

Synthesis and evaluation of CA clan cysteine inhibitors

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Tarek Lawson Millar



University of Canterbury
Christchurch
New Zealand

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Abstract

This investigation involved the synthesis of potential CA clan cysteine inhibitors of m-calpain and cathepsin B. Inhibitors 2.1.3a-j were based on the SJA-6017 construct containing the *N*-(4-fluorobenzenesulfonyl) moiety at the P₃ address region. The inhibitor 2.1.3k was based on CAT-0059 a novel dipeptide dialdehyde inhibitor containing the 5-formyl pyrrole moiety at the P₃ address region.

Chapter 1 introduces proteases in particular m-calpain and cathepsin B implicated in human pathologies cataract and tumour metastasis respectably. Structure, disease processes and known inhibitors for m-calpain and cathepsin B are presented and described. The chapter also describes drug design and rational including the requirement of the β -strand conformation for enzyme substrate binding.

Chapter 2 details the synthesis of m-calpain and cathepsin B inhibitors, *N*-(4-fluorobenzenesulfonyl) peptide aldehyde 2.1.3a-j and the dipeptide dialdehyde 2.1.3k. The synthesis involved the preparation of the *N*-(4-fluorobenzenesulfonyl) α -amino acids 2.1.8a-f, the *N*-(4-fluorobenzenesulfonyl) peptide esters of 2.1.10a-g, the peptide alcohols 2.1.11a-k and the peptide aldehydes 2.1.3a-k. Specific coupling reagents for amide bond formation are also discussed. The oxidation of the alcohols 2.1.11a-k with sulfur trioxide and pyridine complex are also addressed. The results from molecular modelling and enzymatic assays of the inhibitors 2.1.3a-k with m-calpain and cathepsin B are presented and discussed.

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Abbreviations

Ala	alanine (<i>S</i>)-2-aminopropanoic acid
Alk	alkane chain
app t	apparent triplet (NMR)
Avs	allyl vinyl sulfone
boc	<i>t</i> -butoxycarbonyl group
b s	broad singlet (NMR)
calcd.	calculated
cbz	benzyloxycarbonyl group
Chg	L-cyclohexaglycine [(<i>S</i>)-2-amino-2-cyclohexylacetic acid]
Chx	1-aminocyclohexanecarboxylic acid
gCOSY	gradient correlation spectroscopy (NMR)
d	doublet (NMR)
dd	doublet of doublets (NMR)
DCE	1,2-dichloroethane
DCM	dichloromethane
decomp.	decomposition
DIPEA	diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
EDC.HCl	<i>N</i> -ethyl- <i>N</i> '-[3-(dimethylamino)propyl] carbodiimide hydrochloride
Et ₃ N	triethylamine
EtOAc	ethyl acetate
equiv.	equivalent
<i>N</i> -(4-FBS)	<i>N</i> -(4-fluorobenzenesulfonyl)
g	grams
HATU	2-(3 <i>H</i> -[1,2,3]triazolo[4,5- <i>b</i>]pyridin-3- yl)-1,1,3,3-tetramethylisouronium hexafluorophosphate

HBTU	2-(1 <i>H</i> -benzo[d][1,2,3]triazol-1-yl)- 1,1,3,3-tetramethylisouronium hexafluorophosphate
HOBt.H ₂ O	1-hydroxybenzotriazole hydrate
hr	hour
Hz	hertz (NMR)
IR	infra red
Ile	<i>iso</i> L-leucine [(2 <i>S</i> ,3 <i>S</i>)-2-amino-3-methylpentanoic acid]
<i>J</i>	coupling constant (NMR)
Leu	L-leucine [(<i>S</i>)-2-amino-4-methylpentanoic acid]
lit.	literature
m	multiplet (NMR)
MeOH	methanol
Met	L-methionine [(<i>S</i>)-2-amino-4-(methylthio)butanoic acid]
mg	milligrams
min	minutes
mL	millilitre
μL	microlitre
mmol	millimole
m.p.	melting point
N	mole strength
NMR	Nuclear Magnetic Resonance
Pet ether	petroleum ether
Pgl	L-phenylglycine [(<i>S</i>)-2-amino-2-phenylacetic acid]
Phe	L-phenylalanine [(<i>S</i>)-2-amino-3-phenylpropanoic acid]
PMA	phosphomolybdic acid
Pyo	pyrrole

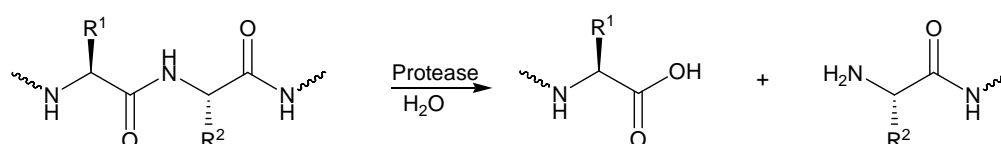
q	quartet (NMR)
rt	room temperature
s	singlet (NMR)
SAR	Structural Activity Relationship
SOCl ₂	thionyl chloride
SO ₃ .Pyr	sulfur trioxide pyridine complex
t	triplet (NMR)
temp.	temperature
THF	tetrahydrofuran
THP	tetrahydrophranyl ether
TLC	thin layer chromatography
Tle	<i>tert</i> L-leucine [(<i>S</i>)-2-amino-3,3-dimethylbutanoic acid]
Trp	tryptophan [(<i>S</i>)-2-amino-3-(1 <i>H</i> -indol-3-yl)propanoic acid]
Tyr	L-tyrosine [(<i>S</i>)-2-amino-3-(4-hydroxyphenyl)propanoic acid]
Val	L-valine [(<i>S</i>)-2-amino-3-methylbutanoic acid]

Chapter 1

Introduction

1.1 Proteases and the cysteine protease family

The protease super family are a vast group of enzymes that control protein synthesis, turnover and function and thereby regulate the normal physiological responses including digestion,^{1,2} cell degradation^{3,4} tissue remodelling^{5,6} blood pressure regulation⁷ and defensive roles.⁸ The proteases are also responsible for disease propagation in numerous pathologies including; cancers,⁹ (tumour metastasis)^{10,11} viral infections (e.g. HIV),¹² cardiovascular diseases,¹³ and neurological disorders including Alzheimer's disease¹⁴ (Table 1.1 below). These biochemical processes all involve selective cleavage and catalysis of specific peptide (amide) bonds on target substrates (Figure 1.1).



R¹ and R²= amino acid side chain

Figure 1.1 Peptide bond cleavage from a protease via catalysed hydrolysis

The majority of proteases are sequence specific therefore to describe these binding positions standard (Schechter and Berger) nomenclature is used (Figure 1.2).^{15,16}

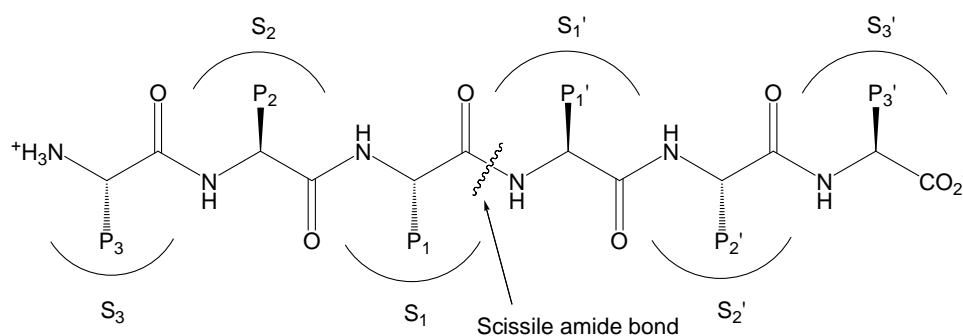


Figure 1.2 Schechter and Berger nomenclature, substrate/inhibitor P regions with the enzyme subsites S regions.^{15,16} Standard polypeptide substrate sequence where the standard substrate/inhibitor regions are designated as P₃, P₂, P₁, P₁', P₂', P₃'. These P regions will bind to their corresponding enzyme subsites S₃, S₂, S₁, S₁', S₂', S₃'.

Protease classification

The protease super family comprises of five classes,¹⁷ cysteine, threonine, serine, aspartic acid, and metalloproteases (Table 1.1). The classification system defines the active functional group within the active site of each enzyme class. The most common characteristic of the proteases is they require a nucleophile for the hydrolysis of a peptide bond.¹⁷ However; the catalytic mechanisms vary between the classes. For cysteine, threonine and serine proteases the nucleophile is a component of the side chain. For aspartic acid and metalloproteases an activated water molecule acts as nucleophile.¹⁷

Table 1.1 Protease classification based on catalytic properties, regular function and dysfunctions

Protease Family	Selected Enzymes	Function	Dysfunction
Cysteine	Calpains μ and m (calcium activated proteases)	Bone resorption ¹⁸ Regulation in cell death ^{19,20} Cytoskeletal remodelling ²¹ Signal transduction ²²	Cataract ²⁶ Muscular dystrophy ²⁷ Alzheimer's disease ¹⁴
	Cathepsins B, C, F, H, K, L, S, O, V, W and X (human cathepsins)	Intracellular lysosomal protein degradation ²³⁻²⁵	Tumour invasion and metastasis ¹⁰
	Papain	Defence ⁸	
Threonine	Proteasome	Degradation of ubiquitinated proteins ²⁸	
Serine	Cathepsin G	Cellular degradation ^{3,4}	
	Chymotrypsin	Digestion ¹	
	Elastase	Elastin degradation ²⁹ Digestion ¹	
	Plasma Kallikrein	Pro-urokinase activation ³⁰	Cardiovascular disease ¹³
	Thrombin	Procoagulant and anticoagulant ³¹	Pulmonary fibrosis ³²
Aspartic acid	HIV protease		HIV replication ¹²
	Cathepsin D and E	Intracellular degradation ⁹	Gastric carcinoma ⁹
	Pepsin	Digestion ²	Peptidic ulcers ²⁷
	Renin	Blood pressure regulation ⁷	
Metallo	Astacin	Animal morphogenesis ³³	
	Collagenase	Tissue remodelling ⁵	Arthritis ³⁴
	Gelatinase	Tissue remodelling ⁶	Tumour invasion ¹¹

The cysteine protease family can be divided into three major clans (see Table 1.2) defined by their structure. The CA or papain like clan is the most abundant of the cysteine proteases. This clan consists of three major groups; mammalian proteases (*e.g.*, the lysosomal human cathepsins B, C, F, H, K, L, O, S, V, W and X), parasitic proteases (*e.g.*, falcipain), and cytosolic proteases (*e.g.*, μ -calpain and m-calpain). The PA or picornain family (*e.g.*, PA(C), SARS virus) consist of viral proteases which carry more than one of the following amino acids at their active sites serine, threonine, or cysteine. The CD or interleukin 1 β converting enzyme (ICE-like) proteases, is the second most abundant clan and consists of mammalian (*e.g.*, the caspases 1 and 3) and bacterial proteases (*e.g.*, the gingipains K and R).^{35,36}

This study will focus on the inhibition of two cysteine proteases of the CA clan, m-calpain and cathepsin B.

Table 1.2 Classification of cysteine proteases^{35,36}

Clan	Family	Example of cysteine proteases
CA	C1 (mammalian)	Cathepsins B, C, F, H, K, L, O, S, V, W and X
CA	C1 (parasite)	Falcipain
CA	C2 (cytosolic)	m-calpain, μ-calpain
PA(C)	C3 (viral)	Picornain 3C (<i>e.g.</i> , Hepatitis A virus, human rhinovirus)
PA(C)	C30 (viral)	SARS virus
CD	C14 (mammalian)	Caspase 1 (ICE) Caspase 3
CD	C25 (bacterial)	Gingipain K and R

The general mechanism of catalysis for the cysteine proteases

Along with a nucleophile in the active site, a proton donor is also required. In the case of cysteine and serine proteases histidine acts as donor.³⁷ Shown below in Figure 1.3 is the mechanism for the catalysis of a suitable substrate with a cysteine protease. The reaction mechanism can be divided into two distinct processes, acylation and deacylation. The first reaction requires a powerful nucleophile to attack a carbonyl carbon of the peptide. The weak nucleophilic thiol group on cysteine is activated to the thiolate anion.³⁸

The reaction begins with the deprotonation of the cysteine's sulfhydryl functional group by an adjacent histidine imidazole group (A) (Figure 1.3). The SH proton on cysteine is transferred to the adjacent histidine to form a Wheland intermediate which is stabilised by hydrogen bonding with a nearby asparagine or aspartic acid residue (B). The thiolate anion on cysteine undergoes nucleophilic attack on to the carbonyl carbon of the peptide substrate. This forms a high energy tetrahedral intermediate with a thioethanolate linkage (C). The thioethanolate anion subsequently deprotonates the imidazole functionality on histidine to give the thioester; consisting of cysteine and the peptide substrate (D). This also affords the first cleaved segment of the substrate as the free amine NH_2R (D).³⁸

The commencement of deacylation phase (to yield the free the cysteine protease) begins with the hydrolysis of the thioester (E). This reaction involves the proton abstraction of a water molecule via the imidazole group on histidine to provide the hydroxyl ion necessary for the nucleophilic attack on carbonyl group on the thioester; this gives rise to the second tetrahedral intermediate (F). This is followed by a nucleophilic attack from the oxyanion hole to the histidine which breaks the $\text{S}\gamma\text{-C}$ bond on cysteine to release the activated enzyme and the second cleaved substrate (G) as the free acid. Then protonation of the cysteine's thiol group (H) from histidine gives the deactivated enzyme (A).³⁸⁻⁴⁰

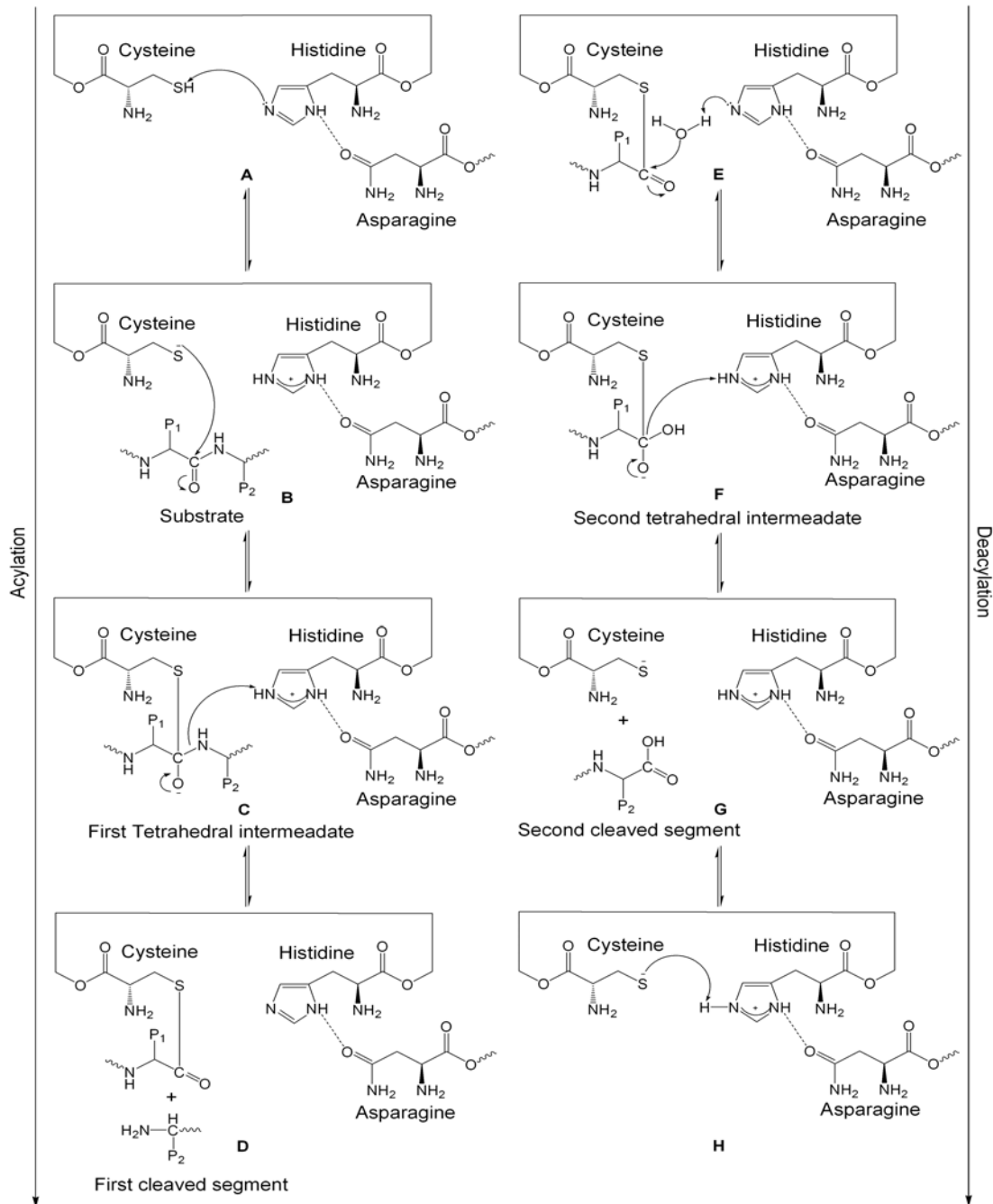


Figure 1.3 Mechanism for the catalysis of peptides with cysteine proteases³⁸⁻⁴⁰

1.2 The calpains and m-calpain structure

The calpains are a diverse family of cysteine proteases present in all mammalian species and first identified in 1964 from rat brain.⁴¹ In the late 1970s the enzymes were isolated and characterised.⁴² They play important beneficial roles in the regulation of bone resorption,¹⁸ signalling transduction pathways,²² remodelling of cytoskeletal attachments to the plasma

membrane²¹ and cell death^{19,20} (Table 1.1). The catalytic action unlike other proteases is limited to the cleavage of amide bonds between domains as opposed to certain amino acids residues or sequences. This form of substrate selectivity leaves large substrate fragments suggesting that calpains are bio-modulators instead of being involved in a digestive function.⁴³ However, calpains are known to play an important role in numerous human pathologies including cataract,²⁶ Alzheimer's disease,¹⁴ and muscular dystrophy,²⁷ as addressed in Table 1.1.

The calpain family group comprises of a number of different isoforms which can be classified into two distinct groups, according to Suzuki and co-workers. The first group are the typical calpains comprising of domains I-IV and the atypical calpains displaying alterations in these domains.⁴⁴ All these isoforms are activated by Ca^{2+} ; this form of activation is unique to the calpain family. The two most abundant isoforms are μ -calpain and m-calpain. The difference between the two isoforms is the concentration of Ca^{2+} required for their activation: 1.0-100 μmol and 0.1-1.0 mmol Ca^{2+} respectively.⁴⁵ Occurrence of both these isoforms in mammals is ubiquitous and the ratios between μ and m-calpain vary from cell to cell.⁴³

m-Calpain is a flat oval disk shaped enzyme made up of a heterodimeric subunit. The crystallographic study of inactivate human lens m-calpain (Figure 1.4) shows it has a total of six subunits (I-VI). The larger catalytic subunit (80 KDa) consists of domains I-IV and the smaller regulatory subunit (28 KDa) comprises of domains V and VI; both subunits are encoded by CAPN 2 and CAPN 4 genes respectively.⁴⁴ Positioned in the centre of the enzyme in Figure 1.4 below (in green folds) is domain I (dI) (an NH_2 terminal domain) this represents the start of the larger catalytic subunit where dI acts as the anchoring helix contained in a vacant cavity in domain VI (dVI) (orange folds); This domain folds into the first part of catalytic domain II (dII), subdomain IIa (dIIa) (shown in gold folds) via a domain I→II linker. Both dI and dIIa form a part of the left hand side of the enzymes architecture which resembles papain. Then this folds into the second subdomain IIb (dIIb) (shown in red folds). This domain has a barrel like structure which descends (via an II→III linker loop) into domain III (dIII) indicated by blue folds. This further descends into the calmodulin-like domain IV (dIV) (the terminus of the larger catalytic subunit) (yellow folds) through an extended III→IV linker, shown in magenta. Domain V (dV) or S domain (30 KDa) coloured in magenta is positioned above dIIa. This domain folds into domain VI (dVI) indicated in orange the left-side calmodulin domain. The catalytic residues (which form the active catalytic triad) are positioned on the two

subdomains dIIa cysteine and dIIb histidine and aspartate or asparagine (not shown); (this is common to all calpains).⁴⁶

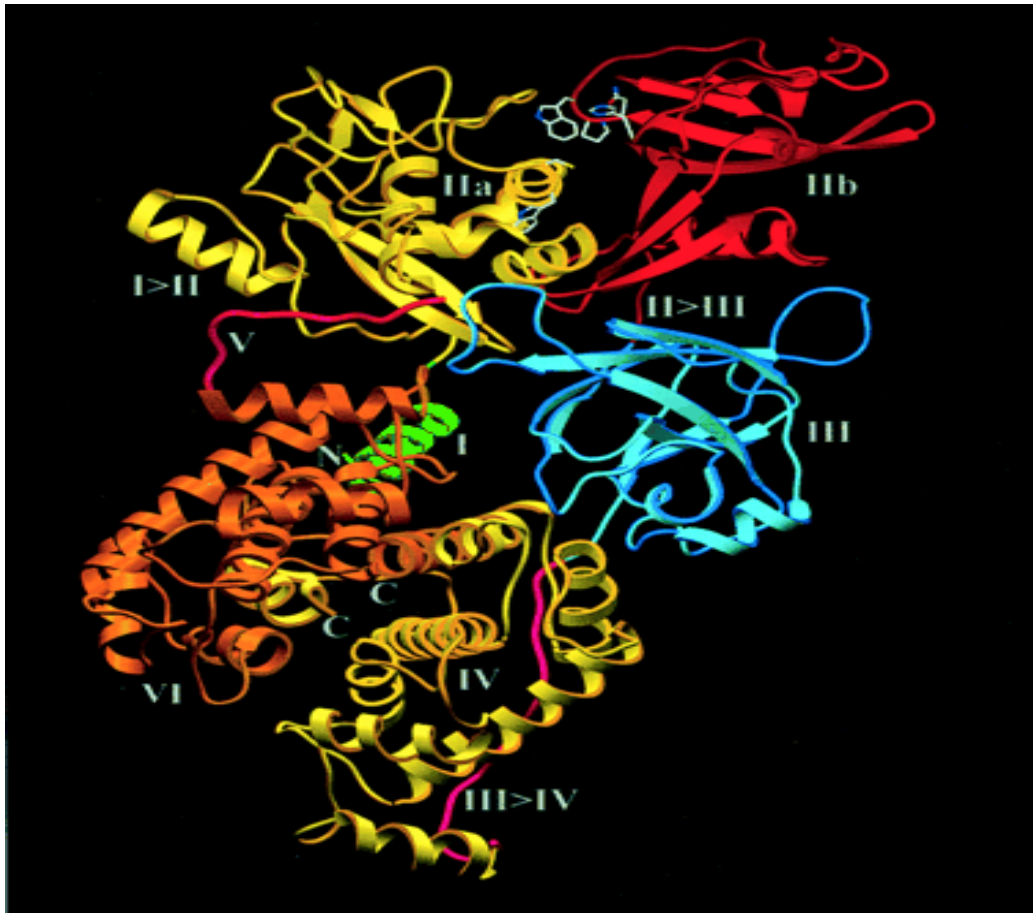


Figure 1.4 Crystallographic construct of human m-calpain in the inactivated state⁴⁶

The EF-hand motifs (calcium binding proteins)

The EF-hand motifs are one of the most common of the calcium binding proteins that were first described by Kretsinger and Nockolds in parvalbumin.⁴⁷ These motifs receive their name by the protein forming the symbolic shape of a human hand, shown in Figure 1.5a. The E helix winds down the index finger to link the F helix winding up through the thumb, (coloured light grey) known as the apo-protein (closed conformation). The calcium bound state or holo-protein displays the open conformation (dark grey). The calcium bound conformer (Figure 1.5b) forms ligands between the amino acid side chains of asparagine or aspartic acid (X and Y), asparagine, aspartic acid or serine (Z). In addition to a peptide carbonyl oxygen (-Y) and a water molecule at (-X) there is also a conserved bidentate ligand at (-Z). This consists of glutamic acid or aspartic acid. Figure 1.5c displays a modified EF-hand loop (without calcium) showing the hydrogen bond

scaffolding. The calpains consist of two sets of five EF-hand motifs where each set occurs on both the large subunit and small subunits.⁴⁸ These motifs are similar to that occurring in calmodulin which are important in the activation of calpains to induce proteolytic activity.⁴⁶

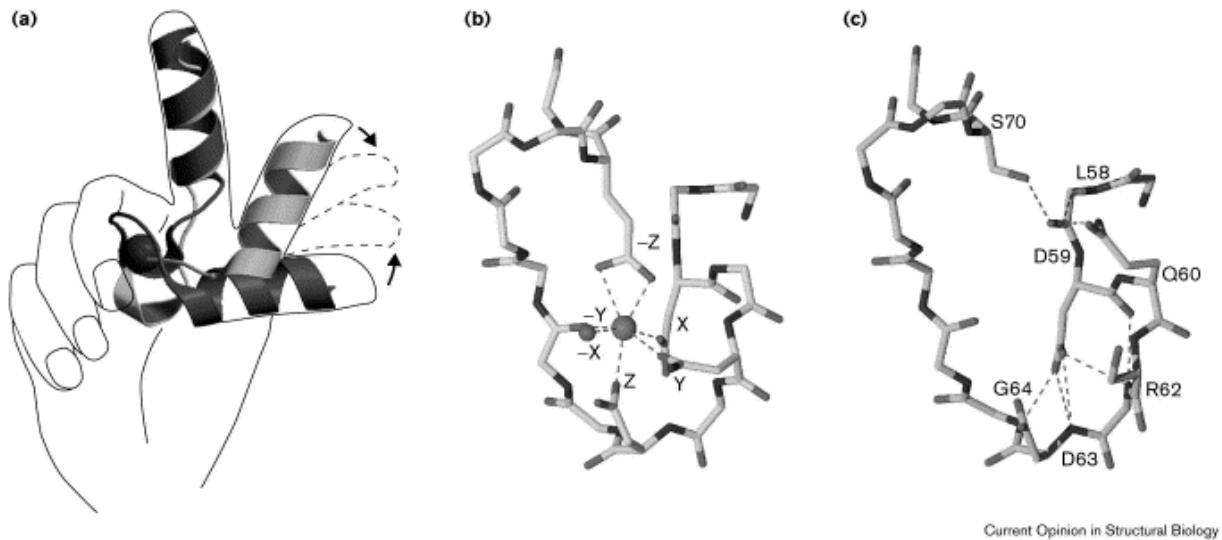


Figure 1.5 The EF-hand motif. (a) Symbolic representation (b) Geometry of the calcium ligands (c) Modified EF-hand motif displaying hydrogen bonding⁴⁸

Calpain activation begins with an influx of Ca^{2+} which binds to the EF-hand motifs and subdomains of dIIa and dIIb. These accommodations of calcium collectively cause conformational changes in the overall m-calpain construct (see Figures 1.6 and 1.7). These changes are thought to be caused (in part) through a rotational turn of Trp₂₈₈ in dIIb which results in twisting of the dIIb subdomain. It is thought that this conformational twisting will reduce the initial gap between the catalytic residues of Cys₁₀₅, His₂₆₈ and Asp₂₈₆ from 8.5Å to a functionally active distance of 3.7Å which forms the catalytic triad complex.⁴⁹

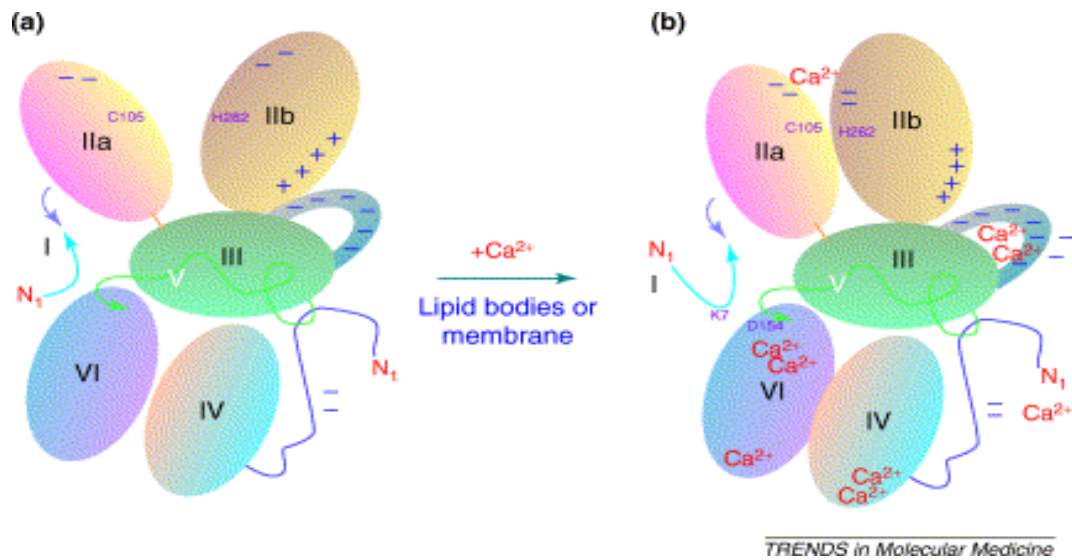


Figure 1.6 Schematic representation of m-calpains main binding events.⁵⁰ (a) m-Calpain in the inactivated state. (b) m-Calpain in the activated state with Ca²⁺ bound.

Then once a suitable substrate is found, catalysis as previously mentioned and illustrated in Figure 1.3 occurs. For m-calpain the cysteine residue containing the thiol group will participate directly in covalent catalytic cleavage of the peptide (amide) bond. The histidine residue, which is in close spatial proximity to the cysteine residue, will also take part in the catalytic cleavage acting as the proton donor. In addition the other nearby residues within the active site will provide functional groups for general acid–base catalysis.

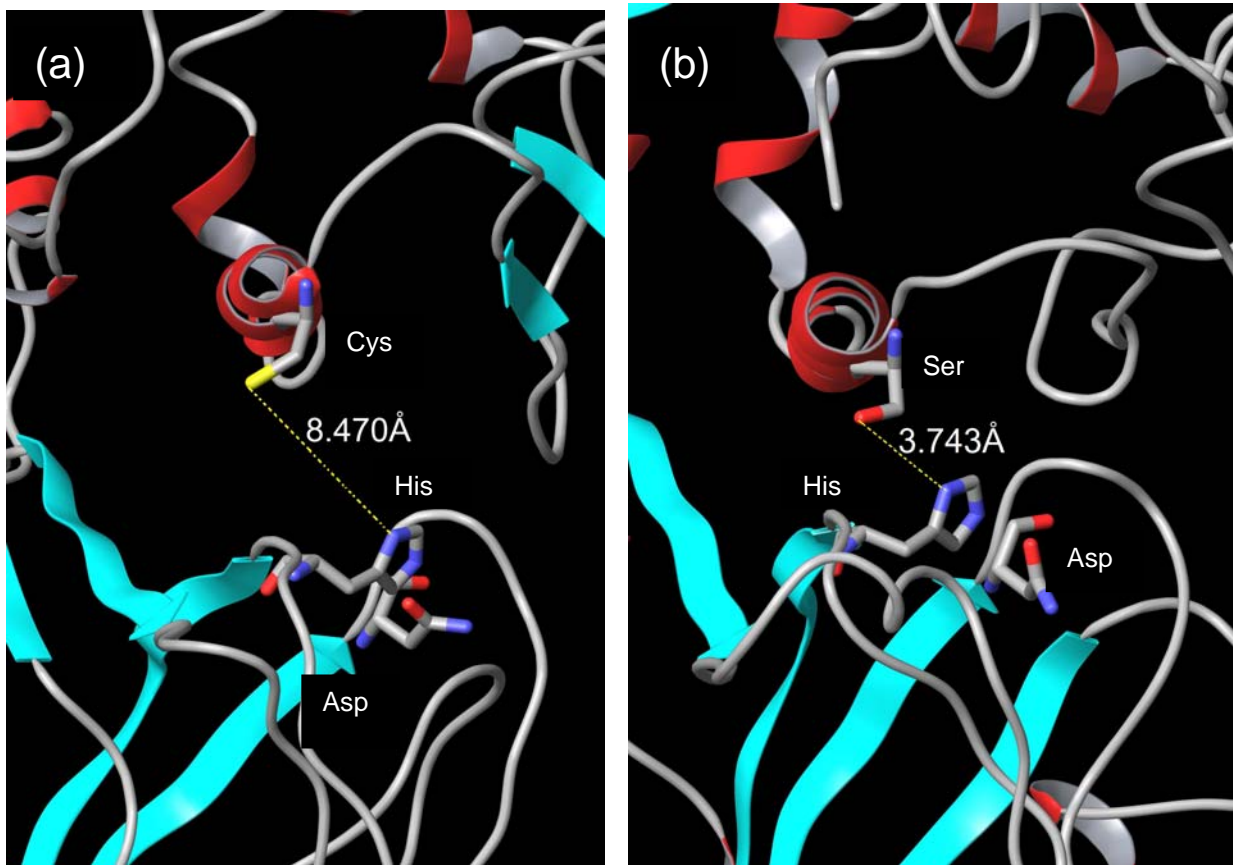


Figure 1.7 Crystallographic structures of the calpains (a) Inactivated human m-calpain⁵¹ (b) Activated (calcium induced) rat m-calpain⁵² bearing the serine (Ser) mutant on dIIa (red folds) and histidine and aspartic acid on dIIb (blue folds). In the inactivated construct (a) the distance between the Cys₁₀₅ and His₂₆₂, Asp₂₈₆ residues is 8.5 Å; this gap is too far to support a functionally active catalytic complex. However upon activation with Ca²⁺ the gap between Cys₁₀₅ and His₂₆₈ closes to 3.7 Å, close enough to perform proteolysis^{49,51}

1.3 Mode of action on m-calpain in cataracts

Cataract is a condition where the eye lens is opaque or cloudy. The normal human eye lens (Figure 1.8) is designed to allow light to pass through and focus to the back of the retina. This direct focusing is achieved through of three types of crystallins (α , β , γ) where the β form is the most abundant. The names are given in order from their elution from gel chromatography. Existence of these forms and their generic sequences have been confirmed and characterised in previous reports.⁵³⁻⁵⁸

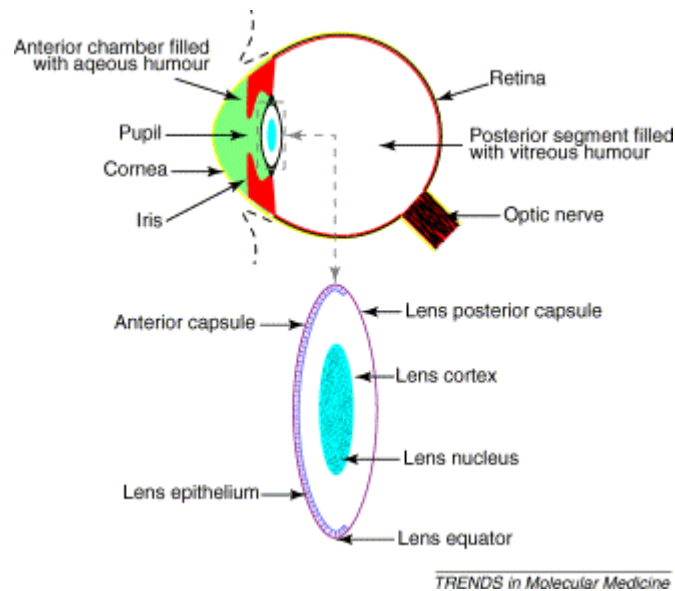


Figure 1.8 Schematic of the human eye showing the lens⁵⁰

The crystallins make up 90% of the water soluble proteins of the lens and are arranged in a tight aligned packing. This arrangement increases the refractive index and transparency to allow light to pass to the retina.⁵⁹ However this level of transparency may degrade with one or more of the following insults, (see Figure 1.9) diabetes,⁶⁰ ageing,⁶¹ oxidants,⁶² selenite,⁶³ galatose,⁶⁴ and other causes.

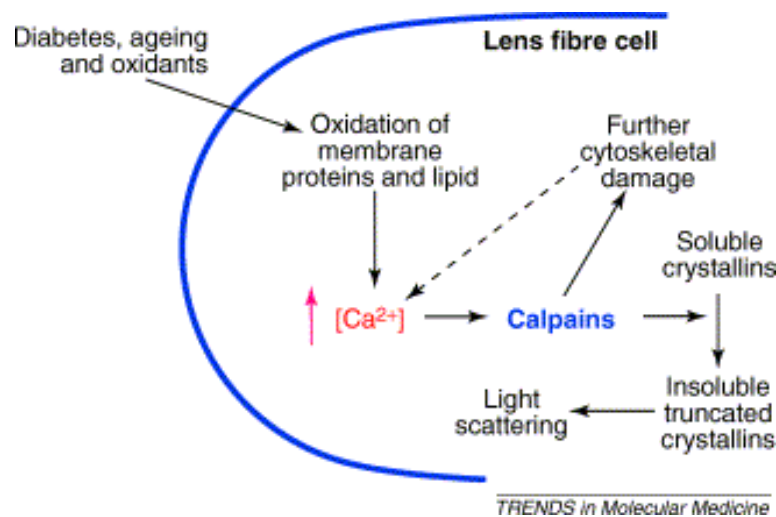


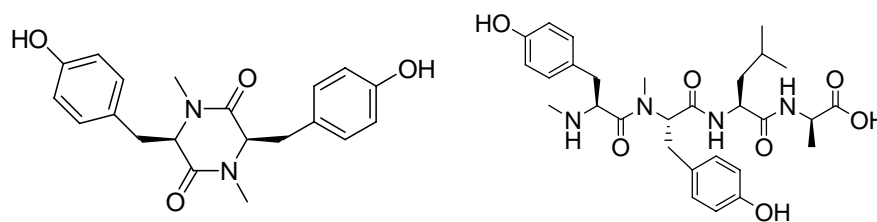
Figure 1.9 Processes of cataract formation initiated from varying outside insults⁵⁰

These insults can lead to increases in Ca^{2+} concentration in the lens; calpains present in the lens can become pathologically over activated and in a deregulatory manner will proteolyse the crystallins. The proteolysis will disrupt or truncate the packing order of the soluble crystallins resulting in insolubilisation and development of opacity. This leads to a decrease in overall reflective index and lens transparency; which causes incoming light to scatter rather than being focused to the back of the retina.⁶⁵

1.4 Known inhibitors of the calpains

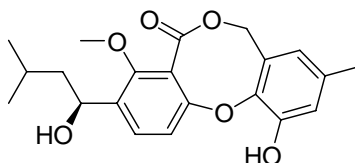
The search for selective inhibitors of calpain is ongoing. In the past 35 years since the enzymes discovery⁴¹ a significant number of inhibitors have been either developed from natural or synthetic sources. Unfortunately a large number of them show little selectivity for other cysteine proteases, for example cathepsin B. This apparent lack of selectivity may be due to similarities of the catalytic triad and surrounding subsite regions of the cysteine proteases. Two of first identifiable calpain inhibitors to appear was the calcium chelators; EDTA and EGTA.⁴⁵ However these were found to be neither potent nor selective for the calpains; a list of calpain inhibitors is summarised below.

Naturally derived inhibitors of calpain (from the plant and fungal kingdoms)



1.4.1. Diketopiperazine

1.4.2. Tetrapeptide



1.4.3. Penicillide

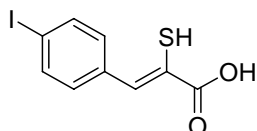
The *Streptomyces* species has yielded many naturally occurring calpain inhibitors; the main groups isolated are the diketopiperazines, peptide aldehydes and the pyrazinones. The inhibitors diketopiperazine **1.4.1** and tetrapeptide **1.4.2** have an N-methyl tyrosine moiety and exhibit

moderate potency for the calpains. Extracted from an actinomycete strain of *Streptomyces griseus* these have respective inhibitions for μ -calpain $IC_{50} = 800$ nM and 1.2 μ M.⁶⁶ Penicillide **1.4.3** is a weak calpain inhibitor $7.1 = \mu$ M for μ -calpain occurring from the *Penicillium* species, this compound is an example of a non-peptide calpain inhibitor however; the compound has 28-fold selectivity towards papain.⁴⁵

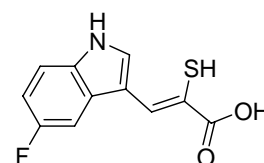
Irreversible calpain inhibitors (from chemical synthesis)

Irreversible inhibitors (non active site directed)

The majority of irreversible inhibitors target the active site (active site directed); however, there are a number of non active site directed inhibitors such as the α -mercaptoacrylate derivatives (PD150606 **1.4.4** $IC_{50} = 370$ nM for m-calpain and $IC_{50} = 0.128$ μ M for cathepsin B and PD151746 **1.4.5** $IC_{50} = 5.33$ nM for m-calpain and $IC_{50} > 0.200$ μ M for cathepsin B). These were first reported by Wang *et al*⁶⁷ and are one of the earliest known non active site directed inhibitors to block calcium ions from binding to calcium binding domains in calpains.⁶⁷



1.4.4. PD-150606

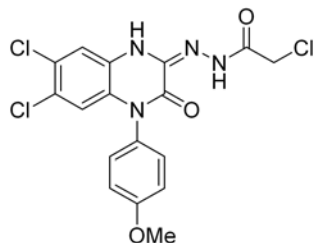


1.4.5. PD-151746

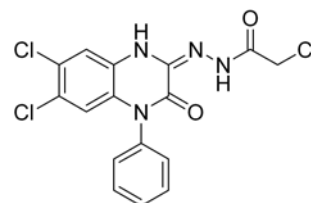
Irreversible inhibitors (active site directed)

The rational of irreversible inhibitors of the calpains such as; peptide diazoketones,^{68,69} halo methyl ketones,⁷⁰ Michael acceptors (α and β -vinyl sulfones)⁷¹ and other derivatives employ an activated electrophile which can be displaced within the active site of calpain by a nucleophile, for example the cysteine thiolate group. This displacement leads to the formation of a covalent bond and can permanently inactivate the enzyme; therefore these activated electrophiles can be regarded as active warheads. The majority of the irreversible inhibitors are selective towards serine proteases. The development of active irreversible inhibitors for calpain is somewhat limited due to the reactive nature of these warheads reacting to other thiol groups occurring throughout the human body.

The non-peptides SJA-7019 **1.4.6** ($IC_{50} = 64$ nM for μ -calpain and $IC_{50} = 1.5$ nM for cathepsin L and SJA-7029 **1.4.7** ($IC_{50} = 170$ nM for m-calpain and $IC_{50} = 4.2$ nM for cathepsin L) displayed below are highly potent against μ -calpain and m-calpain and cathepsin L these compounds are known to prevent extracellular Ca^{2+} influxes.

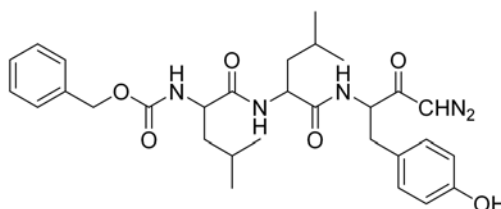


1.4.6. SJA-7019



1.4.7. SJA-7029

Diazomethyl ketones



1.4.8. Cbz leucine leucine tyrosine CHN₂

These inhibitors are generally selective for the calpains and have no observable activity for the serine proteases.⁶⁸ It has been suggested the mechanism of enzymatic inactivation is generated from nucleophilic attack of the carbonyl carbon on the diazo group by thiolate on cysteine to give the hemithioketal. (Figure 1.10) Subsequent protonation of the hemithioketal from the imidazole group on histidine **1.4.11** (rate determining step) gives concomitant cleavage of nitrogen and the thiol ether end product **1.4.12**. The compound cbz Leu Leu Tyr-CHN₂ **1.4.8** gives a second rate order of inhibition constants for calpain ($k_{2nd\ rate} 230,000\ M^{-1}s^{-1}$) and cathepsin B ($k_{2nd\ rate} 1,300\ M^{-1}s^{-1}$).⁶⁹

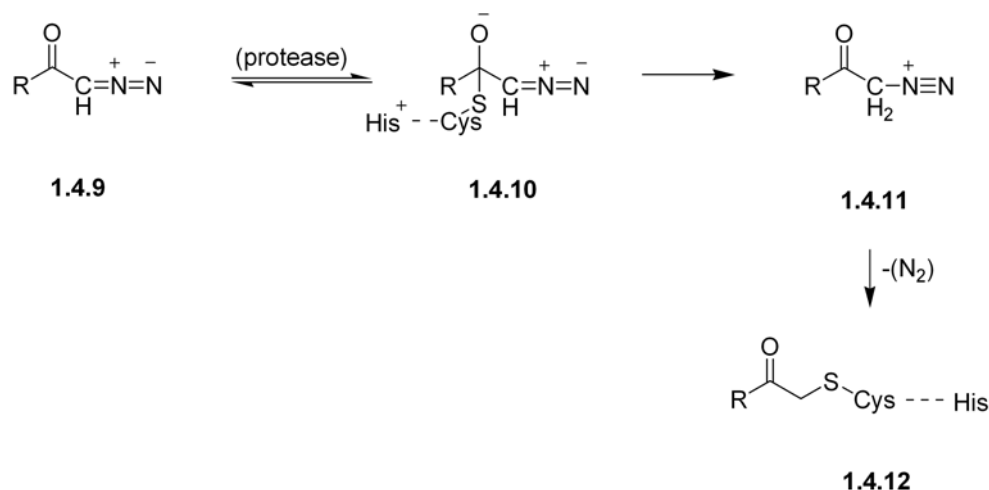
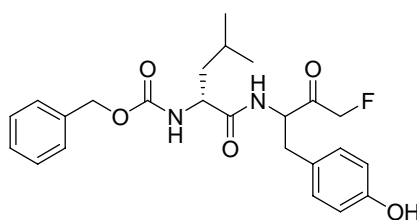


Figure 1.10 Inhibition with diazo methyl ketone inhibitors

Halo methyl ketones

From X-ray crystal structures this group of compounds are known to form irreversible alkylating adducts with cysteine active site residues of the cysteine proteases for example papain⁷² and cathepsin B⁷³. The compound cbz L-leucine D,L-tyrosine CH₂F **1.4.13** has moderate potency for the calpains IC₅₀ = 80 nM and a second order constant ($k_{2\text{nd rate}} 17,000 \text{ M}^{-1}\text{s}^{-1}$) for chicken gizzard m-calpain.⁷⁰ The fluoroketones display superior potency compared to the chloro analogues.⁷⁰



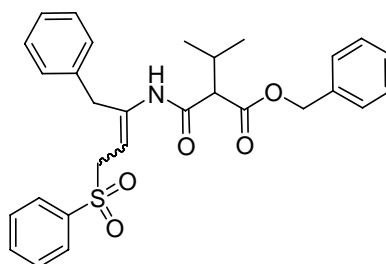
1.4.13. Cbz L-leucine D,L-tyrosine CH₂F

Allyl vinyl sulfones

The Michael acceptors (α and β vinyl sulfones) are generally selective for the calpains with the β (allyl) analogues displaying the greater potency and selectivity towards to cathepsin B compared to the calpains.⁷¹ Nevertheless the α -vinyl sulfones are found to be non-reactive

towards other thiol proteases occurring through the human body and are only active towards the cysteine residue.

The compound cbz-Val-Phe-Avs-Ph (*E&Z*) **1.4.14** displays reasonable inhibition for μ -calpain ($IC_{50} = 3.0$ nM for μ -calpain) and no observable inhibition for cathepsin B. Generally the allyl vinyl sulfones exhibit greater potency than the vinyl sulfones.⁷¹



1.4.14

Synthetic reversible calpain inhibitors

Examples of reversible inhibitors that inhibit calpains have been produced from; peptide aldehydes,^{16,74,75} the ketones (α -ketoacids, α -ketoamides, α -ketoesters and α -ketoacids) and other derivatives. These moieties inactivate the enzyme in a reversible reaction by the reversible formation of a covalent bond. In the case of the peptide aldehydes a hemithioacetal or with the ketones a thioketal would form with the cysteine's thiol group in the active site of calpain.

