed resonances which correspond well with data reported in the literature.[110] The instalment of the benzyloxy group to the amine **154** proceeded with the addition of triethylamine and benzyl chloroformate to a solution of the amine **154**. The ESI (+ve) mass spectrum of the carbamate **155** exhibited the sodiated molecular ion at *m/z* 276 as the base peak. Conversion of the alcohol **155** to the bromide **156** was carried out in the presence of triphenylphosphine and *N*-bromosuccinimide. The ^{1}H NMR spectrum of the bromide **156** displayed resonances which correspond well with data reported in the literature.[109] A mixture of the bromide **156**, potassium fluoride:celite and *N*-(*t*-butoxycarbonyl)-1,3-diaminopropane **(157)** was heated at reflux to give the azalysinate **150**. The ESI (+ve) mass spectrum of azalysinate **150** displayed a peak for the sodiated molecular ion at *m/z* 410.

The two remaining steps in the synthesis of the diamine **136** from the azalysinate **150** were carried out following the reaction sequence depicted in Scheme 38.



(150) **(158)** **(136)**

Scheme 38. Synthesis of (*S*)-N^2-benzyloxycarbonyl-2,7-diamino-4-azaheptanoic acid **(136)**

Hydrolysis of the ester **150** with lithium hydroxide gave the acid **158**. The high resolution ESI (+ve) mass spectrum of the acid **158** displayed a peak corresponding to the protonated molecular ion at *m/z* 396.2135. The successful synthesis of the azaheptanoic acid **136** proceeded *via* treatment of the amide **158** with 4M hydrochloric acid in 1,4-dioxane. The high resolution ESI (+ve) mass spectrum of the diamine **136** exhibited a peak for the protonated molecular ion at *m/z* 296.1618.

The esters **139** and **150** were prepared following the literature methods employed in the syntheses of the same compounds, which were reported to be homochiral.[105,109] The deprotection reaction conditions employed in the preparation the diamines **135** and **136** from the esters **139** and **150**, respectively, are not expected to result in racemisation as peptidic systems exposed to similar conditions are reported to be homochiral.[111] Thus both the diamines **135** and **136** are assumed to be homochiral. The optical rotations of the azahexanoic acid **135** and the azaheptanoic acid **136** were not determined as their syntheses were conducted on only a small quantity of starting materials.

8.3 Interactions of the diamines 135 and 136 with PAM

The binding affinities of the diamines **135** and **136** with PAM were established following the protocol described in Chapter Two. The azahexanoic acid **135** inhibited the activity of the enzyme, with a determined IC_{50} value of 1.9 mM.

Interestingly, the diamine **136** behaved in a similar manner to the two β-oxa acids **67** and **68** discussed in Chapter Four. According to the results from the binding studies, the percentage of the substrate **22** turned over increased as the concentration of the diamine **136** in the sample increased. At concentrations of 0.1, 1.0 and 5.0 mM of the azaheptanoic acid **136**, the yields of the amidated product **23** were greater by factors of 1.3, 2.8 and 7.8 respectively, relative to the sample with no diamine **136** present.

As suggested with the two β-oxa acids **67** and **68**, the effect on the enzyme's activity displayed by the diamine **136** may also be due to an activating allosteric effect imposed on PAM by the compound, which resulted in modifications to the active site that improved the binding of the substrate **22**.

The poor binding affinity exhibited by the diamine **135** may be attributed to the position of the diamino ligand, as substituents at the α-carbon of glycine-based substrates have been established to disrupt binding with PAM.[99]

In addition, the poor binding affinity displayed by the diamine **135** may be associated with the ability of both amino groups to coordinate to the enzyme's copper. As illustrated in the PHM-substrate crystal structure in Figure 1, the two coppers (Cu_H and Cu_M) are held in place by coordination with residues within the enzyme active site. Copper H (Cu_H) is coordinated with the nitrogens of three histidine residues (His107, His108 and His172), while Copper M (Cu_M) is coordinated to the sulfur of the methionine (Met314) and the nitrogens of two histidine (His242 and His244) residues. In order for the amino groups of the diamine **135** to effectively coordinate to either one of the coppers in the enzyme active site, at least one of the original copper coordinating ligands will have to be displaced. This may not have been possible as both sulfur-containing substituents and imidazole groups have been established to coordinate effectively with copper.[112,113]

In summary, the poor binding affinity displayed by the diamine **135** may be affected by a number to factors including the position of the diamino substituent, as well as the ability of the amino groups of the substituent to coordinate with the copper in the enzyme active site. In contrast, the diamine **136** appeared to act as an activator of the enzyme, by inducing the production of the amidated product **23**. This suggests that the diamine **136** may also be useful in the development of therapeutic remedies for conditions associated with low hormone levels.

Chapter Nine

Conclusions

The results presented in this thesis have shown that binding affinity with PAM is influenced by a number of factors including the presence and absence of particular functional groups, and the relationships between the various features of a compound with the recognition points within the enzyme active site.

The results from the preliminary investigation into the effects of recognition features on binding affinity with PAM established that the incorporation of a hydrophobic group or a sulfur containing substituent was beneficial towards preserving binding affinity with the enzyme. The high binding affinities to PAM observed with the compounds possessing aromatic or aliphatic groups are in agreement with the presence of a hydrophobic pocket within the enzyme's active site, observed in the crystal structure of a PHM-substrate complex shown in Figure 1.[9] The hydrophobic moiety of a compound is likely to reside in the hydrophobic pocket, enhancing the compound's binding affinity to the enzyme. The presence of copper in the enzyme active site was also confirmed in the crystal structure in Figure 1 and the high binding affinity observed with the sulfur containing compounds to PAM is likely to be due to the coordination of the sulfur to the enzyme's copper. As a consequence of the results from this section of work, compounds investigated in subsequent sections of the thesis all possessed either a hydrophobic moiety or a copper coordinating group.

In addition, the results presented in Chapter Two of the Results and Discussion established the importance of the distance between key substrate recognition features such as a free carboxyl group, the hydrophobic substituent and the amido moiety in maintaining high binding affinity with the enzyme. Deviation from the desired configuration preferred for tight binding with PAM led to a loss in binding affinity, as observed with the β-alanyl derivatives in contrast to the analogous acylglycines.

The results described in Chapter Three of the Results and Discussion highlighted the importance of a compound's geometry in contributing to binding affinity to PAM. While both glycolic acid derivatives and γ-keto acids were established to be inhibitors of PAM, the glycolic acid derivatives displayed significantly higher binding affinity to the enzyme. The binding affinities displayed by the glycolic acid derivatives were comparable to those of the corresponding acylglycines. The geometric configurations adopted by the two classes of compounds may closely mimic the configuration desired by PAM for tight binding. Given the effective inhibition of PAM displayed by the glycolic acid derivatives, investigations into their *in vivo* effects have been taking place. The results from the preliminary experiments showed that the two glycolic acid derivatives **58** and **59** actively reduced the levels of the peptide hormone Substance P produced by rat DRG cells.[19] Future work will involve the development of more potent inhibitors and testing the effectiveness of those compounds in cell-based assays and potentially in animal and human models.

The results from the research described in Chapter Four of the Results and Discussion highlight the importance of the presence of the ester carbonyl group of glycolic acid derivatives in preserving binding affinity with PAM. The β-oxa acid **67** displayed binding that was by over two orders of magnitude less strong than that observed with the analogous glycolic acid derivative **55**. In contrast, the two β-oxa acids **68** and **69** appeared to enhance the activity of PAM. Future work may concentrate on the elucidation of the interactions of the two β-oxa acids **68** and **69** with PAM that are resulting in the observed activation effect. Furthermore, future work may also involve the development of analogues of the two β-oxa acids **68** and **69**, which may be useful in the development of drug therapies to treat conditions associated with low hormone levels.

The results from the research described in Chapter Five of the Results and Discussion indicate that the high binding affinity observed with the α,β-unsaturated γ-keto acid **80** may be attributed to the similarities in geometry of the olefinic carbon adjacent to the carboxyl group in the acid to the geometry of the α-carbon centred radical formed from the corresponding acylglycine **13**. Alternatively, as the acrylic

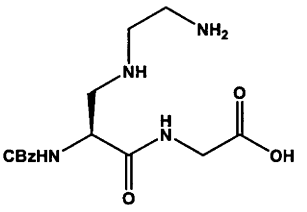
acid **80** has been established as an inactivator of PAM,[14] the tight binding displayed by the acid may be related to the mechanism of enzyme inactivation. Nevertheless, the enhanced binding affinity observed with the acid **80** could not be generalised to other compounds in its class. The leucine derivative **81** exhibited binding affinity which was by around one order of magnitude less than that observed with the corresponding acylglycine **14** and glycolic acid derivative **56**.

The results presented in Chapter Six of the Results and Discussion, established that the combination of two features - a hydrophobic moiety and a *trans*-β,γ-unsaturated acid - which have been determined to preserve binding affinity with PAM produced two compounds **93** and **94** that were effective inhibitors of PAM. The two acids **93** and **94** were the most effective inhibitors of PAM studied as part of the research conducted for this thesis. Given the effective inhibition of PAM displayed by *trans*-β,γ-unsaturated acids, future work may focus on peptidic derivatives of the acids more closely resembling particular peptide prohormones, which may be useful in the development of therapeutic agents that can be administered to treat conditions linked with the over production of peptide hormones.

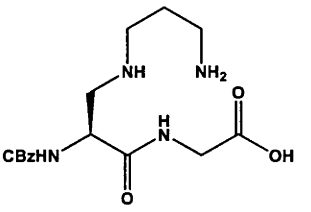
The results described in Chapter Seven of the Results and Discussion highlighted the significance of the thio ether substituent in preserving binding affinity with the enzyme. As indicated by the differences in binding affinities observed between the α-alkylated dipeptides **124** and **122**, the binding affinity of the dipeptide bearing a copper coordinating functionality **124** was not affected to the extent of the corresponding derivative with a non copper-binding substituent **122**. The poor binding affinity of the methionyl glycolic acid **125** to PAM also highlighted the significance of the arrangement of a compound's functional groups with respect to key recognition features of the enzyme active site in influencing binding affinity to PAM, even in the presence of a copper-binding group which has been established to preserve binding affinity with the enzyme. Based on the results from this section of work, future studies may investigate analogues of peptide prohormones with a sulfur-containing group at the penultimate position, which may be useful towards the development of drug treatments for conditions associated with an excess of peptide hormones. A number of sulfur-containing compounds have been reported to inhibit

the production of peptide hormones including the benzyl ester **6** discussed in the Introduction section, which inhibited the production of Substance P, displaying an IC_{50} value of 3 μM.[39]

The results presented in Chapter Eight of the Results and Discussion section suggested that the poor binding affinity exhibited by the diamine **135** could be associated with the position of the diamino ligand, which may have prevented tight binding with the enzyme. It may therefore be of interest to compare the binding affinities of the compounds **159** and **160** to that of the diamines **135** and **136**. The compounds **159** and **160** are glycine-extended versions of the diamines **135** and **136**, respectively.



(159)



(160)

However, the compounds **159** and **160** are not anticipated to display tight binding with the enzyme, as the binding of the diamine **135** to PAM may also be affected by the ability of both the amino groups of the diamine **135** to coordinate with the enzyme's copper.

In contrast to the diamine **135**, the diamine **136** behaved in a similar manner to the two β-oxa acids **68** and **69** discussed in Chapter Four, and appeared to activate the enzyme's activity. As suggested with the two β-oxa acids **68** and **69**, future work may entail establishing the interactions between the diamine **136** and the enzyme, as well as in the development of derivatives of the diamine **136** which may also be useful towards the development of drug remedies to treat conditions associated with depleted levels of hormones.

In conclusion, the results from the research presented in this thesis have established the importance of the presence of a number of functional groups including a copper-

binding substituent and a hydrophobic moiety, as well as the compound's geometric configuration in preserving binding affinity with the enzyme. These results provide valuable information that can be used in the design of compounds to be investigated in future studies. In addition, the results also provide a number of possible leads towards the development of compounds which may be useful in treating conditions associated with an over-production of peptide hormones, as well as compounds that may potentially be used as therapeutic treatments for conditions linked with low hormone levels.

Chapter Ten

Experimental

10.1 General

Melting points were performed on a MPA 100 Optimelt Automated Melting Point System – Digital Image Processing Technology by Stanford Research Systems and on a Kofler hot-stage melting point apparatus and visualised with a Reichert microscope. ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini 300 and Inova 300 spectrometers. Chemical shifts are reported as δ in parts per million and coupling constants are reported as *J* values in Hz. The multiplicity of signals is abbreviated as follows: br = broad signal, d = doublet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dt = doublet of triplets, m = multiplet, q = quartet, s = singlet, t = triplet. Methanol-d_4 with an isotopic purity of 99.8% was purchased from Apollo Scientific Ltd. DMSO-d_6 with an isotopic purity of 99.9%, chloroform-*d* with an isotopic purity of 99.8% and D_2O with an isotopic purity of 99.8% were purchased from Cambridge Isotope Laboratories, Inc. Chloroform-*d* ($CDCl_3$) was referenced at δ 7.26 for ^{1}H and 77.0 for ^{13}C spectroscopy. D_2O was referenced at δ 4.79 for ^{1}H spectroscopy. DMSO-d_6 ($(CD_3)_2SO$) was referenced at δ 2.50 for ^{1}H and 39.5 for ^{13}C spectroscopy. Methanol-d_4 (CD_3OD) was referenced at δ = 3.31 for ^{1}H and 49.0 for ^{13}C spectroscopy.

Electron impact (EI) mass spectra were recorded using a VG AutoSpec M series sector (EBE) mass spectrometer. Electrospray ionisation (ESI) spectra were recorded on a VG Quattro II triple quadrupole mass spectrometer. Microanalyses were carried out on a Carlo Erba 1106 automatic analyser, by the Research School of Chemistry Microanalytical Service at the Australian National University. Optical rotations were determined using a Perkin Elmer 241 polarimeter.

Semi-preparative high performance liquid chromatography (HPLC) was conducted using a Waters 600 controller with a Waters Alliance 2996 photodiode array sampler, together with a Waters 717plus autosampler and a Waters fraction collector III. A Phenomenex Luna C18 5 μm column (250 x 10 mm) and a YMC-Pack ODS AQ C18 5 μm column (250 x 20 mm) were used in preparative separations. Analytical HPLC was carried out on a Waters Alliance 2695 separations Module, with a Waters 2996 photodiode array detector. A Waters Symmetry C18 5 μm column (250 x 4.6 mm) was used in analytical separations. Both HPLC systems were used in conjunction with a IBM data station, and the data were collected and processed with the Empower Pro–Empower 2 software.

Preparative flash chromatography was performed with Scharlau silica gel 60 (230-400 mesh ASTM). Visualisation was either by ultra-violet (UV) radiation or development in phosphomolybdic acid or potassium permanganate dips.

Recombinant solutions of peptidylglycine alpha-amidating enzyme (PAM) were purchased from Wako Pure Chemical Industries. Tripeptide (*R*)-Tyr-(*S*)-Val-Gly-OH **(22)** used in the enzyme assays was acquired from Bachem AG. (*R*)-Alanine, β-alanine, (*S*)-ascorbic acid, (*S*)-asparagine, bovine liver catalase, 6-bromohexanoic acid, *t*-butyl bromoacetate, diacetoxyiodobenzene, *N,N'*-dicyclohexylcarbodiimide, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide, dimethyl methylphosphonate, *N,O*-dimethylhydroxylamine, di-*t*-butyl dicarbonate, glycine, glycine methyl ester hydrochloride, 4M hydrochloric acid solution in 1,4-dioxane, (*S*)-leucine, lithium hydroxide monohydrate, 2-[*N*-morpholino]ethanesulfonic acid (MES) hydrate, (*S*)-methionine, methyltriphenylphosphonium chloride, 7-oxooctanoic acid, (*S*)-phenylalanine, potassium fluoride on celite (50% wt), (*S*)-serine, sodium thiomethoxide, (*S*)-tartaric acid, 3-(trimethylsilyl)propargyl bromide, Tween® 20 and (*S*)-valine were purchased from Sigma-Aldrich, Inc. Castro's reagent (benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate, BOP) was bought from Auspep Pty Ltd and *N*-(*t*-butoxycarbonyl)-1,3-diaminopropane was purchased from TCI America Organic Chemicals. All HPLC solvents were purchased from Labscan Asia Co., Ltd. Water was purified by a ELGA Purelab Classic UV water purification system.

10.2 Enzyme assay conditions and HPLC traces

PAM enzyme inhibition assays were performed with 2 μM copper sulfate, 0.1 mM substrate (*R*)-Tyr-(*S*)-Val-Gly **(22)**, 3 mM ascorbic acid, 100 μg/mL bovine liver catalase, 0.01% Tween® 20 and 1% ethanol, in the presence of nominated concentrations of inhibitors in 100 mM MES buffer at pH 6.6.

The assays were initiated by the addition of a 30 μL enzyme solution (3.75 μg of enzyme in a 550 μL MES buffer solution) to give a total volume of 60 μL and at a final enzyme concentration of 43.7 nmol L^{-1}. The assay mixtures were incubated at 37 °C for 1 hour. The assays were quenched by the addition of 30 μL of 1M sodium hydroxide, followed by the addition of 30 μL of 1M hydrochloric acid to neutralise the assay solutions. Two aliquots of 20 μL of each of the assay solutions were taken and diluted to 110 μL with Milli-Q water and the duplicate solutions were analysed by HPLC. The ratio of substrate (*R*)-Tyr-(*S*)-Val-Gly **(22)** to the product (*R*)-Tyr-(*S*)-Val-NH_2 **(23)** was identified by integration of percentage peak areas appearing on HPLC traces, which determined the amount of substrate **22** processed at specific inhibitor concentrations.

The following Figures 4, 5, 6, and 7 are representative HPLC traces from enzyme assays of the compound **80** at concentrations 0, 0.1, 1.0, 10 μM, respectively. The peak at t_R 8.7 minutes corresponds to the amide product **23** and the peak observed at t_R 9.1 minutes corresponds to the starting material **22**.

