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THE SYNTHESIS AND EVALUATION OF POTENTIAL TRANSGLUTAMINASE INHIBITORS

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A thesis submitted in partial fulfilment of the requirements of The Nottingham Trent University for the degree of Doctor of Philosophy

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by

Robert Edward Saint

Abstract

Transglutaminases are a group of widely distributed enzymes which catalyse the post-transitional modification of proteins by covalent cross-linking of protein-bound glutamine residues with primary amines.

A number of transglutaminase inhibitors have been previously developed to assist in the investigation of transglutaminases in biological processes, but many are non-selective, toxic to whole cells or insoluble in biological media. The aim of the present research was to improve the synthesis of some of the best known inhibitors and to develop new compounds with improved potency, selectivity and solubility in minimally toxic media.

The main area of research centred on known inhibitors derived from *N*-benzyloxycarbonyl-L-phenylalanine. Existing preparations of these compounds necessitate the use hazardous diazomethane. Novel synthetic routes to these compounds which avoided the use of this reagent were established. In particular a short route to 6-diazo-5-oxo-L-norleucine (DON) (**22**) a key intermediate was developed, involving low temperature ring-opening of *N*-protected pyroglutamates by lithium trimethylsilyldiazomethane; a summary of this work has been published. The ring-opening has also been applied to the preparation of protected 2-aminoadipates.

An alternative approach to related chloromethyl ketone inhibitors by the reaction of pyroglutamates with halogeno-esters and lithium chloromethyltrimethyl-silane was only partially successful, but gave rise to interesting by-products.

Potential irreversible inhibitors, containing vinyl sulfone or α,β-unsaturated ester electrophilic sites, not previously used in transglutaminase inhibitors, were prepared in short synthetic sequences again using ring-opening of γ-lactams.

These last compounds showed no inhibitory activity, but a number of the earlier compounds were active with IC_{50} values of less than 500μM. The most promising compound, a Z-phenylalanyl sulfonium bromide (**56**) has an IC_{50} value of approximately 100μM, is water soluble and of low toxicity, comparable with mercaptoimidazole **7**, a known good transglutaminase inhibitor.

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CONTENTS

ABBREVIATIONS

ALOC	allyloxycarbonyl
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BOC	*tert*-butyloxycarbonyl
DCC	1,3-dicyclohexylcarbodiimide
DCM	dichloromethane
DEAD	diethyl azodicarboxylate
DIBAL	diisobutylaluminium hydride
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DON	6-diazo-5-oxo-L-norleucine
EDTA	ethylenediaminetetraacetic acid
FMOC	9-fluorenylmethoxycarbonyl
GABA	4-aminobutyric acid
GDP	guanosine 5'-diphosphate
Gln	glutamine
Gly	glycine
GTP	guanosine 5'-triphosphate
KF	potassium fluoride
LDA	lithium diisopropylamine
Leu	leucine
LiTMSD	lithium trimethylsilyldiazomethane
Lys	lysine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate buffered saline
Phe	phenylalanine
PKC	protein kinase C
Pro	proline
TCA	trichloroacetic acid
TEA	triethylamine
Tfa	trifluoroacetamide
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMEDA	*N*,*N*,*N'*,*N'*-tetramethylethylenediamine
TMSD	trimethylsilyldiazomethane
TROC	trichloroethoxycarbonyl
Val	valine
Z	benzyloxycarbonyl

INTRODUCTION

1. INTRODUCTION

1.1 INTRODUCTION TO THE BIOLOGY OF TRANSGLUTAMINASES

1.1.1 OCCURRENCE AND ROLE OF TRANSGLUTAMINASES

Transglutaminases are a group of enzymes which occur widely in nature. In vertebrates, transglutaminases are distributed throughout tissue and body fluids where they modify a wide range of proteins, both extracellularly as well as in the cytosol. Examples of the former include the formation of fibrin clots in haemostasis and wound healing, and of the latter the stiffening of erthythrocyte membrane and the formation of the cornifying envelope in keratinocytes. They are distinguishable from each other to a large extent by their physical properties and distribution.

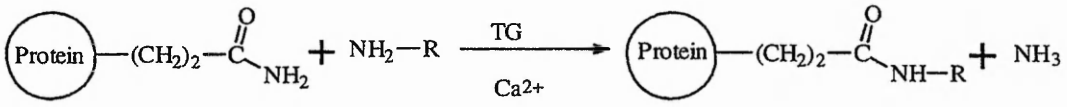
Transglutaminases are also found in invertebrates, plants, unicellular eukaryotes and bacteria. Their mode of action is similar to that of transglutaminases in higher animals except that the requirement for Ca^{2+} ions is reduced. This similarity among transglutaminases suggests that these enzymes appeared early in evolution. However, despite their widespread occurrence, the physiological function of the various transglutaminases is not well understood, with the exception of factor XIIIa which has been extensively studied.

The role of transglutaminases is to catalyse the post-translational modification of proteins leading to formation of an isopeptide bond either within or between polypeptide chains. Since there is no known enzyme which can cleave these peptide bonds, formation is essentially irreversible and, as a result, the action of transglutaminase often leads to the formation of large insoluble supramolecular compounds. This irreversiblility, combined

with the widespread occurrence and high degree of conservation among transglutaminases, indicates that covalent protein cross-linking plays an important role in nature.

1.1.2 MODE OF ACTION

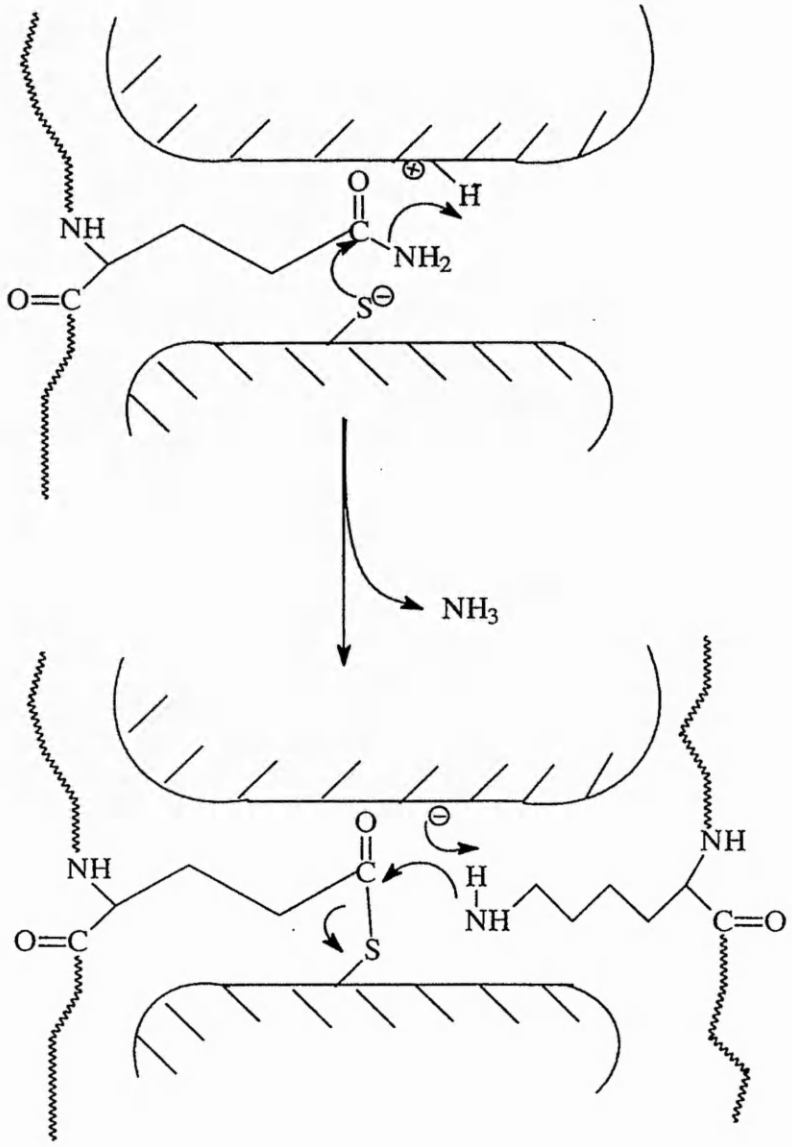
Tranglutaminases catalyse an acyl transfer reaction resulting in the formation of new γ-amide bonds between the γ-carboxamide group of peptide bound glutamine residues and various primary amines[1,2] (**scheme 1**). In higher organisms this reaction is Ca^{2+} dependent.



Scheme 1 The transglutaminase-catalysed acyl transfer reaction.
(R represents either a protein bound lysine residue or an aliphatic amine)

The actual catalytic reaction is a two-step process. The first step is the formation of an acyl-enzyme intermediate between the thiol group, which is in the ionic form at physiological pH, of the active site cysteine and the γ-carboxamide group of a glutamine residue. Protonation results in loss of ammonia, which occurs readily under physiological conditions and provides the thermodynamic basis for the reaction. The second step is attack by the primary amine on the intermediate to yield the peptide bond and regenerate the enzyme (**scheme 2**, p. 3). This second step proceeds with Michaelis-Menton type saturation kinetics.[1,2]

Peptide-bound glutamine residues that act as substrates are very restricted, as specificity of protein-bound glutamine residues is determined both by primary structure



Scheme 2 Reaction mechanism for transglutaminase catalysis.

(including charge) and by conformation.[1,3,4,5] This allows selection of a particular glutamine residue present in the substrate. In addition, specificity or affinity for a glutamine residue present in a substrate differs between transglutaminases. This has been demonstrated by the different fibrin structures produced by the action of tissue transglutaminase and of factor XIII,[6] and by the different affinities for amine incorporation by these enzymes into synthetic

peptide mimics of the amine acceptor site in β-casein.[3,4]

The requirements for the primary amine are less specific, preference being shown for branched chain aliphatic amines with a methylene chain length similar to that of a lysine residue.[1,2] Unlike the glutamyl substrates, adjacent amino acid residues do not greatly affect substrate properties, and this explains why both protein-bound lysine residues and small primary amines can serve as amine donors.

The choice of amine substrate affects biological activity. When the primary amine is an ε-amino group of a peptide-bound lysine residue the reaction results in γ-glutamyl-ε-lysine cross-links either within or between polypeptide chains. When the primary amino groups of some naturally occurring polyamines, such as putrescine and spermidine, are incorporated there is no polymer formation[1,2] although protein modifications occur which may affect biological activity or target protein turnover.

1.2 STRUCTURES OF TRANSGLUTAMINASES

The structures of a number of transglutaminases, mainly from higher organisms, have been established using techniques such as molecular cloning, electron microscopy and sedimentation equilibrium centrifugation.

Comparison of the amino acid sequences between transglutaminases show that they are clearly related, and that the closest similarities are found around the active site. Many of the differences in structure are adaptations to the different roles and biological conditions in which the individual transglutaminases operate. The following examples of vertebrate transglutaminases demonstrate relationships between structure and physiological role.

1.2.1 FACTOR XIII (PLASMA TRANSGLUTAMINASE)

Factor XIII is a plasma protein which circulates in the blood as a tetramer, a_2b_2, composed of two filamentous non-catalytic b-subunits and a dimer of non-covalently associated catalytic a-subunits. Factor XIII is a zymogen which is activated to factor XIIIa by thrombin cleavage of the a-subunit at the NH_2 terminal end of the protein.[6]

The a-subunit consists of 731 amino acids with a molecular mass of approximately 83 kDa[7,8] with the active site cysteine located at position 314.[2] In the intra-cellular environment it exists as the a-subunit dimer[9] which has no disulfide bonding[8] and contains no carbohydrate, despite the presence of a number of free cysteines and potential sites for *N*-linked glycosylation.[7] This is typical of proteins found within the cell, and, indeed, the dimeric a-subunits of factor XIII are found within monocytes, macrophages, megakaryocytes, platelets, uterus and placenta.[9]

The primary structure of the b-subunit is composed of 641 amino acids, about 8.5% carbohydrate, and has a molecular mass of approximately 80 kDa.[10] Sequence analysis shows the b-subunit to be entirely composed of ten tandem repeats of about sixty amino acids. In addition, a 20 amino acid leader sequence commonly found in secretory proteins is present.[11] The tetramic plasma enzyme (a_2b_2) results from secretion of the a-subunit dimer by an unknown pathway into the plasma where it combines with the b-subunits.[9]

1.2.2 TISSUE TRANSGLUTAMINASE

Tissue transglutaminase is widely distributed in varying amounts in different tissues such as the lung, heart, kidney, red blood cells, but is most abundant in the liver and spleen. It exists as a monomeric polypeptide of between 685-691 amino acids with a molecular mass ranging from 75-80 kDa.[12,13]

Tissue transglutaminase contains 17 cysteine residues and 5-6 possible sites for *N*-bonded carbohydrates, but again there are no disulfide bridges[14,15] and no *N*-bound sugar molecules.[1] In addition, the enzyme contains no hydrophobic leader sequence, and has its NH_2 terminus blocked by removal of the initiator methionine and acetylation of the following alanine residue.[16] As previously noted with factor XIII, these are features of proteins of the cytosol. However, tissue transglutaminase is thought to be active in both the extracellular space and interior of the cell, and, in common with factor XIII, secretion is thought to occur via an unknown pathway.[17] It has been speculated that the unusual *N*-acetylation of the alanine residue may be a signal for this unknown secretory pathway, as it has no other known role and no influence on the catalytic activity of the enzyme.[18]

1.2.3 KERATINOCYTE TRANSGLUTAMINASE

Studies of rat and human keratinocyte transglutaminase by cDNA cloning have revealed that the protein consists of 824 amino acids in the rat and 817/813 amino acids in humans, with a molecular mass of approximately 89-90 kDa.[19,20] These studies have also found a high degree of amino acid sequence identity (92%) between rats and humans.

The enzyme has an NH_2 terminal extension comprising a cluster of 5 cysteine residues and a repeating 5-amino acid sequence containing serine and arginine residues.[19] These features are believed to play an important role in anchoring the enzyme to the cytoplasmic side of the plasma membrane.[21] The cysteine cluster attach the enzyme via thioester-linked fatty acylation to palmitic and myristic acid residues,[21,22] while the serine-arginine repeat is similar to that found in phosphorylation sites of protein kinase C.[19,23]

1.2.4 EPIDERMAL TRANSGLUTAMINASE

Epidermal transglutaminase is a proenzyme that requires proteolytic activation and is the least understood of the common vertebrate transglutaminases. Its structure has only recently been described using cDNA cloning from human and mouse epidermis. It was found that the enzyme consists of 692 amino acids, and has a molecular mass of about 77 kDa.[24] The sequence identity between the two species was found to be low at around 75%.

1.2.5 PROSTATE TRANSGLUTAMINASE

Prostate transglutaminase is a dimer of two identical monomers giving a protein of 150 kDa. Each monomer has a molecular mass of 71 kDa and a sequence of 668 amino acids.[25,26] The enzyme is unique to rodents, being a major secretory protein of rat dorsal prostate and coagulating gland, and yet it has no leader sequence and the NH_2 terminal is blocked; both of these being features of intracellular proteins.[26] Additionally, it has been shown to contain mannosyl residues and a phosphatidylinositol anchor. Secretion is thought to occur in apocrine secretory vesicles which are pinched off the apical plasma membrane into the lumen where their contents are released after rupture of the vesicle.[27]

1.2.6 ERYTHROCYTE MEMBRANE PROTEIN BAND 4.2

Band 4.2 is a component of the cytoskeletal structural network which underlies the red blood cell plasma membrane. The protein consists of 691 amino acids with a molecular mass of 77 kDa.[21] The amino acid sequence is similar to other transglutaminases except that an alanine residue has been substituted for the active site cysteine, so rendering the enzyme catalytically inactive.[28] Membrane association is facilitated by acylation via myristic acid residues and phosphorylation.[29]

1.3 REGULATION OF TRANSGLUTAMINASE ACTIVITY AND EXPRESSION

Transglutaminases are controlled by a wide variety of chemical and biological materials. Each transglutaminase has its own unique regulation system. This allows selective induction of the enzyme, either in the presence of other transglutaminases, or at a particular step in a biological cascade process.

1.3.1 SIGNALLING MOLECULES

Of the transglutaminases, two are known to be regulated by signalling molecules, namely tissue transglutaminase and keratinocyte transglutaminase. Their induction is partly controlled by a number of molecules which are specific for the individual enzymes. Indeed, in the case of retinoic acid, tissue transglutaminase is induced while keratinocyte is inhibited. Both proteins are found in the cytoplasm, and may exist in the same cell leading to the evolution of separate induction pathways.

1.3.1.1 TISSUE TRANSGLUTAMINASE

Tissue transglutaminase expression has been shown to be increased several-fold by retinoic acid both *in vitro* and *in vivo*. Retinoids are known to have a wide effect on tissue development such as eye and epithelial formation. Retinoic acid has been shown to act as a morphogen in vertebrate limb development. The induction of tissue transglutaminase occurs at physiological levels of retinoic acid[30] and leads to an intracellular accumulation of the enzyme.[31] This occurs in a number of cell types, such as resident peritoneal macrophages,[30] aortic endothelial cells[32] and hepatocytes.[31]

The actual mediators of tissue transglutaminase induction are the nuclear receptors

for trans-retinoic acid, RAR-β and RAR-γ, and cis-retinoic acid, RXRs. Expression of these receptors, which occur at physiological levels of retinoic acid, lead to an intercellular accumulation of the enzyme.[33] Co-regulation of the retinoid and protein may be due to an oestrogen-type receptor binding site which has been discovered in the promoter region of tissue transglutaminase.[34]

The promoter region was also found to contain response elements for interleukin-6 which is involved in the regulation of the immune response, haemopoiesis and inflammatory reactions and in addition induces differentiation in certain cell types, such as macrophages.[34] Interleukin-6 probably induces concurrent tissue transglutaminase expression in the above biological reactions, but so far its effect on enzyme expression has only been demonstrated in hepatocytes, where increased levels of mRNA and protein have been observed and attributed to this cytokine.[35]

A third molecule shown to induce tissue transglutaminase is the pharmacological agent sodium butyrate.[36] Butyric acid is believed to influence chromatin structure by inhibiting histone deacetylase, thereby causing extensive acetylation of histones.[37] This has numerous effects on cells, such as arrest of growth, and specific induction of various proteins. Another outcome is promotion of differentiation of certain cells into chromaffin like cells, and it is this effect which is accompanied by an increased expression of tissue transglutaminase.[36]

Cells with high levels of the enzyme, due to induction by retinoic acid or sodium butyrate, show a different morphology when compared to their normal counterparts. The cells are flattened and with a spindle-like shape; these features are probably caused by an increased adhesion of the cells to the substratum.[32,36]

1.3.1.2 KERATINOCYTE TRANSGLUTAMINASE

Keratinocyte transglutaminase plays a major role in the cross-linking of the cornified envelope during terminal differentiation of keratinocytes. Regulation of the enzyme is believed to be via the phospholipase C and protein kinase C (PKC) dependent signal transduction pathway. This route involves phosphoinositide turnover, intracellular Ca^{2+} concentration and diacylglycerol. The promoter region of both rabbit and human keratinocyte transglutaminase was found to contain a number of regulatory sites, in particular an AP1 site. AP1 sites are transcriptional regulatory elements for PKC activators such as phorbol esters which are, along with glucocorticoids, known to induce the enzyme.[38]

Tissue transglutaminase has a number of regulation sites in common with keratinocyte transglutaminase.[34] However the AP1 site is absent and this, combined with the inhibitory effect of retinoic acid on keratinocyte tranglutaminase, allows the two enzymes to co-exist within the same cell and yet be differentially regulated.[38,39,40]

1.3.2 PROTEOLYSIS OF ZYMOGENS

Several transglutaminases exist as zymogens and require proteolysis to convert them to their catalytic form. Of these factor XIII and epidermal transglutaminase are the most well characterised.

1.3.2.1 FACTOR XIII

Activation of the factor XIII tetramic proenzyme (a_2b_2) to the active dimeric factor XIIIa (a_2) occurs in the plasma and is a two step process. The initial step is thrombin-catalysed cleavage of the a-subunits at an arginine residue at position 37. This yields two

37 amino acid fragments and the modified tetramer (a'_2b_2).[7,8] The second step is Ca^{2+} mediated dissociation of the b-subunits to afford the active enzyme.[41] Intracellular factor XIII (a_2), as found in platelets for example, is activated by thrombin cleavage alone.[41]

Thrombin has also been found to cleave factor XIIIa at a second specific site releasing a 24kDa fragment from the COOH terminus of the molecule.[7] This cleavage occurs between a lysine and a serine residue at positions 513 and 514 respectively. This peptide bond is not a particularly good substrate for thrombin and the effect of cleavage at this site is not well understood.[7,9]

1.3.2.2 EPIDERMAL TRANSGLUTAMINASE

The epidermal transglutaminase zymogen is cleaved at a serine residue at position 469. The cleavage site is a unique insert of approximately 9 amino acids which varies widely between species.[24] Cleavage can be accomplished by a number of proteases including trypsin, thrombin, proteinase K and dispase; however, in the epidermis, the Ca^{2+} dependent calpains are probably responsible.[42]

Proteolysis affords a 50 kDa fragment which contains the active site residue and a second 27kDa carboxy-terminal fragment. Both fragments remain non-covalently associated which suggests that cleavage leads to a conformational change which activates the enzyme possibly by exposing the active site cysteine.[24,42]

1.3.3 DIVALENT CATIONS AND NUCLEOTIDES

Ca^{2+} binding is mandatory for activation of all vertebrate transglutaminases but this requirement is less strict in plants and micro-organisms. Sequence analysis of

transglutaminase genes revealed no typical Ca^{2+} binding pattern, such as the EF-hand structure which comprises the calcium binding site of many other proteins such as calmodulin. The Ca^{2+} binding sites in transglutaminases are of low affinity and probably have a different structure to proteins with high Ca^{2+} binding affinity.[7,13,20,26]

Tissue transglutaminase was found to bind 3-4 Ca^{2+} ions with a K_d of 1.0 ± 0.6 $x10^{-3}$ M by equilibrium dialysis. Binding leads to a conformational change and, as there are at least two sites with different affinities, this leads to the possibility of several enzyme-metal complexes. Ca^{2+} bound to only one site produces an enzyme capable of ester hydrolysis, whereas for the acyl transfer reaction Ca^{2+} ions must be bound to both sites. Although this ester hydrolysis reaction has been demonstrated *in vitro* at very low calcium levels, at physiological Ca^{2+} concentrations the enzyme will have all its binding sites occupied.[43] Specificity for cation binding is high and only Sr^{2+}, albeit at a 10-fold greater concentration, can replace Ca^{2+} and activate the enzyme.[43]

Some metals such as Fe^{2+}, Cu^{2+}, Zn^{2+} and Hg^{2+} strongly inhibit transglutaminase activity in the presence of Ca^{2+}.[2,15,43] Inactivation of tissue transglutaminase by Cu^{2+} has been shown to be due to oxidation of free thiol groups to intramolecular disulfide bonds, but intermolecular bonds have not been observed.[15] Zn^{2+} has been shown to be a competitive inhibitor of factor XIII and tissue transglutaminase[2] with a K_i of approximately 10^{-7} M. The mechanism of inhibition is different from other metal ions in that no disulfide bonds are formed. This may indicate a role for Zn^{2+} in transglutaminase regulation, as this ion shows an inverse intra- to extracellular concentration relationship with Ca^{2+}, and so it may contribute toward maintaining the intracellular enzyme in an inactive state.[44]

Tissue transglutaminase appears to have its activity regulated by guanosine nucleotides.[45] Guanosine 5'-triphosphate (GTP) and guanosine 5'-diphosphate (GDP) bind to tissue transglutaminase in a reversible manner but do not affect the activity of other transglutaminases such as factor XIII. This binding leads to conformational changes which give inhibition of enzyme activity. The inhibitory effect can be partially reversed by addition of Ca^{2+}, suggesting that the local concentration of GTP and Ca^{2+} may play a part in the regulation of the enzyme activity *in vivo*.[46]

1.4 PHYSIOLOGICAL FUNCTIONS OF TRANSGLUTAMINASES

1.4.1 FACTOR XIII

Factor XIII is involved in the formation of the fibrin clot and wound healing.[2] It binds to the Aα- and Bβ-chains of the D domain of fibrinogen with high affinity, and thus is found in close association with fibrinogen in the blood plasma.[47,48] The zymogen (factor XIII) is activated to the enzyme (factor XIIIa) in the last stages of the vertebrate coagulation cascade.[2] Its main role is first to catalyse cross-linking between fibrin γ-chains to form γ-chain dimers,[49] then to covalently stabilise the α- and γ-chains.[50,51] These reactions lead to the formation of an insoluble fibrin clot of defined physical properties which cannot be degraded by proteolytic enzymes or other biological agents.

A second reaction catalysed by factor XIIIa is cross-linking of α_2-plasmin inhibitor to the α-chain of fibrin,[52] which results in protection of the fibrin clot from attack by plasmin.[53] Factor XIIIa may also be responsible for cross-linking a number of proteins which occur in the blood plasma or vascular walls. These proteins include fibronectin,[8]

collagen[54] and thrombospondin[55] which, when cross-linked, may contribute to stability of the platelet-fibrin-endothelium association and to wound healing.[56]

1.4.2 TISSUE TRANSGLUTAMINASE

Tissue transglutaminase is involved in the stabilisation of extracellular matrices and in formation of cross-linked cell envelopes in programmed cell death (apoptosis). Tissue transglutaminase was the first transglutaminase described,[57] and is present in large amounts in a broad range of vertebrate tissues.[58,59] Despite this, its physiological function is not fully understood. It is thought to have two main functions, one inside the cell and the other in the extracellular space.

In the extracellular space Ca^{2+} concentration is high enough for enzyme activity and inhibitors such as GTP and Zn^{2+} are largely missing. In addition, a number of proteins, such as fibrinogen,[48] fibronectin,[8,60] vitronectin,[61,62] collagen type III[63] and collagen type II,[59] which have been shown to be substrates for the enzyme *in vitro*, are present. It is not clear, however, whether these proteins are cross-linked *in vivo*. An *in vitro* experiment has shown that fibrinogen and fibronectin bind to the surface of hepatocytes and endothelial cells and are cross-linked by tissue transglutaminase into the pericellular matrix.[64,65] Additionally, tissue transglutaminase has been shown to be involved in laminin-nidogen stability in basement membranes,[58] matrix cross-linking in long bone development,[59] and events in wound healing and excessive tissue repair such as formation of the highly insoluble matrix found in cirrhotic liver.[66]

The second function, apoptosis, is an important process in a number of biological events such as metamorphosis, embryonic morphogenesis and hormone-induced tissue

remodelling. The role of tissue transglutaminase in apoptosis is well established in events such as the cross-linking of plasma membrane and cytoskeletal components in the terminal steps of erythrocyte maturation.[2] It is believed that the enzyme cross-links intracellular proteins band 3, band 4.1, spectrin, actin and ankyrin and this results in the changed morphology of the cell.[2]

Activation of tissue transglutaminase within the cell is probably due to an increase in Ca^{2+} concentration in the cytoplasm. There are two ways of increasing Ca^{2+} concentration within the cell. The first is via intracellular Ca^{2+} compartments within the cells; this has been observed in terminal differentiation of keratinocytes. The second is by influx of Ca^{2+} from the extracellular spaces. This is the route observed in terminal maturation of erythrocytes.[2]

1.4.3 KERATINOCYTE AND EPIDERMAL TRANSGLUTAMINASE

Keratinocyte and epidermal transglutaminases are involved in the terminal differentiation of epithelia and formation of the cornified envelope in the epidermis. When epidermal cells undergo terminal differentiation several proteins such as keratolinin, involucrin, cornifin and sciellin are believed to be cross-linked to form the cornified envelope, a 10-20 nm thick deposit of protein on the intracellular surface of the plasma membrane. Both disulfide bonding (between keratins) and transglutaminase cross-links are present. The transglutaminases are activated by influx of Ca^{2+} into the cytoplasm when the cell membranes lose their integrity during the final maturation steps.[2]

Keratinocyte transglutaminase is membrane bound and found in close association with its substrate proteins within the supra-basal layers of the epidermis. It is thought to be the main transglutaminase involved in squamous differentiation of epithelia[9,67] because of

its abundance in these tissues[24] but is also present in epithelia that do not normally keratinise, such as the lung, liver and intestine.[39]

Epidermal transglutaminase is found in solution in the cytosol of epidermal cells which are in the advanced stages of terminal differentiation and in embryologically related hair follicle cells.[24,42,68] Tissue transglutaminase probably contributes to the formation of the cornified envelope in the latter stages of epidermal differentiation and may have a role in hair shaft formation by cross-linking proteins like trichohyalin.[69]

1.4.4 PROSTATE TRANSGLUTAMINASE

Prostate transglutaminase of rodents such as the rat and guinea pig is responsible for the rapid coagulation of the seminal fluid to form a copulatory plug.[25] The copulatory plug is rich in γ-glutamyl-ε-lysine cross-links which are formed between proteins derived from seminal vesicles secreted by the dorsal prostate and coagulation gland.[26,70] Prostate transglutaminase is rich in these tissues and constitutes up to 25% of the total intracellular protein.[25,27] Prevention of premature coagulation of semen is probably achieved by the presence of excess polyamines, for example spermine and spermidine, which compete with the seminal vesicle proteins. The polyamines have been shown to be produced in the ventral prostate of certain species.[70]

1.4.5 BAND 4.2 PROTEIN

Band 4.2 protein is a catalytically inactive transglutaminase. It is a structural component of the cytoskeletal network underlying the erythrocyte plasma membrane, and is also present in other cells and tissues such as platelets, kidney and brain. Band 4.2 is found in association with band 3, an anion exchanger protein, and may also interact with ankyrin

and band 4.1 protein.[28,44] These proteins are known to be substrates for tissue transglutasminase. This suggests that band 4.2 may still have a transglutaminase protein binding site and so it may have a role in the inhibition of other transglutaminase from premature activity within the cell.

1.5 TRANSGLUTAMINASE INHIBITORS

1.5.1 ROLE OF ENZYME INHIBITORS

Enzyme inhibitors play important roles in the study of physiological and pharmacological phenomena. They are widely employed in affinity labelling and active site mapping, which give information about the active site, the location and requirements of substrate binding areas and conformational changes which occur during the catalysis cycle.

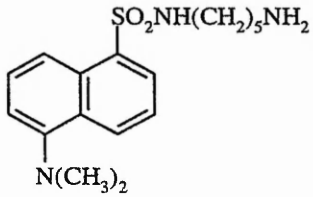
Two types of inhibitor are employed, namely competitive and irreversible. Competitive inhibitors mimic the enzyme substrate and allow features of the binding site to be explored. These inhibitors are incorporated into the enzyme product, and the relative rate of their uptake allows conclusions to be drawn about structure-affinity relationships between the inhibitor and the binding site. Irreversible inhibitors bind covalently to some region, often the active site, of the enzyme. The resulting enzyme-inhibitor complex gives access to the active site and can also report on conformational changes that occur in the presence or absence of enzyme activators.

Although much information can be gained *in vitro* from inhibitors which are general in their action, the design and synthesis of highly specific inactivators is required if the role of a particular enzyme is to studied *in vivo*.

1.5.2 COMPETITIVE AMINE INHIBITORS

1.5.2.1 DANSYLCADAVERINE AND RELATED INHIBITORS

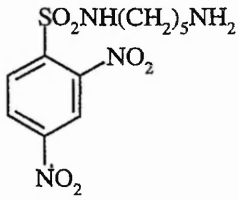
Although a wide range of primary amines can act as inhibitors, early studies on competitive amine inhibition of fibrin cross-linking found a high degree of specificity for the amine. These studies showed that the best amine substrates required a suitably positioned hydrophobic substituent and an alkylamine side chain of specific length. This arrangement was believed to mimic the lysyl residue of the protein substrates and led to the design and synthesis of one of the first specific inhibitors, *N*-(5-aminopentyl)-5-(dimethylamino)-1-naphthalenesulfonamide or dansylcadaverine.[71,72]



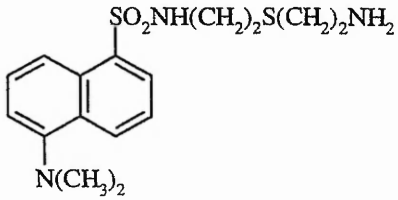
dansylcadaverine

Later studies attempted to optimise the structure-affinity relationship of the amine for the enzyme.[73] A number of known and novel synthetic substrates were studied, and for convenience their relative affinities for the enzyme were compared with the relative affinity of dansylcadaverine. Of the compounds tested, three had affinities essentially the same as dansylcadaverine; they were (2,4-dinitrobenzenesulfonyl)cadaverine, (2,4,6-triisopropyl-benzenesulfonyl)cadaverine and dansyl-3-thiacadaverine.

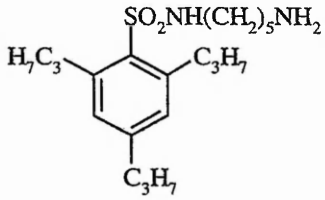
Modification of the alkylamine chain length of dansylcadaverine showed that optimum activity was achieved with 5 methylene groups, a feature which was common to



(2,4-dinitrobenzenesulfonyl)cadaverine

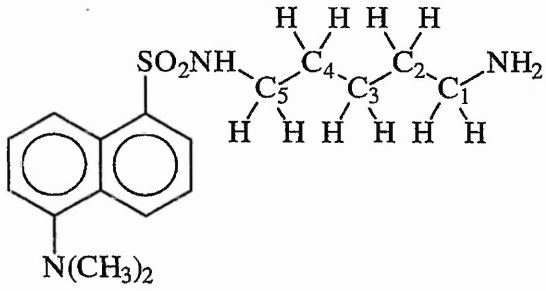


dansylthiacadaverine



(2,4,6-triisopropylbenzenesulfonyl)cadaverine

all the most potent compounds. Atomic modelling of dansylcadaverine and dansyl-3-thiacadaverine indicated the chain length between the terminal primary amine group and the C-5 methylene carbon to be 7.2Å in dansylcadaverine and 7.6Å in dansylthiacadaverine. These distances are comparable to the length (7.5Å) between the ε-amino group and α-carbon of lysyl side chains in polypeptides.



Three other important factors which affect amine-enzyme affinity were determined in this study. The first was the effect of the cross-sectional area of the alkylamine side chain on activity. To this end, a number of amines were tested which had modification at the C3-

position on the side-chain. Even relatively small modifications to the cross-sectional dimensions, such as substitution of -S- (3.4Å) for -CH_2- (2.8Å), led to reduced amine-enzyme affinity. Introduction of very much bulkier substituents, such as gem-dimethyl branching (4.7Å), led to a dramatic reduction in affinity. This information suggests that the primary binding site for the amine is a narrow crevice into which the alkylamine side-chain fits precisely.

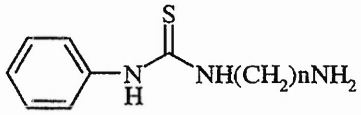
The second factor found was that at around 6 atoms distant from the primary amine group the primary binding site can accommodate much larger substituents such as the -SO_2-NH- group found in the best inhibitors. In addition, replacement of the sulfonamide link with other substituents such as an amide or amine group reduced affinity.

The third factor was the effect of a hydrophobic locus on the molecule. The significance of a hydrophobic area on the amine was demonstrated by the 200-fold difference in affinity between dansylcadaverine and *n*-butylamine. The hydrophobic area corresponds to a secondary binding site, and probably mimics the area around the protein-bound lysyl residue in the transglutaminase substrate.

1.5.2.2 *N*-PHENYL-*N'*-(ω-AMINOALKYL)THIOUREA INHIBITORS

Although dansylcadaverine and its analogues are effective inhibitors of transglutaminase, they have also been found to inhibit other enzymes, such as 17-ketosteroid reductase,[74] phosphatidylcholine synthetase[75] and the calmodulin activation of cyclic nucleotide phosphodiesterase.[76] Consequently, competitive amine inhibitors specific for transglutaminases in cellular systems have been developed. A number of phenylthiourea derivatives of ω-aminoalkanes have been prepared, and initial biological studies showed

them to have no adverse affects on several cellular processes.[77]



***N*-Phenyl-*N* '-(ω-aminoalkyl)thioureas**

n = 2, 3, 4, 5 and 6

These compounds were designed with three structural features: firstly, a primary amino group to compete as a substrate for the acyl-enzyme complex; secondly, having a hydrophobic region to facilitate binding to the secondary binding site and; thirdly, a thiourea group to increase solubility in physiological media.

The compounds were tested for inhibition of both guinea-pig liver transglutaminase-catalysed amine incorporation into various glutamine containing substrates, and plasma transglutaminase-catalysed (factor XIIIa) amine incorporation into fibrin and fibrin cross-linking. The peak in inhibitory activity was achieved with the pentylamine side chain, which is in agreement with the results reported for the dansylcadaverine analogues. The inhibitory activity of the best phenylthiourea diaminoalkane derivative was reportedly to be some two orders of magnitude greater than that of previously reported amines. In addition, as indicated by their LD_{50} values in mice, these compounds were found to be far less toxic than dansylcadaverine; surprisingly, this class of inhibitor has not been further developed.

1.5.3 COMPETITIVE GLUTAMINE INHIBITORS

1.5.3.1 ALIPHATIC AMIDE INHIBITORS

Folk and Gross[78] undertook an investigation into the factors which contribute toward the specificity of transglutaminase toward amide substrates. To this end, a series of aliphatic amides were tested for transglutaminase-mediated [^{14}C] methylamine incorporation. Although all the amides were found to be poor substrates, the data did show some distinctive trends.

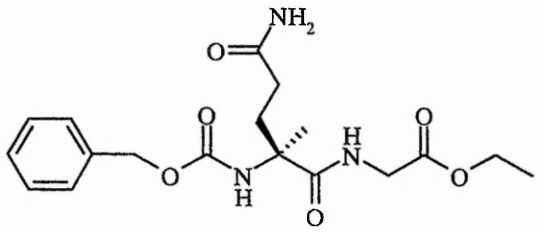
The straight chain amides acetamide, propionamide, *n*-butyramide, *n*-valeramide and *n*-caproamide were all found to be transglutaminase substrates. Compounds with branching α or β to the amide group (α-methylpropionamide and β-methylbutyamide) showed no activity even after extended incubation times. Conversely amides with branching at the γ-position did show substrate properties. The affinity of the straight and γ-branched chain amides for the enzyme suggested to Folk and Gross that the active site thiol residue was located at the end of a pocket or crevice into which the carboxamide and α- and β-methylene groups must fit for productive binding. Atomic modelling estimated the dimensions of this pocket to be approximately 5 x 5 Å.

1.5.3.2 DIPEPTIDE INHIBITORS

Among the first competitive glutamine inhibitors used in the study of transglutaminase were the benzyloxycarbonyl (Z)-containing dipeptides; Z-glycylglycine, Z-L-methionylglycine and Z-γ-ethyl-α-L-glutamylglycine. These compounds were found by Folk and Gross[79] to inhibit hydroxylamine incorporation into Z-L-glutamylglycine with significantly lower K_m values than those of the analogous non-Z-containing dipeptides,

N-benzyloyl-L-glutaminylglycine, *N*-formyl-L-glutaminylglycine and *N*-methyloxycarbonyl-L-glutaminylglycine. These results suggest that the compounds have a common binding site which does not include the active site. The nature of this site was postulated to be the benzyloxycarbonyl group, a hydrophobic region similar to that found in the secondary amine binding site discussed in the previous section.

In addition to this study, a series of dipeptide derivatives of α-, β- and γ-methyl-glutamine containing the Z-group was tested for substrate properties. Of the peptides, only Z-L-α-methylglutamylglycine ethyl ester showed any significant activity. Comparison of this compound with Z-L-glutamylglycine showed that the presence of an α-methyl group hindered reaction with the enzyme. These results confirm those obtained with aliphatic amides where branching in the glutamine side chain prevents constructive binding, and the cysteine in the active site is located at the end of a crevice or pocket.



Z-L-α-methylglutamylglycine ethyl ester

1.5.3.3 PEPTIDE INHIBITORS

A number of peptide inhibitors have been developed from the analysis of amino acid sequences found around the glutamine residues of transglutaminase substrates. An early synthetic peptide inhibitor described by Folk and Gorman[80] contained the sequence; Leu-

Gly-Leu-Gly-Gln-Gly-Lys-Val-Leu-GlyNH_2. This compound was a modified version of the peptide sequence found around the glutamine residue of β-casein, a very strong macromolecular substrate for factor XIIIa. The modification involved substitution of three glycine residues for serine. The resulting compound showed a decrease in activity toward factor XIIIa, but it was considered to represent the minimal structural elements required for efficient expression of the enzyme and so to provide a basis for the design of active site directed peptide inhibitors. Additionally, it considerably simplified the synthesis by removing the reactive side chain (aliphatic hydroxyl group) of serine which could undergo side reactions.

Synthetic peptides were developed by Greenberg *et al.* [81] which enabled the regions within factor XIIIa which are involved in substrate recognition to be determined. The group synthesised a number of peptide sequences from various regions of factor XIIIa and studied their effects on the cross-linking of fibrin, *N,N'*-dimethylcasein or fibronectin. It was found that two peptides, corresponding to regions Asn^{72}-Asp^{97} and Asp^{190}-Phe,[230] inhibited factor XIIIa cross-linking of these substrates. Addition of excess substrate reversed the inhibition of cross-linking, suggesting that the peptides were not interacting with the enzyme but instead were binding to the substrate. No evidence was found that the peptides were affecting the active site or any other region of factor XIIIa. The peptides were also not found to be cross-linked to fibrin. These results led to the conclusion that the two peptide sequences corresponded to regions outside the active site pocket which are important for substrate recognition in factor XIIIa.

A recent paper by Kim *et al.*[82] describes the action against keratinocyte transglutaminase of a number of small peptides derived by simplification of six synthetic

peptides designed from domains of known substrates of the keratinocyte enzyme. The molecules work in a competitive manner, with the two most effective compounds being tetramers with the sequences Pro-Val-Lys-Gly and Val-Lys-Gly-Arg and IC_{50} values of 165 and 144 μM, respectively. These compounds both contain lysine residues as the competitive inhibitor site. The best compound with a glutamine residue as the inhibitory site is also a tetramer, with the sequence Gly-Gln-Asp-Pro and an IC_{50} of 220 μM.

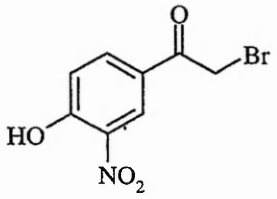
1.5.4 IRREVERSIBLE INHIBITORS

1.5.4.1 IODOACETAMIDE

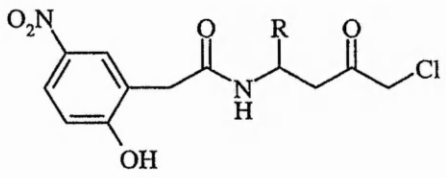
The first irreversible inhibitor used in the study of transglutaminase was the simple alkylating agent iodoacetamide. Iodoacetamide was found to alkylate the active site thiol rapidly and selectively, even though transglutaminase contains 17 or 18 cysteine residues with their associated thiol groups. Alkylation occurred in the presence of calcium ions at between pH 6 and 7. These results gave initial information on the active site and mode of catalysis of transglutaminase.[79,83]

1.5.4.2 PHENOL-CONTAINING HALOMETHYL KETONE INHIBITORS

Another early series of irreversible inhibitors, synthesised[84] specifically to explore the environment in the catalytic centre of transglutaminase, were the nitrophenol-containing halomethyl ketones α-bromo-4-hydroxy-3-nitroacetophenone (BHNA), methyl *N*-(2-hydroxy-5-nitrophenylacetyl)-L-2-amino-4-oxo-5-chloropentanoate (PACK) and 1-chloro-4-(2-hydroxy-5-nitrophenylacetyl)amidobutan-2-one (PBCK) as shown overleaf.



BHNA



PACK R = CO_2CH_3
PBCK R = H

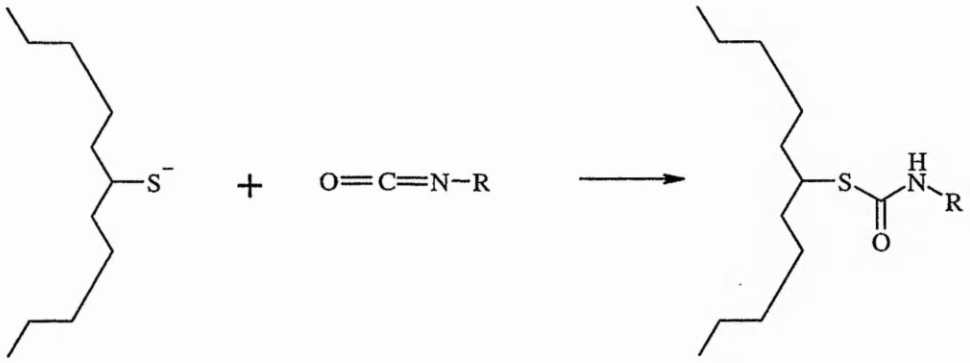
The inhibitor-enzyme complexes obtained with the above compounds enabled a number of features of the active site of transglutaminase to be explored via the induced changes in the visible spectrum and pK_a of the phenol group.

The changes in the spectrum and pK_a of the enzyme-inhibitor complex showed that the active site thiol was located in a hydrophobic region in the Ca^{2+}-activated enzyme, whereas, in the absence of Ca^{2+}, the complex was shown to be in a hydrophilic environment. This was early evidence of the Ca^{2+} ion-mediated conformational change which is important for enzyme activity.[85]

1.5.4.3 ALKYL ISOCYANATE INHIBITORS

The use of alkyl isocyanates as active site inhibitors of transglutaminase has been demonstrated. These compounds, however, have poor selectivity and have been shown to react with a wide variety of enzymes.

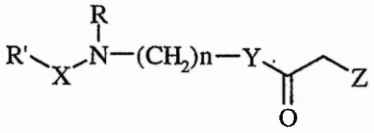
Irreversible inhibition by alkyl isocyanates was shown to proceed via *N*-alkyl thiocarbamoyl ester formation with the active site thiol group[85] as shown in **scheme 3** overleaf.



Scheme 3 Formation of the *N*-alkyl thiocarbamyl ester inactivation complex.

1.5.4.4 α-HALOMETHYLCARBONYL INHIBITORS

Among the first specific inhibitors synthesised using information gleaned from the earlier studies was a class of compounds of the general formula shown below.



α-Halomethylcarbonyl inhibitors of Factor XIIIa

(R' = aryl; R = H, alkyl, benzyl; X = -SO_2-, -CO-; Y= -O-, -NH-, -CH_2; Z = Cl, Br)

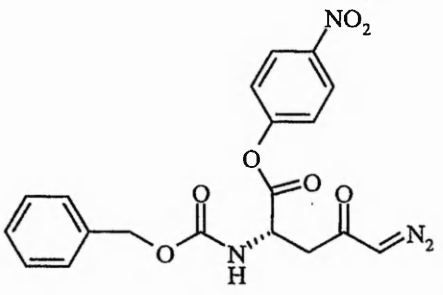
The R' and X portion of the molecule were designed to mimic the secondary binding site, thought to be the primary recognition site for the enzyme. The ground work for these features were the early studies on dansylcadaverine. The α-halomethyl ketone (-CO-CH_2-Z) portion of the molecule was incorporated as the inhibitory site, and was chosen because of the known ready inactivation of active site cysteine groups by this moiety. The Y groups were incorporated to explore the effect of substituents at that

position on binding and enzyme inactivation.[86]

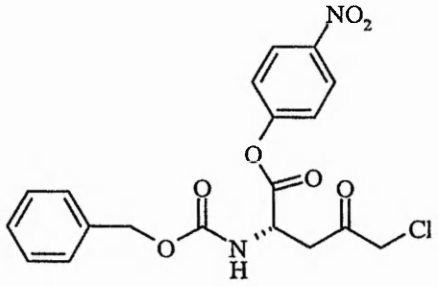
The inhibitory activities of the various compounds were shown to be strongly dependent on their structure. Replacement of $-SO_2-$ with -CO- at position X and substitution of $-CH_2-$ with either -NH- or -O- at position Y led to reductions in activity. As was previously observed in the studies on dansylcadaverine, the optimum side chain length is 5-methylene groups; increasing or decreasing the chain length leads to loss of activity. 1-Chloro-7-tosylamidoheptan-2-one was shown to be a good inhibitor, causing inhibition of fibrin stabilisation at concentrations of approximately 20 μM.