Peptide aldehydes

Many examples of peptide aldehydes have been synthesised for the calpains in which the mechanism of inhibition is illustrated in Figure **1.11** below. This reaction forms a reversible covalent adduct which mimics the active transition states of the thiolate complex **1.4.15** generated from normal substrate proteolysis. This type of inhibitor is considered competitive due to tight binding.⁷⁴

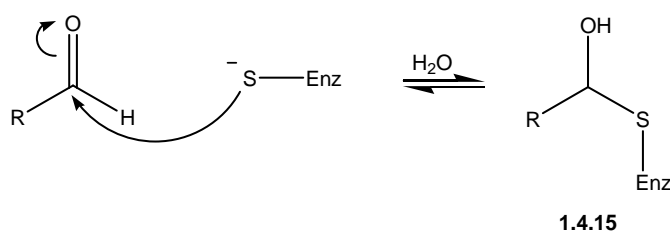
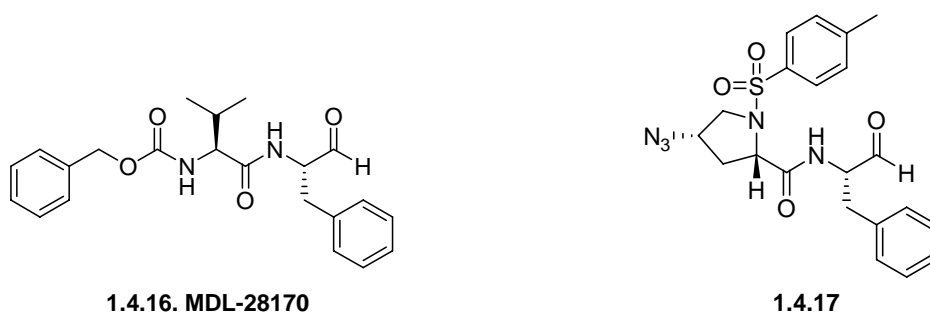


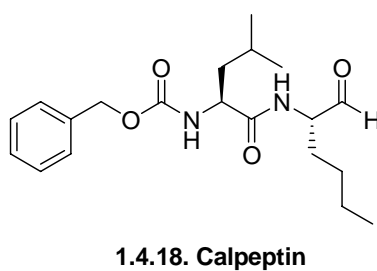
Figure 1.11 The mechanism of reversible inhibition of aldehyde inhibitors forming the hemithioacetal⁷⁴

The compound MDL-28170 **1.4.16** also referred to as calpain III inhibitor, $IC_{50} = 7$ nM for calpain¹⁶ and $IC_{50} = 21$ nM for cathepsin B. MDL-28170 exhibits good potency for both μ -calpain and m-calpain and the cathepsins. The compound **1.4.16** is used as a template compound for other potential inhibitors.



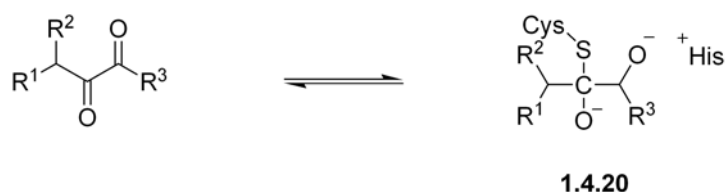
The aldehyde compound **1.4.17** is a potent and inhibitor for μ -calpain $IC_{50} = 28$ nM and $IC_{50} > 10,000$ nM for cathepsin B which exerts good selectivity for μ -calpain over cathepsin B. Compound **1.4.17** consists of a $P_2 =$ azo-proline moiety at P_2 which gives added constraint to the inhibitor.⁷⁵

The calpain inhibitor calpeptin **1.4.18** is a potent, cell penetrating inhibitor which is known to stop the activity of μ -calpain proteolysis in rat platelets after 30 min of incubation, $IC_{50} = 40$ nM for platelet μ -calpain.^{76,77}



The ketones, α -ketoacids, α -ketoamides, α -ketoesters

The ketones are generally non selective inhibitors for the cysteine proteases, their order of potency for the cysteine proteases decreases from the α -ketoacids > α -ketoamides > α -ketoesters.^{45,78} The mechanism of inhibition for the ketones α -ketoacids and α -ketoesters is given in Figure 1.12 to give products **1.4.20**. The reaction with the α -ketoamide gives structure **1.4.21** shown in Figure 1.13 below.⁷⁹



R³= Alk ketone

R³= OH α -ketoacid

R³= OAlk α -ketoester

Figure 1.12 Inhibition of the ketones, α -ketoacids and α -ketoesters⁷⁹

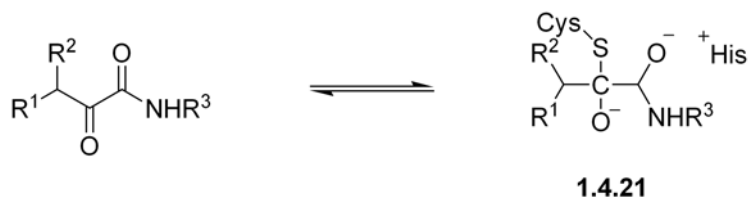
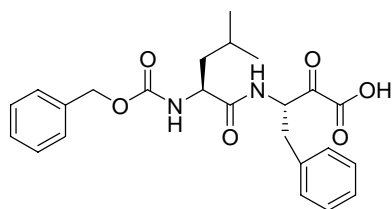
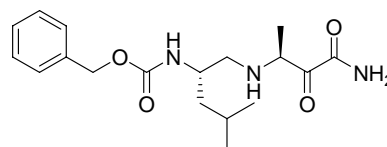


Figure 1.13 Inhibition for the α -ketoamides⁷⁹

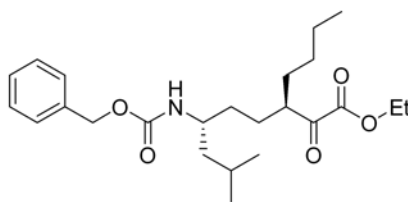
A number of ketones inhibitors based from the cathepsin B inhibitor MDL-28170 (**1.4.16**) were synthesised by Li and co-workers⁷⁸ which found the α -ketoacid **1.4.22** to be one of the most potent and selective (selectivity for m-calpain over cathepsin B 790 fold) examples of the ketones, $IC_{50} = 5.7$ nM, for m-calpain $IC_{50} = 4.5$ μ M for cathepsin B and $IC_{50} = 7.0$ μ M, for papain.⁷⁸

1.4.22. Cbz L-leucine L-phenylalaine α -keto acid

1.4.23

The α -ketoamide inhibitor **1.4.23** has high potency and a good selectivity (190-fold) for m-calpain over cathepsin B, $IC_{50} = 19$ nM for m-calpain, $IC_{50} = 3.4$ μ M for cathepsin B and $IC_{50} = 190$ μ M for papain.⁷⁸

The compound **1.4.24** is an example of a α -ketoester which gives good inhibition and (180-fold) selectivity for m-calpain over cathepsin B $IC_{50} = 180$ nM for m-calpain, $IC_{50} = 0.02$ μ M and $IC_{50} = 0.19$ μ M for papain. α -Ketoesters provide the greatest permeability and selectivity compared to the other ketones.⁷⁸



1.4.24

1.5 The cathepsins and cathepsin B structure

The cathepsins are a large group of ubiquitously expressed proteases that span three main protease groups (cysteine, aspartic and serine).⁸⁰ The largest group is of the cysteine cathepsins these consist of 11 lysosomal (human) cathepsins B, C, F, H, K, L, O, S, V, W and X.⁸¹ The other family members of the cathepsins occur in the aspartic family (cathepsin D and E) and the serine family (cathepsin G)⁸⁰ (Table 1.1). In the past 15 years the number of known beneficial and pathological roles of human cathepsins has increased two fold. The human cathepsins are involved with normal cellular functions for example, protein breakdown in lysosomes, normal protein turnover, bone remodelling and antigen processing.⁸¹ However, they play many significant roles in much human pathology, including Alzheimer's disease, arthritis, atherosclerosis and multiple sclerosis.⁸¹

Cathepsin B is the most abundant and widely studied of the human cathepsins. The enzyme is responsible for the formation of insulin from the precursor proinsulin,⁸² and intracellular protein turnover over. The enzyme has also been implicated in tumour metastasis⁸³ many other human pathologies.

Cathepsin B structure

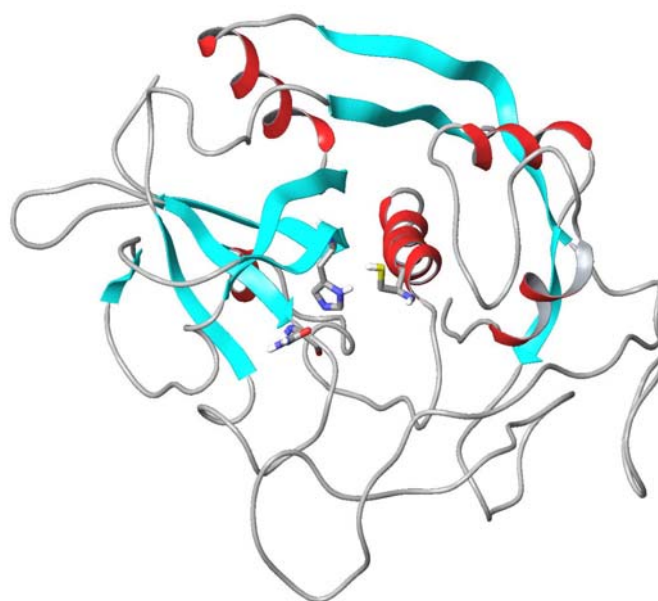


Figure 1.14 Crystallographic construct of human cathepsin B (generated by Steve McNabb). The active site (centred) contains the catalytic triad of residues asparagine (bottom left), histidine (top left) situated on R domain and cysteine (right) on the L domain

The overall structure of cathepsin B is a 30 kDa bilobal lysosomal protease (see Figure 1.14) which is disc shaped of approximately 50 Å in diameter and 30 Å thick. Cathepsin B has two discrete domains; the L and R domain these can interact together through an extended polar interface this opens out to the V-shaped active site cleft (see Figure 1.15 below).⁸⁴ The active functional thiol group belonging to cysteine (coloured yellow) can be clearly seen positioned at the bottom of the active site cleft.

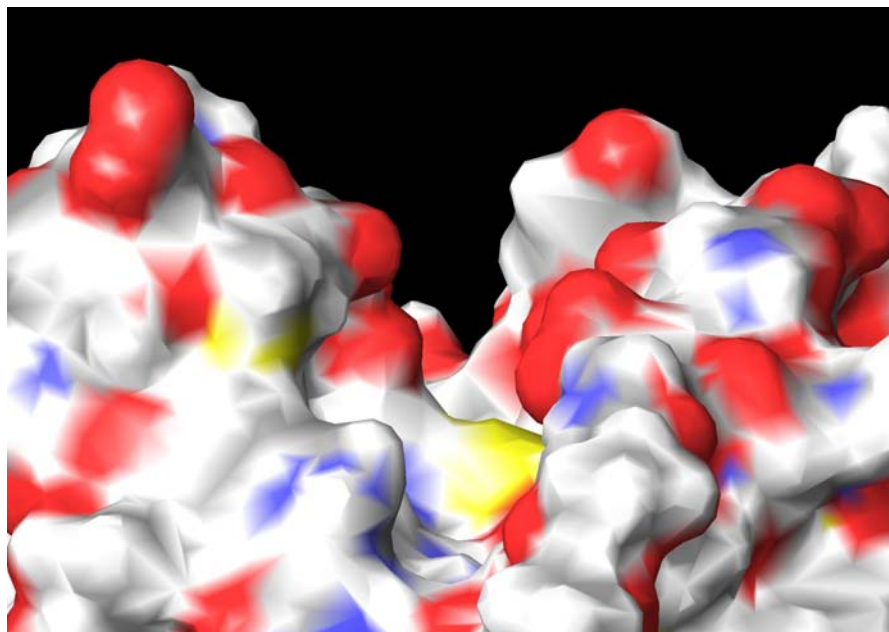


Figure 1.15 V-Shaped active site cleft of cathepsin B (generated by Steve McNabb). Colours denoting individual elements. White = carbon, red = oxygen, blue = nitrogen and yellow = sulfur

Human and rat cathepsin B consists of seven disulfide bridges, however bovine cathepsin B contains an eighth disulfide bridge. Peptide bond cleavage is catalysed by the Cys₂₉ residue which is located in the L domain. Both Cys₂₉ and His₁₉₉ form an active ion pair at pH 4.0-8.5 which initiates the peptide bond cleavage analogous to the calpains.

1.6 Inhibitors of cathepsin B

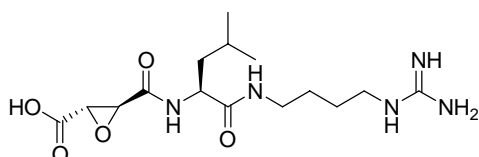
Natural inhibitors from the plant kingdom

There are three main groups of inhibitors that have been isolated from natural sources these are the aziridinyls peptides,⁸⁵ peptide epoxysuccinyls⁸⁶ and the peptide aldehydes.^{74,87,88} The majority of these inhibitors are non selective towards calpain over papain. However with the alteration of amino acids at the P₁ and P₂ address positions selectivity is gained.

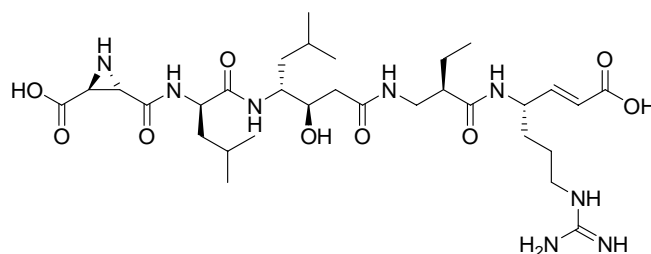
Natural irreversible inhibitors from the plant kingdom

Epoxysuccinyl peptides

The compound E-64 (**1.6.1**) was first identified and characterised by Hanada and co-workers from extracts of *Aspergillus japonicus*.⁸⁶ This natural inhibitor contains an epoxide warhead and gives reverse binding to an enzymes active site.⁸⁹ The compound has low selectivity for the cathepsins for example cathepsin B $IC_{50} = 55 \text{ nM}$ ⁹⁰ ($k_{2nd \text{ rate}} 89,400 \text{ M}^{-1}\text{s}^{-1}$)⁹¹ and cathepsin L $IC_{50} = 68 \text{ nM}$ ⁹⁰ ($k_{2nd \text{ rate}} 96,250 \text{ M}^{-1}\text{s}^{-1}$).⁹¹ E-64 is used as a template compound for the design of other potential inhibitors.⁷⁴



1.6.1. E-64



1.6.2. Mizaridine

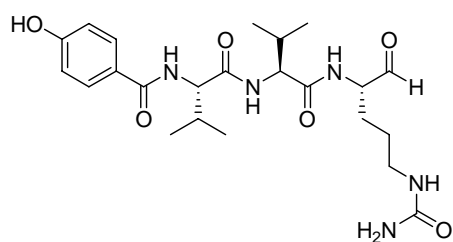
Aziridinyl peptides

Mizaridine **1.6.2** extracted from a marine sponge *Theonella mirabilis* containing the aziridinyl war head. This natural product is non selective with inhibition of $IC_{50} = 2.05 \text{ }\mu\text{M}$ for cathepsin B.⁸⁵

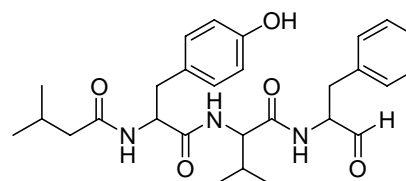
Natural reversible inhibitors from the plant kingdom

Peptide aldehydes

Many peptide aldehydes have been isolated from the plant kingdom such as tokaramide A **1.6.3** which gives potent inhibition for cathepsin B, $IC_{50} = 62.4 \text{ nM}$. The natural product is extracted from the marine sponge *Theonella mirabilis* containing the aldehyde war head. Tokaramide A is known to be a non selective inhibitor.^{74,87}



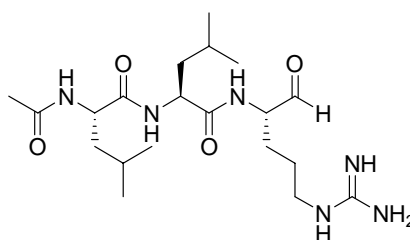
1.6.3. Tokaramide A



1.6.4. YM-51084

The natural product YM-51084 (**1.6.4**) is extracted from *Streptomyces* species known to be a potent inhibitor against cathepsin B $IC_{50} = 12.0$ nM and cathepsin L $IC_{50} = 9.6$ nM.⁷⁴

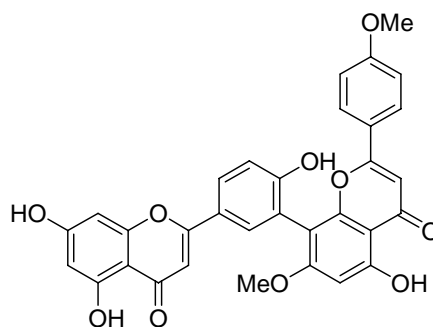
The compound leupeptin **1.6.5** has been shown to be a slow tight binder to cathepsin B with a K_i value of 4-5 nM. This natural product is one of the most highly potent, non selective, natural aldehyde inhibitors known⁸⁸ and has been used as a template.²⁰



1.6.5. Leupeptin

Flavones

Dimethylamentoflavone **1.6.6** is extracted from *Ginkgo biloba* and *Hypericum perforatum* (St John's wort). These compounds can be classified as non-peptide inhibitors which are strong inhibitors of cathepsin B ($IC_{50} = 550$ nM).⁹²



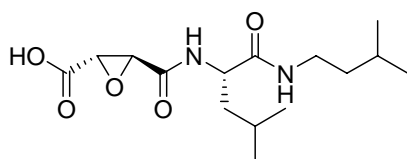
1.6.6. Dimethylamentoflavone

Synthetic irreversible cathepsin B inhibitors

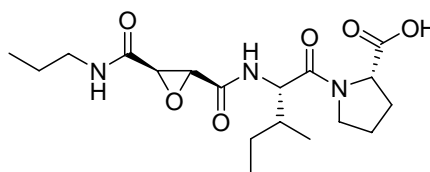
Irreversible cathepsin B inhibitors include the; epoxysuccinyls peptides,^{89,93} cyclic sulfates,^{79,94,95} aziridine peptides,⁹⁶ thiadiazoles,⁹⁷ acylomethyl ketone peptides,⁹⁸ to name a few.

Expoxy succinyl peptide

The epoxysuccinyls peptide inhibitors represent a well studied group of inhibitors; this group is based on the non selective inhibitor E-64 **1.6.1**. Many attempts to improve the selectivity of this group have been performed but without success, for example E-64c **1.6.7** for rat cathepsin B $IC_{50} = 8.70$ nM and rat cathepsin L $IC_{50} = 3.5$ nM. However, the inhibitor CA-0074 **1.6.8** containing the *iso* L-leucine-proline moiety, is selective for cathepsin B over cathepsin L with respective activities $IC_{50} = 1.94$ nM and $IC_{50} = 233$ μ M.⁹⁹ Normal substrate binding occurs with **1.6.8** and cathepsin B preferring the RR ring configuration.^{89,93}



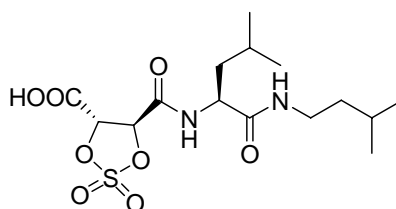
1.6.7. E-64c



1.6.8. CA-0074

Cyclic sulfates

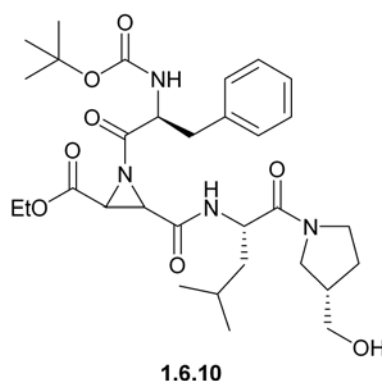
Compound **1.6.9** is a derivative of E-64 in which is highly potent for cathepsin B ($IC_{50} = 0.7$ nM). Steric hindrance of the cyclic sulfate may play a role in the lack of inhibition observed for the serine proteases and calpain $IC_{50} = 300$ nM.^{79,94,95}



1.6.9

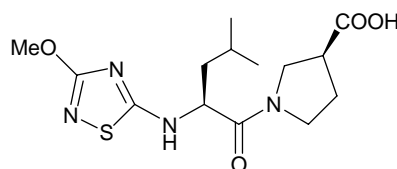
Aziridine peptides

Other derivatives to the epoxysuccinyls peptides are the aziridines where the synthesis employs the electrophilic building block, aziridine-2,3-dicarboxylate. However compared to epoxides the aziridines offer greater variability as potential inhibitors, where the nitrogen atom on the aziridine ring offers extra derivatation and variability on structural and activity relationship (SAR) studies⁹⁶ Generally, inhibitors synthesised with the aziridine ring are very selective for cathepsin B over other cathepsins, in a centralised position. The compound **1.6.10** has the nitrogen substituted aziridine ring in a central position which gives good inhibition and selectivity. The second order rates of inhibition are as follows, $k_{2nd\ rate}$ 6,859 ($M^{-1}\ min^{-1}$) for cathepsin B and cathepsin L $k_{2nd\ rate}$ 212 ($M^{-1}\ min^{-1}$).^{89,96}



Thiadiazoles

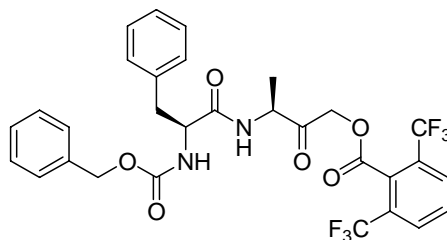
Compound **1.6.11** is a moderately potent cathepsin B inhibitor 2.6 μM . This 1,2,4-thiadiazole heterocyclic ring acts as a good thiol trapping pharmacophore and is known to form a disulfide adduct upon inhibition.⁹⁷



1.6.11. MeO-NH L-leucine L-proline

Acyloymethyl ketone peptides

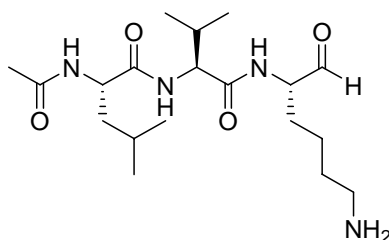
These groups of compounds use a P₁ and P₂ address region recognition sequence, in this case cbz-Phe-Ala and a space filling leaving group. The compound **1.6.12** has proven to be cathepsin B selective over m-calpain with a second order rate of inhibition ($k_{2nd\ rate} 1,600,000\ (M^{-1}\ s^{-1})$) for bovine spleen cathepsin B compared to no observed inhibition for chicken smooth muscle m-calpain. It has been proposed that m-calpain may have low tolerance for the bulky space filling (aryloxy) group.⁹⁸



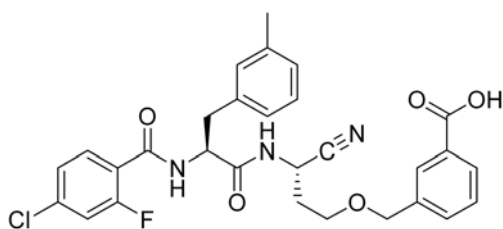
1.6.12

Synthetic reversible cathepsin B inhibitors

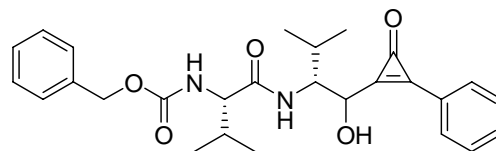
Examples of reversible inhibitor groups are peptide aldehydes,²⁰ nitriles^{74,100,101} and cyclopropanones.¹⁰²



1.6.13



1.6.14



1.6.15. Cbz L-valine D-valine cyclopropanone-Ph

Aldehyde peptides

For the peptide aldehydes the mechanism of inhibition for cathepsin B is analogous to the calpains. Many aldehyde inhibitors have been synthesised over the last 15 years however few have proven to be cathepsin B selective.⁷⁴ For example the compound **1.6.13** is a derivative of leupeptin **1.6.5** of high potency for the cysteine proteases (cathepsin B $IC_{50} = 4$ nM, trypsin $IC_{50} = 45$ nM, and plasmin $IC_{50} = 13$ nM). However like the majority of the aldehydes it is non selective.²⁰

Nitrile peptides

Evidential NMR studies show the nitrile mechanism of inhibition is through the formation of the reversible thiomidate intermediate¹⁰⁰ with mutation studies suggesting **1.6.16** is stabilised with a gly₁₉ residue¹⁰¹ (Figure 1.16). An example of the nitrile peptides is the compound **1.6.16** highly potent inhibitor of cathepsin B $IC_{50} = 2.0$ nM.⁷⁴

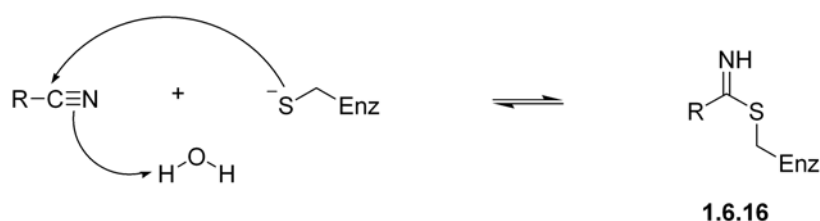


Figure 1.16 The mechanism of inhibition of cathepsin B from nitriles⁷⁴

Cyclopropenones

The cyclopropenone ring present in this group of compounds gives rise to amphiphilic properties. Reversible inhibitors can be obtained by exploiting the cyclopropenone rings electrophilic properties (Figure 1.17). Or conversely the design of inhibitors that allow the protonation of the carbonyl group of the cyclopropenone ring to give a stable 2π -aromatic hydroxycyclopropenium cation **1.6.17** will give rise to irreversible inhibitors. The compound cbz L-valine cyclopropenone-Ph **1.6.17** synthesised by Ando and colleges exhibited high potency towards the cysteine proteases such as the calpains, papain, cathepsin B and L.^{74,102}

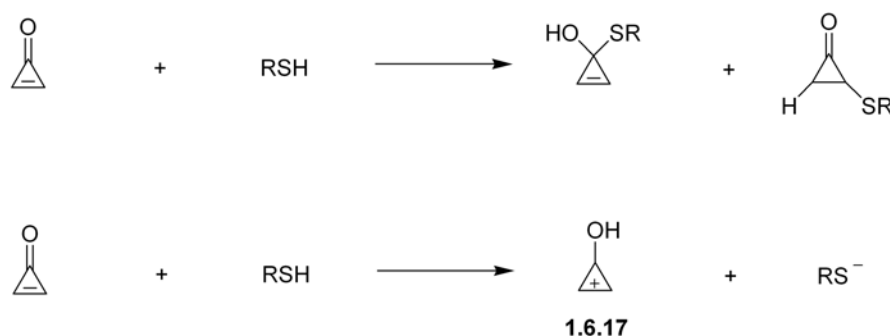
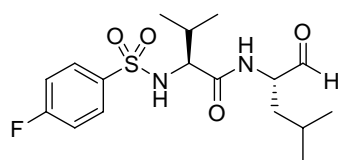


Figure 1.17 The amphiphilic properties of the cyclopropanone ring^{79,102}

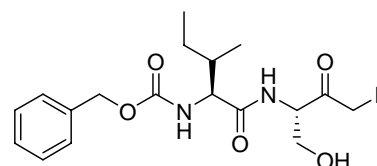
1.7 Drug design and rational

Since toxicity of irreversible inhibitors is greater than reversible inhibitors, the search for reversible, potent, selective, cell permeable calpain and cathepsin B inhibitors is highly sort. The reversible inhibitors commonly employ a peptide address region on P₂ and P₃ for enzyme recognition and an electron deficient warhead, for example, an aldehyde.¹⁰³

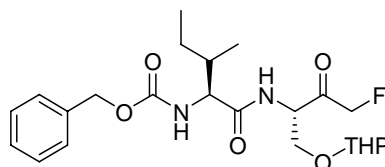
Structural activity relationship studies (SAR) on the calpains demonstrated a preference for substrate binding of L-valine and L-leucine at the P₂ address region. However, Tripathy and co-workers⁷⁵ demonstrated bulk tolerance for the S₂ subsite of μ -calpain by synthesising the compound **1.4.17** containing the azo-proline moiety at P₂ address region.⁷⁵ The S₁ subsite in calpains has shown tolerate a wide range of amino acid types bulky groups, for example L-phenylalanine in **1.4.16** and **1.4.17**, to non-bulky groups such as L-leucine in SJA-6017 **1.7.1**¹⁰³ However studies have demonstrated the S₁ subsites incompatibility to amino acids with polar groups, for example the insertion of D,L-tyrosine at the P₁ address region for the compound **1.4.13** ($k_{2nd\ rate} 17,000\ (M^{-1}\ s^{-1})$ m-calpain)⁷⁰ compared to the phenylalanine analogue $k_{2nd\ rate} 136,300\ (M^{-1}\ s^{-1})$ for m-calpain.¹⁰⁴ Later Chatterjee and co-workers¹⁰⁵ inserted L-serine into the P₁ address region to give compound **1.7.2** $k_{2nd\ rate} 21,000\ (M^{-1}\ s^{-1})$ μ -calpain), compared to the serine (THP) protected compound **1.7.3** ($k_{2nd\ rate} 100,000\ (M^{-1}\ s^{-1})$ μ -calpain). This confirmed the μ -calpain isoform also had similar incompatibilities to polar amino acids.¹⁰⁵



1.7.1. SJA-6017



1.7.2



1.7.3

THP= Tetrahydropyranyl ether

The main focus of this study was the synthesis of selective, reversible, first generation peptide aldehyde m-calpain and cathepsin B inhibitors. The method adopted throughout this investigation was using an aldehyde warhead and variations of α -amino acid residues inserted into the P₁ and P₂ address regions (Figure 1.18). The compounds synthesised in this study were based on the SJA-6017 construct¹⁰³ bearing the *N*-(4-fluorobenzenesulfonyl) moiety at the P₃ address region.

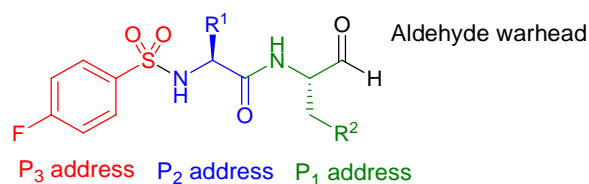
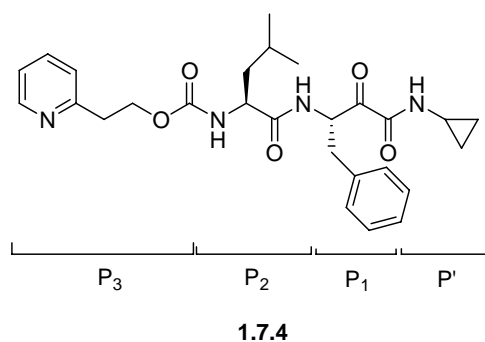


Figure 1.18 The first generation peptide aldehyde construct based on SJA-6017¹⁰³

The first generation construct consists of three main binding regions and a covalent or non covalent warhead region. These binding regions are known as the P₁, P₂ and the P₃ address these regions bind to the active site of calpain and cathepsin B via the S₁, S₂ and the S₃ region respectively.

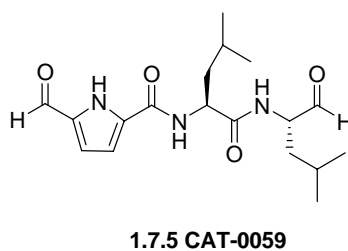
The peptide aldehyde SJA-6017 is one of the most well studied calpain inhibitors which was originally developed in 1997 by Fukiage *et al* at Senju Pharmaceutical Corporation Limited.¹⁰³ They developed their compound from MDL-28170 **1.4.16**, and found the inhibitor to be of higher permeability and potency compared to the natural epoxide based inhibitor E-64 **1.6.1**. Recently SJA-6017 was found to be active against selenite induced cataract in rats and cataract induced porcine lenses.¹⁰⁶ Despite SJA-6017's potency to the calpains, selectivity towards other cysteine proteases such as the cathepsins for example cathepsin B is even greater

(calpain $IC_{50} = 80$ nM and cathepsin B $IC_{50} = 1.6$ nM). Permeability is problematic and entry into the cell is limited; furthermore bioavailability is compromised by the inhibitor reacting with other proteins. To improve the selectivity, potency and bioavailability Senju Pharmaceuticals developed further SJA-6017 analogues by varying the P_1 and P_3 address positions.¹⁰⁷ Solubility was optimised by the insertion of a pyridineethanol moiety at the P_3 address region and the warhead was made more metabolically stable by using a novel ketoamide with an extended P prime cyclopropyl moiety.¹⁰⁸



Potential first generation inhibitors based on the CAT-0059 construct¹⁰⁹

The synthesis of novel calpain inhibitors consisting with the 5-formyl pyrrole moiety were based on the calpain inhibitor 5-formyl pyrrole 2-carboxylate L-leucine L-leucinal **1.7.5** ($IC_{50} = 25$ nM for m-calpain) synthesised by Miyamoto at the University of Canterbury.¹⁰⁹



Modelling

The potential inhibitors selected for synthesis were the result of modelling preformed by Axel Neffe, Blair Stuart and Wangting Jiao using macro model (water simulation) to generate the conformers set within a 12 kJ window of the global minimum. The low energy conformers were docked into the rigid active site of μ -calpain. From the results of rigid docking studies the following parameters were established warhead distance, glide score, and H-bonding contacts.

The warhead distance is the distance between the carbonyl carbon of the aldehyde functionality of an inhibitor in question to the cysteine sulfur atom in the active site of μ -calpain or cathepsin B. In addition the direction angle is also important. For nucleophilic attack from the cysteine residue to occur, a distance of $<5\text{\AA}$ is required. The glide score parameter can be translated into the binding energy between the hydrogen bonds of the enzyme and the ligands of the inhibitor. However good glide scores may not necessarily result in a low IC_{50} concentration.

Requirement of the extended β -strand conformation

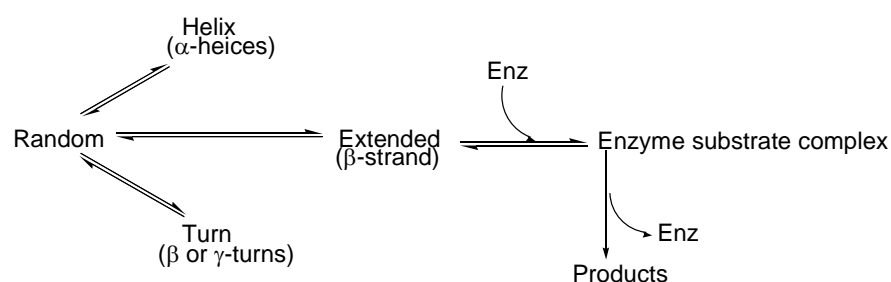


Figure 1.19 Conformational enzymatic selection of substrate/inhibitor by the proteases¹¹⁰

The significance of the extended β -strand conformation (see Figure 1.19) is necessary to facilitate the proteolysis of substrates, inhibitors, including peptide and non peptide forms. This was demonstrated by Fairlie *et al*¹¹⁰ who analysed 266 protease/inhibitor crystal structures spanning from the aspartic, serine, cysteine and metalloprotease families. The non-extended conformations (random, helix and turns) on substrate/inhibitors have their scissile amide bonds protected from proteolysis due to folding of the substrate/inhibitor. Therefore the information gleaned from this paper may suggest that enzyme binding affinities can be increased if the general methods in the organisation of conformational constraining substrate/inhibitors into the extended β -strand conformer.¹¹⁰

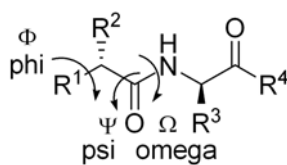


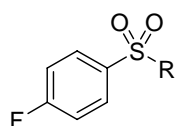
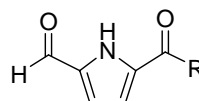
Figure 1.20 Nomenclature of torsion angles.^{111,112} Fairlie *et al*¹¹⁰ found that the Ramachandran plot for ten cyclic HIV-1 protease inhibitors fell in a typical for extended β -strands with torsion angles of $\Phi = -56.9^\circ$ to -169.5° $\Psi = 35.2^\circ$ to 161.7° and a fixed $\Omega = 180^\circ$

Chapter 2

Results and Discussion

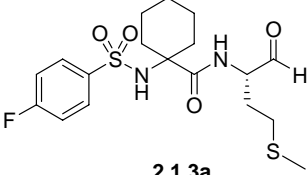
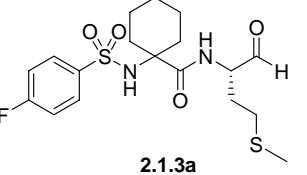
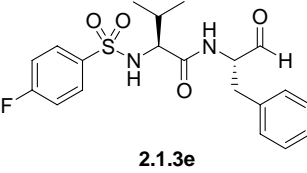
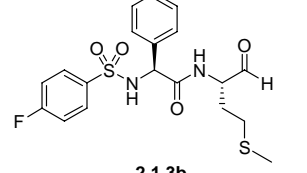
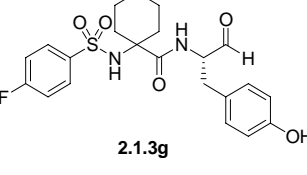
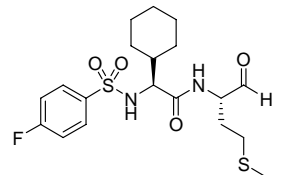
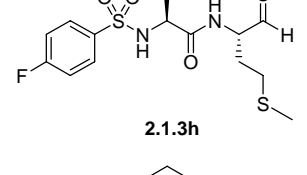
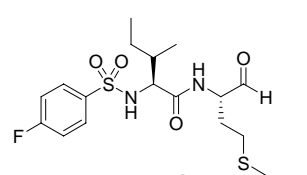
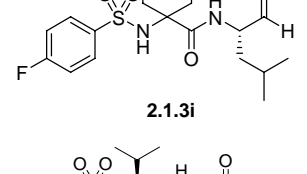
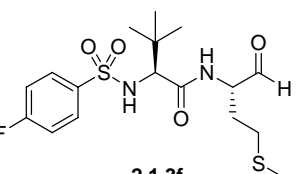
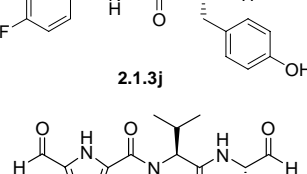
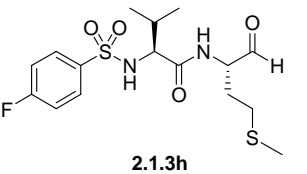
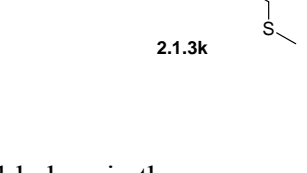
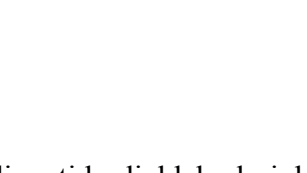
2.1 Synthesis of CA clan inhibitors (m-calpain and cathepsin B)

Throughout the duration of this study the following peptide aldehyde inhibitors have been successfully synthesised for the inhibition of m-calpain and cathepsin B (in Table 2.1 below). These compounds were selected from vigorous modelling studies involving the μ -calpain and cathepsin B active site constructs (addressed in sections 1.7 and 2.5). These synthesised inhibitors were based on using a rigid P₃ address region of either *N*-(4-fluorobenzenesulfonyl) **2.1.1** or 5-formyl pyrrole **2.1.2** moieties. As previously mentioned in Chapter 1 the variations of natural and unnatural amino acid residues at the P₁ and P₂ address region are summarised in Table 2.1 below. Note some of these inhibitors can be classified into both themes.

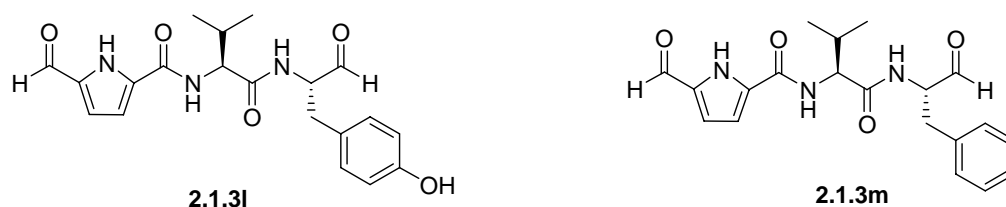
**2.1.1****2.1.2**

The synthesis of the first generation inhibitors commonly involved either the free or boc protected α -amino acids **2.1.4** where the carbonyl group was first protected as the ester **2.1.5**, followed by reaction with *N*-(4-fluorobenzenesulfonyl) sulfonyl chloride **2.1.6**. The sulfonamide methyl esters **2.1.7** were hydrolysed and the resulting acids **2.1.8** coupled to the esters of L-tyrosine, **2.1.9a** L-methionine **2.1.9b** and L-phenylalanine **2.1.9c**. Reduction of the corresponding sulfonamide peptide esters **2.1.10** gave the corresponding alcohols **2.1.11** and these were oxidised to the target aldehydes **2.1.3**.

Table 2.1 Synthesised peptide aldehyde inhibitors (variations at P₁ and P₂ address regions)

P ₁ Variations	P ₂ Variations
 <p style="text-align: center;">2.1.3a</p>	 <p style="text-align: center;">2.1.3a</p>
 <p style="text-align: center;">2.1.3e</p>	 <p style="text-align: center;">2.1.3b</p>
 <p style="text-align: center;">2.1.3g</p>	 <p style="text-align: center;">2.1.3c</p>
 <p style="text-align: center;">2.1.3h</p>	 <p style="text-align: center;">2.1.3d</p>
 <p style="text-align: center;">2.1.3i</p>	 <p style="text-align: center;">2.1.3f</p>
 <p style="text-align: center;">2.1.3j</p>	 <p style="text-align: center;">2.1.3g</p>
 <p style="text-align: center;">2.1.3k</p>	 <p style="text-align: center;">2.1.3h</p>

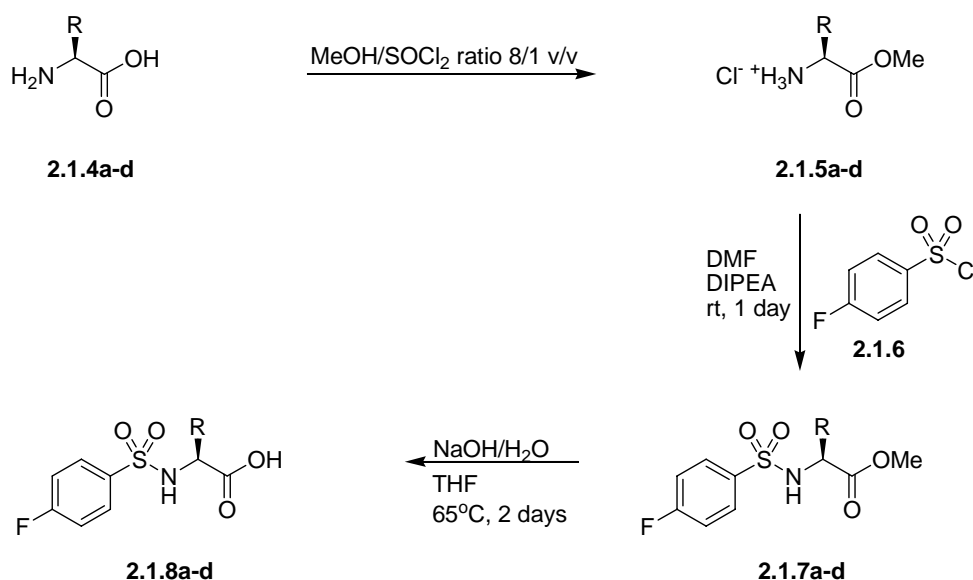
Displayed below is the unsuccessful synthesis of two dipeptide dialdehyde inhibitors these are the L-tyrosinal **2.1.3l** and the L-phenylalaninal **2.1.3m**. These potential inhibitors are included in the P₁ variation theme containing the 5-formyl pyrrole **2.1.2** functionally at the P₃ address region, L-valine at P₂ and L-tyrosine or L-phenylalanine at P₁ respectively.



Synthesis of *N*-(4-fluorobenzenesulfonyl) α -amino acids **2.1.8a-f**

Synthesis of **2.1.8a-d**

The starting α -amino acids 1-aminocyclohexanecarboxylic acid **2.1.4a**, L-phenylglycine **2.1.4b**, L-cyclohexylglycine **2.1.4c** and boc *iso* L-leucine **2.1.4d** were esterified with MeOH and SOCl₂ to give the methyl ester hydrochlorides **2.1.5a-d** (Scheme 2.1). The hydrochloride salts were reacted with the sulfonyl chloride **2.1.6** to give in near quantitative yields of the sulfonyl α -amino acid methyl esters **2.1.7a-d**. The methyl esters were hydrolysed with aqueous sodium hydroxide in THF, under reflux for one day to give the corresponding free acids **2.1.8a-d**. The acids **2.1.8a-d** were confirmed with ¹H NMR and mass spectra displaying characteristic peaks with chemical shifts in deuterated DMSO for **2.1.8b** $\delta_{\text{H}} = 13.10$ -12.50 for the acid proton (COOH $\underline{\text{H}}$). The acid **2.1.8c** in CD₃OD displayed two multiplets for the CH₂'s from $\delta_{\text{H}} = 1.71$ -1.56 and $\delta_{\text{H}} = 1.28$ -1.00. Mass spectra for **2.1.8a-d** displayed the hydrogen and sodium adducts for conformation.



2.1.8a R= 1,1 cyclohexane

2.1.8b R= Ph

2.1.8c R= cyclohexane

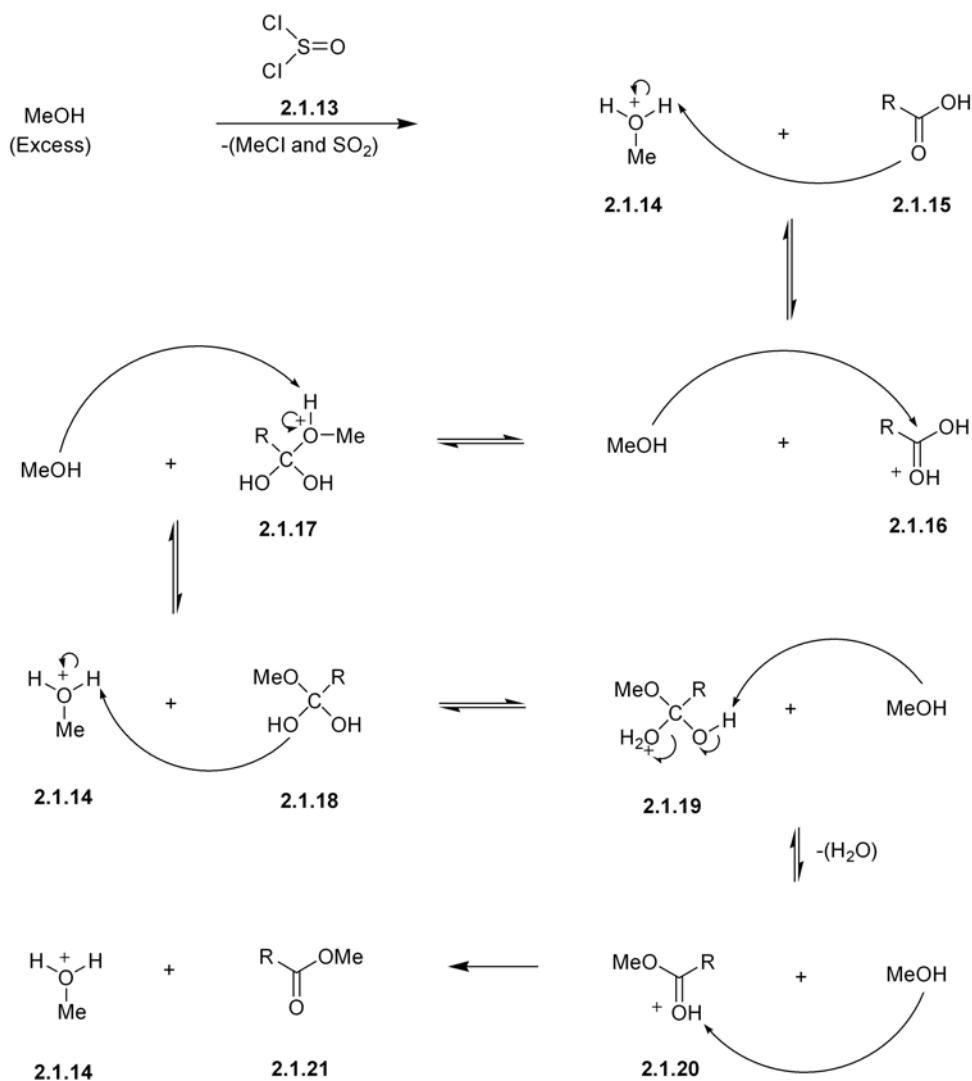
2.1.8d R= (*S*)-*i*-Bu

Scheme 2.1 Synthesis of the sulfonamide α -amino acids 2.1.8a-d

Carbonyl group protection via Fisher esterification

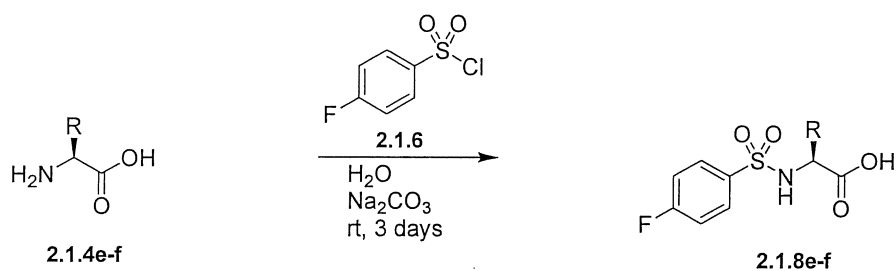
Esterification of the following α -amino acids, 2.1.4a-d and L-tyrosine 2.1.12a to their corresponding methyl esters was carried out by standard methods using L-methanol and eight equivalents of thionyl chloride (SOCl_2). Other esterification reactions involving optimised methods were performed on L-methionine 2.1.12b and boc L-methionine 2.1.12c (as detailed later).