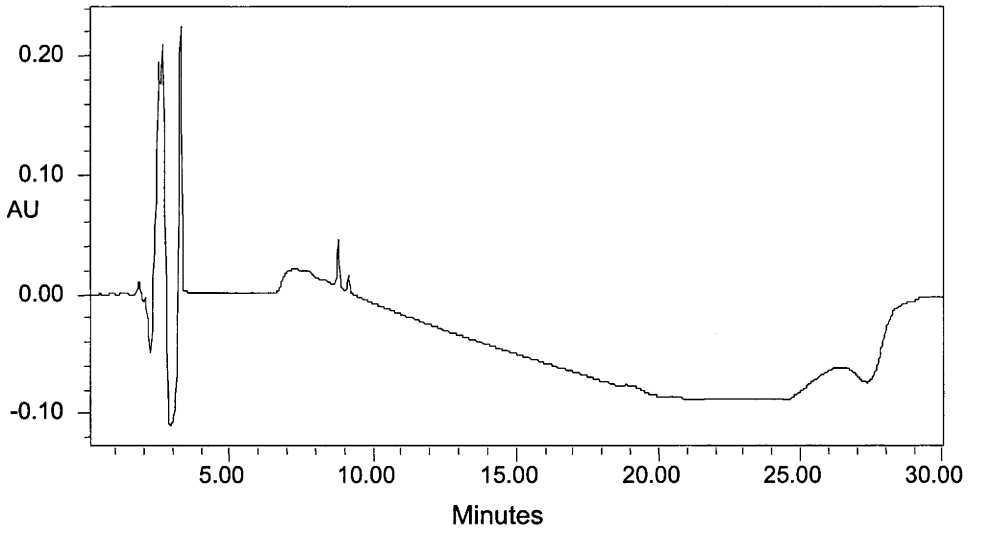


Figure 4. HPLC trace of inhibitor **80** at concentration 0 μM

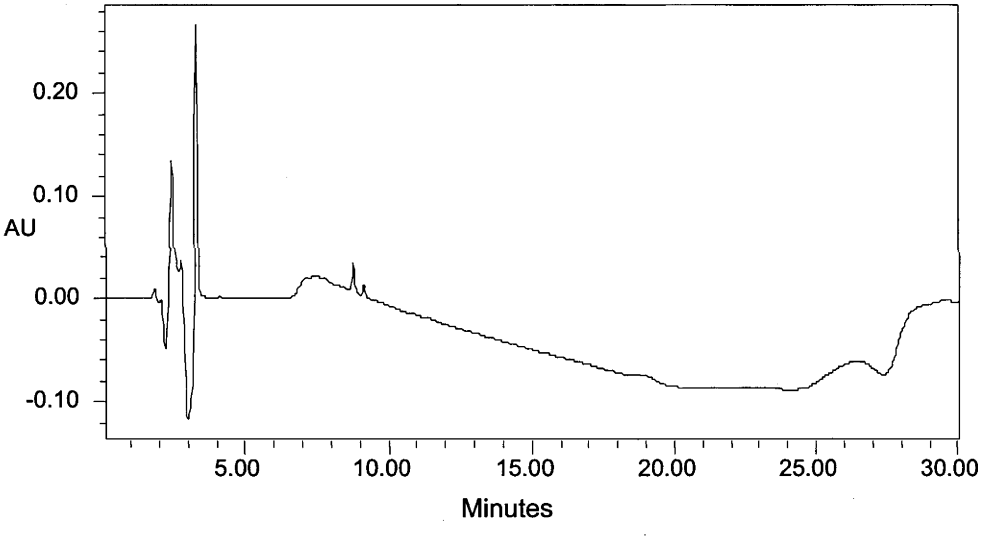


Figure 5. HPLC trace of inhibitor **80** at concentration 0.1 μM

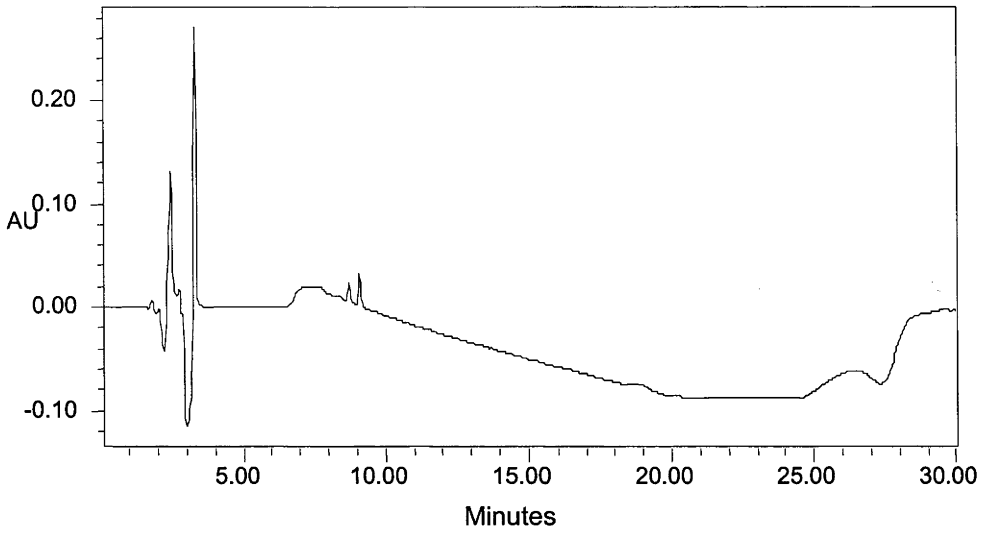


Figure 6. HPLC trace of inhibitor **80** at concentration 1.0 μM

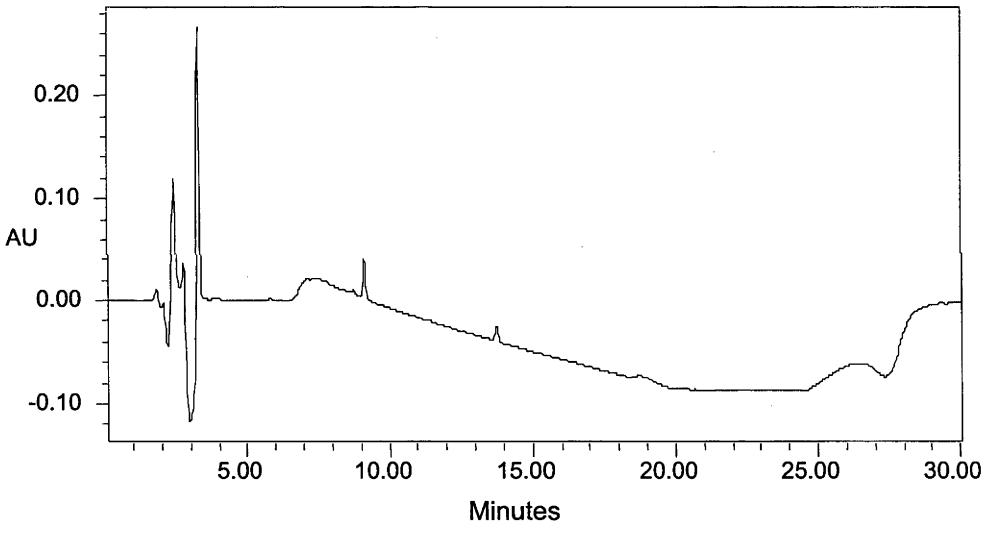


Figure 7. HPLC trace of inhibitor **80** at concentration 10 μM

10.3 Inhibitory concentration (IC_{50}) value determinations

Integration of the peaks of the substrate **22** and the amidated product **23** in the HPLC traces were used to determine the percentage of substrate turnover in the enzyme assays.

A Dixon plot is generated from plotting the reciprocal of the average rate of turnover of substrate against the concentration of the inhibitor. The Dixon plot for the PAM assay with the inhibitor **80** is provided in Figure 8.

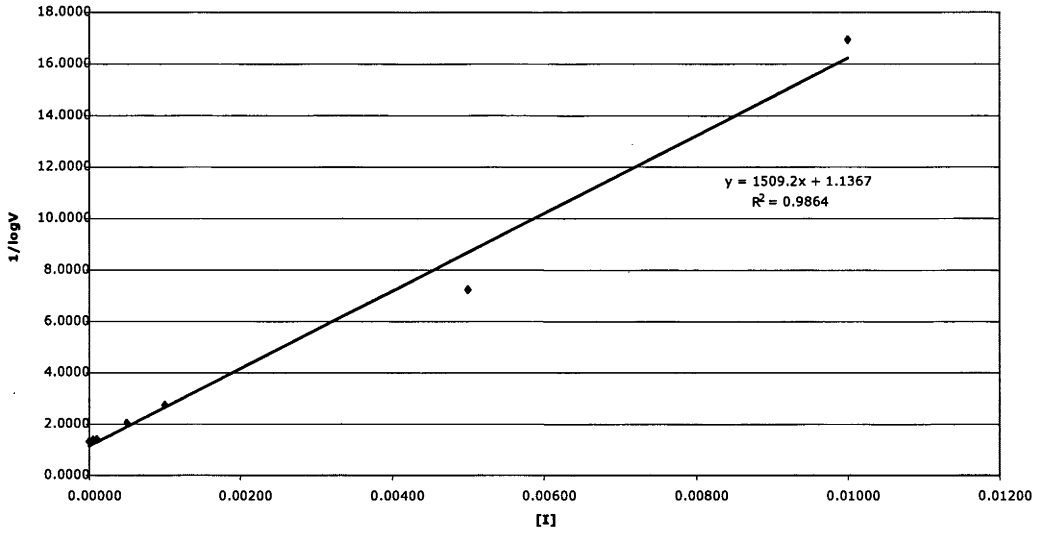


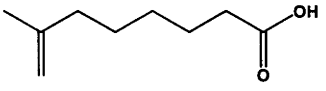
Figure 8. Dixon plot for the activity of the inhibitor **80** against PAM

Using the equation generated from the line of best fit illustrated in Figure 8, the IC_{50} of an inhibitor is determined as the value of –x when y = 0. The IC_{50} value of the inhibitor **80** was established to be 0.0007 mM.

10.4 Synthetic Procedures

10.4.1 Experimental for Chapter Two

7-methyloct-7-enoic acid (20)

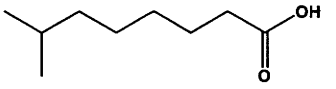


(20)

n-Butyl lithium (1.6M in hexanes, 6.2 mL, 9.9 mmol) was added dropwise to a stirred suspension of methyltriphenylphosphonium chloride **(18)** (3.0 g, 9.7 mmol) in tetrahydrofuran at –78 °C under a nitrogen atmosphere. The mixture was stirred at –78 °C for 45 minutes, then treated with a solution of 7-oxooctanoic acid **(19)** (0.8 g, 4.7 mmol) in tetrahydrofuran (10 mL). The mixture was warmed to room temperature and stirred for 90 minutes. The reaction was quenched by the addition of water (15 mL). The solution was extracted with diethyl ether (3 x 50 mL) and the combined organic extracts were washed with 0.1M sodium hydroxide solution (3 x 35 mL). The combined aqueous extracts were acidified to pH 2 with 1M hydrochloric acid then washed with diethyl ether (3 x 50 mL). The organic extracts were dried, filtered and concentrated under reduced pressure to give a colourless oil. The residue was subjected to flash silica gel column chromatography, eluting with hexanes/ethyl acetate (9:1 to 3:2) to give the title compound **20** (0.4 g, 52%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.23-1.50 (m, 5H), 1.64 (m, 4H), 2.09 (t, J = 7.4 Hz, 2H), 2.36 (t, J = 7.5 Hz, 2H), 4.62 (s, 1H), 4.69 (s, 1H). **MS (ESI)** (-ve): *m/z* 155 ($M\text{-}H^+$, 100%). **HRMS (ESI)** calcd. for $C_9H_{15}O_2$ $[M\text{-}H]^+$ *m/z* 155.1072, found 155.1065. The ^{1}H NMR spectral data obtained are consistent with reported information.[53]

7-methyl-7-octanoic acid (16)

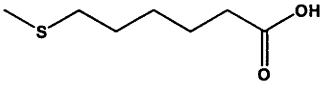


(16)

7-Methyl-oct-7-enoic acid **(20)** (70 mg, 0.5 mmol) was added to a suspension of activated palladium on carbon (10%, 5 mg) in ethanol (5 mL) under a hydrogen atmosphere. The mixture was stirred overnight at room temperature then filtered through a pad of celite. The filtrate was concentrated under reduced pressure to give a yellow oil. The residue subjected to Kugelrohr vacuum distillation to give the title compound **16** (40 mg, 56%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (d, J = 8.4 Hz, 6H), 1.14-1.19 (m, 1H), 1.28-1.35 (m, 5H), 1.47-1.68 (m, 3H), 2.35 (t, J = 7.5 Hz, 2H). **MS (ESI)** (-ve): *m/z* 157 ($M\text{-}H^+$, 100%). **HRMS (ESI)** calcd. for $C_9H_{17}O_2$ $[M\text{-}H]^+$ *m/z* 157.1228, found 157.1221. The ^{1}H NMR spectral data collected are consistent with literature data.[53]

6-Methylthiohexanoic acid (17)

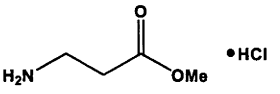


(17)

A mixture of 6-bromohexanoic acid **(21)** (1.9 g, 10 mmol) and sodium thiomethoxide (1.4 g, 20 mmol) in methanol (10 mL) was stirred and heated to reflux for 6 hours. The mixture was cooled to 25 °C, diluted with water (10 mL) and acidified to pH 1 with 1M hydrochloric acid. The solution was washed with chloroform (3 x 50 mL) and the combined organic extracts were dried, filtered and solvent evaporation under reduced pressure gave a yellow oil. The oil was subjected to distillation on a Kugelrohr instrument to yield the title compound **17** (1.2 g, 72%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.37-1.48 (m, 2H), 1.54-1.69 (m, 4H), 2.07 (s, 3H), 2.35 (t, J = 7.5 Hz, 2H), 2.48 (t, J = 7.2 Hz, 2H). **MS (EI)**: *m/z* 162 (M^+, 53%). **HRMS (EI)** calcd. for $C_7H_{14}O_2S$ $[M]^{+\bullet}$ *m/z* 162.0715, found 162.0714. The acquired ^{1}H NMR spectral information are consistent with reported data.[54]

β-Alanine methyl ester hydrochloride (29)

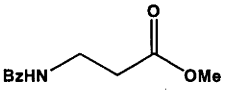


(29)

Thionyl chloride (3.3 mL, 44.9 mmol) was added dropwise to a suspension of β-alanine **(28)** (1.0 g, 11.2 mmol) in methanol (30 mL). The mixture was stirred overnight at room temperature before it was concentrated under reduced pressure to yield a white solid. The solid was redissolved in methanol (20 mL) and evaporated under reduced pressure twice to remove excess hydrochloric acid, to give the title compound **29** (1.0 g, 64%) as a white solid.

Mp. 109-110 °C (lit.,[55] 110-112 °C). **^{1}H NMR** (300 MHz, D_2O): δ 2.66 (t, J = 6.4 Hz, 2H), 3.13 (t, J = 6.4 Hz, 2H), 3.59 (s, 3H). **MS (ESI)** (+ve): *m/z* 104 ($M+H^+$, 100%). **HRMS (EI)** calcd. for $C_4H_9NO_2$ $[M]^{+\bullet}$ *m/z* 103.0633, found 103.0632. The ^{1}H NMR spectral characteristics are consistent with literature values.[55]

***N*-Benzoyl-β-alanine methyl ester (30)**



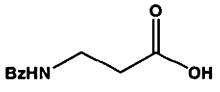
(30)

β-Alanine methyl ester hydrochloride **(29)** (1.0 g, 7.2 mmol) was dissolved in ethyl acetate (20 mL), to which a solution of sodium bicarbonate (1.2 g, 14.5 mmol) in

water (20 mL) and benzoyl chloride (1.3 mL, 10.7 mmol) were added. The mixture was stirred at room temperature overnight and then extracted with ethyl acetate (2 x 50 mL). The combined organic extracts were washed successively with saturated sodium bicarbonate solution (3 x 25 mL), 1M hydrochloric acid (3 x 25 mL), brine solution (3 x 25 mL) then dried and filtered. The filtrate was concentrated under reduced pressure to give a white solid. The solid was recrystallised from diethyl ether, to afford the title compound **30** (0.7 g, 47%) as white crystals.

Mp. 66-67 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 2.66 (t, J = 6.0 Hz, 2H), 3.70-3.76 (m, 2H), 3.71 (s, 3H), 6.89 (br s, 1H), 7.40-7.50 (m, 3H), 7.75-7.78 (m, 2H). **MS (ESI)** (+ve): *m/z* 230 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{11}H_{13}NO_3Na$ $[M+Na]^+$ *m/z* 230.0793, found 230.0791. The ^{1}H NMR spectral information are consistent with previously reported data.[56]

***N*-Benzoyl-β-alanine (24)**



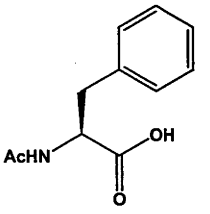
(24)

A solution of lithium hydroxide (50 mg, 2.0 mmol) in water (15 mL) was added to a solution of *N*-benzoyl-β-alanine methyl ester **(30)** (300 mg, 1.5 mmol) in tetrahydrofuran (15 mL). The mixture was stirred overnight at room temperature and acidified to pH 3 with 1M hydrochloric acid. The solution was extracted with ethyl acetate (3 x 40 mL) and the combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **24** (260 mg, 90%) as a white solid.