1.5.4.5 DIAZOKETONE AND CHLOROMETHYL KETONE INHIBITORS DERIVED FROM ASPARTIC AND GLUTAMIC ACID

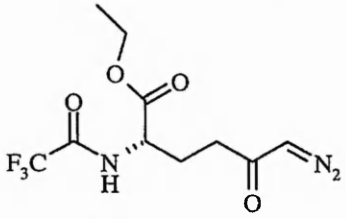
Levitzki *et al.*[87] reported the action of *N*-benzyloxycarbonyl-5-diazo-4-oxo-norvaline *p*-nitrophenyl ester (BDONV) and its homologous chloro-derivative (BClONV) in the inhibition of clustering and internalisation of α_2-macroglobin in rat kidney cells. The reported concentration which gave 50% inhibition of α_2-macroglobin was observed as 0.10 mM for BDONV. Although the value for BClONV was not disclosed, it was reported to



BDONV



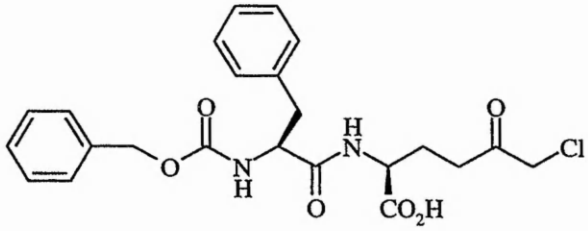
BClONV



TDONL

give strong inhibition. Comparison of these compounds with amine inhibitors showed them to be superior to all except dansycadaverine (50% inhibition at 0.015 mM). The closely related compound *N*-trifluoroacetyl-6-diazo-5-oxonorleucine ethyl ester (TDONL) gave no significant inhibition.

A dipeptide chloromethyl ketone inhibitor of the structure shown below has been prepared and evaluated by Dr El Aloui[88] at the University of Lyon as part of his Ph.D. dissertation.

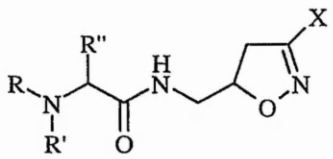


The incorporation of the Z-phenylalanine group is a feature of a number of recent inhibitors (see following sections). This moiety seems to alter the chain length required between the irreversible binding site and the hydrophobic locus of the molecule to one which corresponds to a glutamine mimic.

1.5.4.6 DIHYDROISOXAZOLE INHIBITORS

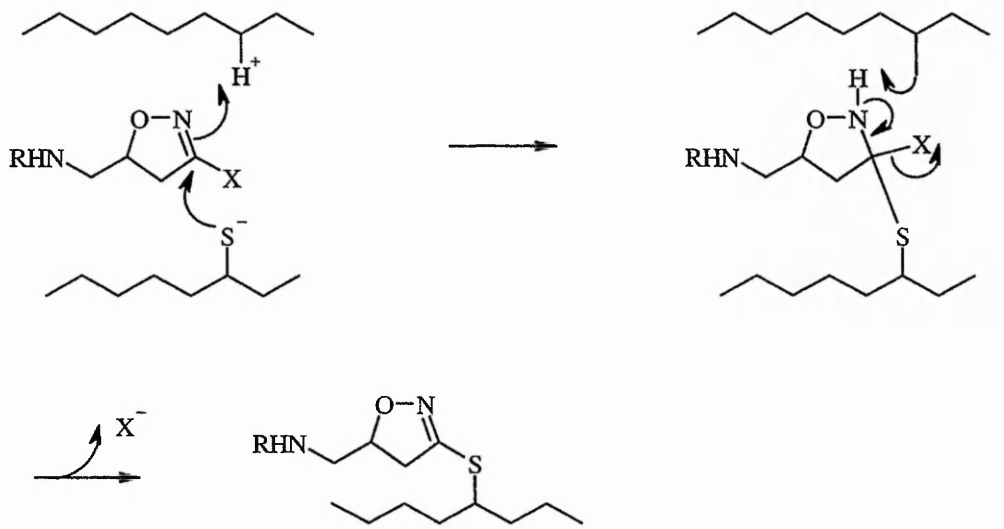
3,5-Disubstituted-4,5-dihydroisoxazoles were synthesised by a group at Syntex and shown to be specific transglutaminase inhibitors. They are particularly effective against epidermal transglutaminase, and have been patented as a treatment for disease states characterised by increased transglutaminase activity, in particular acne and psoriasis.[89]

The patent describes the dihydroisoxazole ring as attached via substitution at C-5 to an amino acid, in which the *N*-terminus is protected by a range of aryl or acyl groups (R and R') and the side chain consists of an aryl group (R").



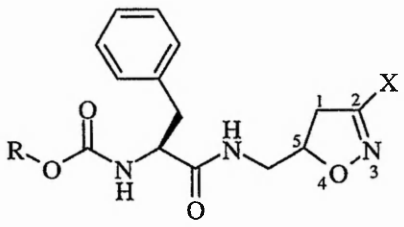
Dihydroisoxazoles

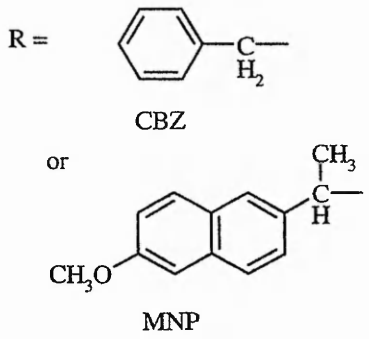
The C-3 substituent, where X is either a halogen or an ether grouping, is attacked by the active site thiol to give a tetrahedral intermediate which then breaks down to give a stable thioimine adduct (**scheme 4**).



Scheme 4 Mechanism for the inactivation of transglutaminase by dihydroisoxazoles.

The published literature dealt mainly with the compounds shown below.[90]

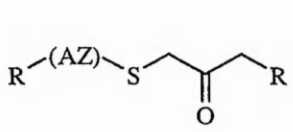


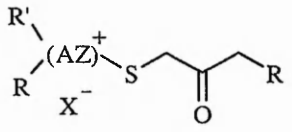


Chirality at C-5 of the oxazole was found to be important, with the S-isomer having the greater potency. The bromo-substituted compounds were most effective at inhibiting bovine epidermal transglutaminase. However, no significant differences in potency were observed between bromo- and chloro-substitution in the inhibition of cross-linked envelope formation by human SCC-9 malignant keratinocytes. The reverse was found for *N*-terminal substitution. The 2-(S)-(6-methoxy-2-naphthyl)propanyl group (MNP) improved inhibition of envelope formation, but there was no significant difference between the Z and MNP groups in enzyme inactivation.

1.5.4.7 AZOLES AND AZOLIUM SALTS AS INHIBITORS

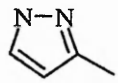
A novel class of azole compounds were found by Merck chemists to inhibit transglutaminase activity and in particular factor XIIIa. These compounds have been patented as potential agents in the treatment of thrombosis and related diseases.[91] The azole compounds and their salts are represented by the general formulas which are displayed on p. 32.



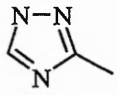


X = Cl
CF_3SO_3

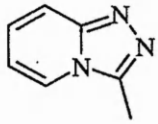
The (AZ) grouping comprises mono *N*-alkyl-substituted pyrazole, triazole, triazolopyridine and tetrazole rings as shown below.



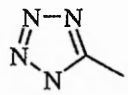
Pyrazole



Triazole

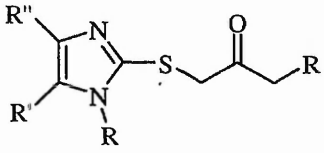


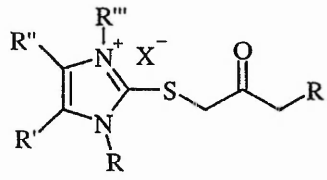
Triazolopyridine



Tetrazole

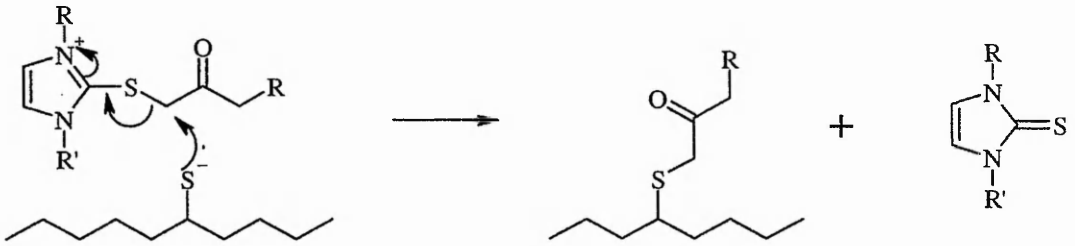
In addition, the Merck chemists prepared and evaluated a series of related thioimidazoles. These compounds have also been patented as factor XIIIa inhibitors, with pharmacological applications in the treatment of thrombosis and fibrinolysis.[92] The general structure of the compounds and their salts are shown below.





X = Cl
CF_3SO_3

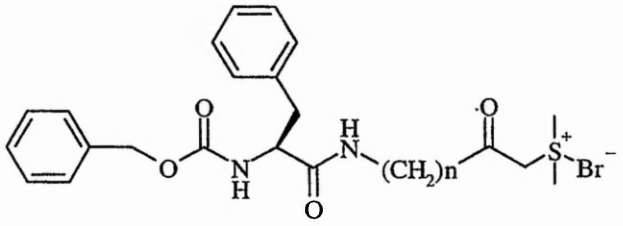
Later studies[93] found the mechanism of inactivation by these compounds to be acetonylation of the active site cysteine residue, with concomitant release of the thione (**scheme 5**, p. 33).



Scheme 5 Mechanism of inactivation of transglutaminase by thioimidazole inhibitors.

1.5.4.8 SULFONIUM METHYLKETONES

A related series of inhibitors, of the structure shown below, were prepared and evaluated as transglutaminase inhibitors by Syntex.[94]

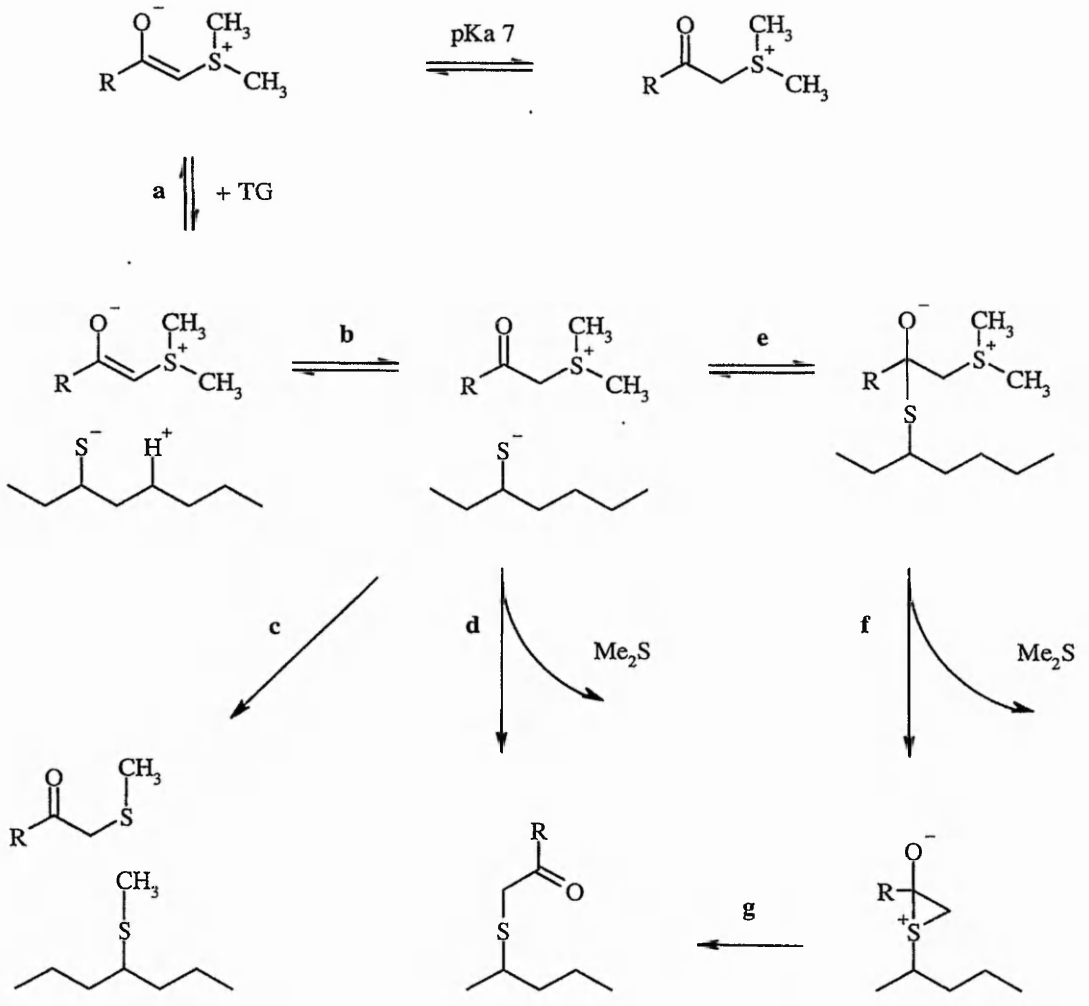


Sulfonium methylketones, n = 1-5

Inactivation by these compounds proceeds via alkylation of the active site thiol residue, which may occur via a number of possible mechanisms[94] as outlined in **scheme 6**, p. 34. It is assumed that initial binding occurs between the enzyme and the resonance-stabilised ylide (**a**). Protonation in the active site then yields the β-keto sulfonium salt (**b**). Inactivation of transglutaminase may then occur via one of three pathways: 1) methylation by the trialkylsulfonium salt (**c**); 2) displacement of dimethylsulfide (**d**) or; 3) by initial hemithioketal formation (**e**) followed by loss of dimethylsulfide to give the episulfonium ion

intermediate (**f**) which rearranges to give the final alkylated enzyme (**g**).

Sulfonium methylketones had originally been studied as inhibitors of the cysteine proteinases papain and cathespin B.[95] It was found, however, that alterations in the chain length could greatly affect specificity for a particular enzyme. Where n=1 the compound was found to be a strong inhibitor of cathepsin B but not of transglutaminase. Conversely where n=3 no inhibition of cathespin B was observed, but transglutaminase was strongly inactivated.



Scheme 6 Proposed mechanisms of inactivation of transglutaminase by sulfonium methylketones.[95]

1.5.5 SUMMARY OF FEATURES OF TRANSGLUTAMINASE INHIBITORS

The early work on transglutaminase inhibitors gave a better understanding of the physical features of the enzyme active site, and the structural requirements of a molecule for binding. The importance of chain length, hydrophobic regions for secondary binding and lack of branching of the alkyl chain near the reactive centre of the inhibitor, were shown.

Later studies discovered more reactive and specific thiol-alkylating agents such as the thioimidazoles and sulfonium methylketones. Additionally it was demonstrated how specificity for transglutaminase over other enzymes could be achieved using structural features such as methylene spacers and specific binding determinants such as the arylsulfonamide or Z-phenylalanine groups.

CHEMICAL DISCUSSION

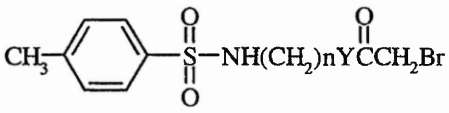
2. CHEMICAL DISCUSSION

2.1 BROMOMETHYL AMIDE DERIVATIVES

Initial studies centred on the halomethyl carbonyl compounds described by Reinhardt (section 1.5.4.4, p. 27). These compounds were relatively easy to prepare and thus enabled an initial investigation into the requirements for specific, non-cytotoxic transglutaminase inhibitors. It is also of note that these compounds have potential for further modification.

2.1.1 ARYL SULFONIUM BROMOMETHYL AMIDE DERIVATIVES

The most potent Reinhardt[86] compounds have the structures shown below.

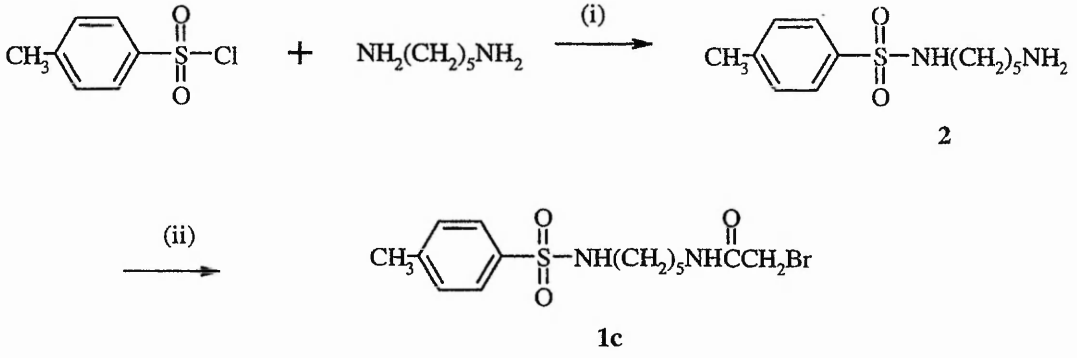


1a Y=CH_2, n=4
b Y=NH, n=4
c Y=NH, n=5
d Y=NH, n=6

These compounds (**1a-d**) all have the same inhibitory activity, i.e., K_i = 0.13. However, initial work was concentrated on the amido compound **1c** which is slightly easier to prepare than the keto compound **1a**.

The reported route to compound **1c** is via *N*-tosylation of 1-amino-5-phthalylaminopentane, followed by hydrazinolysis of the phthalimide group to give the free amine, followed by bromoacetylation to afford the inhibitor. The alternative route reported in this thesis (**scheme 7**, p. 37) uses a statistical approach in which

4-toluenesulfonyl chloride was reacted with a 2.5-fold excess of the diamine to give the mono-tosylated amine **2** in moderate yield (49%).[77] Treatment of **2** with the appropriate anhydride (prepared from bromoacetyl bromide with bromoacetic acid) gave **1c** in 69% yield.



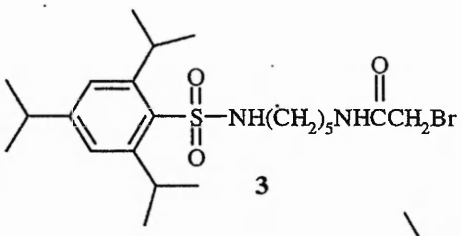
Scheme 7 *Reagents & Conditions*: i. DCM, room temp.; ii. bromoacetyl bromide, bromoacetic acid, pyridine, DCM, 0°C→room temp.

Compound **1c** was found by colleagues in the Life Sciences Department to be a good inhibitor of transglutaminase *in vitro*, with an IC_{50} of 1μM, but, unfortunately, was less potent *in vivo* (see chapter 3, p. 105).

Attempts to improve the inhibitory properties of these compounds were initially focused on the hydrophobic sulfonamide group. As previously discussed (section 1.5.2.1, p. 18), Lorand *et al.*[72] have shown that 2,4,6-triisopropylbenzenesulfonamide has similar properties to the 5-dimethylamino-1-napthalenesulfonamide group found in the best competitive amine inhibitors. Accordingly, compound **3** was prepared in the same manner as **1c**, starting from 2,4,6-triisopropylbenzenesulfonyl chloride. Unfortunately compound **3** did not show a marked improvement in specificity or inhibitory potency and was found to be less effective *in vivo*, and to have an IC_{50} of

3 μM against transglutaminase in common with compound **1c** *in vitro* (see chapter 3, p. 105).

These results suggested that, although there were a number of features built into these molecules to improve their affinity for transglutaminase, they were still able to act as general alkylating agents for other cysteine containing systems. It was hoped that replacement of the bromomethyl group with more selective alkylating moieties would reduce the cytotoxicity of the inhibitors. To this end, compounds **4** and **5** (containing the sulfonium ylides reported by Shaw[95] and the Syntex group[94] (section 1.5.4.8, p. 33)) were prepared via reaction of the corresponding halogenated compounds **1** and **3** with dimethyl sulfide.



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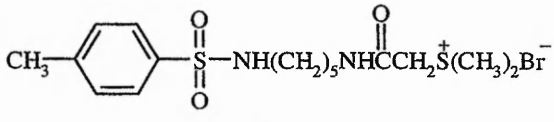

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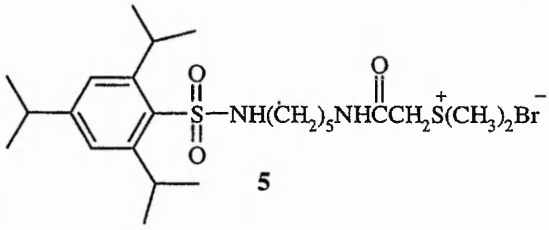
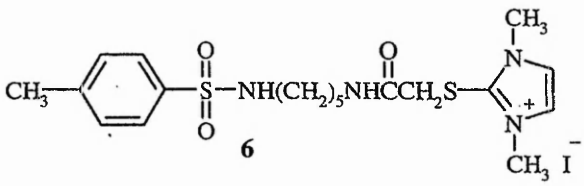


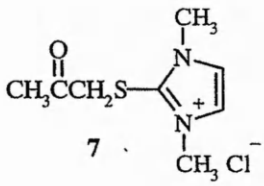
5

Enzyme assay of compounds **4** and **5** showed them to have very poor inhibitory properties. Although disappointing, these results suggested that for a sulfonium salt to have potential as a specific and selective inhibitor substitution is required (such as the Z-phenylalanine residue used by the Syntex group) which might confer strong binding to the transglutaminase.

The mercaptoimidazoles reported by Merck (Section 1.5.4.7, p. 31) also have potential for modifications. The sulfonamide derivative **6** and the known inhibitor **7** were prepared in order to evaluate the effect of the sulfonamide moiety on the inhibitory properties of this class of compound.



6



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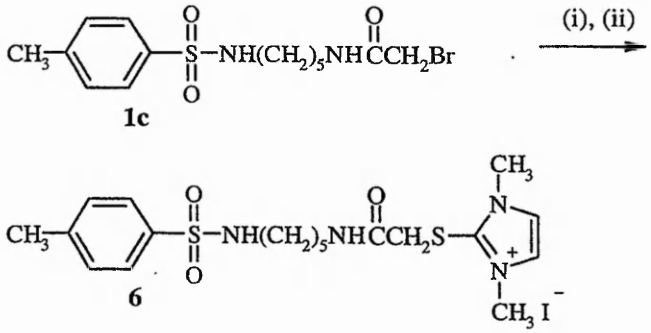
Compound **6**, a hybrid of **7** and **1c**, was synthesised from the bromoamide **1c** and *N*-methyl-2-mercaptomethylimidazole as shown in **scheme 8**.



1c

6

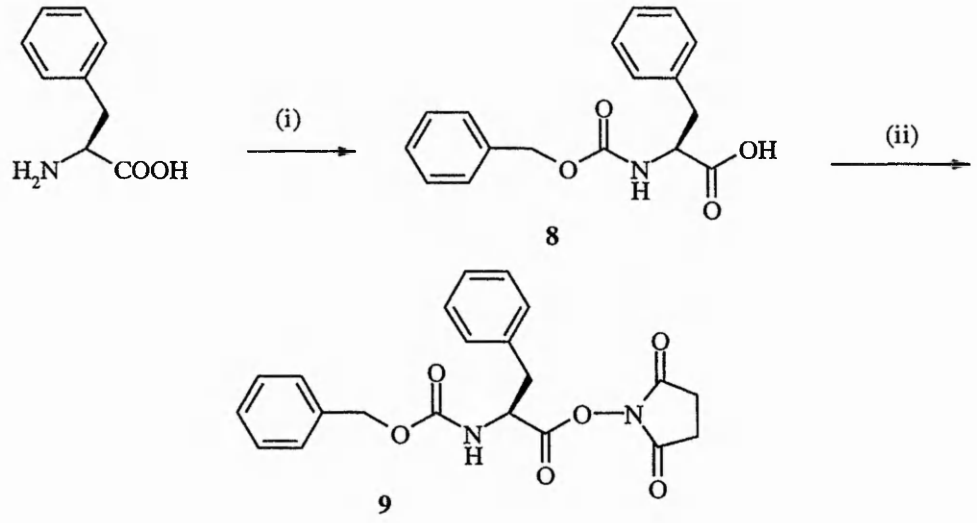
Scheme 8 *Reagents & Conditions*: i. *N*-methyl-2-mercaptomethylimidazole, TEA, DCM; ii. MeI, DCM.

The novel thioether **6** was found to have an IC_{50} of 250μM, while compound **7** was shown to be both a moderately good inhibitor of transglutaminase (IC_{50} of 100μM) and to have low cytotoxicity. This latter result, coupled with the lack of inhibition shown by compounds **4** and **5**, suggests that the 4-toluenesulfonamide group

is not a particularly good binding determinant and so compounds containing alternative recognition groups were prepared.

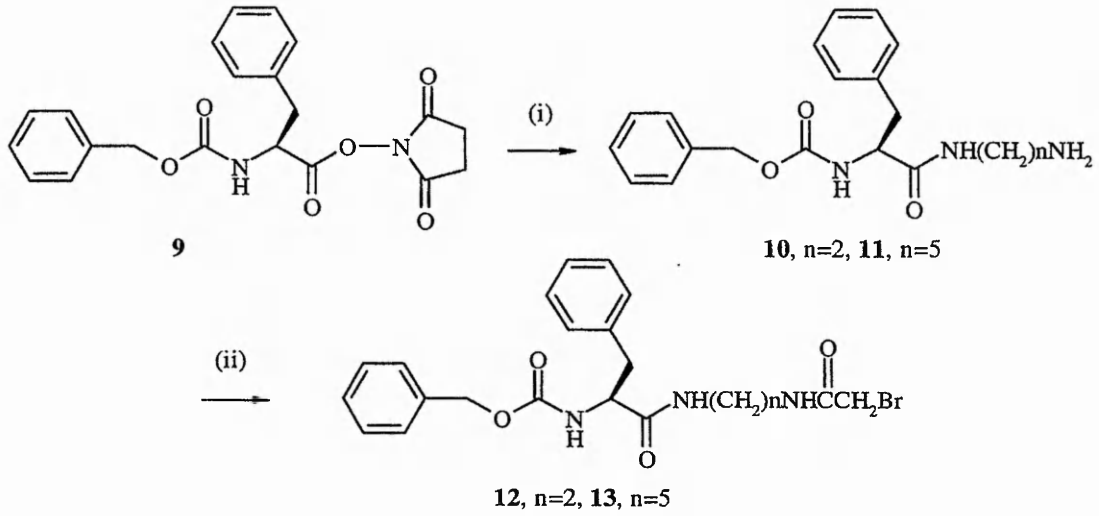
2.1.2 Z-PHENYLALANYL BROMOMETHYL AMIDE DERIVATIVES

A possible modification was the substitution of the sulfonamide grouping of **1c** with the Z-phenylalanine function found in inhibitors such as the dihydroisoxazole (Section 1.5.4.6, p 29) and sulfonium compounds (Section 1.5.4.8, p. 33). It was envisaged that the use of the diamine strategy would give simplified access to compounds with structures similar to those of the previously mentioned inhibitors. The synthesis first required the preparation of Z-phenylalanine **8**, and activation of its carboxyl group. The Z-phenylalanine derivative of choice was the *N*-hydroxysuccinimide active ester **9**, an isolable intermediate which can be prepared in a relatively simple manner in two steps from phenylalanine (**scheme 9**).[96]



Scheme 9 *Reagents & Conditions*: i. benzyl chloroformate, 2M NaOH; ii. *N*-hydroxy-succinimide, DCC, THF, 0°C→room temp.

Reaction of active ester **9** with 5 equivalents of either diaminoethane or diaminopentane afforded the mono-amines **10** and **11** in 37 and 45% yields, respectively (**scheme 10**).



9

10, n=2, **11**, n=5

12, n=2, **13**, n=5

Scheme 10 *Reagents & Conditions*: i. diaminoalkane, DCM, 0°C→room temp; ii. bromoacetyl bromide, bromoacetic acid, pyridine, DCM, 0°C→room temp.

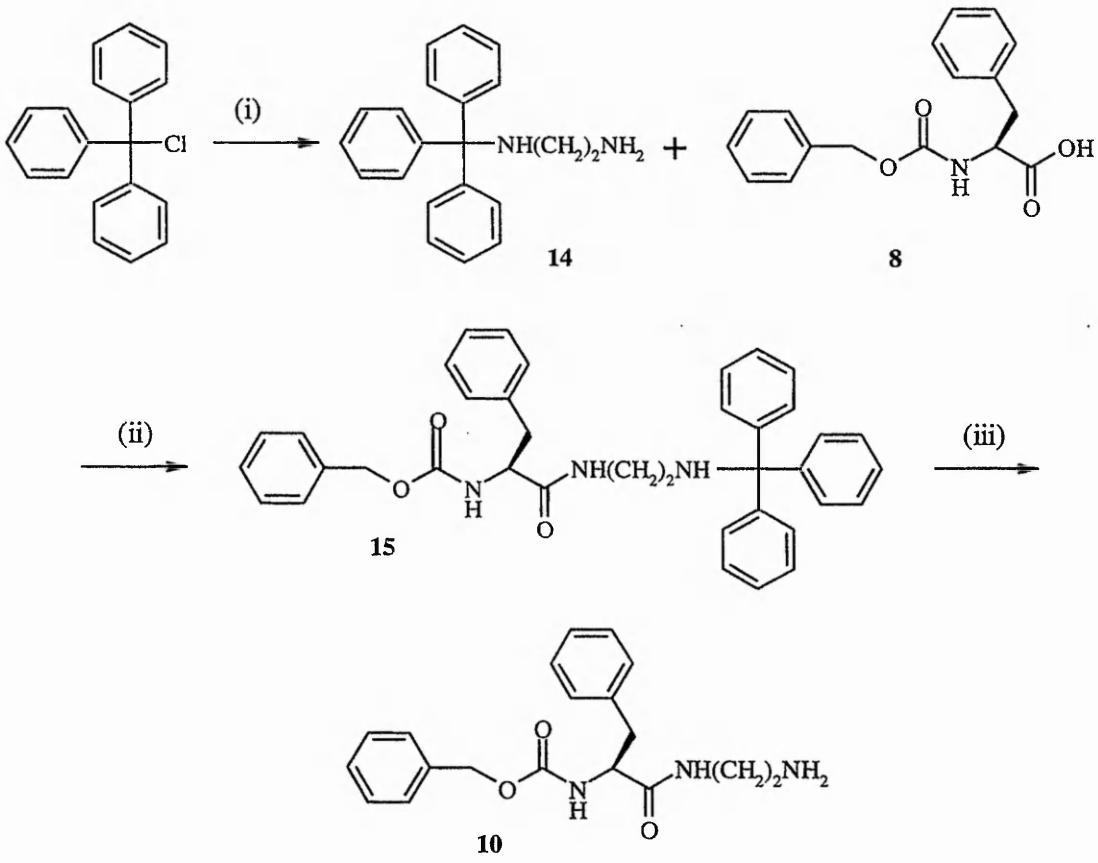
Conversion of **10** and **11** to the bromomethyl ketones **12** and **13**, in 65 and 59% yields, respectively, was accomplished by the method outlined in **scheme 10**.

An alternative synthesis (**scheme 11**, p. 42) was devised based on the mono-trityl-protection of diamines which has been recently described by Papaionnou *et al.*[97] on polyamine preparation. It was hoped that this would improve yields in the coupling step, and so reduce the loss of Z-phenylalanine (**8**).

Mono-protection of trityl chloride to give unsymmetrical diamine **14** was accomplished using an excess of ethylenediamine in rather low yield (37%). Subsequent coupling of **14** with **8**, employing dicyclohexylcarbodiimide, gave the

N-trityl derivative **15** in high yield (88%). Removal of the trityl group with trifluoroacetic acid afforded **10**, again in high yield (83%).

The routes shown in **schemes 10** and **11** have a similar number of steps, although the use of monotrityl-protection gives a better yield overall the method did require greater use of column chromatography.



Scheme 11 *Reagents & Conditions*: i. ethylenediamine, DCM, 0°C→room temp; ii. DCC, DCM; iii. trifluoroacetic acid, triethylsilane, DCM.

The synthesis of the two potential inhibitors **12** and **13** was undertaken to determine the role of chain length and its relation to the two binding determinants, i.e., Z-phenylalanyl (**8**) and the aryl sulfonamide groups. The former was expected to mimic Syntex type inhibitors **16** and the latter was a derivative of the sulfonamide compound **1c**, ***Figure 1***. Enzyme assay showed these compounds to be moderate inhibitors but, unfortunately they have poor solubility in solvents suitable for biological studies.

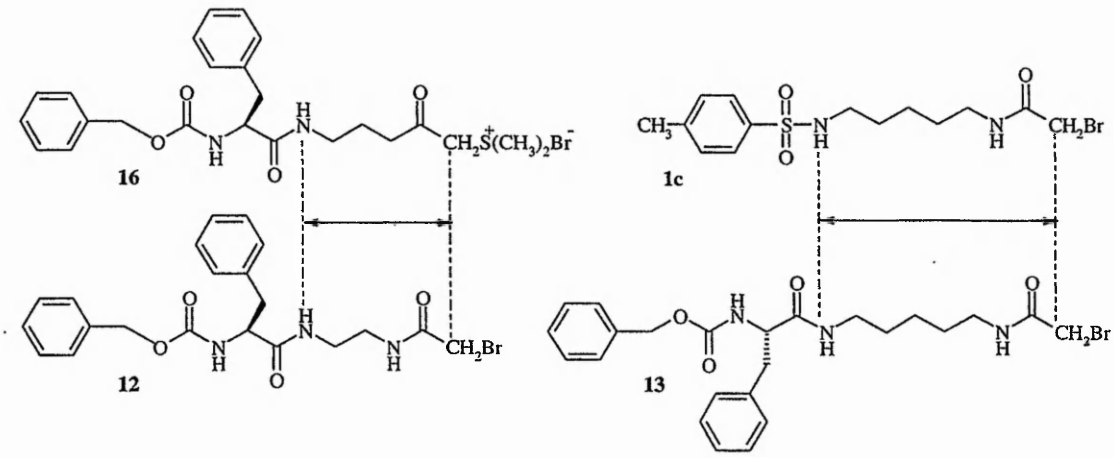
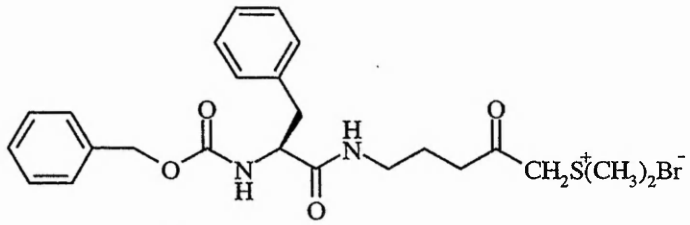


Figure 1 Structural comparison of known inhibitors **1c** and **16** with potential inhibitors **12** and **13**.

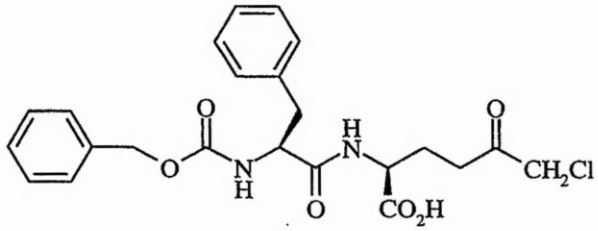
These series of compounds demonstrate both the effectiveness and limitations of the type of inhibitors described by Reinhardt. Compounds containing the Z-phenylalanyl binding determinant appear to have greater potential for modification than the corresponding sulfonamides. Compounds of this type are discussed in the next section.

2.2 Z-PHENYLALANYL HALOMETHYL KETONE DERIVATIVES

The poor inhibitory properties or cytotoxicity of most of the previously described inhibitors led to the preparation of the peptide inhibitor **16** developed by Syntex (Section 1.5.4.8, p. 33) and its analogue **17** prepared by Dr. El-Aloui (Section 1.5.4.5, p. 28).



16

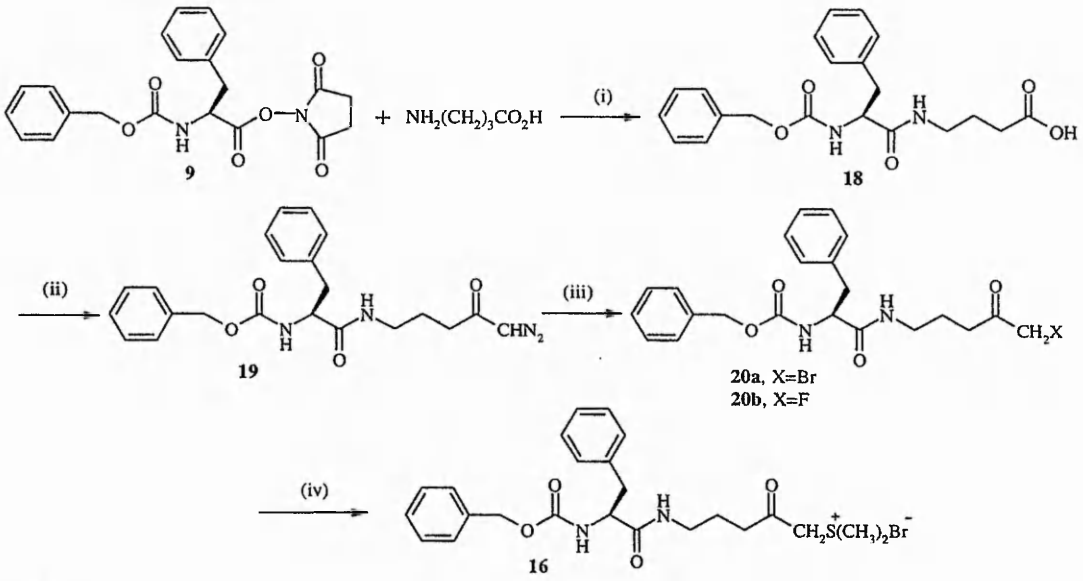


17

2.2.1 SULFONIUM METHYL KETONE **16** AND ANALOGUES

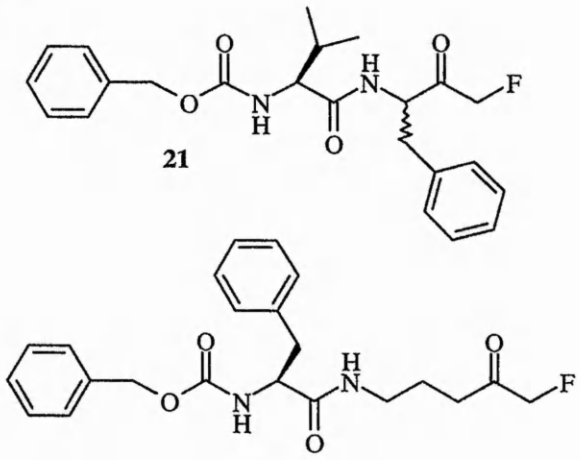
Compound **16** was prepared by the literature method outlined in **scheme 12**.[94] The first step involved the coupling of Z-phenylalanine active ester **9** with 4-aminobutyric acid (GABA) giving acid **18** in high yield (86%). Treatment of **18** with *n*-butyl chloroformate followed by excess of diazomethane gave the diazoketone **19** in low yield (24%). Decomposition of the diazo group with HBr afforded the bromomethylketone **20a** in good yield (79%) which, when treated with

dimethylsulfide, afforded the Syntex inhibitor **16** in 65% yield. Significantly, this methodology affords the key diazoketone intermediate **19** in only low yield.[94]



Scheme 12 *Reagents & Conditions*: i. TEA, THF, H_2O; ii. *n*-butyl chloroformate, *N*-methyl-morpholine, -78°C, then CH_2N_2, →room temp.; iii. HX, DCM; iv. **20a**, dimethyl-sulfide, acetone.

Biological evaluation of both the Syntex compound **16** and the bromomethyl ketone **20a** is reported in chapter 3, p. 105. The diazoketone **19** was used to prepare a



further potential inhibitor, the fluoromethylketone **20b** because dipeptides containing this grouping have been shown[98] to be effective inhibitors of the cysteine protease calpain I. Compounds effective against calpains have the irreversible binding site adjacent to the amide bond of the peptide, for example **21**. It was hoped that the fluoromethyl ketone binding group located γ- to the amide bond in **20b** might provide a novel, specific transglutaminase inhibitor.

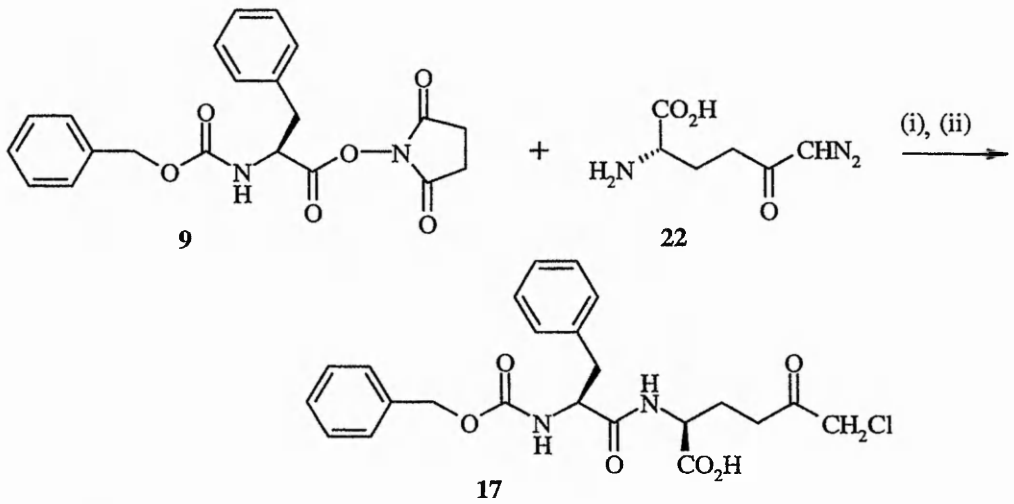
Formation of α-fluoromethyl ketones from diazoketones is usually achieved by treatment of the diazo compound with hydrogen fluoride, the low. boiling point and highly corrosive nature of which make it a difficult material to handle. Pyridinium poly(hydrogen fluoride), also known as Olah's reagent, has been developed to overcome these drawbacks.[99] Olah's reagent consists of a solution of anhydrous hydrogen fluoride in pyridine containing about 9 equivalents of the acid to 1 equivalent of pyridine. The solution is characterised by its stability up to 55°C and its versatility as a fluorinating agent, including decomposition of diazoketones to form α-fluoromethyl ketones. The reagent, however, still requires care in handling and the use of polytetrafluoroethylene (PTFE) reaction vessels. A more recently introduced reagent, triethylamine *tris*(hydrogen fluoride) also known as TREAT HF is much less corrosive and can be used in borosilicate glassware without etching.[100] Initial attempts at the synthesis of **20b** utilised this latter reagent, but no reaction was observed and starting material was recovered even after prolonged reaction times (48 h.). In contrast, Olah's reagent gave the desired product in 82% yield after 15 min. at 0°C.

The above syntheses yielded the Syntex compound **16** and the halomethyl ketones **20a** and **20b** providing authentic samples for future chemical and biological

comparisons. However the overall yields were low and in terms of scaling up to provide quantities sufficient for whole cell work an alternative synthesis was still required.

2.2.2 Z-PHENYLALANYL CHLOROMETHYL KETONE **17**

The synthesis of **17** is complicated by the presence of the α-carboxyl group of the glutamic acid residue. Although the method of El-Aloui[88] is relatively straight forward (**scheme 13**) involving the coupling of commercially available 6-diazo-5-oxo-L-norleucine (DON) (**22**) with Z-phenylalanine active ester **9**, the high price of DON (currently around £1 per milligram) precludes this as a possible route to a series of derivatives, especially with the requirement for inhibitors on a gram scale. However the synthesis was repeated on a small scale (0.15 mmol) providing an authentic sample of **17** in an overall yield of 78%.

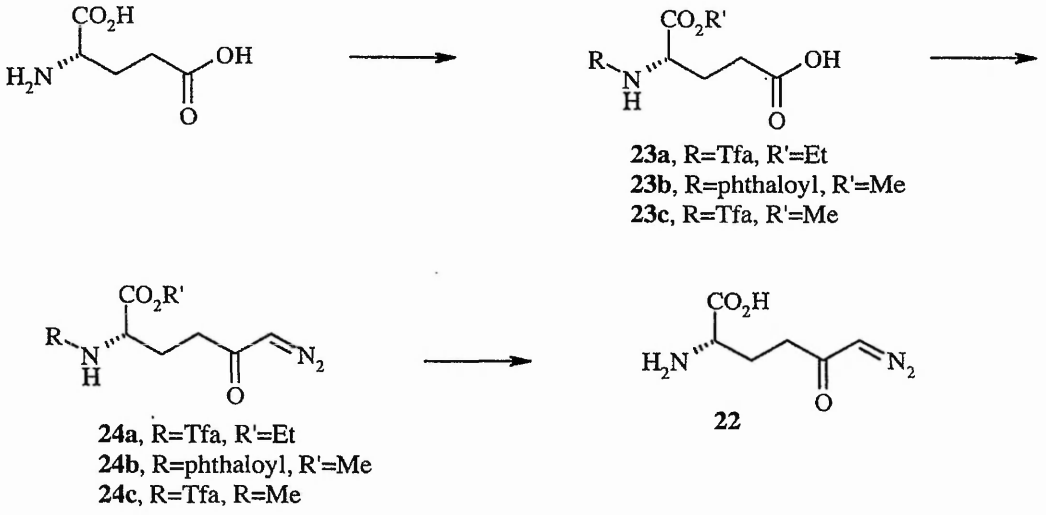


9 **22** **17**

Scheme 13 *Reagents & Conditions*: i. TEA, THF, H_2O; ii. HCl, ethyl acetate.

DON (**22**) is a naturally occurring substance first isolated from an unidentified member of *Streptomyces*[101] found in Peruvian soil and later from *Streptomyces ambofaciens*.[102] Its antineoplastic and antibiotic properties have led to intermittent clinical trials which began in the 1960s and have continued until the present day.[103,104,105]

The first reported preparations of DON (**22**) were by Weygand *et al.*[106] and by DeWald and Moore,[107] both in 1958; a later procedure was published by Pettit and Nelson.[103] All the reported methods were similar and are outlined in **scheme 14**.



Scheme 14

The main difference between the three procedures is the method used to form intermediates **23a-c**. The synthesis described by both Weygand (**23a**) and DeWald and Moore (**23b**) uses ring-opening of *N*-protected glutamic acid anhydride to give the appropriate partially protected glutamic acid derivative. The method employed by Pettit and Nelson uses commercially available γ-benzyl glutamate which was *N*-protected with the trifluoroacetyl group, then esterified at the α-carboxyl group to give

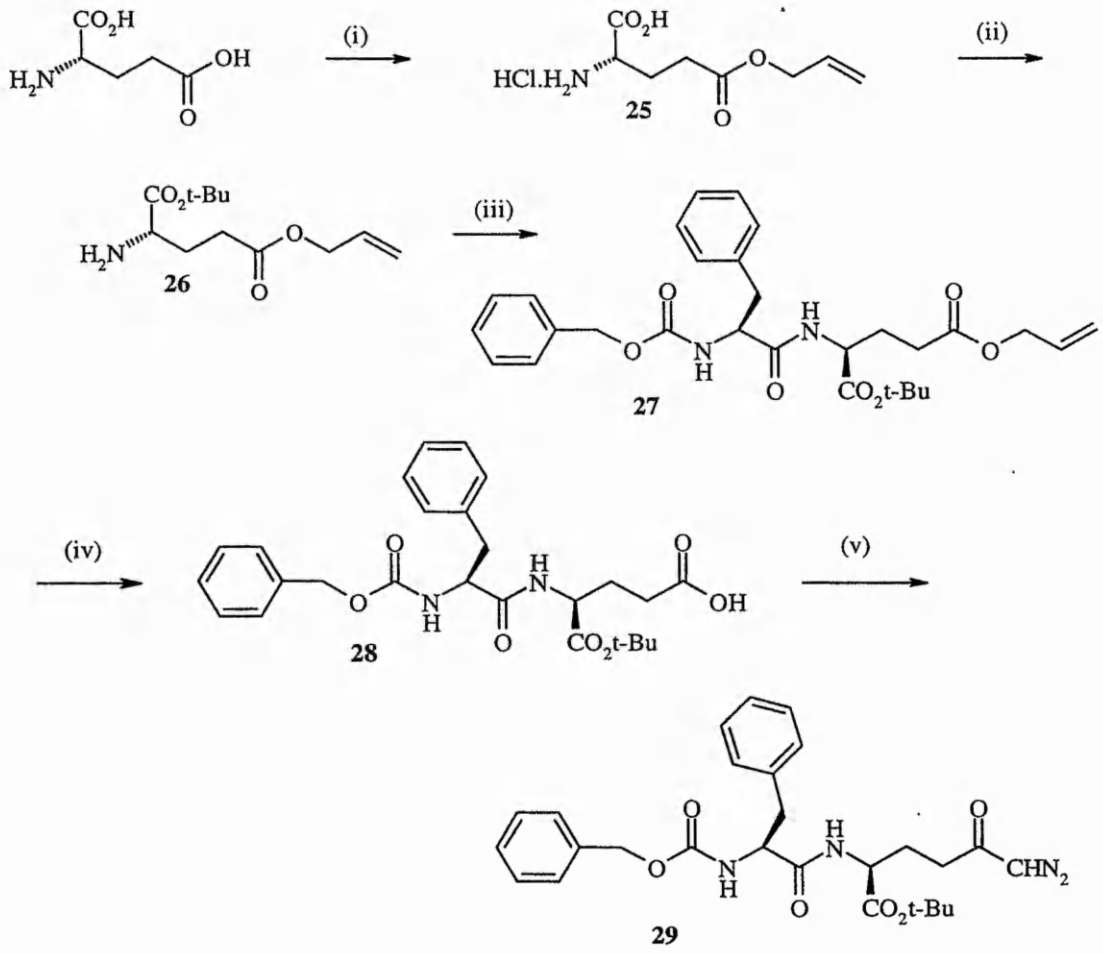
the benzyl ester which was subsequently catalytically hydrogenated to afford compound **23c**.