Esterification was effected under Fisher acid catalysed conditions (Scheme 2.2). The Fisher esterification mechanism starts with the reaction of alcohol with thionyl chloride to afford a proton source. This proton source takes the form of the methyloxonium ion 2.1.14 which protonates the carbonyl group of the α -amino acid 2.1.15 to give the oxonium ion 2.1.16. This generates reactive electrophile for the subsequent nucleophilic attack by methanol to give a tetrahedral intermediate 2.1.17. This intermediate 2.1.17 will generate the more of the ion 2.1.14 and the methoxydiol compound 2.1.18. Protonation of the compound 2.1.18 will lead to 2.1.19 and subsequent elimination of water to afford 2.1.20. Nucleophilic attack with methanol to gives the methyl ester 2.1.21 and regenerate methyloxonium ion 2.1.14.



Scheme 2.2 Carbonyl group protection via Fischer esterification

The α -amino acids L-valine and *tert* L-leucine did not require the esterification step and therefore were reacted directly with the sulfonyl chloride **2.1.6** to give the sulfonyl L-valine **2.1.8e** and the sulfonyl *tert* L-leucine **2.1.8f** (Scheme 2.3), with respective yields of 79% and 77%. The compounds were confirmed with ^1H NMR and mass spectra (Figures 2.1 and 2.2). Compound **2.1.8e**, displayed the characteristic peaks of two doublets for the methyl protons on L-valine ($\delta_{\text{H}} = 0.92$ and $\delta_{\text{H}} = 0.88$). For compound **2.1.8f** a nine proton singlet was observed for the equivalent *t*-butyl protons on *tert* L-leucine ($\delta_{\text{H}} = 0.97$).



2.1.8e. R= *i*-Pr

2.1.8f. R= *t*-Bu

Scheme 2.3 Synthesis of *N*-(4-fluorobenzenesulfonyl) L-valine **2.1.8e** and *N*-(4-fluorobenzenesulfonyl) *tert* L-leucine **2.1.8f**

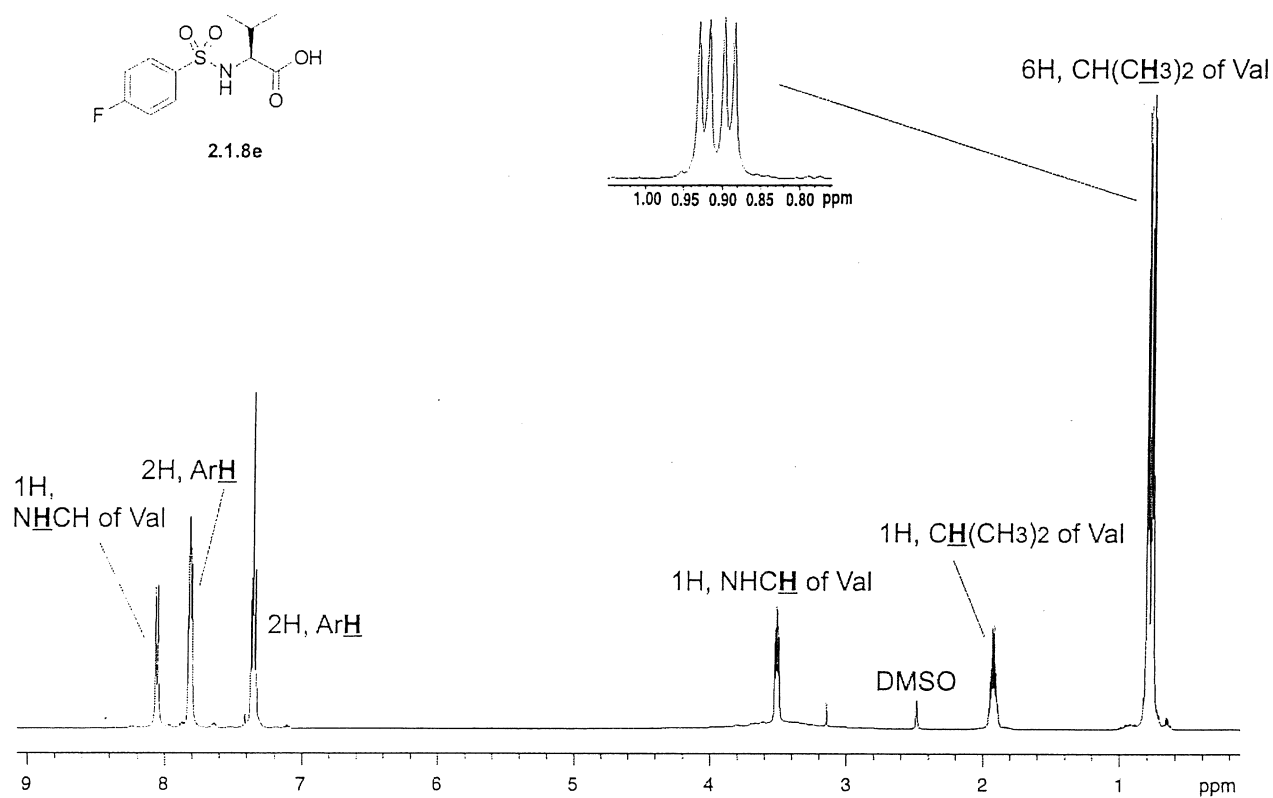


Figure 2.1 ^1H NMR spectra for *N*-(4-fluorobenzenesulfonyl) L-valine **2.1.8e**, in deuterated DMSO with L-valine methyl inset

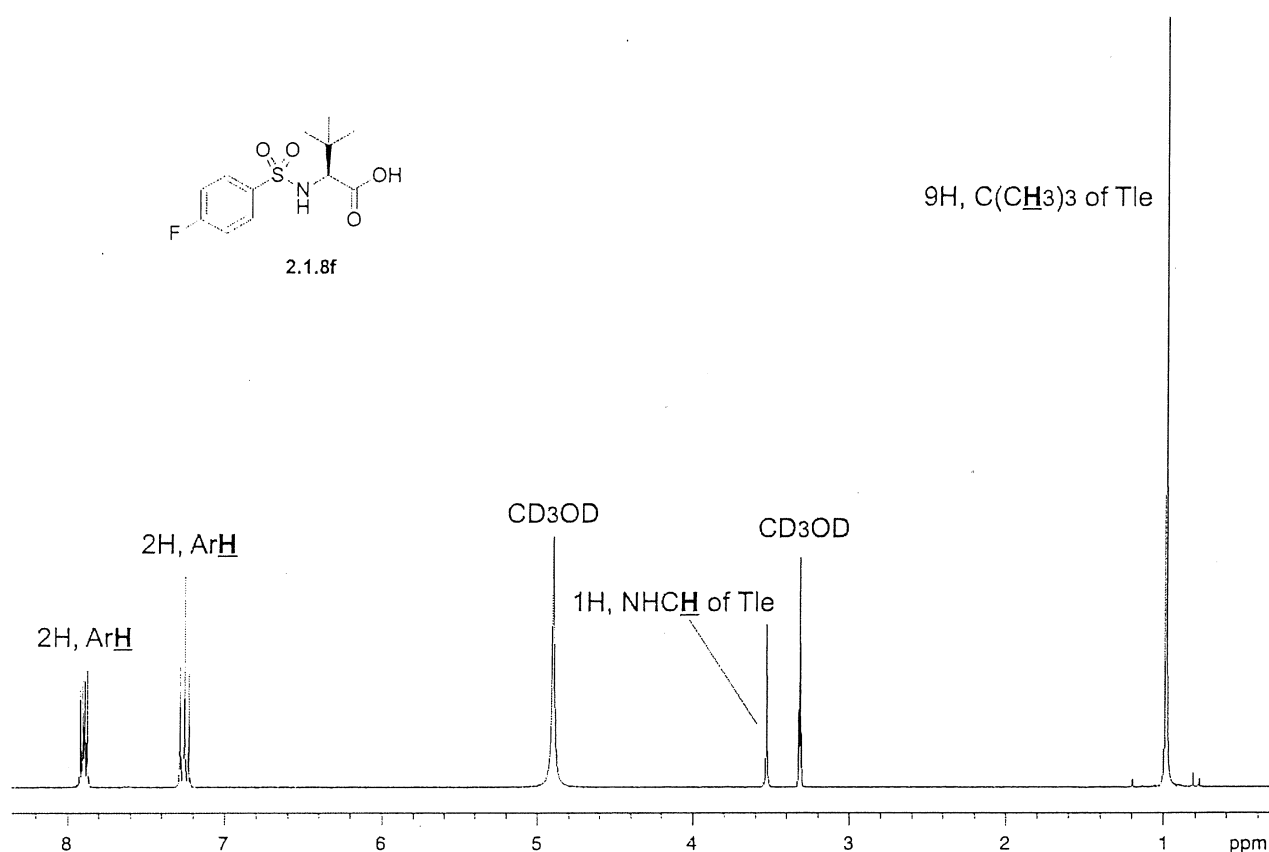
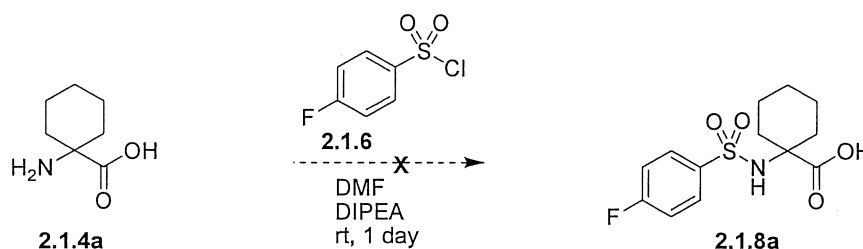


Figure 2.2 ^1H NMR spectra for *N*-(4-fluorobenzenesulfonyl) *tert* L-leucine **2.1.8f** in CD_3OD

Attempted synthesis of 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylic acid **2.1.8a**

The synthesis of **2.1.8a** first involved reaction of the carboxylic acid **2.1.4a** and the sulfonyl chloride **2.1.6** (see Scheme 2.4), but ^1H NMR and mass spectra, showed a complex product mixture and this mixture was not purified further. It was found necessary to protect the carboxyl group as the ester.

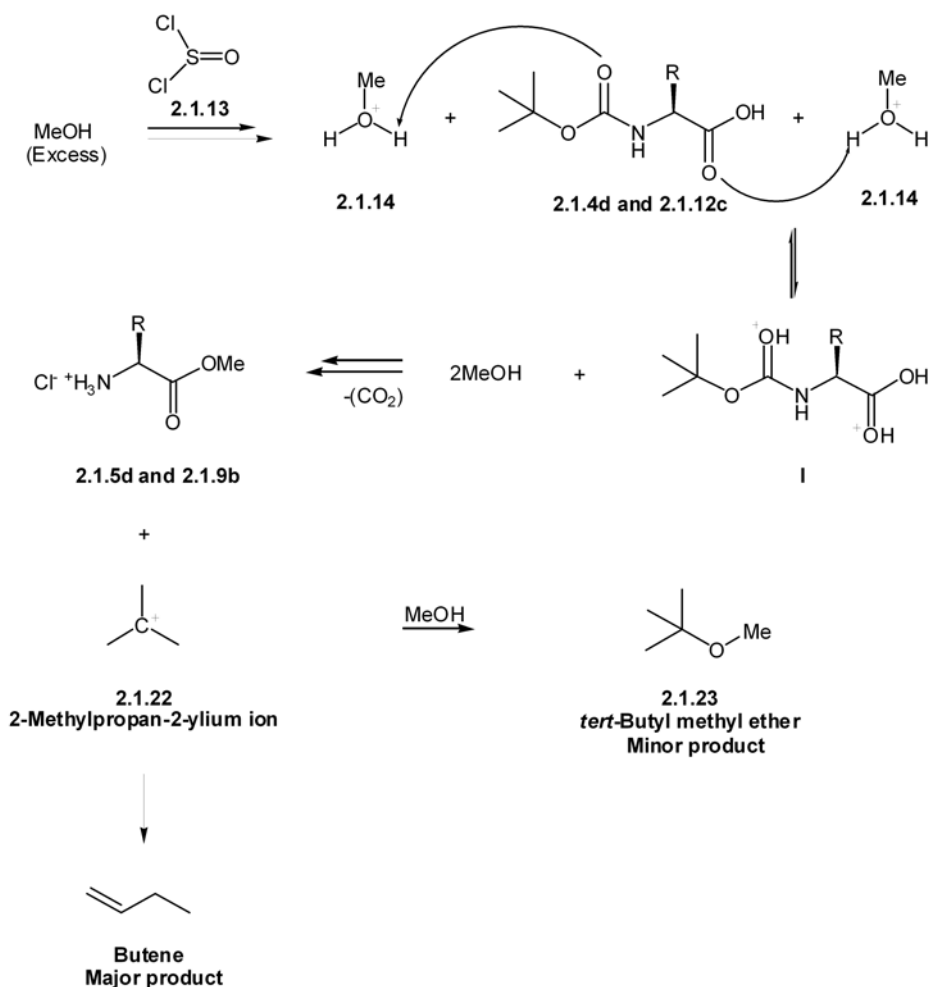


Scheme 2.4 The attempted synthesis of 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylic acid **2.1.8a** (without esterification)

The second synthesis involved esterification of the acid **2.1.4a** using standard literature procedures with MeOH and SOCl₂ and gave a quantitative yield of the corresponding methyl ester hydrochloride salt (Scheme 2.1). The hydrochloride salt was reacted with the sulfonyl chloride **2.1.6** in the presence of DMF to give compound **2.1.7a** which was hydrolysed with aqueous sodium hydroxide in THF for 21 hr to give in near quantitative yield of **2.1.8a**. The acid **2.1.8a** was confirmed with ¹H NMR spectra displaying characteristic peaks with chemical shifts in deuterated DMSO $\delta_{\text{H}} = 12.50$ for the acid proton (COOHH of Chx), and the singlet observed at $\delta_{\text{H}} = 8.01$ as the NHC proton.

Boc deprotection via Fisher esterification

This reaction was performed on the α -amino acids that permitted boc deprotection and esterification in one step (Scheme 2.5 below) on boc *iso* L-leucine **2.1.4d** and previously mentioned boc L-methionine **2.1.12c**. This reaction is similar to Fisher esterification (Scheme 2.2) using the proton source of **2.1.14**, but nucleophilic attack of MeOH (I) and the activated electrophile allows for decomposition of the boc group to carbon dioxide, the 2-methylpropan-2-ylum cation **2.1.22** and the hydrochloride salt of the α -amino methyl esters **2.1.9b** and **2.1.5d**. The cation **2.1.22** reacts with methanol to give butene as the major product and the *tert*-butyl methyl ether **2.1.23** as the minor product and regenerates a proton. The *tert*-butyl methyl ether is easily removed *in-vacuo* due to its low boiling point.



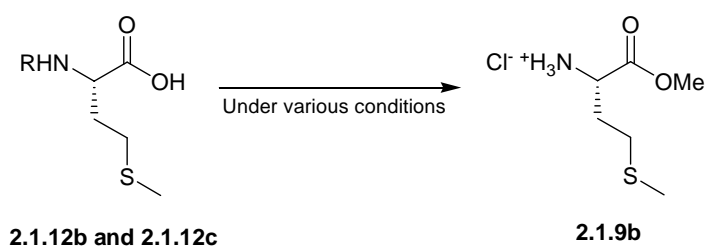
2.1.4d. R= (*S*)-*i*-Bu

2.1.12c. R= $\text{CH}_2\text{CH}_2\text{SCH}_3$

Scheme 2.5 Boc deprotection via Fischer esterification for 2.1.4d and 2.1.12c

Synthesis of L-methionine methyl ester hydrochloride 2.1.9b

Esterification of boc L-methionine and L-methionine was not possible under standard conditions using 8 equivalents SOCl_2 . The ^1H NMR spectra was complex. It was necessary to perform a number of small scale reactions under varying conditions to optimise reaction conditions. These involved the use of two compounds (shown in Scheme 2.6) boc protected L-methionine and L-methionine. Four reactions were carried out on each starting material as summarised in Tables 2.2 and 2.3.



2.1.12b. R= H
2.1.12c. R= boc

Scheme 2.6 The esterification of L-methionine **2.1.12b** and boc L-methionine **2.1.12c** under different conditions

Esterification of the α -amino acid (Table 2.2) was unsuccessful. The ^1H NMR spectra showed the product was present along with other products and starting material. The route was abandoned.

Table 2.2 Optimisation reactions for the esterification of L-methionine

Equivalence of L-Met	SOCl_2	MeOH	Reaction time & temp.	Purity ^a (%)
8	1	4	4 hr, -20°C → 66 hr, rt	65
1.83	1	6	3 hr, -20°C → 13 hr, rt	97
1.23	1	8	3 hr, -20°C → 13.5 hr, rt	98
1.1	1	25	3 hr, -20°C → 3 hr, rt	91

^a Purity estimated on peak heights (^1H NMR)

The esterification reaction involving boc L-methionine was successful under the given experimental conditions as highlighted in Table 2.3 and detailed in Chapter 3.

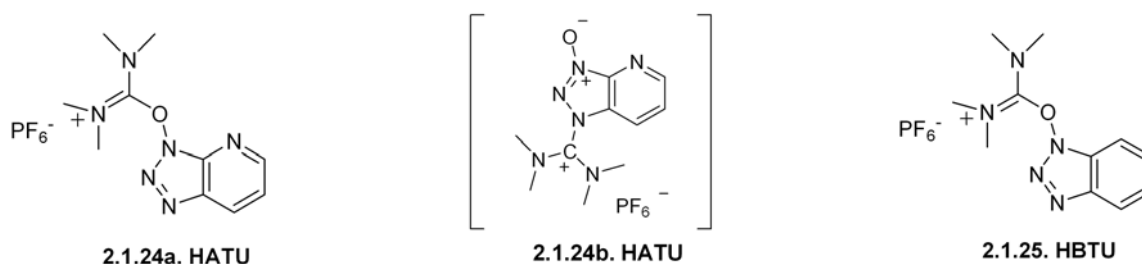
Table 2.3 Optimisation reactions for the esterification of boc L-methionine

Equivalence of boc L-Met	SOCl ₂	MeOH	Reaction time & temp.	Purity ^a (%)
8	1	4	1 hr, 0°C → 19 hr, rt	95
1.2	1	24.6	2 hr, -20°C → 3 hr, rt	100
1.83	1	16.2	2 hr, -20°C → 3 hr, rt	98
2.4	1	11.2	2 hr, -20°C → 3 hr, rt	93

^a Purity estimated on peak heights (¹H NMR)

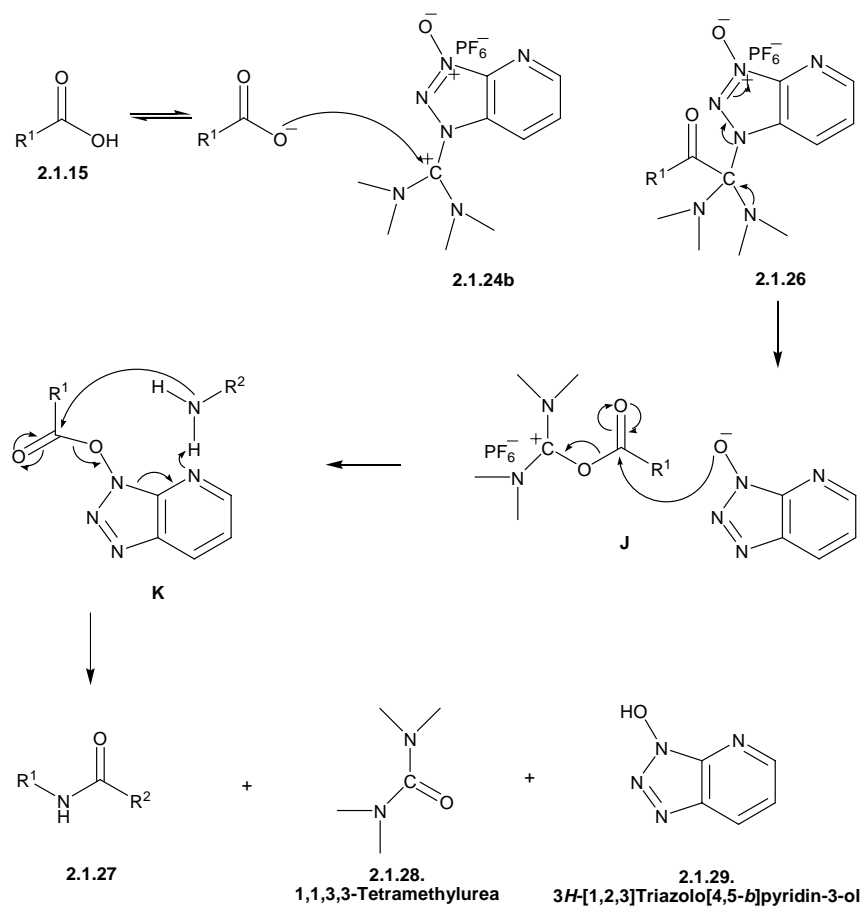
Synthesis of *N*-(4-fluorobenzenesulfonyl) peptide alcohols from HATU coupling

The synthesis of sulfonyl peptide alcohols requires the formation of an amide bond, using a coupling reagent such as HATU **2.1.24a** (**2.1.24b**). The advantages of HATU over other coupling reagents such as HBTU **2.1.25** is the generation (from α -amino acids) of highly reactive 3*H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-olate esters (COOAt) compared to the 1*H*-benzo[*d*][1,2,3]triazol-1-olate esters (COOBt) from HBTU. The higher reactivity of the COOAt ester coupled with the active participation of the nitrogen atom on the pyridine moiety in the amide bond formation offers less enantiomerisation with higher reported yields.^{113,114}



The mechanism for peptide bond formation with HATU (Scheme 2.7) occurs by deprotonation of a α -amino acid **2.1.15** under basic conditions of DIPEA. The deprotonated α -amino acid reacts with the tetramethylisouronium moiety of HATU **2.1.24b** to form a tetrahedral ester intermediate **2.1.26**. The resulting ester intermediate reacts to form the products in (J). Then the following reaction in (J) gives the highly reactive COOAt ester product shown in (K) where an

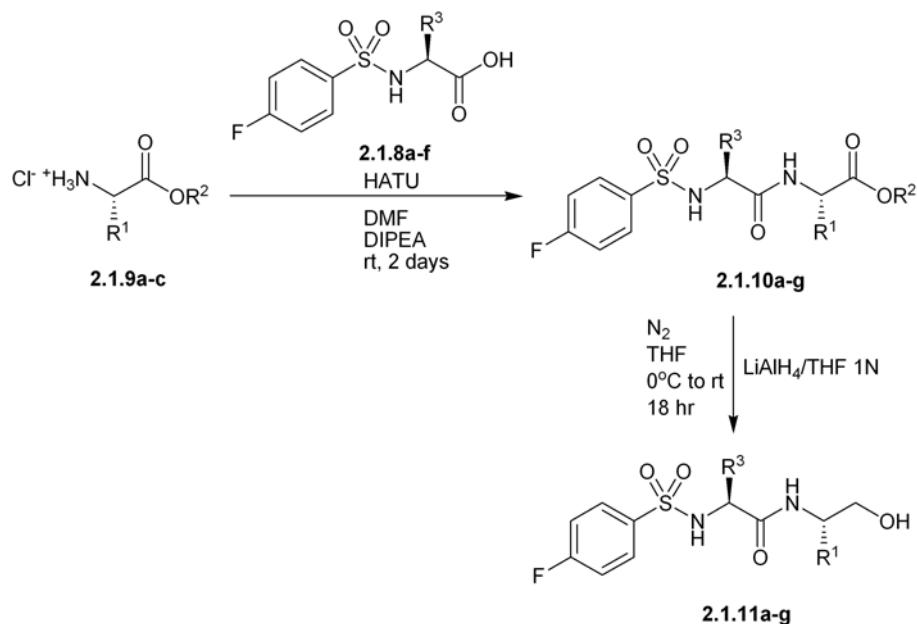
intermolecular reaction in (K) between the free amine functionally of an α -amino acid and the pyridine nitrogen gives the amide product **2.1.27**, 1,1,3,3-tetramethylurea **2.1.28** and 3*H*-[1,2,3]triazolo[4,5-*b*]pyridin-3-ol (HOAt) **2.1.29**. The formation of 1,1,3,3-tetramethylurea is the driving force for the decomposition of HATU during amide bond formation in this reaction.



Scheme 2.7 Synthesis of the peptide bond by application of HATU **2.1.24b** coupling reagent

Synthesis of *N*-(4-fluorobenzenesulfonyl) peptide alcohols from HATU coupling and reduction

Synthesis of the sulphonamide peptide alcohols for compounds **2.1.11a-g** involved the coupling of the hydrochloride salt of the α -amino methyl or ethyl ester **2.1.9a-c** to a sulfonyl α -amino acid **2.1.8a-f**, using HATU over 2 days. These reactions obtained yields between 45-92%. The resulting methyl or ethyl esters were reduced to alcohols **2.1.11a-g** with 1*N* LiAlH₄/THF at 0°C for 1 hr (Scheme 2.15). Confirmation of these sulfonyl peptide alcohols **2.1.11a-g** were carried out using a 1D ¹H NMR experiment in deuterated DMSO, ¹³C NMR and mass spectrometry.



2.1.11a. R¹ = CH₂CH₂SCH₃ R² = Me R³ = 1,1 cyclohexane

2.1.11b. R¹ = CH₂CH₂SCH₃ R² = Me R³ = Ph

2.1.11c. R¹ = CH₂CH₂SCH₃ R² = Me R³ = cyclohexane

2.1.11d. R¹ = CH₂CH₂SCH₃ R² = Me R³ = (*S*)-*i*-Bu

2.1.11e. R¹ = Bn R² = Et R³ = *i*-Pr

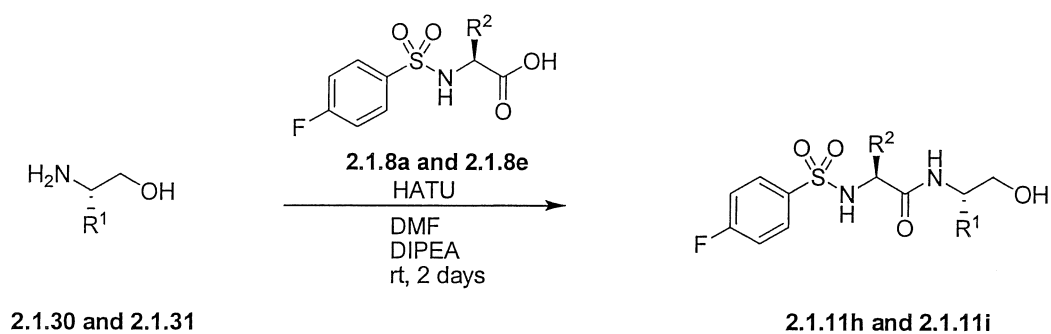
2.1.11f. R¹ = CH₂CH₂SCH₃ R² = Me R³ = *t*-Bu

2.1.11g. R¹ = *p*-CH₂PhOH R² = Me R³ = 1,1 cyclohexane

Scheme 2.8 Synthesis of *N*-(4-fluorobenzenesulfonyl) peptide alcohols from HATU coupling and reduction

Synthesis of 2.1.11h and 2.1.11i

The sulfonyl peptide alcohols **2.1.11h** and **2.1.11i** were directly coupled between sulfonyl α -amino acids and unprotected α -amino alcohols L-methioninol **2.1.30** and **2.1.31** (Scheme 2.9). The acids **2.1.8a** and **2.1.8e** were coupled to L-methioninol **2.1.30** in the presence of DIPEA and HATU to give **2.1.11h** and **2.1.11i**. The alcohol **2.1.11i** was confirmed with ¹H NMR spectra (Figure 2.3) with signals displaying chemical shifts in deuterated DMSO $\delta_{\text{H}} = 7.81$ for the singlet sulfonamide proton which correlated closely to the singlet sulfonamide proton observed in the acid **2.1.8a**, and for compound **2.1.11i** $\delta_{\text{H}} = 0.95$ and $\delta_{\text{H}} = 0.91$ (3H, d, $J = 6.3$ Hz, **CH**₃ of Leu) 3.44-3.40 a multiplet signal for the **CH**₂OH on L-leucinol. Mass spectra displayed the hydrogen adduct for confirmation.



Scheme 2.9 Synthesis of *N*-(4-fluorobenzenesulfonyl) L-valine L-methioninol **2.1.11h** and (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(1-hydroxy-4-methylpentan-2-yl)cyclohexanecarboxamide, **2.1.11i**

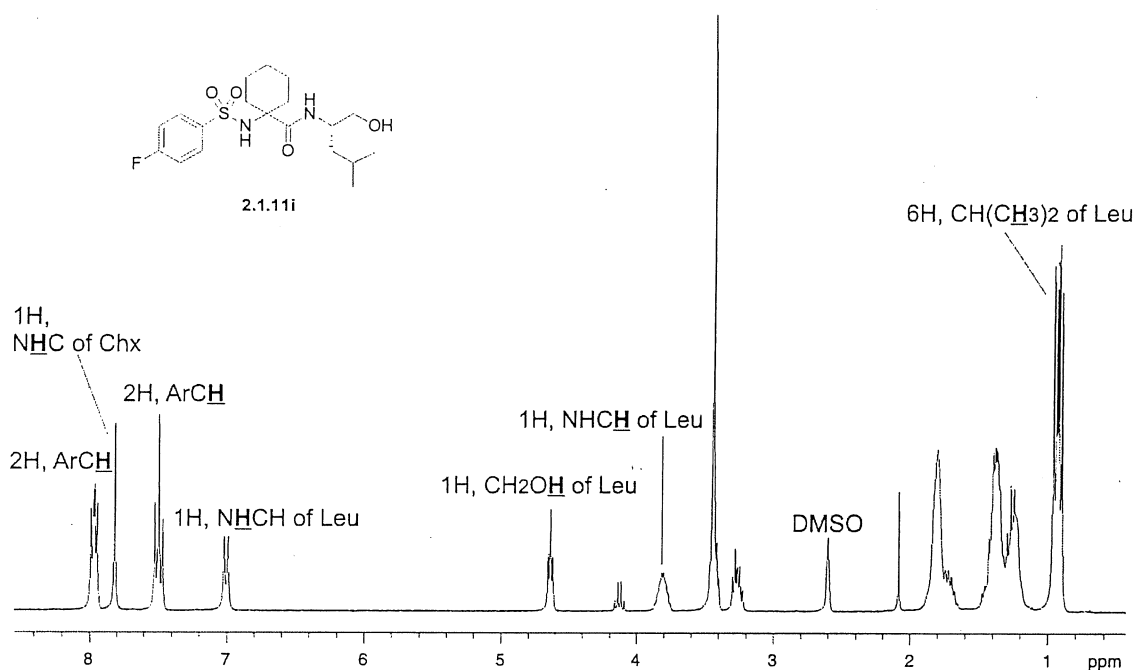
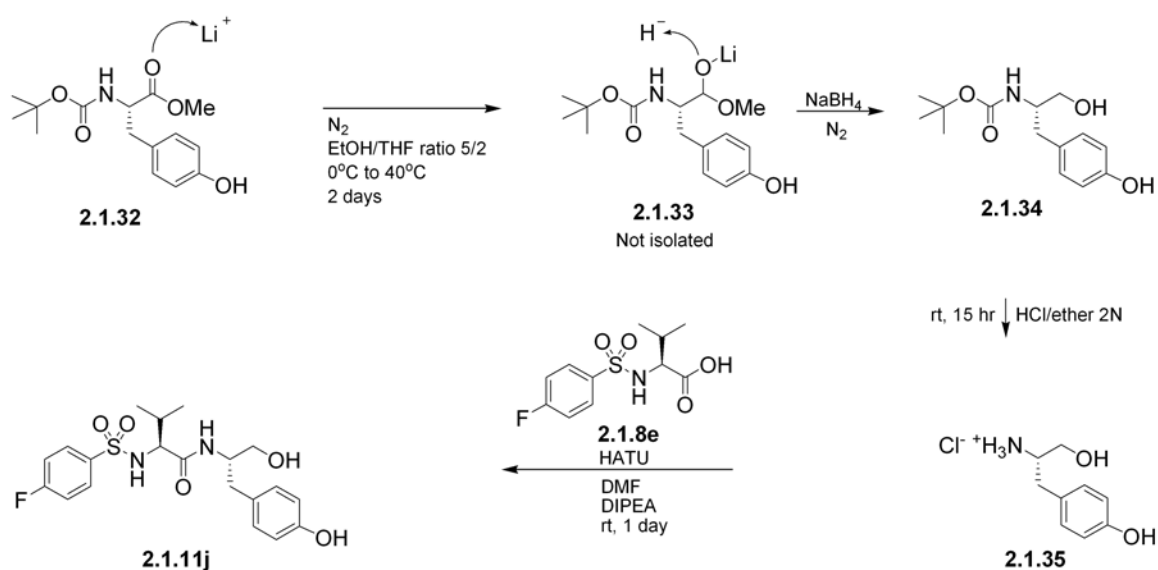


Figure 2.3 ¹H NMR spectra for (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(1-hydroxy-4-methylpentan-2-yl)cyclohexanecarboxamide, **2.1.11i**

Synthesis of *N*-(4-fluorobenzenesulfonyl) L-valine L-tyrosinol **2.1.11j**

The methyl ester boc L-tyrosine methyl ester **2.1.32** was reduced to the corresponding alcohol **2.1.34** (Scheme 2.10). The reduction was performed by dissolving the methyl ester **2.1.32** in a cooled solution at 0°C of EtOH/THF and LiCl to act as a Lewis acid. This initially produced the lithiated product **2.1.33** which was not isolated or its presents confirmed. The compound **2.1.33** was reduced to the corresponding alcohol **2.1.34** from the treatment of a cooled solution of

NaBH₄ dissolved in distilled water. Removal of the boc protection with 2N HCl/ether gave the hydrochloride salt **2.1.35** which was coupled with compound **2.1.8e** in the presence of HATU to give the sulfonyl L-valine L-tyrosinol **2.1.11j**.



Scheme 2.10 Synthesis of *N*-(4-fluorobenzenesulfonyl) L-valine L-tyrosinol **2.1.11j**

The alcohol **2.1.11j** was confirmed with ¹H NMR spectra (Figure 2.4) with signals displaying chemical shifts in deuterated DMSO $\delta_{\text{H}} = 3.29\text{-}3.19$ for the two protons (CH₂OH) on tyrosine, the two methyl (CH₃'s) on valine appeared as a multiplet $\delta_{\text{H}} = 0.88\text{-}0.86$ as opposed to the two doublets observed for the acid precursor **2.1.8e**. However, the presence of compound **2.1.11j** was confirmed with mass spectra observed as the hydrogen adduct within the 5 ppm tolerance range required.

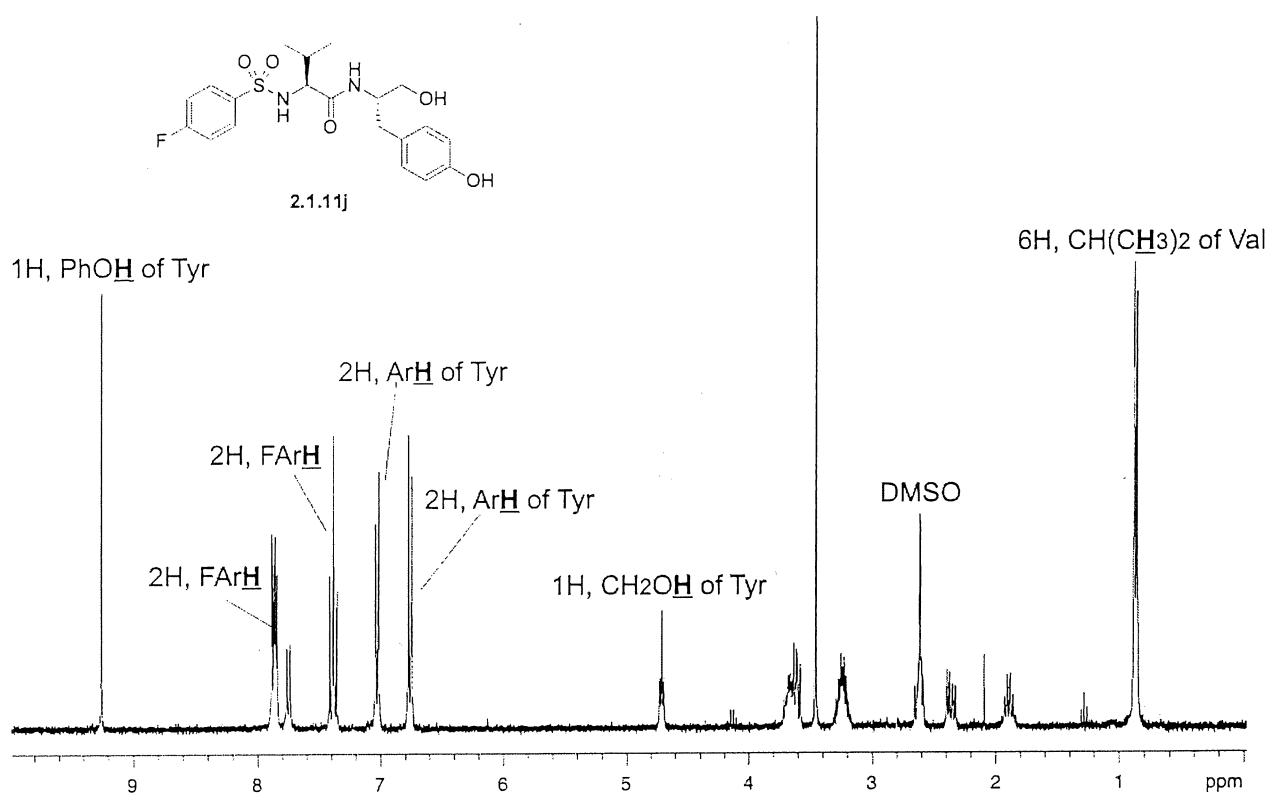
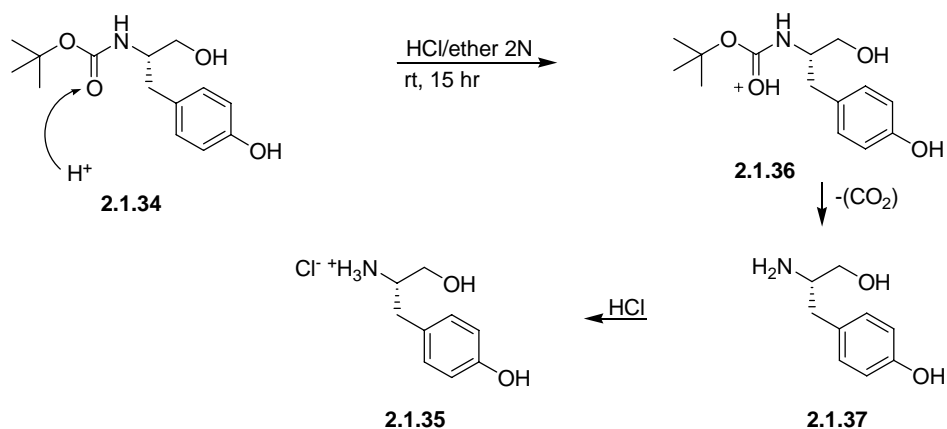


Figure 2.4 ¹H NMR spectra for **2.1.11j**

Boc deprotection for the synthesis of L-tyrosinol 2.1.11j

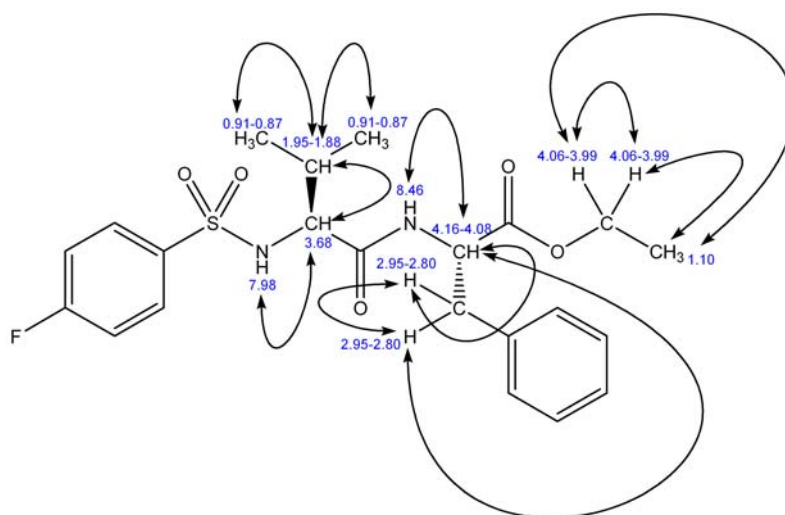
The deprotection of the boc group was performed under acidic conditions. Protonation of the carbonyl group on boc L-tyrosinol **2.1.34** results in a suitable leaving group **2.1.36** to give L-tyrosinol hydrochloride **2.1.35**. The confirmation of the deprotection of the boc group was observed through ¹H NMR spectra where the disappearance of boc group peak can be observed at an approximate chemical shift in CD₃OD of $\delta_{\text{H}} = 1.40$ (9H, s, C(CH₃)₃).



Scheme 2.11 Boc deprotection of boc L-tyrosinol **2.1.34** to L-tyrosinol hydrochloride **2.1.35**

2D gCOSY

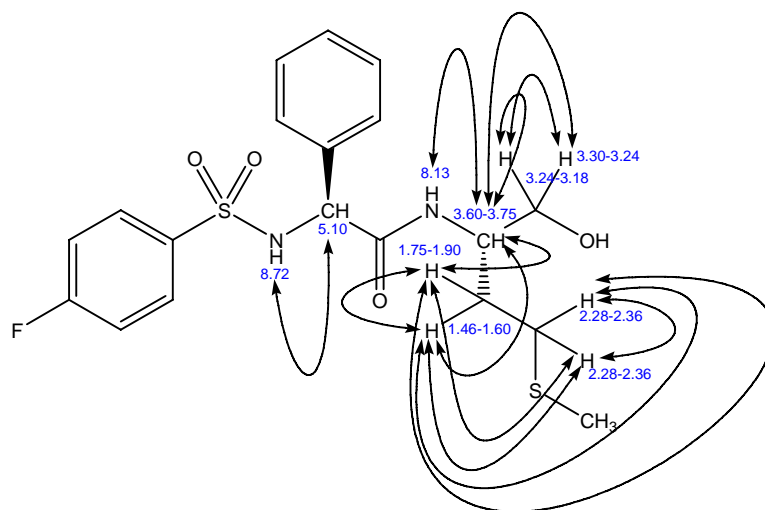
2D gCOSY was used to resolve complex multiplets observed from the following compounds, **2.1.10e**, and **2.1.11b**. The compound *N*-(4-fluorobenzenesulfonyl) L-valine L-phenylalanine ethyl ester consisted of two multiplets occurring at $\delta_{\text{H}} = 4.16\text{--}3.99$ and $0.91\text{--}0.87$ and an unknown triplet at $\delta_{\text{H}} = 1.10$. These were assigned as the NHCH of Phe proton at $\delta_{\text{H}} = 4.16\text{--}4.08$, OCH_2CH_3 of Phe protons at $\delta_{\text{H}} = 4.06\text{--}3.99$ and OCH_2CH_3 of Phe $\delta_{\text{H}} = 1.10$ for the unknown triplet.



2.1.10e. *N*-(4-Fluorobenzenesulfonyl) L-valine L-phenylalanine ethyl ester

The 2D gCOSY experiment of the alcohol **2.1.11b** resolved multiplets occurring the first from $\delta_{\text{H}} = 3.75\text{--}3.19$ and the second from $\delta_{\text{H}} = 1.90\text{--}1.46$. The first multiplet was later assigned as the

α -hydrogen on methioninol $\delta_{\text{H}} = 3.75$ - 3.60 and $\delta_{\text{H}} = 3.32$ - 3.19 as the CH_2OH on methioninol and the second multiplet $\delta_{\text{H}} = 1.90$ - 1.75 and $\delta_{\text{H}} = 1.60$ - 1.46 as the CHCH_2 's on methioninol.

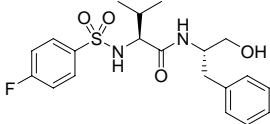
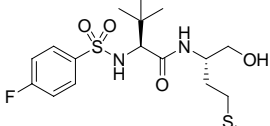
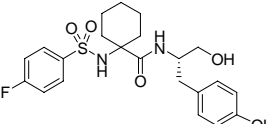
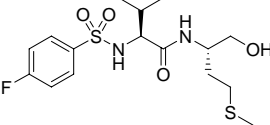
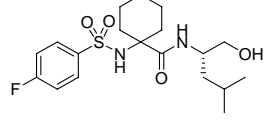
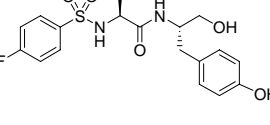
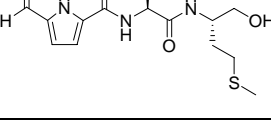


2.1.11b. *N*-(4-Fluorobenzenesulfonyl) L-phenylglycine L-methioninol

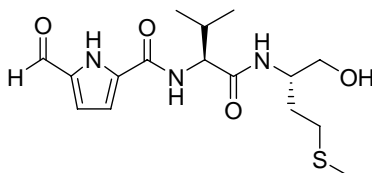
Table 2.4 shows the HATU coupling yields for the peptide alcohols **2.1.11a-k**. This table includes the yields from the coupling reactions involving the peptide acids and α -amino alcohol or α -amino esters. Overall there was no advantage in using carbonyl group protected α -amino esters over the α -amino alcohols and the α -amino alcohols can be used under these coupling conditions without esterification.

Table 2.4 HATU coupling yields for the peptide alcohols **2.1.11a-k**

Compound Number	Compound Structure	Coupling Yield (%)
2.1.11a		66
2.1.11b		69
2.1.11c		66
2.1.11d		57

2.1.11e		64
2.1.11f		71
2.1.11g		73
2.1.11h		56
2.1.11i		82
2.1.11j		28
2.1.11k		84

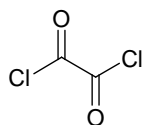
The yields of the peptide alcohols ranged from 84% for *N*-(5-pyrrole 2-carboxylate L-valine L-methioninol **2.1.11k** to 28% for *N*-(4-fluorophenylsulfonyl) L-valine L-tyrosinol **2.1.11j**. The synthesis of compound **2.1.11k** differed from the synthesis of the sulfonyl derivatives, however, the second coupling reaction with EDCI.H₂O and HOBt to give a low yield (7%) of **2.1.11k**. The discussion on synthesis of **2.1.11k** and its corresponding dialdehyde **2.1.3k** is address in section 2.2 below.



2.1.11k. 5-Formyl pyrrole 2-carboxylate L-valine L-methioninol

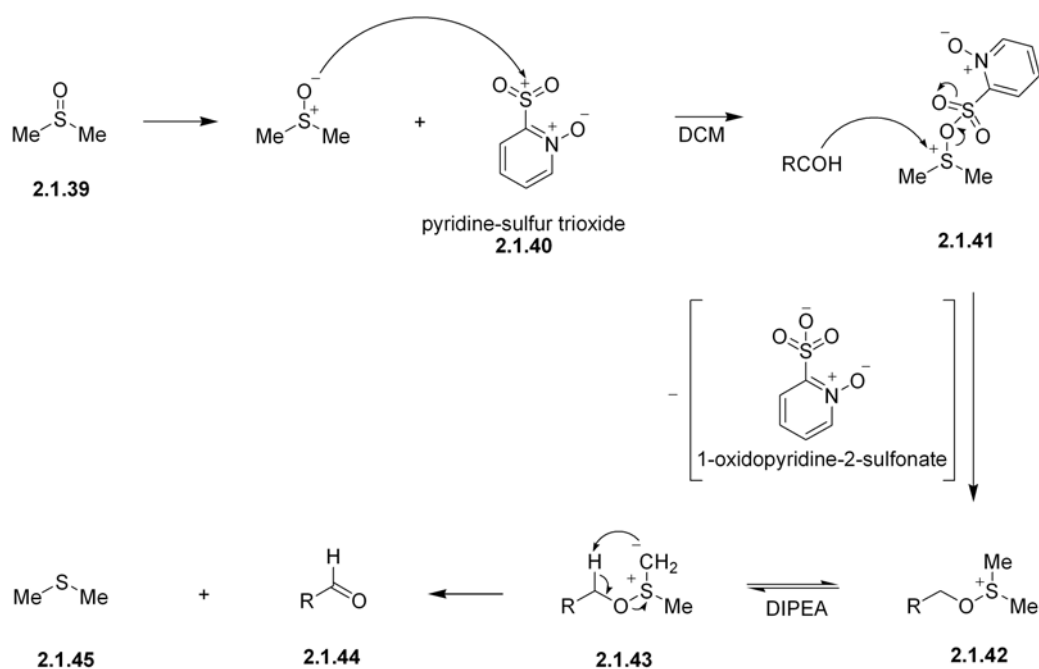
Synthesis of *N*-(4-fluorobenzenesulfonyl) peptide aldehydes 2.1.3a-j by DMSO oxidation

Oxidation to the target aldehydes was performed through Parikh-Doering oxidation¹¹⁵ of the corresponding alcohols. The major advantage to this method is the reaction can be carried out at room temperature or below room temperature (0°C-10°C). Unlike Swern involving the reactive oxalyl dichloride **2.1.38** to perform the oxidation therefore requiring low temperatures (-78°C) to prevent further oxidation.