Mp. 117-118 °C (lit.,[114] 117-118 °C). **^{1}H NMR** (300 MHz, CD_3OD): δ 2.63 (t, J = 6.9 Hz, 2H), 3.62 (t, J = 6.9 Hz, 2H), 7.42-7.60 (m, 2H), 7.77-7.81 (m, 3H). **MS (ESI)** (-ve): *m/z* 192 ($M-H^+$, 79%). **HRMS (ESI)** calcd. for $C_{10}H_{10}NO_3$ $[M-H]^+$ *m/z*

192.0661, found 192.0654. The acquired ^{1}H NMR spectral data are consistent with data previously reported.[57]

(*S*)-*N*-Acetylphenylalanine (32)

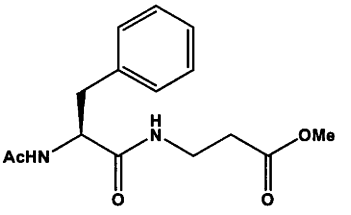


(32)

Triethylamine (5.9 mL, 42.4 mmol) and acetic anhydride (8.6 mL, 90.8 mmol) were added to a solution of (*S*)-phenylalanine **(31)** (5.0 g, 30.2 mmol) in water (350 mL). The mixture was stirred at room temperature overnight then acidified to pH 1 with 1M hydrochloric acid and extracted with ethyl acetate (3 x 200 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give a white solid, which was recrystallised from ethanol to give the title compound **32** (3.2 g, 51%) as a white solid.

Mp. 171-172 °C (lit.,[58] 171-172 °C). **^{1}H NMR** (300 MHz, $CDCl_3$/$(CD_3)_2SO$): δ 1.78 (s, 3H), 2.89 (dd, J = 6.6, 13.8 Hz, 1H), 3.01 (dd, J = 5.4, 13.8 Hz 1H), 4.68 (dd, J = 5.1, 5.1 Hz, 1H), 6.56 (br d, J = 7.5 Hz, 1H), 6.99-7.12 (m, 5H). **MS (ESI)** (+ve): *m/z* 208 ($M+H^+$, 100%). **HRMS (EI)** calcd. for $C_{11}H_{13}NO_3$ $[M]^{+\bullet}$ *m/z* 207.0895, found 207.0898. The obtained ^{1}H NMR spectral data are consistent with values reported in literature.[115]

(*S*)-*N*-Acetylphenylalanyl-β-alanine methyl ester (33)

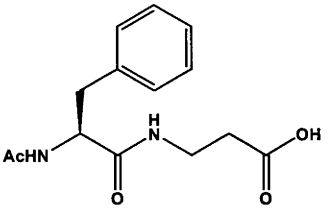


(33)

β-Alanine methyl ester hydrochloride **(29)** (0.5 g, 3.6 mmol), BOP reagent (2.4 g, 5.3 mmol), and *N,N*-diisopropylethylamine (3.4 mL, 19.4 mmol) were added to a solution of (*S*)-*N*-acetylphenylalanine **(32)** (1.1 g, 5.3 mmol) in dichloromethane (15 mL). The mixture was stirred overnight at room temperature then partitioned between brine (100 mL) and ethyl acetate (100 mL). The organic extract was washed with 0.3M citric acid (3 x 40 mL), saturated sodium bicarbonate solution (3 x 40 mL) and brine solution (3 x 40 mL) then dried and filtered. The filtrate was concentrated under reduced pressure to give the title compound **33** (0.3 g, 29%) as a light yellow solid.

Mp. 146-147 °C. $[\alpha]^{23}_{D}$ +2.0 (*c* 0.5, EtOH). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.99 (s, CH_3), 2.24-2.47 (m, CH_2), 2.96 (dd, J = 8.4, 13.5 Hz, 1H), 3.07 (dd, J = 6.3, 13.5 Hz, 1H), 3.26-3.38 (m, 1H), 3.41-3.52 (m, 1H), 3.64 (s, 3H), 4.52-4.59 (m, 1H), 6.11 (br s, 1H), 6.18 (br d, J = 6.9 Hz, 1H), 7.18-7.32 (m, 5H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 23.8, 34.2, 35.4, 39.5, 52.5, 55.4, 127.6, 129.3, 129.9, 137.2, 170.7, 171.5, 173.1. **MS (ESI)** (+ve): *m/z* 293 ($M+H^+$, 67%). **HRMS (EI)** calcd. for $C_{15}H_{20}N_2O_4$ [$M^{+\bullet}$] *m/z* 292.1423, found 292.1426.

(*S*)-*N*-Acetylphenylalanyl-β-alanine (25)

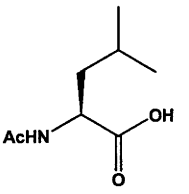


(25)

(*S*)-*N*-Acetylphenylalanyl-β-alanine methyl ester **(33)** (100 mg, 0.3 mmol) was dissolved in tetrahydrofuran (10 mL) to which a solution of lithium hydroxide (11 mg, 0.5 mmol) in water (10 mL) was added. The resulting mixture was stirred at room temperature for 2 hours, acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (3 x 30 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give a pink solid, which was recrystallised from chloroform to yield the title compound **25** (32 mg, 34%) as a white solid.

Mp. 158-159 °C. **^{1}H NMR** (300 MHz, $(CD_3)_2SO$): δ 1.73 (s, 3H), 2.30 (t, J = 6.9 Hz, 2H), 2.69 (dd, J = 9.9, 13.5 Hz, 1H), 2.91 (dd, J = 4.8, 13.5 Hz, 1H), 3.14-3.30 (m, 2H), 4.38-4.43 (m, 1H), 7.14-7.27 (m, 5H), 8.04-8.11 (m, 2H). **MS (ESI)** (-ve): *m/z* 277 ($M\text{-}H^+$, 14%). **HRMS (EI)** calcd. for $C_{14}H_{18}N_2O_4$ $[M]^{+\bullet}$ *m/z* 278.1267, found 278.1269. The ^{1}H NMR spectral characteristics are consistent with literature data.[59]

(*S*)-*N*-Acetylleucine (35)

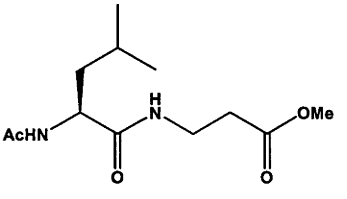


(35)

Triethylamine (7.5 mL, 53.4 mmol) and acetic anhydride (3.8 mL) were added to a suspension of (*S*)-leucine **(34)** (5.0 g, 38.1 mmol) in water (200 mL). The mixture was stirred overnight at room temperature, acidified to pH 1 with 2M hydrochloric acid and extracted with ethyl acetate (3 x 200 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give a white solid, which was recrystallised from ethanol/hexanes to give the title compound **35** (2.3 g, 35%) as white crystals.

Mp. 189-190 °C (lit.,[61] 189-190 °C). **^{1}H NMR** (300 MHz, CD_3OD): δ 0.92 (d, J = 6.3 Hz, 3H), 0.96 (d, J = 6.6 Hz, 3H), 1.58-1.78 (m, 3H), 1.98 (s, 3H), 4.38-4.42 (m, 1H). **^{13}C NMR** (75 MHz, CD_3OD): δ 21.7, 22.3, 23.4, 26.0, 41.5, 52.1, 173.4, 176.1. **MS (ESI)** (+ve): *m/z* 196 ($M+Na^+$, 70%). **HRMS (EI)** calcd. for $C_8H_{15}NO_3$ $[M]^{+\bullet}$ *m/z* 173.1052, found 173.1053. **Anal.** Calcd. for $C_8H_{15}NO_3$: C, 55.47; H, 8.73; N, 8.09. Found: C, 55.46; H, 8.35; N, 7.83%.

(*S*)-*N*-Acetylleucyl-β-alanine methyl ester (36)



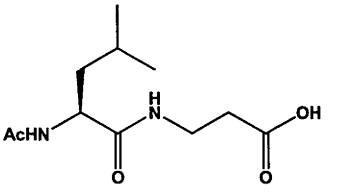
(36)

A suspension of β-alanine methyl ester hydrochloride **(29)** (1.0 g, 7.2 mmol) in *N,N*-dimethylformamide (50 mL) was treated with *N,N*-diisopropylethylamine (1.7 mL,

9.7 mmol) and the mixture was stirred at room temperature for 5 minutes under a nitrogen atmosphere. (*S*)-*N*-Acetylleucine **(35)** (1.8 g, 10.7 mmol), BOP reagent (4.7 g, 10.7 mmol) and *N,N*-diisopropylethylamine (5.1 mL, 29.1 mmol) were then added to the mixture, which was stirred overnight at room temperature. The mixture was treated with brine solution (100 mL) and extracted with ethyl acetate (4 x 50 mL). The combined organic extracts were washed successively with 0.3M citric acid (3 x 30 mL), saturated sodium bicarbonate solution (3 x 30 mL) and brine solution (3 x 30 mL). The solution was dried, filtered and concentrated under reduced pressure to give a yellow solid. The crude product was recrystallised from dichloromethane to yield the title compound **36** (0.2 g, 11%) as a white solid.

Mp. 125-126 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.92 (d, J = 1.8 Hz, 3H), 0.94 (d, J = 1.8 Hz, 3H), 1.47-1.64 (m, 3H), 2.00 (s, 3H), 2.54 (t, J = 6.1 Hz, 2H), 3.45-3.57 (m, 2H), 3.70 (s, 3H), 4.36-4.43 (m, 1H), 6.08 (br d, J = 8.1 Hz, 1H), 6.66 (br s, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 22.2, 22.7, 22.9, 23.0, 24.7, 33.7, 34.9, 41.3, 51.8, 170.3, 172.4, 172.5. **MS (ESI)** (+ve): *m/z* 281 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{12}H_{22}N_2O_4Na$ $[M+Na]^+$ *m/z* 281.1477, found 281.1476.

(*S*)-*N*-Acetylleucyl-β-alanine (26)



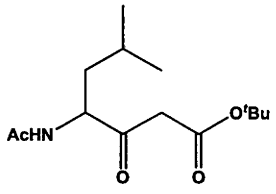
(26)

(*S*)-*N*-Acetylleucyl-β-alanine methyl ester **(36)** (50 mg, 0.2 mmol) was dissolved in tetrahydrofuran (5 mL), to which a solution of lithium hydroxide (10 mg, 0.3 mmol) in water (5 mL) was added. The resulting suspension was stirred overnight at room temperature. The mixture was acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (4 x 20 mL) then dried, filtered and concentrated under reduced pressure. The crude product was recrystallised from dichloromethane to yield the title compound **26** (19 mg, 41%) as a white solid.

Mp. 158-159 °C. **^{1}H NMR** (300 MHz, CD_3OD): δ 0.91 (d, J = 6.4 Hz, 3H), 0.94 (d, J = 6.4 Hz, 3H), 1.51-1.56 (m, 2H), 1.59-1.68 (m, 1H), 1.97 (s, 3H), 2.48 (t, J = 6.7 Hz, 2H), 3.37-3.44 (m, 2H), 4.29-4.34 (m, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 21.5, 22.1, 22.4, 24.4, 33.3, 34.8, 40.6, 51.5, 171.3, 172.8, 174.1. **MS (ESI)** (+ve): *m/z* 267 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{11}H_{20}N_2O_4Na$ $[M+Na]^+$ *m/z* 267.1321, found 267.1326.

10.4.2 Experimental for Chapter Three

_t_-Butyl (_N_-acetylleucyl)acetate (64)



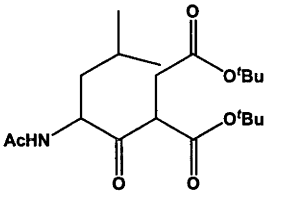
(64)

A suspension of (*S*)-*N*-acetylleucine **(35)** (2.0 g, 11.5 mmol) and 1,1'-carbonyldiimidazole (1.9 g, 11.5 mmol) in tetrahydrofuran (40 mL) was stirred at room temperature under a nitrogen atmosphere for 2 hours. It was then added dropwise to a solution that had been prepared from tetrahydrofuran (40 mL), diisopropylamine (9.7 mL, 69 mmol), *n*-butyl lithium (1.6M in hexanes, 29 mL, 46 mmol) and *t*-butyl acetate (6.2 mL, 46.0 mmol), maintained at -78 °C. The resulting mixture was stirred for 40 minutes, then 1M hydrochloric acid (100 mL) was added and the solution was warmed to room temperature. It was then extracted with ethyl acetate (3 x 100 mL) and the combined organic extracts were dried, filtered and concentrated under reduced pressure to give a yellow oil. The oil was chromatographed on flash silica gel, eluting with hexanes/ethyl acetate (2:1), to give the title compound **64** (1.6 g, 53%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91-0.95 (m, 6H), 1.45 (s, 9H), 1.63-1.67 (m, 3H), 2.01 (s, 3H), 3.41 (d, J = 15.6 Hz, 1H), 3.51 (d, J = 15.6 Hz, 1H), 4.69-4.76 (m, 1H), 6.08 (br d, J = 6.0 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 21.3, 22.7, 23.1, 24.6,

27.7, 39.2, 47.5, 56.7, 81.9, 166.1, 170.2, 202.9. **MS (ESI)** (+ve): *m/z* 294 ($M+Na^+$, 100%). **HRMS (EI)** calcd. for $C_{14}H_{25}NO_4$ $[M]^{+\bullet}$ *m/z* 272.1862, found 272.1851. **Anal.** Calcd. for $C_{14}H_{25}NO_4$: C, 61.97; H, 9.29; N, 5.16. Found: C, 61.71; H, 9.29; N, 5.18%.

t-Butyl 3-(*N*-acetylleucyl)-3-(*t*-butoxycarbonylmethyl)propionate (65)



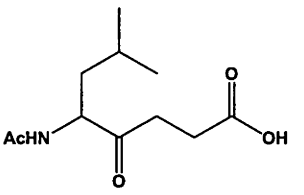
(65)

A solution of *t*-butyl (*N*-acetylleucyl)acetate **(64)** (1.0 g, 3.7 mmol) in tetrahydrofuran (15 mL) was added to a suspension of sodium hydride (100 mg, 5.5 mmol) in tetrahydrofuran (25 mL) maintained at 0 °C under a nitrogen atmosphere. After 10 minutes a solution of *t*-butyl bromoacetate (1.1 mL, 7.4 mmol) in dichloromethane (15 mL) was added dropwise to the mixture, which was then stirred overnight at room temperature. 1M Hydrochloric acid (100 mL) was then added and the solution was extracted with ethyl acetate (3 x 70 mL). The combined organic extracts were washed with brine solution (100 mL), dried and filtered through flash silica gel. The filtrate was concentrated under reduced pressure and the residue was chromatographed on flash silica gel, eluting with hexanes/ethyl acetate (4:1 to 1:1), to give a *ca.* 1:1 mixture of the diastereomers of the title compound **65** (500 mg, 36%) as colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86-0.93 (m, 12H), 1.34 (s, 9H), 1.35 (s, 9H), 1.36 (s, 9H), 1.39 (s, 9H), 1.55-1.69 (m, 6H), 1.94 (s, 3H), 1.96 (s, 3H), 2.61-2.81 (m, 4H), 4.00-4.08 (m, 2H), 4.74-4.81 (m, 1H), 4.96-5.04 (m, 1H), 6.22 (br d, J = 9.0 Hz, 1H), 6.35 (br d, J = 9.0 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 22.1, 22.3, 23.7, 23.8, 25.6, 25.8, 28.4, 28.7, 33.9, 34.7, 39.8, 41.5, 52.6, 53.1, 56.7, 57.0, 81.8, 81.9, 83.1, 83.9, 167.5, 167.9, 170.5, 170.9, 171.1, 171.4, 204.4, 205.5. **MS (ESI)** (+ve): *m/z* 386 ($M+H^+$, 100%). **HRMS (EI)** calcd. for $C_{20}H_{35}NO_6$ $[M]^{+\bullet}$ *m/z*

385.2464, found 385.2468. **Anal.** Calcd. for $C_{20}H_{35}NO_6$: C, 62.31; H, 9.15; N, 3.63. Found: C, 62.39; H, 8.94; N, 3.85%.

3-(*N*-Acetylleucyl)propionic acid (63)



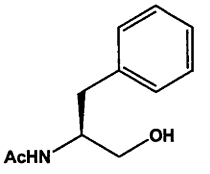
(63)

A solution of *t*-butyl 3-(*N*-acetylleucyl)-3-(*t*-butoxycarbonylmethyl)propionate **(65)** (100 mg, 0.3 mmol) and trifluoroacetic acid (3 mL) in dichloromethane (30 mL) was heated at reflux overnight, then it was cooled and concentrated under reduced pressure to yield the title compound **63** (50 mg, 84%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.94 (d, J = 6.0 Hz, 3H), 0.97 (d, J = 6.0 Hz, 3H), 1.39-1.49 (m, 1H), 1.59-1.65 (m, 2H), 2.11 (s, 3H), 2.66-2.70 (m, 2H), 2.81-2.89 (m, 2H), 4.71-4.76 (m, 1H), 6.56 (br d, J = 9.0 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 21.9, 23.1, 23.5, 25.2, 27.8, 34.9, 40.4, 56.9, 171.3, 177.0, 208.5. **MS (ESI)** (+ve): *m/z* 252 ($M+Na^+$, 100%). **HRMS (EI)** calcd. for $C_{11}H_{19}NO_4$ $[M]^{+\bullet}$ *m/z* 229.1314, found 229.1320. **Anal.** Calcd. for $C_{11}H_{19}NO_4$: C, 57.63; H, 8.35; N, 6.11. Found: C, 57.50; H, 8.11; N, 5.99%.

10.4.3 Experimental for Chapter Four

(*S*)-*N*-Acetylphenylalaninol (70)

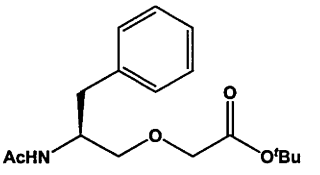


(70)

(*S*)-*N*-Acetylphenylalanine **(32)** (5.0 g, 24.1 mmol) was dissolved in tetrahydrofuran (20 mL), to which triethylamine (4.1 mL, 28.9 mmol) and ethyl chloroformate (2.3 mL, 24.1 mmol) were added. The resulting mixture was stirred at –5 °C under a nitrogen atmosphere for 30 minutes then filtered, and the precipitate was washed with tetrahydrofuran (30 mL). The filtrate was added to a suspension of sodium borohydride (2.3 g, 60.3 mmol) in water (20 mL). The resulting suspension was stirred at 0 °C for 4 hours, extracted with diethyl ether (2 x 50 mL) and dichloromethane (2 x 50 mL). The combined organic extracts were dried and filtered, and solvent evaporation gave the title compound **70** (4.4 g, 94%) as a white solid.