The essential feature common to all of the methods is the use of the base-labile protecting groups *N*-phthaloyl, *N*-trifluoroacetyl and the methyl- and ethyl ester which allow deprotection in the final step without destroying the diazo group. Another important factor are the multiple steps required in the synthesis of mono-α-ester derivatives of glutamic acid **23**, a common problem found when working with the aspartic and glutamic amino acids.

2.2.3 ATTEMPTED ALTERNATIVE ROUTE TO Z-PHENYLALANYL CHLOROMETHYL KETONE **17**

These factors prompted a search for an alternative synthesis not requiring DON (**22**). An attempted synthesis of compound **17** was undertaken via a route similar to that described by Syntex, as indicated in **scheme 15**, p. 50. It was decided that the differential protection of glutamic acid should incorporate a *t*-butyl ester at the α-carbonyl because: 1) this allows for the synthesis of an ester derivative of **17** and; 2) this grouping can easily be removed by mild acid, which is not expected to affect other groups present in the molecule.

Of the various methods available for the selective unsymmetrical diesterification of glutamic and aspartic acid, a modification of a recently published method[108] for the preparation of α-*t*-butyl *N*-blocked glutamates through γ-allyl protection was adopted. The attractiveness of this route lies in the ease in which a γ-allyl ester can be introduced[109] and the selectivity with which it can be removed with palladium(0) complexes.[108]



Scheme 15 *Reagents & Conditions*: i. chlorotrimethylsilane, allyl alcohol; ii. 70% aq. $HClO_4$, *t*-butyl acetate; iii. **8**, DCC, DCM; iv. $Pd(PPh_3)_4$, pyrrolidine, DCM; v. *n*-butyl chloroformate, *N*-methylmorpholine, -78°C, then CH_2N_2 →room temp.

Thus, the γ-allyl ester of glutamic acid **25** was prepared in 76% yield via the chlorotrimethylsilane-mediated esterification of glutamic acid with allyl alcohol.[109] The α-carboxylic acid group of **25** was esterified in 73% yield by the perchloric acid-catalysed transesterification reaction with *t*-butyl acetate.[110] DCC mediated coupling of the unsymmetrical glutamate diester **26** with Z-phenylalanine **8** to give **27** was accomplished in 88% yield. Subsequent selective removal of the allyl group with *tetrakis*triphenylphosphinepalladium(0) gave the mono-acid **28** in 86% yield.[108]

Attempts to prepare the diazoketone **29** from the reaction of diazomethane with the mixed anhydride, generated *in situ* from **28** and *n*-butylchloroformate, gave none of the desired product.

2.2.4 ALTERNATIVE ROUTES TO Z-PHENYLALANYL HALOMETHYL KETONES

The need to use the highly unstable and toxic reagent diazomethane coupled with the poor yields obtained with the above syntheses (**schemes 12** and **15**, p. 45 and p. 50, respectively) demonstrated that the classical approach to diazoketones is not satisfactory when applied to the preparation of the inhibitors **16** and **17**. An obvious approach would be to prepare DON (**22**) by the literature procedures but the use of diazomethane, the variable yields and multiple steps reported make this undesirable. Therefore an alternative method was sought to afford a more convenient and less hazardous route to the desired halomethyl ketone transglutaminase inhibitors.

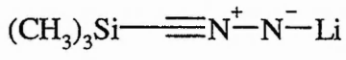
2.2.4.1 TRIMETHYLSILYLDIAZOMETHANE (TMSD) AS A SUBSTITUTE FOR DIAZOMETHANE

A variety of alternative preparative methods of diazocompounds have been developed. These methods have been extensively reviewed[111] because of their synthetic importance. Of these the most attractive appeared to be the reagent trimethylsilyldiazomethane (TMSD).

Recently TMSD has been introduced as a safe substitute for diazomethane.[112] The stability of TMSD has been attributed to $p\pi$-$d\pi$ resonance between the silicon atom and the diazomethyl group. This effect has also been observed with other hetero atoms such as sulfur and phosphorus, but the silicon derivative has become the most

widely used reagent as it is commercially available, or can be conveniently prepared in one step from commercially available starting materials.[113]

The reagent has many of the properties of diazomethane, as well as some synthetic applications of its own. Examples of the former include one carbon homologation of carboxylic acids and ketones,[114] and of the latter oxidation with *m*-chloroperbenzoic acid to give α-keto silanes.[115] In addition, the lithium salt (LiTMSD) is easily prepared by the action of a suitably strong base such as *n*-butyllithium or lithium diisopropylamine. Interestingly, the LiTMSD is stable at room temperature in solution. Theoretical and experimental studies have suggested the most stable isomer to be the *N*-lithiated silylnitrile imine **30** rather than the *C*-lithiated trimethylsilyldiazomethane **31**.[116]



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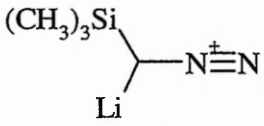
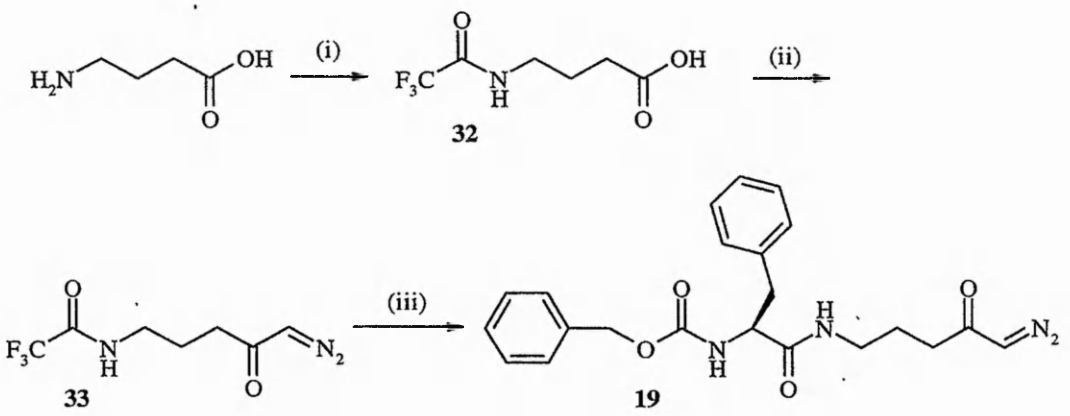


31

The Weygand synthesis of DON (**22**) was modified using TMSD in place of diazomethane to synthesise the Syntex intermediate **19** (**Scheme 16**, p. 53). Protection of 4-aminobutyric acid was achieved by the known procedure[103] using methyl trifluoroacetate in the presence of 1,1,3,3-tetramethylguanidine to give the trifluoroacetamide **32** in high yield (83%). Treatment of **32** with oxalyl chloride, followed by addition of trimethylsilydiazomethane, afforded the diazoketone **33** in good yield (63%).[112] Attempts to remove the trifluoroacetyl group and to isolate the aminodiazoketone under standard conditions[103,106] with aqueous base failed.

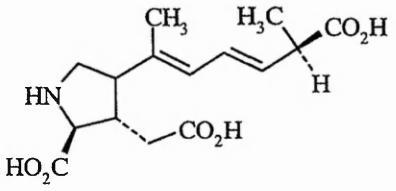
However deprotection in the presence of the Z-phenylalanine *N*-hydroxysuccinimide ester (**9**) gave the desired product (**19**) albeit in low yield (22%).



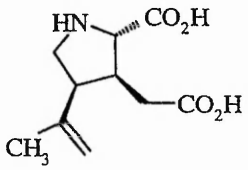
Scheme 16 *Reagents & Conditions*: i. 1,1,3,3-tetramethylguanidine, methyl trifluoroacetate; ii. a) CO_2Cl_2, DCM 0°C; b) TMSD, THF/acetonitrile; iii. 1M NaOH, THF, then **9**.

Extension of this method to the synthesis of the dipeptide derivative **17** required the differential elaboration of the two carboxylic acid groups of glutamic acid. Although this problem had been satisfactorily addressed in a previous synthesis (**scheme 15**, p. 50), it was decided to use an alternative procedure based on pyroglutamic acid. This methodology would allow a greater range of analogues to be prepared in fewer steps.

Pyroglutamic acid provides an internal protection of the γ-carboxyl group of glutamic acid, allowing an easy differentiation of the two carboxyl groups. *N*-protected pyroglutamate esters have been employed in a number of pyrrolidine-containing natural product syntheses such as (-)-domic acid, (-)-kainic acid, (+)-deoxynojirimycin, and the *Monomorium minutum* ant venom alkaloids.[117]



(-)-Domoic acid

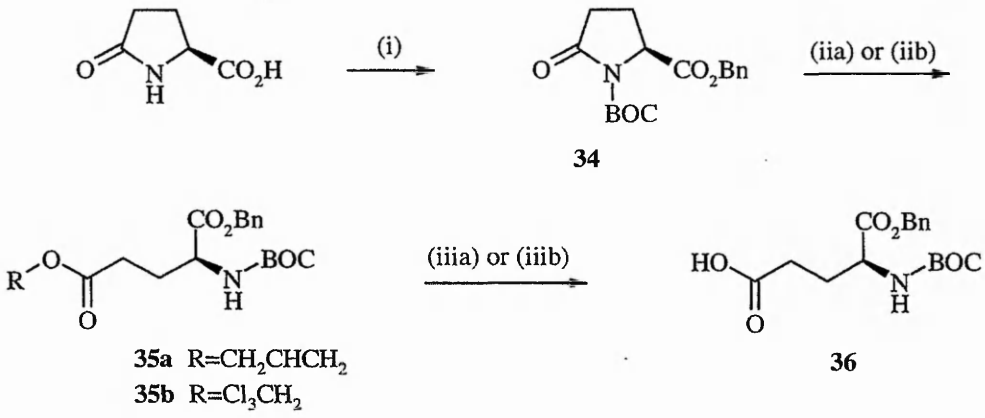


(-)-Kainic acid

In addition, *N*-alkoxycarbonyl-protected pyroglutamates show an enhanced reactivity at the lactam bond, allowing ring-opening reactions with a number of nucleophiles, with excellent regioselectivity in most cases; for example various γ-keto derivatives can be obtained from reaction with Grignard reagents[118] or with ester lithium enolates.[119] Of particular interest in the current context is the preparation of unsymmetrical glutamate diesters that can be formed by cyanide-catalysed alcoholysis.[120] This allows for the elaboration of glutamic acid selectively esterified at the α-carboxylic acid, which can then be transformed to the desired acid as illustrated in **scheme 17**, p. 55.

The known compound benzyl *N*-BOC pyroglutamate **34**[118] was chosen as starting material for initial studies and was synthesised as shown in **scheme 17**, p. 55. 4-Dimethylaminopyridine-catalysed (DMAP) esterification of the acid with benzyl alcohol and dicyclohexylamine afforded the ester in 56% yield, and subsequent *N*-acylation with di-*tert*-butyl dicarbonate and DMAP using the procedure pioneered by Grieco *et al.*[121] and Grehn and Ragnarsson[122] gave the carbamate **34** in excellent yield (98%). Reaction of **34** with two equivalents of allyl alcohol gave the desired diester **35a** in 70% yield but under the same conditions trichloroethanol gave the glutamate **35b** in low yield (22%). Increasing the reaction time failed to improve the

yield of **35b** suggesting that in this case the reaction was reversible, probably due to the good leaving group properties of the trichloroethoxide ion.



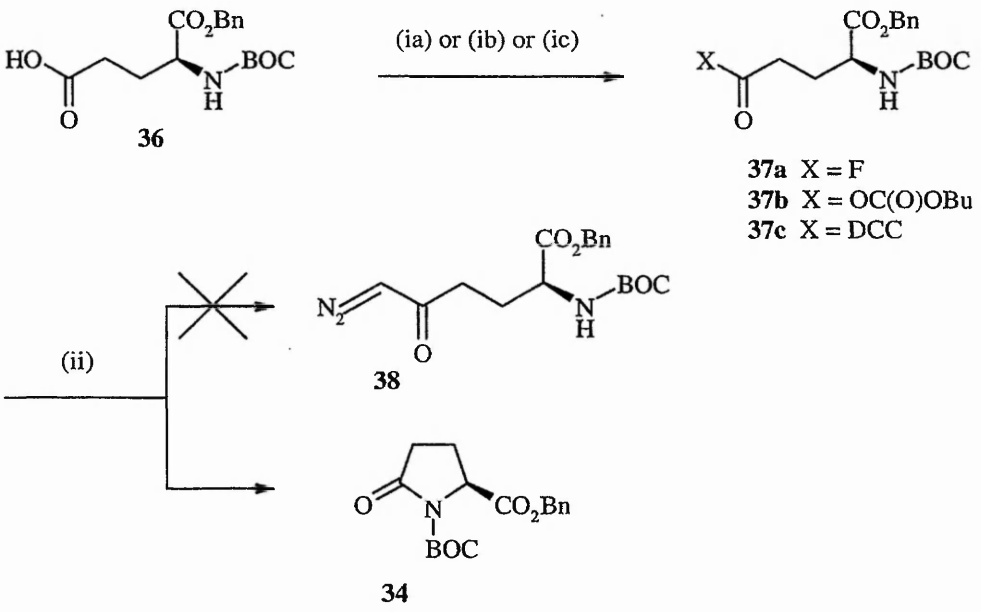
Scheme 17 Reagents & Conditions: i. a) benzyl alcohol, DCC, DMAP, DCM; b) di-*t*-butyl-dicarbonate, DMAP, TEA, DCM; ii. a) allyl alcohol, KCN, THF (**37a**) or; b) 2,2,2-trichloroethanol, KCN, THF (**37b**); iii. a) $Pd(PPh_3)_4$, pyrrolidine, DCM or; b) zinc dust, 1M ammonium acetate, THF.

Removal of the allyl group from **35a** was accomplished under the conditions as described previously (**scheme 17**) to give the desired acid **36** in good yield (76%). The trichloroethyl ester grouping of compound **35b** is stable to both acid and base and was selectively cleaved by zinc dust in buffered aqueous tetrahydrofuran (THF).[123] This last method afforded the mildest deprotection conditions and, using ammonium acetate as buffer, gave **36** in high yield (79%).

The formation of acid chlorides from compounds such as **36** has been reported to lead to serious side reactions in which pyroglutamates are readily formed.[124] The use of acid fluorides has been advocated[124] as an alternative carboxylic acid activating group which overcomes this problem. As shown in **scheme 18**, p. 56, treatment of **36** with cyanuric fluoride gave the acid fluoride **37a** in good yield (60%). However, **37a**

failed to react with trimethylsilyldiazomethane even when subjected to longer reaction times (48 h.) and yielded only the pyroglutamate **34**. This result is likely to be due to the reactivity difference between acid chlorides and fluorides. The latter are known to be more resistant to hydrolysis and have been found to be ineffectual in coupling reactions of highly hindered amines, whereas the corresponding acid chlorides couple easily.[125]

In an attempt to increase the reactivity of the γ-acid function two other methods of carboxylic acid activation were tried (**scheme 18**). Firstly the mixed anhydride approach was adopted, in which reaction of the acid **36** with *n*-butyl chloroformate to give **37b** was followed by treatment with trimethylsilyldiazomethane.

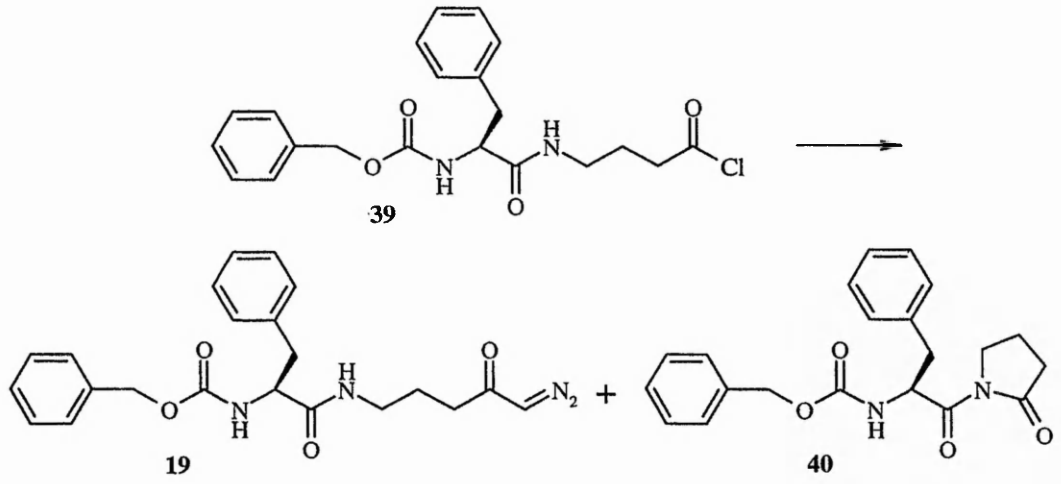


Scheme 18 *Reagents & Conditions*: i. a) cyanuric fluoride, pyridine, DCM -30°C or; b) *n*-butyl chloroformate, *N*-methylmorpholine, THF, -15°C→room temp. or; c) DCC, DCM; ii. TMSD, THF.

No reaction was observed other than extensive cyclisation to the pyroglutamate. A similar result was obtained from the addition of dicyclohexylcarbodiimide to a mixture

of the acid and trimethylsilyldiazomethane.

An endeavour was made to overcome the problem of cyclisation of carbamate-protected glutamic acids by treating the mixed anhydride of acid intermediate **18**, which has previously been shown to react with diazomethane (section 2.2.1, p. 44), with TMSD, but no reaction occurred. The acid chloride **39** however gave the product **19** in low yield (12%) along with the side product **40** caused by the cyclisation of the 4-aminobutyric acid residue (**scheme 19**).

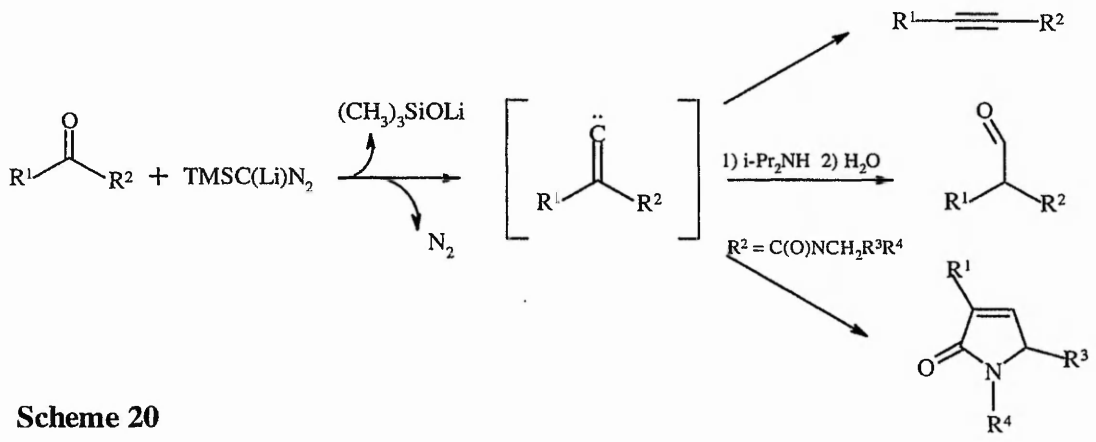


Scheme 19

These results demonstrate that although the reagent TMSD has improved stability, unfortunately, its reactivity is reduced. Only the highly activated acid chlorides appear sufficiently reactive to compensate for the reduction in TMSD reactivity, but these were found to be incompatible with the urethane protecting groups and the chain length found in 4-aminobutyric acid and glutamic acid.

2.2.4.2 ROUTES TO DIAZOKETONES USING LITHIUM TRIMETHYLSILYL-DIAZOMETHANE (LiTMSD)

The results in the above section led to an investigation of LiTMSD in an attempt to improve the reactivity of the reagent. LiTMSD has found use in a number of synthetic areas, in particular the preparation of heterocycles, where it has been extensively studied by a number of groups. The reagent acts as a 1,3-dipole in a similar manner to diazomethane and has been used in the preparation of a number of trimethylsilyl-containing pyrazole and triazole derivatives.[126] Shioiri *et al.*[127] have also studied the carbene chemistry of the reagent (**scheme 20**). They report that treatment

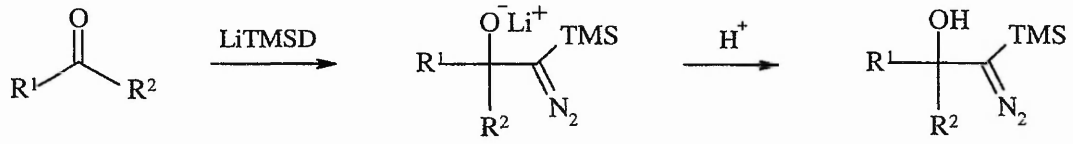


Scheme 20

of an aldehyde or ketone with the reagent yields a species which can lose lithium trimethylsilyloxide to form a diazoalkene. This intermediate spontaneously loses nitrogen to form a carbene which then rearranges to give one of a number of products such as homologated alkynes, homologated aldehydes and heterocycles, depending upon the reaction conditions.

Although much of the chemistry of this reagent involves elimination of TMSOLi from the α-diazoalkoxide intermediate (**scheme 20**) to generate a carbene,

the intermediate has been trapped by Schöllkopf and Scholz[128] to yield a diazoalcohol (**scheme 21**).



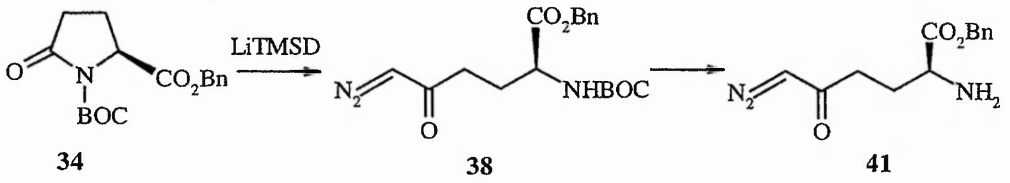
Scheme 21

It was therefore considered that reaction of LiTMSD with a suitably activated carboxyl group, such as those described above, might give the required diazoketone. The acid chloride derivative of *N*-trifluoroacetyl-GABA **32** (**scheme 16**, p. 53) gave the desired diazoketone in low yield (10%) upon treatment with LiTMSD, formed by the reaction of one equivalent of lithium diisopropylamide (LDA) with TMSD at -78°C. Reaction of *N*-BOC glutamic acid derivatives such as the acid fluoride **37a** or the mixed anhydride **37b** (**scheme 18**, p. 56) gave none of the desired product. Additionally, the acid chloride **39** (**scheme 19**, p. 57) afforded no product under the described reaction conditions and this may be attributable to the presence of acidic carbamate and amide hydrogens which could possibly reprotonate the reagent thus preventing any reaction.

2.2.4.3 RING-OPENING OF URETHANE-PROTECTED PYROGLUTAMATES WITH LiTMSD

As previously discussed (section 2.2.4.1, p. 51), suitable pyroglutamic acid derivatives can undergo highly regioselective reaction with carbanion nucleophiles. This suggests that direct reaction of LiTMSD with a carbamate-protected

pyroglutamic acid derivative such as **34** may provide a direct route through to the diazoketone **38** (**scheme 22**), deprotection of which could give key intermediate **41**.



34 **38** **41**

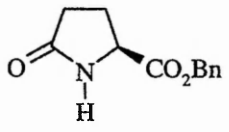
Scheme 22

Treatment of TMSD with LDA at -78°C followed by addition of the pyroglutamate **34** afforded the desired diazoketone **38** in very low (5%) yield. The simpler method of Schöllkopf and Scholz,[128] in which TMSD is treated with *n*-butyllithium at -100°C, was then employed to eliminate any possible side reactions caused by the presence of diisopropylamine in the reaction mixture. The insertion of diisopropylamine into the carbene to form an enamine, which is subsequently converted to the homologated aldehyde, has been previously demonstrated by Shiori *et al.* (**scheme 20**, p. 58). The addition of pyroglutamate **34** to a solution of LiTMSD at -100°C and maintenance of the resulting mixture at -85°C afforded the diazoketone **38** in a 21% yield. However, it was subsequently discovered that by maintaining the temperature at -100°C or below at all times the diazoketone **38** could be isolated in up to 71% yield.

Temperature was found to play a critical role in this reaction since by allowing it to rise to -95°C during the addition step the yield was reduced by approximately 10%. The reaction was found to be virtually complete (by tlc.) shortly after the end of the addition step, and the resulting solution remained stable providing that the

temperature was maintained at -100°C, as demonstrated by the 60% yield of **38** obtained after 4 h. Conversely, it was found that at temperatures below -105°C the reaction became sluggish. Although this may have been due to the solvent (THF) freezing at this temperature, addition of a lower freezing point co-solvent, such as ether, did not improve the reaction rate. The reduction in yield evident at higher temperatures (particularly at -78°C) may be attributed to carbene formation by elimination of TMSOLi in a manner analogous to that described by Shiori *et al.*

The potential of this reaction to prepare a wide range of *N*-carbamate-protected DON ester derivatives was evident. Therefore the pyroglutamate ester derivatives shown below were synthesised.



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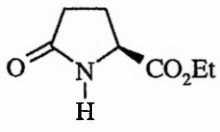

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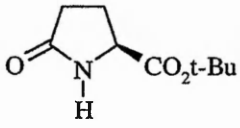


44

The standard route to benzyl pyroglutamate[129] (**42**), as outlined in **scheme 17**, p. 55, gave the desired product in 58-60% yield. However, purification required column chromatography to remove excess benzyl alcohol which, when working on a large scale, proved inconvenient. Modification of a reported procedure[130] in which Z-pyroglutamic acid was esterified with benzyl bromide and triethylamine gave the benzyl pyroglutamate **42** in 64% yield. More importantly, the purification procedure using this method was much simplified.

Although ethyl pyroglutamate **43** is commercially available, it is quite expensive and is also hygroscopic and is therefore better freshly prepared before use. The usual method of its preparation, in which thionyl chloride is added to a suspension of

glutamic acid in ethanol, gave the ester in moderate (48%) yield.[131] A modification of this procedure in which thionyl chloride is added to a suspension of pyroglutamic acid in ethanol afforded the ester in 34% yield.[132] Interestingly, when the reaction was heated under reflux for 2 h. this latter method gave the hydrochloride salt of diethylglutamate, possibly formed via acid-catalysed alcoholysis of the lactam bond. A third method, involving H_2SO_4-catalysed esterification with azotropic removal of water gave **43** in an improved yield of 54%.[133] Further improvement in yield (66%) was obtained by the reaction of pyroglutamic acid with ethyl bromide and triethylamine as previously described for the preparation of benzyl pyroglutamate. However, the best yield (83%) of **43** was obtained by treatment of a suspension of pyroglutamic acid in ethanol with oxalyl chloride.

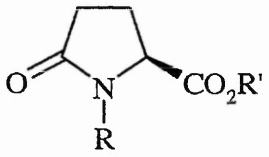
The *t*-butyl ester **44** was prepared in good yield (71%) via the acid-catalysed transesterification of pyroglutamic acid with *t*-butyl acetate.[110]

The requirement for *N*-carbamate-protection to increase the activity of the lactam bond toward LiTMSD led to a range of *N*-carbamate-protected pyroglutamates (**34** and **45a-k**) being prepared (***Table 1***, p. 63) using the DMAP-catalysed acylation method of Grieco and Ragnarsson,[121,122] i.e., Method A. Both the BOC and trichloroethoxycarbonyl (TROC) groups were introduced in high yield. The poor result for the allyloxycarbonyl (ALOC) group is perhaps due to the instability of allyl chloroformate towards DMAP since the closely related reagent allyl dicarbonate has been reported to be decomposed by this reagent.[134]

Alternative procedures for the *N*-carbonylation of amides have been reported, generally involving formation of the nitrogen anion with a suitably strong base before

reaction with the acylating reagent.[135] Recently, it has been reported that lithium *bis*(trimethylsilyl)amide helps prevent racemisation due to its steric bulk, unlike bases such as LDA and sodium hydride.[136] Treatment of pyroglutamate esters with this base, followed by addition of the appropriate chloroformate or dicarbonate, furnished the

Table 1. **N-Protection of pyroglutamate esters (34 and 45a-k) via DMAP-catalysed acylation, (Method A), and *N*-anion acylation, (Method B).**



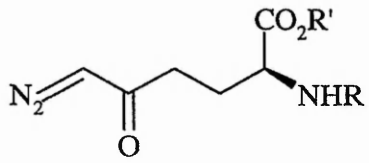
Method	Compound	R	R'	Yield (%) Method A	Yield (%) Method B
A	**45a**	BOC	Et	95	-
A and B	**34**	BOC	Bn	92	76
A and B	**45b**	BOC	*t*-Bu	98	80
A and B	**45c**	TROC	Et	88	67
A and B	**45d**	TROC	Bn	96	66
A and B	**45e**	TROC	*t*-Bu	98	74
B	**45f**	ALOC	Et	-	78
A and B	**45g**	ALOC	Bn	20	64
B	**45h**	ALOC	*t*-Bu	-	72
B	**45i**	FMOC	Et	-	83
B	**45j**	FMOC	Bn	-	75
B	**45k**	FMOC	*t*-Bu	-	84

compounds listed in ***Table 1***, i.e., Method B.

Yields of the BOC and TROC compounds were good although slightly lower than in Method A. The ALOC derivatives were prepared in good yield and the previously inaccessible fluorenylmethoxycarbonyl-protected (FMOC) pyroglutamates were also prepared in high yield.

Ring-opening of the pyroglutamates with LiTMSD gave the diazoketones **38** and **46a-i** in the yields shown in ***Table 2***. As discussed earlier (section 2.2.4.2, p. 58),

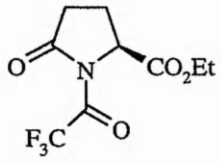
Table 2. **Yields of diazoketones (38 and 46a-i) obtained from the ring-opening of *N*-protected pyroglutamates with LiTMSD.**



N-Protected pyroglutamate	Diazoketone	R	R'	Yield (%)
45a	**46a**	BOC	Et	66
34	**38**	BOC	Bn	71
45b	**46b**	BOC	*t*-Bu	75
45c	**46c**	TROC	Et	20
45e	**46d**	TROC	*t*-Bu	16
45f	**46e**	ALOC	Et	63
45g	**46f**	ALOC	Bn	60
45i	**46g**	FMOC	Et	75
45j	**46h**	FMOC	Bn	61
45k	**46i**	FMOC	*t*-Bu	73

optimum yields were achieved when the reaction temperature was maintained between -100 and -105°C for BOC, FMOC and ALOC derivatives. However, the TROC derivatives (**46c,d**) did not react at this temperature although at -70°C a low yield was obtained.

Ring-opening of alternatively *N*-protected lactams has been cited in the literature.[137] This led to an investigation into the potential of protecting groups other than carbamate. Of interest were protecting groups which could be removed under basic conditions as reported by Weygand or by other similarly mild conditions. The reported protection of ethyl pyroglutamate as the *N*-trifluoroacetamide[138] **47** was carried out but, unfortunately, the product is highly labile (cleaved by methanol in one minute) limiting its synthetic use.



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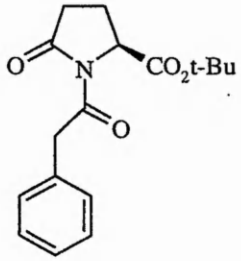

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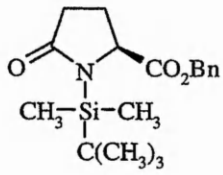

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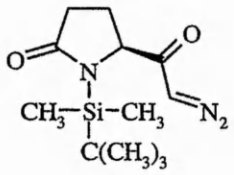


50

Compound **48**, containing the phenylacetyl group, was prepared in 69% yield, as ring-opening would give the *N*-phenylacetyl-protected diazoketone from which the blocking group might be enzymatically removed under neutral conditions.[139] Unfortunately, ring-opening of **48** with LiTMSD was not observed.

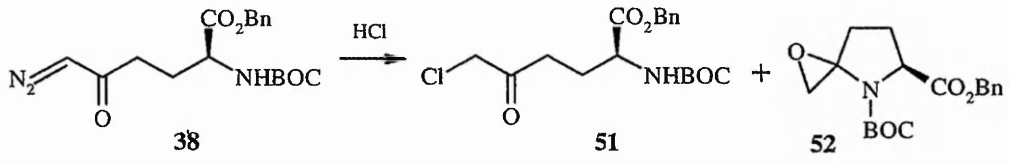
The *t*-butyldimethylsilyl group has been reported in the protection of amides.[140] Compound **49** was prepared but, although with LiTMSD no ring-opening was observed, interestingly, a moderate yield (55%) of the α-diazoketone **50** was obtained

after stirring at low temperature (-100°C) for 4 h. Also of note is the failure to isolate any α-diazoketone from the attempted ring-opening of the *N*-phenylacetate-protected pyroglutamate **48**. These results show that: 1) pyroglutamates undergo ring cleavage with LiTMSD if the lactam nitrogen is also part of a carbamate and; 2) benzyl esters may undergo an alternative reaction in which the side chain carbonyl group reacts to give a diazoketone, although *t*-butyl esters do not appear to be susceptible.

2.2.4.4 REMOVAL OF URETHANE-*N*-PROTECTING GROUPS

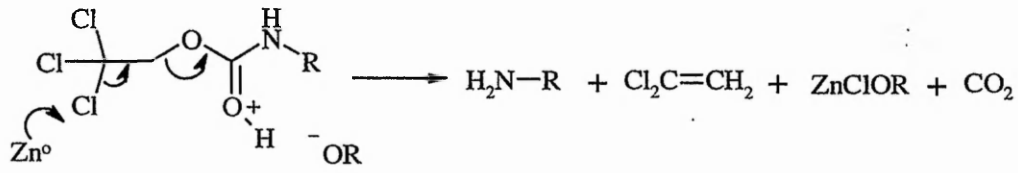
DON (**22**) has been reported to be most stable over the pH range of 4.5-6.5.[103] However, the usual methods for the removal of *N*-BOC protecting groups commonly employ TFA or HCl and are therefore considered unsuitable for the deprotection of **38**. It was thought that the diazo group of **38** might be stable under the more recently reported neutral conditions used for deprotection. An investigation of the deprotection reaction of **38** was carried out systematically with trimethylsilyliodide,[141] trimethysilyltrifluoromethanesulfonate,[142] tin(IV) chloride[143] or one equivalent of 4-toluenesulfonic acid.[144] All of the deprotection reagents employed gave complex reaction products from which none of **41** could be isolated (**scheme 22**, p. 60).

An alternative approach was to convert the diazoketone into an acid-stable base-labile halomethyl ketone before deprotection. Treatment of **38** with HCl afforded a low yield of the halomethyl ketone **51** and also epoxide **52** formation via a Darzens type reaction (**scheme 23**, p. 67). Interestingly, it was observed by tlc that samples of ketone **51** in solution showed conversion to epoxide **52** upon standing. This result demonstrates the remarkable tendency toward cyclisation of *N*-BOC-glutamic acid derivatives.



Scheme 23

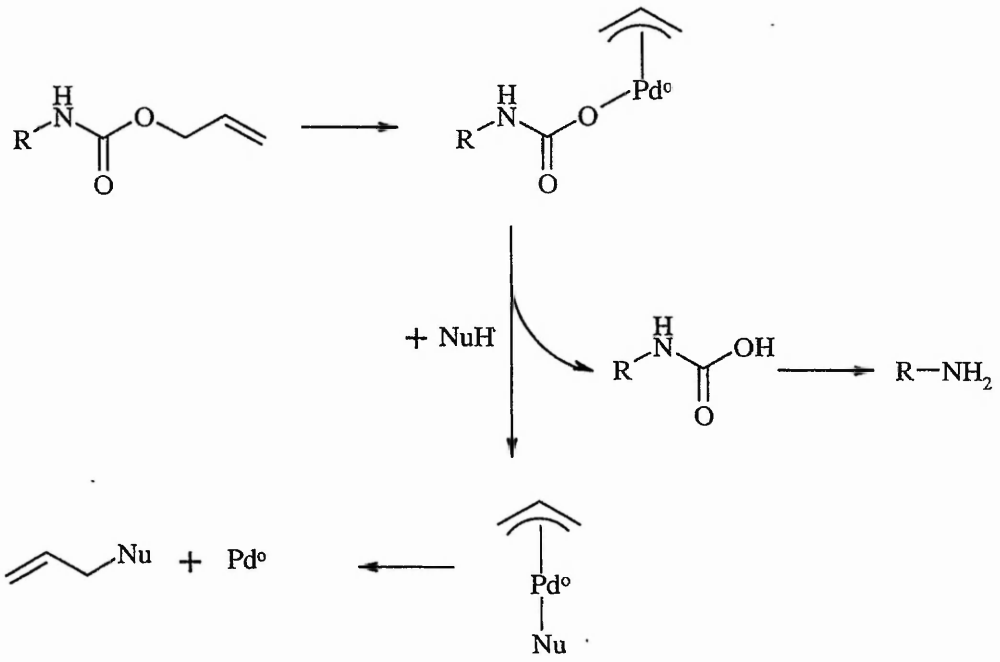
The TROC group is stable to both acid and base but, as mentioned previously, is cleaved by zinc dust in protic solvents such as aqueous THF, ethanol and acetic acid. The process is reductive in that electrons are added to the system. However mechanistically it is similar to a β-elimination type reaction in which electrons are pushed through a two carbon system leading to alkyl-oxygen fission (**scheme 24**).[145]



Scheme 24

The deprotection of the TROC diazoketones (**46c,d**) were unsuccessful. The most attractive method for the removal of the TROC group, i.e., zinc in buffered aqueous tetrahydrofuran, gave no reaction although a similar method had smoothly removed a trichloroethyl ester (**scheme 17**, p. 55). An alternative method, using activated zinc in ethanol under reflux, afforded deprotection as shown by the i.r. spectrum, but disappearance of the diazo group, probably via either a zinc-mediated reductive pathway or by simple thermolysis, was also evident.

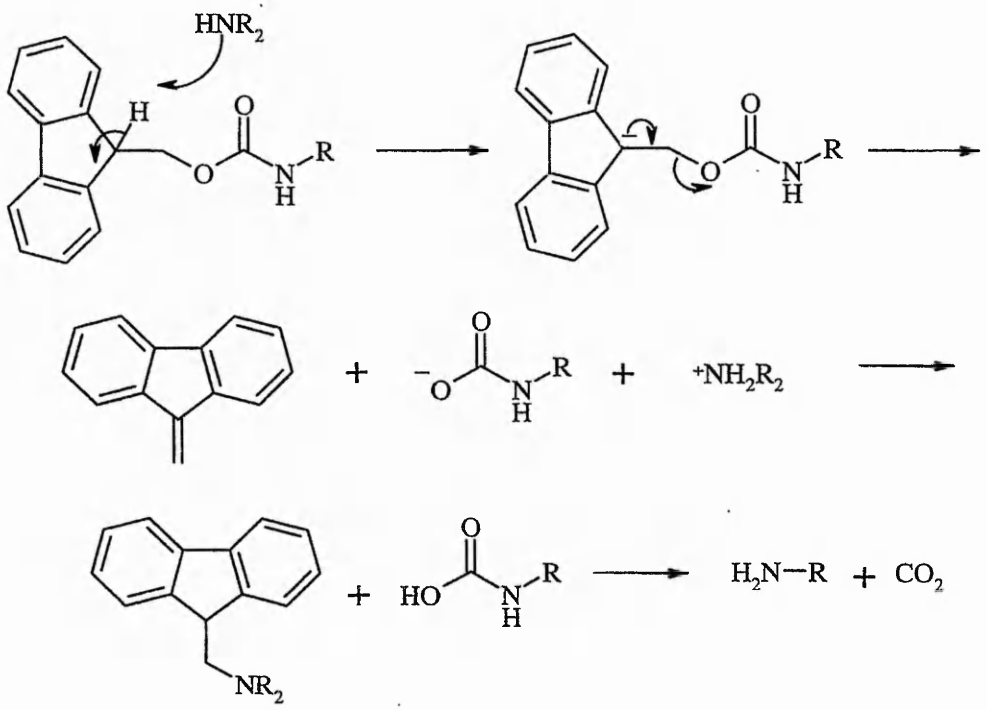
ALOC protection has been used in a number of syntheses, such as glycopeptide and carbapenem work, in which mild deprotection is a prerequisite. The group is generally removed through palladium(0)-catalysed allyl group transfer in which π-allyl Pd complexes are intermediates[145] (**scheme 25**).



Scheme 25

The allyl acceptor is often an amine, although the deprotection of carbamates can be achieved by employing the mildly acidic reagent dimedone or, occasionally, 1,3-dimethylbarbituric acid.[145] Attempts to remove the ALOC group using either this method or the previously successful method used to remove an allyl ester (**schemes 15** and **17**, p. 50 and 55, respectively) were both unsatisfactory. It was suspected that the palladium(0) complex may have coordinated to the diazo group causing decomposition of the diazoketone. Complexes of this type are known, the stability of which is dependent on the coordinating metal and the nature of the diazo group.[111]

Cleavage of FMOC groups occurs through a β-elimination pathway under mild basic conditions by amines or fluoride ion (**scheme 26**).[145]



Scheme 26

The standard deprotection procedure of a 5-10% solution of piperidine in a suitable solvent such as acetonitrile or DMF failed[145] to give the aminodiazoketone. Recently KF in the presence of 18 crown 6 has been advocated as a mild method for FMOC removal.[146] Application of this procedure to the ethyl and benzyl diazoketones **46g** and **46h** afforded no product as shown by the complete disappearance of the diazoketone peak in the i.r. spectrum of the reaction mixture. The *t*-butyl compound **46i** yielded the *t*-butyl ester of DON, which was found to be unstable decomposing on standing overnight even when stored at 0°C. The ester was again obtained when **46i** was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU),[147] whilst **46g** and **46h**

failed to yield any product.

The stability of the diazoketone function was tested against the reagents used to remove the FMOC group. This was achieved by allowing solutions of the BOC-protected DON derivative **38** to stand in the presence of the reagents, piperidine, DBU and tetra-*n*-butylammonium fluoride. No significant decomposition was observed over several days. These results suggested that either the ester derivatives of DON were inherently unstable, or that the diazoketone function may have been reacting with the dibenzofulvene side product, which is known to couple with nucleophiles.

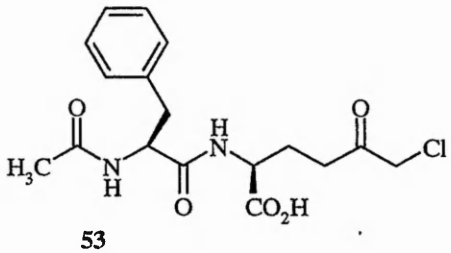
Subsequently, modification of the procedure of Weygand *et al.*[106] and Pettit and Nelson[103] for the removal of TFA groups with 1M NaOH was carried out. This would allow saponification of the benzyl and ethyl esters from compounds **46g** and **46h**, but not **46i,** to yield DON (**22**) which is known to be stable and additionally precipitating the dibenzofulvene by-product from the reaction. Using this method both **46g** and **46h** did, indeed, yield DON (**22**). Compound **46i** again yielded the unstable *t*-butyl ester derivative of DON. However, the drawback to this method was the difficulty in separating the highly water soluble DON (**22**) from the inorganic salts of the reaction mixture. This problem had previously been overcome either by Sephadex chromatography, which separates molecules by differences in size, or by difficult recrystallisation procedures. In an attempt to simplify the purification step NaOH was replaced by an organic base (piperidine). Under these conditions the reaction was somewhat slow and low yielding, probably due to solubility problems. However, adopting the original method described by Carpino and Han[148] the FMOC-substituted DON derivatives **46g** and **46h** were dissolved in a large excess of neat piperidine for

between one and two minutes followed by pouring the mixture into cold water which precipitated the dibenzofulvene-piperidine adduct. Fortuitously, the resulting aqueous piperidine solution was sufficiently basic to saponify both the benzyl ester of **46h** and the ethyl ester of **46g**. The subsequent removal of volatile components yielded crude DON (**22**), which was purified by recrystallisation. The optical activity of **22** is in accordance with that reported for the natural product[103,106] suggesting that little racemisation has occurred during the synthesis. A report of this synthesis has been published.[149]

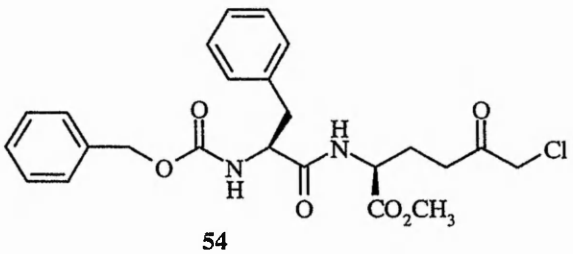
2.2.4.5 SYNTHESIS OF TRANSGLUTAMINASE INHIBITORS DERIVED FROM DON (22)

The development of a short, reliable synthesis of DON (**22**) allowed a range of inhibitors based on Dr. El-Aloui's compound (**17**) (section 2.2, p. 44) to be prepared and evaluated.

The importance of the Z-group was determined by the preparation of the *N*-acetyl derivative **53** from the *N*-hydroxysuccinimide active ester of commercially available *N*-acetyl phenylalanine and DON (**22**). This compound was found to have poor inhibitory properties against transglutaminase which confirmed the significance of the Z-group.



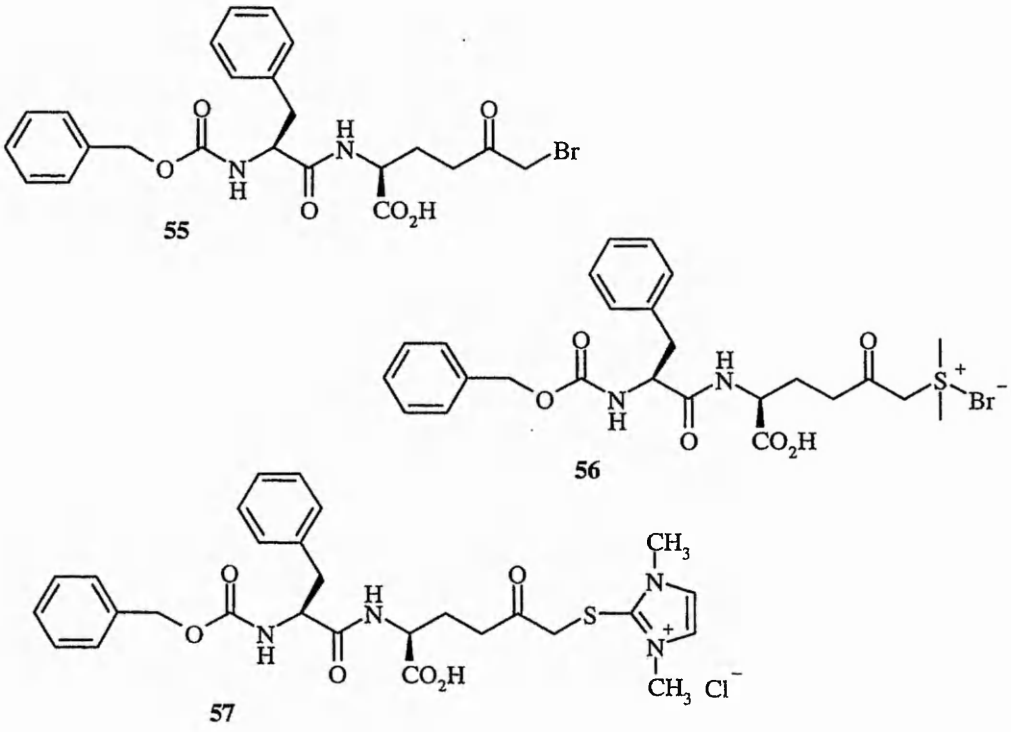
53



54

Although the attempted synthesis of ester derivatives of DON had been unsuccessful, the reaction of **17** with TMSD in methanol[150] afforded the methyl ester **54**, which proved to be a good inhibitor of transglutaminase.

Alteration of the electrophilic portion of the molecule was undertaken in an attempt to improve the affinity of the inhibitor for the enzyme. The bromomethyl ketone **55** had inhibitory properties similar to those of the chloromethyl ketone **17**, but it was less soluble in the media used for enzyme assay. The Syntex derivative **56**, prepared via reaction of dimethylsulfide and bromomethyl ketone **55**, was found to be a moderately good, specific inhibitor but, more importantly, was found to be water soluble and thus had potential to be an effective compound for whole cell work. This result demonstrates that compounds containing a salt have the capability to meet the required target of water soluble inhibitors.