2.1.38. Oxalyl dichloride

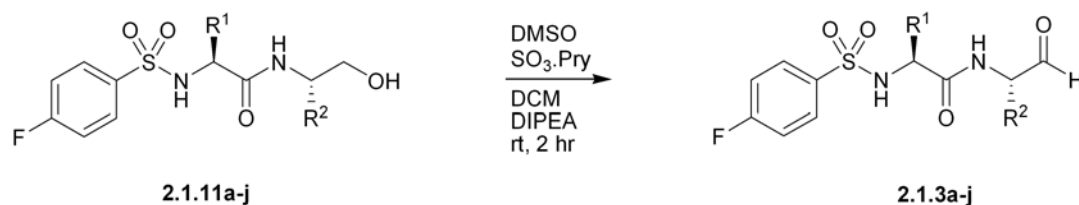
A disadvantage of the Parikh-Doering oxidation is that the reaction for some unknown reason requires a minimum of 200 mg alcohol for the reaction to proceed. For smaller scale reactions a sacrificial alcohol such as isopropyl alcohol (IPA) can be used.



Scheme 2.12 Parikh-Doering oxidation via the formation of an active DMSO.SO₃ complex

The first step in the Parikh-Doering oxidation (Scheme 2.12) involves activation of DMSO **2.1.39** by addition of the SO₃.Pyr complex **2.1.40**, with DCM as solvent resulting in the sulfonium species **2.1.41** where the electrophilic nature of the sulfur atom is increased and

provides a 1-oxidopyridine-2-sulfonate leaving group. The alcohol reacts at sulfur with displacement of the sulfonate anion to give an alkoxydimethylsulfonium salt **2.1.42**. This salt **2.1.42** is deprotonated by DIPEA to form **2.1.43** which reacts through an intramolecular reaction to form the aldehyde **2.1.44** and dimethyl sulphide **2.1.45**. The oxidations of the sulfonyl peptide alcohols **2.1.3a-j** are summarised in Scheme 2.13.



2.1.3a. R¹ = 1,1 cyclohexane R² = CH₂CH₂SCH₃

2.1.3b. R¹ = Ph R² = CH₂CH₂SCH₃

2.1.3c. R¹ = cyclohexane R² = CH₂CH₂SCH₃

2.1.3d. R¹ = (*S*)-*i*-Bu R² = CH₂CH₂SCH₃

2.1.3e. R¹ = *i*-Pr R² = Bn

2.1.3f. R¹ = *t*-Bu R² = CH₂CH₂SCH₃

2.1.3g. R¹ = 1,1 cyclohexane R² = *p*-CH₂PhOH

2.1.3h. R¹ = *i*-Pr R² = CH₂CH₂SCH₃

2.1.3i. R¹ = 1,1 cyclohexane R² = CH₂*i*-Pr

2.1.3j. R¹ = *i*-Pr R² = *p*-CH₂PhOH

Scheme 2.13 Parikh-Doering oxidation¹¹³ of the *N*-(4-fluorobenzenesulfonyl) peptide alcohols **2.1.3a-j**

The compound **2.1.3a** (Figure 2.5) was confirmed with ¹H NMR and mass spectra displaying the aldehyde proton of Met at δ_H = 9.45, the singlet NH proton of Chx (δ_H = 7.91) and the two multiplets of the benzlic group at δ_H = 8.01-7.96 and δ_H = 7.81-7.47 in deuterated DMSO.

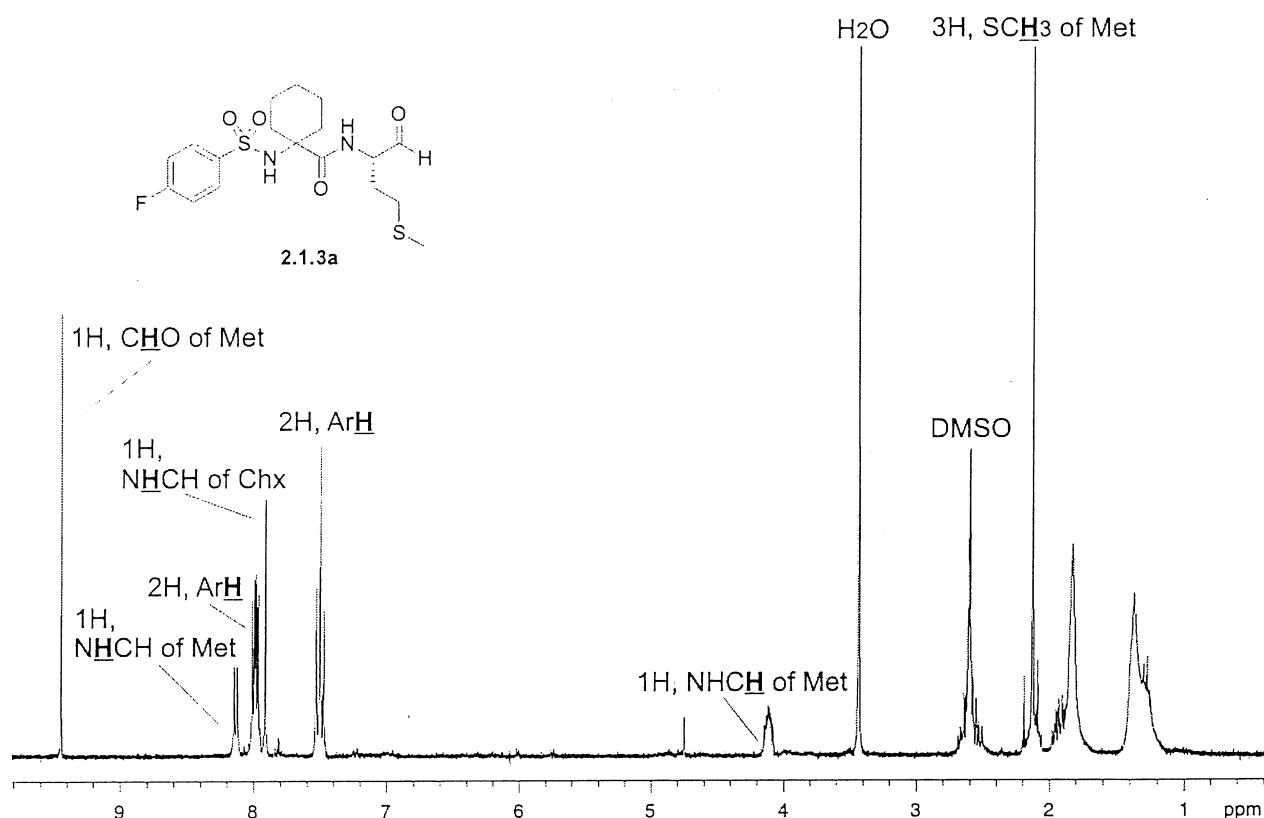
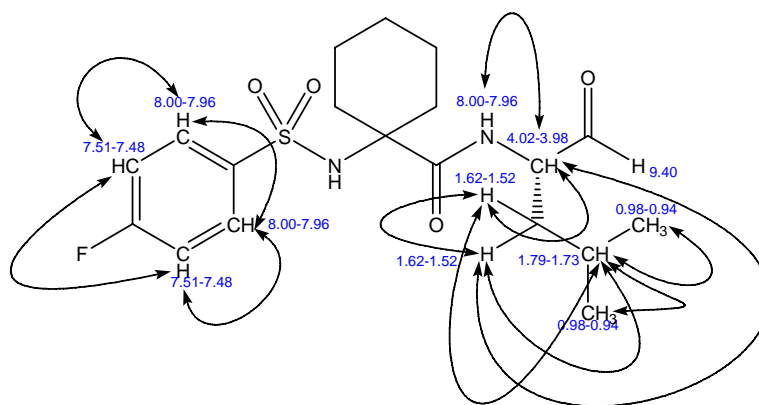


Figure 2.5 ^1H NMR spectra for (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(4-(methylthio)-1-oxobutan-2-yl)cyclohexanecarboxamide **2.1.3a**, in deuterated DMSO

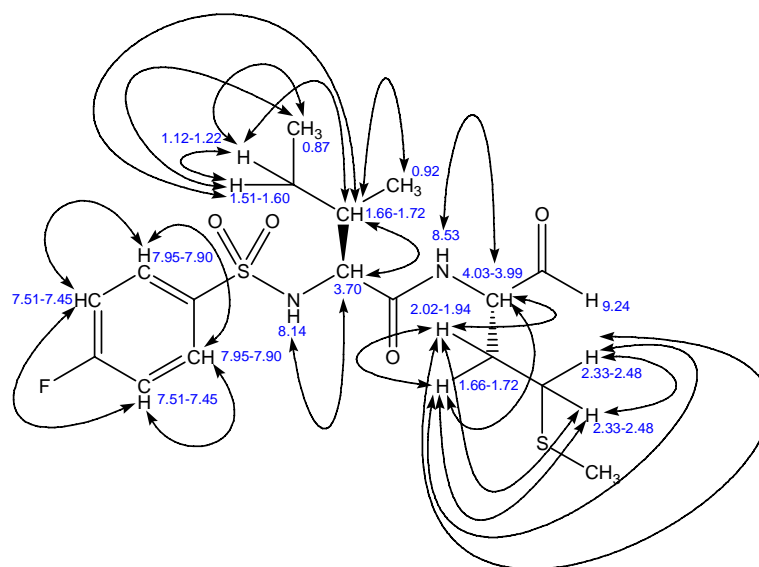
2D gCOSY

The compound **2.13i** 2D gCOSY was used to resolve three observable multiplets from $\delta_{\text{H}} = 1.26\text{-}1.88$. The first multiplet $\delta_{\text{H}} = 1.73\text{-}1.88$ resolved the $\text{CH}(\text{CH}_3)_2$ of Leu proton as a multiplet at $\delta_{\text{H}} = 1.73\text{-}1.79$. The second multiplet $\delta_{\text{H}} = 1.52\text{-}1.62$ was shown to be the CHCH_2CH of Leu protons. The protons CHCH_2CH of Leu displayed strong couplings with NHCH of Leu and $\text{CH}(\text{CH}_3)_2$ of Leu protons. The third multiplet $\delta_{\text{H}} = 1.26\text{-}1.38$ displayed coupling to the first multiplet at $\delta_{\text{H}} = 1.88$. The coupling and integral information suggested that this is CH_2 's of 1,1-cyclohexane protons.



2.1.3i. (S)-1-(4-Fluorophenylsulfonamido)-N-(4-methyl-1-oxopentan-2-yl)cyclohexanecarboxamide

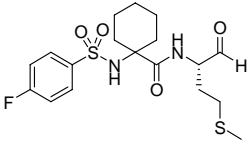
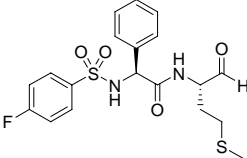
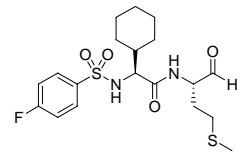
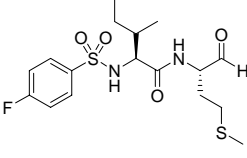
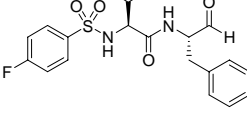
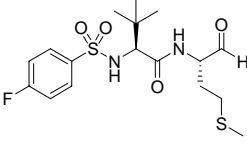
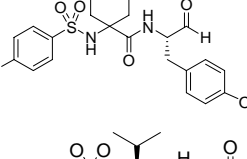
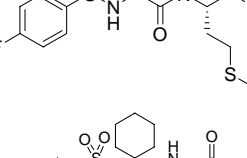
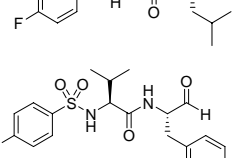
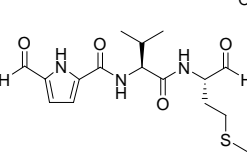

The compound **2.13d** the 2D gCOSY helped to resolved the multiplets occurring from $\delta_{\text{H}} = 1.76-1.51$. This multiplet was later assigned as the α -hydrogen on *iso* L-leucine and a proton of the CHCH_2 's on methioninal $\delta_{\text{H}} = 1.76-1.66$ and $\delta_{\text{H}} = 1.60-1.51$ for one CH_2 on *iso* L-leucine.



2.1.3d. N-(4-Fluorobenzesulfonyl) L-*iso*-leucine- L-methioninal

In Table 2.5 the oxidation yields which range from 24% for the aldehyde **2.1.3h** to near quantitative yield for the sulfonyl *tert* L-leucine L-methioninal **2.1.3f** (99%).

Table 2.5 Yields for the peptide aldehydes

Compound Number	Compound Structure	Oxidation Yield (%)
2.1.3a		84
2.1.3b		41
2.1.3c		95
2.1.3d		96
2.1.3e		90
2.1.3f		99
2.1.3g		45
2.1.3h		24
2.1.3i		71
2.1.3j		70
2.1.3k		25

¹³C NMR spectra

The sulfonyl compounds containing the 4-fluorinated aromatic *N*-(4-fluorobenzenesulfonyl) group) all displayed the characteristic C-F signal splitting into a doublet with an approximate chemical shift (δ_C) in deuterated DMSO $\delta_C = 164.0$ and approximate coupling constant ($J = 250$ Hz). This effect can extend throughout the aromatic ring of the *N*-(4-fluorobenzenesulfonyl) group where it can be observed that carbons positioned further away from the fluorine atom show an overall decrease in the J value. Typical chemical shifts and coupling constant values found for the other *N*-(4-fluorobenzenesulfonyl) aromatic carbons are summarised in Table 2.6 below.

Table 2.6 Experimental ¹³C NMR chemical shift (δ) data found for compounds containing the *N*-(4-fluorobenzenesulfonyl) group. **2.1.7a-d, 2.1.8a-f, 2.1.10a-g, 2.1.11a-j and 2.1.3a-j**

Distance from the fluorine atom	Functionality	Chemical shift range deuterated DMSO (δ)	Coupling constant range deuterated DMSO (J) (Hz)
F ↓ ↓ ↓ ↓	F- <u>C</u>	164.0-164.2	248.3-251.1 (doublet)
	F-C= <u>C</u>	116.2-115.9	22.0-23.0 (doublet)
	S-C= <u>C</u>	129.5-129.8	8.7-9.9 (doublet)
	S- <u>C</u> =	137.6-139.8	Singlet-3.3

Other significant chemical shifts (δ) such as aldehydes (CHO), amides (CONH) and the carbonyl methyl esters (COOCH₃), tertiary butyl groups C(CH₃)₃ and methyl carbons on valine C(CH₃)₂ and thiol methyl on Met (SCH₃) are summarised in Table 2.17 below.

The Aldehydes (CHO)

The aldehydes typically display a singlet with a down field chemical shift (δ) ranging from $\delta_C = 199.7$ -202.0 in deuterated DMSO. Compounds containing the 5-formyl pyrrole group consist of a formyl carbon which can be observed as a singlet in either CD₃OD at $\delta_C = 182.6$ or deuterated DMSO at $\delta_C = 181.3$.

Table 2.7 Experimental ^{13}C NMR chemical shift (δ) data for the non-aromatic carbons for **2.1.7a-d, 2.1.8a-f, 2.1.10a-g, 2.1.11a-j** and **2.1.3a-j**

Functionality	Chemical shift range DMSO-d6 (δ)
Aldehydes ($\underline{\text{C}}\text{HO}$)	199.7-202.0
Carbonyl methyl esters ($\underline{\text{C}}\text{OOCH}_3$)	168.9-171.9
Amides ($\underline{\text{C}}\text{ONH}$)	168-6-170.8
Tertiary butyl groups $\text{C}(\underline{\text{C}}\text{H}_3)_3$	26.5-34.1
Methyl carbons on valine $\text{C}(\underline{\text{C}}\text{H}_3)_2$	$\text{C}\underline{\text{C}}\text{H}_3 = 17.9-19.6$ $\text{C}\underline{\text{C}}\text{H}_3 = 19.1-20.2$
Thiol methyl on methionine ($\text{S}\underline{\text{C}}\text{H}_3$)	14.4-15.6

The tertiary butyl group $\text{C}(\underline{\text{C}}\text{H}_3)_3$ (on *tert* L-leucine)

Compounds containing the group tertiary butyl group $\text{C}(\underline{\text{C}}\text{H}_3)_3$ for example in *tert* L-leucine display the characteristic tall singlet approximately at DMSO $\delta_{\text{C}} = 26.6$ in deuterated.

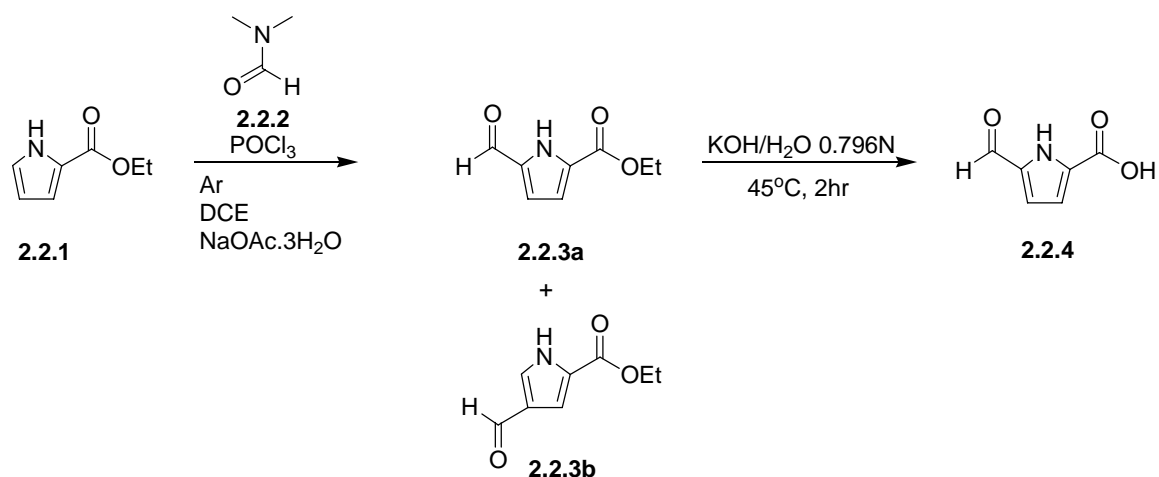
Methyl carbons on valine $\text{C}(\underline{\text{C}}\text{H}_3)$

The experimental chemical shifts (δ) for the methyl carbons on valine $\text{C}(\underline{\text{C}}\text{H}_3)$, where observed as non equivalent entities ranging from $\delta_{\text{C}} = 17.9-20.2$ with a separation of 1 ppm. These peaks were not resolved any further and were assigned as $\underline{\text{C}}\text{H}_3$ of Val in the experimental Chapter 3.

2.2 Synthesis of a novel 5-formyl pyrrole dipeptide dialdehyde inhibitor

The synthesis of the L-methioninal **2.1.3k** differed from the synthesis of the other sulfonyl analogues, where two coupling reactions were required. The first involved HATU for coupling the P₂ address region of boc L-valine **2.2.11** to L-methioninol **2.1.30**, (Scheme 2.17) then the second involved coupling the pre-synthesised acid **2.2.4** (for the P₃ address region) and L-valine L-methioninol with (EDC.HCl) and 1-hydroxybenzotriazole (HOBt.H₂O) (Scheme 2.16).

Synthesis of 5-formyl pyrrole 2-carboxylic acid



Scheme 2.14 The synthesis of 5-formyl pyrrole 2-carboxylic acid *via* the Vilsmeier-Haack formylation¹¹⁶

Synthesis of the ethyl ester **2.2.4** was prepared by Vilsmeier formylation;¹¹⁶ this initially involved the synthesis of the DMF-POCl₃ complex **2.2.5** at 0°C (Scheme 2.15a). Then the complex was reacted by careful addition of pyrrole 2-carboxylate ethyl ester dissolved in DCE. The reaction mixture was heated under reflux at 115°C for 15 min and the reaction mixture was refluxed with NaOAc.3H₂O at 120°C for 20 min to give the 4 and 5-formyl pyrrole 2-carboxylate ethyl esters **2.2.3b** and **2.2.3a**. This reaction was regioselective yielding a 2:1 ratio in favour of the 5 isomer. These acquired regioisomers were separated and their presence confirmed with ¹H NMR spectra. As expected the two regioisomers displayed markedly different spectra. In one case both pyrrole protons displayed similar chemical shifts, this was identified as the 5 isomer **2.2.3a** (Figure 2.6) ($\delta_{\text{H}} = 7.03$ and $\delta_{\text{H}} = 6.95$). The other spectra displayed an apparent separation of the pyrrole protons (Figure 2.7) ($\delta_{\text{H}} = 7.90$ for the NHCH₂ proton and $\delta_{\text{H}} = 7.22$ for the singlet C=CHC proton), this indicated the presence of the 4 isomer **2.2.3b**. Then after confirmation the ethyl ester **2.2.3a** (5 isomer) was hydrolysed with aqueous potassium hydroxide to give a quantitative yield of the carboxylic acid.

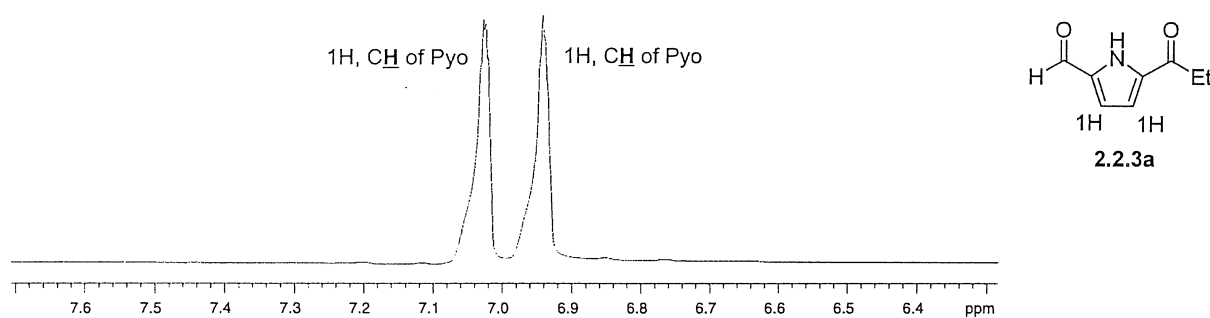


Figure 2.6 ^1H NMR spectra of **2.2.3a** (5-isomer) showing the CH protons on pyrrole in deuterated DMSO

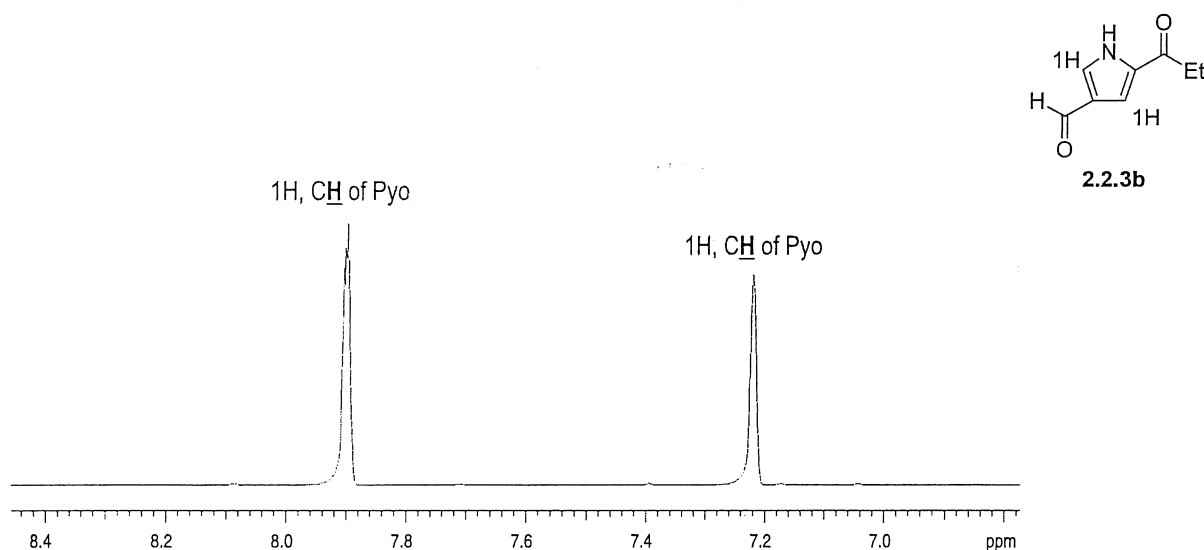
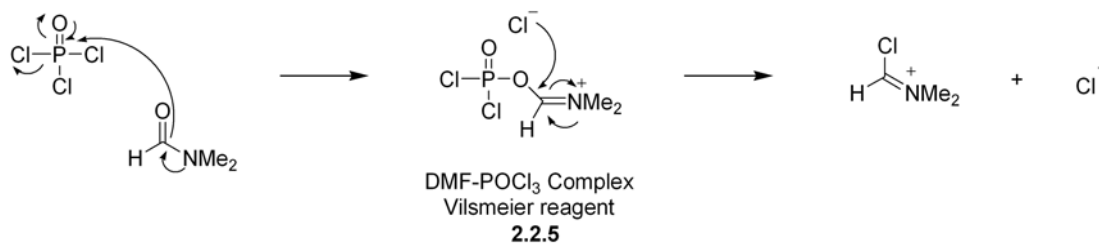


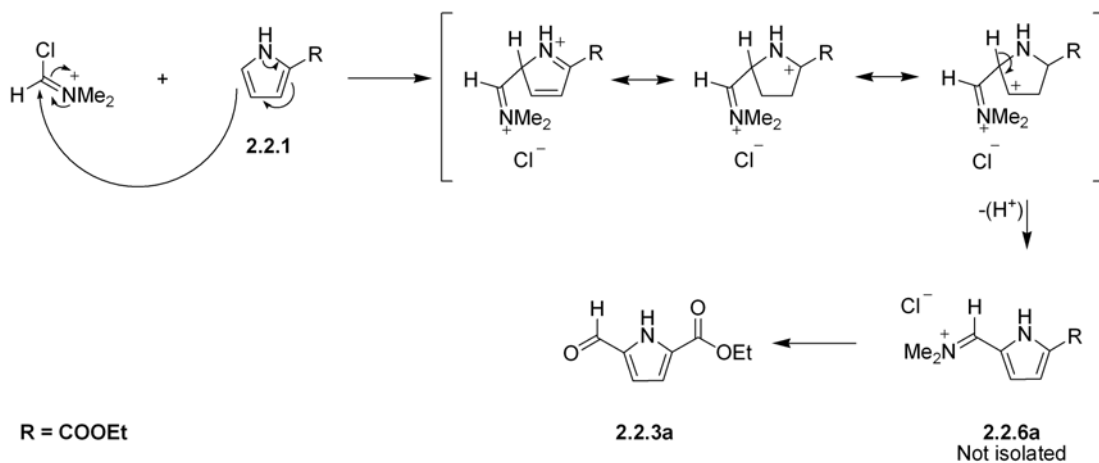
Figure 2.7 ^1H NMR spectra of **2.2.3b** (4-isomer) showing the CH protons on pyrrole in deuterated DMSO

The Vilsmeier formylation mechanism

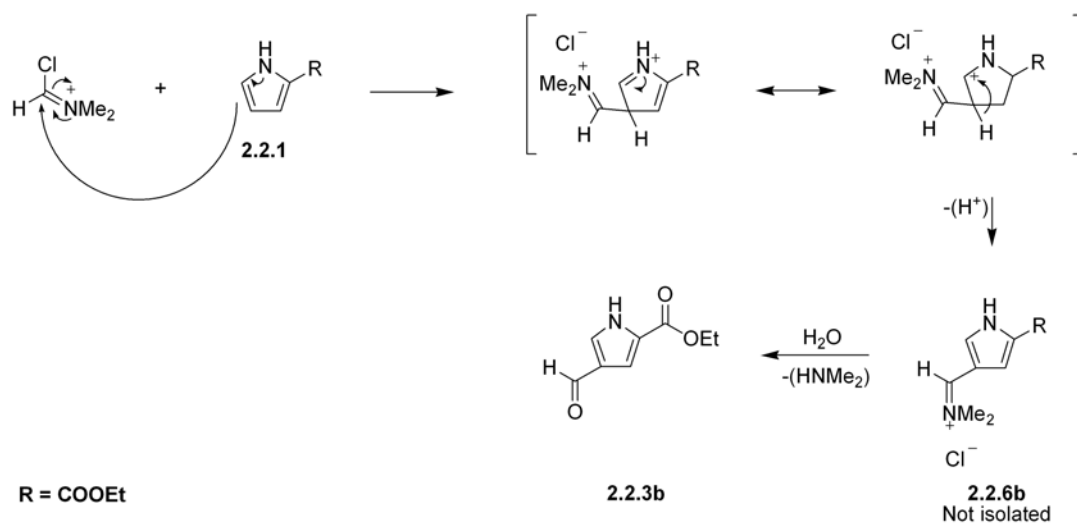
The Vilsmeier or sometimes called Vilsmeier-Haack formylation¹¹⁶ has been extensively used since its discovery in 1927. The mechanism involves reaction of DMF with phosphorus oxychloride (POCl_3) (Scheme 2.15a) to form chloro(dimethylamino)methyl phosphorodichloridate **2.2.5** known as Vilsmeier reagent. This reacts with pyrrole 2-carboxylate ethyl ester **2.2.1** to give either 4 or 5-formyl pyrrole 2-carboxylate ethyl ester. The α -substituted product is the major product (Scheme 19) the transition state to its formation receiving extra resonance stabilisation. The ethyl 4 and 5 ((dichlorophosphoryloxy)(dimethylamino)methyl)-1*H*-pyrrole-2-carboxylates reacts with acid to give the corresponding esters **2.2.3a** and **2.2.3b**.



Scheme 2.15a The mechanism for the formation of the Vilsmeier salt



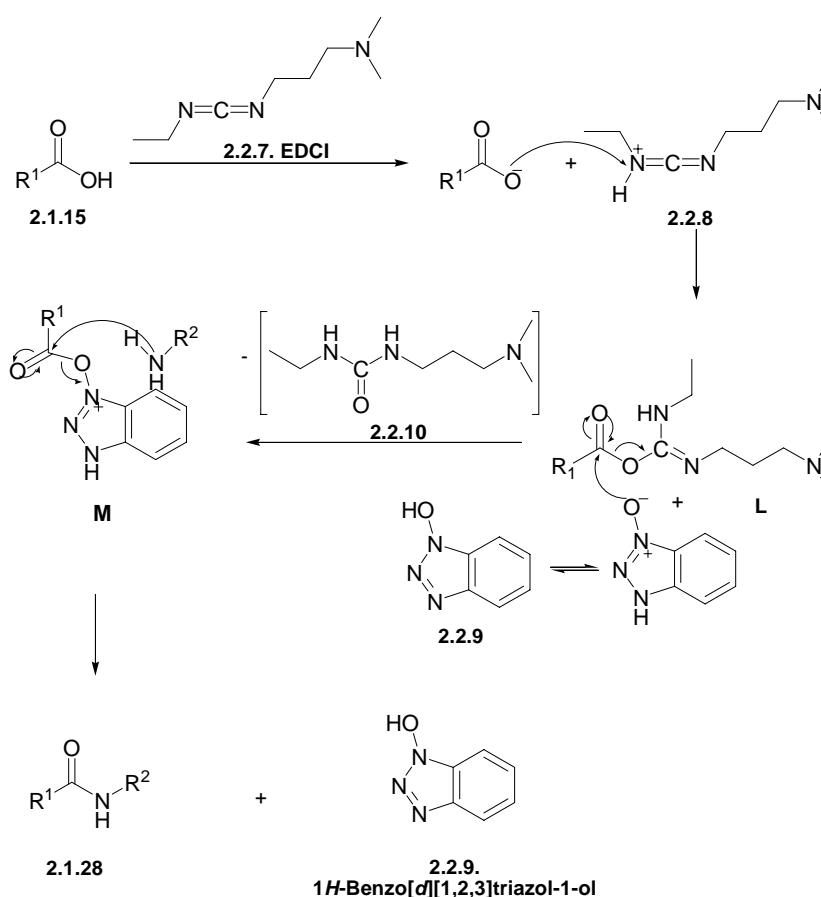
Scheme 2.15b The mechanism for the formation of 5-formyl pyrrole 2-carboxylate ethyl ester



Scheme 2.15c The mechanism for the formation of 4-formyl pyrrole 2-carboxylate ethyl ester

EDCI.HCl and HOBt.H₂O coupling

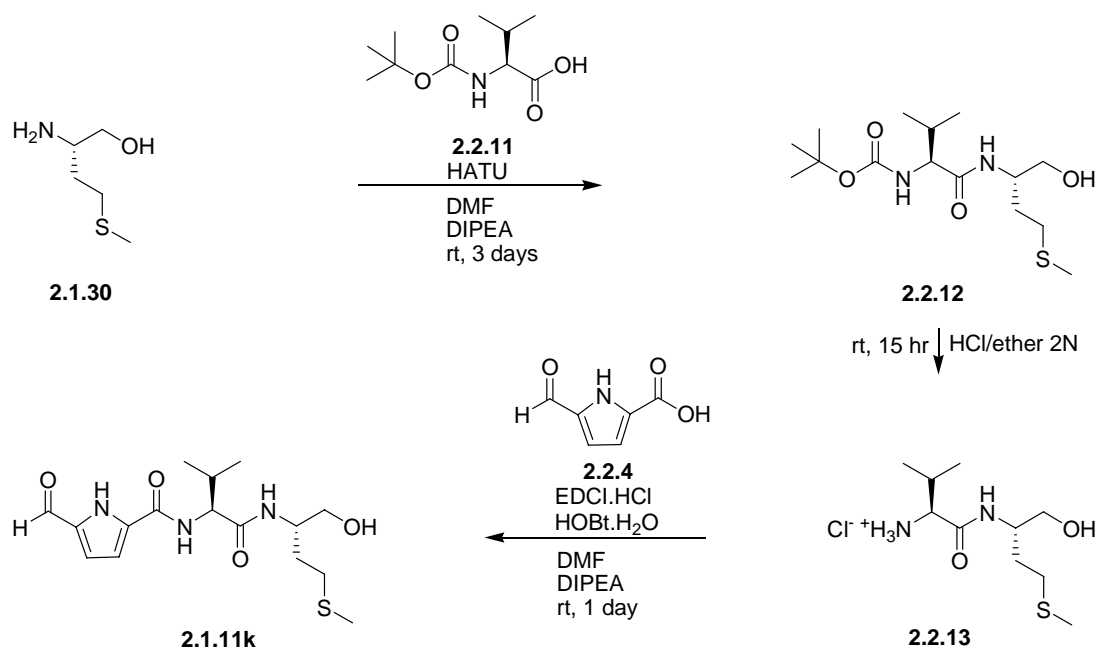
As mentioned earlier the synthesis of the m-calpain inhibitor 5-formyl pyrrole 2-carboxylate L-valine L-methioninol **2.1.3k** required two coupling reactions, the first with HATU and the second with EDCl.HCl and HOBt.H₂O. The mechanism for EDCl.HCl and HOBt.H₂O coupling (Scheme 2.16) requires initial activation of the α -amino acid with EDCl.HCl **2.2.7** to give the activated ester (L) with a good leaving group facilitating nucleophilic attack from HOBt to give the side product 1-(3-(dimethylamino)propyl)-3-ethylurea **2.2.10** and the complex (M). The free amino group of another α -amino peptide effects nucleophilic attack on the carbonyl group (M) to give rise to the target peptide **2.1.28** and regenerated HOBt **2.2.9**.



Scheme 2.16 Mechanism of peptide bond formation with EDCl.HCl and HOBt.H₂O for the synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-methioninol **2.1.11k**

The synthesis of **2.1.11k** (Scheme 2.17) requires coupling of L-methioninol **2.1.30** and boc L-valine **2.2.11** with HATU to give the boc protected peptide alcohol **2.2.12**. Deprotection to give the hydrochloride salt **2.1.13** (was confirmed through ¹H NMR spectra from the

disappearance of the boc peak at $\delta_{\text{H}} = 1.44$ in CD_3OD) was coupled with EDCI.HCl-HOBt.H₂O to give 5-formyl pyrrole 2-carboxylate L-valine L-methioninol **2.1.11k** with a 7% yield.



Scheme 2.17 The synthesis of 5-formyl pyrrole L-valine L-methioninol **2.1.11k**

Synthesis of 5-formyl pyrrole L-valine L-methioninal **2.1.3k**

Parikh-Doering oxidation of the alcohol **2.1.11k** (Scheme 2.28) was the same as for the sulfonyl analogues previously discussed. The crude product resultant from the oxidation reaction was purified by column chromatography and identified by ¹H NMR spectra and mass spectrometry. The ¹H NMR spectrum in deuterated DMSO showed two aldehyde peaks at $\delta_{\text{H}} = 9.74$ for the $\text{C}\underline{\text{H}}\text{O}$ of methioninal and $\delta_{\text{H}} = 9.54$ $\text{C}\underline{\text{H}}\text{O}$ for the formyl group on pyrrole. The chemical shift for the formyl hydrogen corresponded well to the dipeptide alcohol precursor **2.1.11k**. For mass spectrometry the compound **2.1.3k** was identified as the hydrogen adduct in CH_3CN .



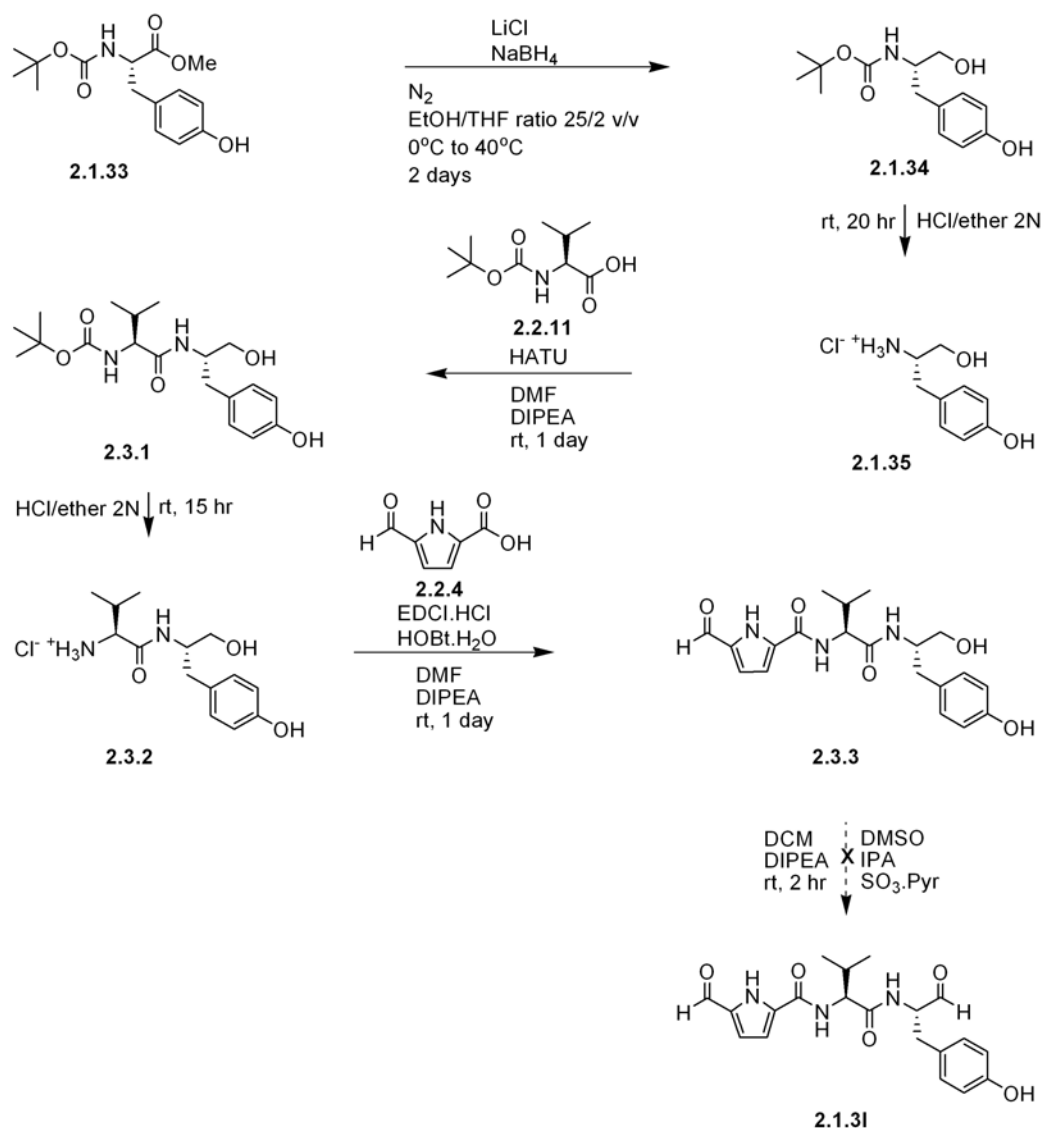
Scheme 2.18 Synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-methioninal from Parikh-Doering oxidation

2.3 Attempted synthesis of dipeptide dialdehydes

Attempted synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal **2.1.31**

In this synthesis, boc L-tyrosine methyl ester **2.1.32** was reduced with NaBH₄ to give the corresponding alcohol **2.1.34**. The boc protecting group was removed using 2N HCl/ether to give L-tyrosinol hydrochloride **2.1.35** which was coupled to boc L-valine **2.2.11**. This gave boc L-valine L-tyrosinol **2.3.1** followed by boc deprotection to give the corresponding hydrochloride salt **2.3.2**. This salt **2.3.2** was coupled to previously prepared 5-formyl pyrrole 2-carboxylic acid **2.2.4** using EDCl.HCl-HOBt.H₂O. This reaction gave only a 5% yield and resulted in us having only 30 mg of the alcohol **2.3.3** to oxidise to aldehyde **2.1.31**. Therefore, the oxidation by DMSO activated by SO₃.Pyr required the use of a sacrificial alcohol, isopropyl alcohol (IPA). The oxidation was unsuccessful and did not provide the target aldehyde 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal **2.1.31**.

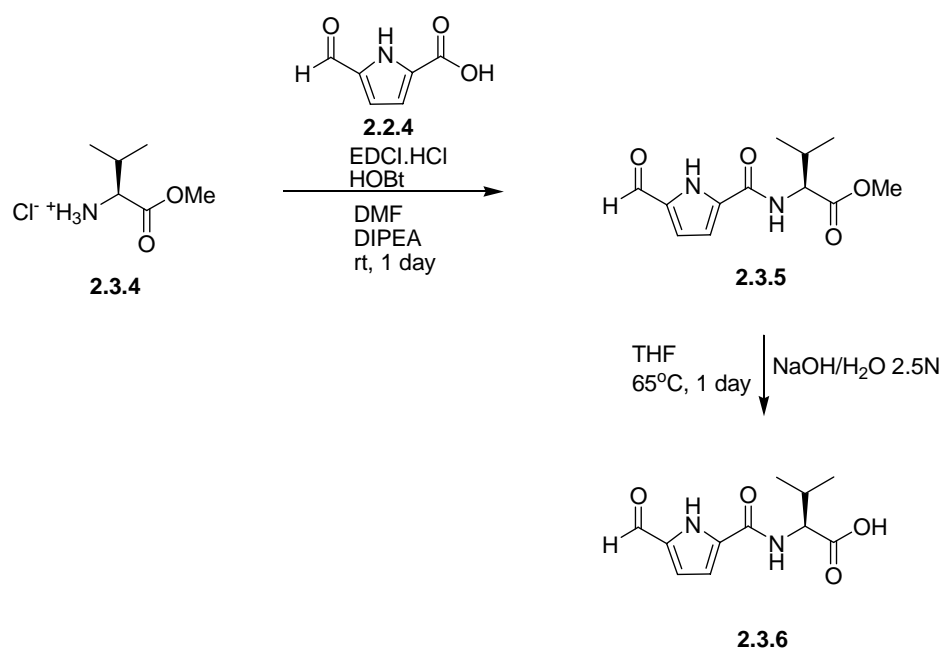
There are two possible reasons for the low yields observed in coupling reactions of L-valine L-tyrosinol hydrochloride **2.3.2** with 5-formyl pyrrole 2-carboxylic acid **2.2.4** and 5-formyl pyrrole 2-carboxylate L-valine L-methioninol **2.2.11k** (7% yield) (Scheme 2.17). Firstly, electronic: the carboxylic acid proton for the acid **2.2.4** may not be acidic enough to react with the coupling reagent and the free amine of L-valine L-tyrosinol hydrochloride **2.3.2**. Secondly, unwanted side reactions may occur between the formyl carbon functionality of **2.2.4** and one or both alcohol groups present in L-tyrosine.



Scheme 2.19 Attempted synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal

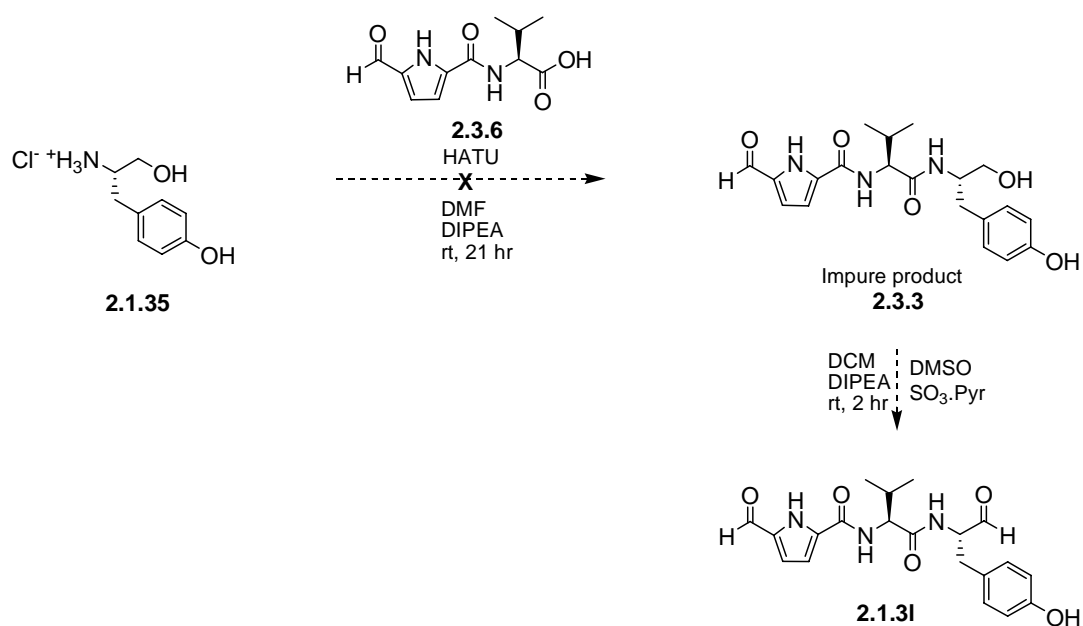
Second attempted synthesis of 2.1.31, synthesis of 5-formyl pyrrole 2-carboxylate L-valine 2.3.6

In this synthesis (see Scheme 2.20), the coupling reaction between L-valine methyl ester hydrochloride **2.3.4** with 5-formyl pyrrole 2-carboxylic acid **2.2.4** was attempted with EDCI.HCl as the activation agent and HOBT.H₂O as the coupling reagent and gave 5-formyl pyrrole 2-carboxylate L-valine methyl ester **2.3.5** as a white solid in 45% yield. Then the methyl ester was hydrolysed to the free acid 5-formyl pyrrole 2-carboxylate L-valine **2.3.6** with aqueous sodium hydroxide. Confirmation of the acid **2.3.6** was confirmed with ¹H NMR spectra. Mass spectra detected the acid **2.3.6** as both the hydrogen and sodium adducts.



Scheme 2.20 Synthesis of 5-formyl pyrrole 2-carboxylate L-valine **2.3.6**

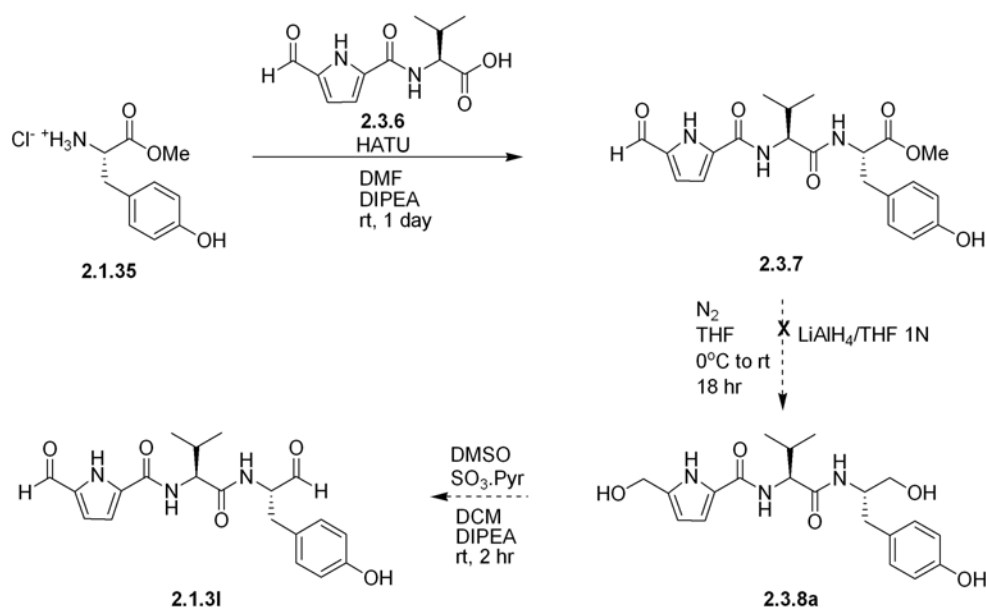
The free acid **2.3.6** was reacted with L-tyrosinol hydrochloride **2.1.35** in Scheme 2.21 in the presence of HATU to give 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinol **2.3.3** as crude. The extent of the impurity in the crude was confirmed with TLC producing many spots of unknown compounds and ¹H NMR as complex spectra. The compound of interest **2.3.3** could not be purified to obtain sufficient alcohol **2.1.31** for oxidation.