Mp. 99-100 °C (lit.,[65] 100-102 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.98 (s, 3H), 2.89 (d, J = 7.2 Hz, 2H), 3.59 (dd, J = 5.1, 11.1 Hz, 1H), 3.69 (dd, J = 3.6, 11.1 Hz, 1H), 4.14-4.19 (m, 1H), 6.11 (br d, J = 7.5 Hz, 1H), 7.30-7.36 (m, 5H). **MS (ESI)** (+ve): *m/z* 232 ($M+K^+$, 100%). The ^{1}H NMR spectral characteristics reported are consistent with literature data.[65]

(*S*)-*t*-Butyl-5-acetamido-3-oxa-6-phenylhexanoate (71)

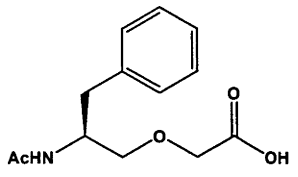


(71)

(*S*)-*N*-Acetylphenylalaninol **(70)** (0.8 g, 3.8 mmol) was added to suspension of sodium hydride (95%) (0.3 g, 12.5 mmol) in *N,N*-dimethylformamide (25 mL) under a nitrogen atmosphere. The mixture was stirred at room temperature for 2 hours then cooled to 0 °C. *t*-Butyl bromoacetate (2.3 mL, 15.5 mmol) was added to the mixture and stirred at 0 °C for 30 minutes, and stirred at room temperature for a further 2 hours. The mixture was diluted with saturated ammonium chloride solution (25 mL) and washed with ethyl acetate (100 mL). The organic extract was washed successively with saturated ammonium chloride solution (3 x 20 mL), saturated sodium bicarbonate solution (3 x 20 mL) and brine solution (3 x 20 mL), dried, filtered and concentrated under reduced pressure to give a yellow oil. The crude product was chromatographed on flash silica gel, eluting with hexanes/ethyl acetate (2:1 to 1:2) then ethyl acetate, to give the title compound **71** (0.4 g, 31%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.48 (s, 9H), 1.99 (s, 3H), 2.84 (dd, J = 9.0, 13.5 Hz, 1H), 3.00 (dd, J = 6.0, 13.5 Hz, 1H), 3.39 (dd, J = 3.3, 9.6 Hz, 1H), 3.47 (dd, J = 3.6, 9.6 Hz, 1H), 3.88 (d, J = 16.5 Hz, 1H), 4.03 (d, J = 16.5 Hz, 1H), 4.15-4.22 (m, 1H), 6.65 (br d, J = 6.3 Hz, 1H), 7.18-7.31 (m, 5H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 23.1, 27.9, 36.9, 50.6, 68.4, 71.2, 81.8, 126.1, 128.2, 129.2, 138.0, 169.7, 170.0. **MS (ESI)** (+ve): *m/z* 330 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{17}H_{25}NO_4Na$ $[M+Na]^+$ *m/z* 330.1681, found 330.1681.

(*S*)-5-Acetamido-3-oxa-6-phenylhexanoic acid (67)

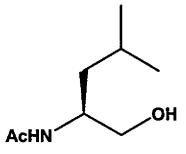


(67)

A solution of (*S*)-*t*-butyl-5-acetamido-3-oxa-6-phenylhexanoate **(71)** (250 mg, 0.8 mmol) and trifluoroacetic acid (3.5 mL) in dichloromethane (20 mL) was stirred overnight at room temperature then concentrated under reduced pressure to give a brown oil. The residue was subjected to preparative high performance liquid chromatography to give the title compound **67** (35 mg, 17%) as a colourless oil.

^{1}H NMR (300 MHz, CD_3OD): δ 1.94 (s, 3H), 2.82 (dd, J = 7.8, 13.8 Hz, 1H), 2.95 (dd, J = 7.2, 13.8 Hz, 1H), 3.52 (d, J = 4.2 Hz, 2H), 4.12 (s, 2H), 4.16-4.26 (m, 1H), 7.19-7.33 (m, 5H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 21.7, 36.5, 51.8, 67.6, 70.7, 126.8, 128.6, 129.1, 136.9, 173.5, 174.4. **MS (ESI)** (-ve): *m/z* 250 ($M\text{-}H^+$, 30%). **HRMS (ESI)** calcd. for $C_{13}H_{16}NO_4$ $[M\text{-}H]^+$ *m/z* 250.1079, found 250.1081. **HPLC**: t_R 22.2 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (75:25) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

(*S*)-*N*-Acetylleucinol (72)



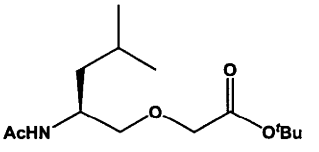
(72)

Triethylamine (3.9 mL, 27.7 mmol) and ethyl chloroformate (2.2 mL, 23.1 mmol) were added to a suspension of (*S*)-*N*-acetylleucine **(35)** (4.0 g, 23.1 mmol) in tetrahydrofuran (20 mL) at 0 °C under a nitrogen atmosphere. The mixture was stirred at –5 °C for 30 minutes, the white precipitate was filtered and washed with

tetrahydrofuran (30 mL). The filtrate was added to a cooled suspension of sodium borohydride (2.2 g, 57.7 mmol) in water (20 mL). The mixture was stirred at 0 °C for 4 hours then extracted with diethyl ether (2 x 50 mL) and dichloromethane (4 x 50 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure. The crude product was recrystallised from diethyl ether to yield the title compound **72** (1.8 g, 50%) as a white solid.

Mp. 66-67 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.91 (d, J = 3.5 Hz, 3H), 0.93 (d, J = 3.5 Hz, 3H), 1.27-1.42 (m, 2H), 1.56-1.67 (m, 1H), 2.01 (s, 3H), 3.51 (dd, J = 5.7, 11.1 Hz, 1H), 3.67 (dd, J = 3.6, 11.1 Hz, 1H), 3.98-4.07 (m, 1H), 6.44 (d, J = 8.4 Hz, 1H). **MS (ESI)** (+ve): *m/z* 160 ($M+H^+$, 88%). **HRMS (ESI)** calcd. for $C_8H_{18}NO_2$ $[M+H]^+$ *m/z* 160.1338, found 160.1334.

***(S)-t*-Butyl-5-acetamido-7-methyl-3-oxaoctanoate (73)**

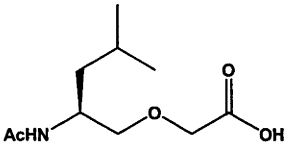


(73)

(*S*)-*N*-Acetylleucinol **(72)** (1.0 g, 6.3 mmol) was added to a suspension of sodium hydride (0.3 g, 13.8 mmol) in *N,N*-dimethylformamide (25 mL) under a nitrogen atmosphere. The mixture was stirred at room temperature for 2 hours and cooled to 0 °C. *t*-Butyl bromoacetate (3.7 mL, 25.2 mmol) was added to the mixture and stirred at 0 °C for 30 minutes, and stirred at room temperature for a further 2 hours. The solution was treated with saturated ammonium chloride solution (20 mL) and extracted with ethyl acetate (150 mL). The organic extract was washed with saturated ammonium chloride solution (3 x 30 mL), saturated sodium bicarbonate solution (3 x 30 mL) and brine solution (3 x 30 mL). The solution was dried, filtered and concentrated under reduced pressure. The residue was subjected to flash silica gel column chromatography, eluting with hexanes/ethyl acetate (2:1 to 1:1 to 1:2) to give the title compound **73** (0.4 g, 25%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (d, J = 5.4 Hz, 6H), 1.30-1.66 (m, 3H), 1.45 (s, 9H), 1.97 (s, 3H), 3.45 (dd, J = 3.3, 9.4 Hz, 1H), 3.56 (dd, J = 3.6, 9.4 Hz, 1H), 3.93 (d, J = 16.8 Hz, 1H), 3.97 (d, J = 16.8 Hz, 1H), 4.06-4.13 (m, 1H), 6.25 (br d, J = 8.1 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 22.2, 22.7, 23.1, 24.6, 27.8, 40.4, 47.0, 68.6, 73.5, 81.7, 169.7, 169.8. **MS (ESI)** (+ve): *m/z* 296 ($M+Na^+$, 82%). **HRMS (ESI)** calcd. for $C_{14}H_{27}NO_4Na$ $[M+Na]^+$ *m/z* 296.1838, found 296.1839.

(*S*)-5-Acetamido-7-methyl-3-oxaoctanoic acid (68)

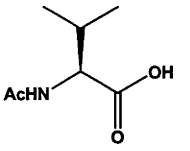


(68)

Trifluoroacetic acid (3 mL) was added to a suspension of *(S)-t*-butyl-5-acetamido-7-methyl-3-oxaoctanoate **(73)** (200 mg, 0.7 mmol) in dichloromethane (20 mL) and stirred overnight at room temperature. The mixture was concentrated under reduced pressure to give a brown oil. The residue was subjected to preparative high performance liquid chromatography to give the title compound **68** (25 mg, 16%) as a colourless oil.

^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 0.80 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H), 1.24-1.30 (m, 2H), 1.52-1.60 (m, 1H), 1.77 (s, 3H), 3.26-3.38 (m, 2H), 3.87-3.94 (m, 1H), 3.97 (s, 2H), 7.67 (br d, J = 8.4 Hz, 1H). **^{13}C NMR** (75 MHz, CD_3OD): δ 22.4, 22.7, 23.6, 25.9, 41.3, 52.2, 68.9, 74.7, 172.9, 174.2. **MS (ESI)** (-ve): *m/z* 216 ($M-H^+$, 73%). **HRMS (ESI)** calcd. for $C_{10}H_{18}NO_4$ $[M-H]^+$ *m/z* 216.1236, found 216.1235. **HPLC**: t_R 28.2 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (80:20) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

(*S*)-*N*-Acetylvaline (75)

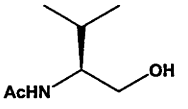


(75)

A suspension of (*S*)-valine **(74)** (5.0 g, 42.7 mmol) in water (150 mL) was treated with triethylamine (8.3 mL, 59.7 mmol) and acetic anhydride (12.1 mL, 128.0 mmol). The mixture was stirred overnight at room temperature, acidified to pH 1 with 2M hydrochloric acid and washed with ethyl acetate (3 x 100 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **75** (4.2 g, 62%) as a white solid.

Mp. 162-163 °C (lit.,[66] 164-165 °C). **^{1}H NMR** (300 MHz, D_2O): δ 0.77 (d, J = 3.6 Hz, 3H), 0.80 (d, J = 3.6 Hz, 3H), 1.88 (s, 3H), 1.95-2.06 (m, 1H), 4.05 (d, J = 5.7 Hz, 1H). **MS (ESI)** (-ve): *m/z* 158 ($M\text{-}H^+$, 100%). The obtained ^{1}H NMR spectral characteristics are consistent with literature data.[116]

(*S*)-*N*-Acetylvalinol (76)



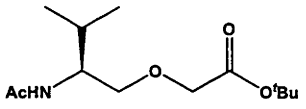
(76)

Triethylamine (4.2 mL, 30.1 mmol) and ethyl chloroformate (2.4 mL, 25.1 mmol) were added to a cooled suspension of (*S*)-*N*-acetylvaline **(75)** (4.0 g, 25.1 mmol) in tetrahydrofuran (20 mL) under a nitrogen atmosphere. The mixture was stirred at –5 °C for 30 minutes, and the white precipitate was filtered and washed with tetrahydrofuran (30 mL). The filtrate was added dropwise to a suspension of sodium borohydride (2.4 g, 62.7 mmol) in water (20 mL) and the resulting mixture was stirred at 0 °C for 4 hours and extracted with diethyl ether (2 x 50 mL) and

dichloromethane (2 x 50 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **76** (2.4 g, 66%) as a white solid.

Mp. 75-76 °C (lit.,[117] 76-77 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.93 (d, J = 6.6 Hz, 3H), 0.95 (d, J = 6.6 Hz, 3H), 1.81-1.92 (m, 1H), 2.04 (s, 3H), 3.60-3.76 (m, 3H), 5.92 (br d, J = 8.4 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 18.8, 19.4, 23.4, 28.9, 57.1, 63.7, 171.3. **MS (ESI)** (+ve): *m/z* 146 ($M+H^+$, 78%). **HRMS (ESI)** calcd. for $C_7H_{15}NO_2Na$ $[M+Na]^+$ *m/z* 168.1000, found 168.0993.

***(S)-t*-Butyl-5-acetamido-6-methyl-3-oxaheptanoate (77)**



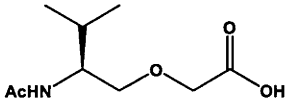
(77)

(*S*)-*N*-Acetylvalinol **(76)** (0.8 g, 5.2 mmol) was added to a stirred suspension of sodium hydride (95%) (0.4 g, 14.6 mmol) in *N,N*-dimethylformamide (25 mL) under a nitrogen atmosphere. The mixture was stirred at room temperature for 2 hours, then cooled to 0 °C. *t*-Butyl bromoacetate (3.1 mL, 20.7 mmol) was added to the mixture and stirred at 0 °C for 30 minutes, and stirred at room temperature for a further 2 hours, then treated with saturated ammonium chloride solution (20 mL) and washed with ethyl acetate (100 mL). The organic extract was washed with saturated ammonium chloride solution (3 x 20 mL), saturated sodium bicarbonate solution (3 x 20 mL) and brine solution (3 x 20 mL) then dried, filtered and concentrated under reduced pressure. The residue was subjected to flash silica gel column chromatography, eluting with hexanes/ethyl acetate (2:1 to 1:2) then ethyl acetate, to give the title compound **77** (0.2 g, 18%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (d, J = 7.1 Hz, 3H), 0.95 (d, J = 7.1 Hz, 3H), 1.44 (s, 9H), 1.84-1.97 (m, 1H), 1.99 (s, 3H), 3.37-3.41 (m, 1H), 3.69-3.79 (m, 3H), 3.88 (d, J = 16.5 Hz, 1H), 3.94 (d, J = 16.5 Hz, 1H), 6.34 (br d, J = 8.4 Hz, 1H). **^{13}C**

NMR (75 MHz, $CDCl_3$): δ 19.5, 23.6, 28.3, 29.5, 54.6, 69.0, 71.9, 82.1, 170.2, 170.3. **MS (ESI)** (+ve): *m/z* 282 ($M+Na^+$, 55%). **HRMS (ESI)** calcd. for $C_{13}H_{25}NO_4Na$ $[M+Na]^+$ *m/z* 282.1681, found 282.1669.

(S)-5-Acetamido-6-methyl-3-oxaheptanoic acid (69)



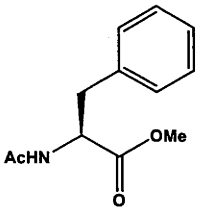
(69)

Trifluoroacetic acid (3 mL) was added to a stirred suspension of *(S)*-*t*-butyl-5-acetamido-6-methyl-3-oxaheptanoate **(77)** (200 mg, 0.8 mmol) in dichloromethane (20 mL). The mixture was stirred overnight and concentrated under reduced pressure to give a brown oil. The residue was subjected to preparative high performance liquid chromatography to give the title compound **69** (30 mg, 19%) as a colourless oil.

^{1}H NMR (300 MHz, CD_3OD): δ 0.91 (d, J = 6.7 Hz, 3H), 0.94 (d, J = 6.7 Hz, 3H), 1.83-1.94 (m, 1H), 1.97 (s, 3H), 3.51 (dd, J = 4.4, 9.9 Hz, 1H), 3.62 (dd, J = 5.4, 9.9 Hz, 1H), 3.74-3.79 (m, 1H), 4.06 (s, 2H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 18.8, 18.9, 21.4, 28.9, 56.1, 67.6, 70.9, 174.4, 174.8. **MS (ESI)** (-ve): *m/z* 202 ($M-H^+$, 30%). **HRMS (ESI)** calcd. for $C_9H_{16}NO_4$ $[M-H]^+$ *m/z* 202.1079, found 202.1076. **HPLC**: t_R 16.0 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (82:18) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

10.4.4 Experimental for Chapter Five

(*S*)-*N*-Acetylphenylalanine methyl ester (82)

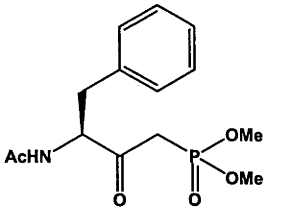


(82)

Thionyl chloride (2.8 mL, 38.6 mmol) was added to a suspension of (*S*)-*N*-acetylphenylalanine **(32)** (2.0 g, 9.7 mmol) in methanol (60 mL) and the mixture was stirred at room temperature for 5 hours. The solution was concentrated under reduced pressure to yield the title compound **82** (2.1 g, 98%) as a white solid.