55

56

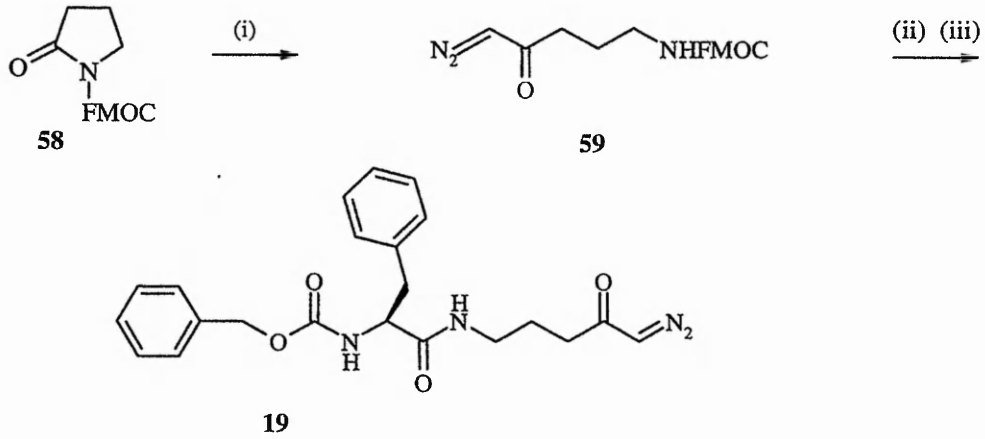
57

Obvious candidates were the *N*-methyl mercaptoimidazole compounds developed by Merck. A synthesis of **57** was therefore attempted via the reaction of chloromethyl ketone **17** with 2-mercapto-1-methylimidazole but no product was isolated, possibly due to complications arising from the presence of the acid grouping. Attempts to prepare the methyl ester derivative were not undertaken due to time constraints but for a possible alternative route to compounds of this type see miscellaneous section.

A more detailed discussion of the biological evaluation of the transglutaminase inhibitors synthesised is given in chapter 3, p. 105.

2.2.4.6 RING-OPENING OF LACTAMS

Extention of this procedure to the synthesis of Syntex compounds and derivatives was attempted via ring-opening of the related *N*-FMOC-pyrrolidinone **58** with LiTMSD (**scheme 27**).

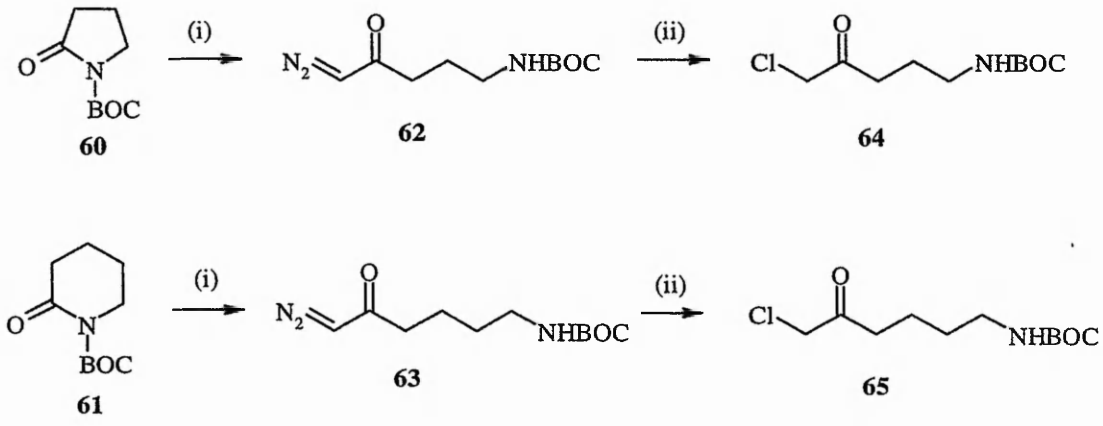


58 **59** **19**

Scheme 27 *Reagents & Conditions*: i. LiTMSD, THF, -100°C; ii. piperidine; iii. **9**, THF.

Pyrrolidinone was converted in 87% yield to carbamate **58** by the lithium *bis*(trimethylsilyl)amide-mediated reaction with 9-fluorenylmethyl chloroformate. Subsequent ring-opening with LiTMSD afforded the diazoketone **59** in 69% yield. Removal of the protecting group with piperidine, followed by coupling with Z-phenylalanine-*N*-hydroxysuccinimide active ester **9**, without isolation of the aminodiazoketone, afforded the Syntex intermediate **19**.

An alternative procedure was also undertaken in which the diazoketones **62** and **63** were prepared from the *N*-BOC lactams **60** and **61** and LiTMSD in 67 and 73% yield, respectively (**scheme 28**). Subsequent decomposition with acid to afford the halomethyl ketones (**64** and **65**) was attempted in a similar manner to that previously described for the *N*-BOC pyroglutamates. In this instance the halomethyl ketones were isolable by chromatography in moderate yield. Deprotection was undertaken with trifluoroacetic acid and the salts were used without purification. Unfortunately, attempts to couple with Z-phenylalanine failed to give the chloro derivative of Syntex intermediate **20a** (**scheme 12**, p. 45) or its higher chain length homologue.



Scheme 28 *Reagents & Conditions*: i. LiTMSD, THF, -100°C; ii. HCl in ethyl acetate.

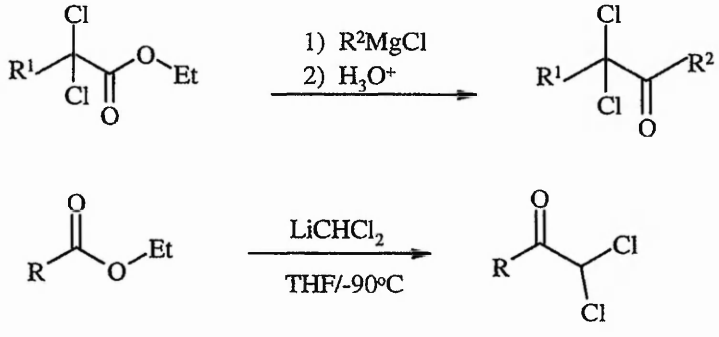
2.3 DIRECT ROUTES TO HALOMETHYL KETONES

The reaction of LiTMSD with *N*-FMOC ethyl pyroglutamate gave access to the key intermediate DON (**22**). However, the synthesis did have the drawback that it did not allow easy access to a range of ester derivatives of **17**. It was therefore considered of interest to seek a route to halomethyl ketones which avoids the use of diazoketone intermediates but which gives the desired ester derivatives.

2.3.1 α-HALOMETHYL KETONES FROM CARBOXYLIC ACIDS

There are a number of methods available for the formation of chloro- and bromomethyl ketones and these have been reviewed.[151] The methods fall into two broad categories, namely: methods for the direct halogenation of ketones and; functional group interconversions. Direct methods tend to yield multiple halogenation or mixtures of products. Of the number of functional group interconversions, direct formation of halomethyl ketones from carboxylic acid derivatives seem most suitable.

The direct formation of α-halomethyl ketones from carboxylic acid derivatives has been known for some time, but early examples have tended to produce

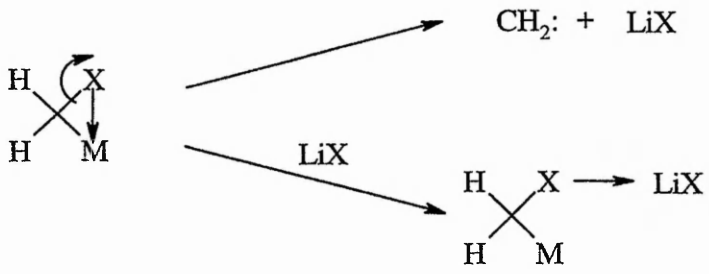


Scheme 29

dihaloketones, and are fairly limited in scope. These include the treatment of α,α-dichloroesters with Grignard reagents and the action of dichloromethyllithium on esters (**scheme 29**, p. 75).[151]

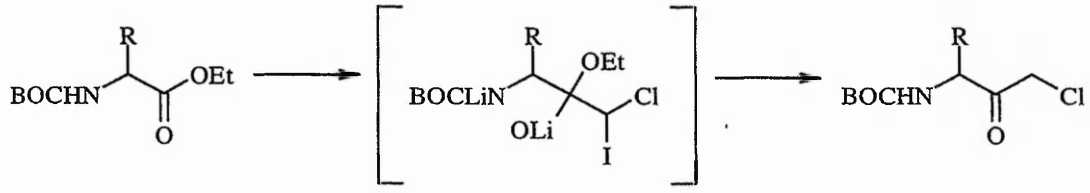
The use of monohalomethyl lithium intermediates has been subjected to reinvestigation. Syntheses have been reported covering a number of applications, such as homologation of boronic esters,[152] synthesis of epichlorohydrins and allyl alcohols,[153] and the preparation of halohydrins, epoxides and α-halomethyl ketones.[154]

The main difficulty encountered in the use of monohalomethyllithium intermediates is their extreme thermal instability and ready decomposition to carbenes. This has led to two general techniques being employed in their generation. The first, and most commonly adopted procedure, involves the generation of the intermediate formed in the presence of the substrate at low temperatures such as -78°C.[153] The second, employed by Villieras *et al.*, uses extremely low temperatures, typically < -115°C, and, in addition, the presence of a suitable salt such as lithium bromide to generate the monohalomethyllithiun intermediate to which the substrate is added.[154] This latter requirement is of particular importance as, in the absence of the salt, decomposition occurs even at temperatures as low as -130°C. The role of the salt is to overcome the intramolecular coordination between the halogen and the metal which has been suggested by a number of groups to contribute to the instability of the carbenoid (**scheme 30**, p. 77). Thus the presence of one equivalent of a mild Lewis acid such as LiX is sufficient to stabilise the carbenoid by coordination of the halogen to the electron acceptor.



Scheme 30

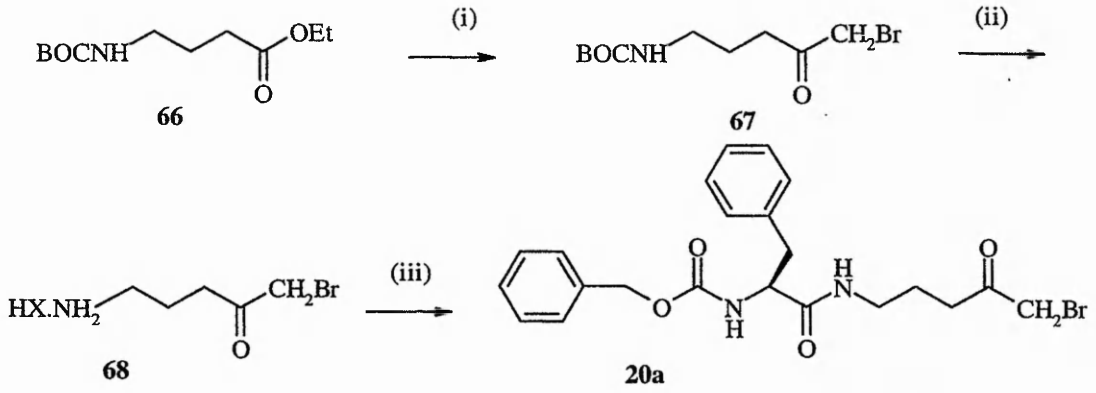
A very recent paper describes the preparation of α-chloroketones of *N*-carbamate-protected α-amino acids.[155] This procedure is based on earlier reports by Kowalski *et al.*[156] on the chemistry of enolate anions generated by the reaction of lithium dibromomethane with esters. The use of lithium dibromomethane was found to be unsatisfactory when applied to *N*-carbamate-protected α-amino acids, however optimisation of the reaction under the conditions shown in **scheme 31** yielded a number of amino acid α-chloroketones in yields ranging from 50-86%. It was reported that four equivalents of CH_2ICl/LDA were necessary to carry out the addition/metalation/elimination via the intermediate shown in **scheme 31** and drive the reaction to completion. Additionally, one equivalent of LDA was required to deprotonate the carbamate nitrogen.



Scheme 31

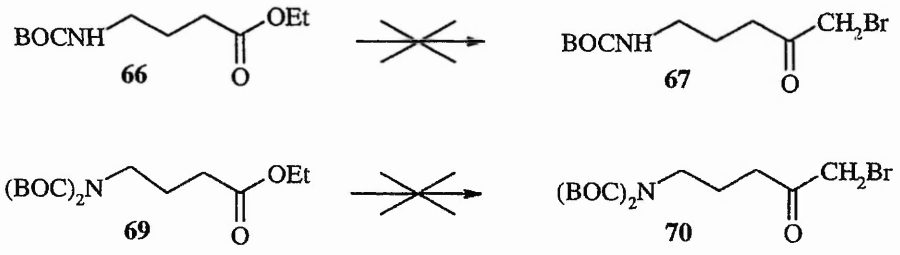
Our initial attempted synthesis was based around the method of Villieras, in which an ester is treated with lithium bromomethane at low temperature in the

presence of LiBr (**scheme 32**). The ethyl ester of 4-aminobutyric acid (GABA) was chosen as the initial starting material because of the complication of two carboxylic groups present in glutamic acid. The attempted synthesis involved reaction of the ester **66** with bromomethyllithium to give the bromomethyl ketone **67**. Deprotection followed by coupling to Z-phenylalanine would then furnish the desired product **22a.**



Scheme 32 *Reagents & Conditions*: i. dibromomethane, *n*-BuLi, LiBr, THF/pentane/ether, -115°C; ii. trifluoroacetic acid, DCM; iii. **9**, TEA, DCM.

Unfortunately no bromomethyl ketone was isolated perhaps due, either to failure to trap the carbenoid intermediate or to reaction of the carbanion with the acidic carbamate hydrogen.



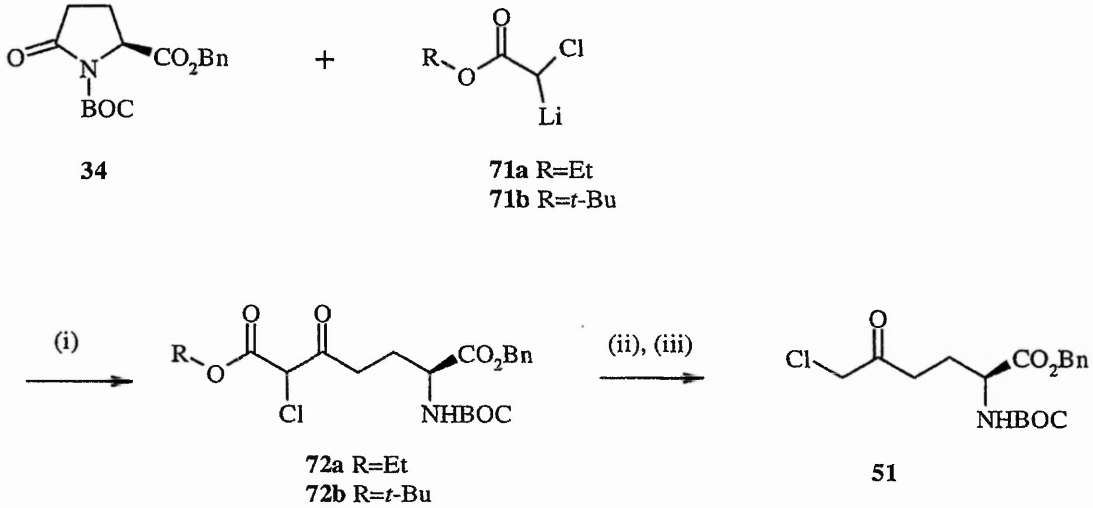
An alternative strategy using the *bis*-protected[108] aminobutyrate **69** to overcome problems caused by deprotonation, also failed to give the desired product.

2.3.2 HALOMETHYL KETONES VIA RING-OPENING OF *N*-PROTECTED PYROGLUTAMATES AND RELATED LACTAMS

These results suggested employing the previously successful use of the ring-opening reactions of pyroglutamates. In addition, a procedure for stabilising the lithium halomethane intermediate was sought.

2.3.2.1 ATTEMPTED RING-OPENING WITH LITHIUM HALOACETATES

The first synthetic approach using this methodology is outlined in **scheme 33**. Since reaction of pyroglutamates with ester lithium enolates has been reported to give β-keto ester derivatives of glutamic acid in good yield, (section 2.2.4.1, p. 51) it was hoped to apply this to the synthesis of the α-halo-β-keto esters **72** by utilising lithium α-haloacetates **71a** and **71b**. It was envisaged that the presence of a carbonyl group adjacent to the lithium halomethane moiety in the intermediates **71a** and **71b** may assist in stabilisation of the anion. Saponification, followed by decarboxylation of **72a**



34

71a R=Et
71b R=*t*-Bu

72a R=Et
72b R=*t*-Bu

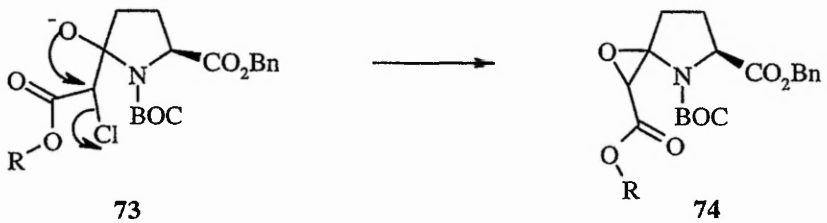
51

Scheme 33 *Reagents & Conditions*: i. THF, -78°C; ii. 1M NaOH or trifluoroacetic acid; iii. heat, toluene.

or **72b** using standard methods, would then furnish the desired γ-halomethyl ketone derivative of glutamic acid **51** which after deprotection and subsequent coupling to the required amino acid **8** would give the benzyl ester of **17**.

Ring opening of **34** with **71a** or **71b** at -78°C gave complex mixtures from which the product could not be isolated except in the case of lithium *t*-butyl chloroacetate when an impure sample of **72b** (R=*t*-Bu) was obtained in a 30% yield.

Failure of these reactions may have been due to a number of factors. The lithium haloacetate **71** may have eliminated LiX to give a carbene species, although the aromatic portion of both the ^{1}H and ^{13}C NMR spectra of the reaction product showed no evidence of attack by an electrophilic species such as a carbene. Alternatively, the intermediate **73** may have undergone a Darzens type reaction as shown in **scheme 34** to yield an epoxide **74**.



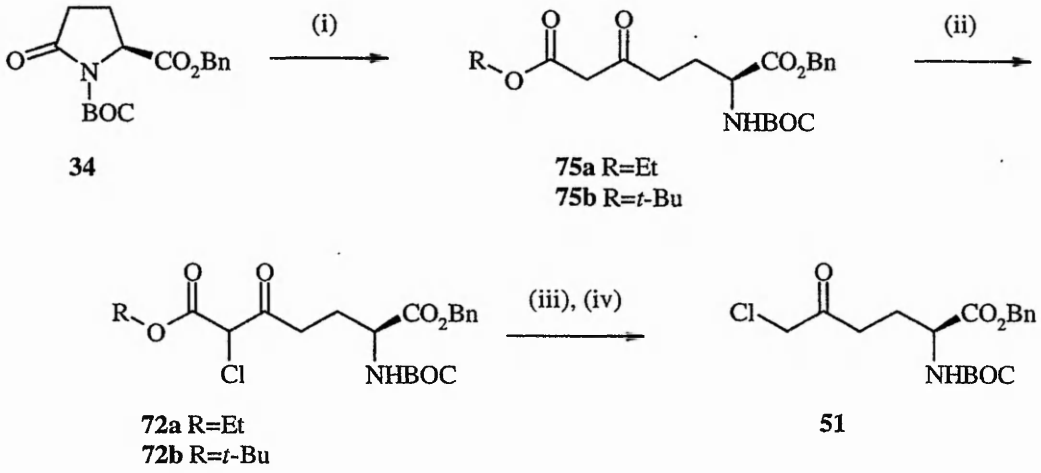
73 **74**

Scheme 34

A third possible side reaction is attack by the nitrogen anion on the α-halogen resulting in polymerisation. Analysis of the n.m.r. spectrum of the reaction mixture suggests that both epoxide formation and polymerisation were the more likely side reactions.

2.3.2.2 ATTEMPTED CHLORINATION OF β-KETO ESTER DERIVATIVES OF GLUTAMIC ACID

The possible involvement of the anion intermediate **73** in the side reaction led us to alter our procedure (**scheme 35**). Using this protocol the compounds **75a** and **75b** were synthesised as described in the literature[119] in moderate to good yield (60% and 75%, respectively). The increased activity of the α-hydrogen situated between the ester and keto functions was expected to allow regioselective chlorination with a suitable chlorinating agent such as sulfonyl chloride.[157] However the products isolated from the reactions were identical, by n.m.r., to those previously obtained for compounds **72a** and **72b**. This led to the conclusion that compounds of the type **72** are insufficiently stable to be useful synthetic intermediates.



34

75a R=Et
75b R=*t*-Bu

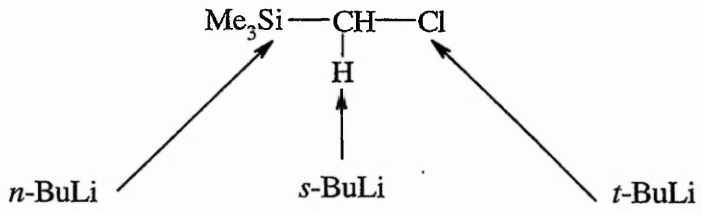
72a R=Et
72b R=*t*-Bu

51

Scheme 35 *Reagents & Conditions*: i. ethyl acetate or *t*-butyl acetate, LDA, THF, -78°C; ii. SO_2Cl_2, DCM; iii. 1M NaOH or trifluoroacetic acid; iv. heat, toluene.

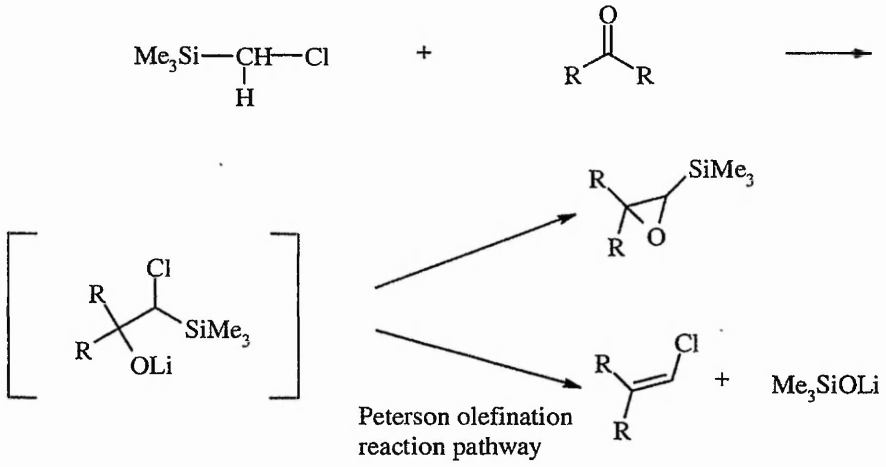
2.3.2.3 RING-OPENING OF PYROGLUTAMATES WITH LITHIUM CHLOROMETHYLTRIMETHYLSILANE

Another synthon for $LiCH_2Cl$ has been reported in which the carbanion is stabilised by a neighbouring silyl group.[158] The reagent chloromethyltrimethylsilane is commercially available and has found application in a wide range of synthetic manipulations.[159] It is readily converted to chloromethyl(trimethylsilyl)lithium by *sec*-butyllithium in the presence of tetramethylethylenediamine in high yield. Treatment with other bases was found to be much less efficient, possibly due to steric factors.[158] For example, *n*-buyllithium was considered small enough to attack the silicon atom giving an "ate" complex that can migrate alkyl groups with loss of Cl^-, whereas the steric bulk of *t*-butyllithium was found to give Cl-Li exchange.



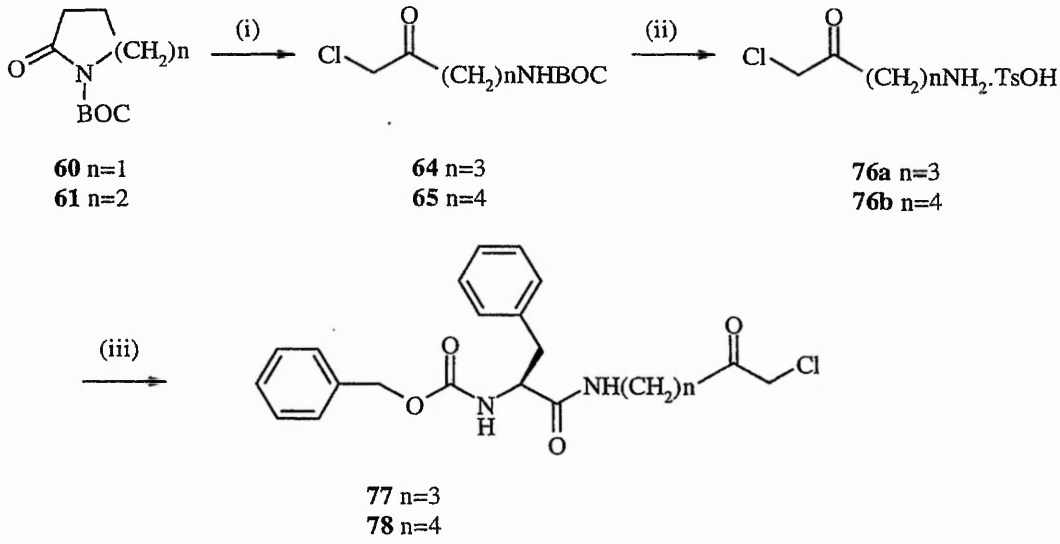
Reaction of this reagent with aldehydes and ketones (**scheme 36**, p. 83) was found to give α,β-epoxyalkylsilanes rather than the expected vinylchlorides that would arise from the elimination of trimethylsilanate via a Peterson reaction pathway.[158]

This led to speculation that reaction of this reagent with a carbonyl function bearing a leaving group might yield chloromethyl ketones (**scheme 37**, p. 83). Again *N*-carbamate-protected lactams were chosen as starting materials and, for the initial experiments, the *N*-BOC-pyrrolidinone **60** was used because of its simplicity. Reaction of **60** with lithium chloromethyltrimethylsilane at -78°C gave a low yield (31%) of the



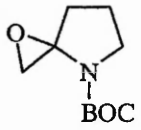
Scheme 36

the ring-opened product **64**. Similarly the piperidone **61** afforded the same yield of the homologous chloroketone **65**. Deprotection was accomplished with one equivalent of 4-toluenesulfonic acid[140] in high yield and the salts **76a** and **76b** were used without further purification in the dicyclohexylcarbodiimide mediated coupling reaction with Z-phenylalanine. None of **77** was isolated but dipeptide **78** was obtained in low yield (11%).

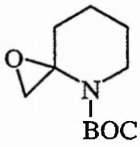


Scheme 37 *Reagents & Conditions*: i. chloromethyltrimethylsilane, *s*-BuLi, THF, -78°C; ii. 4-toluenesulfonic acid, ether; iii. **9**, TEA, THF.

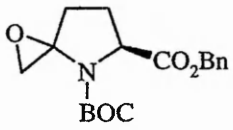
The initial ring-opening reactions were performed at low temperatures (< -65°C) over a short time scale in an attempt to minimise side reactions. The chloromethyl ketones **64** and **65** were isolated in low yield (20-30%) along with the corresponding epoxide (**79** and **80**, respectively) obtained in approximately 10% yield. Additionally, around 35% of the starting materials were recovered. Interestingly the epoxides contained no trace of the silyl group.



79



80



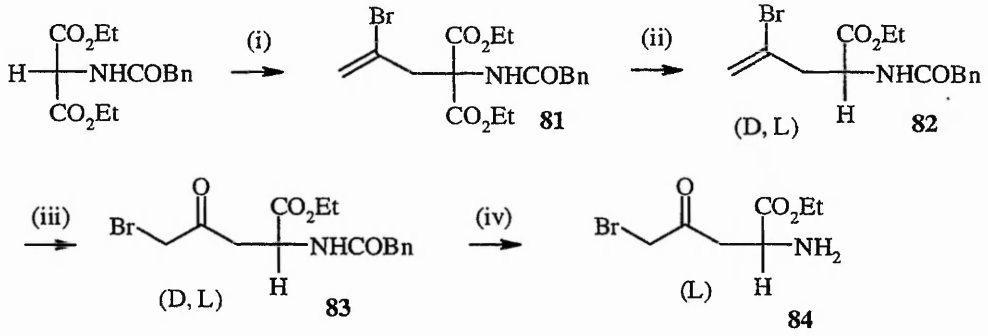
52

Expanding the reaction to include pyroglutamate derivatives yielded no isolable products, as it proved difficult to separate the epoxide side product from the desired chloromethyl ketone. Attempts to improve the yield by conducting the reaction at the higher temperature of -55°C or by extending the reaction time favoured the formation of the epoxide **52**. Indeed, stirring the mixture for 1.5 h. at -55°C afforded the epoxide **52** exclusively, as an equal mixture of diastereomers, in 55% yield.

The poor yields evident in the coupling step may possibly be attributed to a combination of reactivity of the chloromethyl ketones toward basic reaction conditions and the tendency of **76a** and **76b** to cyclise. The reactivity toward nucleophiles of α-halomethyl ketones has been shown to be greater than or equal to the corresponding alkyl halide depending on the nucleophiles, in the case of amines activity is about the same.[151] Hence, the presence of base and secondary amines (the amino groups of the substrate) in the reaction mixture would allow side reactions to occur so limiting formation of the product.

2.3.3 OXIDATIVE HYDROLYSIS OF VINYL BROMIDES

A recent paper details the *N*-bromosuccinimide-mediated oxidative hydrolysis of vinyl bromides to bromomethyl ketones.[160] The synthesis of a lower chain length homologue of compound **17** utilising this methodology was attempted as part of an undergraduate project as outlined in **scheme 38**.[161]



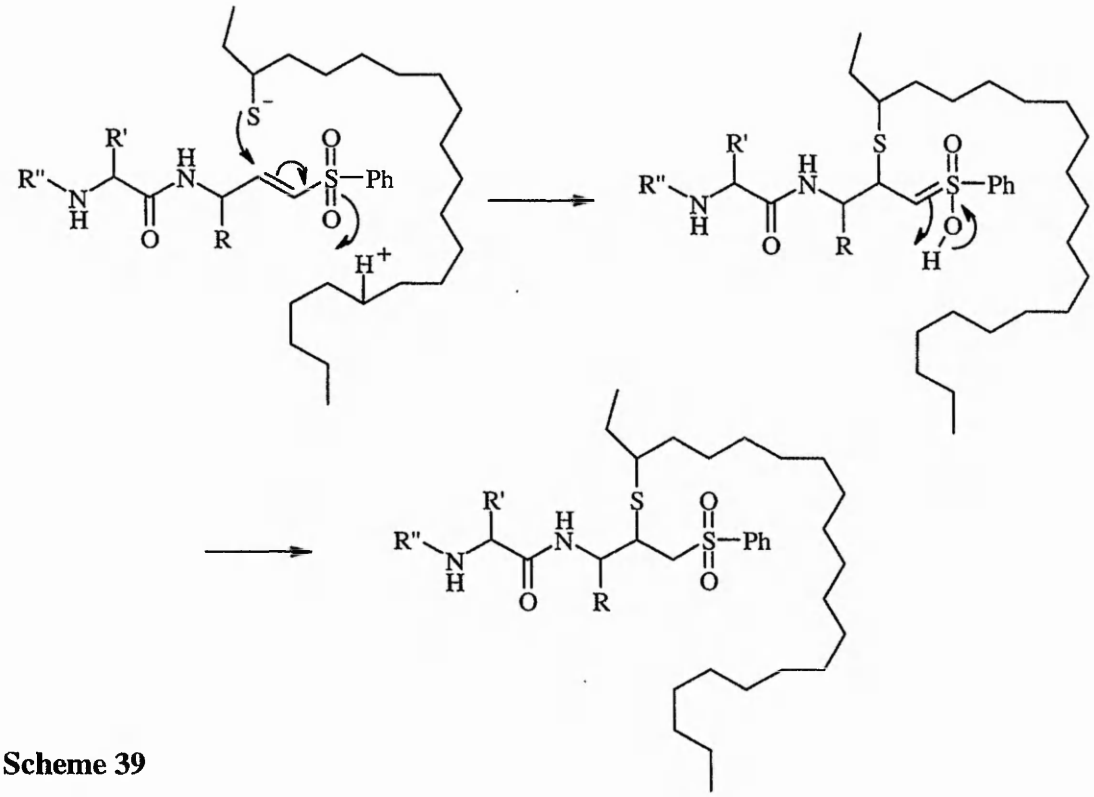
Scheme 38 *Reagents & Conditions*: i. 2,3-dibromopropene, NaH, THF; ii. LiBr, DMF, H_2O; iii. *N*-bromosuccinimide, acetonitrile/H_2O; iv. penicillin amidase, sodium phosphate buffer/methanol, pH 8.1.

The alkylation of *N*-acyl-protected diethyl aminomalonate with dibromopropene followed by decarboxylation afforded the racemic amino acid derivative **82**. Oxidation of the vinyl bromide gave bromomethyl ketone **83**. Although the required product could not be obtained at the deprotection step the preparation of **83** demonstrated the potential of this strategy.

Attempts by a colleague[162] to extend this methodology to the synthesis of compound **17** failed, as the protected aminomalonate did not react with 2,4-dibromo-1-butene. This reduced reactivity can be attributed to the stabilised allylic cation intermediate which can be formed by the dibromopropene but not by the dibromobutene substrate, which probably formed 2-bromobutadiene under the basic reaction conditions.

2.4 POTENTIAL INHIBITORS CONTAINING A VINYL SULFONE GROUP

Vinyl sulfones have recently been reported as mechanism-based inhibitors of cysteine proteases.[163] The effectiveness of these compounds against enzymes cathespin B, L, S, and O2, and calpain I and II was demonstrated and the mode of inhibition is understood to involve 1-4 addition to the double bond to yield a β-bound cysteine sulfone (**scheme 39**). Cathepsin S was found to be the most readily inactivated (best k_{obs}/[I] value of 2.9 x 10^7 $M^{-1}s^{-1}$) followed by cathepsin L (2.24 x 10^6 $M^{-1}s^{-1}$), cathepsin O2 (8.33 x 10^4 $M^{-1}s^{-1}$) and then cathepsin B (3.3 x 10^4 $M^{-1}s^{-1}$). Although the calpains

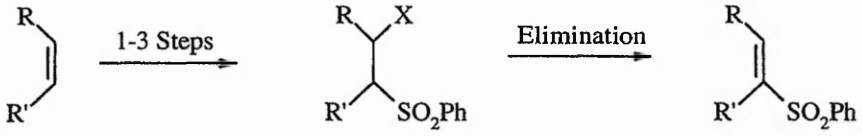


Scheme 39

were found to be quite resistant to inhibition incorporation of known recognition groups for calpain (for example the tripeptide residue; Leu-Leu-Tyr) led to significant inhibition (up to k_{obs}/[I] = 2.43 x10^4 $M^{-1}s^{-1}$).

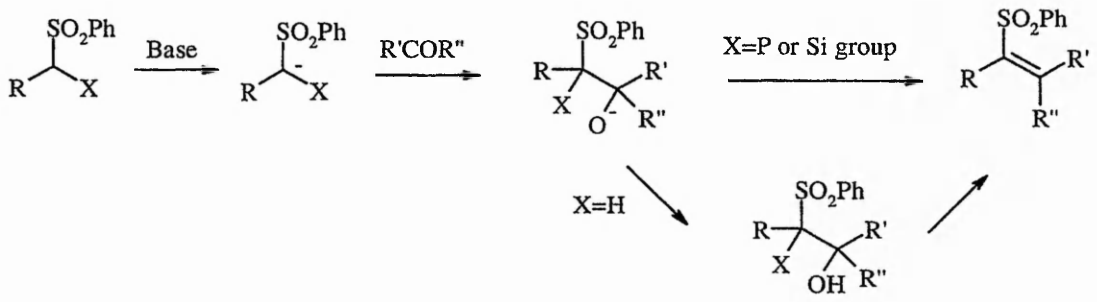
2.4.1 METHODS OF SYNTHESISING VINYL SULFONES

The numerous methods for the preparation of vinyl sulfones have been reviewed[164] although only two are commonly used. The first method, which is broadly applicable, employs ionic and radical additions to alkenes, alkynes and allenes, and involves the construction of a β-hetero-substituted sulfone which can then undergo elimination (**scheme 40**). The nature of X varies depending on the method of construction of the β-hetero-substituted sulfone, in general X= a halogen, a selenide (e.g. SePh) or mercuric chloride.[164]



Scheme 40

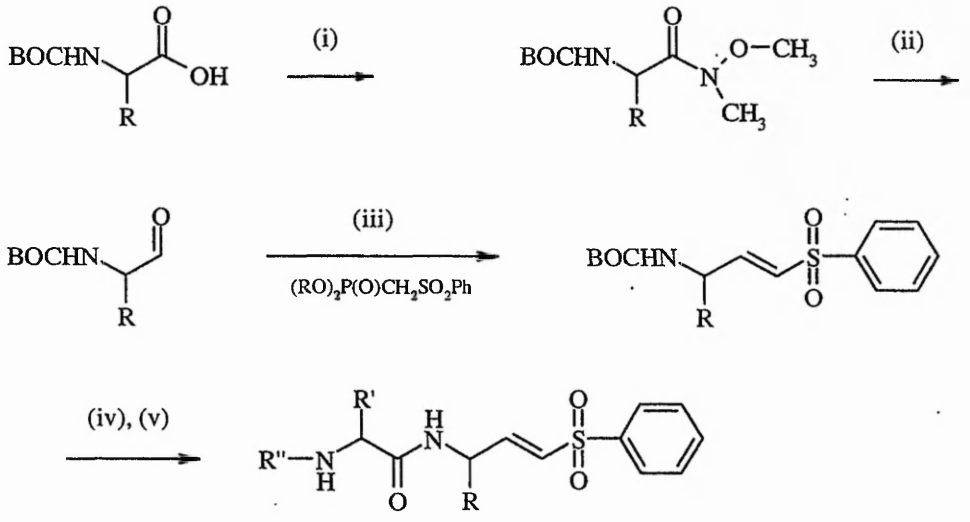
The second method involves the aldol, Wittig, Peterson and related reactions where addition of a sulfonyl carbanion to a carbonyl compound is followed by a subsequent elimination step (**scheme 41**). In the simplest case, where X=H, dehydration of the intermediate hydroxy sulfone is necessary, usually in a separate step,



Scheme 41

whereas the use of phosphorus (e.g. X=PPh_3) or silicon (X=$SiMe_3$) groups allows direct elimination to give the vinyl sulfone.[164] The other methods available are limited in scope[164] and consequently were not used in the course of this work.

The published method[163] for preparation of the cysteine inhibitors is shown in **scheme 42.** Chiral amino aldehydes were prepared via the reduction of *N,O*-dimethylhydroxamate derivatives of *N*-protected amino acids. Wadsworth-Emmons chemistry afforded the vinyl sulfone products, which were subjected to standard deprotection and coupling techniques to afford the peptidyl inhibitors.

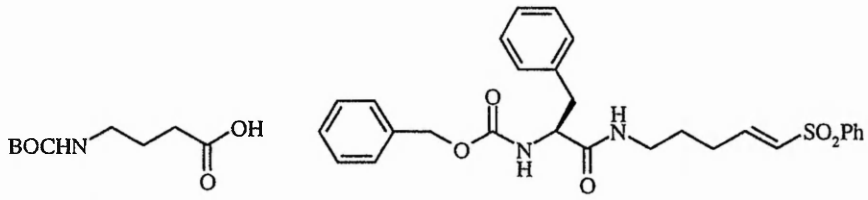


Scheme 42 *Reagents & Conditions*: i. *N,O*-dimethylhydroxylamine, DCC, TEA, DCM, 0°C→room temp.; ii. $LiAlH_4$, ether, 0°C; iii. NaH, THF, 0°C→room temp; iv. HCl, dioxane; v. R"NHCH(R')CO_2H, 4-methylmorpholine, *n*-butylchloroformate, THF, -10°C.

2.4.2 ATTEMPTED SYNTHESIS OF VINYL SULFONE INHIBITORS

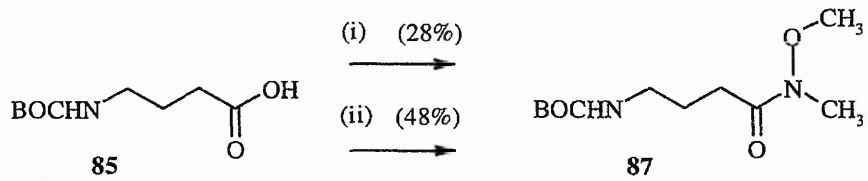
The initial proposed synthesis mirrored the above route (**scheme 42**) employing *N*-protected 4-aminobutyric acid **85** as the starting material. It was hoped that conversion of **85** to the vinyl sulfone followed by coupling to Z-phenylalanine would

furnish the potential inhibitor **86**.



85 **86**

Using the method shown in **scheme 43**, *N*-BOC GABA **85** was converted to the hydroxylamine **87** in low yield (28%). An alternative method[165] utilising the mixed anhydride approach gave an improved yield (48%).

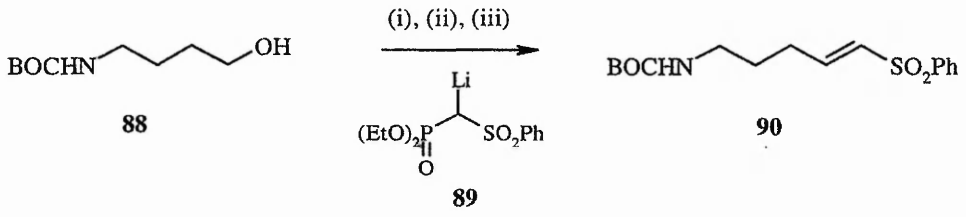


85 **87**

Scheme 43 *Reagents & Conditions*: i. *N,O*-dimethylhydroxylamine, DCC, TEA, DCM, 0°C→room temp.; ii. 4-methylmorpholine, *n*-butylchloroformate, THF, -10°C, then *N,O*-dimethylhydroxylamine.

Reduction of **87** with lithium aluminium hydride gave a complex mixture from which the required aldehyde could not be isolated. A potential route to the aldehyde was the reduction of the acid to the alcohol followed by oxidation.[166] Surprisingly, reduction of the acid with borane-dimethysulfide complex[167] afforded *N*-BOC pyrrolidine in 60% yield and none of the desired alcohol. However, the reduction of **87** was achieved by using the mixed anhydride of the acid[166] which was treated with sodium borohydride to give the desired alcohol **88** in 40% yield. Conversion of the alcohol **88** to the vinyl sulfone **90** was attempted via a two step reaction sequence in which Swern oxidation of the alcohol,[166] without isolation of the aldehyde, was

followed by reaction with **89**, a Wadsworth- Emmons type reagent[168] to give the vinyl sulfone **90**. However no product was isolated from this reaction (**scheme 44**).



Scheme 44 *Reagents & Conditions*: i. oxalyl chloride, DMSO, TEA, DCM, -63°C; ii. methyl phenyl sulfone, *n*-BuLi, diethylchlorophosphate, THF, 0°C; iii. aldehyde from i. and **89** from ii. THF, -78°C.

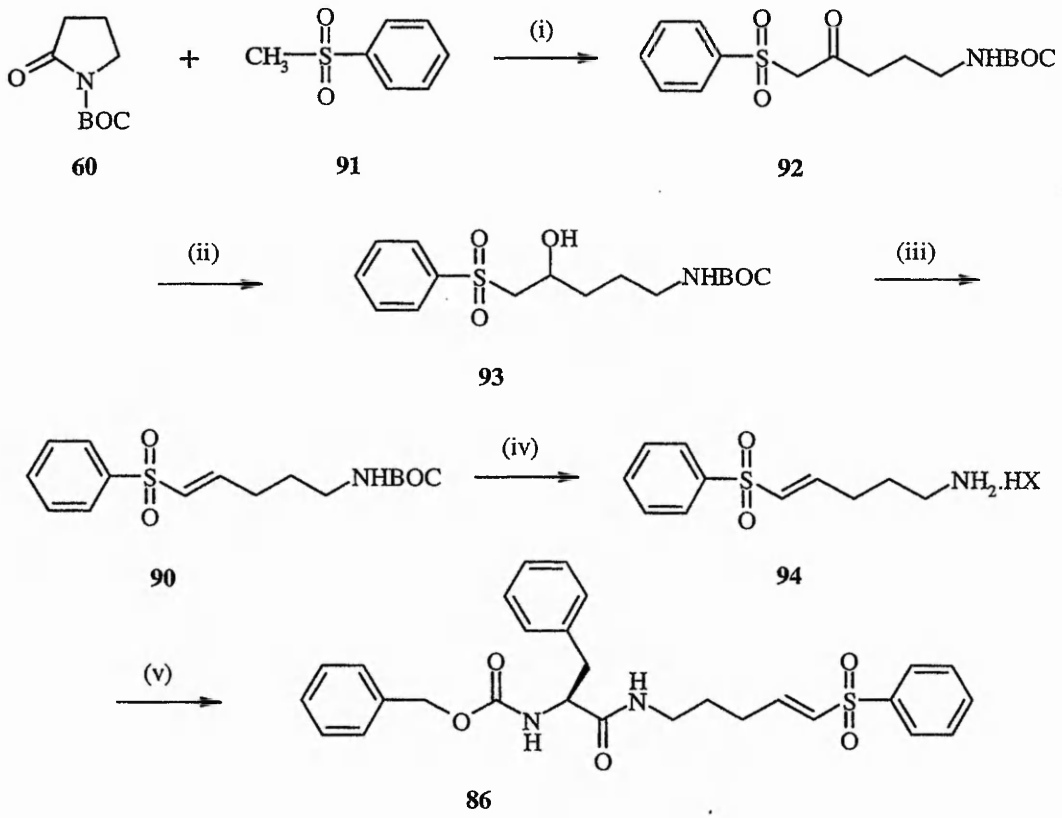
A search of the literature for methods of synthesising γ-alcohols or -aldehydes derived from 4-aminobutyric acid revealed that these transformations are prone to serious side reactions. Olsen *et al.*[169] have reported the ready cyclisation of *N*-BOC-protected glutamic acid γ-alcohols and -aldehydes (compounds which are analogous to **88** except for the presence of an α-carboxyl group) which is in agreement with the findings of this work. The low yields in the preparation of *N,O*-dimethylhydroxamate **87**, the formation of *N*-BOC pyrroldine in the attempted reduction step and the failure of oxidation to the aldehyde were all probably due to cyclisation.

Reduction and oxidation of the γ-terminus of glutamic acid has been described utilising the Z-oxazolidinone method of protection.[167] However this method is not applicable to our intermediate and so the use of an alternative protecting group may be a possibility. The literature reports that the Z group is less prone to cyclisation of glutamic acid derivatives and the FMOC group is completely resistant.[124] However removal of both these groups would be likely to affect the vinyl sulfone group. In the case of the Z group catalytic hydrogenation would reduce the alkene group and the

strongly nucleophilic amines normally used to remove FMOC functions might add across the double bond.

2.4.3 SYNTHESIS OF VINYL SULFONE INHIBITORS VIA RING-OPENING OF *N*-*t*-BUTOXYCARBONYL-2-PYRROLIDINONE

These drawbacks led to the alternative synthesis outlined in **scheme 45**, based on the previously successful ring-opening reactions of carbamate-protected lactams (section 2.2.4.3, p. 59). Addition of the lithium salt of sulfone **91** to BOC-protected



Scheme 45 *Reagents & Conditions*: i. *n*-BuLi, THF, -78°C; ii. DIBAL, THF, -78°C; iii. PPh_3/DEAD, THF, 0°C→room temp.; iv. 4-toluenesulfonic acid, ether, or TFA, DCM; v. **9**, TEA,THF.

pyrrolidinone **60** was expected to yield the β-ketosulfone **92**. Subsequent reduction of **92** would afford the alcohol **93** which could then be dehydrated to give the desired vinyl sulfone **90**.

Lactam **60** smoothly ring-opened upon treatment with lithium methyl phenyl sulfone to give **92** in 73% yield. Selective reduction of **92** was accomplished using diisobutylaluminium hydride (DIBAL), a reagent previously found to be effective in the reduction of keto sulfoxides,[170] yielding the alcohol **93** in 83% yield.

The presence of an acid-labile group required the dehydration step to be performed under mild conditions, and a standard variation of the Mitsunobu reaction[171] in which the alcohol is reacted with triphenylphosphine and DEAD in the absence of a nucleophile allowed **90** to be formed from **93** in high yield (97%). Deprotection using one equivalent of 4-toluenesulfonic acid[140] gave the salt **94** in moderate yield (48%) and subsequent coupling with Z-phenylalanine active ester **9** gave the sulfone **86** in reasonable yield (42%). An alternative procedure in which **90** was first treated with trifluoroacetic acid and then coupled with **9** gave the desired product in an improved overall yield of 49% for the two steps.

The biological results are detailed in chapter 3, p. 105.