Scheme 2.21 Synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal **2.1.31**

Third attempted synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal

This third synthesis involved coupling between pre-synthesised L-tyrosine methyl ester hydrochloride **2.1.35** and 5-formyl pyrrole 2-carboxylate L-valine **2.3.6** with HATU over 21 hr to give a low yield (14%) of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosine methyl ester **2.3.7**. Then the methyl ester could not be reduced with $\text{LiAlH}_4/\text{THF}$ and gave a complex mixture including starting material. Separation on silica (EtOAc/pet ether) was unsuccessful and any further synthesis of this target compound **2.1.31** was abandoned.

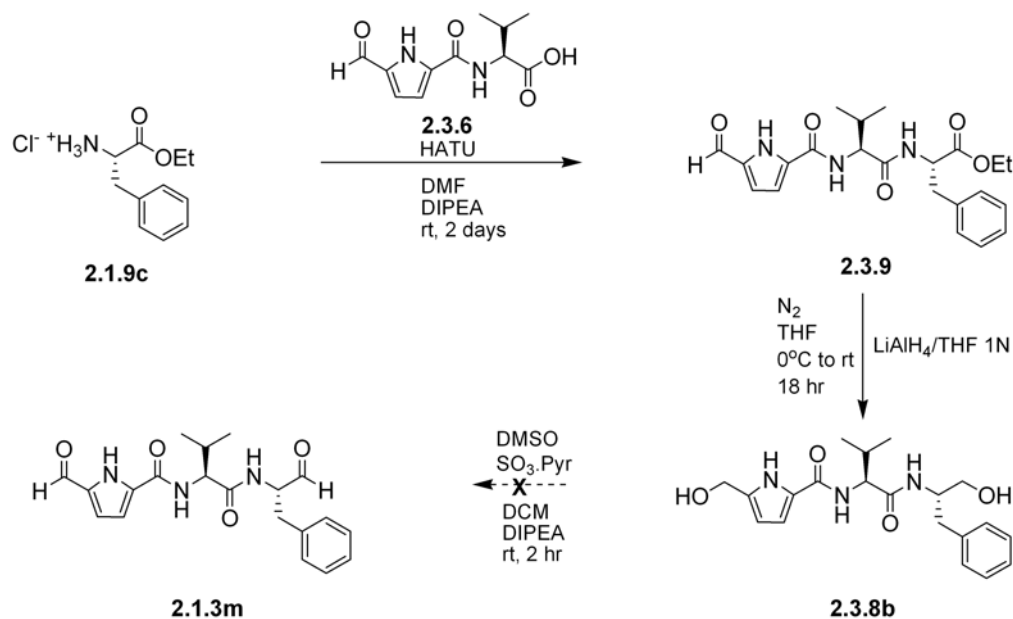


Scheme 2.22 Attempted synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal **2.1.31** via the diol route

Attempted synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-phenylalaninal **2.1.3m**

The attempted synthesis of **2.1.3m** (Scheme 2.23) was similar to that of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal **2.1.31**. The only variation to this synthesis was the use of a pre-synthesised ethyl ester hydrochloride **2.1.9c** as opposed to an esterified methyl ester hydrochloride used as the starting material. The coupling reaction between the L-valine compound **2.3.6**, L-phenylalanine ethyl ester hydrochloride **2.1.9c** and HATU was successful to give the ethyl ester **2.3.9** as an orange solid. Reduction reaction of the ethyl ester with LiAlH_4 in THF over 17 hr produced a complex mixture including starting ethyl ester; chromatography on silica give a 29% yield of the diol **2.3.8b** as a white solid. The oxidation of the diol using DMSO

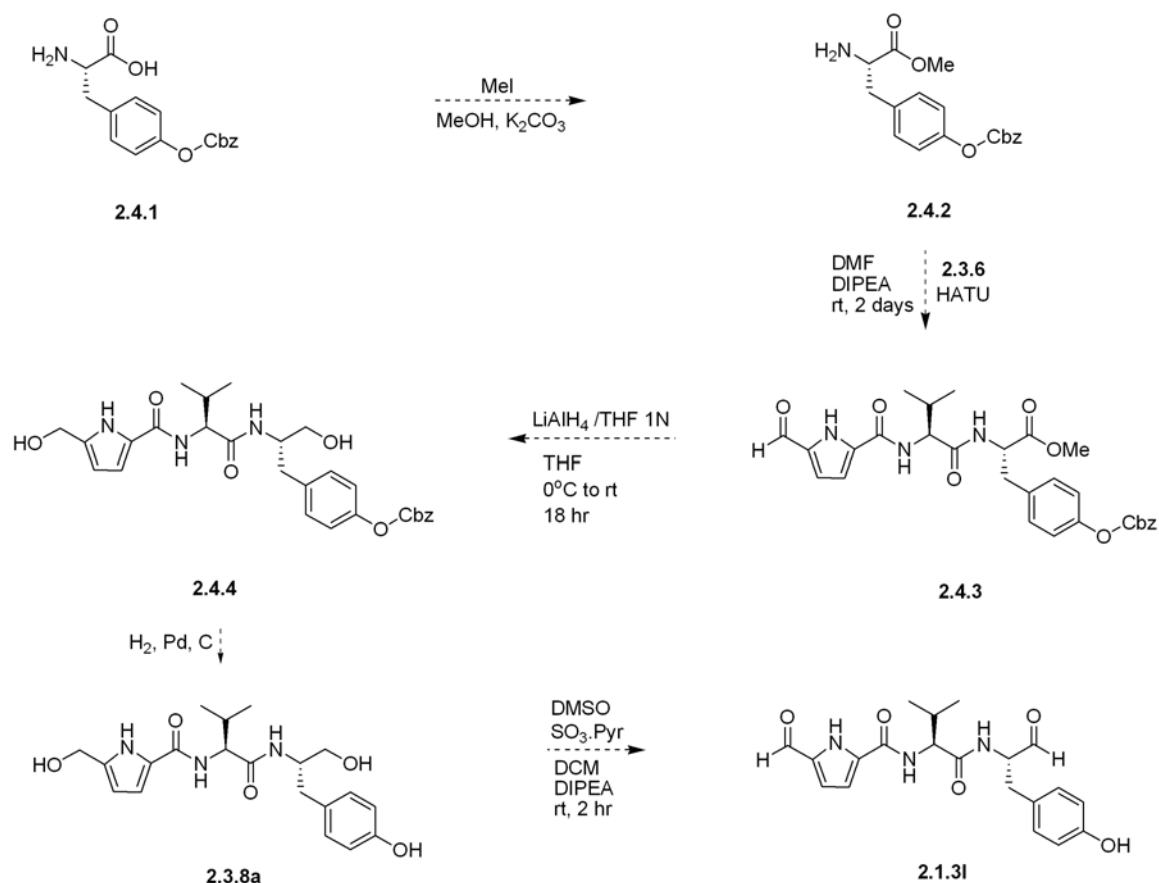
and 8 equivalents of $\text{SO}_3\cdot\text{Pyr}$ was unsuccessful. The ^1H NMR spectra in deuterated DMSO of the reaction mixture was complex.



Scheme 2.23 Attempted synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-phenylalanylal

2.4 Future work

Comparing the unsuccessful synthesis of the L-tyrosinal **2.1.3l** in Schemes 2.19, 2.21 and 2.22 with the successful synthesis of the closely related sulfonyl alcohol **2.1.11j**; may indicate a possible side reaction occurring with the phenolic alcohol in the L-tyrosinol and the 5-formyl pyrrole moiety. This was not clarified experimentally, however, to prevent this potential reaction occurring it would be possible to start with L-tyrosine with its phenolic alcohol protected with a benzyl group **2.4.1**. Then esterification may be possible to the methyl ester **2.4.2** under basic conditions with MeI and K_2CO_3 . Subsequently the methyl ester **2.4.2** would be coupled to 5-formyl pyrrole 2-carboxylate L-valine **2.3.6** using HATU as coupling reagent. The methyl ester **2.4.3** could be reduced to the L-tyrosine protected diol **2.4.4** with $\text{LiAlH}_4/\text{THF}$. Next a deprotection step with H_2/Pd on C of the L-tyrosine protected diol **2.4.4** should afford 5-hydroxymethyl pyrrole 2-carboxylate L-valine L-tyrosinol **2.3.8a**. The final oxidation step with DMSO and 8 equiv. of $\text{SO}_3\cdot\text{Pyr}$ should afford the target aldehyde **2.1.3l**.

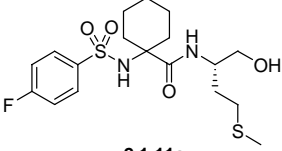
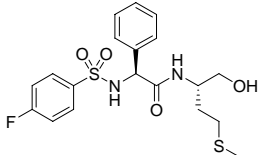
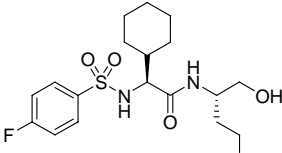
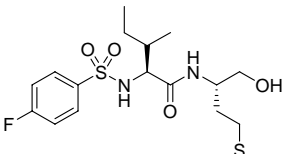
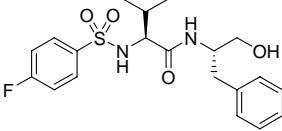
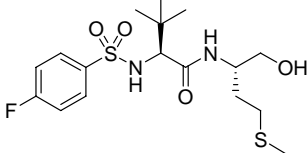
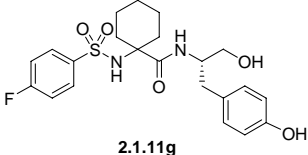
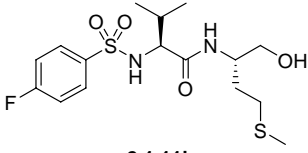


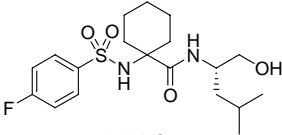
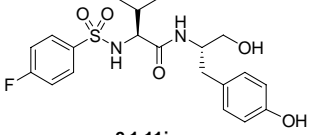
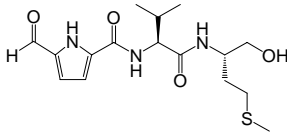
Scheme 2.24 Suggested synthesis of 5-formyl pyrrole 2-carboxylate L-valine L-tyrosinal

2.5 Computational modelling and biological activity

The peptide alcohols **2.1.11a-k** and peptide aldehydes **2.1.3a-k** were docked to μ -calpain and cathepsin B using computational modelling (all modelling studies were performed by Wangting Jiao and Steve McNabb). The modelling results are displayed in Table **2.8**, **2.10**, **2.12** and include glide scores, warhead distance in Å, hydrogen bond contacts to μ -calpain (labelled [A], [B] and [C] where [A] and [B] are hydrogen bonds formed between an inhibitor and Gly₂₀₈ of the enzyme and [C] is a hydrogen bond formed between an inhibitor and Gly₂₇₁ of the enzyme), hydrogen bond contacts to cathepsin B (labelled [A, B] and [C] where [A] and [B] are hydrogen bonds formed between an inhibitor and Gly₇₄ of the enzyme and C is a hydrogen bond formed between an inhibitor and Gly₁₉₈ of the enzyme), and any extra hydrogen bonds.

Table 2.8 Molecular modelling results of peptide alcohols **2.1.11a-k** docked to μ -calpain and [cathepsin B].

Compound	Glide Score	H-bonds A,B,C	Extra H- bonds	Warhead Distance (Å)
 2.1.11a	-5.37 [-4.55]	0 [A]	3 [1]	4.08 [5.78]
 2.1.11b	-5.54 [-6.79]	C [0]	2 [3]	3.95 [3.77]
 2.1.11c	-4.94 [-6.01]	A,C [A,B,C]	0 [1]	3.67 [3.52]
 2.1.11d	-4.72 [-6.33]	A,B,C [A,B,C]	1 [F]	3.34 [3.25]
 2.1.11e	-5.95 [-4.13]	A,B,C [A,B,C]	1 [F]	3.57 [4.19]
 2.1.11f	-2.89 [-5.64]	0 [B,C]	2 [F,+1]	3.99 [3.67]
 2.1.11g	-5.17	0	2	3.89
 2.1.11h	-4.914 [-5.54]	A,B,C [A,B,C]	0 [F,+1]	4.090 [3.49]

	-1.149	B, C, C	0	3.423
2.1.11i				
	-4.799	A,B,C	0	3.917
2.1.11j	[-4.90]	[A, C]	[0]	[4.38]
	-5.677	A,B,C	0	3.505
2.1.11k				

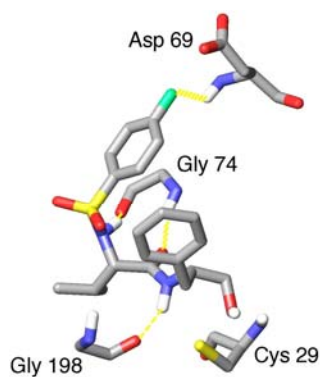
Peptide alcohols

Alcohol **2.1.11d** gave the best modelling results for both μ -calpain and cathepsin B (Table 2.8) as indicated by the smallest warhead distance between the alcohol and the active site cysteine, 3.34 Å and 3.25 Å respectively. A strong F-H hydrogen bond was predicted for compounds **2.1.11d**, **2.1.11e**, **2.1.11f** and **2.1.11h** to Asp₆₉ of cathepsin B. No evidence of an F-H hydrogen bond between any of the compounds and μ -calpain was revealed by molecular modelling inferring that the observed F-H hydrogen bond is unique to cathepsin B.

The peptide alcohols **2.1.11a-k** were assayed against ovine m-calpain (purified from ovine lung tissue) and cathepsin B using a fluorescence-based assay to determine *in-vitro* potency and selectivity (assays were performed by Dr's Matthew Jones and Janna Mehrtens). The assay results are summarised in Table 2.9. The peptide alcohol **2.1.11e** was the most potent inhibitor of m-calpain and cathepsin B ($IC_{50} = 2.3 \mu\text{M}$ for m-calpain and $IC_{50} = 75 \text{ nM}$ for cathepsin B) with good selectivity (30.75 fold) for cathepsin B over m-calpain. The compound was also assayed against μ -calpain and shown to be a good inhibitor ($IC_{50} = 1.5 \mu\text{M}$ for μ -calpain). The selectivity of compound **2.1.11e** for cathepsin B over m-calpain may be due to the predicted formation of the F-H bond between the P₃ address region and Asp₆₉ of cathepsin B (Figure 2.8). The modelling results indicate that compound **2.1.11e** forms a β -strand conformation in the enzymes active site with hydrogen bond to Gly₇₄ [A and B], Gly₂₇₈ [C] and an extra F-H bond to Asp₆₉.

Table 2.9 Biological activity of peptide alcohols **2.1.11a-k**

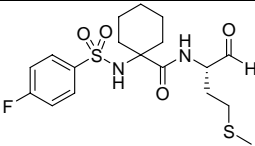
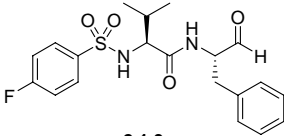
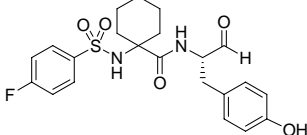
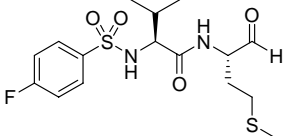
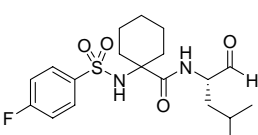
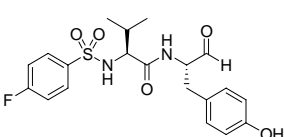
Compound	P ₃	P ₂	P ₁	IC ₅₀	IC ₅₀	Selectivity (mC/CB)
				(μM) m-Cal	(μM) Cath B	
2.1.11a	<i>p</i> FPhSO ₂	Chx	L-MetCH ₂ OH	>50	>50	Zero
2.1.11b	<i>p</i> FPhSO ₂	L-Pgl	L-MetCH ₂ OH	19	>50	0.38
2.1.11c	<i>p</i> FPhSO ₂	L-Chg	L-MetCH ₂ OH	>50	27	1.85
2.1.11d	<i>p</i> FPhSO ₂	L-Ile	L-MetCH ₂ OH	25.7	>50	0.51
2.1.11e	<i>p</i> FPhSO ₂	L-Val	L-PheCH ₂ OH	2.3 1.5 ^a	0.075	30.75
2.1.11f	<i>p</i> FPhSO ₂	L-Tle	L-MetCH ₂ OH	>50	14	3.57
2.1.11g	<i>p</i> FPhSO ₂	Chx	L-TyrCH ₂ OH	50	NA ^b	
2.1.11h	<i>p</i> FPhSO ₂	L-Val	L-MetCH ₂ OH	>50	>50	Zero
2.1.11i	<i>p</i> FPhSO ₂	Chx	L-LeuCH ₂ OH	>50	NA ^b	
2.1.11j	<i>p</i> FPhSO ₂	L-Val	L-TyrCH ₂ OH	>50	>50	Zero
2.1.11k	5-CHO pyrrole	L-Val	L-MetCH ₂ OH	NA ^b	NA ^b	

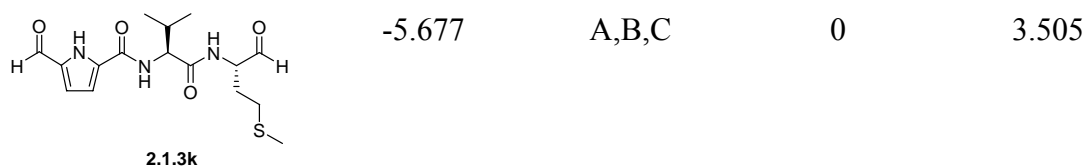
^a Enzyme assay with μ-calpain (nM)^b Not assayed**Figure 2.8** Modelling results of compound *N*-(4-fluorobenzenesulfonyl) L-valine L-phenylalaninol **2.1.11e** in the active site of cathepsin B.

Peptide aldehydes

The modelling results for the peptide aldehydes **2.1.3a**, **2.1.3e**, **2.1.3g**, **2.1.3h**, **2.1.3i**, **2.1.3j** and **2.1.3k** are shown in Table 2.10. Five hydrogen bonds, [A,B,C] and two extra H bonds including a F-H bond, are formed between compound **2.1.3h** and cathepsin B. The modelling results indicate three hydrogen bonds [A, B, C] are formed between compound **2.1.3e** and μ -calpain with the aldehyde warhead in close proximity (3.497 Å) to the active site cysteine.

Table 2.10 Molecular modelling results of the peptide aldehydes **2.1.3a**, **2.1.3e**, **2.1.3g**, **2.1.3h**, **2.1.3i**, **2.1.3j** and **2.1.3k** docked to μ -calpain and [cathepsin B]

Compound	Glide Score	H-bonds A,B,C	Extra H- bonds	Warhead Distance (Å)
 2.1.3a	-6.512 [-4.61]	A,C [A,B,C]	1 [0]	4.016 [3.38]
 2.1.3e	-6.080 [-3.32]	A,B,C [A,B,C]	0 [F,+1]	3.497 [3.64]
 2.1.3g	-5.322	A, B	3	3.958
 2.1.3h	-4.914 [-5.54]	A,B,C [A,B,C]	0 [F,+1]	4.090 [3.49]
 2.1.3i	-1.149	B,C,C	0	3.423
 2.1.3j	-4.799 [-4.90]	A,B,C [A,C]	0 [0]	3.917 [4.38]



The peptide aldehydes **2.1.3a**, **2.1.3e**, **2.1.3g**, **2.1.3h**, **2.1.3i**, **2.1.3j** and **2.1.3k** were assayed against ovine m-calpain (purified from ovine lung tissue) and cathepsin B using a fluorescence-based assay to determine *in vitro* potency and selectivity (Table 2.11). The results revealed three potent inhibitors **2.1.3e**, **2.1.3h** and **2.1.3k** with IC₅₀ constants in the nM range for the calpains. The compounds **2.1.3e**, **2.1.3h** **2.1.3j** was highly potent against cathepsin B. The compound **2.1.3a** was the most potent inhibitor for both isoforms of the calpains and cathepsin B (IC₅₀ = 93 nM for m-calpain, 12 nM for μ -calpain and IC₅₀ = 16 nM for cathepsin B). The most selective inhibitor was compound **2.1.3a**, displaying a 41 fold selectivity for cathepsin B over m-calpain.

Table 2.11 Biological activity of peptide aldehydes **2.1.3a**, **2.1.3e**, **2.1.3g**, **2.1.3h**, **2.1.3i**, **2.1.3j** and **2.1.3k**. (P₁ variations)

Compound	P ₃	P ₂	P ₁	IC ₅₀ (nM) m- cal	IC ₅₀ (nM) Cath B	Selectivity (mC/CB)
2.1.3a	<i>p</i> FPhSO ₂	Chx	L-Met-H	9300	228	40.79
2.1.3e	<i>p</i> FPhSO ₂	L-Val	L-Phe-H	93 12 ^a	16	4.5
2.1.3g	<i>p</i> FPhSO ₂	Chx	L-Tyr-H	1530	NA ^b	
2.1.3h	<i>p</i> FPhSO ₂	L-Val	L-Met-H	94	57	1.65
2.1.3i	<i>p</i> FPhSO ₂	Chx	L-Leu-H	2280	NA ^b	
2.1.3j	<i>p</i> FPhSO ₂	L-Val	L-Tyr-H	NA ^b	27	
2.1.3k	5-CHO pyrrole	L-Val	L-Met-H	135	NA ^b	

^a Enzyme assay with μ -calpain (nM)

^b Not assayed

The selectivity obtained for **2.1.3a** for cathepsin B over m-calpain may be a consequence of numerous effects. A factor that may account for this apparent selectivity is the formation of a stable β -strand when docked into the active site of cathepsin B in Figure 2.9. This is in sharp contrast the docking results of compound **2.1.3a** in the active site of μ -calpain (not shown) where the β -strand fails to form.

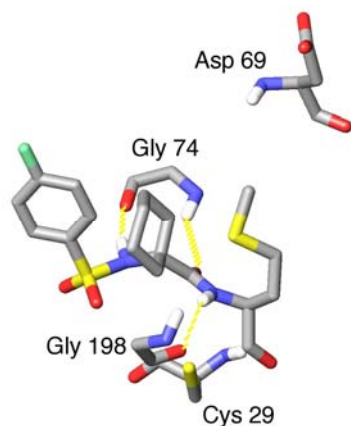


Figure 2.9 Compound (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(4-(methylthio)-1-oxobutan-2-yl)cyclohexanecarboxamide docked in the active site of cathepsin B showing hydrogen bonds [A] and [B] to Gly₇₄ and hydrogen bond [C] to Gly₁₉₈

The dipeptide dialdehyde inhibitor 5-formyl pyrrole 2-carboxylate L-valine L-methioninal **2.1.3k** displayed good inhibition against m-calpain ($IC_{50} = 135$ nM). This was 5.4 fold less potent than the template m-calpain inhibitor CAT-0059 **1.7.5** synthesised by Shigeru Miyamoto^{23,109} ($IC_{50} = 25$ nM). Modelling results of inhibitor **2.1.3k** docked to μ -calpain suggests the compound binds in a unique way to enzyme's active site compared to the sulfonyl analogues; this unique binding mode is illustrated in Figures 2.10 and 2.11 below.

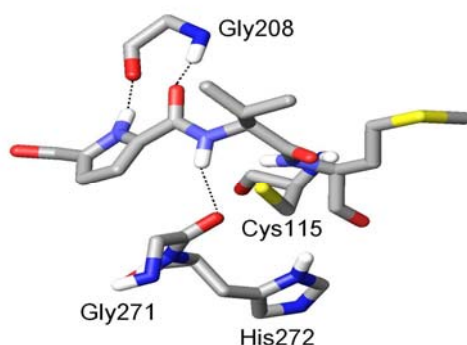
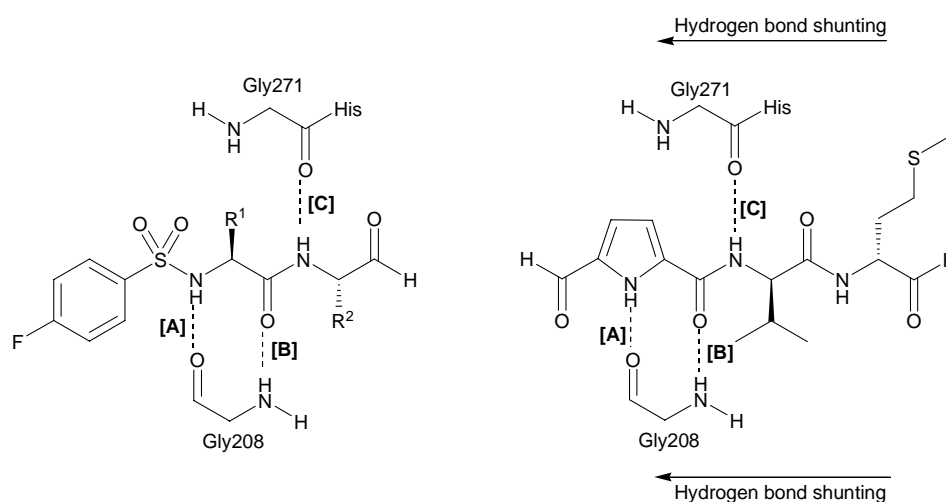


Figure 2.10 5-Formyl pyrrole 2-carboxylate L-valine L-methioninal **2.1.3k** docked in the active site of μ -calpain

Computational modelling of **2.1.3k** to the active site of μ -calpain predicts a shunted binding conformation as shown in Figure 2.10. The compound **2.1.3k** adopts a conformation where hydrogen bond [A] is replaced by a hydrogen bond formed between the pyrrole NH and Gly₂₀₈.



R¹ and R²= α -amino acid

Figure 2.11 Compounds *N*-(4-fluorobenzenesulfonyl) analogues (left) and 5-formyl pyrrole L-valine L-methioninal **2.1.3k** (right), showing the hydrogen bond interactions [A], [B] and [C] with glycine residues

Evaluation of the variations of α -amino acids at P₁ address region

The methionine containing compounds **2.1.3a**, **2.1.3h** and **2.1.3k** were potent inhibitors of both m-calpain and cathepsin B. Compound **2.1.3h** exhibited a 1.65 fold increase in potency for cathepsin B $IC_{50} = 57$ nM over m-calpain $IC_{50} = 94$ nM. The phenylalanine compound **2.1.3e** displayed excellent binding to the assayed enzymes.

Table 2.12 Modelling results for peptide aldehyde inhibitors **2.1.3a**, **2.1.3b**, **2.1.3c**, **2.1.3d**, **2.1.3f** and **2.1.3h** to m-calpain and [cathepsin B]

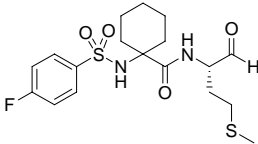
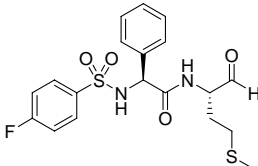
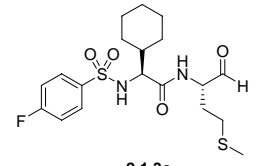
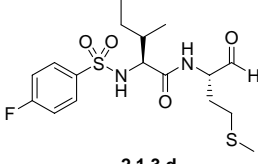
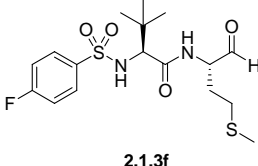
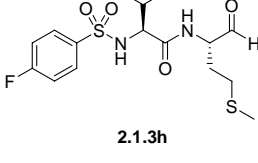
Compound	Glide score	H-bonds A,B,C	Extra H- bonds	War Head Distance (Å)
 2.1.3a	-6.512 [-4.61]	A,C [A,B,C]	1 [0]	4.016 [3.38]
 2.1.3b	-3.925 [-6.79]	C,C [0]	1 [3]	3.759 [3.77]
 2.1.3c	-4.785 [-6.01]	A,C [A,B,C]	0 [1]	3.502 [3.52]
 2.1.3d	-5.371 [-5.79]	A,B,C [B,C]	0 [F]	3.841 [3.70]
 2.1.3f	-4.943 [-6.01]	B,C [A,B,C]	1 [0]	5.491 [3.67]
 2.1.3h	-4.914 [-5.54]	A,B,C [A,B,C]	0 [F,+1]	4.090 [3.49]

Table 2.12 shows the modelling results for the P₂ variations **2.1.3a**, **2.1.3b**, **2.1.3c**, **2.1.3d**, **2.1.3f** and **2.1.3h**. Computational modelling predicted compound **2.1.3d** had a closer warhead distance to Cys₁₁₅ of μ -calpain (3.841 Å) compared to compound **2.1.3h** (4.090 Å). This could be attributed to an extra CH₂ group on the side chain of *iso* L-leucine giving rise to extra extension into the enzymes active site. This may suggest enhanced inhibition for *iso* L-leucine at P₂ address region over its L-valine derivate **2.1.3h**. In addition the modelling suggested **2.1.3d** would take on the β -strand conformation (Figure 2.12). However, the assay results for the aldehyde **2.1.3h** was 3.36 fold more potent than the compound **2.1.3d** at 317 nM (Table 2.13). In this case the assay results did not concur with the modelling predictions made for the P₂ preferences of μ -calpain.

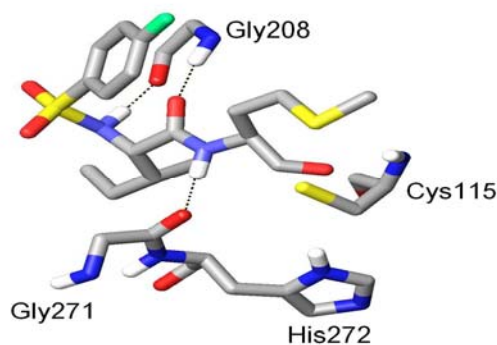


Figure 2.12 *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine L-methioninal docked in the active site of μ -calpain

Table 2.13 Biological activity of peptide aldehydes **2.1.3a**, **2.1.3b**, **2.1.3c**, **2.1.3d**, **2.1.3f** and **2.1.3h** (P₂ variations)

Compound	P ₃	P ₂	P ₁	IC ₅₀	IC ₅₀	Selectivity (mC/CB)
				(nM) m-cal	(nM) Cat B	
2.1.3a	<i>p</i> FPhSO ₂	Chx	L-Met-H	9300	228	40.79
2.1.3b	<i>p</i> FPhSO ₂	L-Pgl	L-Met-H	315	122	2.58
2.1.3c	<i>p</i> FPhSO ₂	L-Chg	L-Met-H	5000	400	12.5
2.1.3d	<i>p</i> FPhSO ₂	L-Ile	L-Tyr-H	317	260	1.22
2.1.3f	<i>p</i> FPhSO ₂	L-Tle	L-Met-H	14500	2800	5.18
2.1.3h	<i>p</i> FPhSO ₂	L-Val	L-Met-H	94	57	1.65

The enzyme assay results for the P₂ variations compounds **2.1.3a**, **2.1.3b**, **2.1.3c**, **2.1.3d**, **2.1.3f** and **2.1.3h** are shown in table (Table 2.13). The results reveal that compounds **2.1.3b**, **2.1.3d** and **2.1.3h** are highly potent with IC₅₀'s in the nM range for m-calpain. Compound **2.1.3h** was more potent against m-calpain (IC₅₀ = 94 nM) compared to cathepsin B (IC₅₀ = 57 nM).

The aldehyde **2.1.3f** displayed low potency against m-calpain and cathepsin B (IC₅₀ = 14.5 μM and 2.8 μM respectively) (Table 2.13). The modelling studies suggested **2.1.3f** bound to m-calpain but didn't display a hydrogen bonding interactions between **NH** of *tert* L-leucine and the carbonyl group on Gly₂₀₈. However, the Gly₂₀₈ residue did form a hydrogen bond to the aldehyde warhead on the L-methioninal moiety as seen in Figure 2.13. The computational modelling reveals the influence of the extra methyl group on *tert* L-leucine moiety of **2.1.3f**. The incorporation of this group results in a reverse binding mode in the active site of μ-calpain and gives an unfavourable warhead distance of 5.491 Å.

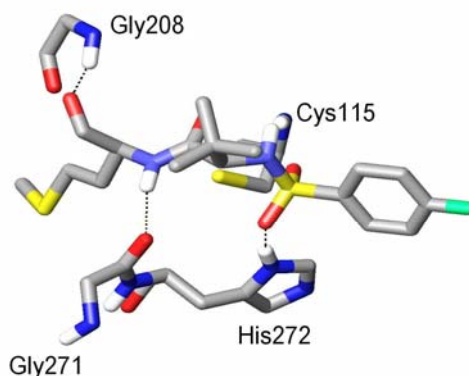


Figure 2.13 Compound *N*-(4-fluorobenzenesulfonyl) *tert* L-leucine L-methioninal **2.1.3f** docked in the active site of μ -calpain revealing a reverse binding mode

Evaluation of the variations of α -amino acids at P_2 address region

m-Calpain was found to favour L-valine, *iso* L-leucine and L-phenylglycine at the P_2 address region with compound **2.1.3h** (containing L-valine) being the most active inhibitor. The inclusion of the bulky unnatural amino acids L-cyclohexaglycine (Chg), *tert* L-leucine (Tle) and 1,1 cyclohexane (Chx) resulted in poor enzyme inhibition of m-calpain. These assays results suggest the S_2 subsite has a low tolerance for bulky amino acids.

The S_2 subsite of cathepsin B seems to have a higher tolerance (gleaned from assay results) for the inclusion of unnatural amino acids used in this investigation such as Chg, with inhibition concentrations in the nM range. However, in agreement with the m-calpain assay results the inclusion of a *tert* L-leucine residue in compound **2.1.3f** results in decreased potency compared to compound **2.1.3h** that contains L-valine at P_2 .

2.6 Conclusion

The CA clan cysteine proteases m-calpain and cathepsin B are implicated in a number of human pathologies including cataract²⁶ and tumour metastases¹⁰ respectively. It's thought inhibiting these enzymes may arrest disease progression.¹¹⁷ In this investigation the successful synthesis peptide aldehydes **2.1.3a-j** based on the SJA-6017 **1.7.1**¹¹⁷ was completed. A potent dipeptide dialdehyde inhibitor **2.1.3k** ($IC_{50} = 135$ nM m-calpain) was successfully synthesised using CAT-0059 **1.7.3** as template.¹⁰³ The variations at the P_1 position produced the most potent inhibitors against the tested enzymes. The compound **2.1.3e** was the most potent inhibitor for μ -calpain and m-calpain with respective IC_{50} concentrations 16 nM and 93 nM. The aldehyde **2.1.3h** ($IC_{50} = 94$ nM for m-calpain) was also a highly active inhibitor. The best cathepsin B inhibitors were the sulfonyl L-valine L-phenylalaninal **2.1.3e** $IC_{50} = 16$ nM and the L-tyrosinal **2.1.3j** $IC_{50} = 27$ nM. Despite their high potency, none of these inhibitors **2.1.3d**, **2.1.3i**, and **2.1.3j** were selective for the enzyme assayed. However selectivity for cathepsin B was marginally improved with the inclusion of bulky aliphatic α -amino acids in the P_2 position of the inhibitor. For example the peptide alcohol **2.1.11c** gave a 13 fold selectivity for cathepsin B ($IC_{50} = 5.0$ μ M for m-calpain and $IC_{50} = 400$ nM for cathepsin B). The synthesis of an m-calpain selective inhibitor was not achieved in this study. Compound **2.1.3k** demonstrated a shunted binding conformation to μ -calpain where the pyrrole **NH** replaced hydrogen bond [A]. Computational modelling predicted the existence of an extra F-H bond for the peptide alcohols **2.1.11b**, **2.1.11d**, **2.1.11g**, **2.1.11j** and the peptide aldehydes **2.1.3b**, **2.1.3d** and **2.1.3j** which were unique to for cathepsin B and seemed to aid binding.

Chapter 3

Experimental

3.1 General experimental methods and procedures

Dichloromethane, ethyl acetate, petroleum ether and triethylamine were distilled over CaH_2 . THF was dried over sodium or potassium metal wire utilising a wetness indicator, benzylphenone. Product mixtures from reactions (when required) were examined by TLC using *Polygram* SIL G/UV₂₅₄ plastic backed silica plates and EtOAc/petroleum ether, EtOAc/MeOH or DCM/MeOH solvent mixtures. Visualisation was achieved by ultra violet light (UV) or using either KMnO_4 or phosphomolybdic acid (PMA) solution. The KMnO_4 solution was prepared with KMnO_4 (3 g), K_2CO_3 (20 g), NaOH (5.0 mL; 0.05 N) and water (300 mL). The PMA solution was prepared with phosphomolybdic acid (12 g) in ethanol (250 mL). Crude mixtures (not including compounds with polar groups such as CO_2H and hydrochloride salts) of >200 mg were either loaded onto a column with dichloromethane or dry loaded onto silica using a *Buchi* rotary evaporator. Separation was achieved by using a *Buchi* C-660 fraction collector.

The common name for compounds is mentioned first followed by the systematic name generated from *ChemDraw Ultra* v. 9.0. Melting points were obtained with an *Electrotherm* melting apparatus using *Samco* 100 mm soda glass capillary tubes. Experimental melting points are reported to the nearest 1°C after any literature melting points when known.

Infrared (IR) experiments were carried out on a *Shimadzu* FTIR-8201PC Fourier Transform Infrared Spectrophotometer and referenced to KBr and reported to the nearest ν_{max} 1.0 cm^{-1} .

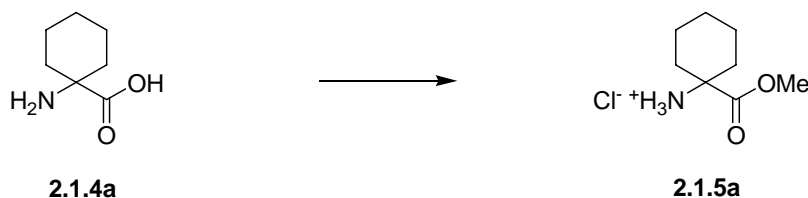
Nuclear Magnetic Resonance (NMR). The NMR solvents used in this study (CDCl_3 , CD_3OD and $(\text{CD}_3)_2\text{SO}$) were supplied by *Cambridge Isotope Laboratories*. ^1H NMR experiments including ^1H and GCOSY spectra were obtained from either a *Varian UNITY* operating at 300 Hz or a *Varian INOVA Unity* operating at 500 Hz. The operating temperature used on both instruments was 23°C, however occasionally a temperature of 25°C was also used. The *INOVA* was equipped with a variable temperature and inverse-detection 5 mm probe or a triple-resonance indirect detection PFG. ^{13}C NMR spectra were recorded on a *Varian UNITY* 300 NMR spectrometer equipped with a variable temperature direct broadband 5 mm probe, operating at 75 MHz. The operating temperatures used were 23°C, however occasionally a

temperature of 25°C was also used. Chemical shifts are expressed in parts per million (ppm) on the δ scale and reported to the nearest ^1H $\delta_{\text{H}} = 0.01$ and for ^{13}C $\delta_{\text{C}} = 0.1$. CDCl_3 is referenced to CHCl_3 at $\delta_{\text{H}} = 7.25$ (^1H); CD_3OD referenced to CHD_2OD at $\delta_{\text{H}} = 3.31$ (^1H) and CD_3OD at $\delta_{\text{C}} = 49.3$ (^{13}C); $(\text{CD}_3)_2\text{SO}$ referenced to $\text{CD}_3(\text{CHD}_2)\text{SO}$ at $\delta_{\text{H}} = 2.50$ (^1H) and $(\text{CD}_3)_2\text{SO}$ at $\delta_{\text{C}} = 39.6$ (^{13}C).

High Resolution Mass Spectra (HRMS) were performed on a *Micromass* LCT spectrometer using a probe voltage of 3,200V, with an operating temperature of 150°C and a source temperature of 80°C. The carrier solvent was 50:50 mixture of $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ at a flow rate of 20 $\mu\text{L}/\text{minute}$. Typically, 10 μL of a 10 $\mu\text{g}/\text{mL}$ solution was injected. Leucine enkephalin was used as the lock mass internal standard at $[\text{M}+\text{H}]^+ = m/z$ 527.2771. Mass spectrometry measurements were reported to $m/z = 0.0001$ and within a tolerance range of 5.0 ppm from its calculated mass.

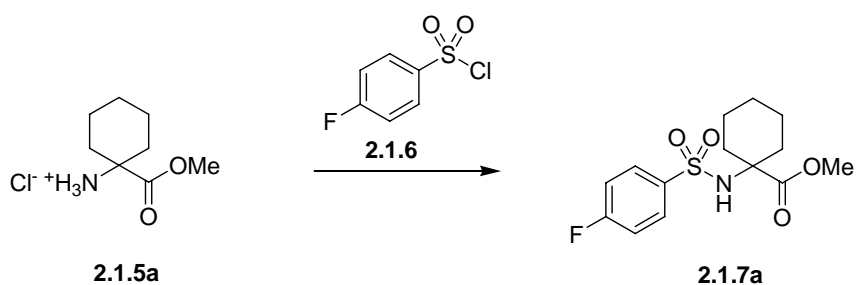
3.2 Experimental

1-(Methoxycarbonyl)cyclohexanaminium chloride



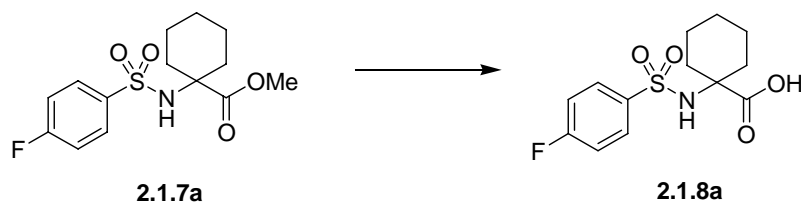
A solution containing 1-aminocyclohexanecarboxylic acid 4.000 g (27.935 mmol, 1 equiv.) in MeOH (65 mL) was cooled to -20°C in a NaCl ice bath. SOCl_2 (16 mL) (223.479 mmol, 8 equiv.) added dropwise over 20 min and the reaction mixture stirred for 1 hr at -20°C and allowed to return to room temperature and stirred for 1 day. The solution was diluted with MeOH (200 mL) and the solvent removed *in-vacuo* to give 1-(methoxycarbonyl)cyclohexanaminium chloride 5.410 g (27.935 mmol, 100%) as a white solid. lit. m.p. $210\text{--}212^\circ\text{C}$ ¹¹⁸ m.p. 209°C ; ^1H NMR ($(\text{CD}_3)_2\text{SO}$) (300 MHz) $\delta_{\text{H}} = 8.87$ (3H, s, CNH_3^+), 3.86 (3H, s, OCH_3), 2.10-1.51 (10H, m, CH_2 's); (HRMS) (CH_3OH) calcd. for the free amine $\text{C}_8\text{H}_{16}\text{NO}_2$ $[\text{M} + \text{H}]^+$: 158.1181, found 158.1186.

Methyl 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylate



A solution containing 1-(methoxycarbonyl)cyclohexanaminium chloride 4.77 g (24.666 mmol, 1 equiv.) in DMF (15 mL) and DIPEA (9.5 mL) (54.266 mmol, 2.2 equiv.) was cooled to 0°C in a ice bath. *N*-(4-Fluorobenzenesulfonyl) chloride 5.760 g (29.598 mmol, 1.2 equiv.) added over 15 min and the reaction mixture was left stirring for 1 day at room temperature. The mixture was partitioned between EtOAc (50 mL) and HCl (50 mL; 1N), and the aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated NaHCO₃ (50 mL) and saturated brine (50 mL) and dried with anhydrous Na₂SO₄ and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:3, R_f = 0.35) gave methyl 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylate 6.768 g (21.459 mmol, 87%) as a white solid. m.p. 139°C; ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 8.20 (1H, s, **NH**C), 7.93-7.89 (2H, m, **ArH**), 7.54-7.48 (2H, m, **ArH**), 3.49 (3H, s, **OCH**₃ of Chx), 1.91-1.75 (4H, m, **CH**₂'s of Chx), 1.39-1.25 (6H, m, **CH**₂'s of Chx); (HRMS) (CH₃OH) calcd. for C₁₄H₁₉FNO₄S [M + H]⁺: 316.1019, found 316.1030.

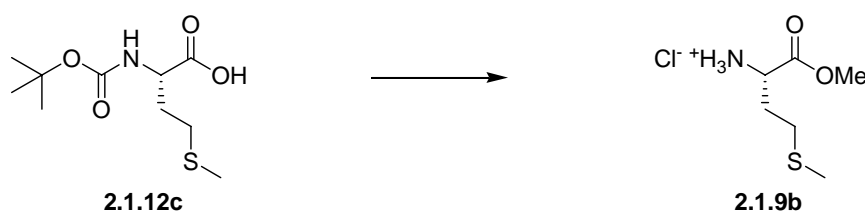
1-(4-Fluorophenylsulfonamido)cyclohexanecarboxylic acid



To methyl 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylate 6.570 g (20.833 mmol, 1 equiv.) in THF (30 mL), a solution of NaOH (10.0 mL; 5.208 N) was added slowly over 5 min. The mixture was heated to 65°C in a silica oil bath and stirred for 21 hr. The mixture was concentrated to half its original volume on a rotary evaporator and the resulting residue dissolved in 80 mL of distilled water. The aqueous phase was washed twice with EtOAc (80 mL) and the organic phase discarded. The reaction mixture was partitioned between EtOAc (80 mL) and HCl (80 mL; 1N) and the aqueous phase washed twice with EtOAc (80 mL). The combined organic phases were washed with saturated brine (80 mL) and dried with anhydrous Na₂SO₄ and the

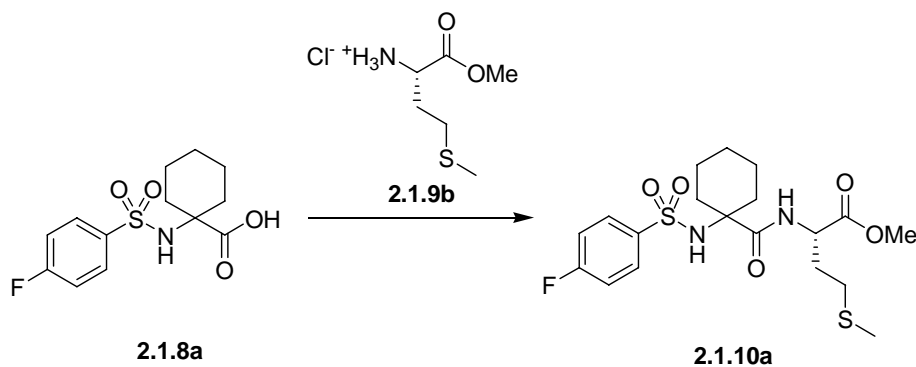
solvent removed *in-vacuo* to give 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylic acid 5.613 g (18.630 mmol, 89%) as a white solid. m.p. 215°C; $^1\text{H NMR}$ ($(\text{CD}_3)_2\text{SO}$) (300 MHz) $\delta_{\text{H}} = 12.50$ (1H, s, COOH of Chx), 8.01 (1H, s, NH of Chx) 7.95-7.90 (2H, m, ArH) 7.52-7.46 (2H, m, ArH) 1.90-1.76 (4H, m, CH₂'s of Chx) 1.50-1.20 (6H, m, CH₂'s of Chx); (HRMS) (CH_3OH) calcd. for $\text{C}_{13}\text{H}_{17}\text{FNO}_4\text{S}$ $[\text{M} + \text{H}]^+$: 302.0862, found 302.0877.

L-Methionine methyl ester hydrochloride [(S)-1-methoxy-4-(methylthio)-1-oxobutan-2-aminium chloride]



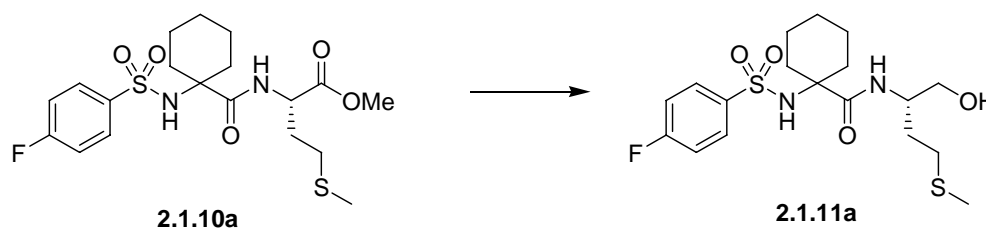
A solution of boc L-methionine 12.500 g (50.134 mmol, 1 equiv.) in MeOH (108 mL) was cooled to -20°C in a NaCl ice bath. SOCl_2 (4.4 mL) (60.161 mmol, 1.2 equiv.) added dropwise over 15 min. The reaction mixture was stirred for 2 hr at -20°C and allowed to come to room temperature for an additional 3 hr. The solution was diluted with MeOH (400 mL) and the solvent removed *in-vacuo* to give L-methionine methyl ester hydrochloride 10.012 g (50.134 mmol, 100%) as a white solid. lit. m.p. $145\text{-}149^\circ\text{C}^{119}$ m.p. 153°C ; $^1\text{H NMR}$ (CD_3OD) (300 MHz) $\delta_{\text{H}} = 4.05$ (1H, t, $J = 6.5$ Hz, CHCO), 3.68 (3H, s, OCH₃), 2.52-2.47 (2H, m, SCH₂), 2.09-1.94 (5H, m, CHCH₂CH₂ and SCH₃); (HRMS) (CH_3OH) calcd. for $\text{C}_6\text{H}_{14}\text{NO}_2\text{S}$ $[\text{M} + \text{H}]^+$: 164.0745, found 164.0740.