Mp. 90-91 °C (lit.,[118] 90-91 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 2.08 (s, 3H), 3.08 (dd, J = 9.0, 13.8 Hz, 1H), 3.18 (dd, J = 5.7, 13.8 Hz, 1H), 3.72 (s, 3H), 4.79-4.87 (m, 1H), 6.55 (br s, 1H), 7.03-7.12 (m, 2H), 7.21-7.31 (m, 3H). **MS (ESI)** (+ve): *m/z* 244 ($M+Na^+$, 22%). The acquired ^{1}H NMR spectral data are consistent with previously reported data.[76]

(*S*)-Dimethyl-3-acetamido-2-oxo-4-phenylbutylphosphonate (83)



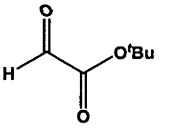
(83)

n-Butyl lithium (1.6M in hexanes, 28.3 mL, 45.2 mmol) was added dropwise to a solution of dimethyl methylphosphonate (4.9 mL, 45.2 mmol) in tetrahydrofuran (50 mL) maintained at -78 °C under a nitrogen atmosphere, and stirred for 30

minutes. A solution of (*S*)-*N*-acetylphenylalanine methyl ester **(82)** (2.0 g, 9.0 mmol) in tetrahydrofuran (40 mL) was added dropwise. The mixture was stirred at –78 °C for 1 hour, quenched with 10% aqueous acetic acid (20 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with 10% aqueous sodium bicarbonate (3 x 40 mL) and brine solution (3 x 40 mL). The solution was dried, filtered and concentrated under reduced pressure. The residue was chromatographed on flash silica gel, eluting with acetonitrile/diethyl ether (1:1 to 5:1) then acetonitrile, to yield the title compound **83** (0.5 g, 16%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 2.03 (s, 3H), 2.99-3.39 (m, 4H), 3.78-3.84 (m, 6H), 4.87 (ddd, J = 6.0, 6.0, 6.0 Hz, 1H), 6.54 (br d, J = 7.5 Hz, 1H), 7.24-7.39 (m, 5H). **MS (ESI)** (+ve): *m/z* 336 ($M+Na^+$, 66%). **HRMS (EI)** calcd. for $C_{14}H_{20}NO_5P$ $[M]^{+\bullet}$ *m/z* 313.1079, found 313.1082. The acquired ^{1}H NMR spectral data are consistent with previously reported data.[74]

***t*-Butyl glyoxylate (84)**

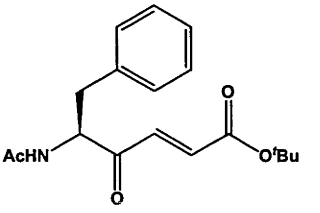


(84)

A mixture of *t*-butyl alcohol (5.6 mL, 58.0 mmol), *N,N'*-dicyclohexylcarbodiimide (10.0 g, 48.5 mmol) and cuprous chloride (0.2 g, 2.0 mmol) was stirred at room temperature over 5 days. (*S*)-Tartaric acid (2.4 g, 16.0 mmol) in dichloromethane (70 mL) was added to the mixture, and stirring was continued at room temperature for 28 hours. The mixture was filtered through a pad of celite and washed with dichloromethane (100 mL). The solution was partitioned between dichloromethane and water (100 mL). The organic extract was dried, filtered and concentrated under reduced pressure to give a yellow solid, which was subjected to flash silica gel column chromatography eluting with diethyl ether/pentane to yield a white solid. The solid (1.0g, 3.8 mmol) was dissolved in methanol (20 mL) and treated with a solution of sodium periodate (980 mg, 4.5 mmol) in water (10 mL). The mixture

was stirred at 0 °C for 80 minutes. The white precipitate was taken up in water (70 mL) and extracted with diethyl ether (3 x 70 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **84** (0.4 g, 99%) as a yellow oil. The residue was used in the synthesis of (*S*,*E*)-*t*-butyl-5-acetamido-4-oxo-6-phenylhex-2-enoate **(85)** without further purification.

(*S*,*E*)-*t*-Butyl-5-acetamido-4-oxo-6-phenylhex-2-enoate (85)



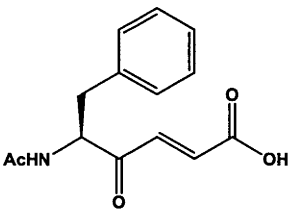
(85)

To a suspension of (*S*)-dimethyl-3-acetamido-2-oxo-4-phenylbutylphosphonate **(83)** (450 mg, 1.4 mmol) in acetonitrile (10 mL) was added a solution of *t*-butyl glyoxylate **(84)** (431 mg, 3.8 mmol) in acetonitrile (10 mL), lithium chloride (61 mg, 1.4 mmol) and triethylamine (0.2 mL, 1.4 mmol). The mixture was stirred at 0 °C under a nitrogen atmosphere for 70 minutes, warmed to room temperature, quenched with 10% aqueous citric acid (8 mL) and extracted with diethyl ether (3 x 50 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure. The residue was chromatographed on flash silica gel, eluting with pentane/diethyl ether (2:1 to 1:1 to 1:2) then diethyl ether, to give the title compound **85** (163 mg, 36%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.55 (s, 9H), 2.00 (s, 3H), 3.08 (dd, J = 5.4, 14.0 Hz, 1H), 3.19 (dd, J = 6.6, 14.0 Hz, 1H), 5.13 (ddd, J = 5.4, 6.6, 6.9 Hz, 1H), 6.11 (br d, J = 6.9 Hz, 1H), 6.70 (d, J = 15.9 Hz, 1H), 7.02 (d, J = 15.9 Hz, 1H), 7.04-7.07 (m, 2H), 7.20-7.31 (m, 3H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 22.9, 27.9, 37.0, 58.2, 82.1, 127.1, 128.5, 129.3, 130.2, 134.6, 135.3, 164.0, 169.7, 197.1. **MS (EI)** (+ve): *m/z*

317 ($M^{+\bullet}$, 100%). **HRMS (EI)** calcd. for $C_{18}H_{23}NO_4$ $[M]^{+\bullet}$ *m/z* 317.1627, found 317.1625.

(*S*,*E*)-5-Acetamido-4-oxo-6-phenylhex-2-enoic acid (80)

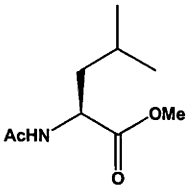


(80)

A suspension of (*S*,*E*)-*t*-butyl-5-acetamido-4-oxo-6-phenylhex-2-enoate **(85)** (50 mg, 0.2 mmol) dissolved in dichloromethane (10 mL) was treated with trifluoroacetic acid (1.0 mL). The mixture was stirred overnight at room temperature and concentrated under reduced pressure. Recrystallisation of the product from ethyl acetate/hexanes yielded the title compound **80** (20 mg, 38%) as a white solid.

Mp. 105-106 °C (lit.,[74] 105-107 °C). **^{1}H NMR** (300 MHz, ($CDCl_3$): δ 1.87 (s, 3H), 2.99 (dd, *J* = 6.3, 13.9 Hz, 1H), 3.16 (dd, *J* = 6.3, 13.9 Hz, 1H), 4.88-4.96 (m, 1H), 6.73 (d, *J* = 15.9 Hz, 1H), 6.99-7.01 (m, 1H), 7.11 (d, *J* = 15.9 Hz, 1H), 7.13-7.30 (m, 5H). **MS (EI)** (+ve): *m/z* 261 ($M^{+\bullet}$, 63%). **HRMS (EI)** calcd. for $C_{14}H_{15}NO_4$ $[M]^{+\bullet}$ *m/z* 261.1001, found 261.0997. **Anal.** Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.35; H, 5.90; N, 5.19%. The ^{1}H NMR spectral information are consistent with literature information.[74]

(*S*)-*N*-Acetylleucine methyl ester (86)

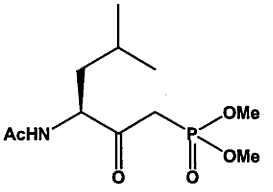


(86)

Thionyl chloride (1.7 mL, 23.1 mmol) was added to a suspension of (*S*)-*N*-acetylleucine **(35)** (2.0 g, 11.5 mmol) dissolved in methanol (25 mL) at 0 °C. The mixture was warmed to room temperature and stirred overnight under a nitrogen atmosphere. Solvent evaporation under reduced pressure gave the title compound **86** (2.0 g, 93%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.91 (d, J = 5.8 Hz, 3H), 0.94 (d, J = 5.8 Hz, 3H), 1.65-1.81 (m, 3H), 2.41 (s, 3H), 3.72 (s, 3H), 4.57 (m, 1H). **MS (ESI)** (+ve): *m/z* 188 ($M+H^+$, 27%). **HRMS (ESI)** calcd. for $C_9H_{18}NO_3$ $[M+H]^+$ *m/z* 188.1287, found 188.1281. The ^{1}H NMR spectral information obtained are consistent with previously reported data.[78]

(*S*)-Dimethyl-3-acetamido-5-methyl-2-oxohexylphosphonate (87)



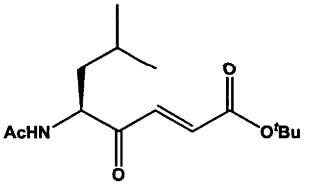
(87)

n-Butyl lithium (1.6M in hexanes, 40.1 mL, 64.1 mmol) was added to a mixture of dimethyl methylphosphonate (6.9 mL, 64.1 mmol) in tetrahydrofuran (40 mL) maintained at –78 °C under a nitrogen atmosphere. After 30 minutes a solution of (*S*)-*N*-acetylleucine methyl ester **(86)** (1.5 g, 8.0 mmol) in tetrahydrofuran (20 mL) was added dropwise and stirred at –78 °C for 1 hour, quenched with 10% aqueous

acetic acid (25 mL), warmed to room temperature and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with 10% aqueous sodium bicarbonate (3 x 50 mL) and brine solution (3 x 50 mL). The solution was dried, filtered and concentrated under reduced pressure. The residue was chromatographed on flash silica gel, eluting with acetonitrile to give the title compound **87** (0.6 g, 28%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.90 (d, J = 2.1 Hz, 3H), 0.92 (d, J = 2.1 Hz, 3H), 1.58-1.76 (m, 3H), 2.04 (s, 3H), 2.99-3.38 (m, 2H), 3.68-3.78 (m, 6H), 4.63-4.70 (m, 1H), 6.57 (br d, J = 8.1 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 21.2, 22.6, 22.9, 24.5, 36.8, 38.6, 38.8, 57.2, 57.3, 170.3, 201.5. **MS (ESI)** (+ve): *m/z* 280 ($M+H^+$, 100%). **HRMS (ESI)** calcd. for $C_{11}H_{23}NO_5P$ $[M+H]^+$ *m/z* 280.1314, found 280.1317.

(*S*,*E*)-*t*-Butyl-5-acetamido-7-methyl-4-oxooct-2-enoate (88)

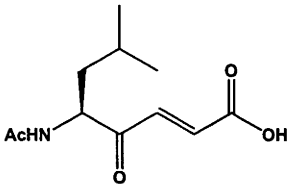


(88)

A suspension of (*S*)-dimethyl-3-acetamido-5-methyl-2-oxohexylphosphonate **(87)** (450 mg, 1.6 mmol) in acetonitrile (15 mL) was treated with freshly prepared *t*-butyl glyoxylate **(84)** (431 mg, 3.8 mmol) in acetonitrile (10 mL), lithium chloride (68 mg, 1.6 mmol) and triethylamine (0.2 mL, 1.6 mmol). The mixture was stirred at 0 °C under a nitrogen atmosphere for 70 minutes, warmed to room temperature, quenched with 10% aqueous citric acid (10 mL) and extracted with diethyl ether (3 x 50 mL). The combined organic extracts were dried, filtered then concentrated under reduced pressure. The residue was chromatographed on flash silica gel, eluting with hexanes/ethyl acetate (2:1 to 1:1 to 1:2), to yield the title compound **88** (160 mg, 35%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.92 (d, J = 6.3 Hz, 3H), 0.99 (d, J = 6.3 Hz, 3H), 1.21-1.73 (m, 3H), 1.51 (s, 9H), 2.03 (s, 3H), 4.98-4.91 (m, 1H), 6.01 (br d, J = 7.8 Hz, 1H), 6.75 (d, J = 15.9 Hz, 1H), 7.07 (d, J = 15.9 Hz, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 21.6, 22.8, 23.1, 24.8, 27.8, 40.2, 55.6, 82.1, 134.3, 135.5, 164.2, 170.1, 198.5. **MS (ESI)** (+ve): *m/z* 306 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{15}H_{25}NO_4Na$ $[M+Na]^+$ *m/z* 306.1681, found 306.1680.

(*S*,*E*)-5-Acetamido-7-methyl-4-oxooct-2-enoic acid (81)



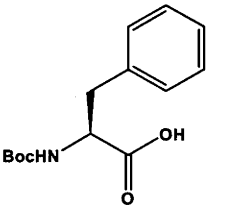
(81)

Trifluoroacetic acid (1.5 mL) was added dropwise to a solution of (*S*,*E*)-*t*-butyl-5-acetamido-7-methyl-4-oxooct-2-enoate **(88)** (100 mg, 0.4 mmol) in dichloromethane (15 mL). The mixture was stirred overnight at room temperature and solvent evaporation under reduced pressure gave a yellow oil. The residue was subjected to preparative high performance liquid chromatography to give the title compound **81** (30 mg, 37%) as a yellow oil.

^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 0.84 (d, J = 6.6 Hz, 1H), 0.87 (d, J = 6.6 Hz, 1H), 1.42 (dd, J = 7.2, 7.5 Hz, 2H), 1.56-1.62 (m, 1H), 1.85 (s, 3H), 4.45 (dt, J = 7.2, 7.5 Hz, 1H), 6.57 (d, J = 15.6 Hz, 1H), 7.10 (d, J = 15.6 Hz, 1H), 8.35 (d, J = 7.2 Hz, 1H). **^{13}C NMR** (75 MHz, $(CD_3)_2SO$): δ 21.3, 22.2, 23.0, 24.3, 37.9, 55.8, 131.9, 136.3, 166.3, 169.7, 198.6. **MS (ESI)** (-ve): *m/z* 226 ($M-H^+$, 8%). **HRMS (ESI)** calcd. for $C_{11}H_{16}NO_4$ $[M-H]^+$ *m/z* 226.1079, found 226.1074. **HPLC**: t_R 20.5 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (80:20) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

10.4.5 Experimental for Chapter Six

(*S*)-*N*-(*t*-Butoxycarbonyl)phenylalanine (95)

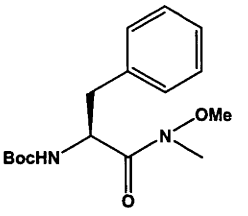


(95)

Sodium hydroxide (1.5 g, 38.5 mmol) and di-*t*-butyl dicarbonate (4.2 g, 19.2 mmol) were added to a mixture of (*S*)-phenylalanine **(31)** (2.9 g, 17.5 mmol) in tetrahydrofuran/water (1:1) (58 mL). The mixture was stirred at room temperature overnight and the organic layer was concentrated under reduced pressure. The aqueous layer was extracted with dichloromethane (3 x 50 mL) and the combined organic extracts were dried, filtered and concentrated under pressure to give the title compound **95** (3.4 g, 73%) as a white solid.

Mp. 86-87 °C (lit.,[119] 85-87 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.27 (s, 9H), 3.08 (dd, *J* = 6.6, 13.8 Hz, 1H), 3.20 (dd, *J* = 5.7, 13.8 Hz, 1H), 4.57-4.64 (m, 1H), 4.94 (d, *J* = 7.8 Hz, 1H), 7.17-7.34 (m, 5H). **MS (ESI)** (-ve): *m/z* 264 (6%, $M\text{-}H^+$). The obtained ^{1}H NMR spectral data are consistent with literature information.[120]

(*S*)-*N*-(*t*-Butoxycarbonyl)phenylalanine *N*-methoxy-*N*-methylamide (97)

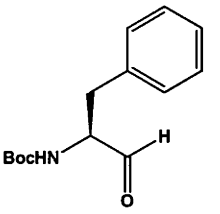


(97)

A suspension of (*S*)-*N*-(*t*-butoxycarbonyl)phenylalanine **(95)** (2.7 g, 10.0 mmol) in dichloromethane (40 mL) was treated with triethylamine (1.4 mL, 10.0 mmol). The mixture was treated with BOP (4.4 g, 10.0 mmol), *N,O*-dimethylhydroxylamine **(96)** (1.1 g, 11.0 mmol) and triethylamine (1.5 mL, 11.0 mmol), and stirred at room temperature for 1 hour. The mixture was diluted with dichloromethane (200 mL) and washed successively with 3M hydrochloric acid (3 x 30 mL), saturated sodium bicarbonate solution (3 x 30 mL), and brine solution (3 x 30 mL). The organic extract was dried, filtered and concentrated under reduced pressure. The crude product was subjected to flash silica gel column chromatography, eluting with hexanes/ethyl acetate (1:1) to give the title compound **97** (2.9 g, 95%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.37 (s, 9H), 2.86 (dd, J = 6.9, 13.3 Hz, 1H), 3.04 (dd, J = 6.0, 13.3 Hz, 1H), 3.15 (s, 3H), 3.64 (s, 3H), 4.90-4.97 (m, 1H), 5.19 (br d, J = 8.4 Hz, 1H), 7.14-7.30 (s, 5H). **MS (ESI)** (+ve): *m/z* 309 ($M+H^+$, 98%). **HRMS (ESI)** calcd. for $C_{16}H_{25}N_2O_4$ $[M+H]^+$ *m/z* 309.1814, found 309.1813. The obtained ^{1}H NMR spectral characteristics are consistent with previously reported data.[84]

(*S*)-*N*-(*t*-Butoxycarbonyl)phenylalaninal (98)

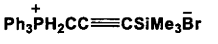


(98)

Lithium aluminium hydride (1.0M in tetrahydrofuran, 3.3 mL, 3.3 mmol) was added dropwise to a solution of the (*S*)-*N*-(*t*-butoxycarbonyl)-phenylalanine *N*-methoxy-*N*-methylamide **(97)** (820 mg, 2.7 mmol) in tetrahydrofuran (20 mL) at 0 °C under a nitrogen atmosphere. After 20 minutes, the reaction was quenched by the addition of a solution of potassium hydrogen sulfate (620 mg, in 10 mL water). The mixture was washed with diethyl ether (3 x 30 mL). The combined organic extracts were washed with 3M hydrochloric acid (3 x 20 mL), saturated sodium bicarbonate solution (3 x 20 mL) and brine solution (3 x 20 mL). The extracts were dried, filtered and concentrated under reduced pressure to give the title compound **98** (530 mg, 82%) as a white solid.