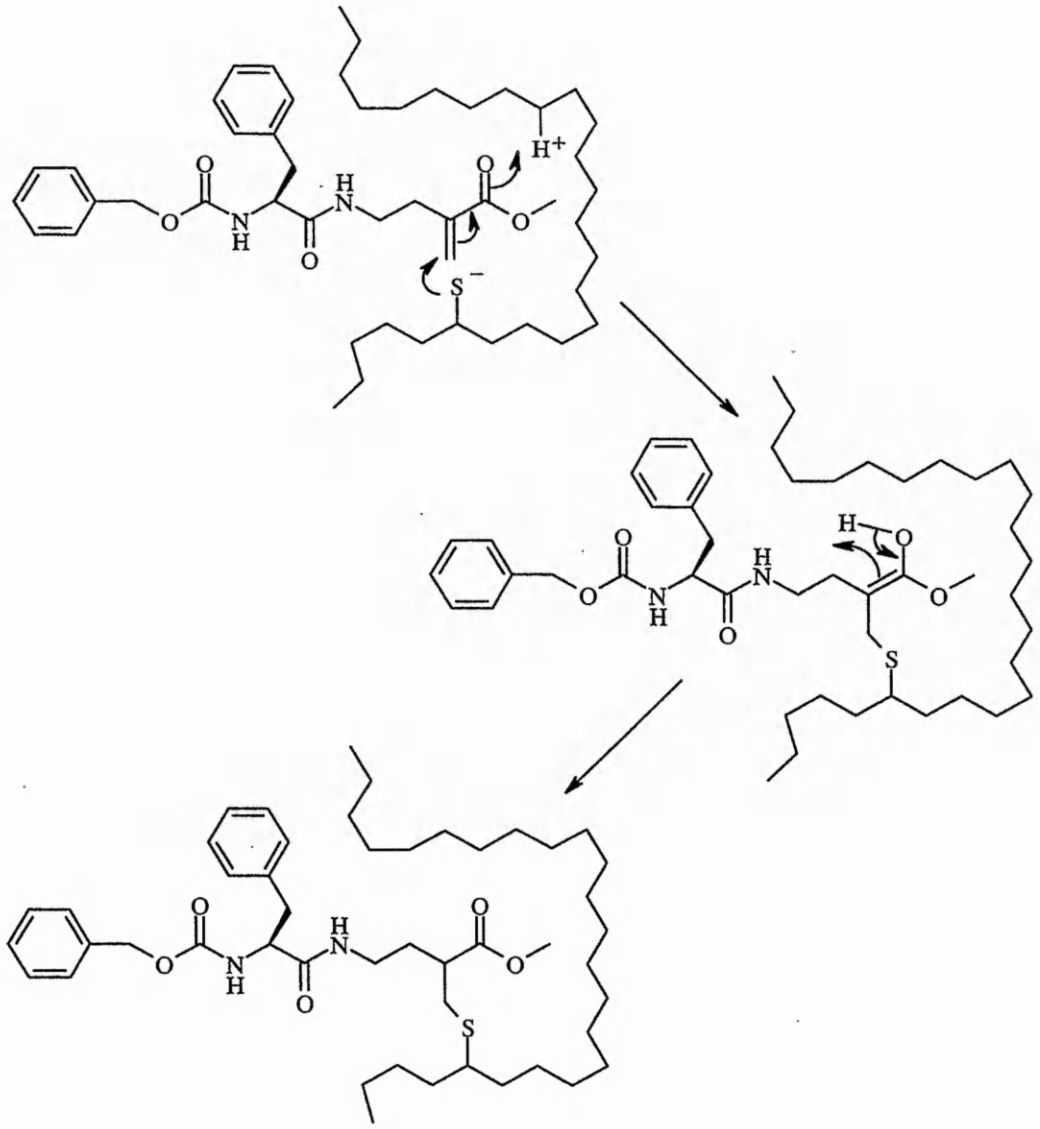
2.5 POTENTIAL INHIBITORS CONTAINING A METHYLENE-GLUTAMIC OR -BUTYRIC ACID RESIDUE

Methyleneglutamic acid is a naturally occurring amino acid, first isolated from germinated peanuts, and subsequently found in many other plants. The compound has received attention due to its potent and selective interaction with glutamate receptors in the mammalian central nervous system. Additionally, it has been shown to inhibit the vitamin K-mediated γ-carboxylation of glutamic acid residues, which is important in the blood clotting process.[172]

The literature, reviewed in section 1.5.1, p. 17, has shown that branching α- or β- to the amide terminal group in the glutamine side chain inhibits its ability to function as a substrate. However, it is still of interest to observe whether the presence of a group capable of irreversibly binding to the active site may be tolerated at this position. The mechanism for binding at the active site may possibly be via a 1,4-Michael addition (**scheme 46**, p. 94) in which attack by the thiol on the double bond yields an intermediate enol which rearranges to the inhibitor-enzyme adduct.

2.5.1 SYNTHESIS OF METHYLENEGLUTAMIC ACID

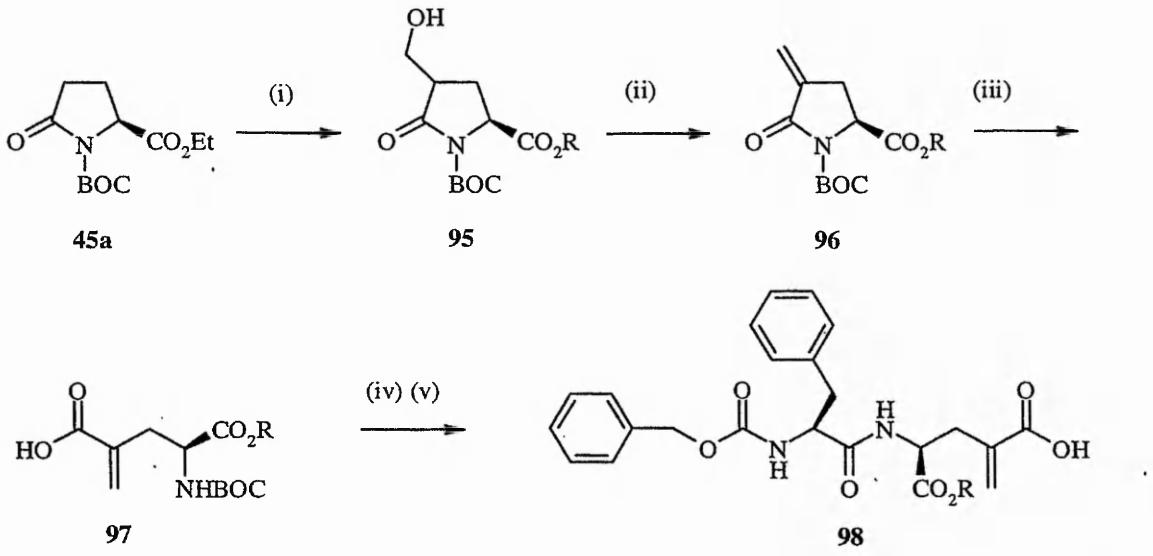
The first syntheses of the racemic methyleneglutamic acid, from diethylmalonate, formaldehyde and diethyl acetamidomalonate, were similar in many respects.[173,174] The preparation of the L-enantiomer has been reported by two groups. Baldwin has described two routes: 1) involves the nucleophilic ring-opening of aziridine-2-carboxylates with Wittig reagents[175] and; 2) a route via reaction of a glycidyl radical equivalent with 2-functionalised stannanes.[176] The method reported by



Scheme 46

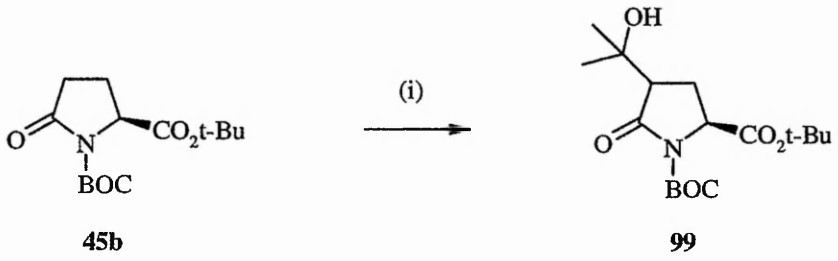
Ohfune *et al.*[177] involves an amino-carbonylation of an optically active propargylglycine equivalent. All three methods allow the preparation of a number of derivatives, but are multi-step procedures.

The previously successful use of pyroglutamic acid in the formation of glutamic acid derivatives led to the design of a potential synthesis based on the pyroglutamate synthon (**scheme 47**, p. 95).



Scheme 47 *Reagents & Conditions*: i. lithium *bis*(trimethylsilyl)amide, THF, -78°C, then CH_2O; ii. PPh_3/DEAD, THF; iii. LiOH, THF/H_2O; iv. TFA, DCM; v. **9**, TEA, DCM.

Initial attempts to quench the anion formed by the reaction of lithium *bis*(trimethylsilyl)amide and pyroglutamate **45a** with the cyclic formaldehyde trimer, 1,3,5-trioxane, failed. Similarly, no reaction was observed when *p*-formaldehyde was used. These results suggested that either the anion was not forming, or that the formaldehyde was not sufficiently reactive. The formation of the anion was checked by substitution of acetone as electrophile (**scheme 48**). Under these conditions **99** was obtained in 58% yield.

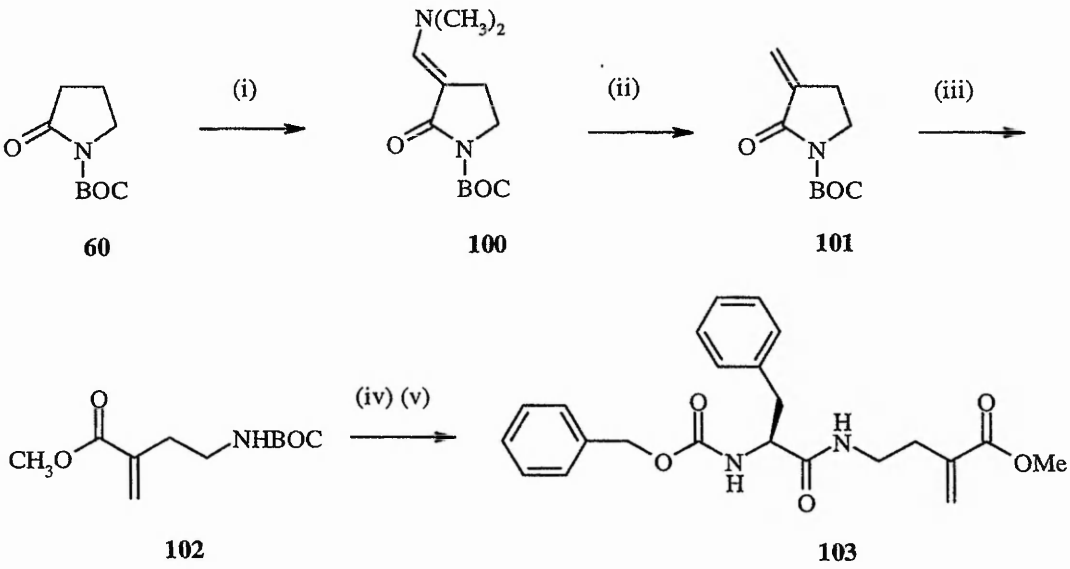


Scheme 48 *Reagents & Conditions*: i. lithium *bis*(trimethylsilyl)amide, THF, -78°C, then acetone.

A review of the literature revealed that many of the reported aldol reactions of pyroglutamates are limited in scope, good yields only being obtained with reactive electrophiles such as benzylbromide.[178] More recently it has been shown that this limitation can be overcome if the reaction is mediated by boron trifluoride diethyl ether complex.[179] Using this method the acetone adduct **99** was obtained in 78% yield, however no reference was made to the reaction with formaldehyde, or equivalent, to form **95**.

2.5.2 SYNTHESIS OF A METHYLENEBUTYRIC ACID-CONTAINING POTENTIAL INHIBITOR

Although this method looked promising, an alternative procedure for the preparation of **96** was also uncovered.[180] The use of Bredereck's reagent (*t*-butoxy*bis*-[dimethylamino]methane) was central to the stereospecific synthesis of a range of



Scheme 49 *Reagents & Conditions*: i. *t*-butoxy*bis*(dimethylamino)methane, dimethoxyethane, reflux; ii. DIBAL, THF, -78°C; iii. $NaOCH_3$, methanol, THF; iv. TFA, DCM; v. **9**, TEA, DCM.

4-alkylideneglutamic acids, 4-alkylglutamates and 4-alkylprolines, including the 4-methyleneglutamic acid. The synthesis of potential inhibitor **103** was carried out using this new methodology as outlined in **scheme 49**.

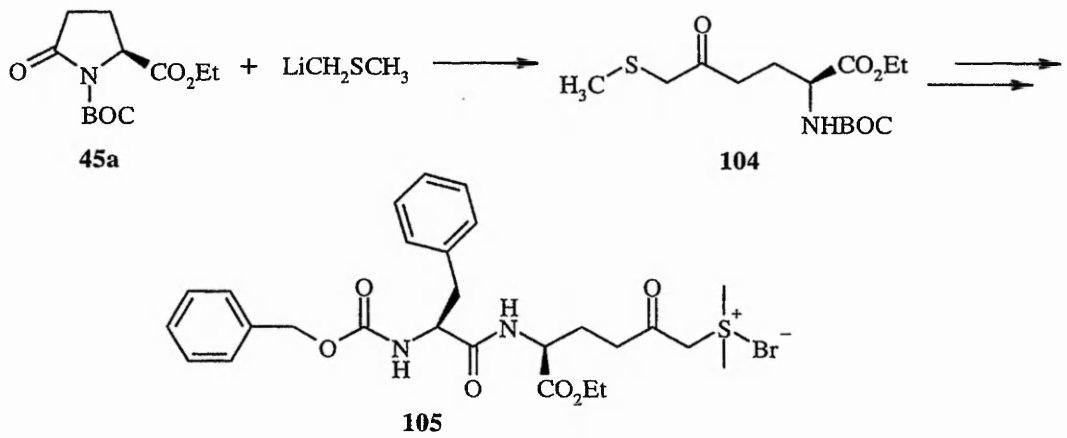
The simplified pyrrolidinone system **60** was chosen as starting material and prepared in high yield as previously described (section 2.2.4.2, p. 52). Reaction of **60** with Bredereck's reagent afforded the enaminone **100** in high yield (80%). Subsequent reduction with diisobutylaluminium hydride (DIBAL) gave the methylene derivative **101** in good (70%) yield. Unsaturated pyroglutamates such as **101** are not susceptible to ring-opening with aqueous base requiring more reactive nucleophiles such as alkoxide ions.[180] Thus reaction with methoxide generated the ring-opened compound **102** in 17% yield. Removal of the BOC group with 4-toluenesulfonic acid followed by coupling of the resulting crude salt with Z-phenylalanine-*N*-hydroxysuccinimide active ester **9** gave a low yield (11% overall for the combined deprotection and coupling steps) of compound **103**. Biological assay of this compound showed it to be a poor inhibitor of transglutaminase, therefore extension of this synthetic route to the preparation of methyleneglutamic acid derivatives such as **98** was not undertaken.

Biological evaluation is described in more detail in chapter 3, p. 105.

2.6 MISCELLANEOUS METHODS

2.6.1 SYNTHESIS OF N-*t*-BUTYLOXYCARBONYL-6-METHYLTHIO-5-OXO-L-NORLEUCINE ETHYL ESTER

The relative ease of ring-opening of carbamate-protected pyroglutamates led to a number of experiments in which carbanions stabilised by an adjacent sulfur atom were employed as nucleophiles. A reaction of this type has been reported by Ezquerra *et al.*[119] in which suitably protected pyroglutamates were ring-opened with the 2-lithio-derivative of 1,3-dithiane. An analogous reaction using the lithium salt of dimethylsulfide would allow a more direct route to the Syntex homologue **105** (**scheme 50**).



Scheme 50

Although dimethyl sulfide is not as reactive towards strong base as 1,3-dithiane its use as a nucleophile in reaction with aldehydes and ketones is known.[181] The lithium salt was prepared by the action of a solution of *n*-butyllithium and tetramethylethylenediamine (TMEDA) in hexane on dimethyl sulfide at room temperature.[182] Addition of the resulting mixture to a cold (-78°C) solution of the

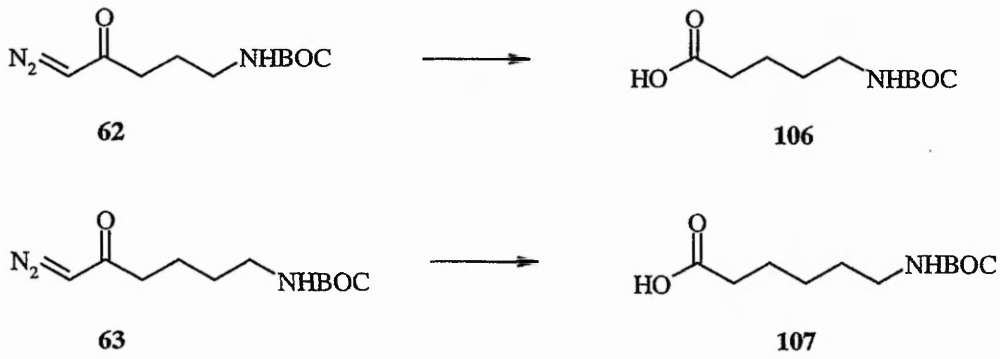
pyroglutamate **45a** gave the product **104** in 20% yield. Attempts to repeat this procedure on the *N*-FMOC pyroglutamate *t*-butyl ester **45k** were unsuccessful. Decomposition of the FMOC protecting group was observed by tlc., probably due to the presence of TMEDA in the reaction medium.

2.6.2 SYNTHESIS OF 2-AMINOADIPIC ACID DERIVATIVES

The potential of the carbamate-protected DON derivatives to prepare α-aminoadipic acid derivatives via Arndt-Eistert homologation was briefly investigated.

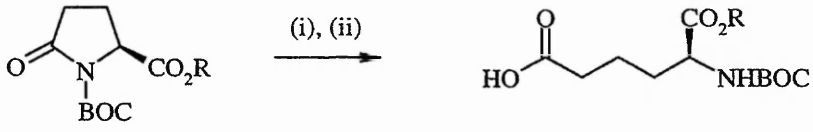
The literature preparations[183] of 2-aminoadipic acid can be classified into five groups: 1) the oxidation of lysine and lysine derivatives; 2) the homologation of glutamic and aspartic acid; 3) the resolution of 2-aminoadipic acid; 4) enzymatic reductive amination and; 5) via an Ugi reaction.

In initial experiments the ω-carbamoyl-substituted diazoketones **62** and **63** underwent silver benzoate-mediated rearrangement (**scheme 51**) to give the homologated acids **106** and **107** in moderate yield (52% and 56% respectively).



Scheme 51

Application of the above method to the glutamic acid derivatives **45a** and **45b**, **scheme 52**, afforded the 2-aminoadipic acid compounds **108a** and **108b** in good yields (70 and 75%, respectively). Notably, this method gives direct access to the α-ester derivatives.



45a R=Et
45b R=*t*-Bu

108a R=Et
108b R=*t*-Bu

Scheme 52 *Reagents & Conditions*: i. LiTMSD, -100°C; ii. $C_6H_5CO_2Ag$, H_2O.

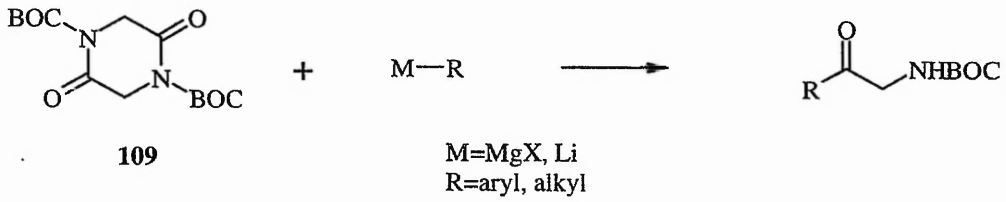
This possibly represents the most versatile and convenient route to a variety of *N*-protected 2-aminoadipates. It may be possible to achieve the preparation of unsymmetrical diesters if the Arndt-Eistert reaction is performed using an alcohol as solvent.

2.6.3 RING-OPENING OF *N*-*t*-BUTOXYCARBONYL PIPERAZINE-2,5-DIONES

The ease with which the lactam bond of *N*-carbamate-protected pyroglutamates reacts with nucleophiles led to a short study into the scope of this methodology. An obvious extension was to examine the reaction of LiTMSD with lactams of varying sizes. The previous section describes the preparation of 2-aminoadipate derivatives which have potential to allow access to derivatives of pyroaminoadipate, i.e., the higher homologue of pyroglutamic acid, but unfortunately there was insufficient time to explore this further. It may be significant that Ezquerra *et al.* have reported lower yields for KCN-catalysed ring-opening of pyroaminoadipates with nucleophiles

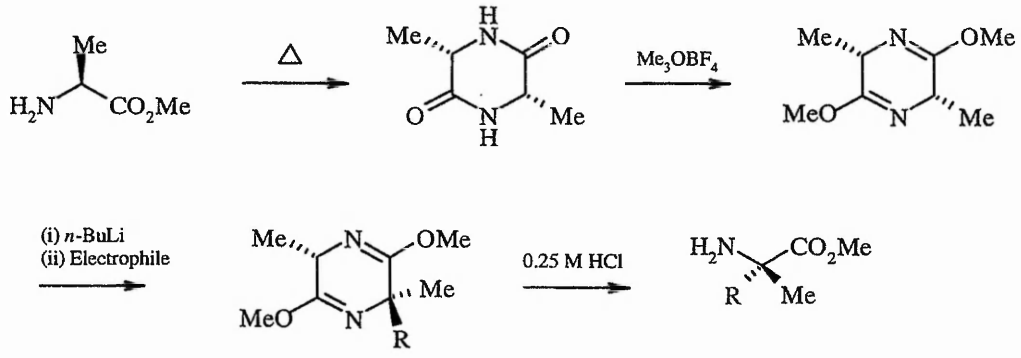
compared with pyroglutamates.[184] However piperidone **61** reacted readily with LiTMSD suggesting that pyroaminoadipates would undergo smooth ring-opening.

Among smaller lactams the appropriate β-lactams are relatively difficult[185] to prepare and α-lactams are unstable and can be formed only if suitably protected by bulky protecting groups.[186] There is, however, another ring system derived from α-amino acids, i.e., the piperazine-2,5-diones or diketopiperazines. The potential of this class of compound to provide amino acid derivatives is evident if a similar reactivity was induced upon introduction of a carbamate protecting group (**scheme 53**).



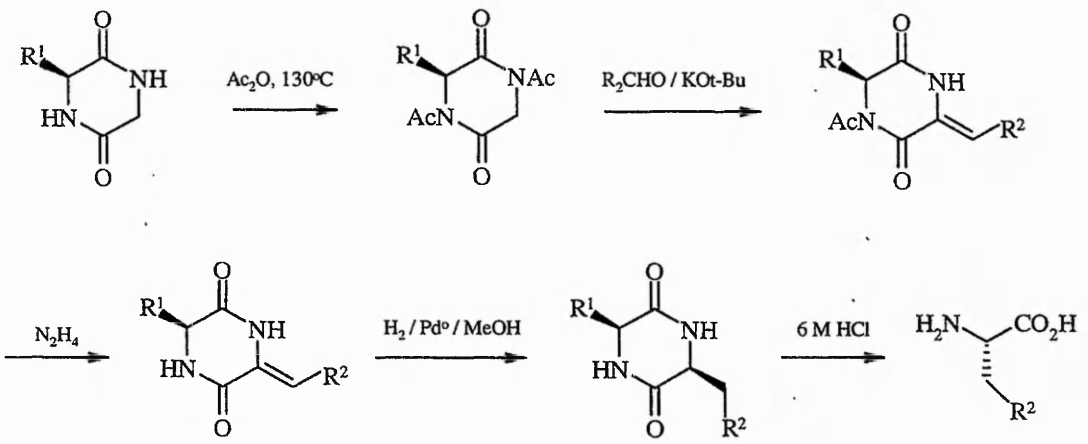
Scheme 53

Piperazine-2,5-diones have been studied extensively due to their importance in natural product synthesis.[187] They are also used in asymmetric synthesis of amino acids as intermediates in the formation of the *bis*-lactim ethers of Schollkopf[117] (**scheme 54**).



Scheme 54

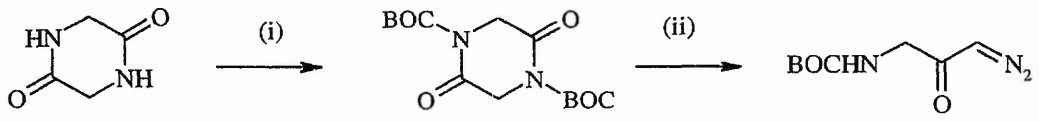
Additionally the *N*-acetyl unsymmetrical derivatives, **scheme 55**, have been directly alkylated to give dehydropiperazinediones which are stereoselectively reduced, subsequent hydrolysis affording the amino acid product.[188]



Scheme 55

Although the *N*-acylation of piperazine-2,5-dione compounds has been reported,[187] the role of the introduced group has been either to facilitate alkylation, as described in **scheme 55**, or simply to protect the amide nitrogen. The only reported procedure for the formation of the di-BOC-protected piperazinedione[189] **109** is low yielding (22%) and uses a large excess of catalyst. The DMAP-catalysed method of acylation used to synthesise *N*-BOC-protected pyroglutamates gave the desired compound in improved yield (48%).

Ring opening was attempted with LiTMSD as this would furnish α-aminodiazoketones which are important precursors to α-amino-α'-chloromethyl ketones,[164] which serve as irreversible enzyme inhibitors, and β-amino acids. Under the previously described conditions used for the ring-opening of pyroglutamates the diazoketone **110** was obtained in 50% yield (**scheme 56**, p. 103).

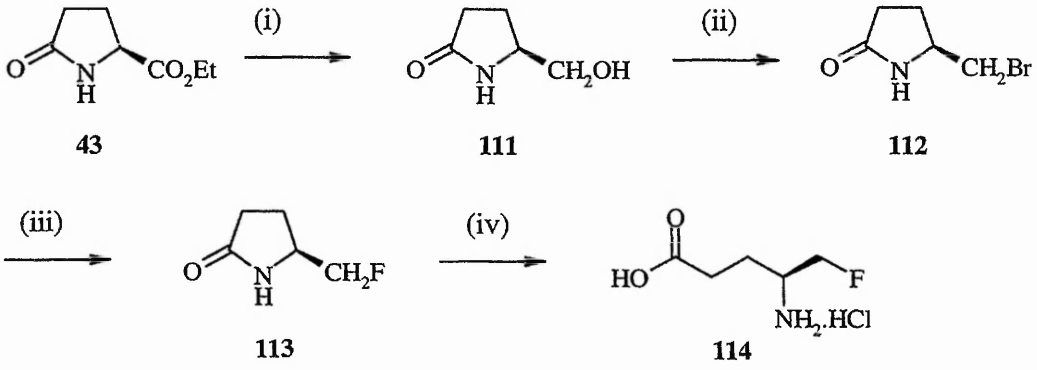


109 **110**

Scheme 56 *Reagents & Conditions*: i. di-*t*-butyl dicarbonate, DMAP, acetonitrile; ii. LiTMSD, THF, -100°C.

2.6.4 ATTEMPTED SYNTHESIS OF A POTENTIAL INHIBITOR BASED ON 4-AMINO-5-FLUOROPENTANOIC ACID

The preparation of 4-amino-5-fluoropentanoic acid (AFPA) was undertaken as a joint collaboration with the Life Sciences Department at Nottingham Trent University. The synthesis was part of an investigation into the relationship between potato greening and the concomitant increase in toxic steroidal glycoalkaloid production.[190] The glutamic acid-based structure of AFPA was also considered of interest as a potential transglutaminase inhibitor, although the reactive centre was not situated at the γ-end of the molecule. AFPA was synthesised in a manner similar to that described by Silverman and Levy[151] (**scheme 57**).

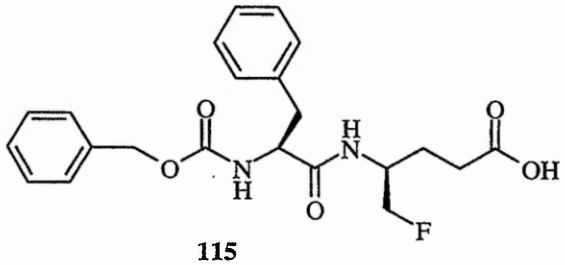


43 **111** **112**

113 **114**

Scheme 57 *Reagents & Conditions*: i. sodium borohydride, THF/methanol; ii. PPh_3, *N*-bromo-succinimide, DCM; iii. AgF, acetonitrile; iv. 1M HCl, reflux.

Reduction of the ethyl ester of pyroglutamate **43** afforded the alcohol **111** in good yield (74%). The conversion of alcohol **111** to bromide **112** gave erratic results, although **112** could be obtained in a 35% yield using triphenylphosphine and *N*-bromosuccinimide.[191] Subsequently, halogen exchange to form compound **113** was achieved in 50% yield by the reported procedure as was the final ring-opening step of **113** which yielded AFPA **114** in 81% yield. However, attempts to couple AFPA to Z-phenylalanine active ester **9** to give compound **115** gave none of the desired product. This may be attributed to the instability of the fluoroalkyl group to the basic conditions required in the coupling step.



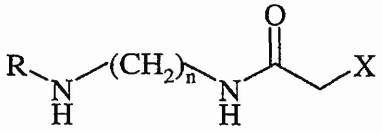
115

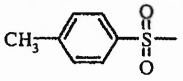
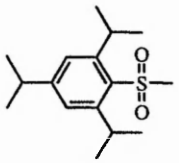
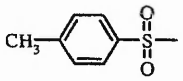
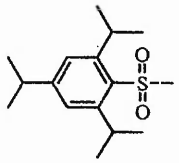
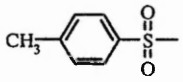
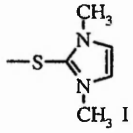
BIOLOGICAL DISCUSSION

3. BIOLOGICAL DISCUSSION

Biological assays of the inhibitors were carried out by A. Cox of the Department of Life Sciences. All compounds were initially screened using an assay which determines the inhibition by the test substance of transglutaminase-catalysed

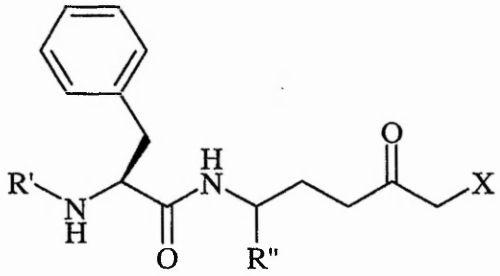
Table 3. **IC_{50} values observed for bromomethyl amide inhibitors and derivatives.**



Compound number	R	X	n	Solvent system	IC_{50} (μM)
1c		Br	5	ethanol	1
3		Br	5	ethanol	3
4		$S(CH_3)_2Br$	5	1:1 DMSO/ethanol	>500
5		$S(CH_3)_2Br$	5	1:1 DMSO/ethanol	>500
6			5	5% aqueous ethanol	300
12	Z-Phenylalanine	Br	2	DMSO	>500
13	Z-Phenylalanine	Br	5	DMSO	5

incorporation of ^{14}C-labelled putrescine. ***Tables 3*** and ***4***, respectively, show the IC_{50} values obtained for bromomethyl amide and Z-phenylalanyl halomethyl ketone inhibitors and their derivatives.

Table 4. **IC_{50} values observed for Z-phenylalanyl halomethyl ketone inhibitors and derivatives.**



Compound number	R'	R"	X	Solvent	IC_{50} μM
16	Z	H	$S(CH_3)_2$	DMSO	6
20a	Z	H	Br	DMSO	>500
20b	Z	H	F	DMSO	>500
17	Z	CO_2H	Cl	5% aqueous ethanol	80
17	Z	CO_2H	Cl	10% aqueous ethanol	20
17	Z	CO_2H	Cl	10% aqueous DMSO	150
53	CH_3CO	CO_2H	Cl	5% aqueous ethanol	>500
54	Z	CO_2CH_3	Cl	5% aqueous DMSO	12
55	Z	CO_2H	Br	insoluble	-
56	Z	CO_2H	$S(CH_3)_2$	5% aqueous ethanol	200
56	Z	CO_2H	$S(CH_3)_2$	H_2O	100

Of the bromomethyl amide inhibitors and their derivatives (see section 2.1, p. 36) both the 4-toluenesulfonamide and triisopropylbenzenesulfonamide compounds **1c** and **3** were found to be good inhibitors of transglutaminase *in vitro* with IC_{50} values of 1 and 3 μM respectively. Substitution for the alternative electrophilic group, dimethylsulfonium bromide, gave compounds **4** and **5** which were found not to inhibit transglutaminase. Alternatively replacement of the bromomethyl group with 2-mercapto-dimethylimidazole iodide yielded **6** which was found to be a moderate inhibitor. Changing the *N*-terminus substitution from a sulfonamide to a Z-phenylalanine grouping resulted in compounds **12** and **13**. Interestingly the shorter chain length derivative gave no reaction with the enzyme whereas C_5 compound **13** was an effective inhibitor with an IC_{50} figure of 5μM.

The results obtained for the phenylalanyl-substituted inhibitors (section 2.2, p. 44). cover a range of IC_{50} values from 6μM for compound **16** up to 200μM (***Table 4***) for compound **56**. These values must be treated with caution as inhibition was seen to vary with solvent suggesting that several of the compounds were at their limit of solubility in the solvent system quoted.

No inhibition was observed for the bromomethyl ketone **20a**, the fluoromethyl ketone **20b** and the *N*-acetyl-substituted chloromethyl ketone **53**. The bromomethyl ketone derivative **55** was not sufficiently soluble in the assay solvent system and so was not tested.

The results obtained for **20a** and **20b** are interesting especially in view of the strong inhibitory properties of the closely related dimethylsulfonium bromide **16**. This observation is similar to that described by Reinhardt (section 1.5.4.4, p. 27) in which it

was noted that transglutaminase is not particularly prone to inhibition by halomethyl carbonyl compounds (such as iodoacetamide) but that other structural features are required to make inhibitors of this type effective (for example the arylsulfonamide groupings of **1c** and **3**). This is especially borne out when compounds such as **17**, which differ from **20a** and **20b** only by a carboxyl group, are taken into account.

The failure of **53** can be attributed to the substitution of the acetyl moiety for the benzyloxycarbonyl (Z) group. This was undertaken in an attempt to improve the solubility of the compounds in aqueous media. Although the importance of a hydrophobic region within the inhibitor has been demonstrated (section 1.5.3.2, p. 22), the complete lack of inhibition of the enzyme by **53** also shows the importance of the location of the hydrophobic site. The presence of the hydrophobic benzyl side chain of phenylalanine was apparently not sufficient to allow constructive enzyme-inhibitor binding.

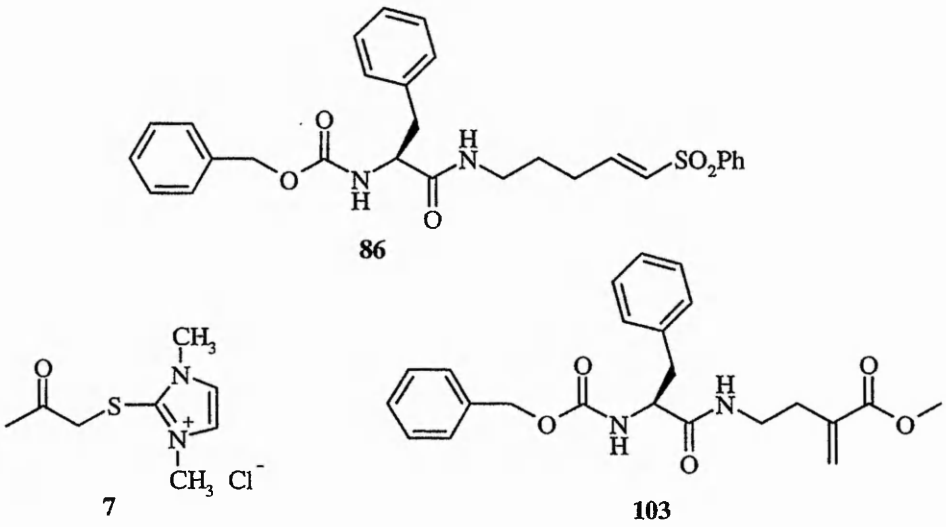


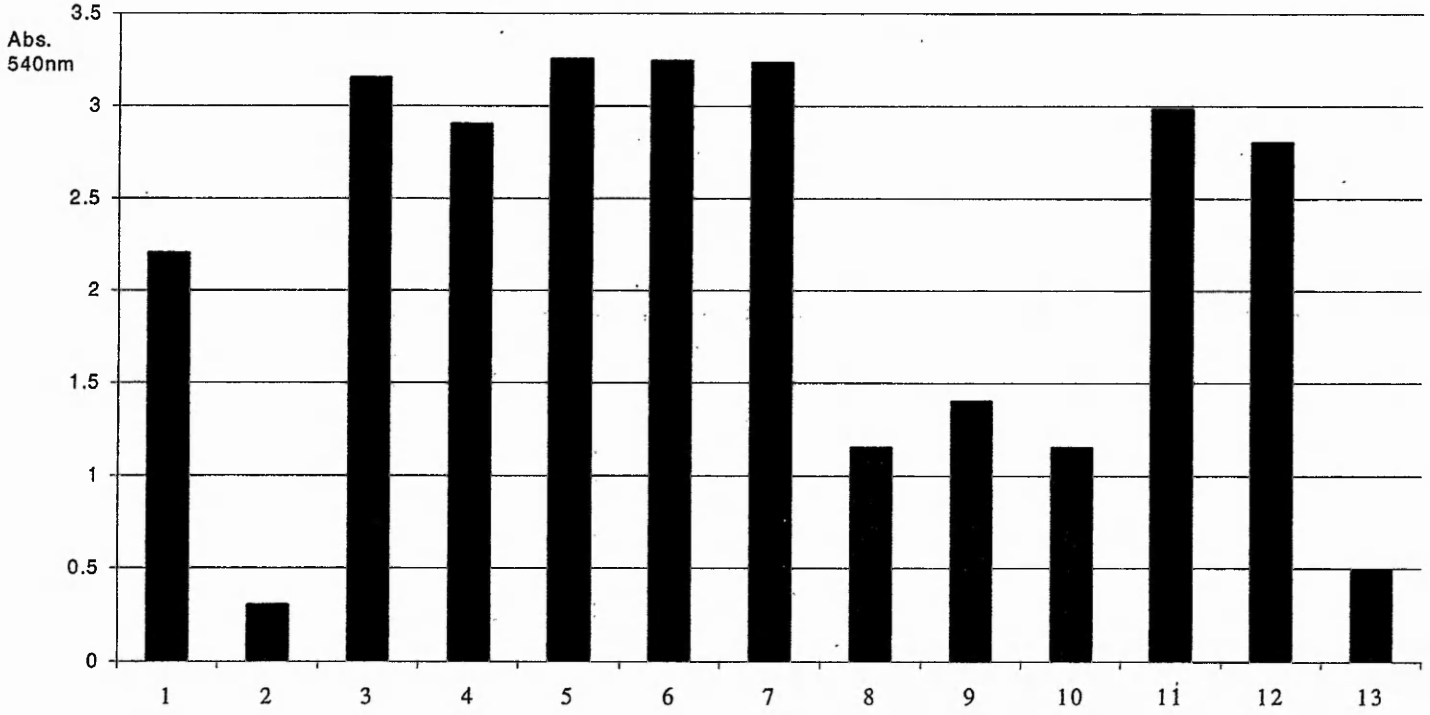
Figure 2. Miscellaneous inhibitors.

The known azole inhibitor **7** (section 1.5.4.7, p. 31) was prepared and found to have an IC_{50} of 100μM which agrees with the value given in a recent paper[192] which describes the effects of the inhibitor on the appearance and development of the fertilisation envelope in sea urchin eggs. The methylene glutamic acid derivative **103** did not inhibit transglutaminase activity, presumably due to the position of the methylene group preventing binding with the enzyme. The vinyl sulfone **86** similarly showed no reaction with transglutaminase whether this was due to the presence of the bulky phenyl sulfone group or lack of reactivity of the vinylsulfone function with the active site cysteine is unclear.

Compounds **1c**, **3**, **13**, **16** and **17** were tested for cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This colourimetric procedure uses the ability of cells to reduce MTT as an indication of mitochondrial integrity and activity and so is indicative of cell viability. The results are shown in ***Figure 3***. The compounds were tested at various concentrations in either DMSO or ethanol. The compounds tested in ethanol appear non toxic; in DMSO however cell viability was reduced. Toxicity in this case is probably due to the solvent, DMSO, as can be seen for inhibitor **17** which was tested in both ethanol and DMSO with different results. Therefore compounds soluble only in DMSO are not regarded as candidates for whole cell work.

The sulfonamide inactivators **1c** and **3**, chloromethyl ketone **17**, sulfonium methylketones **16** and **56** and mercaptomethylimidazole **7** were selected for a quick check in a fluorescein cadaverine assay. This method uses a cell line expressing high levels of transglutaminase and monitors the both the effect of the inhibitor on cell

Figure 3. MTT assay showing cell viability in the presence of various concentrations of potential transglutaminase inhibitors **1c**, **3**, **4**, **13**, **16** and **17** in DMSO or ethanol.



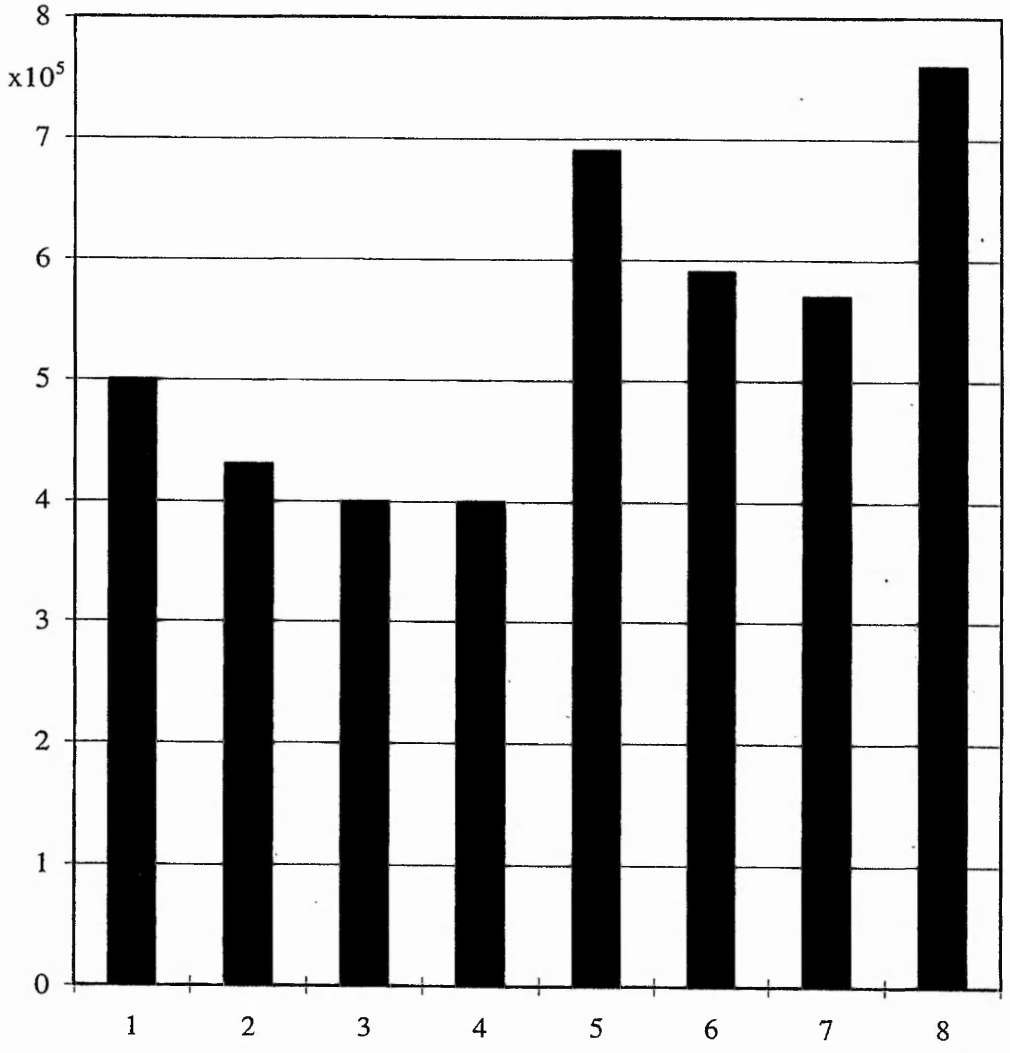
1=Control	4=**1c** 5μM Ethanol	7=**3** 10μM Ethanol	10=**16** 6μM 6% DMSO	13=**17** 100μM DMSO
2=Control DMSO	5=**1c** 10μM Ethanol	8=**4** 10μM DMSO	11=**17** 80μM Ethanol	
3=Control ethanol	6=**3** 5μM Ethanol	9=**13** 5μM DMSO	12=**17** 20μM Ethanol	

viability and cellular transglutaminase activity. Using this assay compounds **1c**, **3**, **16** and **17** gave poor inhibitory results and further **3** appeared cytotoxic. These results were not considered conclusive however because of the use of DMSO as solvent. Inhibitors **7** and **56** were both effective against transglutaminase activity and appeared to have no cytotoxic effects. Significantly both these inactivators were tested using water as solvent.

The most promising compounds, **7** and **56**, were further tested for toxicity using the trypan blue assay. The results of this assay are shown in ***Figure 4***. No morphological changes or significant drop in the average count of viable cells was observed after 2 hours or overnight for either inhibitor. This observation suggests these compounds have low cytotoxicity.

Although many of the compounds tested were shown to be good inhibitors of transglutaminase *in vitro* most were found to be poorer *in vivo* often due to their solubility characteristics. Even though they were not the most potent two inhibitors, mercaptoimidazole **7** and dipeptide sulfonium methylketone **56**, were found to have the desired characteristics of water solubility and selectivity. These compounds are considered the most likely candidates for use in future *in vivo* studies on transglutaminase.

Figure 4. Average count of viable cells determined by trypan blue assay after 2 h. and 24 h. in the presence of transglutaminase inhibitors **7** (100μM) and **56** (200μM) in either ethanol or water.



Average cell count after harvesting	
2 h.	24 h.
1=Control	5=Control
2=**56** in Ethanol	6=**56** in Ethanol
3=**56** in Water	7=**56** in Water
4=**7** in Water	8=**7** in Water

EXPERIMENTAL

4. EXPERIMENTAL

4.1 CHEMICAL EXPERIMENTAL

General procedures. Microanalyses were performed by The Microanalysis Unit, University of Nottingham, Nottingham. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. IR spectrum were recorded on a Perkin-Elmer 1600 series FT-IR instrument. ^{1}H spectra were recorded on a JEOL E-270 instrument at 270Mz. ^{13}C spectra were recorded on the same instrument at 67.8 MHz. All NMR samples were prepared in deuteriochloroform unless otherwise stated. Chemical shifts are reported relative to the internal standard tetramethylsilane and quoted as ppm.

Tetrahydrofuran (THF) was freshly distilled from sodium benzophenone ketyl before use. Ether was distilled from lithium aluminium hydride and stored over sodium wire. Methanol and ethanol were distilled and stored over 5 Å molecular sieves. *N*,*N*-dimethylformamide was distilled from calcium hydride and stored over 5 Å molecular sieves. Chloroform, dichloromethane and acetone were dried over granular calcium chloride. Light petroleum refers to petroleum ether (b.p. 40-60°C). Solvents used for flash column chromatography were distilled before use.

Flash chromatography was carried out using Fluka silica gel 60, 220-240 mesh size. Thin layer chromatography was carried out using Whatman silica gel 60A F254 precoated glass plates.

Metalation reactions were performed in oven or flame-dried glassware under nitrogen, using syringe-septum cap techniques.

Procedure 1: Preparation of mono-N-substituted α-ω-diamines.

To a 1M solution of freshly distilled diamine (2.5 equivalents) and triethylamine (1.5 equivalents) in DCM was added a 0.5M solution of the appropriate sulfonyl chloride or carboxylic acid derivative in DCM dropwise. The reaction was stirred for 2 h. and then filtered. The filtrate was washed with 2M HCl (3 x 10 ml) and the phases separated. The aqueous phase was basified with 10% sodium carbonate solution and back extracted with chloroform (3 x 20 ml). The combined chloroform portions were washed with water (20 ml), brine (20 ml) and dried (Na_2SO_4). Removal of the solvent afforded the product which was either converted to the hydrochloride salt or used without further purification.

Procedure 2: Preparation of N-bromoacetamides.