(S)-Methyl 2-(1-(4-fluorophenylsulfonamido)cyclohexanecarboxamido)-4-(methylthio)butanoate



To a solution (*S*)-methyl 2-(1-(4-fluorophenylsulfonamido)cyclohexanecarboxamido)-4-(methylthio)butanoate 1.800 g (5.974 mmol, 1 equiv.) in DMF (10 mL) kept under N₂, L-methionine methyl ester hydrochloride 1.312 g (6.571 mmol, 1.1 equiv.) added followed by DIPEA (2.3 mL) (13.143 mmol, 2.2 equiv.). HATU 2.498 g (6.571 mmol, 1.1 equiv.) added over 15 min and the reaction stirred for 2 days at room temperature. The reaction mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N), and the aqueous phase was washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO₃ (25 mL) and saturated brine (25 mL) and dried with anhydrous Na₂SO₄ and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:1, R_f = 0.43) gave (*S*)-methyl 2-(1-(4-fluorophenylsulfonamido)cyclohexanecarboxamido)-4-(methylthio)butanoate 2.460 g (5.509 mmol, 92%) as a white solid. m.p. 113°C; IR ν max (KBr) 1717 (COOCH₃), 1663 (CONH); ¹H NMR (CD₃OD) (300 MHz) δ_H = 7.96-7.91 (2H, m, ArH), 7.80 (1H, d, *J* = 7.5 Hz, NHCH of Met), 7.31-7.24 (2H, m, ArH), 4.57-4.50 (1H, m, NHCH of Met), 3.73 (3H, s, OCH₃ of Met), 2.70-2.54 (2H, m, SCH₂ of Met), 2.21-1.73 (9H, m, CH₂'s of Chx, SCH₃ of Met and CHCH₂CH₂ of Met), 1.42-1.00 (6H, m, CH₂'s of Chx); ¹³C NMR ((CD₃)₂SO) δ_C = 173.6, 172.2, 164.1 (d, *J* = 249.4 Hz, F-C=), 139.5 (=C-S), 129.6 (2C, d, *J* = 9.3 Hz, C=C-S), 116.1 (2C, d, *J* = 22.6 Hz, F-C=C), 61.7, 52.0, 51.4, 33.7, 32.2, 30.7, 29.6, 24.8, 21.1, 14.7 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₉H₂₈FN₂O₅S₂ [M + H]⁺: 447.1424, found 447.1439.

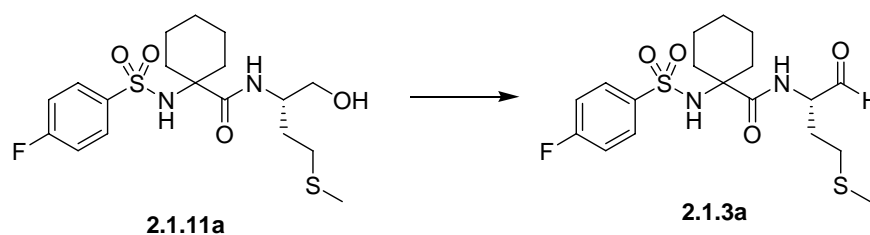
(*S*)-1-(4-Fluorophenylsulfonamido)-*N*-(1-hydroxy-4-(methylthio)butan-2-yl)cyclohexanecarboxamide



A two necked round bottom flask containing a solution of **2.1.10a** 2.222 g (4.976 mmol, 1 equiv.) in THF (7.2 mL) kept under N₂, was cooled to 0°C in a ice bath. Then a solution of LiAlH₄/THF (5.5 mL; 1N) added dropwise over 3 min. The reaction mixture was stirred at 0°C for 1 hr and allowed to come to room temperature and stirred for a further 17 hr. The reaction mixture was partitioned between EtOAc (25 mL) and KHSO₄ (25 mL; 1N), and the aqueous phase was washed twice with CHCl₃ (10 mL). The combined organic phases were washed with

saturated brine and dried with anhydrous MgSO_4 and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:1, $R_f = 0.28$) gave (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(1-hydroxy-4-(methylthio)butan-2-yl)cyclohexanecarboxamide 1.367 g (3.266 mmol, 66%) as an opaque solid. m.p. 108°C; IR ν_{max} (KBr) 3514 (H_2COH), 1651 (CONH), 1030 (H_2COH); ^1H NMR (CD_3OD) (300 MHz) $\delta_{\text{H}} = 7.96\text{--}7.91$ (2H, m, ArH), 7.30-7.25 (2H, m, ArH), 4.01-3.98 (1H, m, NHCHH of Met), 3.61-3.51 (2H, m, CH2OH of Met), 2.66-2.51 (2H, m, SCH2 of Met), 2.08 (1H, s, SCH3 of Met), 1.93-1.80 (6H, m, CH2's of Chx and CHCH₂CH₂ of Met), 1.43-1.05 (6H, m, CH2's of Chx); ^{13}C NMR ($(\text{CD}_3)_2\text{SO}$) $\delta_{\text{C}} = 173.4$ (CCONH of Chx), 164.0 (d, $J = 248.9$ Hz, F-C=), 139.8 (d, $J = 2.8$ Hz, =C-S), 129.6 (2C, d, $J = 9.4$ Hz, C=C-S), 116.1 (2C, d, $J = 22.5$ Hz, F-C=C), 62.9, 62.0, 50.3, 33.4, 32.6, 30.8, 30.1, 24.8, 21.2, 14.8 (SCH₃ of Met); (HRMS) (CH_3OH) calcd. for $\text{C}_{18}\text{H}_{28}\text{FN}_2\text{O}_4\text{S}_2$ [$\text{M} + \text{H}$]⁺: 419.1475, found 419.1484.

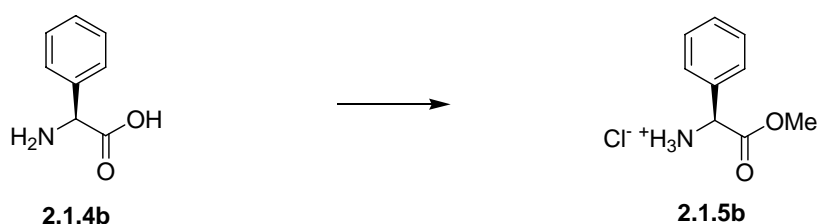
(*S*)-1-(4-Fluorophenylsulfonamido)-*N*-(4-(methylthio)-1-oxobutan-2-yl)cyclohexanecarboxamide



(*S*)-1-(4-Fluorophenylsulfonamido)-*N*-(1-hydroxy-4-(methylthio)butan-2-yl)cyclohexanecarboxamide 975 mg (2.330 mmol, 1 equiv.) in DMSO (8 mL) and DCM (11 mL) was kept under N_2 and DIPEA (1.6 mL) (9.318 mmol, 4 equiv.) was added. A separate vial containing $\text{SO}_3\cdot\text{Pyr}$ 1.483 g (9.318 mmol, 4 equiv.) dissolved in DMSO (4 mL) was heated and added slowly to the reaction mixture over 5 min. The reaction was stirred for 2 hr at room temperature. The reaction mixture was partitioned between EtOAc (20 mL) and HCl (20 mL; 1N), and the aqueous phase washed twice with EtOAc (20 mL). The combined organic phases were washed with saturated NaHCO_3 (20 mL) and saturated brine (20 mL) and dried with anhydrous Na_2SO_4 . The solvent was removed *in-vacuo*, to give (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(4-(methylthio)-1-oxobutan-2-yl)cyclohexanecarboxamide 820 mg (1.969 mmol, 84%) as white solid. m.p. 137°C; IR ν_{max} (KBr) 1719 (COH), 1650 (CONH); ^1H NMR ($(\text{CD}_3)_2\text{SO}$) (300 MHz) $\delta_{\text{H}} = 9.45$ (1H, s, CHO of Met), 8.13 (1H, d, $J = 6.9$ Hz, NHCH of Met), 8.01-7.96 (2H, m, ArH), 7.91 (1H, s, NHC of Chx), 7.81-7.47 (2H, m, ArH), 4.15-4.08 (1H, m, NHCHH of Met), 2.69-2.51 (2H, m, SCH2 of Met), 2.20-2.06 (4H, m, CHCH₂CH₂ of Met

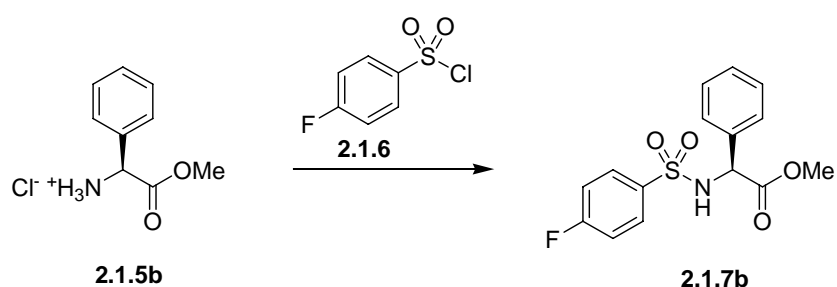
and SCH₃ of Met), 1.98-1.83 (5H, m, CHCH₂CH₂ of Met and CH₂'s of Chx), 1.40-1.25 (6H, m, CH₂'s of Chx); ¹³C NMR ((CD₃)₂SO) δ_C = 201.1 (CHO of Met), 174.2 (CONH of Chx), 164.0 (d, J = 248.9 Hz, F-C=), 139.7 (d, J = 2.8 Hz, =C-S), 129.5 (2C, d, J = 9.4 Hz, C=C-S), 116.1 (2C, d, J = 22.5 Hz, F-C=C), 61.5, 57.7, 33.3, 32.8, 29.5, 27.6, 24.7, 21.1, 14.5 (SCH₃ of Met); (HRMS) (CH₃CN) calcd. for C₁₈H₂₆FN₂O₄S₂ [M + H]⁺: 417.1318, found 417.1310.

L-Phenylglycine methyl ester hydrochloride [(S)-2-methoxy-2-oxo-1-phenylethanaminium chloride]



L-Phenylglycine 3.000 g (19.845 mmol, 1 equiv.) in MeOH (46 mL) was cooled to 0°C and SOCl₂ (11.5 mL) (158.762 mmol, 8 equiv.) added dropwise over 15 min. The reaction mixture was stirred the first hr at 0°C and for a further 21 hr at room temperature. MeOH (50 mL) added and the solvent removed *in-vacuo* to give L-phenylglycine methyl ester hydrochloride 4.000 g (19.845 mmol, 100%) as a white solid. lit. m.p. 200°C¹²⁰ m.p. 198°C; ¹H NMR (CD₃OD) (300 MHz) δ_H = 9.04 (3H, b s, CHNH₃), 7.53-7.47 (5H, m, ArH), 5.24 (1H, s, CHCO), 3.79 (3H, s, OCH₃); (HRMS) (CH₃OH) calcd. for C₉H₁₂NO₂ [M + H]⁺: 166.0868, found 166.0860.

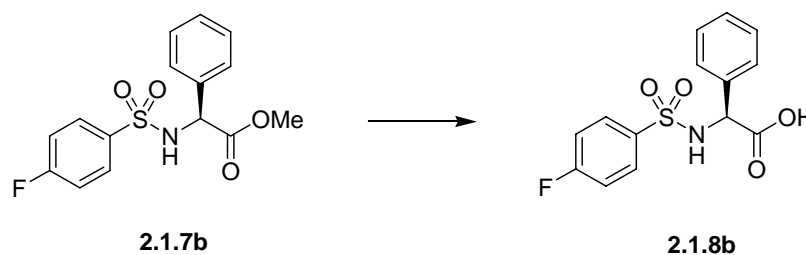
N-(4-Fluorobenzenesulfonyl) L-phenylglycine methyl ester [(S)-methyl 2-(4-fluorophenylsulfonamido)-2-phenylacetate]



L-Phenylglycine methyl ester hydrochloride 3.000 g (14.877 mmol, 1 equiv.) in DMF (17 mL) was cooled to 0°C in an ice bath and DIPEA (5.7 mL) (32.729 mmol, 2.2 equiv.) added followed by the addition of *N*-(4-fluorobenzenesulfonyl) chloride 3.475 g (17.853 mmol, 1.2 equiv.) over 15 min. The reaction mixture was stirred for 1 day and partitioned between EtOAc (50 mL) and HCl (50 mL; 1N). The aqueous phase was washed twice with EtOAc (50 mL). The organic

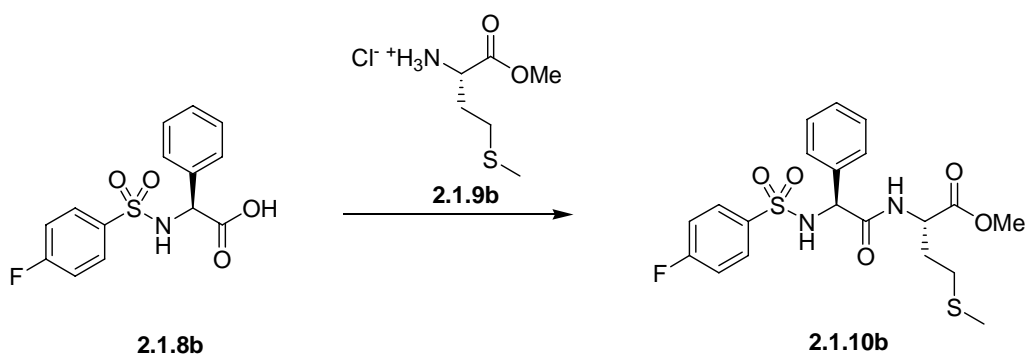
phases were combined and washed with saturated NaHCO₃ (50 mL), saturated brine (50 mL) and dried with anhydrous Na₂SO₄. Solvent was removed *in-vacuo* and the product was recrystallised (EtOAc/petroleum ether 1:3, R_f = 0.29) to give *N*-(4-fluorobenzenesulfonyl) L-phenylglycine methyl ester 3.36 g (10.392 mmol, 70%) as a white solid. m.p. 135°C; IR ν max (KBr) 1734 (COOCH₃); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.08 (1H, d, *J* = 9.3, Hz, NHCH of Pgl), 7.89-7.84 (2H, m, FArH) 7.44-7.36 (7H, m, 2 x FArH and 5 x ArH of Pgl), 5.16 (1H, d, *J* = 9.3 Hz, NHCH of Pgl), 3.58 (3H, s, OCH₃ of Pgl); ¹³C NMR ((CD₃)₂SO) δ_C = 170.2, (COCH₃ of Pgl), 164.2 (d, *J* = 249.4 Hz, F-C=), 137.4 (d, *J* = 2.7 Hz, =C-S), 135.7 (NHCHC= of Pgl), 129.7 (2C, d, *J* = 9.3 Hz, C=C-S), 128.7 (2C, s, C=C-C of Pgl), 128.4 (C=C-C of Pgl), 127.5 (2C, s, C=C-C of Pgl), 116.1 (2C, d, *J* = 22.5 Hz, F-C=C), 59.6 (NHCH of Pgl), 52.5 (OCH₃ of Pgl); (HRMS) (CH₃OH) calcd. for C₁₅H₁₅FNO₄S [M + H]⁺: 324.0706, found 324.0714.

***N*-(4-Fluorobenzenesulfonyl) L-phenylglycine [(*S*)-2-(4-fluorophenylsulfonamido)-2-phenylacetic acid]**



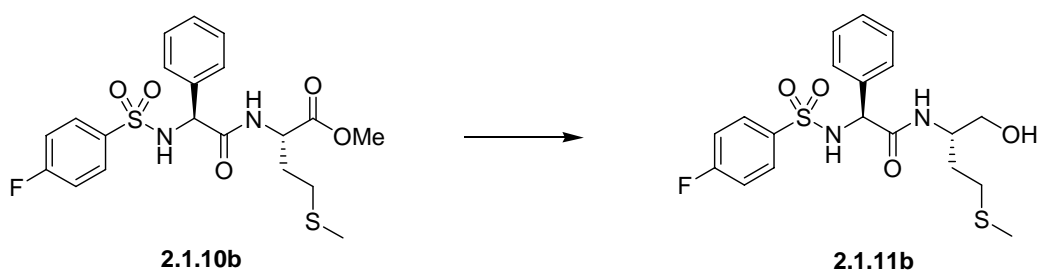
To *N*-(4-fluorobenzenesulfonyl) L-phenylglycine methyl ester 3.00 g (9.278 mmol, 1 equiv.) in THF (30.0 mL), NaOH (10.0 mL; 2.320 N) added over 5 min. The reaction mixture was heated to 65°C and stirred for 1 day. The mixture was concentrated to half its original volume on a rotary evaporator and distilled water (50 mL) added. The aqueous phase was washed twice with EtOAc and the solution was partitioned between EtOAc (50 mL) and HCl (50 mL; 1N), the aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated brine (50 mL) and dried with anhydrous Na₂SO₄ and the solvent removed *in-vacuo* to give *N*-(4-fluorobenzenesulfonyl) L-phenylglycine 2.675 g (8.648 mmol, 93%) as a white solid. lit. m.p. 158-160°C¹²¹ m.p. 166°C. ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 13.09 (1H, b s, COOH), 8.91 (1H, d, *J* = 9.3 Hz, NHCH of Pgl), 7.89-7.85 (2H, m, FArH), 7.42-7.36 (7H, m, 2 x FArH and 5 x ArH of Pgl), 5.03 (1H, d, *J* = 9.6 Hz, NHCH of Pgl); (HRMS) (CH₃OH) calcd. for C₁₄H₁₃FNO₄S [M + H]⁺: 310.0549 found 310.0535.

***N*-(4-Fluorobenzenesulfonyl) L-phenylglycine L-methionine methyl ester [(*S*)-methyl 2-((*S*)-2-(4-fluorophenylsulfonamido)-2-phenylacetamido)-4-(methylthio)butanoate]**



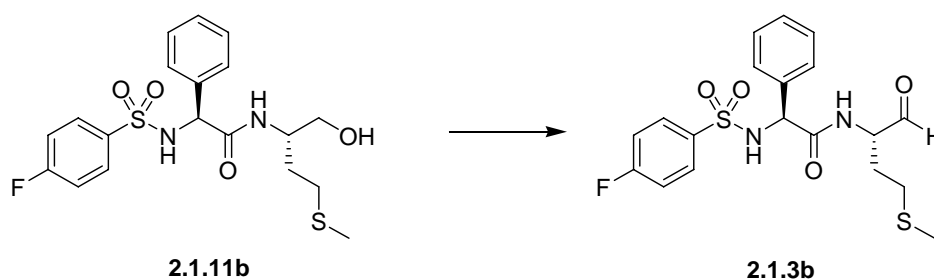
To *N*-(4-fluorobenzenesulfonyl) L-phenylglycine 2.253 g (7.283 mmol, 1 equiv.) in DMF (15 mL) kept under N₂, L-methionine methyl ester hydrochloride 1.600 g (8.012 mmol, 1.1 equiv.) added. DIPEA (2.8 mL) (16.023 mmol, 2.2 equiv.) added and HATU 3.046 g (8.012 mmol, 1.1 equiv.) added over 15 min and the mixture stirred for 2 days at room temperature. The mixture was partitioned between EtOAc (50 mL) and HCl (50 mL; 1N), and the aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated NaHCO₃ (50 mL) and saturated brine (50 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 2:3, R_f = 0.23) gave *N*-(4-fluorobenzenesulfonyl) L-phenylglycine L-methionine methyl ester 1.718 g (3.780 mmol, 52%) as a white solid. m.p. 150°C; IR ν max (KBr) 1728 (COOH₃), 1649 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 8.81 (1H, d, *J* = 9.6 Hz, NHCH), 8.74 (1H, d, *J* = 7.5 Hz, NHCH), 7.91-7.80 (2H, m, FArH), 7.44-7.29 (7H, m, 2 x FArH and 5 x ArH of Pgl), 5.19 (1H, *J* = 9.6 Hz, NHCH of Pgl), 4.33-4.26 (1H, m, NHCH of Met), 3.60 (3H, s, OCH₃ of Met), 2.44-2.39 (2H, m, SCH₂ of Met), 2.12 (3H, s, SCH₃ of Met), 1.97-1.82 (2H, m, CHCH₂CH₂ of Met); ¹³C NMR ((CD₃)₂SO) δ_C = 171.6, (C=O of Met), 168.9 (CONH of Pgl), 164.1 (d, *J* = 251.1 Hz, F-C=), 137.6, 137.2, 129.6 (2C, d, *J* = 9.4 Hz, C=C-S), 128.2 (2C, s, C=C-C of Pgl), 127.7 (C=C-C of Pgl), 127.2 (2C, s, C=C-C of Pgl), 115.9 (2C, d, *J* = 22.6 Hz, F-C=C), 59.1 (NHCH of Pgl), 52.0, 51.0, 30.4 (CH₂ of Met), 29.4 (CH₂ of Met), 14.5 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₂₀H₂₄FN₂O₅S₂ [M + H]⁺: 455.1111 found 455.1104.

***N*-(4-Fluorobenzenesulfonyl) L-phenylglycine L-methioninol [(*S*)-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-1-hydroxy-4-(methylthio)butan-2-yl)-2-phenylacetamide]**



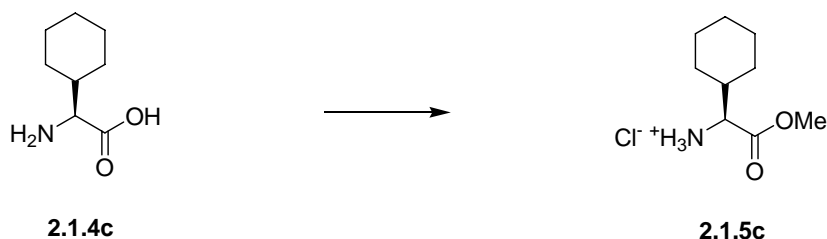
N-(4-Fluorobenzenesulfonyl) L-phenylglycine L-methionine methyl ester 1.600 g (3.520 mmol, 1 equiv.) in THF (7.2 mL) under N₂, was cooled to 0°C and a solution of LiAlH₄/THF (3.9 mL; 1N) added dropwise over 5 min. The reaction mixture was stirred at 0°C for 1 hr and allowed to warm to room temperature and stirred for a further 17 hr. The reaction mixture was partitioned between EtOAc (40 mL) and KHSO₄ (40 mL; 1N), the aqueous phase was washed twice with CHCl₃ (10 mL). The separate organic phase was washed with saturated brine (40 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 7:3 R_f = 0.45,) gave *N*-(4-fluorobenzenesulfonyl) L-phenylglycine L-methioninol 1.034 g (2.424 mmol, 69%) as a white solid. m.p. 163°C; IR ν max (KBr) 1647 (CONH), 1063 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 8.72 (1H, d, *J* = 9.3 Hz, NHCH of Pgl), 8.13 (1H, d, *J* = 8.4 Hz, NHCH of Met), 7.90-7.85 (2H, m, FArH), 7.42-7.32 (7H, m, 2 x FArH and 5 x ArH of Pgl), 5.10 (1H, d, *J* = 9.9 Hz, NHCH of Pgl), 4.78-4.74 (1H, m, CH₂OH of Met), 3.75-3.60 (1H, m, NHCH of Met), 3.32-3.19 (2H, m, CH₂OH of Met), 2.36-2.28 (2H, m, SCH₂ of Met), 2.09 (3H, s, SCH₃ of Met), 1.90-1.75 (1H, m, CHCH₂CH₂ of Met), 1.60-1.46 (1H, m, CHCH₂CH₂ of Met); ¹³C NMR ((CD₃)₂SO) δ_C = 168.6, (CONH of Pgl), 164.0 (d, *J* = 249.4 Hz, F-C=), 137.9, 137.7, 129.6 (2C, d, *J* = 9.9 Hz, C=C-S), 128.2 (2C, s, C=C-C of Pgl), 127.6 (C=C-C of Pgl), 127.1 (2C, s, C=C-C of Pgl), 115.9 (2C, d, *J* = 22.5 Hz, F-C=C), 62.6 (CH₂OH of Met), 59.5 (NHCH of Pgl), 50.2 (NHCH of Met), 30.5 (CH₂ of Met), 29.9 (CH₂ of Met), 14.7 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₉H₂₄FN₂O₄S₂ [M + H]⁺: 427.1162 found 427.1156.

***N*-(4-Fluorobenzenesulfonyl) L-phenylglycine L-methioninal [(*S*)-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-4-(methylthio)-1-oxobutan-2-yl)-2-phenylacetamide]**



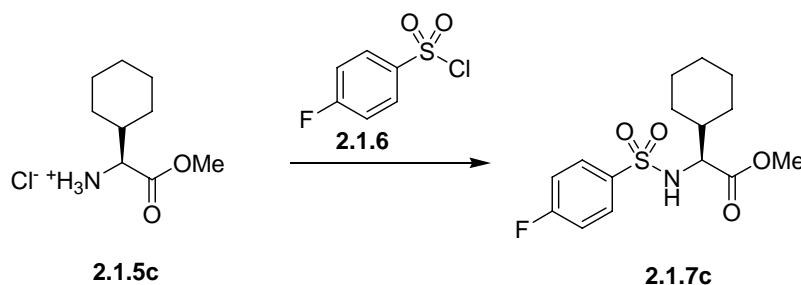
N-(4-Fluorobenzenesulfonyl) L-phenylglycine L-methioninol 843 mg (1.977 mmol, 1 equiv.) was dissolved in DMSO (18 mL) and DCM (18 mL) and then DIPEA (1.4 mL) (7.906 mmol, 4 equiv.) added to the solution. Then in a separate vial containing SO₃.Pyr 1.258 g (7.906 mmol, 4 equiv.) dissolved in DMSO (5 mL), the solution was heated and added slowly to the reaction mixture over 3 min. The reaction was left stirring for 2 hr at room temperature. Then the reaction mixture was partitioned between EtOAc (20 mL) and HCl (20 mL; 1N), the aqueous phase was washed twice more with EtOAc (20 mL). The combined organic phases were washed with saturated NaHCO₃ (20 mL) and saturated brine (20 mL) and dried with anhydrous Na₂SO₄. The solvent was evaporated to dryness *in-vacuo* to give a brown crude. Flash chromatography on silica (EtOAc/pet ether 2:1, R_f = 0.65) gave *N*-(4-fluorobenzenesulfonyl) L-phenylglycine L-methioninal 354 mg (0.813 mmol, 41%) as white yellow solid. m.p. 47°C; IR ν max (KBr) 1734 (CHO), 1653 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.28 (1H, s, CHO of Met), 8.85-8.82 (2H, m, NHCH), 7.90-7.80 (2H, m, FArH), 7.49-7.25 (7H, m, FArH and ArH of Pgl), 5.19 (1H, d, *J* = 9.3 Hz, NHCH of Pgl), 4.17-4.08 (1H, m, NHCH of Met), 2.36-2.30 (2H, m, SCH₂ of Met), 2.04 (3H, s, SCH₃ of Met), 1.85-1.56 (2H, m, CHCH₂CH₂ of Met); ¹³C NMR ((CD₃)₂SO) δ_C = 200.0 (CHO of Met), 169.6 (CONH of Pgl), 164.1 (d, *J* = 248.9 Hz, F-C=), 137.5, 137.3, 129.8 (2C, d, *J* = 9.8 Hz, C=C-S), 129.6, 128.3 (2C, s, C=C-C of Pgl), 127.8 (C=C-C of Pgl), 127.2 (2C, s, C=C-C of Pgl), 116.0 (2C, d, *J* = 23.1 Hz, F-C=C), 59.3, 57.4, 29.2 (CH₂ of Met), 27.3 (CH₂ of Met), 14.4 (SCH₃ of Met); (HRMS) (CH₃CN) calcd. for C₁₉H₂₂FN₂O₄S₂ [M + H]⁺: 425.1005 found 425.1007.

L-Cyclohexylglycine methyl ester hydrochloride [(*S*)-1-cyclohexyl-2-methoxy-2-oxoethanaminium chloride]



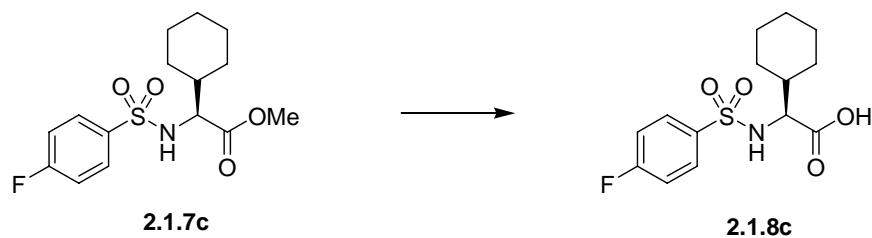
A solution of L-cyclohexaglycine 1.000 g (6.361 mmol, 1 equiv.) in MeOH (15 mL) was cooled to -20°C in a NaCl ice bath, SOCl_2 (3.7 mL) (50.887 mmol, 8 equiv.) added over 15 min, and the mixture was stirred for 1 hr at -20°C and for 18 hr at room temperature. MeOH 2 x (50 mL) added and the solvent removed *in-vacuo* to give L-cyclohexaglycine methyl ester hydrochloride 1.321 g (6.361 mmol, 100%) as a white solid. m.p. 181°C ; ^1H NMR (CD_3OD) (300 MHz) $\delta_{\text{H}} = 3.87$ (1H, d, $J = 5.1$ Hz, CHCO), 3.83 (3H, s, OCH_3), 1.90-1.69 (6H, m, CH_2 's and CH_2CHCH_2), 1.34-1.10 (5H, m, CH_2 's); (HRMS) (CH_3OH) calcd. for $\text{C}_9\text{H}_{18}\text{NO}_2$ $[\text{M} + \text{H}]^+$: 172.1338 found 172.1345.

***N*-(4-Fluorobenzenesulfonyl) L-cyclohexaglycine methyl ester [(*S*)-methyl 2-cyclohexyl-2-(4-fluorophenylsulfonamido)acetate]**



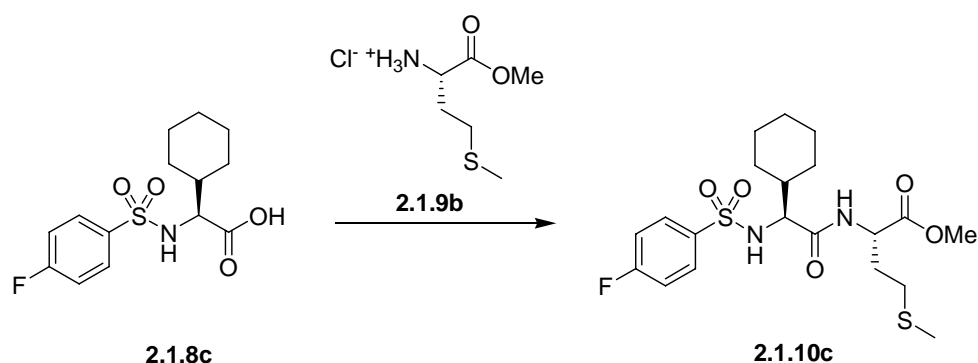
L-Cyclohexaglycine methyl ester hydrochloride 1.163 g (5.599 mmol, 1 equiv.) in DMF (10 mL) was and cooled to 0°C in an ice bath and DIPEA (2.1 mL) (12.318 mmol, 2.2 equiv.) added. *N*-(4-fluorobenzenesulfonyl) chloride 1.308 g (6.719 mmol, 1.1 equiv.) added over 15 min and the reaction stirred for 20 hr at room temperature. The mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N), and the aqueous phase washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO_3 (25 mL) and saturated brine (25 mL) and dried with anhydrous Na_2SO_4 the solvent was removed *in-vacuo*. The product was attained by recrystallisation (EtOAc/petroleum ether 1:3, $R_f = 0.37$) to give *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine methyl ester 1.289 g (3.913 mmol, 70%) as a white solid. m.p. 129°C ; IR ν_{max} (KBr) 1732 (COOCH_3); ^1H NMR (CD_3OD) (300 MHz) $\delta_{\text{H}} = 7.81$ -7.76 (2H, m, ArH), 7.23-7.17 (2H, m, ArH), 3.59 (1H, d, $J = 6.9$ Hz, NHCH of Chg), 3.32 (3H, s, OCH_3 of Chg), 1.65-1.40 (6H, m, CH_2 's of Chg and CH_2CHCH_2 of Chg), 1.20-0.91 (5H, m, CH_2 's of Chg); ^{13}C NMR ($(\text{CD}_3)_2\text{SO}$) $\delta_{\text{C}} = 171.2$ (COOCH_3 of Chg), 164.2 (d, $J = 248.9$ Hz, F-C=), 137.4 (d, $J = 2.8$ Hz, =C-S), 129.7 (2C, d, $J = 9.3$ Hz, C=C-S), 116.2 (2C, d, $J = 22.5$ Hz, F-C=C), 60.9 (NHCH of Chg), 51.7 OCH_3 of Chg), 28.9 (CH_2 of Chg), 28.3 (CH_2 of Chg), 25.6 (CH_2 of Chg), 25.4 (CH_2 of Chg), 25.3 (CH_2 of Chg); (HRMS) (CH_3OH) calcd. for $\text{C}_{15}\text{H}_{21}\text{FNO}_4\text{S}$ $[\text{M} + \text{H}]^+$: 330.1175 found 330.1180.

***N*-(4-Fluorobenzenesulfonyl) L-cyclohexaglycine [(*S*)-2-cyclohexyl-2-(4-fluorophenylsulfonamido)acetic acid]**



To *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine methyl ester 1.142 g (3.467 mmol, 1 equiv.) in THF (30 mL) was treated with a solution of NaOH (10.0 mL; 0.867 N) 346.7 mg (8.668 mmol, 2.5 equiv.) which added over 5 min. The mixture was heated to 65°C in a silica oil bath and stirred for 2 days. The mixture was reduced to half its original volume on a rotary evaporator and was diluted with distilled water (50 mL). The reaction mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N) and the aqueous phase washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated brine (25 mL) and dried with anhydrous Na₂SO₄ and the solvent removed *in-vacuo* to give *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine 1.032 g (3.272 mmol, 94%) as a white solid. m.p. 150°C; ¹H NMR (CD₃OD) (300 MHz) δ_H = 7.90-7.85 (2H, m, ArH), 7.27-7.21 (2H, m, ArH), 3.63 (1H, d, *J* = 6.0 Hz, NHCH of Chg), 1.71-1.56 (6H, m, CH₂'s of Chg and CH₂CHCH₂ of Chg), 1.28-1.00 (5H, m, CH₂'s of Chg).

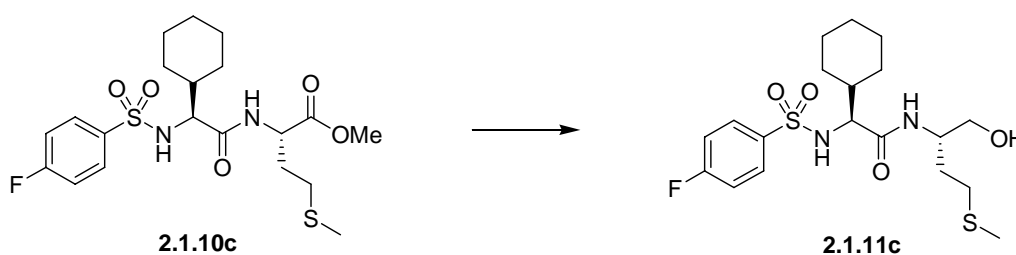
***N*-(4-Fluorobenzenesulfonyl) L-cyclohexaglycine L-methionine methyl ester [(*S*)-methyl 2-(*S*)-2-cyclohexyl-2-(4-fluorophenylsulfonamido)acetamido)-4-(methylthio)butanoate]**



To *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine 843 mg (2.673 mmol, 1 equiv.) dissolved in DMF (10 mL) kept under N₂, L-methionine methyl ester hydrochloride 587 mg (2.940 mmol, 1.1 equiv.) added and dissolved. DIPEA (1.0 mL) (5.881 mmol, 2.2 equiv.) added and HATU 1.118 g (2.940 mmol, 1.1 equiv.) added slowly over 15 min and the reaction was stirred for

2 days at room temperature. The reaction mixture was partitioned between EtOAc (20 mL) and HCl (20 mL; 1N), the aqueous phase was washed twice with EtOAc (20 mL). The combined organic phases were washed with saturated NaHCO₃ (20 mL) and saturated brine (20 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:2, R_f = 0.31) gave *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine L-methionine methyl ester 665 mg (1.444 mmol, 54%) as a white solid. m.p. 158°C; IR ν max (KBr) 1730 (COOCH₃), 1646 (CONH); ¹H NMR ((CD₃)₂SO, 298 K) (300 MHz) δ_{H} = 8.38 (1H, d, *J* = 7.5 Hz, NHCH), 8.02 (1H, d, *J* = 9.3 Hz, NHCH), 7.92-7.87 (2H, m, ArH), 7.49-7.43 (2H, m, ArH), 4.20-4.12 (1H, m, NHCH of Met), 3.70-3.64 (4H, m, NHCH of Chg and OCH₃ of Met), 2.42-2.28 (2H, m, SCH₂ of Met), 2.11 (3H, s, SCH₃ of Met), 1.96-1.58 (8H, m, CHCH₂CH₂ of Met, CH₂'s of Chg and CH₂CHCH₂ of Chg), 1.30-0.91 (5H, m, CH₂'s of Chg); ¹³C NMR ((CD₃)₂SO) δ_{C} = 171.9 (COOCH₃ of Met), 170.2 (CONH of Chg), 164.1 (d, *J* = 248.9 Hz, F-C=), 137.8 (d, *J* = 2.8 Hz, =C-S), 129.5 (2C, d, *J* = 9.3 Hz, C=C-S), 115.9 (2C, d, *J* = 22.0 Hz, F-C=C), 60.5 (NHCH of Chg), 51.9, 50.8, 30.4, 29.4, 28.8, 28.3, 25.9, 25.5, 14.5 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₂₀H₃₀FN₂O₅S₂ [M + H]⁺: 461.1580 found 461.1600.

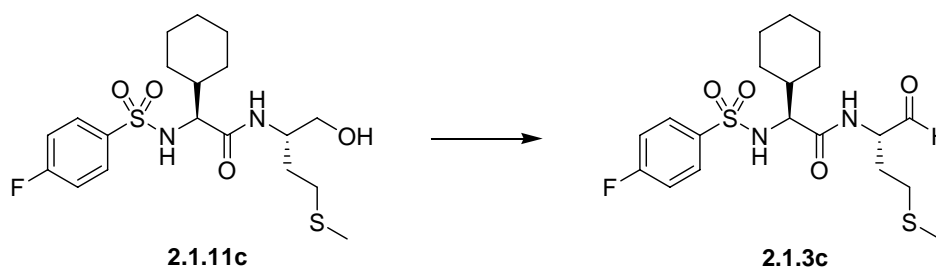
***N*-(4-Fluorobenzenesulfonyl) L-cyclohexaglycine L-methioninol [(*S*)-2-cyclohexyl-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-1-hydroxy-4-(methylthio)butan-2-yl)acetamide]**



N-(4-Fluorobenzenesulfonyl) L-cyclohexaglycine methyl ester 602 mg (1.307 mmol, 1 equiv.) in THF (7.2 mL) under N₂, was cooled to 0°C and LiAlH₄/THF (1.4 mL; 1N) added dropwise over 5 min. The mixture was stirred at 0°C for 1 hr and was allowed to come to room temperature and stirred for a further 17 hr. The mixture was partitioned between EtOAc (20 mL) and KHSO₄ (20 mL; 1N) and the aqueous phase washed twice with CHCl₃ (5 mL). The organic phases were washed with saturated brine (20 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 7:3, R_f = 0.35) gave *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine L-methioninol 373 mg (0.862 mmol, 66%)

as a white solid. m.p. 188°C; IR ν max (KBr) 3528 (H₂COH), 1640 (CONH), 1061 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_{H} = 7.95-7.89 (3H, m, ArH and NHCH), 7.75 (1H, d, J = 8.4 Hz, NHCH), 7.48-7.42 (2H, m, ArH), 4.74 (1H, t, J = 5.3 Hz, CH₂OH of Met), 3.70-3.56 (2H, m, NHCH of Met and NHCH of Chg), 3.38-3.33 (1H, m, CH₂OH of Met), 3.23-3.16 (1H, m, CH₂OH of Met), 2.30-2.08 (5H, m, SCH₂ of Met and SCH₃ of Met), 1.75-1.37 (8H, m, CHCH₂CH₂ of Met, CH₂'s of Chg and CH₂CHCH₂ of Chg), 1.33-0.85 (5H, m, CH₂'s of Chg); ¹³C NMR ((CD₃)₂SO) δ_{C} = 169.6 (CONH of Chg) 164.0 (d, J = 248.3 Hz, F-C=), 137.9 (=C-S), 129.5 (2C, d, J = 9.4 Hz, C=C-S), 116.0 (2C, d, J = 22.0 Hz, F-C=C), 62.8, 61.0, 50.0 (NHCH of Met), 30.4, 29.8, 29.0, 28.3, 25.9, 25.5, 14.6 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₉H₃₀FN₂O₄S₂ [M + H]⁺: 433.1631 found 433.1622.

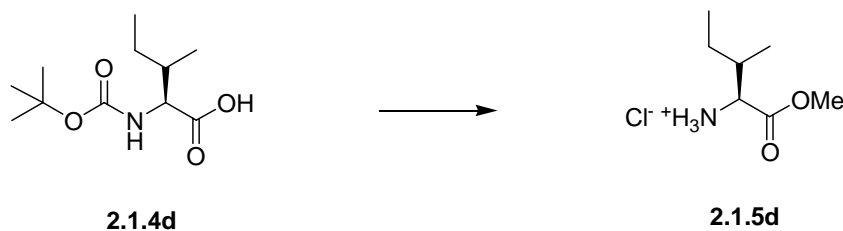
***N*-(4-Fluorobenzenesulfonyl) L-cyclohexaglycine L-methioninal [(*S*)-2-cyclohexyl-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-4-(methylthio)-1-oxobutan-2-yl)acetamide]**



To *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine L-methioninol 321 mg (0.742 mmol, 1 equiv.) in DMSO (3 mL) and DCM (5 mL), DIPEA (0.5 mL) (2.968 mmol, 4 equiv.) added. A separate vial containing SO₃.Pyr 472 mg (2.968 mmol, 4 equiv.) in DMSO (2 mL) was heated and added over 3 min. The reaction was left stirring for 2 hr at room temperature. The mixture was partitioned between EtOAc (10 mL) and HCl (10 mL; 1N), and the aqueous phase was washed twice with EtOAc (10 mL). The combined organic phases were washed with saturated NaHCO₃ (10 mL) and saturated brine (10 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo* to give *N*-(4-fluorobenzenesulfonyl) L-cyclohexaglycine L-methioninal 303 mg (0.704 mmol, 95%) as light yellow solid. m.p. 168°C; IR ν max (KBr) 1735 (CHO), 1643 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_{H} = 9.24 (1H, s, CHO of Met), 8.51 (1H, d, J = 6.6 Hz, NHCH), 8.09 (1H, d, J = 9.3 Hz, NHCH), 7.94-7.89 (2H, m, ArH), 7.50-7.44 (2H, m, ArH), 4.09-4.02 (1H, m, NHCH of Met), 3.71-3.66 (1H, t, J = 8.0 Hz, NHCH of Chg), 2.43-2.31 (2H, m, SCH₂ of Met), 2.09-1.96 (4H, SCH₃ of Met and CHCH₂CH₂ of Met), 1.80-1.55 (7H, m, CHCH₂CH₂ of Met, CH₂'s of Chg and CH₂CHCH₂ of Chg), 1.33-0.90 (5H,

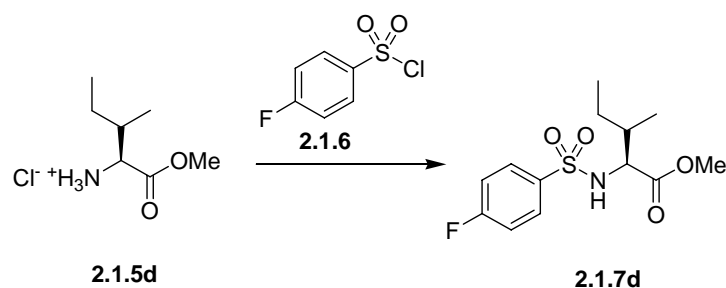
m, CH_2 's of Chg); ^{13}C NMR ($(\text{CD}_3)_2\text{SO}$) $\delta_{\text{C}} = 200.1$ (CHO of Met), 170.7 (CONH of Chg), 164.2 (d, $J = 249.4$ Hz, F-C=), 137.8 ($=\text{C-S}$), 129.7 (2C, d, $J = 8.8$ Hz, C=C-S), 116.0 (2C, d, $J = 22.5$ Hz, F-C=C), 60.7 (NHCH of Chg), 57.5 (NHCH of Met), 29.4 , 29.0 , 28.4 , 27.5 , 25.9 , 25.5 , 20.9 , 14.5 (SCH_3 of Met); (HRMS) (CH_3CN) calcd. for $\text{C}_{19}\text{H}_{28}\text{FN}_2\text{O}_4\text{S}_2$ $[\text{M} + \text{H}]^+$: 431.1475 found 431.1496 .

***Iso* L-leucine methyl ester hydrochloride [(2*S*,3*S*)-1-methoxy-3-methyl-1-oxopentan-2-aminium chloride]**



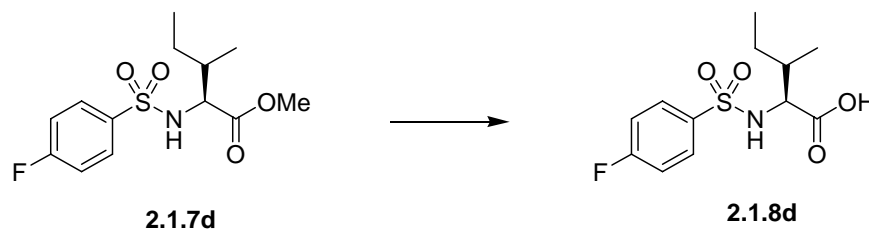
A solution of boc *iso* L-leucine hemi hydrate 4.000 g (16.646 mmol, 1 equiv.) in MeOH (39 mL) was cooled to 0°C in an ice bath and SOCl_2 (9.7 mL) (133.167 mmol, 8 equiv.) added dropwise over 20 min. The mixture was stirred for 1 hr at 0°C and then for 2 days at room temperature. MeOH (400 mL) added and the solvent removed evaporated down to dryness *in-vacuo* to give *iso* L-leucine methyl ester hydrochloride 3.024 g (16.646 mmol, 100%) as a light yellow brown solid. lit. m.p. $98-100^\circ\text{C}^{122}$ m.p. $95-96^\circ\text{C}$; ^1H NMR (CD_3OD) (300 MHz) $\delta_{\text{H}} = 8.45$ (3H, b s, NH_3^+), 3.98 (1H, d, $J = 3.6$ Hz, NHCH), 3.79 (3H, s, OCH_3), $1.98-1.93$ (1H, m, CHCH_3), $1.54-1.45$ (1H, m, CHCH_2CH_3), $1.37-1.27$ (1H, m, CHCH_2CH_3), 0.97 (3H, d, $J = 4.2$ Hz CHCH_3), 0.94 (3H, m, CH_2CH_3); (HRMS) (CH_3OH) calcd. for $\text{C}_7\text{H}_{16}\text{NO}_2$ $[\text{M} + \text{H}]^+$: 146.1181 , found 146.1181 .

***N*-(4-Fluorobenzenesulfonyl) *iso* L-leucine methyl ester [(2*S*,3*S*)-methyl 2-(4-fluorophenylsulfonamido)-3-methylpentanoate]**



Iso L-leucine methyl ester 2.500 g (13.686 mmol, 1 equiv.) was dissolved in DMF (15 mL) and DIPEA (5.2 mL) (30.109 mmol, 2.2 equiv.). The solution was cooled to 0°C in an ice bath and was subsequently treated with the slow addition (over 10 min) of *N*-(4-fluorobenzenesulfonyl) chloride 3.196 g (16.423 mmol, 1.2 equiv.), the reaction mixture was left stirring for 1 day at room temperature. The reaction mixture was partitioned between EtOAc (50 mL) and HCl (50 mL; 1N), the aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated NaHCO₃ (50 mL) and saturated brine (50 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:3, R_f = 0.46) gave *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine methyl ester 3.243 g (10.691 mmol, 78%) as a white solid. m.p. 83°C; ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 8.45 (1H, d, *J* = 9.6 Hz, NHCH of Ile), 7.92-7.88 (2H, m, ArH), 7.54-7.48 (2H, m, ArH), 3.69 (1H, app t, *J* = 7.2 Hz, NHCH of Ile), 3.45 (3H, s, OCH₃ of Ile), 1.81-1.72 (1H, m, CHCH₃ of Ile), 1.52-1.42 (1H, m, CH₂CH₃ of Ile), 1.26-1.16 (1H, m, CH₂CH₃ of Ile), 0.87 (3H, d, *J* = 4.2 Hz, CHCH₃ of Ile), 0.84 (3H, t, *J* = 3.6 Hz, CH₂CH₃ of Ile); (HRMS) (CH₃OH) calcd. for C₁₃H₁₉FNO₄S [M + H]⁺: 304.1019, found 304.1018.