Mp. 80-81 °C (lit.,[121] 82 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.43 (s, 9H), 3.12 (d, J = 6.6, 2H), 4.42 (dt, J = 6.6 Hz, 6.9 Hz, 1H), 5.05 (br d, J = 6.0 Hz, 1H), 7.15-7.34 (m, 5H), 9.63 (s 1H). **MS (EI)** (+ve): *m/z* 249 ($M^{+\bullet}$, 92%). **HRMS (EI)** calcd. for $C_{14}H_{19}NO_3$ $[M]^{+\bullet}$ *m/z* 249.1365, found 249.1370. The obtained ^{1}H NMR spectral information are consistent with previously reported values.[90]

[1-(Trimethylsilyl)propyn-3-yl)]triphenylphosphonium bromide (99)



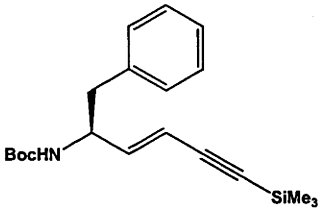
(99)

A mixture of 3-(trimethylsilyl)propargyl bromide (1.1 mL, 7.8 mmol), triphenylphosphine (2.7 g, 10.2 mmol) in benzene (7.5 mL) was stirred in the dark

for 18 hours. The brown precipitate was filtered and washed with hexanes (15 mL), and the filtrate was concentrated under reduced pressure to give the title compound **99** (2.6 g, 74%) as a brown solid.

Mp. 157-158 °C (lit.,[86] 155-159 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ -0.05 (s, 9H), 5.13 (d, J = 15.0 Hz, 2H), 7.27-7.96 (m, 15H). **MS (ESI)** (+ve): *m/z* 373 ($(M-Br)^+$ 100%). The ^{1}H NMR spectral information are consistent with literature data.[92]

(*S*,*E*)-5-[*N*-(*t*-butoxycarbonylamino)]-6-phenyl-1-trimethylsilylhex-3-en-1-yne (100)



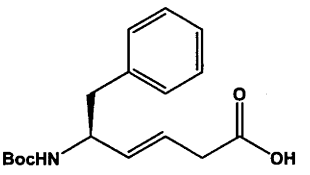
(100)

A suspension of [1-(trimethylsilyl)propyn-3-yl)]triphenylphosphonium bromide **(99)** (295 mg, 0.7 mmol) in tetrahydrofuran (5 mL) was treated with *n*-butyl lithium (1.6M in diethyl ether, 0.4 mL, 0.7 mmol). The mixture was stirred at –78 °C for 1 hour under a nitrogen atmosphere, then treated with a solution of (*S*)-*N*-(*t*-butoxycarbonyl)phenylalaninal **(98)** (163 mg, 0.7 mmol) in tetrahydrofuran (5 mL). The mixture was stirred at –78 °C for 1 hour, warmed to room temperature and stirred overnight. The solution was concentrated under reduced pressure and the residue was triturated with diethyl ether. The organic extract was washed with brine solution (3 x 20 mL) then dried, filtered and concentrated under reduced pressure to give a brown oil. The residue was chromatographed on flash silica gel, eluting with dichloromethane to yield the title compound **100** (98 mg, 45%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.16 (s, 9H), 1.37 (s, 9H), 2.79-2.81 (m, 2H), 4.49 (m, 1H), 5.60 (d, J = 15.9 Hz, 1H), 6.12 (d, J = 15.9 Hz, 1H), 7.17-7.31 (m, 5H). **MS (ESI)** (+ve): *m/z* 366 ($M+Na^+$, 24%). **HRMS (ESI)** calcd. for $C_{20}H_{29}NO_2NaSi$

$[M+Na]^+$ *m/z* 366.1865, found 366.1879. The ^1H NMR spectral information obtained are consistent with literature values.[91]

(*S*,*E*)-5-[*N*-(*t*-Butoxycarbonylamino)]-6-phenylhex-3-enoic acid (93)

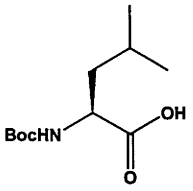


(93)

Cyclohexene (0.2 mL, 1.7 mmol) was added to a suspension of borane-tetrahydrofuran complex (1.0M in tetrahydrofuran, 0.9 mL, 0.9 mmol) and stirred at 0 °C for 1 hour under a nitrogen atmosphere. (*S*,*E*)-5-[*N*-(*t*-Butoxycarbonylamino)]-6-phenyl-1-trimethylsilylhex-3-en-1-yne **(100)** (90 mg, 0.3 mmol) in tetrahydrofuran (1 mL) was added dropwise. The resulting mixture was stirred at 0 °C for 1 hour, and was then treated with methanol (0.3 mL), 2M sodium hydroxide (0.5 mL), hydrogen peroxide (0.3 mL, 30% w/v) and stirred at room temperature for 1 hour. The mixture was poured into water (4 mL) containing 2M sodium hydroxide (0.4 mL) and extracted with diethyl ether (3 x 15 mL). The aqueous layer was acidified to pH 2 with 1M hydrochloric acid and extracted with diethyl ether (3 x 15mL). The organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **93** (60 mg, 74%) as a colourless oil.

^1H NMR (300 MHz, $CDCl_3$): δ 1.38 (s, 9H), 2.83 (d, *J* = 6.9 Hz, 2H), 3.09 (d, *J* = 5.7 Hz, 2H), 4.42 (br s, 1H), 5.45-5.61 (m, 2H), 7.16-7.31 (m, 5H). **MS (ESI)** (-ve): *m/z* 304 ($M-H^+$, 48%). **HRMS (ESI)** calcd. for $C_{17}H_{22}NO_4$ $[M-H]^+$ *m/z* 304.1549, found 304.1554. The ^1H NMR spectral data are consistent with previously reported information.[89]

(*S*)-*N*-(*t*-Butoxycarbonyl)leucine (118)

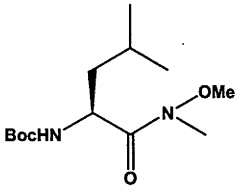


(118)

Sodium hydroxide (3.4 g, 83.8 mmol) and di-*t*-butyl dicarbonate (9.2 g, 41.9 mmol), were added to a suspension of (*S*)-leucine **(34)** (5.0 g, 38.1 mmol) in a 1:1 mixture of tetrahydrofuran and water (127 mL). The mixture was stirred at room temperature overnight and the organic layer was concentrated under reduced pressure. The aqueous layer was extracted with dichloromethane (3 x 70 mL), acidified to pH 4 with 1M hydrochloric acid and extracted with dichloromethane (3 x 70 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **118** (7.8 g, 89%) as a white solid.

Mp. 72-73 °C (lit.,[122] 72-74 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.95 (d, J = 6.3 Hz, 6H), 1.44 (s, 9H), 1.48-1.76 (m, 3H), 4.27-4.35 (m, 1H), 4.89 (br d, J = 8.4 Hz, 1H). **MS (ESI)** (-ve): *m/z* 230 ($M-H^+$, 100%). **HRMS (ESI)** calcd. for $C_{11}H_{20}NO_4$ $[M-H]^+$ *m/z* 230.1392, found 230.1387. The ^{1}H NMR spectral information acquired are consistent with literature values.[123]

(*S*)-*N*-(*t*-Butoxycarbonyl)leucine *N*-methoxy-*N*-methylamide (119)



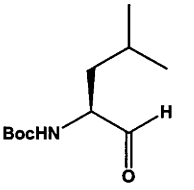
(119)

Triethylamine (2.7 mL, 19.5 mmol) was added to a solution of *N,O*-dimethyl hydroxylamine **(96)** (1.9 g, 19.5 mmol) in dichloromethane (50 mL). The mixture

was treated with *(S)*-*N*-(*t*-butoxycarbonyl)leucine **(118)** (5.0 g, 21.6 mmol) in dichloromethane (30 mL), *N,N'*-dicyclohexylcarbodiimide (5.2 g, 25.3 mmol), triethylamine (5.4 mL, 38.9 mmol) and a catalytic amount of 4-dimethylaminopyridine. The mixture was stirred at room temperature for 1 hour and the white precipitate was filtered. The filtrate was washed successively with 0.3M citric acid (3 x 35 mL), saturated sodium carbonate solution (3 x 35 mL), brine solution (3 x 35 mL), then dried, filtered and concentrated under reduced pressure. The residue was chromatographed on flash silica gel, eluting with hexanes/ethyl acetate (9:1 to 3:2 to 1:1), to give the title compound **119** (1.8 g, 33%) as a light yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.92 (d, J = 6.6 Hz, 3H), 0.96 (d, J = 6.6 Hz, 3H), 1.42 (s, 9H), 1.45-1.82 (m, 3H), 3.19 (s, 3H), 3.78 (s, 3H), 4.67-4.83 (m, 1H), 5.07 (br d, J = 9.0 Hz, 1H). **MS (ESI)** (+ve): *m/z* 297 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{13}H_{26}N_2O_4Na$ $[M+Na]^+$ *m/z* 297.1790, found 297.1792. The acquired ^{1}H NMR spectral information are consistent with reported data.[93]

(*S*)-*N*-(*t*-Butoxycarbonyl)leucinal (120)



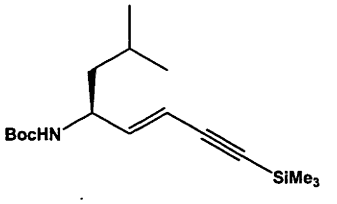
(120)

Lithium aluminium hydride (1.0M in tetrahydrofuran, 13.7 mL, 13.7 mmol) was added dropwise to a suspension of (*S*)-*N*-(*t*-butoxycarbonyl)leucine *N*-methoxy-*N*-methylamide **(119)** (750 mg, 2.7 mmol) in tetrahydrofuran (60 mL) under a nitrogen atmosphere. The mixture was stirred at 0 °C for 30 minutes, and quenched with a potassium hydrogen sulfate solution (1.4 g in 10 mL water). The organic layer was concentrated under reduced pressure, and the aqueous layer was extracted with dichloromethane (3 x 60 mL). The combined organic extracts were washed with 3M hydrochloric acid (3 x 20 mL), brine solution (2 x 30 mL), saturated sodium

bicarbonate solution (3 x 20 mL) then brine solution (2 x 30 mL). The organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **120** (460 mg, 78%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 0.95 (d, J = 6.6 Hz, 6H), 1.23-1.89 (m, 3H), 1.44 (s, 9H), 4.20-4.27 (m, 1H), 4.95 (br s, 1H), 9.58 (s, 1H). **MS (ESI)** (+ve): *m/z* 216 ($M+H^+$, 95%). The ^{1}H NMR spectral information is consistent with published information.[124]

(*S*,*E*)-5-[N-(*t*-Butoxycarbonyl)amino]-7-methyl-1-trimethylsilyoct-3-en-1-yne (121)

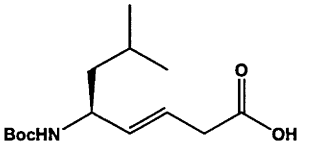


(121)

n-Butyl lithium (1.6M in hexanes, 1.3 mL, 2.1 mmol) was added dropwise to a mixture of [1-(trimethylsilyl)propyn-3-yl)]triphenylphosphonium bromide **(99)** (970 mg, 2.1 mmol) in tetrahydrofuran (40 mL) under a nitrogen atmosphere. The mixture was stirred at –78 °C for 1 hour and treated with a solution of (*S*)-*N*-(*t*-butoxycarbonyl)leucinal **(120)** (460 mg, 2.1 mmol) in tetrahydrofuran (20 mL), then warmed to room temperature. Solvent evaporation under reduced pressure gave a red oil, which was triturated with diethyl ether. The organic extract was concentrated under reduced pressure, treated with 1M sodium bicarbonate solution (30 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were washed with 10% hydrochloric acid (3 x 30 mL) and brine solution (2 x 50 mL), then dried and filtered. Solvent evaporation under reduced pressure gave a red oil, which was chromatographed on flash silica gel, eluting with hexanes/dichloromethane (1:1) then dichloromethane, to yield the title compound **121** (345 mg, 52%) as a yellow solid.

Mp. 89-90 °C (lit.,[92] 89-90 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.17 (s, 9H), 0.91 (d, J = 6.6 Hz, 6H), 1.30-1.35 (m, 2H), 1.43 (s, 9H), 1.61-1.70 (m, 1H), 4.17-4.22 (m, 1H), 4.36-4.38 (m, 1H), 5.64 (d, J = 15.9 Hz, 1H), 6.07 (dd, J = 6.0, 15.9 Hz, 1H). **MS (ESI)** (+ve): *m/z* 332 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{17}H_{31}NO_2SiNa$ $[M+Na]^+$ *m/z* 332.2022, found 332.2016. The acquired ^{1}H NMR spectral data are consistent with literature values.[92]

(*S*,*E*)-5-[*N*-(*t*-Butoxycarbonylamino)]-7-methyloct-3-enoic acid (94)



(94)

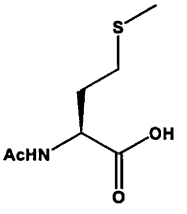
Cyclohexene (0.3 mL, 3.4 mmol) in tetrahydrofuran (2 mL) was added dropwise to a mixture of borane-tetrahydrofuran complex (1.0M in tetrahydrofuran, 1.7 mL, 1.7 mmol) under a nitrogen atmosphere and stirred at 0 °C for 1 hour. (*S*,*E*)-5-[N-(*t*-Butoxycarbonyl)amino]-7-methyl-1-trimethylsilyoct-3-en-1-yne **(121)** (150 mg, 0.5 mmol) in tetrahydrofuran (0.6 mL) was added dropwise. The mixture was stirred for 1 hour at 0 °C, treated with methanol (0.6 mL), 2M sodium hydroxide (0.9 mL) and 30% hydrogen peroxide (w/v) (0.6 mL), and stirred at room temperature for 1 hour. The mixture was poured into water (10 mL) containing 2M sodium hydroxide (0.9 mL) and extracted with diethyl ether (3 x 25 mL). The aqueous layer was acidified to pH 2 with 1M hydrochloric acid and extracted with diethyl ether (3 x 25 mL). The organic extracts were washed with brine solution (3 x 20 mL), then dried and filtered. Solvent evaporation under reduced pressure gave the title compound **94** (82 mg, 63%) as a colourless oil.

$[\alpha]^{23}_D$ -20.6 (*c* 0.5, EtOH). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.91 (d, J = 6.6 Hz, 6H), 1.27-1.37 (m, 2H), 1.44 (s, 9H), 1.58-1.73 (m, 1H), 3.11 (d, J = 6.6 Hz, 2H), 4.13 (m, 1H), 5.51 (dd, J = 5.7, 15.6 Hz, 1H), 5.65-5.75 (m, 1H). **MS (ESI)** (-ve): *m/z* 270 ($M-H^+$, 38%). **HRMS (ESI)** calcd. for $C_{14}H_{24}NO_4$ $[M-H]^+$ *m/z* 270.1705, found

270.1714. The obtained ^{1}H NMR spectral information are consistent with reported data.[92]

10.4.6 Experimental for Chapter Seven

(*S*)-*N*-Acetylmethionine (127)

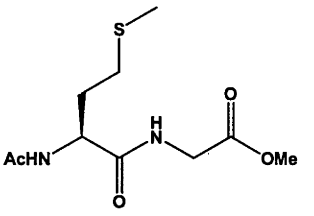


(127)

Triethylamine (6.5 mL, 46.9 mmol) and acetic anhydride (9.5 mL, 100.0 mmol) were added to a suspension of (*S*)-methionine **(126)** (5.0 g, 33.5 mmol) in water (250 mL). The mixture was stirred overnight at room temperature, acidified to pH 1 with 1M hydrochloric acid then extracted with ethyl acetate (3 x 200 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure. The residue was triturated with hexanes to give the title compound **127** (4.3 g, 67%) as a white solid.

Mp. 104-105 °C (lit.,[125] 106 °C). **^{1}H NMR** (300 MHz, ($CDCl_3$): δ 1.88-2.19 (m, 2H), 1.99 (s, 3H), 2.06 (s, 3H), 2.50 (t, J = 7.5 Hz, 2H), 4.58 (dt, J = 4.9, 7.6 Hz, 1H), 6.93 (br d, J = 7.6 Hz, 1H). **MS (ESI)** (-ve): *m/z* 190 ($M-H^+$, 100%). **HRMS (ESI)** calcd. for $C_7H_{13}NO_3SNa$ $[M+Na]^+$ *m/z* 214.0514, found 214.0505. The ^{1}H NMR spectral data obtained are consistent with literature values.[97]

(*S*)-*N*-Acetylmethionylglycine methyl ester (129)

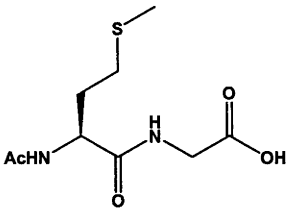


(129)

Triethylamine (1.5 mL, 10.4 mmol) was added to a suspension of glycine methyl ester hydrochloride **(128)** (1.3 g, 10.4 mmol) in dichloromethane (70 mL) and stirred at –5 °C for 30 minutes. The mixture was then treated with (*S*)-*N*-acetylmethionine **(127)** (1.5 g, 7.8 mmol) and *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (1.6 g, 8.1 mmol), stirred at –5 °C for 1 hour, warmed to room temperature and stirred overnight. The mixture was extracted with water (2 x 100 mL) and the combined aqueous extracts were washed with ethyl acetate (3 x 100 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **129** (0.5 g, 26%) as a white solid.

Mp. 109-110 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.94-2.15 (m, 2H), 2.04 (s, 3H), 2.11 (s, 3H), 2.52-2.66 (m, 2H), 3.75 (s, 3H), 3.97 (dd, J = 5.4, 18.3 Hz, 1H), 4.10 (dd, J = 6.0, 18.3 Hz, 1H), 4.70 (dt, J = 6.9, 7.8 Hz, 1H), 6.68 (br s, 1H), 7.11 (br s, 1H). **^{13}C NMR** (75 MHz, $CDCl_3$): δ 15.1, 22.8, 29.8, 31.5, 41.0, 51.8, 52.1, 169.8, 170.6, 172.1. **MS (ESI)** (+ve): *m/z* 285 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{10}H_{18}N_2O_4SNa$ $[M+Na]^+$ *m/z* 285.0885, found 285.0886.

(*S*)-*N*-Acetylmethionylglycine (123)

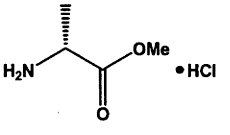


(123)

Lithium hydroxide (38 mg, 1.6 mmol) in water (12 mL) was added to a suspension of (*S*)-*N*-acetylmethionylglycine methyl ester **(129)** (300 mg, 1.1 mmol) in tetrahydrofuran (12 mL) and stirred at room temperature overnight. The mixture was acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure. The crude product was recrystallised from ethyl acetate to yield the title compound **123** (133 mg, 47%) as a white solid.