A 2M solution of bromoacetyl bromide (1 equivalent) in DCM was added to a stirred 0.5M solution of freshly distilled bromoacetic acid (1 equivalent) in DCM. The reaction mixture was stirred at room temperature for 15 min., then cooled to -15°C and a 0.5M solution of the appropriate amine in DCM was added, dropwise, together with portions of pyridine (1.2 equivalents). After stirring for 16 h. at 0°C the mixture was washed successively with 1M HCl, saturated sodium hydrogen carbonate solution, water, brine and dried ($MgSO_4$). Removal of the solvent yielded an oily residue which was purified by flash column chromatography.

Procedure 3: Preparation of sulfonium bromides.

The appropriate bromomethyl carbonyl compound was treated with the minimum amount of acetone to achieve solution. Dimethyl sulfide (2.5 equivalents) was added

and the solution was left stoppered overnight at room temperature. The resulting solid precipitate was collected by filtration and washed with a small amount of cold acetone and ether (5 ml) to yield the product as colourless crystals.

Procedure 4: DMAP-catalysed N*-carbonylation of 2-pyrrolidinone-5-carboxylic acids.*

4a: To a 0.5M solution of the appropriate 2-pyrrolidinone-5-carboxylic acid ester in DCM was added triethylamine (1 equivalent), the appropriate dicarbonate or chloroformate (1.1 equivalents) and DMAP (1 equivalent). The reaction was stirred for 6 h., then washed with 0.5M HCl, brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded a residue which was purified either by recrystallisation or by flash column chromatography.

4b: Di-*t*-butyldicarbonate (1.1 equivalents) was added to a mixture of the appropriate 2-pyrrolidinone-5-carboxylic acid ester and DMAP (0.05 equivalents) in acetonitrile (1M) and the solution was stirred for 6 h. The solvent was removed *in vacuo* and the residue purified by flash column chromatography.

Procedure 5: N-Carbonylation of 2-pyrrolidinone-5-carboxylic acids.

To a cold (-78°C), stirred 0.2M solution of the appropriate 2-pyrrolidinone-5-carboxylic acid ester in THF was added lithium *bis*(trimethylsilyl)amide (1 equivalent) dropwise. The reaction was stirred for a further 20 min. and then a 1M solution of the appropriate dicarbonate or chloroformate (1 equivalent) in THF added dropwise. The mixture was stirred for 0.5 h. at -78°C and the reaction quenched by addition of saturated ammonium chloride solution. The layers were separated and the aqueous

layer was extracted with chloroform and ether. The combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded an oily residue which was purified by flash column chromatography.

*Procedure 6: Preparation of 6-diazo-5-oxo-*N*-urethane-protected norleucinate esters.*

To a cold (-100°C) 0.2M solution of TMSD (1.2 equivalents) in THF in an inert atmosphere of nitrogen was added *n*-BuLi (1.2 equivalents) dropwise. The reaction was stirred for 0.5 h. and then the deep yellow solution was cooled and transferred, via cannula, to a cold (-105°C) and vigorously stirred 0.05M solution of the appropriate 2-pyrrolidinone-5-carboxylic acid ester in THF at such a rate that the internal temperature of the reaction remained below -100°C. The mixture was stirred for a further 10 min. and then quenched by rapid addition to a stirred saturated ammonium chloride solution. The layers were separated and the aqueous layer was extracted with chloroform and ether. The combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave an orange oil which was purified by flash column chromatography.

*Procedure 7: Preparation of 1-benzyl 7-alkyl (2R)-2-*N*-tert-butyloxycarbonyl-amino-5-oxo-heptanedioates*

A cold (0°C), stirred 0.1M solution of diisopropylamine (1.2 equivalents) in THF in an atmosphere of dry nitrogen was treated with *n*-BuLi (1.2 equivalents). After 10 min. the mixture was cooled to -78°C, and the appropriate acetate (1 equivalent) was added, dropwise, and the reaction stirred for 0.5 h. A 1M solution of *N*-BOC

benzyl pyroglutamate in THF was added, dropwise, and the reaction stirred for a further 0.5 h. at -78°C. The reaction was quenched by addition of saturated ammonium chloride solution, the layers were separated and the aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a residue which was purified by flash column chromatography.

Procedure 8: Arndt-Eistert homologation

To a solution of the diazoketone (0.45 g, 2 mmol) in a mixture of dioxane and water (2:1, 30 ml) was added silver benzoate (4 mg, 17 x 10^{-3} mmol). The reaction was protected from light, warmed to 70°C and stirred for 6 h. The reaction was filtered and the solvent removed *in vacuo*. The residue was dissolved in 10% sodium carbonate and the aqueous layer was washed with chloroform (3 x 15 ml) and ether (20 ml). The aqueous layer was then acidified with 2M HCl to pH 2 and back extracted with chloroform (3 x 15 ml). The combined organic layer from the second extraction was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded the product.

N-*(5-Aminopentyl)-4-methyl-1-benzenesulfonamide* **2**

Compound **2a** was prepared by *procedure 1*, using 1,5-diaminopentane (5.86 ml, 50 mmol), triethylamine (4.23 ml, 30 mmol) and 4-toluenesulfonyl chloride (3.81 g, 20 mmol). After work-up, removal of the solvent yielded the sulfonamide **2** as a colourless oil (2.5 g, 49%) which solidified on standing, m.p. 58-61°C (HCl salt 122-124°C, Lit.[192] 123.5-124.5°C). ν_{max} (KBr)/cm^{-1} 2940, 1602, 1313, 1140; δ_H 1.2-1.5 (6

H, m), 2.3 (3 H, s), 2.6 (2 H, t), 2.9 (2 H, t), 7.3 (2 H, dd, ArH), 7.7 (2 H, dd, ArH); δ_C 21.3, 23.6, 29.1, 32.6, 41.5, 42.8, 126.9, 129.5, 137.0, 143.0.

N-*[5-(4-Methylphenylsulfonamido)pentyl]-2-bromoacetamide* **1c**

Compound **1c** was prepared by *procedure 2*, using bromoacetyl bromide (0.875 ml, 10 mmol), bromoacetic acid (1.4 g, 10 mmol), pyridine (1 ml, 12 mmol) and amine **2a** (2.5 g, 10 mmol). After work up, removal of the solvent yielded a pale brown oil which was purified by flash column chromatography (chloroform/methanol 97:3) to afford the bromoacetamide **1c** as a colourless oil (2.4 g, 64%) which crystallised on standing, m.p. 70-71°C (Lit.[86] 66-70°C). ν_{max} (KBr)/cm^{-1} 3276, 1654, 1548; δ_H 1.3-1.6 (6 H, m), 2.4 (3 H, s), 2.9 (2 H, q), 3.2 (2 H, q), 3.9 (2 H, s), 4.9 (1 H, t), 6.6 (1 H, br), 7.3 (2 H, dd, ArH), 7.7 (2 H, dd, ArH); δ_C 21.5, 23.5, 28.6, 28.9, 29.3, 39.8, 42.8, 127.1, 129.7, 136.8, 143.4, 165.7.

N-*[5-(2,4,6-Triisopropylphenylsulfonamido)pentyl]-2-bromoacetamide* **3**

N-*(5-Aminopentyl)-2,4,6-triisopropylbenzenesulfonamide* was prepared by *procedure 1*, using 1,5-diaminopentane (4.1 ml, 14 mmol), triethylamine (2.96 ml, 21 mmol) and 2,4,6-triisopropylbenzenesulfonyl chloride (4.24 g, 14 mmol). After work up, removal of the solvent yielded a colourless solid which was recrystallised from ethanol/ether to give the intermediate hydrochloride salt as colourless crystals (2.85 g, 50%), m.p. 195°C (Lit.[73] 195-197°C). ν_{max} (KBr)/cm^{-1} 2940, 1602, 1313, 1140; δ_H 1.2-1.3 (20 H, m), 1.6 (2 H, m), 1.9 (2 H, m), 2.8 (1H, m), 2.9 (2 H, t), 3.1 (2 H, t), 4.2 (2 H, m), 5.8 (1 H, t), 7.2 (2 H, s, ArH), 8.2 (3 H, br); δ_C 23.1, 23.6, 25.0, 26.6, 28.4, 29.5, 34.1, 39.5, 42.1, 123.7, 132.1, 150.3, 152.5.

Compound **3** was prepared by *procedure 2*, using bromoacetyl bromide (0.35 ml, 4 mmol), bromoacetic acid (0.56 g, 4 mmol), hydrochloride salt obtained above (1.62 g, 4 mmol) and pyridine (0.4 ml). After work up, removal of the solvent yielded a pale yellow oil which was purified by flash column chromatography (chloroform/methanol 97:3) to afford the bromoacetamide **3** as a colourless solid (1.43 g, 73%), m.p. 97.5-98.5°C. (Found: C, 54.19; H, 7.48; N, 5.62. $C_{22}H_{37}BrN_2O_3S$ requires C, 53.98; H, 7.62; N, 5.72%.); ν_{max} (KBr)/cm^{-1} 3276, 1654, 1548; δ_H 1.2-1.3 (18 H, m), 1.4 (2 H, m), 1.5, (4 H, m), 2.9 (1 H, m), 3.0 (2 H, q), 3.2 (2 H, q), 3.8 (2 H, s), 4.2 (2 H, m), 4.5 (1 H, t), 6.6 (1 H, br), 7.2 (2 H, s, ArH); δ_C 23.6, 23.7, 24.9, 28.8, 29.2, 29.3, 29.6, 34.1, 39.8, 42.5, 123.8, 132.2, 150.2, 152.7, 165.5.

Dimethyl[5-(4-methylphenylsulfonamido)pentylcarbamoylmethyl]sulfonium bromide **4**

Compound **4** was prepared by *procedure 3*, using bromomethyl amide **1c** (0.26 g, 0.7 mmol) and dimethyl sulfide (0.11 ml, 1.54 mmol). The sulfonium bromide **4** was obtained as colourless crystals (0.24 g, 78%), m.p. 124-125°C. (Found: C, 43.64; H, 6.28; N, 6.26. $C_{16}H_{27}BrN_2O_3S_2$ requires C, 43.73; H, 6.19; N, 6.37%.); ν_{max} (KBr)/ cm^{-1} 3276, 1654, 1548, 660; δ_H 1.4-1.6 (6 H, m), 2.4 (3 H, s), 2.9 (2 H, q), 3.2-3.4 (8 H, m), 5.0 (2 H, s), 6.6 (1 H, t), 7.3 (2 H, dd, ArH), 7.8 (2 H, dd, ArH), 8.5 (1 H, t); δ_C 21.5, 23.5, 25.7, 27.7, 28.4, 39.5, 42.5, 47.0, 127.1, 129.7, 136.8, 143.2, 162.7.

Dimethyl[5-(2,4,6-triisopropylphenylsulfonamido)pentylcarbamoylmethyl]sulfonium bromide **5**

Compound **5** was prepared by *procedure 3*, using bromomethyl amide **3** (0.34 g, 0.7 mmol) and dimethyl sulfide (0.11 ml, 1.54 mmol). The sulfonium bromide **5** was

obtained as colourless crystals (0.32 g, 83%), m.p.134°C. (Found: C, 52.20; H, 7.92; N, 4.91. $C_{24}H_{43}BrN_2O_3S_2$ requires C, 52.25; H, 7.87; N, 5.06%.); ν_{max} (KBr)/cm^{-1} 3276, 3072, 2937, 1654, 1548, 1446, 1323, 1157, 660; δ_H 1.2-1.3 (20 H, m), 1.6 (4 H, m), 2.8 (1 H, m), 2.9 (2 H, q), 3.2-3.4 (8 H, m), 4.2 (2 H, m), 5.1 (2 H, s), 6.2 (1 H, t), 7.1 (2 H, s, ArH), 8.7 (1 H, t); δ_C 21.5, 23.5, 25.7, 27.7, 28.4, 39.5, 42.5, 47.0, 127.1, 129.7, 136.8, 143.2, 162.7.

1,*3-Dimethyl-2-[5-(4-methylphenylsulfonamido)pentylcarbamoylmethylsulfanyl]-3*H-*1,3-diazol-1-ium iodide* **6**

To a stirred solution of the bromomethyl amide **1c** (0.4 g, 1.06 mmol) in DCM (5 ml) was added 2-mercapto-5-methylimidazole (0.12 g, 1.06 mmol) followed by triethylamine (0.18 ml, 1.27 mmol). The resulting pale yellow solution was stirred for a further 48 h. The mixture was diluted with ethyl acetate (10 ml) and washed with water (2 x 5 ml), 1M sodium hydroxide solution (2 x 5 ml), brine (5 ml) and dried ($MgSO_4$). Removal of the solvent under reduced pressure afforded an oily residue which was purified by flash column chromatography (ethyl acetate/methanol 95:5) to give the imidazole intermediate a colourless oil (0.14 g, 32%). ν_{max} (film)/cm^{-1} 3273, 2933, 2861, 1658; δ_H 1.3 (2 H, m), 1.4 (4 H, m), 2.4 (3 H, s), 2.9 (2H, q), 3.2 (2 H, q), 3.6 (5 H, s), 5.2 (1 H, t), 6.9 (1 H, d), 7.0 (1 H, d), 7.3 (2 H, dd, ArH), 7.7 (2 H, dd, ArH), 8.4 (1 H, t); δ_C 21.5, 23.5, 28.5, 28.9, 33.4, 36.5, 39.2, 42.9, 122.9, 127.0, 128.7, 129.7, 137.1, 141.6, 143.2, 169.4.

The imidazole (0.14 g, 0.34 mmol) obtained above was dissolved in DCM (2 ml) and methyl iodide was added. The flask was tightly stoppered and left to stand for 48 h. Removal of the solvent yielded a waxy gum which was recrystallised (methanol/ether)

to afford the diazolium iodide **6** as a pale yellow solid (0.10 g, 53%), m.p. 142-144°C. (Found: C, 40.98; H, 5.34; N, 10.06. $C_{19}H_{29}N_4O_3S_2I$ requires C, 41.31; H, 5.29; N, 10.14%.); ν_{max} (KBr)/cm^{-1} 3221, 2933, 2860, 1656; δ_H (CD_3OD) 1.3-1.5 (6 H, m), 2.4 (3 H, s), 2.8 (2 H, m), 3.1 (2 H, m) 3.6 (2 H, s), 4.0 (6 H, s), 7.4 (2 H, dd, ArH), 7.7 (2 H, dd, ArH), 7.8 (2 H, s); δ_C (CD_3OD) 21.4, 24.8, 29.5, 30.1, 37.2, 37.8, 40.7, 43.7, 49.1, 126.5, 128.0, 130.7, 136.2, 142.5, 166.7.

*1,3,-Dimethyl-2-(2-oxopropylsulfanyl)-3*H*-1,3-diazol-1-ium chloride* **7**

To a stirred solution of 2-mercapto-1-methylimidazole (1.14 g, 10 mmol) in dry tetrahydrofuran (50 ml) at -12°C was added *n*-butyllithium (6.25 ml, 10 mmol) dropwise. The mixture was warmed to 4°C and stirring continued for 15 min., then chloroacetone (0.8 ml, 10 mmol) was added dropwise. The reaction was allowed to warm to room temperature and stirring continued for another 4 h. Methanol (10 ml) was added and the solvent removed under vacuum. The residue was redissolved in ethyl acetate (25 ml) and the organic phase was washed with 1M sodium hydroxide solution (2 x 10 ml), water (2 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent yielded a pale brown oil (0.55 g, 32%) which was homogeneous by tlc (100% ethyl acetate) and used without further purification. ν_{max} (film)/cm^{-1} 3109, 2947, 1712. 1-Methyl-2-[(2-oxypropyl)thio]imidazole (0.55 g, 3.2 mmol) was dissolved in dichloromethane (3 ml) and methyl iodide (0.2 ml, 3.2 mmol) was added. The reaction vessel was tightly sealed and the mixture allowed to stand for 24 h. Removal of the solvent afforded an oily solid which was crystallised from ethanol to yield the methiodide salt as pale yellow crystals (0.31 g, 31%), m.p. 184-186°C (Lit.[93] m.p. 180-184°C).

The imidazolium chloride salt was prepared from the methiodide salt by Dowex-1 (Cl^-) ion-exchange chromatography (distilled water). Purification by recrystallisation from isopropyl alcohol afforded the diazolium chloride **7** as colourless crystals (0.18 g, 82%), m.p. 172-175°C (Lit.[93] m.p. 174-177°C). ν_{max} (KBr)/cm^{-1} 3110, 2942, 1711; δ_H (CD_3OD) 2.2 (3 H, s), 4.0 (6 H, s), 4.2 (2 H, s), 7.7 (2 H, s); δ_C (CD_3OD) 28.6, 37.1, 45.1, 49.1, 126.2, 202.8.

N-*Benzyloxycarbonyl-L-phenylalanine* **8**

To a solution of L-phenylalanine (10 g, 60 mmol) in 2M sodium hydroxide solution (63 ml, 126 mmol) at 4°C was added freshly distilled benzyl chloroformate (9.4 ml, 66 mmol) dropwise. The mixture was stirred at 10°C for 1 h. then washed with ethyl acetate (2 x 40 ml) before being acidified to pH 2 by addition of 2M HCl. The aqueous phase was extracted with ethyl acetate (3 x 60 ml) and the combined organic extract was washed with brine (40 ml) and dried ($MgSO_4$). Removal of the solvent yielded Z-phenylalanine **8** as an oil which solidified on standing to a colourless solid (14.6 g, 87%), m.p. 85-87°C (Lit.[96] m.p. 85-87°C). ν_{max} (KBr)/cm^{-1} 3320, 3025, 2945, 1715, 1700, 1520; δ_H 3.0 (2 H, m), 3.7 (1 H, q), 5.1 (2 H, s), 5.4 (1 H, d), 7.1-7.3 (10 H, m, ArH), 11.4 (1 H, s, br); δ_C 37.7, 54.7, 67.1, 127.1 128.1, 128.2, 128.5, 128.6, 129.3, 135.7, 136.1, 156.0, 175.9.

N-*Benzyloxycarbonyl-L-phenylalanine* N-*hydroxysuccinimide ester* **9**

To a cold (4°C) stirred solution of Z-phenylalanine **8** (5 g, 16.7 mmol) and *N*-hydroxysuccinimide (1.92 g, 16.7 mmol) in THF (16 ml) was added a solution of dicyclohexylcarbodiimide (3.44 g, 16.7 mmol) in THF (16 ml) dropwise. The reaction

was stirred at 4°C for a further 24 h., then the mixture filtered. Removal of the solvent afforded a solid which was purified by recrystallisation from ethanol to yield the ester **9** as colourless crystals (5.16 g, 78%), m.p. 139-140°C (Lit.[96] m.p. 140-140.5°C). ν_{max} (KBr)/cm^{-1} 3292, 3034, 2927, 1812, 1784, 1746, 1683; δ_H 2.8 (4 H, s), 3.2 (2 H, m), 5.0 (1 H, m), 5.1 (2 H, s), 5.3 (1 H, d), 7.3 (10 H, m, ArH); δ_C 25.5, 37.9, 53.0, 67.2, 127.4, 128.2, 128.3, 128.5, 128.7, 129.6, 134.4, 135.9, 155.4, 167.5, 168.7.

Mono-N-*benzyloxycarbonyl-L-phenylalanyl-1,2-diaminoethane* **10**

Compound **10** was prepared by *procedure 1*, using Z-phenylalanine active ester **9** (0.28 g, 0.7 mmol) and ethylenediamine (0.12 ml, 1.75 mmol). After work up, removal of the solvent afforded the mono-substituted diamine **10** a colourless oil which solidified on standing (0.14 g, 59%), m.p. 135-137°C (Lit.[193] no m.p. given). ν_{max} (KBr)/cm^{-1} 3290, 3025, 2923, 1680, 1648, 1540; δ_H 2.6 (2 H, m), 3.0-3.2 (4 H, m), 4.4 (1 H, q), 5.1 (2 H, s), 5.6 (1 H, d), 6.4 (1 H, s), 7.2 (10 H, m, ArH); δ_C 38.9, 40.9, 42.0, 56.6, 67.0 127.0, 128.0 128.2, 128.6, 128.7, 129.3, 136.2, 136.6, 156.0, 171.0.

Mono-N-*Benzyloxycarbonyl-L-phenylalanyl-1,5-diaminopentane* **11**

Compound **13** was prepared by *procedure 1*, using Z-phenylalanine active ester **9** (0.59 g, 1.5 mmol) and 1,5-diaminopentane (1.75 ml, 15 mmol). After work up, removal of the solvent afforded the diamine **11** a colourless solid (0.26 g, 45%), m.p. 121-126°C (Lit.[193] no m.p. given). ν_{max} (KBr)/cm^{-1} 3297, 3021, 2923, 1684, 1649, 1530; δ_H 1.2-1.4 (6 H, m), 2.6 (2 H, m), 3.0-3.2 (4 H, m), 4.3 (1 H, q), 5.1 (2 H, s), 5.6 (1 H, d), 6.0 (1 H, s), 7.2 (10 H, m, ArH); δ_C 23.9, 29.0, 33.0, 38.9, 39.4, 41.8, 56.5, 67.0 127.0, 128.0 128.2, 128.6, 128.7, 129.3, 136.2, 136.6, 156.0, 170.7.

N'-(N-*Benzyloxycarbonyl-L-phenylalanyl*)-N''-*bromoacetyl-1,2-diaminoethane* **12**

Compound **12** was prepared by *procedure 2*, using bromoacetyl bromide (0.05 ml, 0.56 mmol), bromoacetic acid (0.08 g, 0.56 mmol) amine **10** (0.19 g, 0.56 mmol) and pyridine (0.06 ml). After work-up, removal of the solvent yielded the bromoamide **12** as a colourless solid which was purified by recrystallisation from ethyl acetate (0.17 g, 66%), m.p. 185-187°C. (Found: C, 53.87; H, 5.18; N, 8.82. $C_{21}H_{24}BrN_3O_4$ requires C, 54.55; H, 5.23; N, 9.09%.); ν_{max} (KBr)/cm^{-1} 3298, 3086, 2926, 1686, 1655, 1552, 1288, 1243, 746, 698; δ_H 3.0 (2 H, m), 3.3 (4 H, m), 3.8, (2 H, s), 4.4 (1 H, q), 5.1 (2 H, s), 6.1 (1 H, d), 7.2 (10, m, ArH), 7.5 (1 H, s); δ_C 28.8, 38.6, 38.9, 39.4, 56.2, 66.7, 126.8, 127.9, 128.0, 128.4, 129.3, 136.3, 136.8, 155.9, 166.7, 171.9.

N'-(N-*Benzyloxycarbonyl-L-phenylalanyl*)-N''-*bromoacetyl-1,5-diaminopentane* **13**

Compound **13** was prepared by *procedure 2*, using bromoacetyl bromide (0.06 ml, 0.68 mmol), bromoacetic acid (0.096 g, 0.68 mmol), amine **11** (0.26 g, 0.68 mmol) and pyridine (0.07 ml). After work-up, removal of the solvent yielded the bromomethyl amide **13** as a colourless solid which was purified by recrystallisation from ethyl acetate (0.20 g, 58%), m.p. 135-137°C. (Found: C, 50.64; H, 5.92; N, 8.23. $C_{24}H_{30}BrN_3O_4$ requires C, 51.15; H, 5.99; N, 8.33%.); ν_{max} (KBr)/cm^{-1} 3298, 3086, 2927, 1688, 1652, 1536; δ_H 1.2-1.6 (6 H, m), 3.0-3.3 (6 H, m), 3.9, (2 H, s), 4.3 (1 H, q), 5.1 (2 H, s), 5.5 (1 H, d), 5.9 (1 H, t), 6.6 (1 H, s), 7.2-7.4 (10 H, m, ArH); δ_C 23.6, 28.6, 28.7, 29.3, 38.8, 39.1, 39.7, 56.5, 67.0, 127.0, 128.0, 128.2, 128.6, 128.7, 129.3, 136.1, 136.5, 155.9, 165.6, 170.8.

Mono-N-*triphenylmethyl-1,2-diaminoethane* **14**

To a stirred solution of ethylenediamine (22 ml, 330 mmol) in DCM (30 ml) was added a solution of trityl chloride (9.3 g, 33 mmol) in DCM (15 ml) dropwise. The mixture was stirred for 2.5 h. and then ether was added (50 ml). The organic layer was washed with water (3 x 50 ml) and dried (Na_2SO_4). Removal of the solvent *in vacuo* yielded a yellow oil which was redissolved in ethyl acetate (50 ml) and 0.5M HCl was added in small portions with stirring. The resulting colourless precipitate was filtered, washed with chloroform (3 x 10 ml), evaporated then resuspended in ethyl acetate and washed with 1M sodium hydroxide solution (3 x 20 ml). The organic layer was dried (Na_2SO_4) and removal of the solvent afforded the mono-trityl diamine **14** as a yellow oil (3.7 g, 37%). ν_{max} (film)/cm^{-1} 3058, 3020, 2918, 1598; δ_H 2.1 (2 H, t), 2.7 (2 H, t), 7.1-7.3 (15 H, m, ArH); δ_C 42.6, 46.4, 70.6, 126.2, 127.7, 128.6, 146.1.

N'-*(*N-*Benzyloxycarbonyl-L-phenylalanyl)*-N''-*triphenylmethyl-1,2-diaminoethane* **15**

To a cold (0°C) stirred solution of Z-phenylalanine **8** (1.2 g, 4 mmol) and *N*-trityl-1,2-diaminoethane **14** (1.2 g, 4 mmol) in DCM (40 ml) was added dicyclohexyl-carbodiimide, portionwise, over 10 min. The reaction was stirred for a further 5 h. and filtered. The filtrate was washed with 1M HCl (3 x 15 ml), saturated sodium hydrogen carbonate (3 x 15 ml), water (15 ml), brine (15 ml) and dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded the desired diamine derivative **15** as a pale yellow oil (2.05 g, 88%). ν_{max} (film)/cm^{-1} 3305, 3059, 3029, 2928, 1708, 1653, 1533; δ_H 2.6 (2 H, m), 3.0-3.2 (4 H, m), 4.4 (1 H, q), 5.1 (2 H, s), 5.6 (1 H, d), 6.4 (1 H, s), 7.1-7.4 (25 H, m, ArH); δ_C 38.9, 40.9, 42.0, 56.6, 67.0, 70.7, 126.1, 127.0, 127.8, 128.0 128.2, 128.5, 128.6, 128.7, 129.3, 136.2, 136.6, 146.1, 156.0, 171.0.

Mono-N-*benzyloxycarbonyl-L-phenylalanyl-1,2-diaminoethane* **10**

To a solution of trityl-protected amine **15** (2.0 g, 3.43 mmol) in DCM (7 ml) was added triethylsilane (1.37 ml, 8.6 mmol) and trifluoroacetic acid (3.45 ml, 44.6 mmol). The resulting mixture was stirred at room temperature for 5 h. An excess of ether was then added to the reaction mixture, the resulting precipitate filtered, resuspended in ethyl acetate and repeatedly washed with 1M sodium hydroxide solution. The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* to give the desired mono-substituted diamine **10** as a colourless oil which solidified on standing (0.97 g, 83%), m.p. 135-137°C. The ir and nmr spectra of the product were identical to those recorded earlier for **10**.

N-*(*N-*Benzyloxycarbonyl-L-phenylalanyl)-4-aminobutyric acid* **18**.

To a cold (0°C) solution of Z-phenylalanine *N*-hydroxysuccinimide ester **9** (1.4 g, 3.5 mmol) and GABA (0.37 g, 3.5 mmol) in THF/water (7:4, 55 ml)) was added triethylamine (0.97 ml, 7 mmol). The reaction was stirred at room temperature for 4 h. and the organic solvent removed under vacuum. The residue was acidified to pH 2 by the careful addition of 2M HCl, the aqueous layer was extracted with ethyl acetate (3 x 25 ml), the combined organic fractions were washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a colourless solid which was purified by recrystallisation from ethyl acetate to give the acid **18** as colourless crystals (1.15 g, 86%) m.p. 148-150°C (Lit.[94] no m.p. given). ν_{max} (KBr)/cm^{-1} 3295, 1714, 1689, 1654, 1532; δ_H 1.7 (2 H, quint.), 2.2 (2 H, t), 4.4 (1 H, q), 5.1 (2 H, s), 5.6 (1 H, d), 6.3 (1 H, s), 7.2 (10 H, m, ArH); δ_C 24.2, 31.0, 38.6, 38.8, 56.5, 67.2, 127.1, 128.1 128.3, 128.6, 128.7, 129.3, 136.3, 156.3, 171.4, 176.8.

N-*(*N-*Benzyloxycarbonyl-L-phenylalanyl)-5-amino-1-diazo-2-oxopentane* **19**

N-Methylmorpholine (0.66 ml, 6 mmol) followed by *n*-butylchloroformate (1.1 ml, 7.81 mmol) were added to a cold (-78°C) solution of acid **18** (2 g, 5.21 mmol) in THF (52 ml) in an atmosphere of nitrogen. The reaction was stirred for 0.5 h. and an ethereal solution of diazomethane, prepared from *N*-methyl-*N*-nitroso-4-toluenesulfonamide (6.23 g, 29 mmol), was added, dropwise, and the reaction left to warm to room temperature overnight. Saturated ammonium chloride solution (10 ml) was added and the mixture was stirred vigorously for 5 min., then the layers were separated. The organic layer was washed successively with saturated sodium hydrogen carbonate, brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a green oil which was purified by flash column chromatography (49:1, ethyl acetate/DCM) to afford the desired diazoketone **19** as a pale yellow solid (0.51 g, 24 %) m.p.114-115°C. (Found: C, 64.43; H, 6.16; N, 13.34. $C_{22}H_{24}N_4O_4$ requires C, 64.69; H, 5.92; N, 13.72%.); ν_{max} (KBr)/cm^{-1} 3316, 3063, 2104, 1715, 1685, 1645, 1537; δ_H 1.7 (2 H, m), 2.2 (2 H, t), 3.0 (2 H, m), 3.2 (2H, m), 4.4 (1 H, q), 5.1 (2 H, s), 5.2 (1 H, s), 5.5 (1 H, d), 6.3 (1 H, t), 7.2 (10 H, m, ArH); δ_C 24.3, 38.7, 38.8, 38.9, 54.7, 56.4, 67.0, 127.0, 128.0, 128.2, 128.5, 128.7, 129.3, 136.2, 136.5, 155.9, 171.0, 194.4.

N-*(*N-*Benzyloxycarbonyl-L-phenylalanyl)-5-amino-1-bromo-2-oxopentane* **20a**

To a cold (0°C), stirred solution of the diazoketone **19** (0.2 g, 0.49 mmol) in THF (5 ml) was added a 1:1 solution of 48% HBr/acetic acid (0.8 ml) dropwise. The mixture was stirred for a further 10 min. and saturated sodium hydrogen carbonate was added carefully until gas evolution ceased. The organic layer was separated and the aqueous

layer was extracted with ethyl acetate (3 x 10 ml). The combined organic layer was washed with water, brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded a colourless residue which was purified by flash column chromatography (7:3 ethyl acetate/light petroleum) to afford the bromomethyl ketone **20a** as colourless crystals (0.202 g, 79%) m.p. 129-131°C (Lit.[94] m.p. 129.5-132°C). ν_{max} (KBr)/cm^{-1} 3300, 2925, 1725, 1687, 1653, 1533; δ_H 1.6 (2 H, quint.), 2.5 (2 H, t), 3.0-3.2 (4 H, m), 3.8 (2 H, s), 4.4 (1 H, q), 5.1 (2 H, s), 5.4 (1 H, d), 5.9 (1 H, t), 7.2-7.4 (10 H, m, ArH); δ_C 23.3, 34.3, 36.7, 38.5, 38.9, 56.4, 67.1, 127.1, 128.1, 128.3, 128.6, 128.8, 129.3, 136.1, 136.4, 156.0, 171.0, 201.7.

N-(N-*Benzyloxycarbonyl-L-phenylalanyl)-5-amino-1-fluoro-2-oxopentane* **20b**

To an ice-cold, stirred solution of the diazoketone **19** (0.1 g, 0.245 mmol) in DCM (3 ml) was added Olah's reagent (0.1 ml) dropwise. The reaction was stirred for 15 min. and then 10% sodium carbonate solution was added carefully. The layers were separated and the aqueous extracted with ethyl acetate (3 x 15 ml), the combined organic layer was washed successively with water, 0.5M HCl, saturated sodium hydrogen carbonate, brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded a colourless solid which was purified by flash column chromatography (1:1 ethyl acetate/light petroleum) to give the fluoromethyl ketone **20b** as a colourless solid (80 mg, 82 %) m.p. 126-128°C. (Found: C, 65.99; H, 6.20; N, 6.84. $C_{22}H_{25}FN_2O_4$ requires C, 65.99; H, 6.29; N, 6.99%.); ν_{max} (KBr)/cm^{-1} 3299, 1715, 1687, 1650, 1532; δ_H 1.7 (2 H, quint.), 2.4 (2 H, t), 3.0-3.2 (4 H, m), 3.8 (2H, s), 4.3 (1 H, q), 4.7 (2 H, d, J_{HF}=48 Hz), 5.1 (2 H, s), 5.4 (1 H, d), 5.9 (1 H, t), 7.2-7.4 (10 H, m, ArH); δ_C

22.2, 35.3, 38.6, 38.7, 56.5, 67.1, 84.9 (d, J_{CF}=184 Hz), 127.1, 128.1, 128.3, 128.6,128.8, 129.3, 136.2, 136.4, 156.0, 171.1, 206.6 (d, J_{CF}=19.5 Hz).

N-*(*N-*Benzyloxycarbonyl-L-phenylalanyl)-5-amino-1-dimethylsulfonium-2-oxopentane, bromide salt* **16**

Compound **16** was prepared by *procedure 3*, using bromomethyl ketone **20a** (50 mg, 0.108 mmol) and dimethyl sulfide (0.02 ml, 0.27 mmol). The bromide salt **16** was obtained as colourless crystals (37 mg, 65%) m.p. 116-118°C (Lit.[94] m.p. 118-120°C). ν_{max} (KBr)/cm^{-1} 3298, 3024, 2924, 1708, 1692, 1656, 1540; δ_H (d_6 DMSO) 1.7 (2 H, quint.), 2.6 (2 H, t), 3.0 (6 H, s), 3.1 (4 H, m), 4.4 (1 H, q), 5.0 (2 H, s), 5.1 (2 H, s), 6.8 (1 H, d), 7.2-7.4 (10 H, m, ArH), 7.8 (1 H, t); δ_C (d_6 DMSO) 24.6, 39.3, 39.6, 39.9, 40.2, 53.9, 56.3, 66.2, 126.5, 127.7, 127.9, 128.2, 128.3, 129.3, 136.9, 137.3, 155.5, 172.1, 201.1.

N-*Benzyloxycarbonyl-L-phenylalanyl-6-chloro-5-oxo-L-norleucine* **17**

To a cold (0°C) solution of active ester **9** (58 mg, 0.146 mmol) in a 1:1 mixture of THF/water (3 ml) was added 6-diazo-5-oxo-norleucine **22** followed by triethylamine (0.031 ml, 0.22 mmol). The reaction was stirred for 2 h. and then the solvent was removed under high vacuum at room temperature. The pale yellow residue was dissolved in ethyl acetate (2 ml) and a 2.9 M solution of HCl in ethyl acetate added dropwise until gas evolution ceased. The mixture was stirred for 10 min., diluted with ethyl acetate (10 ml), washed with water, brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded a colourless solid which was recrystallised from chloroform to afford the desired chloromethyl ketone **17** as colourless crystals (52 mg, 78%) m.p.

143-145°C (Lit.[88] m.p. 125°C). ν_{max} (KBr)/cm^{-1} 3308, 3030, 2956, 1724, 1712, 1689, 1650, 1530; δ_H (CD_3OD) 1.9 (2 H, m), 2.2 (2 H, m), 2.6 (2 H, m), 2.8-3.2 (2 H, m), 4.2 (2 H, s), 4.4 (2 H, m), 5.0 (2 H, s), 7.2 (10 H, m, ArH); δ_C (CD_3OD) 27.1, 36.4, 38.8, 49.8, 52.1, 57.9, 67.5, 127.7, 128.6, 128.9, 129.2, 129.4, 130.3, 138.1, 138.5, 157.1, 172.2, 173.3, 203.4.

γ-Allyl-L-glutamate hydrochloride **25**

Chlorotrimethylsilane (10.77 ml, 85 mmol) was added to a rapidly stirred suspension of L-glutamic acid (5 g, 34 mmol) in allyl alcohol (170 ml). The resulting solution was stirred for 18 h., then excess ether was added to give a colourless precipitate which was filtered, washed with ether (50 ml) and dried under vacuum to afford allyl glutamate salt **25** as a colourless solid (5.95 g, 78%) m.p. 131-132°C (Lit.[109] m.p. 130-132°C). ν_{max} (KBr)/cm^{-1} 3080, 2924, 1735, 1645; δ_H (D_2O) 2.1 (2 H, m), 2.6 (2 H, t), 4.1 (1 H, t), 4.6 (2 H, d), 5.3 (2 H, m), 5.9 (1 H, m); δ_C (D_2O) 25.9, 30.6, 53.2, 67.1, 119.7, 132.7, 172.7, 175.1.

α-tert-*Butyl-γ-allyl-L-glutamate* **26**

To a stirred suspension of the allyl glutamate **25** (2.01 g, 9 mmol) in *t*-butyl acetate (56 ml) was added 70% aqueous perchloric acid (0.85 ml, 9 mmol). The flask was tightly stoppered and the reaction mixture stirred for 20 h. The resulting solution was cooled, (4°C), and saturated aqueous sodium hydrogen carbonate solution added carefully until gas evolution ceased. The layers were separated, the aqueous layer extracted with ethyl acetate (3 x 40 ml) and the combined organic layer was washed with brine, dried ($MgSO_4$). The solvent was removed *in vacuo* to afford the glutamate diester **26** as a

colourless oil (1.61 g, 73%) which was used without further purification. ν_{max} (film)/cm^{-1} 3380, 3320, 3080, 2956, 1732, 1645; δ_H 1.5 (9 H, s), 1.8 (2 H, m), 2.4 (2 H, m), 4.1 (1 H, t), 4.6 (2 H, d), 5.2 (2 H, m), 5.9 (1 H, m); δ_C 28.0, 28.1, 30.7, 56.0, 65.2, 118.2, 132.2, 172.9, 177.7.

N-*Benzyloxycarbonyl-L-phenylalanyl*-α-tert-*butyl*-γ-*allyl-L-glutamate* **27**

To an ice-cold, stirred solution of Z-phenylalanine **8** (1.79 g, 6 mmol) and glutamate diester **26** (1.46 g, 6 mmol) in DCM (32 ml) was added, portionwise, dicyclohexyl-carbodiimide (1.37 g, 6.6 mmol). The reaction was allowed to warm to room temperature and stirring was continued for 6 h. The reaction mixture was filtered and the solvent removed *in vacuo* to yield a colourless oil which was purified by flash column chromatography (7:3 light petroleum/ethyl acetate) to afford the dipeptide **27** as a colourless oil which solidified on standing (2.1 g, 68%) m.p. 84-86°C. (Found: C, 66.57; H, 6.97; N, 5.16. $C_{29}H_{36}N_2O_7$ requires C, 66.39; H, 6.92; N, 5.34%.); ν_{max} (KBr)/cm^{-1} 3289, 3034, 2924, 1736, 1732, 1687, 1653, 1538; δ_H 1.9-2.2 (2 H, m), 2.3 (2 H, m), 3.1 (2 H, m), 4.4 (2 H, m), 4.6 (2 H, d), 5.2 (2 H, m), 5.6 (1 H, d), 5.9 (1 H, m), 6.8 (1 H, d), 7.1-7.3 (10 H, m, ArH); δ_C 27.4, 27.9, 30.0, 38.5, 52.1, 56.0, 65.3, 66.9, 82.4, 118.3, 126.9, 127.9, 128.0, 128.4, 128.5, 129.3, 132.0, 136.2, 136.3, 155.9, 170.4, 170.8, 172.3.

N-*Benzyloxycarbonyl-L-phenylalanyl*-α-tert-*butyl -L-glutamic acid* **28**

To a stirred solution of the dipeptide **27** (1.65 g, 3.15 mmol) in DCM (17 ml) was added tetra*kis*triphenylphosphine palladium(0) (0.1 g, 0.09 mmol) followed by pyrrolidine (0.6 ml, 7.2 mmol). The reaction was stirred for a further 0.5 h. with

exclusion of light, ether (50 ml) was added and the organic mixture washed with 0.5M HCl (50 ml). The aqueous layer was extracted with chloroform (2 x 20 ml), then ether (20 ml) and the combined organic phase washed with 10% sodium carbonate solution (3 x 40 ml). The basic aqueous extract was acidified to pH 2 by addition of 1M HCl, extracted with ethyl acetate (3 x 40 ml) and the combined organic extract was washed with brine, dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a solid residue which was recrystallised from DCM/cyclohexane to give the desired acid derivative **28** as colourless crystals (0.92 g, 60%) m.p. 140.5-141.5°C. (Found: C, 64.51; H, 6.69; N, 5.63. $C_{26}H_{32}N_2O_7$ requires C, 64.45; H, 6.66; N, 5.78%.); ν_{max} (KBr)/cm^{-1} 3389, 3060, 2973, 1731, 1713, 1645, 1541; δ_H 1.4 (9 H, s), 1.9- 2.2 (2 H, m), 2.4 (2 H, m), 3.1 (2 H, m), 4.5 (2 H, m), 5.0 (2 H, s), 5.6 (2 H, d), 6.8 (1 H, d), 7.2-7.4 (10 H, m, ArH); δ_C 27.5, 27.9, 30.0, 38.5, 52.2, 56.1, 67.2, 82.4, 127.1, 128.0, 128.2, 128.5, 128.6, 129.3, 136.1, 136.2, 156.2, 171.2, 173.3, 174.7.

Attempted synthesis of N-*benzyloxycarbonyl-L-phenylalanyl-6-diazo-5-oxo-L-norleucine* tert-*butyl ester* **29**

In a similar manner to that described for the synthesis of diazoketone **19** (p. 127) the preparation of **29** was attempted using *N*-methylmorpholine (0.18 ml, 1.45 mmol), *n*-butylchloroformate (0.2 ml, 1.45 mmol), acid **28** (0.7 g, 1.45 mmol) and *N*-methyl-*N*-nitroso-4-toluenesulfonamide (1.73 g, 8 mmol). After work up, removal of the solvent *in vacuo* gave a colourless oil which, by ir spectroscopy, contained none of the desired product. Starting material (0.5 g) was recovered from the basic aqueous extract.

4-Trifluoroacetamidobutyric acid **32**

1,1,3,3-Tetramethylguanidine (1.88 ml, 15 mmol) was added dropwise to a cold (4°C), stirred suspension of 4-aminobutyric acid (1.03 g, 10 mmol) in methyl trifluoroacetate (10 ml). The reaction mixture was allowed to warm to room temperature and the resulting solution was stirred for 24 h., then poured into water (30 ml), acidified to pH 2 by addition of concentrated HCl and extracted with ethyl acetate (3 x 40 ml). The combined organic layer was washed with water (3 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave the acid **32** as a colourless oil (1.65 g, 83%). ν_{max} (film)/cm^{-1} 3304, 3116, 2967, 1705; δ_H (d_6 DMSO) 1.7 (2 H, quint), 2.2 (2 H, t), 3.2 (2 H, q), 9.2(1 H, s), 12.1 (1 H, s); δ (d_6 DMSO) 23.6, 27.4, 38.5, 115.9 (q, J_{CF}=288 Hz), 156.2 (q, J_{CF}=36.6 Hz), 173.9.

1-Diazo-5-trifluoroacetamido-2-oxopentane **33**

Oxalyl chloride (0.196 ml, 2.25 mmol) was added dropwise to a stirred, cold (4°C) solution of **32** (0.3 g, 1.5 mmol) in DCM (3 ml). The reaction mixture was allowed to warm to room temperature, stirring was continued for a further 1 h. and then the solvent was removed *in vacuo*. The residue was redissolved in a 1:1 mixture of THF/acetonitrile (4 ml) and the solution was cooled to 4°C, then trimethylsilyldiazomethane (1.5 ml, 3 mmol) was added. The reaction was stirred for a further 2 h. at 4°C and saturated ammonium chloride solution was then added (4 ml). The layers were separated, the organic layer was washed with saturated sodium carbonate solution, brine and dried ($MgSO_4$). The solvent was removed *in vacuo* and the resulting pale brown oil was purified by flash column chromatography (3:2 light petroleum/ethyl acetate) to afford 1-diazo-5-trifluoroacetamido-2-oxopentane **33** as a

pale green oil (0.21 g, 63%). ν_{max} (film)/cm^{-1} 3337, 3084, 2976, 2104, 1703, 1632,1560; δ_H 1.9 (2 H, m), 2.4 (2 H, t), 3.4 (2 H, q), 5.4 (1 H, s), 7.8 (1 H, s); δ_C 23.4, 37.9, 39.8, 55.4, 115.9 (q, J_{CF}=288 Hz), 157.6 (q, J_{CF}=36.6 Hz), 195.0.

N-*(*N-*Benzyloxycarbonyl-L-phenylalanyl)-5-amino-1-diazo-2-oxopentane* **19**

To a solution of the diazoketone **33** (0.15 g, 0.67 mmol) in THF (5 ml) was added 2M sodium hydroxide solution (1 ml, 2 mmol) dropwise. The mixture was stirred for 2 h., cooled to 0°C and a solution of the Z-phenylalanine active ester **9** (0.265 g, 0.67 mmol) in THF (2.5 ml) was added. The reaction mixture was stirred at 0°C for 5 h., then concentrated *in vacuo*. To the residue was added water (10 ml), the aqueous layer was extracted with chloroform (2 x 10 ml) and then ether (30 ml), and the combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a brown oil which was purified by flash column chromatography (49:1, ethyl acetate/DCM) to afford the diazoketone **19** as a pale yellow solid (60 mg, 22%) m.p.114-115°C, the ir and nmr spectra of which were identical to those of previously prepared **19**.

N-tert-*Butoxycarbonyl-L-2-pyrrolidinone-5-carboxylic acid benzyl ester* **34**

Compound **34** was prepared by *procedure 4a*, using di-*t*-butyldicarbonate (9.6 g, 44 mmol), 2-pyrrolidinone-5-carboxylic acid benzyl ester **42** (8.76 g, 40 mmol), triethylamine (5.64 ml, 40 mmol) and DMAP (4.8 g, 40 mmol). Purification by flash column chromatography (3:2 light petroleum/ethyl acetate) gave the protected pyroglutamate **34** as a colourless solid (12.5 g, 83%) m.p. 71-72°C (Lit.[178] no m.p. given). ν_{max} (KBr)/cm^{-1} 1782, 1739, 1702; δ_H 1.4 (9 H, s), 2.0-2.7 (4 H, m), 4.6 (1 H,

dd), 5.2 (2 H, s), 7.4 (5 H, m, ArH); δ_C 21.5, 27.8, 31.1, 58.9, 67.3, 83.6, 128.5, 128.6, 128.7, 135.1, 149.2, 171.2, 173.3.

N-tert-*Butyloxycarbonyl-L-glutamic acid* γ-*allyl*-α-*benzyl diester* **35a**

To a solution of the protected pyroglutamate **34** (2 g, 6.3 mmol) in THF (12 ml) was added allyl alcohol (0.86 ml, 12.6 mmol) and potassium cyanide (0.1 g, 1.57 mmol). The reaction mixture was stirred for 48 h., filtered and the solvent removed *in vacuo* to give a pale yellow oil. Purification by flash column chromatography (3:1 light petroleum/ethyl acetate) afforded the diester **35a** as a pale yellow oil (1.66 g, 70%). ν_{max} (film)/cm^{-1} 3372, 3032, 2975, 1740, 1714, 1648; δ_H 1.4 (9 H, s), 1.9-2.3 (2 H, m), 2.4 (2 H, m), 4.4 (1 H, q), 4.6 (2 H, dd), 5.1 (2 H, s), 5.2 (2 H, m), 5.8 (1 H, m), 7.3 (5 H, m, ArH); δ_C 27.5, 28.3, 30.2, 53.0, 65.3, 67.1, 79.9, 118.3, 128.2, 128.4, 128.6, 132.0, 135.3, 155.4, 172.1, 172.3.

N-tert-*Butyloxycarbonyl -L-glutamic acid* α-*benzyl* γ-*trichloroethyl diester* **35b**

In a similar manner **35b** was prepared from benzyl *N*-BOC pyroglutamate **34** (5 g, 15.6 mmol), trichloroethanol (3.74 ml, 39 mmol), potassium cyanide (0.1 g, 1.56 mmol) and THF (32 ml) and isolated as a colourless solid (1.6 g, 22%) m.p. 65-66°C (ethanol). (Found: C, 48.99; H, 5.36; N, 3.25. $C_{19}H_{24}Cl_3NO_6$ requires C, 48.68; H, 5.16; N, 2.99%.); ν_{max} (KBr)/cm^{-1} 3380, 1754, 1738, 1700; δ_H 1.4 (9 H, s), 2.0-2.3 (2 H, m), 2.5 (2 H, q), 4.4 (1 H, q), 4.7 (2 H, s), 5.2 (2 H, s), 7.4 (5 H, m, ArH); δ_C 27.4, 28.3, 29.9, 52.8, 67.3, 74.0, 80.2, 94.8, 128.3, 128.5, 128.6, 135.1, 155.4, 171.1, 171.9.