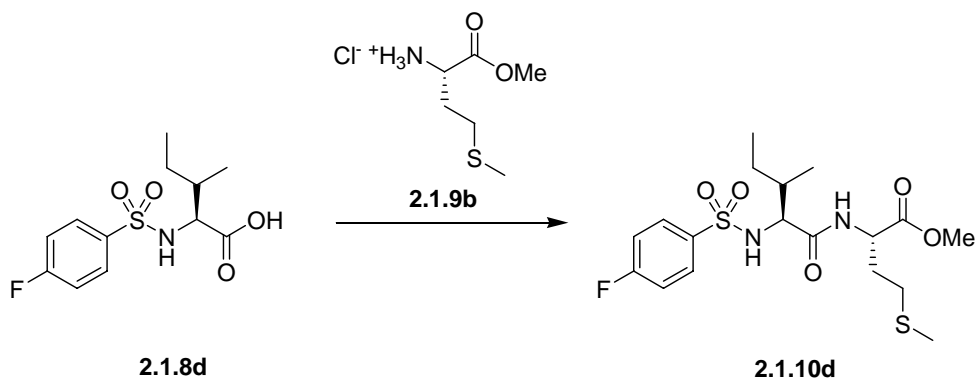
***N*-(4-Fluorobenzenesulfonyl) L-*iso*-leucine [(2*S*,3*S*)-2-(4-fluorophenylsulfonamido)-3-methylpentanoic acid]**



To *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine methyl ester 2.955 g (9.741 mmol, 1 equiv.) in THF (30 mL), NaOH (10 mL; 2.435 N) added over 5 min. The reaction mixture was heated to 65°C and stirred for 1 day. The mixture was concentrated to half its original volume using a rotary evaporator. Distilled water (50 mL) added and the mixture partitioned between EtOAc (50 mL) and HCl (50 mL; 1N). The aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated brine (50 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo* to give *N*-(4-fluorophenylsulfonamido) *iso* L-leucine 2.550 g (8.813 mmol, 90%) as a white solid. m.p. 114°C; ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 12.65 (1H, b s, COOH of Ile), 8.20 (1H, d, *J* = 9.6 Hz, NHCH of Ile), 7.94-7.89 (2H, m, ArH), 7.51-7.45 (2H, m, ArH), 3.64 (1H, app t, *J* = 6.3 Hz, NHCH of Ile), 1.80-1.71 (1H, m, CHCH₃ of Ile), 1.48-1.40 (1H, m, CH₂CH₃ of Ile), 1.26-1.14 (1H, m, CH₂CH₃ of Ile),

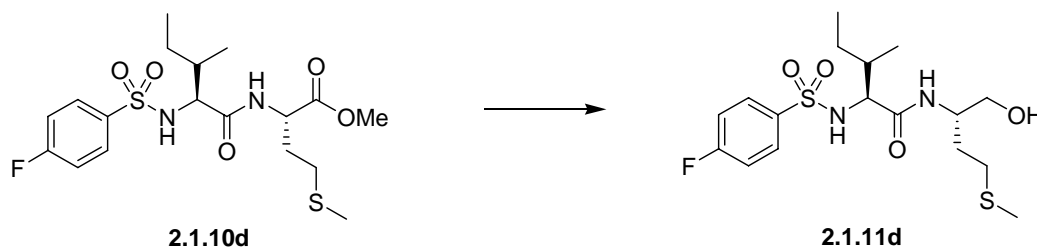
0.90-0.83 (6H, m, 2 x CH_3 of Ile); (HRMS) (CH_3OH) calcd. for $\text{C}_{13}\text{H}_{17}\text{FNO}_4\text{S}$ [$\text{M} + \text{H}$] $^+$: 290.0862, found 290.0873.

***N*-(4-Fluorobenzenesulfonyl) *iso* L-leucine L-methionine methyl ester [(*S*)-methyl 2-((2*S*,3*S*)-2-(4-fluorophenylsulfonamido)-3-methylpentanamido)-4-(methylthio)butanoate]**



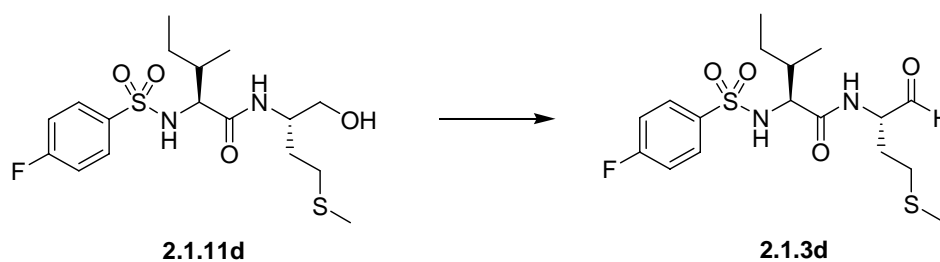
To *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine 2.305 g (7.966 mmol, 1 equiv.) dissolved in DMF (15 mL) kept under N_2 , L-methionine methyl ester hydrochloride 1.750 g (8.763 mmol, 1.1 equiv.) added DIPEA (3.1 mL) (17.525 mmol, 2.2 equiv.) and HATU 3.332 g (8.763 mmol, 1.1 equiv.) added slowly over 15 min. The reaction was stirred for 2 days at room temperature and the mixture partitioned between EtOAc (50 mL) and HCl (50 mL; 1N). The aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated NaHCO_3 (50 mL) and saturated brine (50 mL) and dried with anhydrous Na_2SO_4 the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:2, $R_f = 0.23$) gave *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine L-methionine methyl ester 1.553 g (3.574 mmol, 45%) as a white solid. m.p. 146°C; IR ν_{max} (KBr) 1734 (COOCH_3), 1649 (CONH); ^1H NMR ($(\text{CD}_3)_2\text{SO}$) (300 MHz) $\delta_{\text{H}} = 8.42$ (1H, d, $J = 7.2$ Hz, NHCH of Met) 8.07 (1H, d, $J = 9.3$ Hz, NHCH of Ile), 7.92-7.87 (2H, m, ArH), 7.49-7.43 (2H, m, ArH), 4.13-4.11 (1H, m, NHCH of Met), 3.70-3.64 (4H, m, NHCH of Ile and OCH_3 of Met), 2.39-2.33 (2H, m, SCH_2 of Met), 2.11-1.68 (4H, m, CHCH_3 of Ile, 0.5 x CH_2CH_3 of Ile and CHCH_2CH_2 of Met), 1.20-1.10 (1H, m, CH_2CH_3 of Ile), 0.90 (3H, d, $J = 6.6$ Hz, CHCH_3 of Ile), 0.85 (3H, t, $J = 7.5$ Hz, CH_2CH_3 of Ile); ^{13}C NMR ($(\text{CD}_3)_2\text{SO}$) $\delta_{\text{C}} = 171.9$ (COOCH_3 of Met), 170.4 (CONH of Ile), 164.1 (d, $J = 248.9$ Hz, $\text{F-C}=\text{C}$), 137.8 (d, $J = 3.3$ Hz, $=\text{C-S}$), 129.6 (2C, d, $J = 9.4$ Hz, $\text{C}=\text{C-S}$), 115.9 (2C, d, $J = 22.6$ Hz, $\text{F-C}=\text{C}$), 60.0 (NHCH of Ile), 51.9, 50.9, 37.1, 30.5 (CH_2 of Met), 29.4 (CH_2 of Met), 24.2, 15.0, 14.6, 10.6 (CH_2CH_3 of Ile); (HRMS) (CH_3OH) calcd. for $\text{C}_{18}\text{H}_{28}\text{FN}_2\text{O}_5\text{S}_2$ [$\text{M} + \text{H}$] $^+$: 435.1424, found 435.1438.

***N*-(4-Fluorobenzenesulfonyl) *iso* L-leucine L-methioninol [(2*S*, 3*S*)-2-(4-fluorophenylsulfonamido)-*N*-(*S*)-1-hydroxy-4-(methylthio)butan-2-yl)-3-methylpentanamide]**



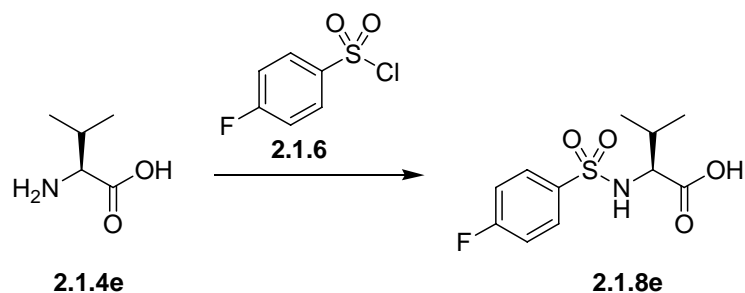
N-(4-Fluorobenzenesulfonyl) *iso* L-leucine L-methionine methyl ester 1.330 g (3.061 mmol, 1 equiv.) in THF (7.2 mL) under N₂ was cooled to 0°C in an ice bath. LiAlH₄/THF (3.4 mL; 1N) added dropwise over 5 min. The reaction mixture was stirred at 0°C for 1 hr and warmed to room temperature and stirred for a further 17 hr. The mixture was partitioned between EtOAc (25 mL) and KHSO₄ (25 mL; 1N), the aqueous phase was washed twice with CHCl₃ (10 mL). The separate organic phases were washed with saturated brine (25 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:2, R_f = 0.25) gave *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine L-methioninol 711 mg (1.749 mmol, 57%) as a white solid. m.p. 168°C; IR ν_{max} (KBr) 1639 (CONH), 1059 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 8.00-7.89 (3H, m, NHCH and 2 x ArH), 7.76 (1H, d, *J* = 8.4 Hz, NHCH), 7.49-7.43 (2H, m, ArH), 4.74 (1H, t, *J* = 5.4 Hz, CH₂OH of Met), 3.63-3.58 (2H, m, NHCH of Met and NHCH of Ile), 3.40-3.32 (1H, m, CH₂OH of Met), 3.23-3.18 (1H, m, CH₂OH of Met), 2.29-2.21 (1H, m, SCH₂ of Met), 2.09 (3H, s, SCH₃ of Met), 1.75-1.40 (4H, m, CHCH₃ of Ile, 0.5 x CH₂CH₃ of Ile and CHCH₂CH₂ of Met), 1.14-1.10 (1H, m, CH₂CH₃ of Ile), 0.87 (3H, d, *J* = 3.0 Hz, CHCH₃ of Ile), 0.86-0.82 (3H, m, CH₂CH₃ of Ile); ¹³C NMR ((CD₃)₂SO) δ_C = 169.9 (CONH of Ile), 164.1 (d, *J* = 248.3 Hz, F-C=), 137.9 (=C-S), 129.6 (2C, d, *J* = 9.3 Hz, C=C-S), 116.0 (2C, d, *J* = 22.6 Hz, F-C=C), 62.8 (CH₂OH of Met), 60.6 (NHCH of Val), 50.1 (NHCH of Met), 37.2, 30.5 (CH₂ of Met), 29.9 (CH₂ of Met), 24.3, 15.3, 14.7, 10.8 (CH₂CH₃ of Ile); (HRMS) (CH₃OH) calcd. for C₁₇H₂₈FN₂O₄S₂ [M + H]⁺: 407.1475, found 407.1484.

***N*-(4-Fluorobenzenesulfonyl) *iso* L-leucine L-methioninal [(2*S*,3*S*)-2-(4-fluorophenylsulfonamido)-3-methyl-*N*-((*S*)-4-(methylthio)-1-oxobutan-2-yl)pentanamide]**



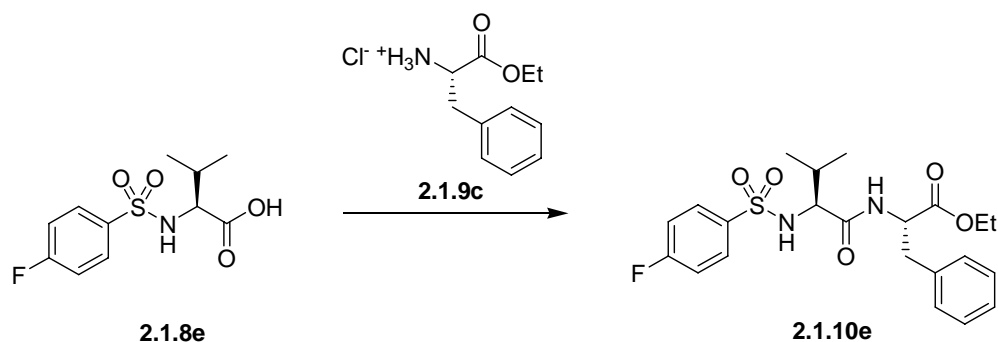
N-(4-Fluorobenzenesulfonyl) *iso* L-leucine- L-methioninol 593 mg (1.459 mmol, 1 equiv.) was dissolved in DMSO (7 mL) and DCM (9 mL) and DIPEA (1.0 mL) (5.835 mmol, 4 equiv.) added. A separate vial containing SO₃.Pyr 929 mg (5.835 mmol, 4 equiv.) dissolved in DMSO (4 mL), was heated and added slowly to the reaction mixture over 3 min. The reaction was left stirring for 2 hr at room temperature and partitioned between EtOAc (20 mL) and HCl (20 mL; 1N). The aqueous phase was washed twice with EtOAc (20 mL) and the combined organic phases were washed with saturated NaHCO₃ (20 mL) and saturated brine (20 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo*, to give *N*-(4-fluorobenzenesulfonyl) *iso* L-leucine L-methioninal 564 mg (1.394 mmol, 96%) as off white solid. m.p. 151°C; IR ν max (KBr) 1730 (CHO), 1643 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_{H} = 9.24 (3H, s, CHO of Met), 8.53 (1H, d, *J* = 6.6 Hz, NHCH of Met), 8.14 (2H, d, *J* = 9.3 Hz, NHCH of Ile), 7.95-7.90 (2H, m, ArH), 7.51-7.45 (2H, m, ArH), 4.03-3.98 (1H, m, NHCH of Met), 3.70 (1H, t, *J* = 8.6 Hz, NHCH of Ile), 2.48-2.33 (2H, m, SCH₂ of Met), 2.10 (3H, s, SCH₃ of Met), 2.02-1.94 (1H, m, CHCH₂CH₂ of Met), 1.76-1.66 (2H, m, CHCH₃ of Ile and CHCH₂CH₂ of Met), 1.60-1.51 (1H, m, CH₂CH₃ of Ile), 1.22-1.12 (1H, m, CH₂CH₃ of Ile), 0.92 (3H, d, *J* = 6.9 Hz, CHCH₃ of Ile), 0.87 (3H, d, *J* = 7.4 Hz, CH₂CH₃ of Ile); ¹³C NMR ((CD₃)₂SO) δ_{C} = 200.1 (CHO of Met), 170.8 (CONH), 164.1 (d, *J* = 249.4 Hz, F-C=), 137.7 (d, *J* = 3.3 Hz, =C-S), 129.7 (2C, d, *J* = 9.3 Hz, C=C-S), 116.0 (2C, d, *J* = 22.6 Hz, F-C=C), 60.1 (NHCH of Val), 57.5 (NHCH of Met), 37.0, 29.4 (CH₂ of Met), 27.4 (CH₂ of Met), 24.3, 15.3, 14.5, 10.6 (CH₂CH₃ of Ile); (HRMS) (CH₃CN) calcd. for C₁₇H₂₆FN₂O₄S₂ [M + H]⁺: 405.1318, found 405.1334.

***N*-(4-Fluorobenzenesulfonyl) L-valine [(*S*)-2-(4-fluorophenylsulfonamido)-3-methylbutanoic acid]**



Five batches of this compound were synthesised. L-valine 1.17 g (10 mmol, 1 equiv.) dissolved in distilled water (10 mL) and was cooled to 0°C in an ice bath. Anhydrous Na₂CO₃ 2.32 g (22 mmol, 2.2 equiv.) added and the mixture stirred for 20 minutes. *N*-(4-fluorobenzenesulfonyl) chloride 2.30 g (12 mmol, 1.2 equiv.) added to this mixture in three portions over one hour and the mixture allowed to return to room temperature and stirred for 2 days. Then the mixture was acidified with HCl (2.5 mL; 9N) to a pH 1.5. This initially gave a clear solution then a white precipitate formed within 5 min. This precipitate was filtered using a sintered glass filter and washed with HCl (3 mL; 1N). The product was dried *in-vacuo* to give *N*-(4-fluorobenzenesulfonyl) L-valine 2.17 g (7.9 mmol, 79%) a white solid. lit. m.p. 116-118°C¹²³ m.p. 131°C. ¹H NMR ((CD₃)₂SO) (500 MHz) δ_H = 8.17 (1H, d, *J* = 9.5 Hz, NHCH of Val), 7.95-7.92 (2H, m, ArH), 7.49-7.45 (2H, m, ArH), 3.63 (1H, dd, *J* = 8.5 Hz and 6.3 Hz, NHCH of Val), 2.06-2.02 (1H, m, CH(CH₃)₂ of Val), 0.92 (3H, d, *J* = 6.5 Hz, CH₃ of Val), 0.88 (3H, d, *J* = 6.5 Hz CH₃ of Val); (HRMS) (CH₃OH) calcd. for C₁₁H₁₄FNO₄SNa [M + Na]⁺: 298.0525, found 298.0536.

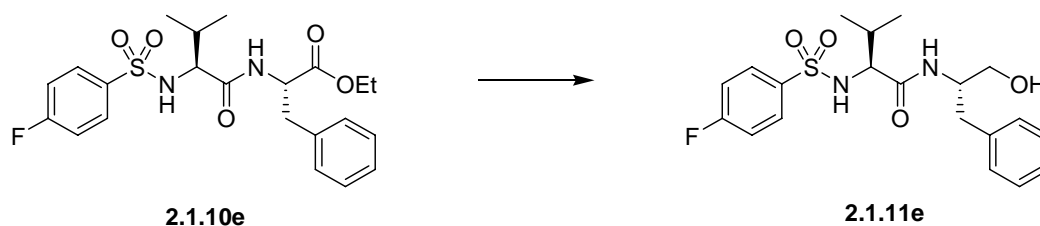
***N*-(4-Fluorobenzenesulfonyl) L-valine L-phenylalanine ethyl ester [(*S*)-ethyl 2-((*S*)-2-(4-fluorophenylsulfonamido)-3-methylbutanamido)-3-phenylpropanoate]**



To *N*-(4-fluorobenzenesulfonyl) L-valine 1.634 g (5.936 mmol, 1 equiv.) in DMF (10 mL) kept under N₂, L-phenylalanine ethyl ester hydrochloride 1.500 g (6.530 mmol, 1.1 equiv.) added.

Then DIPEA (2.3 mL) (13.059 mmol, 2.2 equiv.) and HATU 2.483 g (6.530 mmol, 1.1 equiv.) added over 15 min and the reaction was stirred for 2 days at room temperature. The mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N), and the aqueous phase washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO₃ (25 mL) and saturated brine (25 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 9:11, R_f = 0.44) gave *N*-(4-fluorobenzenesulfonyl) L-valine L-phenylalanine ethyl ester 1.776 g (3.942 mmol, 66%) as a white yellow solid. m.p. 132°C; IR ν max (KBr) 1724 (COOCH₂CH₃), 1649 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ _H = 8.46 (1H, d, *J* = 6.6 Hz, NHCH of Phe), 7.98 (1H, d, *J* = 9.6 Hz, NHCH of Val), 7.84-7.80 (2H, m, FArH), 7.42-7.32 (5H, m, 2 x FArH and 3 x ArH of Phe), 7.25-7.23 (2H, m, ArH of Phe), 4.16-3.99 (2H, m, NHCH of Phe and OCH₂CH₃ of Phe), 3.68 (1H, app t, *J* = 7.2 Hz, NHCH of Val), 2.95-2.80 (2H, m, CHCH₂ArH of Phe), 1.95-1.88 (1H, m, CH(CH₃)₂ of Val), 1.10 (3H, t, *J* = 6.9 Hz, OCH₂CH₃ of Phe), 0.91-0.87 (6H, m, CH(CH₃)₂ of Val); ¹³C NMR ((CD₃)₂SO) (75 MHz) δ _C = 171.2 (COOCH₂ of Phe), 170.2 (CONH of Val), 164.1 (d, *J* = 248.3 Hz, F-C=), 137.7, 136.9, 129.7 (2C, d, *J* = 9.3 Hz, C=C-S), 129.1 (2C, s, C=C-C of Phe), 128.4 (2C, s, C=C-C of Phe), 126.8 (C=C-C of Phe), 115.9 (2C, d, *J* = 22.5 Hz, F-C=C), 61.4, 60.5, 54.0 (NHCH of Phe), 37.0 (CHCH₂ArH of Phe), 31.1 (CH(CH₃)₂ of Val), 19.1 (CH₃ of Val), 18.3 (CH₃ of Val), 13.9 (OCH₂CH₃ of Phe); (HRMS) (CH₃OH) calcd. for C₂₂H₂₈FN₂O₅S [M + H]⁺: 451.1703 found 451.1712.

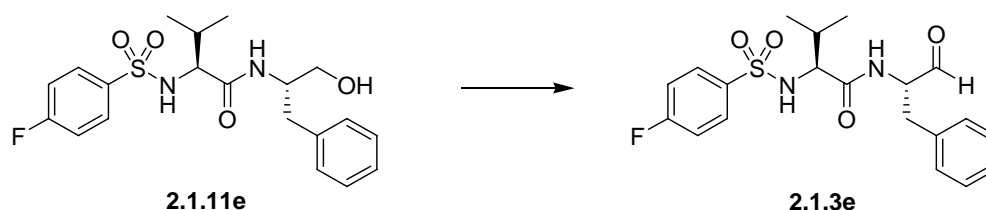
***N*-(4-Fluorobenzenesulfonyl) L-valine L-phenylalaninol [(*S*)-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-1-hydroxy-3-phenylpropan-2-yl)-3-methylbutanamide]**



N-(4-Fluorobenzenesulfonyl) L-valine L-phenylalanine ethyl ester 1.533 g (3.403 mmol, 1 equiv.) in THF (7.2 mL) under N₂, was cooled to 0°C in an ice bath. LiAlH₄/THF (3.7 mL; 1N) added dropwise over 5 min. The mixture was stirred at 0°C for 1 hr and returned to room temperature and stirred for a further 17 hr. The mixture was partitioned between EtOAc (25 mL) and KHSO₄ (25 mL; 1N) and the aqueous phase washed twice with CHCl₃ (10 mL). The organic phases were washed with saturated brine (25 mL) and dried with anhydrous Na₂SO₄ the solvent

was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:2, $R_f = 0.49$) gave *N*-(4-fluorobenzenesulfonyl) L-valine L-phenylalaninol 896 mg (2.193 mmol, 64%) as a white solid. m.p. 185-187°C (decomp.); IR ν_{\max} (KBr) 3591 (H₂COH), 1641 (CONH), 1036 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) $\delta_{\text{H}} = 7.90-7.80$ (4H, 2 x FArH and 2 x NHCH), 7.40-7.22 (7H, m, 2 x FArH and 5 x ArH of Phe), 4.78 (1H, t, $J = 5.3$ Hz, CH₂OH of Phe), 3.80-3.70 (1H, m, NHCH of Phe), 3.60 (1H, t, $J = 8.1$ Hz, NHCH of Val), 3.30-3.20 (2H, m, CH₂OH of Phe), 2.78-2.72 (1H, m, CHCH₂ArH of Phe), 2.51-2.44 (1H, m, CHCH₂ArH of Phe), 1.92-1.85 (1H, m, CH(CH₃)₂ of Val), 0.86-0.84 (6H, m, CH(CH₃)₂ of Val); ¹³C NMR ((CD₃)₂SO) $\delta_{\text{C}} = 169.6$ (CONH of Val), 164.1 (d, $J = 248.3$ Hz, F-C=), 139.0 (CH₂-C= of Phe), 137.7 (=C-S), 129.7 (2C, d, $J = 9.3$ Hz, C=C-S), 129.2 (2C, s, C=C-C of Phe), 128.3 (2C, s, C=C-C of Phe), 126.1 (C=C-C of Phe), 115.9 (2C, d, $J = 22.5$ Hz, F-C=C), 62.0, 61.7, 52.6 (NHCH of Phe), 36.4 (CHCH₂ArH of Phe), 31.1 (CH(CH₃)₂ of Val), 19.2 (CH₃ of Val), 18.1 (CH₃ of Val); (HRMS) (CH₃OH) calcd. for C₂₀H₂₆FN₂O₄S [M + H]⁺: 409.1597 found 409.1586.

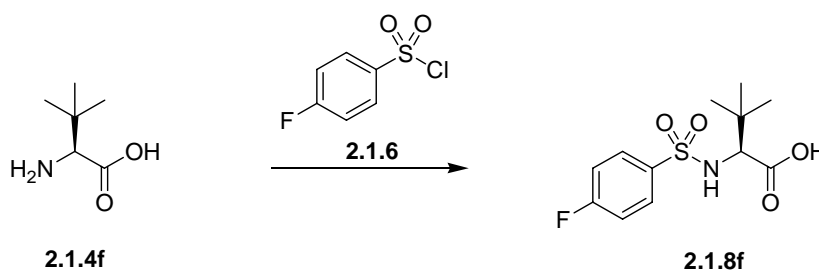
***N*-(4-Fluorobenzenesulfonyl) L-valine L-phenylalaninal [(*S*)-2-(4-fluorophenylsulfonamido)-3-methyl-*N*-((*S*)-1-oxo-3-phenylpropan-2-yl)butanamide]**



N-(4-Fluorobenzenesulfonyl) L-valine L-phenylalaninal 748 mg (1.831 mmol, 1 equiv.) was dissolved in DMSO (8 mL) and DCM (11 mL) and DIPEA (1.3 mL) (7.325 mmol, 4 equiv.) added. A separate vial containing SO₃.Pyr 1.166 g (7.325 mmol, 4 equiv.) in DMSO (5 mL) was heated and added over 3 min. The mixture was stirred for 2 hr at room temperature. The mixture was partitioned between EtOAc (20 mL) and HCl (20 mL; 1N), and the aqueous phase washed twice with EtOAc (20 mL). The combined organic phases were washed with saturated NaHCO₃ (20 mL) and saturated brine (20 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo* to give *N*-(4-fluorobenzenesulfonyl) L-valine L-phenylalaninal 673 mg (1.656 mmol, 90%) as white yellow solid. lit. m.p. 109.9-111.1°C¹⁰⁷ m.p. 144°C (decomp.); IR ν_{\max} (KBr) 1730 (CHO), 1638 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) $\delta_{\text{H}} = 9.18$ (1H, s, CHO of Phe), 8.54 (1H, d, $J = 6.3$ Hz, NHCH of Phe), 8.02 (1H, d, $J = 8.7$ Hz, NHCH of Val), 7.89-7.84 (2H, m, FArH), 7.69-7.25 (7H, m, 2 x FArH and 5 x ArH of Phe), 4.14-4.11 (1H, m, NHCH of Phe), 3.69 (1H, t, $J = 7.7$ Hz, NHCH of Val), 3.15-3.09 (1H, m, CHCH₂ArH of Phe),

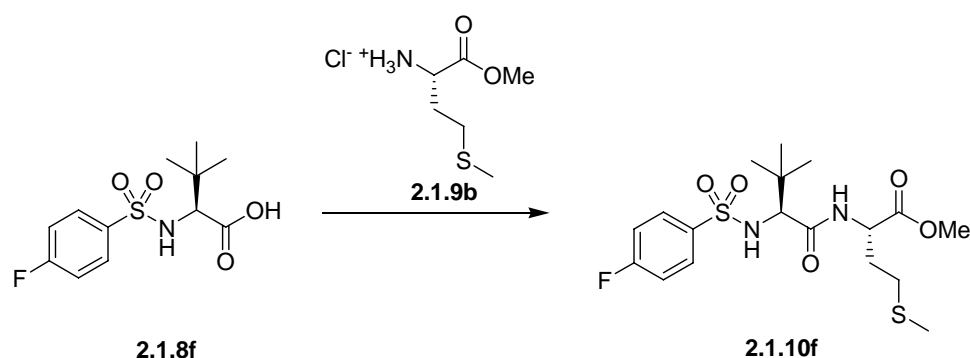
2.77 (1H, m, CHCH₂ArH of Phe), 1.93-1.86 (1H m, CH(CH₃)₂ of Val), 0.88 (3H, d, $J = 3.6$ Hz, CH₃ of Val), 0.86 (3H, d, $J = 3.6$ Hz, CH₃ of Val); ¹³C NMR ((CD₃)₂SO) $\delta_c = 199.7$ (CHO of Phe), 170.7 (CONH of Val), 164.1 (d, $J = 248.9$ Hz, F-C=), 137.6 (d, $J = 2.8$ Hz, =C-S), 137.6 (CH₂-C= of Phe), 129.7 (2C, d, $J = 9.3$ Hz, C=C-S), 129.2 (2C, s, C=C-C of Phe), 128.4 (2C, s, C=C-C of Phe), 128.3, 126.5, 115.9 (2C, d, $J = 22.5$ Hz, F-C=C), 61.3, 59.9, 33.4 (CHCH₂ArH of Phe), 31.0 (CH(CH₃)₂ of Val), 19.2 (CH₃ of Val), 17.9 (CH₃ of Val); (HRMS) (CH₃CN) calcd. for C₂₀H₂₄FN₂O₄S [M + H]⁺: 407.1441 found 407.1439.

***N*-(4-Fluorobenzenesulfonyl) *tert* L-leucine [(*S*)-2-(4-fluorophenylsulfonamido)-3,3-dimethylbutanoic acid]**



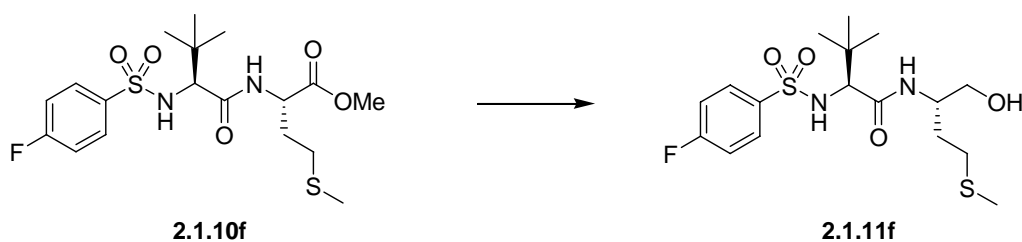
Tert L-leucine 1.75 g (13.341 mmol, 1 equiv.) was dissolved in (20 mL) of distilled water and was cooled to 0°C in an ice bath. Anhydrous Na₂CO₃ 3.099 g (29.350 mmol, 2.2 equiv.) was added to the solution and the mixture stirred until the carbonate was dissolved. *N*-(4-fluorobenzenesulfonyl) chloride 3.116 g (16.010 mmol, 1.2 equiv.) added in three portions over one hour and the mixture allowed to return to room temperature and stirred for 3 days. The mixture was acidified with HCl (4 mL; 9 N) to a pH 1.5 to give a clear solution which in 10 min to give a white precipitate. The precipitate was filtered with a sintered glass filter to give *N*-(4-fluorobenzenesulfonyl) *tert* L-leucine 2.985 g (10.317 mmol, 77%) a white solid. m.p. 205°C; IR ν_{\max} (KBr) 1701 (COOH); ¹H NMR (CD₃OD) (300 MHz) $\delta_H = 7.91$ -7.86 (2H, m, ArH), 7.27-7.21 (2H, m, ArH), 3.51 (1H, s, NHCH of Tle), 0.97 (9H, s, C(CH₃)₂ of Tle); ¹³C NMR (CD₃OD) $\delta_c = 173.7$ (COOH of Tle), 166.7 (d, $J = 250.7$ Hz, F-C=), 138.5 (d, $J = 3.1$ Hz, =C-S), 131.6, (2C, d, $J = 9.3$ Hz, C=C-S), 117.2 (2C, d, $J = 22.7$ Hz, F-C=C), 66.2 (NHCH of Tle), 35.3, (C(CH₃)₃ of Tle), 27.3 (3C, s, C(CH₃)₃ of Tle); (HRMS) (CH₃OH) calcd. for C₁₂H₁₇FNO₄S [M + H]⁺: 290.0862, found 290.0852.

***N*-(4-Fluorobenzenesulfonyl) *tert* L-leucine L-methionine methyl ester [(*S*)-methyl 2-((*S*)-2-(4-fluorophenylsulfonamido)-3,3-dimethylbutanamido)-4-(methylthio)butanoate]**



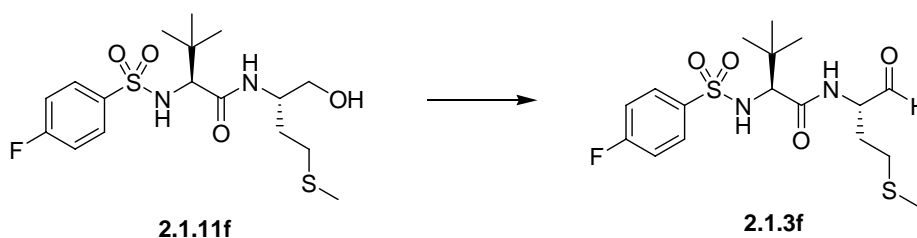
To *N*-(4-fluorobenzenesulfonyl) *tert*-L-leucine 2.305 g (7.966 mmol, 1 equiv.) dissolved in DMF (15 mL) kept under N₂, L-methionine methyl ester hydrochloride 1.750 g (8.763 mmol, 1.1 equiv.) added. DIPEA (3.1 mL) (17.525 mmol, 2.2 equiv.) added and HATU 3.332 g (8.763 mmol, 1.1 equiv.) was slowly added over 15 min. The reaction mixture was stirred for two days at room temperature and partitioned between EtOAc (50 mL) and HCl (50 mL; 1N). The aqueous phase was washed twice with EtOAc (50 mL) and the combined organic phases were washed with saturated NaHCO₃ (50 mL) and saturated brine (50 mL, dried with anhydrous Na₂SO₄ and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:2, R_f = 0.28) gave *N*-(4-fluorobenzenesulfonyl) *tert*-L-leucine L-methionine methyl ester 2.724 g (6.269 mmol, 79%) as a white solid. m.p. 98°C; IR ν max (KBr) 1742 (COOCH₃), 1665 (CONH); ¹H NMR (CD₃OD) (300 MHz) δ_H = 8.22 (1H, d, *J* = 6.3 Hz, NHCH), 7.91-7.86 (2H, m, ArH), 7.28-7.22 (2H, m, ArH), 4.19-4.17 (1H, m, NHCH of Met), 3.66 (3H, s, OCH₃ of Met), 3.56 (1H, s, NHCH of Tle), 2.29-2.06 (2H, m, SCH₂ of Met), 2.05 (3H, s, SCH₃ of Met), 1.87-1.82 (1H, m, CHCH₂CH₂ of Met), 1.72-1.68 (1H, m, CHCH₂CH₂ of Met), 0.98 (9H, s, C(CH₃)₃ of Tle); ¹³C NMR ((CD₃)₂SO) δ_C = 171.8 (COOCH₃ of Met), 169.3 (CONH of Tle), 164.1 (d, *J* = 249.5 Hz, F-C=), 137.8 (d, *J* = 3.3 Hz, =C-S), 129.7 (2C, d, *J* = 9.3 Hz, C=C-S), 115.9 (2C, d, *J* = 22.6 Hz, F-C=C), 63.5 (NHCH of Tle), 51.9, 51.0, 34.4 (C(CH₃)₃ of Tle), 30.4 (CH₂ of Met), 29.4 (CH₂ of Met), 26.5 (3C, s, C(CH₃)₃ of Tle) 14.6 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₈H₂₈FN₂O₅S₂ [M + H]⁺: 435.1424, found 435.1416.

***N*-(4-Fluorobenzenesulfonyl) *tert*-L-leucine L-methioninol [(*S*)-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-1-hydroxy-4-(methylthio)butan-2-yl)-3,3-dimethylbutanamide]**



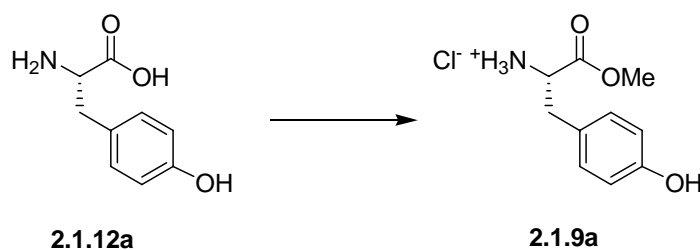
N-(4-Fluorobenzenesulfonyl) *tert* L-leucine L-methionine methyl ester 2.400 g (5.523 mmol, 1 equiv.) dissolved in THF (7.2 mL) under N₂ was cooled to 0°C. LiAlH₄/THF (6.1 mL; 1N) added dropwise over 5 min. The reaction mixture was stirred at 0°C for 1 hr and returned to room temperature and stirred for a further 17 hr. The reaction mixture was partitioned between EtOAc (50 mL) and KHSO₄ (50 mL, 1N), and the aqueous phase was washed twice with CHCl₃ (10 mL). The organic phase was washed with saturated brine (50 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:2, R_f = 0.39) gave *N*-(4-fluorobenzenesulfonyl) *tert* L-leucine L-methioninol 1.605 g (3.948 mmol, 71%) as a white solid. m.p. 183°C; IR ν max (KBr) 1641 (CONH), 1030 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 7.96-7.91 (2H, m, ArH), 7.86 (1H, d, *J* = 9.9 Hz, NHCH), 7.72 (1H, d, *J* = 8.4 Hz, NHCH), 7.47-7.41 (2H, m, ArH), 4.71 (1H, t, *J* = 5.1 Hz, CH₂OH of Met), 3.62-3.59 (2H, m, NHCH of Tle and NHCH of Met), 3.38-3.33 (1H, m, CH₂OH of Met), 3.23-3.15 (1H, m, CH₂OH of Met), 2.16-2.12 (2H, m, SCH₂ of Met), 2.07 (3H, s, SCH₃ of Met); 1.75-1.65 (1H, m, CHCH₂CH₂ of Met), 1.40-1.30 (1H, m, CHCH₂CH₂ of Met), 0.97 (9H, s, C(CH₃)₃ of Tle); ¹³C NMR ((CD₃)₂SO) δ_C = 168.7 (C=O of Tle), 164.1 (d, *J* = 248.9 Hz, F-C=), 138.0 (d, *J* = 2.7 Hz, =C-S), 129.6 (2C, d, *J* = 9.4 Hz, C=C-S), 116.0 (2C, d, *J* = 22.5 Hz, F-C=C), 63.9, 62.7, 50.1 (NHCH of Met), 34.1 (C(CH₃)₃ of Tle), 30.3 (CH₂ of Met), 29.9 (CH₂ of Met), 26.7 (3C, s, C(CH₃)₃ of Tle), 14.6 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₇H₂₈FN₂O₄S₂ [M + H]⁺: 407.1475, found 407.1476.

***N*-(4-Fluorobenzenesulfonyl) *tert*-leucine L-methioninal [(*S*)-2-(4-fluorophenylsulfonamido)-3,3-dimethyl-*N*-((*S*)-4-(methylthio)-1-oxobutan-2-yl)butanamide]**



N-(4-Fluorobenzenesulfonyl) *tert* L-leucine-L-methioninol 1.363 g (3.353 mmol, 1 equiv.) was dissolved in DMSO (28 mL) and DCM (28 mL) and DIPEA (2.3 mL) (13.410 mmol, 4 equiv.) added. A separate vial containing SO₃.Pyr 2.135 g (13.410 mmol, 4 equiv.) dissolved in DMSO (7 mL), was heated and added to the reaction mixture over 3 min. The reaction was left stirring for 2 hr at room temperature. The mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N), the aqueous phase was washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO₃ (25 mL) and saturated brine (25 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo* to give *N*-(4-fluorobenzenesulfonyl) *tert* L-leucine L-methioninal 1.343 g (3.319 mmol, 99%) as a white solid. m.p. 49-50°C; IR v max (KBr) 1732 (CHO), 1663 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.18 (1H, s, CHO of Met), 8.48 (1H, d, *J* = 6.0 Hz, NHCH), 8.03 (1H, d, *J* = 10.2 Hz, NHCH), 7.98-7.93 (2H, m, ArH), 7.51-7.45 (2H, m, ArH), 3.95-3.90 (1H, m, NHCH of Met), 3.71 (1H, d, *J* = 9.6 Hz, NHCH of Tle), 2.40-2.30 (2H, m, SCH₂ of Met), 2.10 (3H, s, SCH₃ of Met), 2.00-1.85 (1H, m, CHCH₂CH₂ of Met), 1.73-1.62 (1H, m, CHCH₂CH₂ of Met), 1.03 (9H, s, C(CH₃)₃ of Tle); ¹³C NMR ((CD₃)₂SO) δ_C = 199.9 (CHO of Met), 169.7 (CONH of Tle), 164.2 (d, *J* = 249.4 Hz, F-C=C), 137.8 (d, *J* = 3.3 Hz, =C-S), 129.7 (2C, d, *J* = 9.375 Hz, C=C-S), 129.6, 116.0 (2C, d, *J* = 22.6 Hz, F-C=C), 63.6 (NHCH of Tle), 57.5 (NHCH of Met), 34.1 (C(CH₃)₃ of Tle), 29.4 (CH₂ of Met), 27.4 (CH₂ of Met), 26.6 (3C, s, C(CH₃)₃ of Tle), 14.4 (SCH₃ of Met); (HRMS) (CH₃CN) calcd. for C₁₇H₂₆FN₂O₄S₂ [M + H]⁺: 405.1318, found 405.1331.

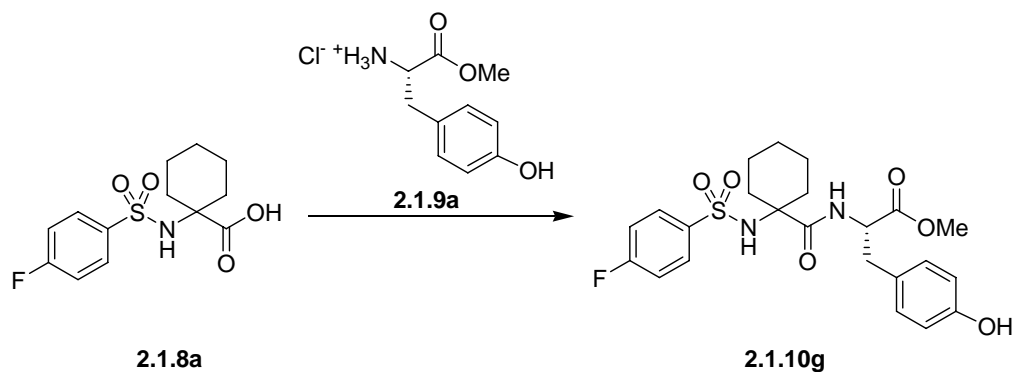
L-Tyrosinol methyl ester hydrochloride [(*S*)-3-(4-hydroxyphenyl)-1-methoxy-1-oxopropan-2-aminium chloride]



To a solution of L-tyrosine 1.500 g (8.278 mmol, 1 equiv.) in MeOH (19.2 mL), at -20°C was slowly added SOCl₂ (4.8 mL) (66.225 mmol, 8 equiv.) over 15 min and the mixture stirred for 1 hr at 0°C and was returned to room temperature and stirred for a further 18 hr. MeOH (100 mL) added and the solvent removed *in-vacuo* to give L-tyrosine methyl ester hydrochloride 1.774 g (7.657 mmol, 93%) as a white solid. lit. m.p. 190°C¹²⁴ m.p. 192°C. ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.60 (1H, s, PhOH), 8.75 (3H, s, CHNH₃⁺) 7.10 (2H, d, *J* = 8.4 Hz, ArH), 6.82

(2H, d, $J = 8.4$ Hz, ArH), 4.25-4.21 (1H, m, COCHH), 3.75 (3H, s, OCH3) 3.20-3.04 (2H, m, CHCH2ArH); (HRMS) (CH₃OH) calcd. for the free amine C₁₀H₁₄NO₃ [M + H]⁺: 196.0974, found 196.0972.

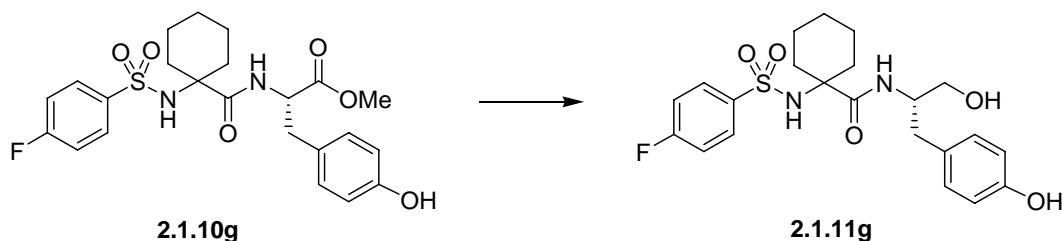
(S)-Methyl 2-(1-(4-fluorophenylsulfonamido)cyclohexanecarboxamido)-3-(4-hydroxyphenyl)propanoate



To a solution of 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylic acid 1.800 g (5.974 mmol, 1 equiv.) in DMF (10 mL) kept under N₂ L-tyrosine methyl ester hydrochloride 1.522 g (6.571 mmol, 1.1 equiv.) added. DIPEA (2.3 mL) (13.143 mmol, 2.2 equiv.) added followed by the slow addition of HATU 2.498 g (6.571 mmol, 1.1 equiv.) over 15 min and the reaction stirred for 2 days at room temperature. The reaction mixture was partitioned between EtOAc (40 mL) and HCl (40 mL; 1N), and the aqueous phase washed twice with EtOAc (40 mL). The combined organic phases were washed with saturated NaHCO₃ (40 mL) and saturated brine (40 mL) and dried with anhydrous MgSO₄ and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:1, R_f = 0.29) gave (S)-methyl 2-(1-(4-fluorophenylsulfonamido)cyclohexanecarboxamido)-3-(4-hydroxyphenyl)propanoate 2.315g (4.838 mmol, 81%) as a light yellow solid. m.p. 57-58°C. IR ν_{max} (KBr) 1736 (COOCH₃), 1655 (CONH), 1040 (ArCOH); ¹H NMR ((CD₃)₂SO, 298 K) (300 MHz) δ_{H} = 9.33 (1H, s, PhOHH of Tyr), 7.92-7.87 (3H, m, FArH and NHH), 7.50-7.43 (3H, m, FArH and NHH), 7.04 (2H, d, $J = 8.4$ Hz, ArH of Tyr), 6.78-6.74 (2H, m, ArH of Tyr), 4.27-4.20 (1H, m, NHCHH of Tyr), 2.91 (2H, d, $J = 6.6$ Hz, CHCH2ArH of Tyr), 1.87-1.72 (4H, m, CH2's of Chx), 1.42-1.25 (6H, m, CH2's of Chx); ¹³C NMR ((CD₃)₂SO) δ_{C} = 173.1 (CONH of Chx), 171.8 (COOCH₃ of Tyr), 164.1 (d, $J = 249.5$ Hz, F-C=), 156.4 (HO-C= of Tyr), 139.2 (d, $J = 2.8$ Hz, =C-S), 130.4 (2C, s, HO-C=C-C of Tyr), 129.8 (2C, d, $J = 9.4$ Hz, C=C-S), 126.7 (CH₂-C= of Tyr), 116.1 (2C, d, $J = 22.6$ Hz, F-C=C), 115.3 (2C, s, HO-C=C of Tyr), 61.6, 54.1,

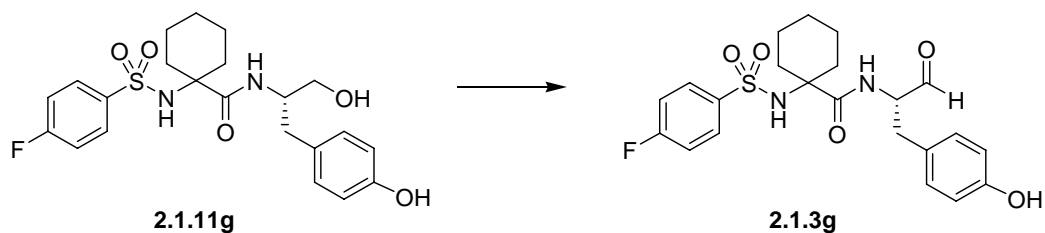
51.9, 36.5, 32.9, 24.9, 21.1; (HRMS) (CH₃OH) calcd. for C₂₃H₂₈FN₂O₆S [M + H]⁺: 479.1652, found 479.1647.