Mp. 112-113 °C. $[\alpha]^{23}_{D}$ –3.8 (*c* 2.0, EtOH). **^{1}H NMR** (300 MHz, D_2O): δ 1.91-2.17 (m, 2H), 2.03 (s, 3H), 2.09 (s, 3H), 2.51-2.70 (m, 2H), 3.97 (s, 2H), 4.44-4.49 (m, 1H). **^{13}C NMR** (75 MHz, CD_3OD): δ 15.2, 22.5, 30.9, 32.7, 41.7, 53.8, 172.7, 173.4, 174.4. **MS (ESI)** (-ve): *m/z* 247 ($M\text{-}H^+$, 100%). **HRMS (ESI)** calcd. for $C_9H_{16}N_2O_4S$ $[M\text{-}H]^+$ *m/z* 247.0752, found 247.0751. **Anal.** Calcd. for $C_9H_{15}N_2O_4S$: C, 43.54; H, 6.49; N, 11.28. Found: C, 43.60; H, 6.36; N, 11.04%. The obtained ^{1}H NMR spectral information are consistent with information previously reported.[126]

(*R*)-Alanine methyl ester hydrochloride (131)



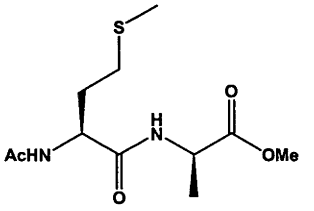
(131)

Thionyl chloride (6.5 mL, 89.8 mmol) was added dropwise to a mixture of (*R*)-alanine **(130)** (2.0 g, 22.4 mmol) in methanol (40 mL). The mixture was stirred at

room temperature overnight and concentrated under reduced pressure. Recrystallisation of the crude product from dichloromethane yielded the title compound **131** (2.1 g, 67%) as white crystals.

Mp. 108-109 °C (lit.,[127] 109-110 °C). $[\alpha]^{23}_{D}$ –0.4 (c 2.0, EtOH). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.72 (d, J = 7.2 Hz, 3H), 3.81 (s, 3H), 4.26-4.30 (m, 1H), 4.88 (br s, 2H). **MS (ESI)** (+ve): *m/z* 104 ($M+H^+$, 100%). **HRMS (ESI)** calcd. for $C_4H_9NO_2Na$ $[M+Na]^+$ *m/z* 126.0531, found 126.0526. The ^{1}H NMR spectral characteristics are consistent with literature data.[128]

(*S*,*R*)-*N*-Acetylmethionylalanine methyl ester (132)



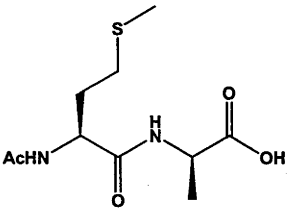
(132)

N,*N*-Diisopropylethylamine (2.0 mL, 11.6 mmol) was added dropwise to a mixture of (*S*)-*N*-acetylmethionine **(127)** (612 mg, 3.2 mmol), (*R*)-alanine methyl ester hydrochloride **(131)** (300 mg, 2.1 mmol) and BOP reagent (1.4 g, 3.2 mmol) in dichloromethane (20 mL). The mixture was stirred overnight at room temperature and treated with brine solution (100 mL). The mixture was extracted with ethyl acetate (4 x 50 mL) and the combined organic extracts were washed successively with 0.3M citric acid (3 x 50 mL), saturated sodium bicarbonate solution (3 x 50 mL) and brine solution (3 x 50 mL). The organic material was dried, filtered and concentrated under reduced pressure to give the title compound **132** (117 mg, 20%) as a white solid.

Mp. 130-131 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.41 (d, J = 6.9 Hz, 3H), 1.88-2.15 (m, 2H), 2.02 (s, 3H), 2.10 (s, 3H), 2.44-2.58 (m, 2H), 3.74 (s, 3H), 4.47-4.56 (m, 1H), 4.59-4.80 (m, 1H), 6.56 (br d, J = 6.9 Hz, 1H), 7.05 (br d, J = 7.2 Hz, 1H).

MS (EI) (+ve): *m/z* 276 ($M^{+\bullet}$, 3%). **HRMS (EI)** calcd. for $C_{11}H_{20}N_2O_4S$ $[M]^{+\bullet}$ *m/z* 276.1144, found 276.1145.

(*S*,*R*)-*N*-Acetylmethionylalanine (124)

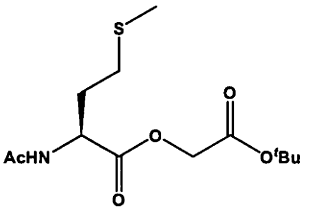


(124)

A suspension of (*S*,*R*)-*N*-acetylmethionylalanine methyl ester **(132)** (150 mg, 0.5 mmol) in tetrahydrofuran (5 mL) was treated with lithium hydroxide (18 mg, 0.5 mmol) in water (5 mL). The mixture was stirred at room temperature for 2 hours, acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (3 x 40 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure. The solid was recrystallised from ethyl acetate/hexanes to give the title compound **124** (30 mg, 21%) as a white solid.

Mp. 148-149 °C. $[\alpha]^{23}_D$ –12.0 (*c* 0.5, EtOH). **^{1}H NMR** (300 MHz, D_2O): δ 1.22 (d, *J* = 7.5 Hz, 3H), 1.73-1.96 (m 2H), 1.84 (s, 3H), 1.91 (s, 3H), 2.31-2.48 (m, 2H), 4.17 (q, *J* = 7.5 Hz, 1H), 4.25 (dd, *J* = 6.0, 6.0 Hz, 1H). **MS (ESI)** (+ve): *m/z* 285 ($M+Na^+$, 52%). **HRMS (EI)** calcd. for $C_{10}H_{18}N_2O_4$ $[M]^{+\bullet}$ *m/z* 262.0987, found 262.0987. The acquired ^{1}H NMR spectral information are consistent with literature data.[98]

_t_-Butyl (_S_)-O^{α}-(_N_-acetylmethionyl)glycolate (133)

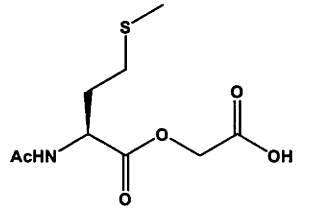


(133)

(_S_)-_N_-Acetylmethionine **(127)** (500 mg, 2.6 mmol) and _t_-butyl bromoacetate (0.4 mL, 2.6 mmol) were added to a mixture of potassium carbonate (360 mg, 2.6 mmol) in acetone (60 mL). The mixture was heated at reflux overnight under a nitrogen atmosphere, cooled to room temperature and extracted with ethyl acetate (2 x 50 mL). The organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **133** (703 mg, 89%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.45 (s, 9H), 1.98-2.29 (m, 2H), 2.02 (s, 3H), 2.09 (s, 3H), 2.59 (t, J = 7.3 Hz, 2H), 4.43 (d, J = 15.6 Hz, 1H), 4.64 (d, J = 15.6 Hz, 1H), 4.77-4.84 (m, 1H), 6.35 (br d, J = 8.1 Hz, 1H). **^{13}C NMR** (75 MHz, CD_3OD): δ 15.4, 23.1, 27.9, 29.8, 31.6, 51.5, 61.7, 82.8, 166.1, 169.9, 171.5. **MS (ESI)** (+ve): _m/z_ 328 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{13}H_{24}NO_5S$ $[M+H]^+$ _m/z_ 306.1375, found 306.1392.

(_S_)-O^{α}-(_N_-acetylmethionyl)glycolic acid (125)



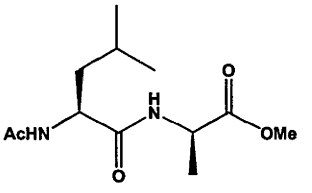
(125)

A suspension of _t_-butyl (_S_)-O^{α}-(_N_-acetylmethionyl)glycolate **(133)** (200 mg, 0.7 mmol) in dichloromethane (20 mL) was treated with trifluoroacetic acid (2.5 mL).

The mixture was stirred overnight at room temperature and concentrated under reduced pressure to give a colourless oil. The residue was subjected to preparative high performance liquid chromatography to give the title compound **125** (120 mg, 69%) as a colourless oil.

$[\alpha]^{23}_{D}$ –63.8 (*c* 0.5, EtOH). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.94-2.25 (m, 2H), 2.01 (s, 3H), 2.08 (s, 3H), 2.56 (t, *J* = 7.5 Hz, 2H), 4.59 (d, *J* = 16.2 Hz, 1H), 4.75 (d, *J* = 16.2 Hz, 1H), 4.73-4.78 (m, 1H). **^{13}C NMR** (75 MHz, CD_3OD): δ 15.3, 22.7, 29.8, 31.3, 51.4, 61.2, 169.4, 170.9, 171.6. **MS (ESI)** (-ve): *m/z* 248 ($M\text{-}H^+$, 12%). **HRMS (ESI)** calcd. for $C_9H_{15}NO_5SNa$ $[M+Na]^+$ *m/z* 272.0569, found 272.0577. **HPLC**: t_R 19.3 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (85:15) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

(*S,R*)-*N*-Acetylleucylalanine methyl ester (134)

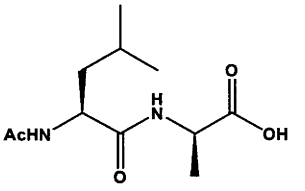


(134)

(*R*)-Alanine methyl ester hydrochloride **(131)** (0.5 g, 3.6 mmol) in *N,N*-dimethylformamide (30 mL) was treated with *N,N'*-diisopropylethylamine (0.8 mL, 4.8 mmol). The mixture was stirred at room temperature for 5 minutes under a nitrogen atmosphere and treated with (*S*)-*N*-acetylleucine **(35)** (0.9 g, 5.3 mmol), BOP (2.4 g, 5.3 mmol) and *N,N'*-diisopropylethylamine (2.5 mL, 14.5 mmol). The mixture was stirred overnight at room temperature, treated with brine solution (50 mL) and extracted with ethyl acetate (3 x 40 mL). The combined organic extracts were washed successively with 0.3M citric acid (3 x 30 mL), saturated sodium bicarbonate solution (3 x 30 mL) and brine solution (3 x 30 mL). The organic material was dried, filtered and concentrated under reduced pressure. The crude product was recrystallised from diethyl ether to yield the title compound **134** (0.1 g, 9%) as a white solid.

Mp. 133-134 °C. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 0.91 (d, *J* = 3.3 Hz, 3H), 0.94 (d, *J* = 3.3 Hz, 3H), 1.40 (d, *J* = 7.2 Hz, 3H), 1.48-1.73 (m, 3H), 2.02 (s, 3H), 3.74 (s, 3H), 4.46-4.58 (m, 2H), 6.22 (br d, *J* = 8.4 Hz, 1H), 6.91 (br d, *J* = 7.2 Hz, 1H). **^{13}C NMR** (75 MHz, CD_3OD): δ 17.9, 22.1, 22.8, 22.9, 24.7, 41.0, 48.0, 51.3, 52.3, 170.4, 172.0, 173.1. **MS (ESI)** (+ve): *m/z* 281 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{12}H_{22}N_2O_4Na$ $[M+Na]^+$ *m/z* 281.1477, found 281.1469. **Anal.** Calcd. for $C_{12}H_{22}N_2O_4$: C, 55.80; H, 8.58; N, 10.84. Found: C, 55.92; H, 8.44; N, 10.86%.

(*S*,*R*)-*N*-Acetylleucylalanine (122)



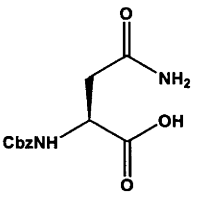
(122)

(*S*,*R*)-*N*-Acetylleucylalanine methyl ester **(134)** (75 mg, 0.3 mmol) in tetrahydrofuran (5 mL) was treated with a solution of lithium hydroxide (10 mg, 0.4 mmol) in water (5 mL). The mixture was stirred overnight at room temperature, acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (4 x 20 mL). The combined organic extracts were dried, filtered, and concentrated under reduced pressure to give the title compound **122** (55 mg, 75%) as a white solid.

Mp. 153-154 °C. **$[\alpha]^{23}_D$** –44.8 (c 0.5, EtOH). **^{1}H NMR** (300 MHz, CD_3OD): δ 0.91 (d, *J* = 6.4 Hz, 3H), 0.95 (d, *J* = 6.4 Hz, 3H), 1.38 (d, *J* = 7.2 Hz, 3H), 1.53-1.67 (m, 3H), 1.98 (s, 3H), 4.33-4.45 (m, 2H). **^{13}C NMR** (75 MHz, CD_3OD): δ 17.7, 22.0, 22.5, 23.4, 25.9, 42.0, 53.0, 173.3, 174.6, 175.8. **MS (ESI)** (+ve): *m/z* 267 ($M+Na^+$, 100%). **HRMS (ESI)** calcd. for $C_{11}H_{20}N_2O_4Na$ $[M+Na]^+$ *m/z* 267.1321, found 267.1316. **Anal.** Calcd. for $C_{11}H_{20}N_2O_4$: C, 54.08; H, 8.25; N, 11.47. Found: C, 54.01; H, 8.42; N, 11.07%.

10.4.7 Experimental for Chapter Eight

(*S*)-*N*-Benzyloxycarbonylasparagine (142)

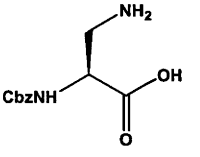


(142)

Benzyl chloroformate (6.5 mL, 45.4 mmol) was added to a mixture of (*S*)-asparagine **(141)** (5.0 g, 37.8 mmol) in saturated sodium bicarbonate (60 mL) and stirred for 3 hours at room temperature then acidified to pH 3 with 2M hydrochloric acid. The white precipitate was filtered, washed with water (15 mL) and a small amount of ice-cold ethanol. The solid was suspended in diethyl ether and filtered to give the title compound **142** (6.4 g, 63%) as a white solid.

Mp. 162-163 °C (lit.,[129] 163-165 °C). **^{1}H NMR** (300 MHz, $(CD_3)_2SO$): δ 2.37-2.57 (m, 2H), 4.28-4.36 (m, 1H), 5.01 (s, 2H), 6.95 (br s, 1H), 7.14-7.36 (m, 6H), 7.49 (br d, J = 8.4 Hz, 1H). **MS (ESI)** (-ve): *m/z* 265 ($M\text{-}H^+$, 5%). **HRMS (ESI)** calcd. for $C_{12}H_{14}N_2O_5Na$ $[M+Na]^+$ *m/z* 289.0800, found 289.0790. The ^{1}H NMR spectral information are consistent with literature data.[130]

(*S*)-*N*-Benzyloxycarbonyl-2,3-diaminopropionic acid (143)



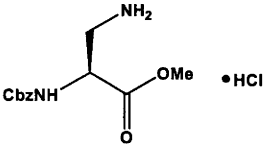
(143)

Diacetoxyiodobenzene (4.3 g, 13.4 mmol) was added to a suspension of (*S*)-*N*-benzyloxycarbonylasparagine **(142)** (3.0 g, 11.2 mmol) in a mixture of ethyl

acetate/acetonitrile/water (48/49/27 mL) and stirred at 15 °C for 30 minutes, warmed to room temperature and stirred for a further 2.5 hours. The mixture was cooled to 5 °C, the white solid was filtered and washed with ethyl acetate (30 mL) to give the title compound **143** (2.2 g, 83%) as a white solid.

Mp. 226-227 °C (lit.,[105] 227-228 °C). **^{1}H NMR** (300 MHz, $(CD_3)_2SO$/TFA): δ 3.01-3.28 (m, 2H), 4.26-4.33 (m, 1H), 5.05 (s, 2H), 7.26-7.34 (m, 5H), 7.69 (d, J = 8.7 Hz, 1H), 7.90 (br s, 3H). **MS (ESI)** (+ve): *m/z* 239 ($M+H^+$, 20%). **HRMS (ESI)** calcd. for $C_{11}H_{15}N_2O_4$ $[M+H]^+$ *m/z* 239.1032, found 239.1024. The ^{1}H NMR spectral data are consistent with those previously reported.[105]

Methyl (*S*)-N^2-benzyloxycarbonyl-2,3-diaminopropionate hydrochloride (137)

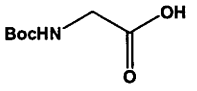


(137)

Thionyl chloride (1.2 mL, 16.7 mmol) was added to a mixture of (*S*)-*N*-benzyloxycarbonyl-2,3-diaminopropionic acid **(143)** (2.0 g, 8.4 mmol) in methanol (35 mL) and stirred overnight at room temperature. Solvent evaporation under reduced pressure gave the title compound **137** (2.1 g, 87%) as a white solid.

Mp. 156-157 °C (lit.,[105] 156-157 °C). **$[\alpha]^{23}_D$** –45.4 (*c* 0.5, EtOH). **^{1}H NMR** (300 MHz, D_2O): δ 3.16 (dd, J = 9.0, 13.6 Hz, 1H), 3.39 (dd, J = 5.1, 13.6 Hz, 1H), 3.70 (s, 3H), 4.50-4.55 (m, 1H), 5.10 (s, 2H), 7.37 (s, 5H). **MS (ESI)** (+ve): *m/z* 253 ($M+H^+$, 20%). **HRMS (ESI)** calcd. for $C_{12}H_{17}N_2O_4$ $[M+H]^+$ *m/z* 253.1188, found 253.1180. The ^{1}H NMR spectral characteristics are consistent with literature data.[105]

_N_-(_t_-Butoxycarbonyl)glycine (147)

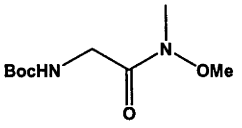


(147)

1M Sodium hydroxide (67 mL) and di-_t_-butyl dicarbonate (14.5 g, 66.6 mmol) were added to a suspension of glycine **(146)** (5.0 g, 66.6 mmol) in tetrahydrofuran (150 mL), stirred overnight at room temperature then acidified to pH 2 with saturated potassium hydrogen sulfate. The mixture was extracted with ethyl acetate (3 x 100 mL), dried, filtered and concentrated under reduced pressure to give the title compound **147** (9.8 g, 84%) as a white solid.