N-tert-*Butoxycarbonyl-L-glutamic acid* α-*benzyl ester* **36**

Method a): To a solution of the allyl glutamate **35a** (1.51 g, 4 mmol) in DCM (16 ml) was added tetra*kis*triphenylphosphine palladium(0) (0.1 g, 0.09 mmol) followed by pyrrolidine (0.67 ml, 8 mmol). The reaction was stirred for a further 0.5 h. with exclusion of light, ether (50 ml) was added and the organic layer was washed with 0.5M HCl (50 ml). The aqueous layer was extracted with chloroform (2 x 20 ml) and ether (20 ml) and the combined organic extract was washed with 10% sodium carbonate solution (3 x 40 ml). The basic aqueous extract was acidified to pH 2 by addition of 1M HCl, extracted with ethyl acetate (3 x 40 ml) and the pooled organic extracts were washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a solid residue which was recrystallised from ethanol to give the α-benzyl ester **36** as colourless crystals (1.02 g, 76%) m.p. 93-95°C (Lit.[195] m.p. 93-94°C). ν_{max} (KBr)/cm^{-1} 3355, 1744, 1700, 1681; δ_H 1.4 (9 H, s), 1.9-2.2 (2 H, m), 2.4 (2 H, q), 4.4 (2 H, q), 5.2 (2 H, s) 5.4 (1 H, s), 7.4 (5 H, m, ArH); δ_C 27.6, 27.9, 30.0, 52.9, 67.3, 80.2, 128.3, 128.5, 128.6, 135.2, 155.5, 172.1, 178.0.

Method b): To a rapidly stirred solution of the trichloroethyl glutamate **35b** (1 g, 2.13 mmol) in THF (6.6 ml) was added zinc dust (1.39 g, 21.3 mmol) followed by 1M aqueous ammonium acetate (1.33 ml, 1.33 mmol). The resultant slurry was stirred for 30 min., filtered and the filtrate stirred with Dowex-50 (H^+) ion-exchange resin for 15 min. The mixture was filtered and concentrated and the residue redissolved in ethyl acetate (20 ml). The organic layer was washed with 10% sodium carbonate solution (3 x 15 ml) and the combined aqueous extract was acidified with 2M HCl to pH 2. The aqueous layer was extracted with chloroform (3 x 20 ml) and the combined organic

extract was washed with brine, dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a residue which was recrystallised from ethanol to give the α-benzyl ester **36** as a colourless solid (0.57 g, 79%) m.p. 94-96°C (Lit.[195] 93-94°C). Spectroscopic data as in Method (a) above.

Attempted synthesis of N-tert-*butoxycarbonyl-6-diazo-5-oxo-L-norleucine benzyl ester* **38**

a) Via acid fluoride **37a**

A solution of the *N*-BOC-α-benzyl glutamate **36** (1 g, 3 mmol) and pyridine (1 ml, 12 mmol) in DCM (9 ml) in an atmosphere of nitrogen was cooled to -30°C and cyanuric fluoride (0.81 ml, 9 mmol) was added dropwise. The reaction was stirred for 1 h. and then crushed ice/DCM (20 ml) was added. The layers were separated and the aqueous layer was extracted with DCM (3 x 10 ml), the combined organic layer was washed with brine, dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded an oily residue which was crystallised from DCM/light petroleum to give the acid fluoride **37a** as a colourless solid (0.65 g, 65%) m.p. 52-54°C (Lit.[124] m.p. 50-51°C). ν_{max} (KBr)/cm^{-1} 3306, 1844, 1747, 1702, 1684; δ_H 1.4 (9 H, s), 1.9-2.2 (2 H, m), 2.6 (2 H, q), 4.4 (2 H, q), 5.2 (2 H, s) 5.8 (1 H, d), 7.4 (5 H, m, ArH).

The acid fluoride **37a** (0.4 g, 1.12 mmol) was added in portions to a cold (0°C) 1:1 mixture of trimethylsilyldiazomethane (0.56 ml, 1.12 mmol) and triethylamine (0.16 ml, 1.12 mmol) in THF (11 ml). The reaction was stirred for 48 h. at (0°C) and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography (1:1 light petroleum/ethyl acetate) to give the pyroglutamate **34** as the main fraction (0.28 g, 79%).

b) Via mixed anhydride **37b**

To a cold (-15°C) solution of the glutamic acid derivative **36** (1 g, 3 mmol) in THF (15 ml) in an atmosphere of nitrogen was added triethylamine (0.42 ml, 3 mmol) followed by ethyl chloroformate (0.29 ml, 3 mmol). The mixture was stirred for 15 min. and allowed to warm to -5°C. A solution of TMSD (1.65 ml, 3.3 mmol) in THF (3 ml) was added and the resulting yellow mixture was stirred at room temperature for 3 h. Removal of the solvent gave an oily residue which when analysed by tlc and ir spectroscopy showed that extensive cyclisation to the pyroglutamate **34** had occurred.

c) Via intermediate **37c**

Dicyclohexylcarbodiimide (0.62 g, 3 mmol) was added in portions to a cooled (4°C) solution of the glutamate **36** (1 g, 3 mmol) and TMSD (1.65 ml, 3.3 mmol) in DCM (6 ml). The reaction was stirred for 5 h. and left to stand overnight at 4°C. After filtration, removal of the solvent under reduced pressure yielded a residue which upon analysis, as in b) above, showed extensive formation of the pyroglutamic acid derivative **34**.

N-*Benzyloxycarbonyl-L-phenylalanyl-5-amino-1-diazo-2-oxopentane* **19**

To a solution of acid **18** (1 g, 2.6 mmol) in THF (13 ml) was added oxalyl chloride (0.35 ml, 3.9 mmol) and DMF (1 drop). The pale yellow solution was stirred for 1 h. and the solvent removed *in vacuo* to give acid chloride **39** as a yellow oil. The oil was redissolved in a 1:1 mixture of THF/acetonitrile (13 ml) and the mixture cooled in an ice bath. TMSD (2.6 ml, 5.2 mmol) was added and the reaction mixture was stirred with cooling for a further 4 h. Saturated ammonium chloride solution (20 ml) was

added and the aqueous layer was extracted with ethyl acetate (3 x 10 ml). The combined organic fraction was washed with brine and dried, ($MgSO_4$). Removal of the solvent *in vacuo* gave a yellow oil which was purified by flash column chromatography (49:1, ethyl acetate/DCM) to give two fractions. Fraction one yielded the diazoketone **19** as a yellow solid (0.13 g, 12%) m.p. 114-115°C with spectroscopic data identical to those of a previously prepared sample of **19**. Fraction two gave a colourless solid which was found to be *N*-(*N*-benzyloxycarbonyl-L-phenylalanyl)-2-oxo-pyrrolidine **40** (0.11 g) m.p. 129-130°C. ν_{max} (KBr)/cm^{-1} 3365, 3030, 2939, 1735, 1716, 1686, 1522; δ_H 2.0 (2 H, m), 2.6 (2 H, q), 2.8 and 3.2 (2 H, m), 3.7 (2 H, m), 4.4 (2 H, q), 5.0 (2 H, s) 5.4 (1 H, s), 5.7 (1 H, m), 7.1 (1 H, m), 7.3 (10 H, m, ArH); δ_C 17.4, 33.4, 38.5, 45.6, 55.3, 66.8, 127.0, 128.0, 128.4, 129.4, 136.0, 136.1, 155.4, 173.1, 175.1.

N-tert-*Butyloxycarbonyl-6-diazo-5-oxo-L-norleucine benzyl ester* **38**

Compound **38** was prepared by *procedure 6*, using TMSD (5.9 ml, 4.8 mmol), *n*-BuLi (3.1 ml, 4.8 mmol) and *N-tert*-butyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid benzyl ester **34** (1.28 g, 4 mmol). Purification by flash column chromatography (3:2 light petroleum/ethyl acetate) yielded the diazoketone as yellow oil which solidified on standing to a light yellow solid (1.03 g, 71%) m.p. 55.5-56.5°C. (Found: C, 60.05; H, 6.63; N, 11.35. $C_{18}H_{23}N_3O_5$ requires C, 59.82; H, 6.41; N, 11.63%.); ν_{max} (KBr)/cm^{-1} 3360, 2104, 1738, 1715, 1636; δ_H 1.4 (9 H, s), 2.0 and 2.2 (2 H, m), 2.4 (2 H, m), 4.3 (1 H, q), 5.1 (2 H, s), 5.2 (1 H, s), 5.3 (1 H, d), 7.3 (5 H, m, ArH); δ_C 27.5, 28.3, 36.5, 53.2, 54.7, 67.2, 80.0, 128.4, 128.5, 128.6, 135.3, 155.5, 172.1, 193.6.

2-Pyrrolidinone-5-carboxylic acid benzyl ester **42**

Method a) To a solution of L-2-pyrrolidinone-5-carboxylic acid (10 g, 77.4 mmol) and benzyl alcohol (9.57 ml, 93 mmol) in DCM (100 ml) was added 4-dimethylamino-pyridine (DMAP) (2.4 g, 19.4 mmol) followed by dicyclohexylcarbodiimide (16 g, 77.6 mmol) in portions. The reaction mixture was stirred for 24 h., filtered and the solvent removed *in vacuo* to give a pale brown oil which was purified by flash column chromatography (100% ethyl acetate) to afford the benzyl ester **42** as a pale yellow oil (9.5 g, 56%). ν_{max} (film)/cm^{-1} 3244, 1740, 1700; δ_H 2.0-2.4 (4 H, m), 4.2 (1 H, m), 5.1 (2 H, s), 7.2 (5 H, m, ArH), 7.7 (1 H, s); δ_C 24.6, 29.3, 55.6, 67.0, 128.2, 128.4, 128.6, 135.3, 172.2, 178.7.

Method b) To a solution of pyroglutamic acid (5 g, 38.8 mmol) in DMF (25 ml) was added benzyl bromide (5 ml, 42 mmol) followed by triethylamine (5.4 ml, 42 mmol) dropwise. The mixture was stirred vigorously for 15 h. and then filtered. The filtrate was diluted with ethyl acetate (200 ml) and the organic layer was washed with water (2 x 100 ml), 1M HCl (3 x 20 ml), brine and dried ($MgSO_4$). Removal of the solvent under high vacuum afforded the product as a colourless oil (5.4 g, 64%) with spectroscopic data identical to those of a sample previously obtained by Method a.

Pyrrolidinone-5-carboxylic acid ethyl ester **43**

Method a) Freshly distilled thionyl chloride (30 ml, 410 mmol) was added to an ice-cold, stirred suspension of glutamic acid (25.6 g, 175 mmol) in absolute ethanol (250 ml). The resulting solution was stirred at room temperature overnight, then the solvent was removed *in vacuo* to give an oily residue which was redissolved in

chloroform (150 ml), neutralised with triethylamine and the mixture was filtered. The solvent was removed *in vacuo* and the crude residue was purified by vacuum distillation to afford the ethyl pyroglutamate **43** as a colourless oil which solidified on standing (13.5 g, 49%) m.p. 53-54°C, b.p. 148-150°C 0.8 mmHg (Lit.[131] m.p. 48-50°C b.p. 159-162°C 2 mmHg). ν_{max} (KBr)/cm^{-1} 3238, 1739, 1704; δ_H 1.3 (3 H, t), 2.2-2.5 (4 H, m), 4.2 (2 H, q), 4.3 (1 H, m), 7.4 (1 H, s); δ_C 14.1, 24.8, 29.4, 55.7, 61.6, 172.3, 178.6.

Method b) Concentrated H_2SO_4 (0.05 ml) was added to a suspension of pyroglutamic acid (2.6 g, 20 mmol) in ethanol (2.5 ml, 54 mmol) and benzene (2 ml). The reaction mixture was heated under reflux in a Dean-Stark apparatus for 1 h. The solvent was removed *in vacuo*, the residue redissolved in chloroform (20 ml), then potassium carbonate (0.5 g) was added and the resulting mixture was stirred for 0.5 h., filtered and the solvent removed to yield an oily residue which was purified as above to afford the ethyl ester **43** as a colourless oil which solidified on standing (1.2 g, 39%) m.p. 52-54°C.

Method c) In a similar manner to that described for the synthesis of benzyl pyroglutamate **42**, pyroglutamic acid (5.16 g, 40 mmol), ethyl bromide (3.3 ml, 44 mmol) and triethylamine (6 ml, 44 mmol) in DMF (40 ml) gave the ethyl pyroglutamate **43** as a colourless solid (3.83 g, 61 %) m.p. 52-54°C after purification as described above.

Method d) To an ice-cold, stirred suspension of pyroglutamic acid (3.87 g, 30 mmol) in absolute ethanol (60 ml) was added oxalyl chloride (2.82 ml, 33 mmol) dropwise. The resulting solution was stirred at room temperature for 1 h. and the solvent

removed *in vacuo*. Purification in the normal manner yielded the product as a colourless solid (3.9 g, 83%) m.p. 53-55°C.

2-Pyrrolidinone-5-carboxylic acid tert-*butyl ester* **44**

To a stirred suspension of pyroglutamic acid (4 g, 31 mmol) in *t*-butyl acetate (62 ml) was added 70% aqueous perchloric acid (0.96 ml) dropwise. The flask was tightly stoppered and the mixture stirred for 48 h. The reaction was neutralised by careful addition to an ice-cold saturated sodium bicarbonate solution. The aqueous layer was extracted with ether (3 x 50 ml) and the combined organic layer was washed with brine, dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded the *t*-butyl pyroglutamate **44** as a colourless solid (4.07 g, 71%) m.p. 103-104.5°C (Lit.[110] m.p. 91-92°C). ν_{max} (KBr)/cm^{-1} 3241, 1738, 1701; δ_H 1.5 (9 H, s), 2.2-2.5 (4 H, m), 4.2 (1 H, m) 7.1 (1 H, s); δ_C 24.8, 27.9, 29.5, 56.2, 82.2, 171.3, 178.3.

N-tert-*Butyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester* **45a**

Compound **45a** was prepared by *procedure 4b*, using ethyl pyroglutamate **43** (1.1 g, 7 mmol), di-*t*-butyl dicarbonate (1.68 g, 7.7 mmol) and DMAP (0.04 g, 0.35 mmol). After purification by flash column chromatography (3:2 light petroleum/ethyl acetate) the desired pyroglutamate **45a** was afforded as a colourless solid (1.71 g, 95 %) m.p. 51-52.5°C (Lit.[179] no m.p. given). ν_{max} (KBr)/cm^{-1} 1793, 1744, 1715; δ_H 1.3 (3 H, t), 2.0 and 2.3-2.6 (4 H, m), 4.2 (2 H, q), (4.6 (1 H, dd); δ_C 14.2, 21.5, 27.9, 31.1, 59.0, 61.7, 83.5, 149.3, 171.4, 173.4.

N-tert-*Butyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid tert-butyl ester* **45b**

Compound **45b** was prepared in the same way as above using *t*-butyl pyroglutamate **44** (1.3 g, 7 mmol), di-*t*-butyl dicarbonate (1.68 g, 7.7 mmol) and DMAP (0.04 g, 0.35 mmol). After purification by flash column chromatography (3:2 light petroleum/ethyl acetate) the *N*-BOC protected pyroglutamate **45b** was obtained as a colourless solid (1.71 g, 95 %) m.p. 55-56.5°C (Lit.[179] no m.p. given). ν_{max} (KBr)/cm^{-1} 1792, 1739, 1717; δ_H 1.5 (2 x 9 H, 2 x s), 2.0 and 2.3-2.6 (4 H, m), 4.5 (1 H, dd); δ_C 21.6, 27.9, 28.0, 31.1, 59.6, 82.2, 83.2, 149.3, 170.4, 173.7.

N-*2,2,2-Trichloroethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester* **45c**

Compound **45c** was prepared by *procedure 4a*, using ethyl pyroglutamate **43** (0.64 g, 4.1 mmol); 2,2,2-trichloroethyl chloroformate (0.62 ml, 4.51 mmol), triethylamine (0.57 ml, 4.1 mmol) and DMAP (0.5 g, 4.1 mmol). The solvent was removed *in vacuo* and the residue was purified by recrystallisation from ethanol to give the pyroglutamate **45c** as a colourless solid (1.06 g, 78 %) m.p. 88-90°C. (Found: C, 36.10; H, 3.72; N, 4.39. $C_{10}H_{12}Cl_3NO_5$ requires C, 36.12; H, 3.64; N, 4.21%.); ν_{max} (KBr)/cm^{-1} 1805, 1785, 1746; δ_H 1.3 (3 H, t), 2.1 and 2.4-2.8 (4 H, m), 4.2 (2 H, q), 4.8 (1 H, dd), 4.9 (2 H, q); δ_C 14.1, 22.1, 30.7, 58.6, 62.1, 75.2, 94.2, 148.9, 170.6, 173.0.

N-2,2,2-Trichloroethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid benzyl ester **45d**

Compound **45d** was prepared as described for **45c** using benzyl pyroglutamate **42** (0.9 g, 4.1 mmol), 2,2,2-trichloroethyl chloroformate (0.62 ml, 4.51 mmol), triethylamine (0.57 ml, 4.1 mmol) and DMAP (0.5 g, 4.1 mmol) to afford the protected

pyroglutamate derivative **45d** as a colourless solid (1.22 g, 76 %) m.p. 85-86°C (ethanol). (Found: C, 45.66; H, 3.61; N, 3.75. $C_{15}H_{14}Cl_3NO_5$ requires C, 45.65; H, 3.57; N, 3.55%.); ν_{max} (KBr)/cm^{-1} 1805, 1791, 1743; δ_H 2.1 and 2.4-2.8 (4 H, m), 4.7 (2 H, q), 4.8 (1 H, dd), 5.2 (2 H, s) 7.3 (5 H, m, ArH); δ_C 22.0, 30.6, 58.6, 67.7, 75.1, 94.1, 128.4 128.5, 128.7, 134.8, 148.8, 170.4, 172.8.

N-2,2,2-*Trichloroethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid* tert-*butyl ester* **45e**

Compound **45e** was prepared as described for the previous carbamate using *t*-butyl pyroglutamate **44** (1.0 g, 5.4 mmol), 2,2,2-trichloroethyl chloroformate (0.82 ml, 5.94 mmol), triethylamine (0.75 ml, 5.4 mmol) and DMAP (0.66 g, 5.4 mmol) and was obtained as a colourless solid (1.52 g, 78 %) m.p. 141-142°C (ethanol). (Found: C, 39.96; H, 4.63; N, 3.98. $C_{12}H_{16}Cl_3NO_5$ requires C, 39.97; H, 4.47; N, 3.88%.); ν_{max} (KBr)/cm^{-1} 1802, 1792, 1746; δ_H 1.5 (9 H, s), 2.1 and 2.4-2.8 (4 H, m), 4.6 (1 H, dd), 4.8 (2 H, q); δ_C 22.2, 28.0, 30.7, 59.2, 75.2, 82.9, 94.2, 149.1, 169.6, 173.0.

N-*Allyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid benzyl ester* **45g**

Compound **45g** was prepared by *procedure 4a,* using benzyl pyroglutamate **42** (1.1 g, 4.97 mmol), allyl chloroformate (0.60 ml, 5.47 mmol), triethylamine (0.69 ml, 4.97 mmol), DMAP (0.58 g, 4.8 mmol). The product was obtained as a colourless solid (0.30 g, 20 %) m.p. 85-86.5°C (ethanol). (Found: C, 63.31; H, 5.67; N, 4.45. $C_{16}H_{17}NO_5$ requires C, 63.36; H, 5.65; N, 4.62%.); ν_{max} (KBr)/cm^{-1} 1798, 1758, 1724; δ_H 2.0 and 2.3-2.7 (4 H, m), 4.6 (2 H, m), 4.7 (1 H, dd), 5.2 (2 H, s), 5.3 (2 H, m), 5.9

(1 H, m); δ_C 21.7, 31.0, 58.7, 67.3, 67.4, 118.9, 128.3, 128.6, 128.7, 131.0, 135.0, 150.8, 170.8, 172.8.

N-tert-*Butyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid*-tert-*butyl ester* **45b**

Compound **45b** was prepared by *procedure 5*, using *t*-butyl pyroglutamate (1.5 g, 8.11 mmol), lithium *bis*(trimethylsilyl)amide (8.11 ml, 8.11 mmol) and di-*t*-butyl dicarbonate (1.78 g, 8.11 mmol). Purification by flash column chromatography (3:2 light petroleum/ethyl acetate) yielded the desired *N*-BOC pyroglutamate **45b** as a colourless solid (1.85 g, 80%) m.p. 55-57°C with spectroscopic data identical to those obtained for a sample prepared using *procedure 4b*.

N-2,2,2-Trichloroethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester **45c**

Compound **45c** was prepared using the above method from ethyl pyroglutamate **43** (1 g, 6.37 mmol), lithium *bis*(trimethylsilyl)amide (6.37 ml, 6.37 mmol) and 2,2,2-trichloroethyl chloroformate (0.88 ml, 6.37 mmol). After purification the pyroglutamate **45c** was obtained as a colourless solid (1.4 g, 67%) m.p. 88-90°C (ethanol). The spectroscopic data are identical to those obtained for a sample prepared using *procedure 4a*.

N-2,2,2-*Trichloroethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester* **45d**

Compound **45d** was prepared as above using benzyl pyroglutamate (1.1 g, 5 mmol), lithium *bis*(trimethylsilyl)amide (5 ml, 5 mmol) and 2,2,2-trichloroethyl chloroformate (0.69 ml, 5 mmol). The desired *N*-protected pyroglutamate **45d** was obtained as a colourless solid (1.3 g, 66%) m.p. 85-86°C (ethanol) with identical spectroscopic data as those obtained for a sample prepared using *procedure 4a*.

N-2,2,2-Trichloroethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester **45e**

Compound **45e** was prepared by *procedure 5*, from *t*-butyl pyroglutamate **44** (1 g, 5.4 mmol), lithium *bis*(trimethylsilyl)amide (5.4 ml, 5.4 mmol), 2,2,2-trichloroethyl chloroformate (0.74 ml, 5.4 mmol). Purification was achieved by recrystallisation from ethanol to afford the desired pyroglutamate derivative **45e** as colourless crystals (1.4 g, 74%) m.p. 141-142°C which were found to have identical spectroscopic data to those obtained from a sample prepared via *procedure 4a*.

N-*Allyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester* **45f**

Compound **45f** was prepared, using the procedure described above, from ethyl pyroglutamate **43** (1 g, 6.37 mmol), lithium *bis*(trimethylsilyl)amide (6.37 ml, 6.37 mmol), allyl chloroformate (0.68 ml, 6.37 mmol). Purified by flash column chromatography (3:2 light petroleum/ ethyl acetate) give the desired pyroglutamate **45f** as a colourless oil (1.2 g, 78%). ν_{max} (film)/cm^{-1} 1799, 1760, 1726, 1648; δ_H 1.3 (3 H, t), 2.0 and 2.3-2.8 (4 H, m), 4.2 (2 H, q), 4.6 (1 H, dd), 4.7 (2 H, m), 5.3 (2 H, m), 5.9 (1 H, m); δ_C 14.1, 21.8, 31.0, 58.8, 61.8, 67.2, 118.9, 131.1, 150.8, 171.1, 173.0.

N-*Allyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid benzyl ester* **45g**

Compound **45g** was prepared using the above procedure from benzyl pyroglutamate (0.7 g, 3.2 mmol), lithium *bis*(trimethylsilyl)amide (3.2 ml, 3.2 mmol) and allyl chloroformate (0.35 ml, 3.2 mmol) and was obtained as colourless solid (0.62 g, 64%) m.p. 85-86.5°C (ethanol) with spectroscopic data identical to those obtained from a sample of **45g** prepared by *procedure 4a*.

N-*Allyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid* tert-*butyl ester* **45h**

Compound **45h** was prepared as above from *t*-butyl pyroglutamate **44** (1 g, 5.4 mmol), lithium *bis*(trimethylsilyl)amide (5.4 ml, 5.4 mmol) and allyl chloroformate (0.57 ml, 5.4 mmol). The desired *N*-protected pyroglutamate **45h** was obtained, following column chromatography (3:2 light petroleum/ethyl acetate), as a colourless oil (1.0 g, 72%). ν_{max} (film)/cm^{-1} 1799, 1760, 1725, 1648; δ_H 1.5 (9 H, s), 2.0 and 2.3-2.8 (4 H, m), 4.6 (1 H, dd), 4.8 (2 H, m), 5.2 (2 H, m), 5.9 (1 H, m); δ_C 21.8, 27.6, 31.0, 59.4, 67.1, 82.5, 118.9, 131.2, 150.8, 170.2, 173.2.

N-*9-Fluorenylmethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid ethyl ester* **45i**

Compound **45i** was prepared by *procedure 5*, using ethyl pyroglutamate (1 g, 6.37 mmol), lithium *bis*(trimethylsilyl)amide (6.37 ml, 6.37 mmol) and 9-fluorenylmethyl chloroformate (1.65 g, 6.37 mmol). Purification by flash column chromatography (3:2 light petroleum/ethyl acetate) afforded the desired pyroglutamate **45i** as a colourless solid (2 g, 83%) m.p. 91-92°C. (Found: C, 69.61; H, 5.67; N, 3.75. $C_{22}H_{21}NO_5$ requires C, 69.65; H, 5.58; N, 3.59%.); ν_{max} (KBr)/cm^{-1} 1749, 1737, 1717; δ_H 1.2 (3 H, t), 2.0 and 2.3-2.8 (4 H, m), 4.2 (2 H, q), 4.3 (1 H, t), 4.4-4.6 (2 H, m), 4.7 (1 H, dd), 7.2 (4 H, m, ArH), 7.6 (4 H, m, ArH); δ_C 14.1, 21.9, 31.2, 46.6, 58.7, 61.9, 69.0, 120.0, 125.4, 127.2, 127.9, 141.3, 143.4, 151.4, 171.0, 172.7.

N-*9-Fluorenylmethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid benzyl ester* **45j**

Compound **45j** was prepared using an identical procedure as described for **45i** from benzyl pyroglutamate **42** (0.7 g, 3.2 mmol), lithium *bis*(trimethylsilyl)amide (3.2 ml, 3.2 mmol), 9-fluorenylmethyl chloroformate (0.83 g, 3.2 mmol). The product was

isolated as a colourless solid (1.06 g, 75%) m.p. 104-106°C (ethanol). (Found: C, 73.35; H, 5.35; N, 3.36. $C_{27}H_{23}NO_5$ requires C, 73.46; H, 5.25; N, 3.17%.); ν_{max} (KBr)/cm^{-1} 1762, 1740, 1721; δ_H 2.0 and 2.3-2.7 (4 H, m), 4.2 (1 H, t), 4.3-4.5 (2 H, m), 4.7 (1 H, dd), 5.1 (2 H, s), 7.3 (9 H, m, ArH), 7.7 (4 H, m, ArH); δ_C 21.8, 31.1, 46.5, 58.7, 67.5, 69.0, 120.0, 125.3, 127.2, 127.9, 128.3, 128.6, 128.7, 135.0, 141.2, 143.3, 151.4, 170.8, 172.6.

N-*9-Fluorenylmethyloxycarbonyl-2-pyrrolidinone-5-carboxylic acid* tert-*butyl ester* **45k**

Compound **45k** was prepared as above from *t*-butyl pyroglutamate **44** (0.81 g, 4.4 mmol), lithium *bis*(trimethylsilyl)amide (4.4 ml, 4.4 mmol) and 9-fluorenylmethyl chloroformate (1.14 g, 4.4 mmol) and the desired pyroglutamate **45k** was obtained as a colourless solid (1.48 g, 83%) m.p. 170°C (ethanol). (Found: C, 70.76; H, 6.22; N, 3.59. $C_{24}H_{25}NO_5$ requires C, 70.75; H, 6.18; N, 3.44%.); ν_{max} (KBr)/cm^{-1} 1749, 1735, 1715; δ_H 1.5 (9 H, s), 2.1 and 2.3-2.8 (4 H, m), 4.3 (1 H, t), 4.4-4.5 (2 H, m), 4.6 (1 H, dd), 7.3 (4 H, m, ArH), 7.7 (4 H, m, ArH); δ_C 21.9, 27.9, 31.2, 46.6, 59.4, 69.1, 82.7, 119.9, 125.4, 127.2, 127.9, 143.4, 143.5, 151.5, 170.1, 172.9.

N-tert-*Butyloxycarbonyl-6-diazo-5-oxo-L-norleucine ethyl ester* **46a**

Compound **46a** was prepared by *procedure 6*, using *N*-BOC ethyl pyroglutamate **45a** (1.1 g, 7 mmol), TMSD (3.85 ml, 7.7 mmol) and *n*-BuLi (4.86 ml, 7.7 mmol). Purification by flash column chromatography (7:3 light petroleum/ethyl acetate) afforded the desired diazoketone **46a** as yellow crystals (1.38 g, 66%) m.p. 87-88°C. (Found: C, 52.04; H, 7.18; N, 13.82. $C_{13}H_{21}N_3O_5$ requires C, 52.16; H, 7.07; N,

14.04%.); ν_{max} (KBr)/cm^{-1} 3359, 2103, 1736, 1712, 1642; δ_H 1.3 (3 H, t), 1.4 (9 H, s), 2.0, 2.2 and 2.4 (4 H, m), 4.2 (2 H, q), 4.3 (1 H, m), 5.2 (1 H, d), 5.3 (1 H, s); δ_C 14.2, 27.8, 28.3, 36.5, 53.1, 54.7, 61.5, 80.0, 155.9, 172.2, 193.9.

N-tert-*Butyloxycarbonyl-6-diazo-5-oxo-L-norleucine* tert-*butyl ester* **46b**

Compound **46b** was prepared similarly from *N*-BOC *t*-butyl pyroglutamate **45b** (0.8 g, 2.8 mmol), TMSD (1.5 ml, 3 mmol) and *n*-BuLi (1.87 ml, 3 mmol). Purification by flash column chromatography (7:3 light petroleum/ethyl acetate) gave the diazoketone **46b** as yellow oil (0.69 g, 75%). ν_{max} (film)/cm^{-1} 3351, 2104, 1734, 1714, 1643; δ_H 1.4 (2 x 9 H, 2 x s), 2.0, 2.2 and 2.4 (4 H, m), 4.2 (1 H, m), 5.2 (1 H, s), 5.3 (1 H, d); δ_C 27.7, 28.0, 28.3, 36.5, 53.1, 54.6, 80.0, 81.2, 156.1, 171.9, 193.4.

6-Diazo-N-*(2,2,2-trichloroethyloxycarbonyl)-5-oxo-L-norleucine ethyl ester* **46c**

Compound **46c** was prepared by the above procedure using *N*-TROC ethyl pyroglutamate **45c** (0.73 g, 2.2 mmol), TMSD (1.32 ml, 2.64 mmol) and *n*-BuLi (1.65 ml, 2.64 mmol). The crude material obtained was purified by flash column chromatography (7:3 light petroleum/ethyl acetate) to give the desired diazoketone **46c** as a yellow oil (0.16 g, 20%). ν_{max} (film)/cm^{-1} 3329, 2105, 1732, 1729, 1640; δ_H 1.3 (3 H, t), 2.0, 2.2 and 2.4 (4 H, m), 4.2 (2 H, q), 4.3 (1 H, m), 4.8 (2 H, s), 5.2 (1 H, s), 5.7 (1 H, d); δ_C 14.3, 27.6, 36.3, 54.0, 54.7, 61.0, 74.5, 95.4, 154.1, 171.0, 193.8.

6-Diazo-N-*(2,2,2-trichloroethyloxycarbony)-5-oxo-L-norleucine* tert-*butyl ester* **46d**

Compound **46d** was prepared using *procedure 6* from *N*-TROC *t*-butyl pyroglutamate **45e** (0.5 g, 1.4 mmol), TMSD (0.84 ml, 1.68 mmol), *n*-BuLi (1.05 ml, 1.68 mmol).

Flash column chromatography (7:3 light petroleum/ethyl acetate) afforded the desired norleucinate **46d** as yellow oil (90 mg, 16%). ν_{max} (film)/cm^{-1} 33249, 2105, 1735, 1730, 1637; δ_H 1.5 (9 H, s), 2.0, 2.2 and 2.4 (4 H, m), 4.3 (1 H, m), 4.8 (2 H, s), 5.3 (1 H, s), 5.8 (1 H, d); δ_C 27.5, 28.0, 36.3, 54.1, 54.8, 74.6, 82.7, 95.4, 154.3, 170.5, 194.1.

N-*Allyloxycarbonyl-6-diazo-5-oxo-L-norleucine ethyl ester* **46e**

Compound **46e** was prepared using *procedure 6* from *N*-ALOC ethyl pyroglutamate **45f** (0.68 g, 2.8 mmol), TMSD (1.68 ml, 3.36 mmol) and *n*-BuLi (2.1 ml, 3.36 mmol). Flash column chromatography (7:3 light petroleum/ethyl acetate) gave the desired diazoketone **46e** as a yellow oil (0.5 g, 63%). ν_{max} (film)/cm^{-1} 3330, 2104, 1720, 1715, 1636; δ_H 1.3 (3 H, t), 2.0, 2.2 and 2.4 (4 H, m), 4.2 (2 H, q), 4.3 (1 H, m), 4.6 (2 H, d), 5.2 (2 H, m), 5.3 (1 H, s), 5.6 (1 H, d), 5.9 (1 H, m); δ_C 14.1, 27.5, 36.3, 53.5, 54.8, 61.7, 65.8, 117.8, 132.6, 156.0, 171.9, 193.6.

N-*Allyloxycarbonyl-6-diazo-5-oxo-L-norleucine benzyl ester* **46f**

Compound **46f** was prepared as previously described using *N*-ALOC benzyl pyroglutamate **45g** (0.45 g, 1.48 mmol), TMSD (0.89 ml, 1.78 mmol) and *n*-BuLi (1.11 ml, 1.78 mmol). Flash column chromatography (7:3 light petroleum/ethyl acetate) yielded the desired norleucinate diazoketone **46f** as yellow oil (0.31 g, 60%). ν_{max} (film)/cm^{-1} 3326, 2104, 1722, 1712, 1640; δ_H 2.0, 2.2 and 2.4 (4 H, m), 4.3 (1 H, m), 4.6 (2 H, d), 5.1 (2 H, s), 5.2 (2 H, m), 5.3 (1 H, s), 5.7 (1 H, d), 5.9 (1 H, m), 7.3 (5 H, m, ArH); δ_C 27.6, 36.3, 53.4, 54.8, 61.7, 67.3, 118.0, 128.3, 128.5, 128.6, 131.9, 135.2, 155.8, 172.1, 194.0.

6-Diazo-N-*(9-fluorenylmethyloxycarbonyl)-5-oxo-L-norleucine ethyl ester* **46g**

Compound **46g** was prepared using *procedure 6*, from *N*-FMOC ethyl pyroglutamate **45i** (1.52 g, 4 mmol), TMSD (2.4 ml, 4.8 mmol) and *n*-BuLi (3 ml, 4.8 mmol). After purification by flash column chromatography (7:3 light petroleum/ethyl acetate) the desired diazoketone **46g** was obtained as a yellow solid (1.28 g, 75%) m.p. 121-122°C. (Found: C, 66.31; H, 5.55; N, 9.86. $C_{23}H_{23}N_3O_5$ requires C, 66.55; H, 5.50; N, 9.97%.); ν_{max} (KBr)/cm^{-1} 3356, 2100, 1740, 1686, 1636; δ_H 1.3 (3 H, t), 2.0, 2.2, 2.4 (4 H, m), 4.3 (3 H, m), 4.4 (3 H, m), 5.3 (1 H, s), 5.8 (1 H, d), 7.5 (4 H, m, ArH), 7.6 (2 H, m, ArH), 7.8 (2 H, m, ArH); δ_C 14.1, 27.5, 36.4, 47.1, 53.5, 54.8, 61.7, 67.0, 120.0, 125.1, 127.1, 127.7, 141.3, 143.8, 156.1, 171.9, 193.0.

6-Diazo-N-*(9-fluorenylmethyloxycarbonyl)-5-oxo-L-norleucine benzyl ester* **46h**

Compound **46h** was prepared as described for DON derivative **46g** using *N*-FMOC benzyl pyroglutamate **45j** (1.77 g, 4 mmol), TMSD (2.4 ml, 4.8 mmol) *n*-BuLi (3 ml, 4.8 mmol). After purification by flash column chromatography (7:3 light petroleum/ethyl acetate), the desired norleucinate derivative **46h** was isolated as a yellow solid (1.18 g, 61%) m.p. 82-84°C. (Found: C, 69.69; H, 5.36; N, 8.41. $C_{28}H_{25}N_3O_5$ requires C, 69.55; H, 5.21; N, 8.69%.); ν_{max} (KBr)/cm^{-1} 3354, 2103, 1741, 1700, 1636; δ_H 2.0, 2.2, 2.4 (4 H, m), 4.1 (1 H, m), 4.2 (1 H, t), 4.4 (2 H, d), 5.1 (2 H, s), 5.2 (1 H, s), 5.6 (1 H, d), 7.2 (9 H, m, ArH), 7.6 (2 H, m, ArH), 7.8 (2 H, m, ArH); δ_C 27.3, 36.3, 47.1, 53.6, 54.8, 60.4, 67.3, 120.0, 125.1, 127.1, 127.7, 128.4, 128.5, 128.6, 135.2, 141.3, 143.7, 156.1, 171.8, 193.5.

6-Diazo-N-*(9-fluorenylmethyloxycarbonyl)-5-oxo-L-norleucine* tert-*butyl ester* **46i**

Compound **46i** was prepared using the above procedure from *N*-FMOC *t*-butyl pyroglutamate **45k** (2 g, 4.9 mmol), TMSD (2.95 ml, 5.9 mmol) and *n*-BuLi (3.7 ml, 5.9 mmol). Flash column chromatography (7:3 light petroleum/ethyl acetate) yielded the desired product **46i** as a yellow solid (1.61 g, 73%) m.p. 83-84°C. (Found: C, 66.98; H, 6.06; N, 9.05. $C_{25}H_{27}N_3O_5$ requires C, 66.80; H, 6.05; N, 9.35%.); ν_{max} (KBr)/cm^{-1} 3354, 2104, 1740, 1602, 1640; δ_H 1.4 (9 H, s), 2.0, 2.2, 2.4 (4 H, m), 4.2 (1 H, m), 4.3 (1 H, t), 4.4 (2 H, d), 5.2 (1 H, s), 5.6 (1 H, d), 7.3 (4 H, m, ArH), 7.6 (2 H, m, ArH), 7.8 (2 H, m, ArH); δ_C 27.4, 28.0, 36.4, 47.2, 53.9, 54.7, 67.0, 82.5, 120.0, 125.1, 127.1, 127.7, 141.3, 143.7, 156.1, 171.0, 193.7.

N-*Phenylacetyl-2-pyrrolidinone-5-carboxylic acid* tert-*butyl ester* **48**

Compound **48** was prepared by *procedure 5*, using *t*-butyl pyroglutamate **44** (1.2 g, 6.5 mmol), lithium *bis*(trimethylsilyl)amide (3.57 ml, 7.15 mmol) and phenyl acetyl chloride (0.95 ml, 7.15 mmol). After purification by flash column chromatography (3:2 light petroleum/ethyl acetate) the desired pyroglutamate **48** was obtained as a colourless oil (1.35 g, 69%). ν_{max} (film)/cm^{-1} 1740, 1736, 1700; δ_H 1.4 (9 H, s), 2.0, 2.2 and 2.5-2.8 (4 H, m), 4.3 (2 H, q), 4.6 (1 H, dd), 7.3 (5 H, m, ArH); δ_C 21.2, 27.8, 32.0, 42.5, 58.9, 82.3, 126.9, 128.3, 129.7, 133.8, 170.1, 171.7, 174.5.

N-tert-*butyldimethylsilyl-2-pyrrolidinone-5-carboxylic acid benzyl ester* **49**

Compound **49** was prepared by *procedure 4* from benzyl pyroglutamate **42** (1.1 g, 5 mmol), *t*-butyldimethylchlorosilane (1.5 g, 10 mmol), triethylamine (1.4 ml, 10 mmol) and DMAP (0.6 g, 5 mmol). After purification by flash column chromatography (3:2

light petroleum/ethyl acetate) the desired *N*-silyl pyroglutamate **49** was obtained as a pale yellow oil (1.2 g, 72%). ν_{max} (film)/cm^{-1} 1744, 1696; δ_H -0.33 (3 H, s), -0.31 (3 H, s), 0.6 (9 H, s), 1.9-, 2.2 (4 H, m), 3.9 (1 H, dd), 4.8 (2 H, s), 7.0 (5 H, m, ArH); δ_C -3.6, 19.2, 26.7, 26.8, 30.8, 60.7, 67.1, 128.5, 128.6, 135.1, 173.5, 174.3.

6-Diazo-5-oxo-L-norleucine (DON) **22**

A suspension of powdered norleucine derivative **46g** (0.73 g, 1.73 mmol) in piperidine (20 ml) was stirred vigorously for 2 min. and the resulting solution was added in one portion to ice/water (80 ml). The mixture was stirred for 10 min., filtered and the solvent removed under high vacuum at room temperature to give a pale brown solid. The crude residue was dissolved in the minimum amount of warm water to achieve solution and crystallised by addition of dry methanol to give DON **22** as pale yellow crystals (0.24 g, 81%) m.p. 144-146°C dec. (Lit.[103] m.p. 146°C dec.); $[\alpha]^{22}_D$ +18.25° *c*, 0.8 in H_2O; (Lit.[103] $[\alpha]^{34}_D$ +19.5° *c*, 2.05 in H_2O). ν_{max} (KBr)/cm^{-1} 3434, 2111, 1628, 1584; δ_H (D_2O) 2.1, (2 H, q), 2.6 (2 H, t), 3.8 (1 H, t), 5.9 (1 H, s); δ_C (D_2O) 26.8, 36.3, 55.0, 58.9, 175.0, 199.0.

N-*Acetyl-L-phenylalanyl-6-chloro-5-oxo-L-norleucine* **53**

To an ice-cold solution of the *N*-hydroxysuccinimide ester of *N*-acetylphenylalanine (91 mg, 0.3 mmol) and DON **22** (50 mg, 0.3 mmol) in a 1:1 mixture of THF/water (6 ml) was added triethylamine (0.064 ml, 0.45 mmol). The reaction mixture was stirred for 2 h. and the solvent removed under high vacuum at room temperature. The residue was dissolved in ethyl acetate (6 ml) and treated with a saturated solution of HCl in ether (4 ml). After stirring for 15 min. the organic layer was washed with water (3 x 5

ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a solid residue which was recrystallised from ethanol/ether to give the desired chloromethyl ketone **53** as colourless crystals (67 mg, 61%) m.p. 155-156°C. (Found: C, 54.93; H, 5.87; N, 7.53. $C_{17}H_{21}ClN_2O_5$ requires C, 55.36; H, 5.74; N, 7.60%.); ν_{max} (KBr)/cm^{-1} 1720, 1658, 1655; δ_H (d_6 acetone) 1.8 (3 H, s), 2.0, 2.2 and 2.6 (4 H, m), 2.9-3.2 (2 H, m), 4.3 (2 H, s), 4.4 (1 H, m), 4.6 (1 H, m), 7.2 (5 H, m, ArH); δ_C (d_6 acetone) 22.9, 27.0, 36.1, 38.6, 49.8, 51.7, 57.2, 127.3, 129.1, 130.2, 136.6, 168.6, 169.8, 173.4, 201.6.

N-*Benzyloxycarbonyl-L-phenylalanyl-6-chloro-5-oxo-L-norleucine methyl ester* **54**

A solution of the acid **17** (115 mg, 0.25 mmol) in THF (1 ml) was treated with methanol (0.5 ml) and TMSD (0.5 ml, 1 mmol) and the reaction mixture was stirred for 3 h. Removal of the solvent gave an oily residue which was purified by flash column chromatography (1:1 light petroleum/ethyl acetate) to afford the methyl ester **54** as a colourless solid (70 mg, 59%) m.p. 131-134°C. (Found: C, 61.01; H, 5.83; N, 5.97. $C_{24}H_{27}ClN_2O_3$ requires C, 60.70; H, 5.73; N, 5.90%.); ν_{max} (KBr)/cm^{-1} 3296, 1732, 1688, 1655; δ_H 1.9, 2.2 and 2.6 (4 H, m), 3.1 (2 H, m), 3.7 (2 H, s), 4.0 (2 H, s), 4.4 (1 H, m), 4.5 (1 H, m), 5.1 (2 H, s), 5.3 (1 H, d), 6.4 (1 H, d), 7.3 (10 H, m, ArH); δ_C 26.2, 35.2, 38.1, 48.1, 51.3, 52.6, 56.3, 67.1, 127.1, 128.0, 128.3, 128.6, 128.8, 128.9, 136.0, 155.9, 171.0, 171.6, 201.6.

N-*Benzyloxycarbonyl-L-phenylalanyl-6-bromo-5-oxo-L-norleucine* **55**

A cold (0°C) solution of Z-phenylalanine active ester **9** (238 mg, 0.6 mmol) and DON **22** (103 mg, 0.6 mmol) in a 1:1 mixture of THF/water was treated with triethylamine (0.127 ml, 0.9 mmol). The reaction was stirred at 0°C for 2.5 h. and the solvent

removed under high vacuum at room temperature. The residue was redissolved in ethyl acetate (20 ml), then a 1:1 mixture of HBr and acetic acid (1.5 ml) was added and the reaction mixture was stirred for 15 min. Subsequently, ethyl acetate (20 ml) was added and the organic layer was washed with water (3 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave the crude residue which was recrystallised from ethyl acetate to give the dipeptide **55** as colourless crystals (195 mg, 64%) m.p. 132-133°C. (Found: C, 54.42; H, 5.14; N, 5.44. $C_{23}H_{25}BrN_2O_6$ requires C, 54.66; H, 4.99; N, 5.54%.); ν_{max} (KBr)/cm^{-1} 3294, 1719, 1689, 1655; δ_H (d_6 acetone) 1.9, 2.2 and 2.7 (4 H, m), 2.9-3.2 (2 H, m), 4.2 (2 H, s), 4.5 (2 H, m), 5.0 (2 H, s), 6.6 (1 H, d), 7.3 (10 H, m, ArH) 7.6 (1 H, d); δ_C (d_6 acetone) 27.0, 36.0, 36.3, 38.6, 51.8, 57.2, 66.7, 127.3, 128.5, 128.6, 129.1, 129.2, 130.2, 137.9, 138.6, 153.5, 172.4, 173.7, 201.0.

N-*Benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine, bromide salt* **56**

Compound **56** was prepared using *procedure 3* from bromomethyl ketone **55** (50 mg, 0.1 mmol) and dimethylsulfide (0.032 ml, 0.44 mmol) to give the desired bromide salt **56** as a colourless hygroscopic solid (35 mg, 62%) m.p. 90-92°C. (Found: C, 52.89; H, 4.89; N, 5.05. $C_{25}H_{31}BrN_2O_6S$ requires C, 52.91; H, 5.51; N, 4.94%.); ν_{max} (KBr)/cm^{-1} 3296, 1715, 1700, 1661; δ_H (d_6 acetone) 1.9, 2.2 and 2.7 (4 H, m), 2.9-3.1 (2 H, m), 3.2 (6 H, s), 4.6 (2 H, m), 5.0 (2 H, s), 5.1 (2 H, s), 5.4 (1 H, d), 7.3 (10 H, m, ArH), 7.4 (1 H, d); δ_C (d_6 acetone) 25.2, 36.1, 38.5, 41.8, 51.9, 56.3, 60.6, 66.3, 128.4, 128.6, 129.0, 129.2, 130.3, 138.2, 138.6, 153.3, 168.7, 173.2, 202.0.

N-*9-Fluorenylmethyloxycarbonyl-2-oxo-pyrrolidine* **58**

Compound **58** was prepared by *procedure 5*, using pyrrolidin-2-one (0.31 ml, 4 mmol), lithium *bis*(trimethylsilyl)amide (4 ml, 4 mmol) and 9-fluorenylmethyl chloroformate (1.05 g, 4 mmol). The desired pyrrolidine **58** was isolated as a colourless solid (1.07 g, 87%) m.p. 134-135°C. (Found: C, 74.18; H, 5.45; N, 4.69. $C_{19}H_{17}NO_3$ requires C, 74.25; H, 5.57; N, 4.56%.); ν_{max} (KBr)/cm^{-1} 1770, 1690; δ_H 2.0 (2 H, quint.), 2.6 (2 H, t), 3.8 (2 H, t), 4.3 (1 H, t), 4.5 (2 H, d), 7.3 (4 H, m, ArH), 7.7 (4 H, m, ArH); δ_C 17.5, 32.9, 46.4, 46.6, 68.5, 120.0, 125.3, 127.2, 127.9, 141.2, 143.5, 151.7, 173.9.

1-Diazo-5-N-*(9-fluorenylmethyloxycarbonylamino)-2-oxopentane* **59**

Compound **59** was prepared using *procedure 6*, from *N*-FMOC pyrrolidin-2-one **58** (0.62 g, 2 mmol), TMSD (5 ml, 2.4 mmol) and *n*-BuLi (1.5 ml, 2.4 mmol). The desired diazoketone **59** was obtained as a yellow solid (0.48 g, 69%) m.p. 109-110°C. (Found: C, 68.39; H, 5.60; N, 11.69. $C_{20}H_{19}N_3O_3$ requires C, 68.75; H, 5.48; N, 12.03%.); ν_{max} (KBr)/cm^{-1} 3357, 1698, 1634; δ_H 1.8 (2 H, quint.), 2.4 (2 H, t), 3.2 (2 H, q), 4.2 (1 H, t), 4.4 (2 H, d), 5.0 (1 H, br), 5.3 (1 H, s), 7.3 (4 H, m, ArH), 7.6 (2 H, m, ArH), 7.8 (2 H, m, ArH); δ_C 25.1, 37.8, 40.4, 47.3, 54.7, 66.6, 120.0, 125.0, 127.0, 127.7, 141.3, 143.9, 156.5, 194.0.

N-*Benzyloxycarbonyl-L-phenylalanyl-5-amino-1-diazo-2-oxopentane* **19**

Piperidine (2.5 ml) was added to powdered diazoketone **59** (0.35 g, 1 mmol) and the mixture was stirred vigorously for 2 min. The reaction was poured into ice water (5 ml) filtered and the solvent removed under high vacuum at room temperature to give an oily residue which was dissolved in THF (5 ml), cooled in an ice bath and treated

with a solution of the Z-phenylalanine active ester **9** (0.39 g, 0.98 mmol) in THF (2.5 ml). The resulting solution was stirred for 4 h. at room temperature and the solvent removed to give a residue which was purified by flash column chromatography (49:1 ethyl acetate/DCM) to give the diazoketone **19** as a pale yellow solid (118 mg, 29%) m.p. 114-115°C with spectroscopic data identical to a sample previously prepared by a literature procedure.

N-tert-*Butyloxycarbonyl-2-pyrrolidinone* **60**

Compound **60** was prepared using *procedure 4b*, from pyrrolidin-2-one (1.7 g, 20 mmol), di-*t*-butyldicarbonate (4.8 g, 22 mmol) and DMAP (0.12 g, 1 mmol). After purification by flash column chromatography (1:1 light petroleum/ethyl acetate) the pyrrolidinone **60** was obtained as a pale yellow oil (3.66 g, 99%). ν_{max} (film)/cm^{-1} 1785, 1753, 1715; δ_H 1.5 (9 H, s), 2.0 (2 H, quint.), 2.5 (2 H, t), 3.8 (2 H, t); δ_C 17.4, 28.0, 32.9, 46.5, 82.5, 150.1, 174.4.

N-tert-*Butyloxycarbonyl-2-piperidinone* **61**

Compound **61** was prepared using a similar procedure to that described above from δ-valerolactam (2.48 g 25 mmol), di-*t*-butyldicarbonate (6 g, 27.5 mmol) and DMAP (0.15 g, 1.25 mmol). After purification by flash column chromatography (3:2 light petroleum/ethyl acetate) the desired piperidinone **61** was obtained as a pale yellow oil (4.48 g, 90%). ν_{max} (film)/cm^{-1} 1768, 1711; δ_H 1.5 (9 H, s), 1.8 (4 H, 2 x quint.), 2.5 (2 H, t), 3.6 (2 H, t); δ_C 20.5, 22.8, 28.0, 34.9, 46.3, 82.7, 152.6, 171.3.

5-N-tert-*Butyloxycarbonylamino-1-diazo-2-oxopentane* **62**

Compound **62** was prepared by *procedure 6* using *N*-BOC pyrrolidin-2-one **60** (1.02 g, 5.5 mmol), TMSD (3 ml, 6 mmol) and *n*-BuLi (3.8 ml, 6.1 ml). The diazoketone **62** was obtained, after purification by flash column chromatography (3:2 light petroleum/ethyl acetate), as a yellow oil (0.84 g, 67%). ν_{max} (film)/cm^{-1} 3357, 2103, 1704, 1639; δ_H 1.4 (9 H, s), 1.8 (2 H, quint.), 2.2 (2 H, t), 3.2 (2 H, q), 4.8 (1 H, s), 5.4 (1 H, s); δ_C 25.4, 28.4, 38.0, 40.0, 54.6, 79.1, 156.1, 194.6.

6-N-tert-*Butyloxycarbonylamino-1-diazo-2-oxohexane* **63**

Compound **63** was prepared using *procedure 6* from lactam **61** (1.09 g, 5.5 mmol), TMSD (3 ml, 6 mmol) and *n*-BuLi (3.8 ml, 6.1 ml). After purification by flash column chromatography (3:2 light petroleum/ethyl acetate) the diazoketone **63** as obtained as a yellow solid (0.97 g, 73%) m.p. 44-45.5°C. (Found: C, 54.66; H, 8.14; N, 16.98. $C_{11}H_{19}N_3O_3$ requires C, 54.76; H, 7.94; N, 17.41%.); ν_{max} (KBr)/cm^{-1} 3357, 2104, 1698, 1634; δ_H1.4 (9 H, s), 1.5 (2 H, quint.), 1.6 (2 H, quint.), 2.4 (2 H, t), 3.1 (2 H, q), 4.6 (1 H, s), 5.3 (1 H, s); δ_C 22.1, 28.4, 29.5, 40.0, 40.2, 54.5, 79.1, 156.0, 194.8.

5-N-tert-*Butyloxycarbonylamino-1-chloro-2-oxopentane* **64**

To a cold (0°C) solution of diazoketone **62** (0.57 g, 2.5 mmol) in ethyl acetate (2.5 ml) was added a 2.9M solution of HCl in ethyl acetate (2.6 ml, 7.5 mmol) dropwise. The reaction was stirred for 15 min., washed with saturated sodium hydrogen carbonate and dried ($MgSO_4$). The solvent was removed *in vacuo* to give an oily residue which was purified by flash column chromatography (3:2 light petroleum/ethyl acetate) to give the chloromethyl ketone **64** as a colourless solid (0.20 g, 34%) m.p. 67-68°C

(cyclohexane). (Found: C, 51.05; H, 7.82; N, 5.75. $C_{10}H_{18}ClNO_3$ requires C, 50.96; H, 7.70; N, 5.94%.); ν_{max} (KBr)/cm^{-1} 3365, 1715, 1700; δ_H 1.4 (9 H, s), 1.8 (2 H, quint.), 2.6 (2 H, t), 3.2 (2 H, q), 4.1 (2 H, s), 4.7 (1 H, s); δ_C 24.1, 28.4, 36.7, 39.6, 48.3, 79.3, 156.2, 202.3.

6-N-tert-*Butyloxycarbonylamino-1-chloro-2-oxohexane* **65**

The chloromethyl ketone **65** was prepared in the above manner from diazoketone **63** (0.60 g, 2.5 mmol), 2.9M solution of HCl in ethyl acetate (2.6 ml, 7.5 mmol) and ethyl acetate (2.5 ml). After purification by flash column chromatography (3:2 light petroleum/ethyl acetate) the desired ketone **65** was obtained as a colourless solid (0.36 g, 58%) m.p. 71-72°C (cyclohexane). (Found: C, 53.07; H, 8.21; N, 5.45. $C_{11}H_{20}ClNO_3$ requires C, 52.90; H, 8.07; N, 5.61%.); ν_{max} (KBr)/cm^{-1} 3359, 1717, 1702; δ_H 1.4 (9 H, s), 1.5 (2 H, quint.), 1.7 (2 H, quint.), 2.6 (2 H, t), 3.1 (2 H, q), 4.1 (2 H, s), 4.5 (1 H, s); δ_C 20.5, 28.2, 28.4, 29.4, 39.1, 48.2, 79.2, 156.0, 202.4.

*Attempted preparation of 1-benzyl 7-ethyl (2*R*)-2-*N-tert-*butyloxycarbonylamino-6-chloro-5-oxo-L-heptanedioate* **72a**

An attempted synthesis of compound **72a** was undertaken using *procedure 7* from benzyl *N*-BOC pyroglutamate **34** (1.28 g, 4 mmol), ethyl chloroacetate (0.43 ml, 4 mmol), diisopropylamine (0.68 ml, 4.8 mmol) and *n*-BuLi (3.1 ml, 4.8 mmol). Removal of the solvent *in vacuo* gave an oily residue which did not yield any of the desired product after attempted purification by flash column chromatography.

*Attempted preparation of 1-benzyl 7-*tert*-butyl (2R)-2-*N*-tert-butyloxycarbonylamino-6-chloro-5-oxo-L-heptanedioate* **72b**

In a similar manner preparation of **72b** was attempted using *procedure 7* from benzyl *N*-BOC pyroglutamate **34** (1.28 g, 4 mmol), *t*-butyl chloroacetate (0.57 ml, 4 mmol), diisopropylamine (0.68 ml, 4.8 mmol) and *n*-BuLi (3.1 ml, 4.8 mmol) with a similar outcome.

*1-Benzyl 7-ethyl (2R)-2-*N*-tert-butyloxycarbonylamino-5-oxo-L-heptanedioate* **75a**

Compound **75a** was prepared as described above from *N*-BOC pyroglutamate **34** (1.28 g, 4 mmol), ethyl acetate (0.39 ml, 4 mmol), diisopropylamine (0.68 ml, 4.8 mmol) and *n*-BuLi (3.1 ml, 4.8 mmol) and was obtained as a pale green oil (0.98 g, 60%). ν_{max} (film)/cm^{-1} 3372, 1738, 1715, 1700; δ_H 1.3 (3 H, t), 1.4 (9 H, s), 1.9, 2.1 and 2.6 (4 H, m), 3.4 (2 H, s), 4.1 (2 H, q), 4.3 (1 H, m), 5.1 (2 H, s), 5.2 (1 H, m), 7.4 (5 H, m, ArH); δ_C 14.1, 26.3, 28.3, 38.7, 49.2, 52.8, 61.4, 67.2, 80.0, 128.3, 128.5, 128.6, 135.3, 155.5, 167.0, 172.1, 201.7.

*1-Benzyl 7-*tert*-butyl (2R)-2-*N*-tert-butyloxycarbonylamino-5-oxo-L-heptanedioate* **75b**

Compound **75b** was prepared as described above from benzyl *N*-BOC pyroglutamate (1.28 g, 4 mmol), *t*-butyl acetate (0.54 ml, 4 mmol), diisopropylamine (0.68 ml, 4.8 mmol) and *n*-BuLi (3.1 ml, 4.8 mmol) and was obtained as a pale brown oil (1.07 g, 75%). ν_{max} (film)/cm^{-1} 3368, 1740, 1712, 1700; δ_H 1.4 (9 H, s), 1.5 (9 H, s), 1.9, 2.2 and 2.6 (4H, m), 3.4 (2 H, s), 4.3 (1 H, m), 5.1 (2 H, s), 5.4 (1 H, m), 7.3 (5 H, m, ArH); δ_C 26.3, 28.0, 28.3, 38.7, 49.2, 52.8, 67.2, 80.0, 82.1, 128.3, 128.5, 128.7, 135.5, 155.6, 167.2, 172.3, 201.4.

5-N-tert-*Butyloxycarbonylamino-1-chloro-2-oxopentane* **64**

To a cold (-78°C) solution of chloromethyltrimethylsilane (0.83 ml, 5.94 mmol) in THF (12 ml) in an atmosphere of nitrogen was added *s*-BuLi (1.3M, 4.8 ml, 6.24 mmol), dropwise, followed after 5 min. by *N,N,N',N'*-tetramethylethylenediamine (0.94 ml, 6.24 mmol). The mixture was stirred at -78°C for 0.5 h. and a solution of *N*-BOC 2-pyrrolidone **60** (1 g, 5.4 mmol) in THF (5.4 ml) was added dropwise. The reaction was stirred for 0.5 h., quenched by the addition of saturated ammonium chloride solution (20 ml), then the layers were separated and the aqueous layer extracted with chloroform (2 x 10 ml) then ether (25 ml). The combined organic layer was washed with brine, dried ($MgSO_4$) and the solvent removed *in vacuo* to give an oily residue which was purified by flash column chromatography (3:2 light petroleum/ethyl acetate) to give the chloromethyl ketone **64** (0.39 g, 31%) m.p. 67-68°C (cyclohexane). The spectroscopic data were identical to those of a sample prepared from diazoketone **62**.

6-N-tert-*Butyloxycarbonylamino-1-chloro-2-oxo-hexane* **65**

Using the above procedure chloromethyl ketone **65** was obtained from *N*-BOC 2-piperidone **61** (1 g, 5 mmol), chloromethyltrimethylsilane (0.84 ml, 6 mmol), *s*-BuLi (5 ml, 6.5 mmol) and *N,N,N',N'*-tetramethylethylenediamine (0.98 ml, 6.5 mmol) in THF (35 ml) and obtained as a colourless solid (0.41 g, 33%) m.p. 71-72°C (cyclohexane). The spectroscopic data were identical to those of a sample prepared from diazoketone **63**.

Attempted preparation of N-*benzyloxycarbonyl-L-phenylalanyl-5-amino-1-chloro-2-oxo-pentane* **77**

N-BOC protected chloromethyl ketone **64** (0.19 g, 0.8 mmol) was dissolved in ether (4 ml) and 4-toluenesulfonic acid (0.15 g, 0.8 mmol) added. The mixture was stirred vigorously until solution was complete and the flask was stoppered and left to stand overnight. The resulting precipitate was filtered, washed with ether (5 ml), cold ethyl acetate (5 ml) and then dried in a desiccator under vacuum to give the crude 4-toluenesulfonic acid salt **76a** as colourless crystals (0.2 g) m.p. 132-135°C. ν_{max} (KBr)/cm^{-1} 3424, 3236, 2700, 1727, 1224.

To an ice-cold solution of the 4-toluenesulfonic acid salt **76a** (0.2 g, 0.65 mmol) and phenylalanine derivative **9** (0.25 g, 0.65 mmol) in THF/water (1:1, 6.5 ml) was added triethylamine (0.1 ml, 0.71 mmol). The reaction was stirred for 3 h. and the solvent removed to afford a brown oil from which none of the required product could be isolated.

N-*Benzyloxycarbonyl-L-phenylalanyl-6-amino-1-chloro-2-oxo-hexane* **78**

Using the above method crude 4-toluenesulfonic acid salt **76b** was obtained from *N*-BOC chloromethyl ketone **65** (0.3 g, 1.2 mmol) and 4-toluenesulfonic acid (0.225 g, 1.2 mmol) in ether (5 ml) as colourless crystals (0.27 g) 126-129°C. ν_{max} (KBr)/cm^{-1} 3424, 2759, 1730, 1220.

To a cold (0°C) solution of **76b** (0.2 g, 0.62 mmol) and phenylalanine active ester **9** (0.24 g, 0.62 mmol) in DCM (6 ml) was added triethylamine (0.1 ml, 0.71 mmol). The reaction was stirred for 5 h., diluted with ethyl acetate (15 ml), washed with 1M HCl (3 x 5 ml), saturated sodium hydrogen carbonate solution (3 x 5 ml), brine and dried

($MgSO_4$). Removal of the solvent *in vacuo* gave a brown oil which was triturated with light petroleum/ethyl acetate (1:1) to give the desired chloromethyl ketone **78** as a colourless solid (30 mg, 11%) m.p. 133-135°C. (Found: C, 63.89; H, 6.26; N, 6.05. $C_{23}H_{27}ClN_2O_4$ requires C, 64.11; H, 6.31; N, 6.50%.); ν_{max} (KBr)/cm^{-1} 3294, 1715, 1702, 1656; δ_H 1.5 (2 H, m), 1.6 (2 H, m), 2.6 (2H, t), 3.0 (2 H, q), 3.2 (2 H, m), 4.0 (2 H, s), 4.4 (1 H, m), 5.1 (2 H, s), 5.3 (1 H, d), 5.8 (1 H, t), 7.2 (5 H, m, ArH); δ_C 20.3, 25.6, 28.5, 38.7, 48.1, 49.2, 57.4, 67.1, 127.1, 128.1, 128.3, 128.6, 128.7, 129.3, 136.4, 136.5, 156.0, 170.8, 202.8.

N-tert-*Butyloxycarbonyl-4-aminobutyric acid* **85**

To a rapidly stirred suspension of 4-aminobutyric acid (3 g, 29 mmol) in methanol (39 ml) was added triethylamine (4.35 ml, 31.2 mmol) followed by di-*t*-butyl dicarbonate (12.65 g, 58 mmol). The resulting solution was stirred for a further 5 h. and left to stand overnight. The solvent was removed under vacuum and the residue redissolved in ethyl acetate (30 ml) and the organic layer was washed with 2M HCl (3 x 10 ml) and 10% sodium carbonate solution (3 x 20 ml). The basic extract was acidified, by careful addition of 4M HCl, to pH 2 and extracted with ethyl acetate (3 x 20 ml). The combined organic extract was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a colourless oil which crystallised on standing to give the *N*-protected aminobutyric acid **85** as a colourless solid (5.17 g, 88%) m.p. 61-62°C (ether/light petroleum) (Lit.[121] no m.p. given). ν_{max} (KBr)/cm^{-1} 3356, 1715, 1689, 1522; δ_H 1.4 (9 H, s), 1.8 (2 H, quint.), 2.4 (2 H, t), 3.2 (2 H, q), 4.8 (1 H, br), 11.3 (1 H, br); δ_C 25.1, 28.3, 31.3, 39.8, 79.5, 156.2, 178.4.

*4-(*N-tert-*Butyloxycarbonyl)amino*-N',O-*dimethylbutylamide* **87**

To an ice-cold solution of the aminobutyric acid **85** (0.78 g, 3.8 mmol) in DCM (20 ml) was added *N,O*-dimethylamine hydrochloride (0.37 g, 3.8 mmol) and triethylamine (0.59 ml, 4.18 mmol). The mixture was stirred for 5 min. and then dicyclohexylamine (0.83 g, 3.99 mmol) was added. The reaction mixture was allowed to warm to room temperature, stirred for a further 5 h., and then filtered, washed with 1M HCl (3 x 10 ml), saturated sodium hydrogen carbonate solution (3 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* yielded the desired amide **87** as a colourless oil (0.26 g, 28%). ν_{max} (film)/cm^{-1} 3354, 1700, 1648, 1512; δ_H 1.4 (9 H, s), 1.8 (2 H, quint.), 2.5 (2 H, t), 3.1 (2 H, q), 3.2 (3 H, s), 3.7 (3 H, s), 4.1 (1 H, br); δ_C 24.8, 28.4, 29.2, 32.2, 40.2, 61.2, 78.9, 156.1, 174.1.

4-N-tert-*Butyloxycarbonylamino-n-butan-1-ol* **88**

A cold (-12°C) solution of acid **85** (2 g, 10 mmol) in THF (14 ml) was treated with triethylamine (1.67 ml, 12 mmol) followed by a solution of *n*-butyl chloroformate (1.46 ml, 11 mmol) in THF (7 ml). The reaction was warmed to 0°C, stirred for a further 10 min., filtered and then added, dropwise, to a solution of sodium borohydride (0.76 g, 20 mmol) in water (8 ml). The mixture was stirred at 0°C for a further 4 h. and then quenched by the slow addition of 2M HCl until gas evolution ceased. The THF was removed *in vacuo* and the residue extracted with ethyl acetate. The combined ethyl acetate extract was washed with saturated sodium hydrogen carbonate, brine and dried ($MgSO_4$). Removal of the solvent gave a colourless oil which was purified by flash column chromatography (3:2 light petroleum/ethyl acetate) to yield the desired alcohol

88 as a colourless oil (0.75 g, 40%). ν_{max} (film)/cm^{-1} 3430, 1682; δ_H 1.4 (9 H, s), 1.6 (4 H, m), 3.1 (2 H, q), 3.6 (2 H, t), 3.7 (1 H, br), 5.1 (1 H, t); δ_C 26.5, 28.4, 29.6, 40.3, 61.9, 79.1, 156.6.

*Attempted preparation of 5-*N*-tert-butyloxycarbonylamino-pent-1-ene-1-phenyl sulfone* **90**

To a solution of oxalyl chloride (0.47 ml, 5.23 mmol) in DCM (4.8 ml), cooled to -63°C in an atmosphere of nitrogen, was added a solution of dimethylsulfoxide (0.89 ml, 10.46 mmol) in DCM (1.5 ml) dropwise over 10 min. A solution of the alcohol **88** (0.6 g, 3.17 mmol) in DCM (3 ml) was added over 10 min. and the reaction stirred at -63°C for a further 10 min. A solution of triethylamine (2.1 ml, 20.92 mmol) in DCM (8 ml) was added over 15 min., the reaction was stirred for a further 15 min. and then quenched by addition of water (0.4 ml). To the resulting slurry was added ether (30 ml) and the mixture was washed with 20% potassium hydrogen sulfate solution (2 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a pale yellow oil (0.56 g) which was used unpurified in the next stage of the reaction sequence.

To a cold (0°C) solution of methyl phenyl sulfone (0.5 g, 3.17 mmol) in THF (16 ml) in an inert atmosphere of nitrogen was added *n*-BuLi (4.36 ml, 6.97 mmol) dropwise. The resulting yellow suspension was stirred at 0°C for a further 0.5 h. and then a solution of diethyl chlorophosphate (0.55 ml, 3.17 mmol) in THF (6 ml) added dropwise. The yellow solution was stirred for 0.5 h. at -78°C and then a solution of the crude *N*-BOC-4-aminobutanal in THF (5 ml) was added. The reaction was stirred for a further 1 h. at -78°C then quenched with saturated ammonium chloride solution.

The layers were separated, the aqueous layer was extracted with ether (3 x 25 ml) and the combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent afforded a yellow oil from which none of the desired product could be isolated.

5-N-tert-*Butyloxycarbonylamino-2-oxopentanyl phenyl sulfone* **92**

An ice-cold solution of methyl phenyl sulfone (0.78 g, 5 mmol) in THF (40 ml) in an atmosphere of nitrogen was treated with *n*-BuLi (3.12 ml, 5 mmol) and the mixture stirred at 0°C for 0.5 h. The reaction was cooled to -78°C and a solution of the pyrrolidinone **60** (0.92 g, 5 mmol) in THF (10 ml) added dropwise. Stirring was continued for 0.5 h. at -78°C and then the reaction was quenched with saturated ammonium chloride solution. The layers were separated, the aqueous layer was extracted with chloroform (3 x 15 ml), then ether (20 ml) and the combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a pale yellow oil which was purified by flash column chromatography (3:2 light petroleum/ethyl acetate) to afford the phenyl sulfone **92** as a colourless oil which solidified on standing (1.25 g, 73%) m.p. 74-76°C. (Found: C, 55.85; H, 6.46; N, 4.01. $C_{16}H_{23}NO_5S$ requires C, 56.29; H, 6.79; N, 4.10%.); ν_{max} (KBr)/cm^{-1} 3350, 1715, 1678; δ_H 1.4 (9 H, s), 1.8 (2 H, quint.), 2.8 (2 H, t), 3.1 (2 H, q), 4.2 (2 H, s), 4.7 (1 H, br), 7.6 (2 H, m, ArH), 7.7 (1 H, m, ArH), 7.9 (2 H, m, ArH); δ_C 23.7, 28.4, 39.3, 41.4, 66.9, 79.3, 128.2, 129.4, 134.3, 138.7, 156.1, 197.7.

N-tert-*Butyloxycarbonyl-5-amino-2-hydroxypentyl phenyl sulfone* **93**

To a cold (-78°C) solution of keto-sulfone **92** (1.02 g, 3 mmol) in THF (30 ml) under a blanket of nitrogen was added, dropwise, a 1.5M solution of diisobutylaluminium-hydride in toluene (4 ml, 6 mmol). The reaction was stirred at -78°C for 1 h. then quenched with saturated ammonium chloride solution. The layers were separated, the aqueous layer was extracted with chloroform (3 x 10 ml), then ether (20 ml) and the combined organic layer was washed with brine and dried ($MgSO_4$). The solvent was removed under high vacuum and the residue purified by flash column chromatography (1:1 light petroleum/ethyl acetate) to yield the desired sulfone **93** as a colourless oil (0.85 g, 83%). ν_{max} (film)/cm^{-1} 3380, 1690; δ_H 1.4 (9 H, s), 1.5 (4 H, m), 3.2 (2 H, m), 3.6 (1 H, br), 4.7 (1 H, br), 7.6 (2 H, m, ArH), 7.7 (1 H, m, ArH), 7.9 (2 H, m, ArH); δ_C 25.8, 28.4, 33.5, 40.1, 62.3, 65.7, 79.2, 127.9, 129.5, 134.1, 139.2, 156.1.

N-tert-*Butyloxycarbonyl-5-amino-1-pentenyl phenyl sulfone* **90**

To a cold (0°C), stirred solution of the hydroxy sulfone **93** (0.6 g, 1.75 mmol) and diethyl azodicarboxylate (1.1 ml, 7 mmol) in THF (35 ml) in an atmosphere of nitrogen was added triphenylphosphine (1.8 g, 7 mmol). The reaction mixture was allowed to warm to room temperature and stirred for 24 h. The solvent was removed *in vacuo* and the residue purified by flash column chromatography (7:3 light petroleum/ethyl acetate) to afford the desired vinyl sulfone **90** as a colourless oil (55 g, 97%). ν_{max} (film)/cm^{-1} 3242, 1697, 1625; δ_H 1.4 (9 H, s), 1.6 (2 H, quint.), 2.3 (2 H, q), 3.1 (2 H, q), 4.7 (1 H, br), 6.4 (1 H, d), 7.0 (1 H, m), 7.5 (2 H, m, ArH), 7.6 (1 H, m, ArH), 7.9 (2 H, m, ArH); δ_C 25.0, 28.4, 36.4, 39.8, 79.4, 127.3, 129.3, 130.9, 133.5, 140.5, 146.0, 156.2.

N-*Benzyloxycarbonyl-L-phenylalanyl-5-amino-1-pentenyl phenyl sulfone* **86**

A solution of *N*-BOC vinyl sulfone **90** (200 mg, 0.6 mmol) in DCM (1.23 ml, 19 mmol) was treated with triethylsilane (0.24 ml, 1.5 mmol) and trifluoroacetic acid (0.62 ml, 8 mmol) and the reaction mixture was stirred for 0.5 h. The solvent was removed under high vacuum, the residue redissolved in THF (3 ml) and then cooled by means of an ice bath. Z-Phenylalanine active ester **9** (0.24 g, 0.6 mmol) was added followed by triethylamine (0.13 ml, 0.9 mmol) and the mixture stirred for 2 h. at 0°C. The reaction was diluted with ethyl acetate (15 ml) and the organic layer was washed with 1M HCl (3 x 10 ml), saturated sodium hydrogen carbonate solution (3 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a residue which was purified by recrystallisation from aqueous ethanol to give the vinyl phenyl sulfone **86** as a colourless solid (128 mg, 42%) m.p. 102-105°C. (Found: C, 65.84; H, 6.06; N, 5.31. $C_{28}H_{30}N_2O_5S$ requires C, 66.38; H, 5.97; N, 5.53%.); ν_{max} (KBr)/cm^{-1} 3316, 1684, 1653; δ_H 1.5 (2 H, quint.), 2.1 (2 H, q), 3.1 (4 H, m), 4.3 (1 H, q), 5.1 (2 H, s), 5.4 (1 H, br), 5.8 (1 H, t), 6.3 (1 H, m), 6.9 (1 H, m), 7.2 (10 H, m, ArH), 7.5 (2 H, m, ArH), 7.6 (1 H, m, ArH), 7.9 (2 H, m, ArH); δ_C 27.1, 37.8, 38.6, 54.8, 56.5, 67.1, 127.0, 127.3, 128.0, 128.4, 128.6, 129.3, 129.4, 130.9, 133.6, 133.7, 136.0, 136.4, 140.5, 146.0, 156.6, 170.8.

tert-*Butyl* N-tert-*butyloxycarbonyl-3-(2-hydroxy*iso*propyl)pyroglutamate* **99**

To a cold (-78°C), stirred solution of *t*-butyl *N*-BOC pyroglutamate **45b** (0.3 g, 1.05 mmol) in THF (10.5 ml) under an atmosphere of nitrogen was added lithium *bis*(trimethylsilyl)amide (1.05 ml, 1.05 mmol) dropwise. The mixture was stirred for

20 min. and dry acetone (0.084 ml, 1.15 mmol) was added. The reaction mixture was stirred for a further 0.5 h. at -78°C and then quenched with saturated ammonium chloride solution. The aqueous layer was extracted with ethyl acetate (3 x 10 ml) and the combined organic layer was washed with water (3 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave the pyroglutamate derivative **99** as a colourless oil. ν_{max} (film)/cm^{-1} 3390, 1788, 1742, 1712; δ_H 1.2 (3 H, s), 1.3 (3 H, s), 1.4 (9 H, s), 1.5 (9 H, s), 2.1-2.8 (3 H, m), 4.4 (1 H, dd); δ_C 24.8, 25.4, 27.9, 31.1, 51.1, 57.4, 71.2, 82.6, 83.7, 149.0, 170.1, 175.4.

N-tert-*Butyloxycarbonyl-3-(2-dimethylaminomethylene)-2-pyrrolidinone* **100**

A mixture of *N*-BOC pyrrolidinone **60** (1.11 g, 6 mmol) and *t*-butoxy*bis*(dimethyl-amino)methane (1.86 ml, 9 mmol) in dimethoxyethane (6 ml) was heated under reflux for 10 h. The reaction was cooled, an excess of ether added and the resulting precipitate collected by filtration. The crude material was purified by recrystallisation from cyclohexane to afford the desired pyrrolidinone **100** as colourless crystals (1.15 g, 80%) m.p. 127-129°C. (Found: C, 60.04; H, 8.35; N, 11.52. $C_{12}H_{20}N_2O_3$ requires C, 59.98; H, 8.39; N, 11.66%.); ν_{max} (KBr)/cm^{-1} 1703, 1685, 1640; δ_H 1.5 (9 H, s), 2.9 (2H, t), 3.0 (6 H, s), 3.7 (2 H, m), 7.1 (1 H, s); δ_C 21.4, 28.2, 41.9, 43.3, 81.5, 94.0, 145.8, 152.1, 170.7.

3-Methylene-2-pyrrolidinone **101**

To a cold (-70°C), stirred solution of the enaminone **100** (0.96 g, 4 mmol) in THF (40 ml) in an atmosphere of nitrogen was added a 1.5M solution of diisobutylaluminium-hydride in toluene (2.9 ml, 4.4 mmol) dropwise. The reaction was stirred for 1.5 h at

-78°C then quenched by addition of saturated ammonium chloride solution. The layers were separated, the aqueous layer extracted with chloroform (3 x 10 ml), then ether (20 ml) and the combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a pale yellow residue which was purified by flash column chromatography (9:1 chloroform/ethyl acetate) to yield the 4-methylene-2-pyrrolidinone **101** as a colourless solid (0.55 g, 70%) m.p. 72-74°C. (Found: C, 61.07; H, 7.59; N, 6.93. $C_{10}H_{15}NO_3$ requires C, 60.90; H, 7.66; N, 7.10%.); ν_{max} (KBr)/cm^{-1} 1770, 1710, 1658; δ_H 1.5 (9 H, s), 2.7 (2 H, m), 3.7 (2 H, m), 5.5 (1 H, s), 6.2 (1 H, s); δ_C 23.2, 28.0, 43.0, 83.0, 119.7, 139.1, 150.8, 166.4.

N-tert-*Butyloxycarbonyl-4-amino-2-methylene-butyric acid methyl ester* **102**

A cold (-15°C) solution of *N*-BOC methylene pyrrolidinone **101** (0.5 g, 2.5 mmol) in THF (12.5 ml) in an inert atmosphere of nitrogen was treated with a 2M solution of sodium methoxide (1.37 ml, 2.75 mmol). The reaction was warmed to 0°C and stirred for 1.5 h. Brine was added and the aqueous layer was extracted with chloroform (2 x 10 ml) and ether (15 ml). The combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* afforded a pale yellow oil which was purified by flash column chromatography (7:3 light petroleum/ethyl acetate) to yield the methyl ester **102** as a colourless oil (100 mg, 17%). ν_{max} (film)/cm^{-1} 3373, 1715, 1700, 1631; δ_H 1.4 (9 H, s), 2.5 (2 H, m), 3.3 (2 H, m), 3.7 (3 H, s), 4.7 (1 H, br),. 5.6(1 H, s), 6.2 (1 H, s); δ_C 28.4, 32.5, 38.6, 51.9, 79.2, 127.1, 137.5, 155.8, 170.5.

N-*Benzyloxycarbonyl-L-phenylalanyl-4-amino-2-methylene-butyric acid methyl ester* **103**

A solution of the *N*-BOC 4-aminobutyric acid methyl ester **102** (100 mg, 0.44 mmol) in DCM (0.9 ml, 14 mmol) was treated with triethylsilane (0.18 ml, 1.1 mmol) followed by trifluoroacetic acid (0.45 ml, 5.87 mmol) and the reaction mixture was stirred for 1 h. The solvent was removed under high vacuum and the residue redissolved in THF (2.5 ml) and cooled by means of an ice bath. Z-Phenylalanine active ester **9** (0.17 g, 0.44 mmol) and triethylamine (0.09 ml, 0.66 mmol) were added and the mixture stirred for 2 h. at 0°C. The reaction was diluted with ethyl acetate (20 ml) and the organic layer was washed with 1M HCl (3 x 10 ml), saturated sodium hydrogen carbonate solution (3 x 10 ml), brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a residue which was purified by flash column chromatography (1:1 light petroleum/ethyl acetate) to give the desired methyl ester **103** as a colourless solid (58 mg, 32%) m.p. 120-121°C. (Found: C, 66.91; H, 6.52; N, 6.86. $C_{23}H_{26}N_2O_5$ requires C, 67.30; H, 6.38; N, 6.82%.); ν_{max} (KBr)/cm^{-1} 3308, 1717, 1692, 1650, 1627; δ_H 2.4 (2 H, q), 3.0 (2 H, m), 3.3 (2 H, m), 3.8 (3 H, s), 4.3 (1 H, q), 5.1 (2 H, s), 5.3 (1 H, br), 5.4 (1 H, s), 6.0 (1 H, br), 6.2 (1 H, s), 7.1-7.4 (10 H, m, ArH); δ_C 31.7, 38.5, 38.7, 52.0, 56.4, 67.1, 127.1, 127.4, 128.1, 128.2, 128.5, 128.7, 129.3, 136.1, 136.4, 137.1, 156.2, 168.4, 170.6.

N-tert-*Butyloxycarbonyl-6-methylthio-5-oxo-L-norleucine* **104**

Methyl sulfide (0.15 ml, 2 mmol) was added to a stirred mixture of *N,N,N',N'*-tetramethylethylenediamine (0.3 ml, 2 mmol) and *n*-BuLi (1.25 ml, 2 mmol) in an

atmosphere of nitrogen at room temperature. The reaction was stirred for 4.5 h. and added, dropwise, to a cold (-78°C) solution of ethyl *N*-BOC pyroglutamate (0.5 g, 1.94 mmol) in THF (20 ml). Stirring was continued for a further 0.5 h. and the reaction quenched by addition of saturated ammonium chloride solution. The aqueous layer was extracted with ethyl acetate (3 x 30 ml) and the combined organic layer was washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a pale yellow residue which was purified by flash column chromatography (3:2 light petroleum/ethyl acetate) to give the desired norleucinate derivative **104** as a pale yellow oil (0.12 g, 20%). ν_{max} (film)/cm^{-1} 3364, 1736, 1709, 1510; δ_H 1.3 (3 H, t), 1.4 (9H, s), 1.9 and 2.2 (2 H, m), 2.1 (3 H, s), 2.7 (2 H, m), 3.2 (2 H, s), 4.2 (2 H, q), 4.3 (1 H, m), 5.2 (1 H, br); δ_C 14.2, 15.7, 26.8, 28.3, 36.0, 43.0, 52.9, 61.5, 79.9, 155.5, 172.4, 204.4.

5-N-tert-*Butyloxycarbonylaminopentanoic acid* **106**

Compound **106** was prepared using *procedure 8* from diazoketone **62** (0.45 g, 2 mmol), 1,4-dioxan (20 ml), water (10 ml) and silver benzoate (4 mg, 17.4 μmol). The desired acid **106** was obtained as a colourless oil (0.23 g, 53%). ν_{max} (film)/cm^{-1} 3335, 1705, 1690; δ_H 1.4 (9 H, s), 1.5 (2 H, m), 1.7 (2 H, m), 2.4 (2 H, t), 3.1 (2 H, m), 4.7 (1 H, br), 10.8 (1 H, br); δ_C 21.8, 28.4, 29.4, 33.6, 40.1, 79.3, 156.1, 178.8.

6-N-tert-*Butyloxycarbonylamino-hexanoic acid* **107**

Compound **107** was prepared similarly from diazoketone **63** (0.48 g, 2 mmol), 1,4-dioxan (20 ml), water (10 ml) and silver benzoate (4 mg, 17.4 μmol). The desired acid **107** was obtained as a colourless oil (0.26 g, 56%). ν_{max} (film)/cm^{-1} 3330, 1710, 1690,

1525; δ_H 1.3 (2 H, m), 1.4 (9 H, s), 1.5 (2 H, m), 1.7 (2 H, quint.), 2.4 (2 H, t), 3.1 (2 H, m), 4.6 (1 H, br), 10.4 (1 H, br); δ_C 24.3, 26.1, 28.3, 29.6, 33.9, 40.3, 79.2, 156.0, 179.0.

N-tert-*Butyloxycarbonyl-L-2-aminohexanedioic acid 1-ethyl ester* **108a**

Compound **108a** was prepared as before using ethyl *N*-BOC diazoketone **46a** (0.5 g, 1.67 mmol), 1,4-dioxan (16 ml), water (8 ml) and silver benzoate (46 mg, 0.2 mmol) and obtained as a colourless oil (0.34 g, 70%). ν_{max} (film)/cm^{-1} 3240, 1737, 1705, 1696; δ_H 1.3 (3 H, t), 1.5 (9 H, s), 1.7 (4 H, m), 2.4 (2 H, m), 4.2 (2 H, q), 4.3 (1 H, m), 5.3 (1 H, br), 10.6 (1 H, br); δ_C 14.2, 20.9, 28.2, 32.1, 33.3, 53.6, 61.6, 79.9, 155.6, 171.3, 179.0.

N-tert-*Butyloxycarbonyl-L-2-aminohexanedioic acid 1*-tert-*butyl ester* **108b**

Using the above procedure *t*-butyl *N*-BOC amino adipic acid **108b** was prepared from diazoketone **46b** (0.6 g, 1.8 mmol) 1,4-dioxan (18 ml) water (9 ml) and silver benzoate (3.6 mg, 15.7 μmol). The desired adipic acid **108b** was obtained as a pale yellow oil (0.43 g, 75%). ν_{max} (film)/cm^{-1} 3240, 1740, 1710, 1686; δ_H 1.4 (9 H, s), 1.5 (9H, s), 1.7 (4 H, m), 2.4 (2 H, m), 4.2 (1 H, m), 5.2 (1 H, br); δ_C 20.8, 28.0, 32.1, 33.4, 53.6, 79.9, 82.1, 155.5, 171.8, 178.8.

1,4-Di-tert-*butyloxycarbonylpiperazine-2,5-dione* **109**

Compound **109** was prepared using *procedure 4b*, from piperazine-2,5-dione (1.14 g, 10 mmol), acetonitrile (15 ml), DMAP (0.12 g, 1 mmol) and di-*t*-butyl dicarbonate (6.5 g, 30 mmol). The reaction was stirred for 48 h. Removal of the solvent *in vacuo*

gave an orange oily residue which was purified by flash column chromatography (7:3 light petroleum/ethyl acetate) followed by crystallisation from ethyl acetate/light petroleum to afford the protected piperazinedione **109** as a colourless solid (1.5 g, 48%) m.p. 140-141°C (Lit.[189] m.p. 135-137°C). ν_{max} (KBr)/cm^{-1} 2979, 1789, 1729; δ_H 1.5 (9 H, s), 4.4 (4 H, s); δ_C 27.9, 49.5, 85.2, 149.5, 164.5.

N-tert-*Butyloxycarbonyl-3-amino-1-diazo-2-oxopropane* **110**

Compound **110** was prepared using *procedure 6* from protected piperazine-2,5-dione **109** (0.157 g, 0.5 mmol), TMSD (0.5 ml, 1 mmol) and *n*-BuLi (0.62 ml, 1 mmol). Purification by flash column chromatography afforded the desired diazoketone **110** as a yellow oil (98 mg, 49%). ν_{max} (film)/cm^{-1} 3362, 2104, 1703, 1636; δ_H 1.4 (9 H, s), 3.9 (2 H, s), 5.2 (1 H, s); δ_C 27.8, 47.9, 53.5, 153.0, 197.6.

(S)-*5-(Hydroxymethyl)-2-pyrrolidinone* **111**

To a cold (0°C), stirred solution of ethyl pyroglutamate **43** (5 g, 32 mmol) in a 1:1 mixture of THF/methanol (75 ml) was added sodium borohydride (2.41 g, 63.5 mmol) portionwise. The reaction mixture was warmed to room temperature and stirring continued for 1.5 h. The solvent was removed *in vacuo* and the residue redissolved in distilled water (40 ml). The solution was adjusted to pH 6 by addition of glacial acetic acid, an ion exchange resin (Dowex 50 H^+) was added and the mixture was stirred for a further 0.5 h. After filtration, removal of the solvent gave an oil which was purified by vacuum distillation to yield the alcohol **111** as a colourless solid (2.7 g, 74%) b.p. 162-165°C (0.1 mm) (Lit.[131] b.p. 147-149°C (0.06 mmHg); m.p. 65-67°C (Lit.[131] m.p.

66-68°C). ν_{max} (KBr)/cm^{-1} 3225, 1686; δ_H 1.8 and 2.2 (2 H, m), 2.4 (2 H, m), 3.6 (2 H, m), 3.8 (1 H, m), 4.9 (1 H, br), 7.6 (1 H, s); δ_C 22.6, 30.4, 56.6, 65.6, 179.6.

(S)-*5-(Bromomethyl)-2-pyrrolidinone* **112**

A cold (0°C) solution of triphenylphosphine (5.93 g, 22.65 mmol) in DCM (90 ml) was treated with *N*-bromosuccinimide (4.36 g, 24.5 mmol). The reaction mixture was stirred for 10 min. and the alcohol **111** (2 g, 17.4 mmol) added in portions. The reaction was warmed to room temperature and stirring continued for 18 h. Saturated ammonium chloride solution (40 ml) was added and the aqueous layer was extracted with chloroform (3 x 15 ml). The organic layer was combined, washed with brine and dried ($MgSO_4$). Removal of the solvent *in vacuo* gave a purple oil which was purified by flash column chromatography (100% ethyl acetate) to afford the bromide **112** as a colourless solid (1.01 g, 33%) m.p. 69-71°C (Lit.[131] m.p. 71-74°C). ν_{max} (KBr)/cm^{-1} 3236, 1697; δ_H 1.9 and 2.3 (2 H, m), 2.4 (2 H, m), 3.4 (2 H, m), 4.0 (1 H, m), 7.6 (1 H, s); δ_C 25.6, 30.0, 36.8, 55.1, 178.5.

(S)-*5-(Fluoromethyl)-2-pyrrolidinone* **113**

To a stirred suspension of silver fluoride (1.6 g, 12.7 mmol) in acetonitrile (20 ml) protected from light, was added a solution of bromomethyl pyrrolidinone **112** (1 g, 5.6 mmol) in acetonitrile (5 ml) dropwise. The reaction mixture was stirred at room temperature for 12 h. and then filtered through diatomaceous earth. The filtrate was concentrated under reduced pressure to give a brown oil which was triturated with chloroform and again filtered through diatomaceous earth. Concentration of the filtrate gave a yellow oil which was purified by flash column chromatography (100%

ethyl acetate) to yield the (S)-5-(fluoromethyl)-2-pyrrolidinone **113** as a colourless solid (325 mg, 50%) m.p. 26-28°C (Lit.[131] m.p. 24-26°C). ν_{max} (KBr)/cm^{-1} 3228, 1693; δ_H 1.8 and 2.2 (2 H, m), 2.4 (2 H, m), 3.9 (1 H, m), 4.4 (2 H, m), 7.8 (1 H, s); δ_C 21.9, 29.8, 53.9 ($j_{C\text{-}F}$ 20 Hz), 85.4 ($j_{C\text{-}F}$ 173 Hz), 179.1.

(S)-*2-Amino-5-fluoro-pentanoic acid hydrochloride salt* **114**

A solution of fluoromethyl pyrrolidinone **113** (0.3 g, 1.7 mmol) in 1M HCl (6 ml) was heated under reflux for 6 h. The solvent was removed under high vacuum at room temperature to yield a colourless crystalline solid which was recrystallised from acetic acid/ethyl acetate to afford the desired hydrochloride salt **114** as colourless crystals (320 mg, 81%) m.p. 173-175°C (Lit.[131] m.p. 170-171°C). ν_{max} (KBr)/cm^{-1} 3236, 1697; δ_H 2.0 (2 H, q), 2.6 (2 H, t), 3.6 (1 H, m), 4.6 (2 H, m); δ_C 23.9, 30.5, 51.7 ($j_{C\text{-}F}$ 18 Hz), 82.4 ($j_{C\text{-}F}$ 170 Hz), 177.5.

4.2 BIOLOGICAL EXPERIMENTAL

Cell lines: ECV 304 human lung endothelial cell line showing high levels of transglutaminase activity, was supplied by Richard Jones of The Nottingham Trent University. Cells were grown in Dulbecco modified Eagles medium, supplemented with 2mM L-glutamine (50 U/ml), penicillin/streptomycin (50 μg/ml) and 10% (v/v) heat-inactivated foetal calf serum in a humidified atmosphere at 37°C, 5% (v/v) CO_2 and 95% (v/v) air.

Clone 3 of mouse 3T3 Swiss Fibroblast cells, which express elevated levels of transglutaminase regulated by the tetracycline inducible system, were supplied (in the induced form to express high levels of the enzyme) by Dr. Elisabetta Verderio-Edwards. Cells were grown in the same media composition as ECV 304 cells.

^{14}C Putrescine assay

The reactions were carried out at 37°C in 1.5 ml microfuge tubes. The reaction was initiated by addition of 45μl of a known concentration of inhibitor in a suitable solvent to the reaction vials. The vials contained 10μl of the following reagents; 50mM of pH7.4 Tris-HCl, 38.4mM DTT, 12mM stock 1,4-^{14}C-putrescine. In addition, the reaction media contained 5μl of a 50mM solution of $CaCl_2$ in 50mM Tris and 20μl of a stock solution (25 mg/ml in 50mM Tris) of *N,N'*-dimethylcasein.

Aliquots were taken in duplicate at 2, 5 and 10 min. intervals. Each aliquot consisted of 10μl of reaction mixture and was spotted onto 10 mm^2 filter paper squares. The filter papers were placed in ice-cold 10% TCA and after completion of the assay were

washed with ice-cold 10% TCA (x 1), ice-cold 5% TCA (x 3), 1:1 acetone:ethanol (x 1) and acetone (x 1). The filter papers were dried overnight and placed in 2 ml of Optiphase high safe liquid scintillant for counting in the Packard liquid scintillator.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

ECV 304 cells (5 x 10^3/well) were plated in well flat bottom tissue culture plates. Stock solutions of inhibitors in various solvents were added to afford a range of concentrations with total volume per well of 200µl. The cells were incubated overnight and MTT added (50µl/well). After a further 4 h. the medium was aspirated from the wells, replaced with 75µl of DMSO and the absorbance read at 550µM on a ELISA plate reader.

Fluorescein Cadaverine incorporation into 3T3 clone 3 cells

Induced cells were seeded onto 8-well glass chamber slides at a density of $7x10^4$ cells/well in complete media. These were allowed to incubate and settle for 4 h. and inhibitor was added to a final concentration of twice the compounds IC_{50} value. Incubation was continued for a further 45 min. and 0.5mM FITC-cadaverine added. The cells were incubated and collected at 24, 48 and 72 h. intervals. The media was removed and the slides rinsed in phosphate buffered saline (PBS) pH 7.4 then fixed in methanol at -20°C for 10 min. The slides were further washed with PBS (4 x 8 min.) followed by a 5 min. incubation with propidium iodide (5µg/ml). The slides were again washed with PBS, mounted and viewed with a Leica CLSM confocal laser microscope using argon krypton laser at 488 and 560nms for fluorescein and rhodamine excitation.

Trypan blue assay

ECV 304 cells (5 $x10^5$) were plated out into 12-well plates with a total volume of 500µl medium per well. Wells were treated with inhibitor 3 h. after the cells had been plated down. Cells were harvested 2 h. after addition of inhibitor and after 24 h. incubation using trypsin in PBS and 2 mmol ethylenediaminetetraacetic acid (EDTA). The cells were resuspended in complete media and incubated with 0.4% (v/v) prior to counting using a neubauer haemocytometer.

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