Mp. 87-88 °C (lit.,[131] 88-90 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.45 (s, 9H), 3.91-3.98 (m, 2H), 5.07 (br s, 1H), 6.87 (br s, 1H). **MS (ESI)** (+ve): *m/z* 198 ($M+Na^+$, 77%). **HRMS (ESI)** calcd. for $C_7H_{13}NO_4Na$ $[M+Na]^+$ *m/z* 198.0742, found 198.0730. The ^{1}H NMR spectral data are consistent with reported data.[107]

_N_-(_t_-Butoxycarbonyl)glycine _N_-methoxy-_N_-methyl amide (148)



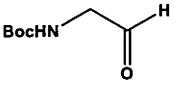
(148)

Triethylamine (4.3 mL, 30.7 mmol) was added to a solution of _N,O_-dimethylhydroxylamine hydrochloride **(96)** (3.0 g, 30.7 mmol) in dichloromethane (40 mL). _N_-(_t_-Butoxycarbonyl)glycine **(147)** (5.9 g, 33.8 mmol) in dichloromethane (30 mL), _N,N'_-dicyclohexylcarbodiimide (8.3 g, 39.8 mmol) and 4-dimethylaminopyridine (3 mg) were added to the mixture and stirred for 1 hour at room temperature. The white precipitate was filtered and the filtrate was diluted with dichloromethane (50 mL) and washed with 0.3M citric acid (3 x 30 mL), saturated sodium bicarbonate solution (3 x 30 mL) and brine solution (3 x 30 mL).

The organic extract was dried, filtered and concentrated under reduced pressure to give a red solid. The crude product was chromatographed on flash silica gel, eluting with hexanes/ethyl acetate (2:1 then 1:1) to give the title compound **148** (2.4 g, 36%) as a white solid.

Mp. 101-102 °C (lit.,[94] 102 °C). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 1.45 (s, 9H), 3.20 (s, 3H), 3.71 (s, 3H), 4.08-4.09 (m, 2H), 5.27 (br s, 1H). **MS (ESI)** (+ve): *m/z* 241 ($M+Na^+$, 35%). **HRMS (ESI)** calcd. for $C_9H_{18}N_2O_4Na$ $[M+Na]^+$ *m/z* 241.1164, found 241.1152. The ^{1}H NMR spectral information are consistent with literature data.[94]

***N*-(*t*-Butoxycarbonyl)glycinal (138)**

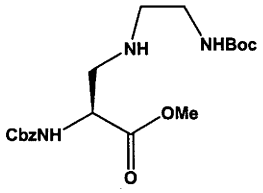


(138)

Lithium aluminium hydride (0.7 g, 18.3 mmol) was added to a mixture of *N*-(*t*-butoxycarbonyl)glycine *N*-methoxy-*N*-methyl amide **(148)** (1.0 g, 4.6 mmol) in tetrahydrofuran (25 mL) under a nitrogen atmosphere. The mixture was stirred for 20 minutes at 0 °C and quenched with a potassium hydrogen sulfate solution (1.4 g in 10 mL of water). The organic layer was concentrated under reduced pressure and the aqueous layer was extracted with dichloromethane (3 x 50 mL). The combined organic extracts were washed with 3M hydrochloric acid (3 x 40 mL), brine solution (3 x 40 mL), saturated sodium bicarbonate solution (3 x 40 mL) and brine solution (3 x 40 mL). The organic extract was dried, filtered and concentrated under reduced pressure to give the title compound **138** (0.7 g, 94%) as a colourless oil. The oil was used in the synthesis methyl (*S*)-N^2-benzyloxycarbonyl-N^6-(*t*-butoxycarbonyl)-2,6-diamino-4-azahexanoate **(139)** without further purification.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.41 (s, 9H), 4.01-4.03 (m, 2H), 9.60 (s, 1H). The ^{1}H NMR spectral characteristics are consistent with those previously reported.[94]

Methyl (*S*)-N^2-benzyloxycarbonyl-N^6-(*t*-butoxycarbonyl)-2,6-diamino-4 -azahexanoate (139)

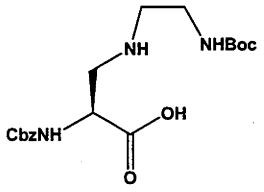


(139)

Methyl (*S*)-N^2-benzyloxycarbonyl-2,3-diaminopropionate hydrochloride **(137)** (1.0 g, 3.5 mmol) was added to a suspension of *N*-(*t*-butoxycarbonyl)glycinal **(138)** (0.7 g, 4.1 mmol) in a 1% acetic acid/methanol solution (25 mL) and stirred at room temperature under a nitrogen atmosphere. Sodium cyanoborohydride (311 mg, 5.0 mmol) was added to the mixture in ten portions over 45 minutes and stirred overnight at room temperature. The mixture was cooled to 0 °C, treated with a 5% sodium bicarbonate solution (100 mL) and washed with ethyl acetate (150 mL). The organic layer was extracted with water (3 x 70 mL), dried, filtered and concentrated under reduced pressure to give a yellow oil which was subjected to flash silica gel column chromatography, eluting with dichloromethane/ethyl acetate (3:1 to 1:1) then ethyl acetate, to yield the title compound **139** (0.6 g, 43%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H), 2.74-2.78 (m, 2H), 3.03-3.11 (m, 2H), 3.19-3.21 (m, 2H), 3.76 (s, 3H), 4.49-4.54 (m, 1H), 5.12 (s, 2H), 4.95, (br s, 1H), 5.99 (br d, J = 7.8 Hz, 1H), 7.31-7.36 (m, 5H). **MS (ESI)** (+ve): *m/z* 396 ($M+H^+$, 100%). The ^{1}H NMR spectral information are consistent with previously reported information.[105]

(*S*)-N^2-Benzyloxycarbonyl-N^6-(*t*-butoxycarbonyl)-2,6-diamino-4-azahexanoic acid (140)

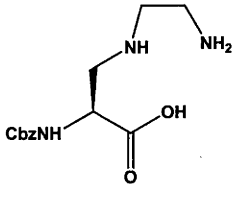


(140)

A suspension of methyl (*S*)-N^2-benzyloxycarbonyl-N^6-(*t*-butoxycarbonyl)-2,6-diamino-4-azahexanoate **(139)** (300 mg, 0.8 mmol) in tetrahydrofuran (15 mL) was treated with lithium hydroxide (25 mg, 1.1 mmol) in water (15 mL) and stirred overnight at room temperature. The mixture was acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **140** (220 mg, 76%) as a colourless oil.

^{1}H NMR (300 MHz, $(CD_3)_2SO$): δ 1.37 (s, 9H), 2.91-2.98 (m, 2H), 3.08-3.24 (m, 4H), 4.19-4.26 (m, 1H), 5.04 (s, 2H), 7.00-7.04 (m, 1H), 7.29-7.37 (m, 5H), 7.55 (br d, J = 8.1 Hz, 1H). **MS (ESI)** (-ve): *m/z* 380 ($M\text{-}H^+$, 55%). **HRMS (ESI)** calcd. for $C_{18}H_{26}N_3O_6$ $[M\text{-}H]^+$ *m/z* 380.1821, found 380.1836.

(*S*)-N^2-Benzyloxycarbonyl-2,6-diamino-4-azahexanoic acid (135)



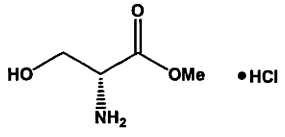
(135)

A mixture of (*S*)-N^2-benzyloxycarbonyl-N^6-(*t*-butoxycarbonyl)-2,6-diamino-4-azahexanoic acid **(140)** (20 mg, 0.06 mmol) in 4M hydrochloric acid in 1,4-dioxane (3 mL) was stirred at 0 °C for 30 minutes and at room temperature for 1 hour.

Solvent evaporation under reduced pressure gave a yellow solid. The crude product was subjected to preparative high performance liquid chromatography to yield the title compound **135** (10 mg, 59%) as a colourless oil.

^{1}H NMR (300 MHz, CD_3OD): δ 3.36-3.42 (m, 5H), 3.54-3.60 (m, 1H), 4.50-4.51 (m, 1H), 5.13 (s, 2H), 7.28-7.39 (m, 5H). **^{13}C NMR** (75 MHz, CD_3OD): δ 36.9, 45.9, 47.6, 67.5, 68.3, 129.0, 129.2, 129.5, 158.9, 171.7, 184.3. **MS (ESI)** (+ve): *m/z* 282 ($M+H^+$, 48%). **HRMS (ESI)** calcd. for $C_{13}H_{20}N_3O_4$ $[M+H]^+$ *m/z* 282.1454, found 282.1462. **HPLC**: t_R 17.6 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (87:13) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

(*S*)-Serine methyl ester hydrochloride (154)

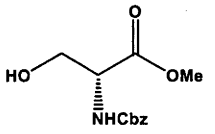


(154)

(*S*)-Serine **(153)** (5.0 g, 47.6 mmol) was added to a mixture of acetyl chloride (9.8 mL, 137.9 mmol) in methanol (65 mL) at 0 °C. The mixture was heated at reflux for 2 hours, cooled to room temperature and solvent evaporation under reduced pressure gave the title compound **154** (5.6 g, 76%) as a white solid.

Mp. 163-164 °C (lit.,[110] 164 °C). **$[\alpha]^{23}_D$** +4.0 (*c* 0.5, EtOH). **^{1}H NMR** (300 MHz, $CDCl_3/(CD_3)_2SO$): δ 3.17 (s, 3H), 3.78-3.83 (m, 2H), 3.93-3.97 (m, 1H), 8.66 (br s, 2H). **MS (ESI)** (-ve): *m/z* 118 ($M-H^+$, 20%). The ^{1}H NMR spectral information are consistent with previously reported data.[110]

(*S*)-2-(*N*-Benzyloxycarbonyl)serine methyl ester (155)

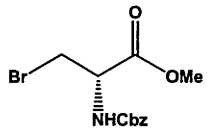


(155)

Triethylamine (14.8 mL, 106.0 mmol) and benzyl chloroformate (7.0 mL, 49.1 mmol) were added to a suspension of (*S*)-serine methyl ester hydrochloride **(154)** (5.0 g, 32.2 mmol) in dichloromethane (100 mL). The mixture was stirred at room temperature for 5 hours and washed with water (3 x 50 mL). The organic extract was dried, filtered and concentrated under reduced pressure to give a yellow oil. The residue was subjected to flash silica gel column chromatography, eluting with hexanes/ethyl acetate (3:1 to 1:1 to 1:2) to yield the title compound **155** (1.1 g, 14%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.76 (s, 3H), 3.88-4.01 (m, 2H), 4.43-4.46 (m, 1H), 5.11 (s, 2H), 5.80 (br s, 1H), 7.31-7.36 (m, 5H). **MS (ESI)** (+ve): *m/z* 276 ($M+Na^+$, 100%). The ^{1}H NMR spectral data are consistent with the literature data.[109]

(*S*)-2-(*N*-Benzyloxycarbonyl)-3-bromo-serine methyl ester (156)



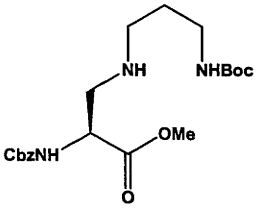
(156)

N-Bromosuccinimide (0.7 g, 3.9 mmol) was added slowly to a mixture of (*S*)-*N*-benzyloxycarbonylserine methyl ester **(155)** (0.5 g, 1.9 mmol) and triphenylphosphine (1.0 g, 3.9 mmol) in *N,N*-dimethylformamide (20 mL). The mixture was heated to 50 °C under a nitrogen atmosphere and stirred for 30 minutes. The mixture was treated with methanol (2 mL), and after 5 minutes, diethyl ether (10 mL) was added. The mixture was washed with water (15 mL), saturated sodium

bicarbonate solution (15 mL) and brine solution (15 mL), then dried and filtered. Solvent evaporation under reduced pressure gave an orange oil. The residue was subjected to flash silica gel column chromatography eluting with hexanes/ethyl acetate (6:1 to 4:1) to yield the title compound **156** (0.2 g, 27%) as a colourless oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 3.72-3.86 (m, 2H), 3.81 (s, 3H), 4.80-4.85 (m, 1H), 5.13 (s, 2H), 5.70 (br d, J = 5.7 Hz, 1H), 7.31-7.38 (m, 5H). **MS (ESI)** (+ve): *m/z* 337 ($M+Na^+$, ^{79}Br, 60%) and 339 ($M+Na^+$, ^{81}Br, 58%). The ^{1}H NMR spectral data obtained are consistent with data previously reported.[109]

Methyl (*S*)-N^2-benzyloxycarbonyl-N^7-(*t*-butoxycarbonyl)-2,7-diamino-4-azaheptanoate (150)



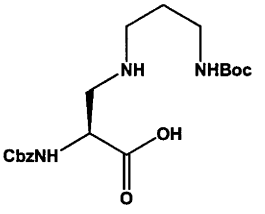
(150)

A mixture of (*S*)-2-(*N*-benzyloxycarbonyl)-3-bromo-serine methyl ester **(156)** (450 mg, 1.4 mmol) in acetonitrile (25 mL) was stirred at room temperature for 1 hour. Potassium fluoride on celite (50% wt) (700 mg) and *N*-(*t*-butoxycarbonyl)-1,3-diaminopropane **(157)** (570 mg, 3.3 mmol) in acetonitrile (2.5 mL) were added to the mixture, which was heated to reflux overnight and cooled to room temperature. The mixture was filtered through a pad of celite, washed with acetonitrile (100 mL) and solvent evaporation under reduced pressure gave a yellow oil. The residue was subjected to flash silica gel column chromatography eluting with chloroform/methanol (99:1 to 97:3) to yield the title compound **150** (220 mg, 27%) as a yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H), 1.56-1.62 (m, 2H), 2.62-2.63 (m 2H), 2.91-3.05 (m, 2H), 3.14-3.16 (m, 2H), 3.75 (s, 3H), 4.42-4.45 (m, 1H), 5.11 (s, 2H),

5.83 (br d, J = 6.6 Hz, 1H), 7.28-7.36 (m, 5H). **MS (ESI)** (+ve): *m/z* 410 ($M+H^+$, 100%). **HRMS (ESI)** calcd. for $C_{20}H_{31}N_3O_6Na$ $[M+Na]^+$ *m/z* 432.2111, found 432.2118. The collected ^1H NMR spectral data are consistent with literature information.[109]

(*S*)-N^2-Benzyloxycarbonyl-N^7-(*t*-butoxycarbonyl)-2,7-diamino-4-azaheptanoic acid (158)

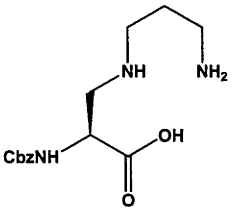


(158)

A suspension of methyl (*S*)-N^2-benzyloxycarbonyl-N^7-(*t*-butoxycarbonyl)-2,7-diamino-4-azaheptanoate **(150)** (300 mg, 0.6 mmol) in tetrahydrofuran (20 mL) was treated with lithium hydroxide (23 mg, 0.9 mmol) in water (20 mL) and stirred overnight at room temperature. The mixture was acidified to pH 3 with 1M hydrochloric acid and extracted with ethyl acetate (3 x 50 mL). The combined organic extracts were dried, filtered and concentrated under reduced pressure to give the title compound **158** (162 mg, 84%) as a colourless oil.

^1H NMR (300 MHz, $(CD_3)_2SO$): δ 1.44 (s, 9H), 1.70-1.75 (m, 2H), 2.86-2.97 (m, 4H), 3.10-3.14 (m, 1H), 3.25-3.30 (m, 1H), 4.30-4.37 (m, 1H), 5.04 (s, 2H), 6.94-6.97 (m, 1H), 7.29-7.36 (m, 5H). 7.71 (d, J = 8. 1 Hz, 1H). **MS (ESI)** (+ve): *m/z* 396 ($M+H^+$, 100%). **HRMS (ESI)** calcd. for $C_{19}H_{30}N_3O_6$ $[M+H]^+$ *m/z* 396.2135, found 396.2120.

(*S*)-N^2-Benzyloxycarbonyl-2,7-diamino-4-azaheptanoic acid (136)



(136)

A mixture of (*S*)-N^2-benzyloxycarbonyl-N^7-(*t*-butoxycarbonyl)-2,7-diamino-4-azaheptanoic acid **(158)** (60 mg, 0.2 mmol) in 4M hydrochloric acid in 1,4-dioxane (4 mL) was stirred at 0 °C for 30 minutes and at room temperature for 1 hour. Solvent evaporation under reduced pressure gave a yellow solid, which was subjected to preparative high performance liquid chromatography to yield the title compound **136** (18 mg, 40%) as a colourless oil.

^{1}H NMR (300 MHz, CD_3OD): δ 2.06-2.14 (m, 2H), 3.01-3.06 (m, 2H), 3.15-3.21 (m, 2H), 3.37-3.40 (m, 1H), 3.54-3.60 (m, 1H), 4.53 (m, 1H), 5.14 (s, 2H), 7.29-7.41 (m, 5H). **^{13}C NMR** (75 MHz, CD_3OD): δ 24.2, 25.1, 30.7, 37.9, 46.1, 68.2, 129.0, 129.2, 129.5, 129.6, (carbonyl carbon signals not detected). **MS (ESI)** (+ve): *m/z* 296 ($M+H^+$, 47%). **HRMS (ESI)** calcd. for $C_{14}H_{22}N_3O_4$ $[M+H]^+$ *m/z* 296.1610, found 296.1618. **HPLC**: t_R 15.2 min (column: YMC-Pack ODS-AQ, 250 x 20 mm; (85:15) water (containing 0.1% TFA): acetonitrile; flow rate: 10.0 cm^3 min^{-1}).

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Now this is not the end
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning.

Sir Winston Churchill (1942)