(S)-1-(4-Fluorophenylsulfonamido)-N-(1-hydroxy-3-(4-hydroxyphenyl)propan-2-yl)cyclohexanecarboxamide



A solution of (S)-methyl 2-(1-(4-fluorophenylsulfonamido)cyclohexanecarboxamido)-3-(4-hydroxyphenyl)propanoate 2.200 g (4.597 mmol, 1 equiv.) in THF (9.2 mL) under N₂, was cooled to 0°C in an ice bath. LiAlH₄/THF (5.1 mL; 1N) added dropwise slowly over 5 min and the mixture stirred at 0°C for 1 hr and allowed to come to room temperature and stirred for a further 17 hr. The mixture was partitioned between EtOAc (50 mL) and KHSO₄ (50 mL; 1N), and the aqueous phase was washed twice with CHCl₃ (10 mL). The organic phase was washed with saturated brine (50 mL) and dried with anhydrous Na₂SO₄ the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:1, R_f = 0.35) gave (S)-1-(4-fluorophenylsulfonamido)-N-(1-hydroxy-3-(4-hydroxyphenyl)propan-2-yl)cyclohexanecarboxamide 1.522 g (3.378 mmol, 73%) as a yellow solid. m.p. 77°C; IR ν max (KBr) 1651 (CONH), 1040 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.22 (1H, s, PhOH of Tyr), 7.97-7.92 (2H, m, FArH), 7.85 (1H, s, NH₂C of Chx), 7.51-7.45 (2H, m, FArH), 7.12-7.07 (3H, m, ArH of Tyr and NHCH of Tyr), 6.74 (2H, d, J = 8.4 Hz, ArH of Tyr), 4.77 (1H, t, J = 5.1 Hz, CH₂OH of Tyr), 3.85-3.75 (1H, m, NHCH of Tyr), 3.42-3.27 (2H, m, CH₂OH of Tyr), 2.81-2.74 (1H, m, CHCH₂ArH of Tyr), 2.65-2.58 (1H, m, CHCH₂ArH of Tyr), 1.85-1.65 (4H, m, CH₂'s of Chx), 1.36-1.20 (6H, m, CH₂'s of Chx); ¹³C NMR ((CD₃)₂SO) δ_C = 173.2 (C=O of Chx), 164.2 (d, J = 250.0 Hz, F-C=), 155.8 (HO-C= of Tyr), 139.7 (=C-S), 130.4 (2C, s, HO-C=C of Tyr), 129.8 (2C, d, J = 9.9 Hz, C=C-S), 129.2 (CH₂-C= of Tyr), 116.2 (2C, d, J = 22.5 Hz, F-C=C), 115.3 (2C, s, HO-C=C of Tyr), 62.1, 61.9, 52.9, 35.7, 33.6, 32.8, 25.0, 21.3; (HRMS) (CH₃OH) calcd. for C₂₂H₂₈FN₂O₅S [M + H]⁺: 451.1703, found 451.1725.

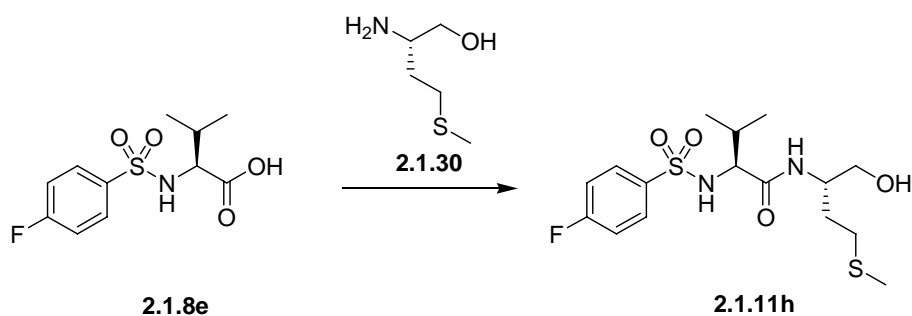
(S)-1-(4-Fluorophenylsulfonamido)-N-(1-(4-hydroxyphenyl)-3-oxopropan-2-yl)cyclohexanecarboxamide



(S)-1-(4-Fluorophenylsulfonamido)-N-(1-hydroxy-3-(4-hydroxyphenyl)propan-2-yl)cyclohexanecarboxamide

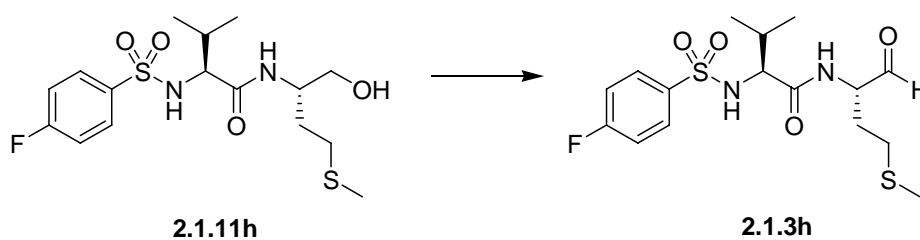
1.170 g (2.597 mmol, 1 equiv.) was dissolved in DMSO (10 mL) and DCM (14 mL), DIPEA (1.8 mL) (10.388 mmol, 4 equiv.) added. A vial containing SO₃.Pyr 1.653 g (10.388 mmol, 4 equiv.) dissolved in DMSO (5 mL), was heated and added slowly to the reaction mixture over 3 min. The reaction was left stirring for 2 hr at room temperature. The reaction mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N) and the aqueous phase washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO₃ (25 mL) and saturated brine (25 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 2:1, R_f = 0.50) gave (S)-1-(4-fluorophenylsulfonamido)-N-(1-(4-hydroxyphenyl)-3-oxopropan-2-yl)cyclohexanecarboxamide 520 mg (1.159 mmol, 45%) as an off white solid. m.p. 86°C. IR ν max (KBr) 1730 (CHO), 1657 (CONH), 1041 (ArCOH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.42 (1H, s, CHO of Tyr), 9.29 (1H, s, PhOH of Tyr), 7.96-7.89 (4H, m, FArH and 2 x NH), 7.50-7.44 (2H, m, FArH), 7.09-7.06 (2H, m, ArH of Tyr), 6.75-6.72 (2H, m, ArH of Tyr), 4.19-4.11 (NHCH of Tyr), 3.08-3.01 (1H, m, CHCH₂ArH of Tyr), 2.89-2.82 (1H, m, CHCH₂ArH of Tyr), 1.85-1.70 (4H, m, CH₂'s of Chx), 1.40-1.20 (6H, m, CH₂'s of Chx); ¹³C NMR ((CD₃)₂SO) δ_C = 200.9 (CHO of Tyr), 173.9 (CONH of Chx), 164.1 (d, J = 249.4 Hz, F-C=), 156.1 (HO-C= of Tyr), 139.6 (d, J = 2.7 Hz, =C-S), 130.4 (2C, s, HO-C=C-C= of Tyr), 129.7 (2C, d, J = 8.7 Hz, C=C-S), 127.5 (CH₂-C= of Tyr), 116.1 (2C, d, J = 23.0 Hz, F-C=C), 115.2 (2C, s, HO-C=C- of Tyr), 61.6, 60.3, 33.1, 33.0, 24.8, 21.2; (HRMS) (CH₃CN) calcd. for C₂₂H₂₆FN₂O₅S [M + H]⁺: 449.1546, found 449.1537.

N-(4-Fluorobenzenesulfonyl) L-valine L-methioninol [(S)-2-(4-fluorophenylsulfonamido)-N-((S)-1-hydroxy-4-(methylthio)butan-2-yl)-3-methylbutanamide]



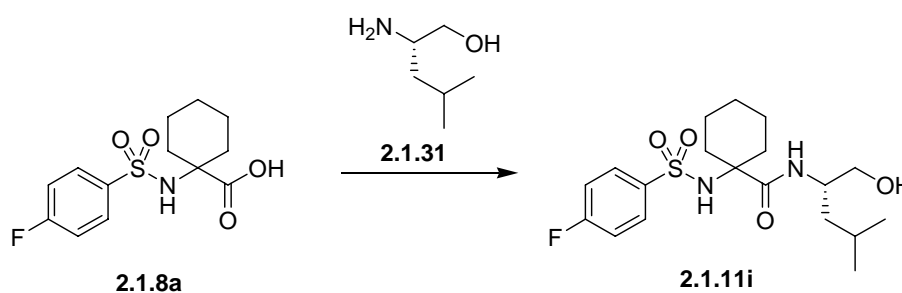
N-(4-Fluorobenzenesulfonyl) L-valine, 1.800 g (6.538 mmol, 1 equiv.) was dissolved in (90 mL) of DMF kept under argon. L-methioninol 972 mg (7.192 mmol, 1.1 equiv.) added and the mixture stirred for 5 min until it was completely dissolved. The mixture was treated with the base DIPEA (2.5 mL) (14.38 mmol, 2.2 equiv.). HATU 2.735 g (7.192 mmol, 1.1 equiv.) added to the mixture slowly over 15 min and the mixture stirred for 24 hr at room temperature. Mixture was partitioned between EtOAc (40 mL) and HCl (40 mL; 1N), the aqueous phase was washed twice with EtOAc (40 mL). The combined organic phases were washed with saturated NaHCO₃ (40 mL) saturated brine (40 mL) and dried with anhydrous MgSO₄ and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:4, R_f = 0.45) gave *N*-(4-fluorobenzenesulfonyl) L-valine L-methioninol 1.440 g (3.67 mmol, 56%) as a white solid. m.p. 177°C; IR ν max (KBr) 3520 (H₂COH), 1638 (CONH), 1057 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ _H = 7.94-7.89 (3H, m, ArH and NHCH), 7.74 (1H, d, *J* = 8.1 Hz, NHCH), 7.48-7.42 (2H, m, ArH), 4.74 (1H, t, *J* = 5.3 Hz, CH₂OH of Met), 3.66-3.55 (2H, m, NHCH of Met and NHCH of Val), 3.36-3.19 (2H, m, CH₂OH of Met), 2.29-2.17 (2H, m, SCH₂ of Met), 2.08 (3H, s, SCH₃ of Met), 1.92-1.77 (2H, m, CH(CH₃)₂ of Val and CHCH₂CH₂ of Met), 1.46-1.42 (1H, m, CHCH₂CH₂ of Met), 0.90 (3H, d, *J* = 6.6 Hz, CH₃ of Val), 0.87 (3H, d, *J* = 6.6 Hz, CH₃ of Val); ¹³C NMR ((CD₃)₂SO) δ _C = 169.8 (CONH of Val), 164.1 (d, *J* = 248.9 Hz, F-C=), 137.8 (=C-S), 129.6 (2C, d, *J* = 8.8 Hz, C=C-S), 116.0 (2C, d, *J* = 22.5 Hz, F-C=C), 62.9, 61.7, 50.1 (NHCH of Met), 31.2, 30.5, 29.9, 19.2 (CH₃ of Val), 18.2 (CH₃ of Val), 14.6 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₆H₂₆FN₂O₄S₂ [M + H]⁺: 393.1318, found 393.1331.

***N*-(4-Fluorobenzenesulfonyl) L-valine L-methioninal [(*S*)-2-(4-fluorophenylsulfonamido)-3-methyl-*N*-((*S*)-4-(methylthio)-1-oxobutan-2-yl)butanamide]**



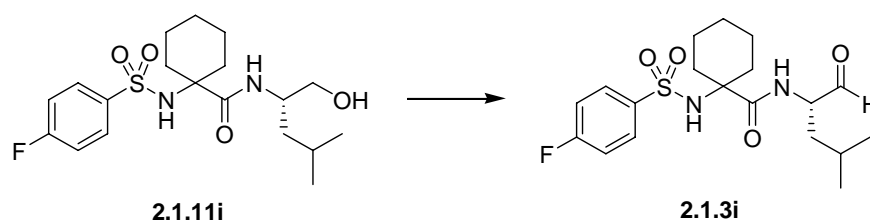
To a solution of *N*-(4-fluorobenzenesulfonyl) L-valine L-methioninol 1.300 g (3.315 mmol, 1 equiv.) in DMSO (20 mL) and DCM (6 mL), DIPEA (2.3 mL) (13.26 mmol, 4 equiv.). A vial containing SO₃.Pyr 2.111 g (13.26 mmol, 4 equiv.) dissolved in DMSO (10 mL) was heated and added slowly over 5 min to the reaction mixture. This mixture was stirred for 2 hr at room temperature. The reaction mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N), and the aqueous phase was washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO₃ (25 mL) and saturated brine (25 mL) and dried with anhydrous MgSO₄ and the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:4, R_f = 0.65) gave *N*-(4-fluorobenzenesulfonyl) L-valine L-methioninal 343 mg (0.879 mmol, 24%) as a white solid. m.p. 159°C; IR ν max (KBr) 1724 (CHO), 1643 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.26 (1H, s, CHO of Met), 8.49 (1H, d, *J* = 6.6 Hz, NHCH), 8.09 (1H, d, *J* = 9.3 Hz, NHCH), 7.94-7.89 (2H, m, ArH), 7.50-7.44 (2H, m, ArH), 4.06-4.02 (1H, m, NHCH of Met), 3.66 (1H, app t, *J* = 7.1 Hz, NHCH of Val), 2.44-2.36 (2H, m, SCH₂ of Met), 2.09-1.91 (5H, m, SCH₃ of Met, CH(CH₃)₂ of Val and CHCH₂CH₂ of Met), 1.74-1.65 (1H, m, CHCH₂CH₂ of Met), 0.95-0.89 (6H, CH(CH₃)₂ of Val); ¹³C NMR ((CD₃)₂SO) δ_C = 200.1 (CHO of Met), 170.8 (CONH of Val), 164.1 (d, *J* = 249.5 Hz, F-C=), 137.7 (=C-S), 129.7 (2C, d, *J* = 9.3 Hz, C=C-S), 116.0 (2C, d, *J* = 22.5 Hz, F-C=C), 61.4 (NHCH of Val), 57.4 (NHCH of Met), 31.0, 29.4, 27.4, 19.1 (CH₃ of Val), 18.2 (CH₃ of Val), 14.5 (SCH₃ of Met); (HRMS) (CH₃CN) calcd. for C₁₆H₂₄FN₂O₄S₂ [M + H]⁺: 391.1162, found 391.1151.

(S)-1-(4-Fluorophenylsulfonamido)-N-(1-hydroxy-4-methylpentan-2-yl)cyclohexanecarboxamide



A solution containing 1-(4-fluorophenylsulfonamido)cyclohexanecarboxylic acid 1.800 g (5.974 mmol, 1 equiv.) dissolved in DMF (8 mL) kept under N₂, added a solution of L-leucinol 770 mg (6.571 mmol, 1.1 equiv.) dissolved in DMF (2 mL). DIPEA (1.2 mL) (7.169 mmol, 1.2 equiv.) added followed by the slow addition of HATU 2.498 g (6.571 mmol, 1.1 equiv.) over 15 min. The solution was stirred for 2 days at room temperature. The reaction mixture was partitioned between EtOAc (40 mL) and HCl (40 mL; 1N), and the aqueous phase washed twice with EtOAc (40 mL). The combined organic phases were washed with saturated NaHCO₃ (40 mL) and saturated brine (40 mL) and dried with anhydrous Na₂SO₄ and the solvent removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:2, R_f = 0.30) gave (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(1-hydroxy-4-methylpentan-2-yl)cyclohexanecarboxamide 1.963 g (4.901 mmol, 82%) as a white solid. m.p. 79-80°C; IR ν max (KBr) 1647 (CONH), 1032 (H₂COH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ _H = 7.99-7.94 (2H, m, ArH), 7.81 (1H, s, NH of Chx), 7.52-7.46 (2H, m, ArH), 7.01 (1H, d, *J* = 8.4 Hz, NHCH of Leu), 4.63 (1H, t, *J* = 5.4 Hz, CH₂OH of Leu), 3.85-3.75 (1H, m, NHCH of Leu), 3.44-3.40 (1H, m, CH₂OH of Leu), 3.30-3.22 (1H, m, CH₂OH of Leu), 1.85-1.67 (5H, m, CH₂'s of Chx and CH(CH₃)₂ of Leu), 1.48-1.24 (8H, m, CH₂'s of Chx, and CHCH₂CH of Leu), 0.95 (3H, d, *J* = 6.6 Hz, CH₃ of Leu), 0.91 (3H, d, *J* = 6.3 Hz, CH₃ of Leu); ¹³C NMR ((CD₃)₂SO) δ _C = 173.1 (CONH of Chx), 164.0 (d, *J* = 248.9 Hz, F-C=), 139.8 (d, *J* = 3.3 Hz, =C-S), 129.5 (2C, d, *J* = 9.4 Hz, C=C-S), 116.1 (2C, d, *J* = 22.6 Hz, F-C=C), 63.5, 62.0, 48.9, 32.9, 24.8, 24.2, 23.4, 22.1, 21.1; (HRMS) (CH₃OH) calcd. for C₁₉H₃₀FN₂O₄S, [M + H]⁺: 401.1910, found 401.1899.

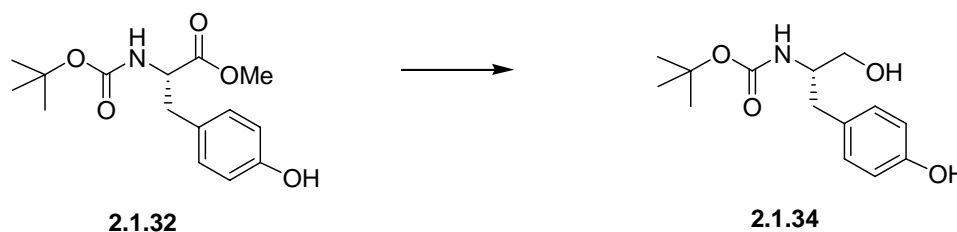
(*S*)-1-(4-Fluorophenylsulfonamido)-*N*-(4-methyl-1-oxopentan-2-yl)cyclohexanecarboxamide



(*S*)-1-(4-Fluorophenylsulfonamido)-*N*-(1-hydroxy-4-methylpentan-2-yl)cyclohexanecarboxamide 1.356 mg (3.386 mmol, 1 equiv.) was dissolved in DMSO (10 mL) and DCM (14 mL), then DIPEA (2.4 mL) (13.544 mmol, 4 equiv.) added. A separate vial containing SO₃.Pyr 2.156 mg (13.544 mmol, 4 equiv.) dissolved in DMSO (5 mL) was heated and added to the reaction mixture over 3 min and the mixture stirred for 2 hr at room

temperature. The reaction mixture was partitioned between EtOAc (25 mL) and HCl (25 mL; 1N) and the aqueous phase was washed twice with EtOAc (25 mL). The combined organic phases were washed with saturated NaHCO₃ (25 mL) and saturated brine (25 mL) and dried with anhydrous Na₂SO₄. The solvent was removed *in-vacuo* to give a yellow solid which was recrystallised from EtOAc/petroleum ether 1:1 (*R_f* = 0.43) to give (*S*)-1-(4-fluorophenylsulfonamido)-*N*-(4-methyl-1-oxopentan-2-yl)cyclohexanecarboxamide 954 mg (2.394 mmol, 71%) as white solid. m.p. 159°C. IR ν max (KBr) 1731 (CHO), 1655 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ _H = 9.40 (1H, s, CHO of Leu), 8.00-7.96 (3H, m, ArH and NHCH of Leu), 7.86 (1H, s, NHC of Chx), 7.51-7.48 (2H, m, ArH), 4.02-3.98 (1H, m, NHCH of Leu), 1.85-1.70 (5H, m, CH₂'s of Chx and CH(CH₃) of Leu), 1.63-1.50 (2H, m, CHCH₂CH of Leu), 1.42-1.25 (6H, m, CH₂'s of Chx), 0.97 (3H, d, *J* = 6.5 Hz, CH₃ of Leu), 0.94 (3H, d, *J* = 6.5 Hz, CH₃ of Leu); ¹³C NMR ((CD₃)₂SO) δ _C = 202.0 (CHO of Leu), 174.0 (CONH of Chx), 164.0 (d, *J* = 248.9 Hz, F-C=), 139.7 (d, *J* = 3.2 Hz, =C-S), 129.6 (2C, d, *J* = 9.3 Hz, C=C-S), 116.1 (2C, d, *J* = 22.6 Hz, F-C=C), 61.5, 57.0, 36.6 (CHCH₂CH of Leu), 33.4, 32.8, 24.7, 24.0, 23.2, 21.6, 21.1; (HRMS) (CH₃CN) calcd. for C₁₉H₂₈FN₂O₄S [M + H]⁺: 399.1754, found 399.1762.

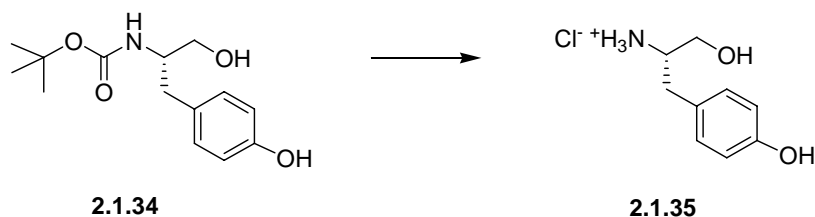
Boc L-tyrosinol [(*S*)-*tert*-butyl 1-hydroxy-3-(4-hydroxyphenyl)propan-2-ylcarbamate]



A solution of boc L-tyrosine methyl ester 4.425 g (15.000 mmol, 1 equiv.) in EtOH (112 mL) and anhydrous THF (75 mL) kept under N₂ was cooled to 0°C in an ice bath. LiCl 2.550 g (60.00 mmol, 4 equiv.) added followed by granulated NaBH₄ 2.250 g (60.00 mmol, 4 equiv.) in one portion and the mixture stirred for 1 hr. The mixture was heated gently to 40°C in a silica oil bath and was stirred for a further 2 days. The reaction mixture was concentrated on a rotatory evaporator to half the original volume and was acidified to pH 3 with 10 % citric acid solution (30 mL). The mixture was partitioned between EtOAc (50 mL) and citric acid (50 mL) (10%) solution and the aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were dried with anhydrous MgSO₄. The solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:1, *R_f* = 0.21) gave boc L-tyrosinol

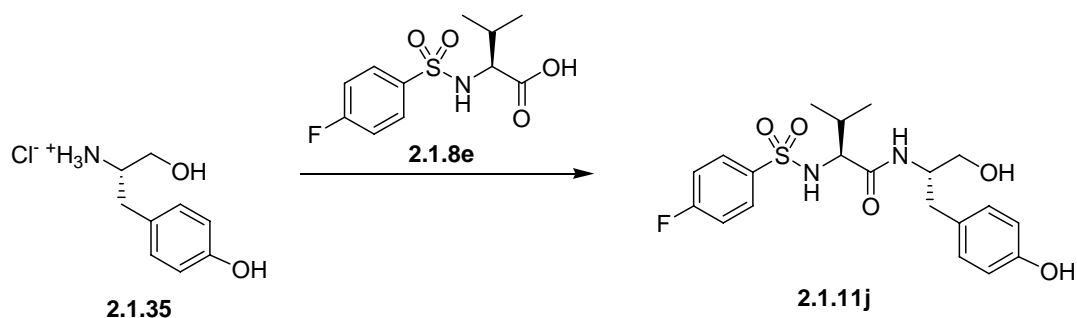
2.807 g (10.580 mmol, 71%) as a white solid. lit. m.p. 120°C¹²⁵ m.p. 124°C. ¹H NMR (CD₃OD) (300 MHz) δ_{H} = 7.03 (2H, d, J = 8.4 Hz, ArH), 6.70-6.67 (2H, m, ArH), 3.70-3.65 (1H, m, NHCH), 3.47-3.45 (2H, m, CH₂OH), 2.78-2.72 (1H, m, CH₂ArH), 2.60-2.55 (1H, m, CH₂ArH), 1.37 (9H, s, C(CH₃)₃ of boc); (HRMS) (CH₃OH) calcd. for C₁₄H₂₂NO₄ [M + H]⁺: 268.1549, found 268.1540.

L-Tyrosinol hydrochloride [(S)-1-hydroxy-3-(4-hydroxyphenyl)propan-2-aminium chloride]



To boc L-tyrosinol 2.501 g (9.355 mmol, 1 equiv.), HCl/ether (30 mL; 2N) added and the mixture stirred for 15 hr at room temperature. The solvent was removed *in-vacuo* to give L-tyrosinol hydrochloride 1.902 g (9.339 mmol, 99%) as a white green solid. m.p. 160°C; ¹H NMR ((CD₃)₂SO) (500 MHz) δ_{H} = 9.52 (1H, s, PhOH), 8.21 (3H, s, NH₃⁺), 7.14 (2H, d, J = 5.1 Hz, ArH) 6.84-6.82 (2H, m, ArH), 5.42 (1H, m, CH₂OH), 3.60-3.45 (2H, m, NCH and CH₂OH), 3.30-3.25 (1H, m, CH₂OH), 2.92 (1H, dd, J = 13.5 Hz and J = 5.0 Hz, CH₂ArH), 2.79 (1H, dd, J = 13.0 Hz and J = 9.5 Hz, CH₂ArH), (HRMS) (CH₃OH) calcd. for the free amine C₉H₁₄NO₂ [M + H]⁺: 168.1025, found 168.1024.

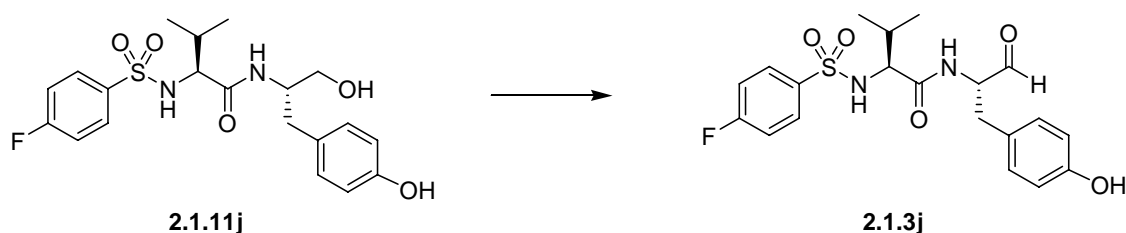
N-(4-Fluorobenzenesulfonyl) L-valine L-tyrosinol [(S)-2-(4-fluorophenylsulfonamido)-N-(S)-1-hydroxy-3-(4-hydroxyphenyl)propan-2-yl]-3-methylbutanamide]



L-Tyrosinol hydrochloride 1.000 g (4.910 mmol, 1.2 equiv.) dissolved in DMF (10 mL) under argon, N-(4-fluorobenzenesulfonyl) L-valine 1.126 g (4.092 mmol, 1 equiv.) added and

dissolved. Subsequently the solution was treated with DIPEA (1.6 mL) (9.002 mmol, 2.2 equiv.). HATU 1.711 g (4.501 mmol, 1.1 equiv.) added slowly over 15 min and was left stirring over night. The reaction mixture was partitioned between EtOAc (20 mL) and HCl (20 mL; 1N), the aqueous phase was washed twice with EtOAc (20 mL). The combined organic phases were washed with saturated NaHCO₃ (20 mL) and saturated brine (20 mL) and dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:1, R_f = 0.40) gave *N*-(4-fluorophenylsulfonyl) L-valine L-tyrosinol 481.1 mg (1.133 mmol, 28%) as a white solid. m.p. 190-192°C (decomp.); IR ν max (KBr) 1643 (CONH), 1040 (H₂COH); ¹H NMR ((CD₃)₂SO, 298 K) (300 MHz) δ_H = 9.26 (1H, s, PhOH of Tyr) 7.89-7.84 (3H, m, FArH and NHCH) 7.75 (1H, d, *J* = 8.1 Hz, NHCH) 7.42-7.36 (2H, m, FArH), 7.03 (2H, d, *J* = 8.4 Hz, ArH of Tyr), 6.76 (2H, d, *J* = 8.4 Hz, ArH of Tyr), 4.71 (1H, t, *J* = 5.1 Hz, CH₂OH of Tyr), 3.72-3.58 (2H, m, NHCH of Tyr and NHCH of Val), 3.29-3.19 (2H, m, CH₂OH of Tyr), 2.66-2.59 (1H, m, CH₂ArH of Tyr), 2.36 (1H, dd, *J* = 13.5 Hz and 6.6 Hz, CH₂ArH of Tyr), 1.93-1.86 (1H, m, CH(CH₃) of Val), 0.88-0.86 (6H, m, CH(CH₃)₂ of Val); ¹³C NMR ((CD₃)₂SO) δ_C = 169.5 (CONH of Val), 164.1 (d, *J* = 249.1 Hz, F-C=), 155.7 (HO-C= of Tyr), 137.8 (d, *J* = 2.6 Hz, =C-S), 130.0 (2C, s, HO-C=C-C- of Tyr) 129.6 (2C, d, *J* = 9.2 Hz, C=C-S), 129.0 (CH₂-C= of Tyr), 115.9 (2C, d, *J* = 22.8 Hz, F-C=C), 115.0 (2C, s, HO-C=C of Tyr), 62.0, 61.6, 52.8 (NHCH of Tyr), 35.5 (CHCH₂ArH of Tyr) 31.1 (CH(CH₃)₂ of Val), 19.2 (CH₃ of Val), 18.2 (CH₃ of Val); (HRMS) (CH₃OH) calcd. for C₂₀H₂₆FN₂O₅S [M + H]⁺: 425.1546, found 425.1526.

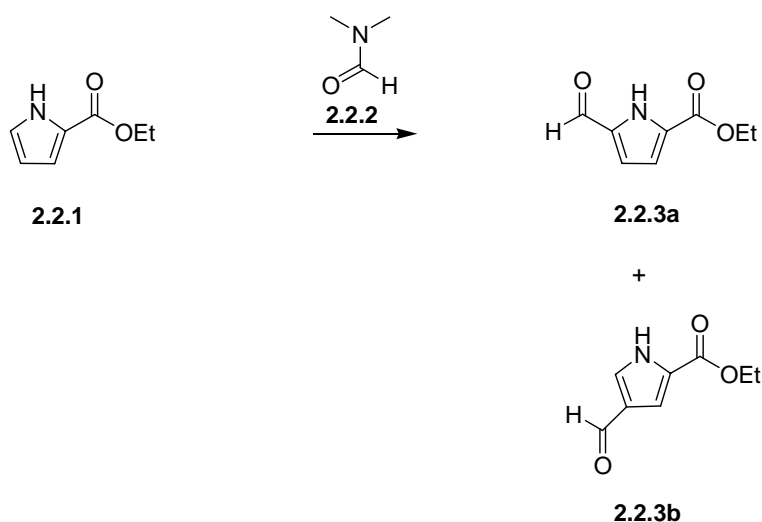
***N*-(4-Fluorobenzenesulfonyl) L-valine L-tyrosinal [(*S*)-2-(4-fluorophenylsulfonamido)-*N*-((*S*)-1-(4-hydroxyphenyl)-3-oxopropan-2-yl)-3-methylbutanamide]**



To a solution of *N*-(4-fluorobenzenesulfonyl) L-valine L-tyrosinol 420 mg (0.990 mmol, 1 equiv.) in DMSO (6 mL) and DCM (10 mL) added DIPEA (0.69 mL) (3.960 mmol, 4 equiv.). A separate vial of SO₃.Pyr 630.2 mg (3.960 mmol, 4 equiv.) dissolved in DMSO (4 mL) was heated and added dropwise to the reaction mixture over 5 min and the reaction stirred for 2 hr at room temperature. The reaction mixture was partitioned between EtOAc (10 mL) and

HCl (10 mL; 1N) and the aqueous phase was washed twice with EtOAc (10 mL). The combined organic phase was washed with saturated NaHCO₃ (10 mL) and saturated brine (10 mL) dried with anhydrous Na₂SO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 3:2, R_f = 0.23) gave *N*-(4-fluorobenzenesulfonyl) L-valine L-tyrosinal 292 mg (0.691 mmol, 70%) as a white solid. m.p. 79°C (decomp.); IR ν max (KBr) 1732 (CHO), 1659 (CONH), 1043 (ArCOH); ¹H NMR ((CD₃)₂SO) (300 MHz) δ _H = 9.34 (1H, s, **CHO** of Tyr), 9.18 (1H, s, **PhOH** of Tyr), 8.45 (1H, d, *J* = 6.9 Hz, **NHCH**) 8.01 (1H, d, *J* = 9.6 Hz, **NHCH**) 7.89-7.83 (2H, m, **FArH**) 7.44-7.36 (2H, m, **FArH**), 7.06-7.00 (2H m, **ArH** of Tyr), 6.78-6.70 (2H m, **ArH** of Tyr), 4.05-4.00 (1H, m, **NHCH** of Tyr) 3.70 (1H, app t, *J* = 6.6 Hz, **NHCH** of Val), 2.82 (1H, dd, *J* = 14.0 Hz and *J* = 5.6 Hz, **CH₂ArH** of Tyr), 2.71-2.59 (1H, m, **CH₂ArH** of Tyr), 1.94-1.87 (1H, m, **CH(CH₃)₂** of Val), 0.90-0.83 (6H, m, **CH(CH₃)₂** of Val); ¹³C NMR ((CD₃)₂SO) δ _C = 200.0 (**CHO** of Tyr), 170.6 (**CONH** of Val), 164.1 (d, *J* = 248.9 Hz, **F-C=**), 156.1 (**HO-C=** of Tyr), 137.7 (d, *J* = 3.3 Hz, **=C-S**), 130.1 (2C, s, **HO-C=C-C=** of Tyr), 129.7 (2C, d, *J* = 9.3 Hz, **C=C-S**), 127.3 (**CH₂-C=** of Tyr), 115.9 (2C, d, *J* = 22.6 Hz, **F-C=C**), 115.2 (2C, s, **HO-C=C-** of Tyr), 61.4, 60.2, 59.9, 32.7, 31.0, 20.9, 19.2 (**CH₃** of Val), 18.0 (**CH₃** of Val); (HRMS) (CH₃CN) calcd. for C₂₀H₂₄FN₂O₅S [M + H]⁺: 423.1390, found 423.1384.

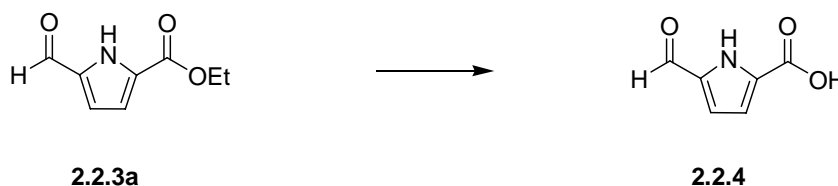
Ethyl 5-formyl-1*H*-pyrrole-2-carboxylate and ethyl 4-formyl-1*H*-pyrrole-2-carboxylate.



The synthesis of 5-formyl pyrrole 2-carboxylate ethyl ester was performed using Vilsmeier-Haack formylation¹¹⁶ of pyrrole 2-carboxylate ethyl ester. To a three necked flask (250 mL) fitted with a reflux condenser and dropping funnel added DMF (6.2 mL) and the solution cooled to 0°C in a ice bath stirring under argon. POCl₃ (7.4 mL) (78.98 mmol, 1.1 equiv.) added slowly over 15 min via a dropping funnel. A separate vial pyrrole

2-carboxylate ethyl ester 10.000 g (71.8 mmol, 1 equiv.) was dissolved in DCE (18 mL) and added to the DMF-POCl₃ complex slowly over 1 hr. The mixture was heated under reflux at 120°C in a silica oil bath for 15 min. The reaction mixture was allowed to return to room temperature and a solution containing NaOAc.3H₂O 48.2 g (359.00 mmol, 5 equiv.) in distilled water (80 mL) was slowly added over 5 min. The mixture was heated under reflux at 120°C in a silica oil bath for 20 min and was slowly returned to room temperature. The reaction mixture was partitioned with ether (200 mL) and the aqueous phase was washed twice more with ether (200 mL). The combined organic phase was washed with saturated brine and dried with anhydrous MgSO₄ the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc/petroleum ether 1:3, R_f = 0.25) gave 5-formyl pyrrole 2-carboxylate ethyl ester 6.20 g (37.08 mmol, 52%) as a light pink solid. lit. m.p. 72-74°C¹²⁶ m.p. 75°C; ¹H NMR ((CD₃)₂SO) (500 MHz) δ_H = 13.10 (1H, s, NH of Pyo), 9.80 (1H, s, CHO), 7.03 (1H, t, *J* = 1.8 Hz, CH of Pyo), 6.95 (1H, s, CH of Pyo), 4.39-4.35 (2H, m, OCH₂CH₃), 1.38 (3H, t, *J* = 7.0 Hz, OCH₂CH₃); (HRMS) (CH₃OH) calcd. for C₈H₁₀NO₃ [M + H]⁺: 168.0661, found 168.0659. Flash chromatography over silica (EtOAc/pet ether 1:2, R_f = 0.20) gave 4-formyl pyrrole 2-carboxylate ethyl ester 3.03 g (18.12 mmol, 25%) as a orange solid m.p. 101-102°C¹²⁷ m.p. 104-105°C; ¹H NMR ((CD₃)₂SO) (500 MHz) δ_H = 12.77 (1H, s, NH of Pyo), 9.85 (1H, s, CHO), 7.90 (1H, t, *J* = 1.8 Hz, NHCH of Pyo), 7.22 (1H, s, C=CHC of Pyo), 4.38-4.34 (2H, m, OCH₂CH₃), 1.40-1.36 (3H, m, OCH₂CH₃); (HRMS) (CH₃OH) calcd. for C₈H₁₀NO₃ [M + H]⁺: 168.0661, found 168.0664.

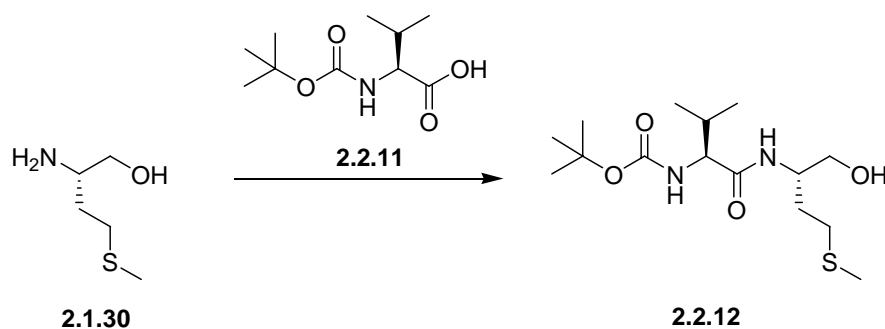
5-Formyl-1H-pyrrole-2-carboxylic acid



A two necked flask, (250 mL) fitted with a reflux condenser containing 5-formyl pyrrole 2-carboxylate ethyl ester 5.32 g (31.82 mmol, 1 equiv.) was treated with a aqueous solution of KOH (160 mL; 0.796 N). The mixture was heated to 45°C on a silica oil bath and stirred for 2 hr. The reaction mixture was partitioned between EtOAc (50 mL) and HCl (50 mL; 1N), the aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated brine (50 mL) and dried with anhydrous MgSO₄ the solvent was removed *in-vacuo* to give 5-formyl pyrrole 2-carboxylic acid 4.06 g (29.06 mmol, 91%) as an orange red

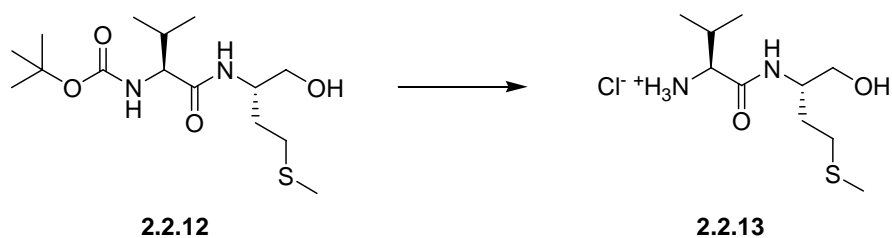
solid lit. m.p. 202-203°C¹²⁸ m.p. 211°C (decomp. to carbon); ¹H NMR ((CD₃)₂SO) (300 MHz) δ_H = 9.78 (1H, s, CHO), 7.03 (1H, app t, *J* = 2.4 Hz, CH of P_{yo}), 6.93 (1H, app t, *J* = 2.4 Hz, CH of P_{yo}).

Boc L-valine L-methioninol [*tert*-butyl (*S*)-1-((*S*)-1-hydroxy-4-(Methylthio)butan-2-ylamino)-3-methyl-1-oxobutan-2-ylcarbamate]



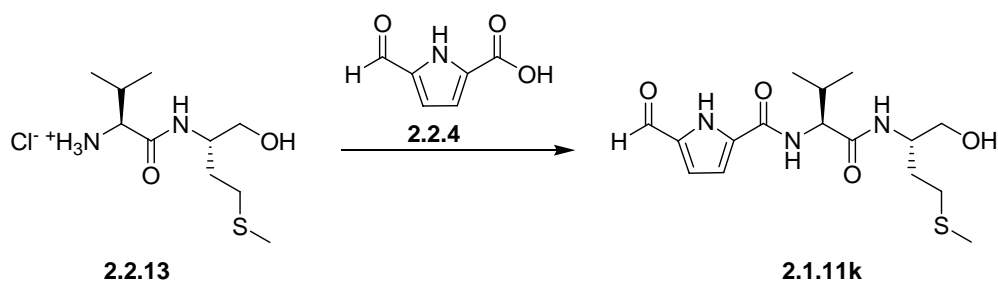
To boc L-valine 6.806 g (31.320 mmol, 1.1 equiv.), L-methioninol 3.850 g (28.480 mmol 1 equiv.) in DMF (40 mL) under argon added DIPEA (10.9 mL) (62.660 mmol, 2.2 equiv.), followed by the slow addition of HATU 11.924 g (31.320 mmol, 1.1 equiv.) over 15 min. The mixture was stirred for 3 days and partitioned between EtOAc (75 mL) and HCl (75 mL; 1N). The aqueous phase was washed twice with EtOAc (75 mL) and the combined organic phases were washed with saturated NaHCO₃ (75 mL) and saturated brine (75 mL) and dried with anhydrous MgSO₄. The solvent was removed *in-vacuo* to give boc L-valine L-methioninol 8.005 g (23.933 mmol, 84%) as a white solid m.p. 91°C; IR ν max (KBr) 1682 (COO*t*-Bu), 1655 (CONH), 1042 (H₂COH); ¹H NMR (CD₃OD) (300 MHz) δ_H = 7.77 (1H, d, *J* = 8.7 Hz, NHCH of Met), 6.55 (1H, d, *J* = 9.0 Hz, NHCH of Val), 4.03-3.98 (1H, m, NHCH of Met), 3.80-3.74 (1H, m, NHCH of Val), 3.56-3.43 (2H, m, CH₂OH of Met), 2.61-2.41 (2H, m, SCH₂ of Met), 2.07-1.88 (5H, m, SCH₃ of Met, CH(CH₃)₂ of Val and CHCH₂CH₂ of Met), 1.76-1.65 (1H, m, CHCH₂CH₂ of Met), 1.44 (9H, s, OC(CH₃)₃ of boc), 0.95-0.91 (6H, m, CH(CH₃)₂ of Val). ¹³C NMR ((CD₃)₂SO) δ_C = 171.4 (CCONH of Val), 155.6 (OCONH), 78.1 (OC(CH₃)₃), 63.3 (CH₂OH of Met), 60.3 (NHCH of Val), 50.0 (NHCH of Met), 30.8, 30.2, 30.1, 28.3 (3C, s, C(CH₃)₃ of boc), 19.4 (CH₃ of Val), 18.5 (CH₃ of Val), 14.8 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₅H₃₁N₂O₄S [M + H]⁺: 335.2005, found 335.2013.

L-Valine L-methioninol hydrochloride [(*S*)-1-((*S*)-1-hydroxy-4-(methylthio)butan-2-ylamino)-3-methyl-1-oxobutan-2-aminium chloride]



A mixture of boc L-valine L-methioninol 4.000 g (11.959 mmol, 1 equiv.) and HCl/ether (50 mL; 2N) was stirred at room temperature for 15 hr. The solvent was removed *in-vacuo* to give L-valine L-methioninol hydrochloride 3.230 g (11.959 mmol, 100%) as a hygroscopic white solid. IR ν max (KBr) 1672 (CONH), 1049 (H₂COH); ¹H NMR (CD₃OD) (300 MHz) δ_{H} = 8.17 (1H d, J = 9.0 Hz, NHCH of Met), 4.07-4.02 (1H, m, NHCH of Met), 3.74-3.53 (3H, m, CH₂OH of Met and CHCO of Val), 2.62-2.46 (2H, m, SCH₂ of Met), 2.20-2.09 (1H, m, CH(CH₃)₂ of Val), 2.08 (3H, s, SCH₃ of Met), 1.95-1.85 (1H, m, CHCH₂CH₂ of Met), 1.81-1.71 (1H, m, CHCH₂CH₂ of Met), 1.09 (3H, d, J = 4.5 Hz, CH₃ of Val), 1.04 (3H, d, J = 4.5 Hz, CH₃ of Val); ¹³C NMR (CD₃OD) δ_{C} = 169.7 (CONH of Val), 64.9 (CH₂OH of Met), 60.1 (CHCO of Val), 52.5 (NHCH of Met), 31.9, 31.8, 31.7, 19.2 (CH₃ of Val), 18.2 (CH₃ of Val), 15.5 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for the free amine C₁₀H₂₃N₂O₂S [M + H]⁺: 235.1480, found 235.1482.

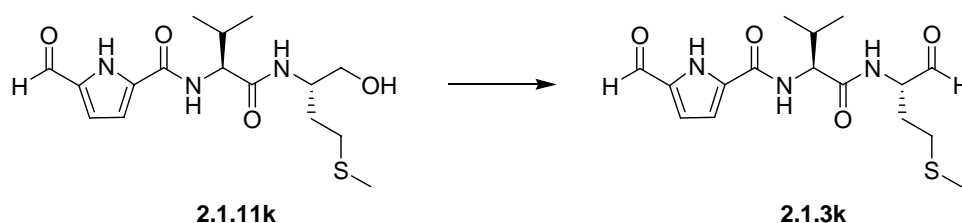
5-Formyl pyrrole 2-carboxylate L-valine L-methioninol [5-formyl-N-((S)-1-((S)-1-hydroxy-4-(methylthio)butan-2-ylamino)-3-methyl-1-oxobutan-2-yl)-1H-pyrrole-2-carboxamide]



To L-valine L-methioninol hydrochloride 3.050 g (11.281 mmol, 1.1 equiv.) in DMF (20 mL) kept under argon, added 5-formyl pyrrole 2-carboxylic acid 1.432 g (10.255 mmol, 1 equiv.). The solution was treated with DIPEA (4.3 mL) (24.612 mmol, 2.4 equiv.). EDCI.HCl 2.359 g (12.306 mmol, 1.2 equiv.) added slowly over 5 min followed by HOBt.H₂O 1.884 g (12.306 mmol, 1.2 equiv.) added slowly to the mixture over 15 min. The mixture was stirred at room temperature for 1 day and partitioned between EtOAc (50 mL) and HCl (50 mL; 1N). The aqueous phase was washed twice with EtOAc (50 mL). The combined organic phases were washed with saturated brine (50 mL) and dried with anhydrous MgSO₄ and the solvent removed

in-vacuo. Flash chromatography on silica (MeOH/DCM 1:19, $R_f = 0.15$.) gave 5-formyl pyrrole 2-carboxylate L-valine L-methioninol 273 mg (0.768 mmol, 7%) as a yellow orange solid. m.p. 148°C; IR ν_{\max} (KBr) 1655 (CONH), 1650 (CONH), 1047 (H₂COH); ¹H NMR (CD₃OD) (300 MHz) $\delta_H = 9.59$ (1H, s, CHO of Pyro), 8.02 (1H, d, $J = 9.0$ Hz, NHCH), 7.01-6.99 (1H, m, CH of Pyro), 6.96 (1H, d, $J = 3.9$ Hz, CH of Pyro), 4.25 (1H, d, $J = 8.4$ Hz, NHCH of Val), 4.06-4.00 (1H, m, NHCH of Met), 3.57-3.47 (2H, m, CH₂OH of Met), 2.58-2.39 (2H, m, SCH₂ of Met), 2.20-2.05 (1H, m, CH(CH₃)₂ of Val), 2.01 (3H, s, SCH₃ of Met), 1.95-1.84 (1H, m, CHCH₂CH₂ of Met), 1.78-1.65 (1H, m, CHCH₂CH₂ of Met), 1.04-0.91 (6H, m, CH(CH₃)₂ of Val); ¹³C NMR (CD₃OD) $\delta_C = 182.6$ (CHO of Pyro), 174.4 (CONH of Val), 163.5 (CONH of Pyro), 134.9 (C=C-CHO of Pyro), 126.7 (C=C-CONH of Pyro), 113.7, 109.5, 65.1 (CH₂OH of Met), 61.0 (NHCH of Val), 52.2 (NHCH of Met), 32.1, 31.9, 31.8, 20.2 (CH₃ of Val), 19.6 (CH₃ of Val), 15.6 (SCH₃ of Met); (HRMS) (CH₃OH) calcd. for C₁₆H₂₆N₃O₄S [M + H]⁺: 356.1644, found 356.1633.

5-Formyl pyrrole 2-carboxylate L-valine L-methioninol [5-formyl-N-((S)-3-methyl-1-((S)-4-(methylthio)-1-oxobutan-2-ylamino)-1-oxobutan-2-yl)-1H-pyrrole-2-carboxamide]



5-Formyl pyrrole 2-carboxylate L-valine L-methioninol 200 mg (0.563 mmol, 1 equiv.) was dissolved in DMSO (6 mL) and DCM (5 mL) and DIPEA (0.39 mL) (2.251 mmol, 4 equiv.) added. A separate vial SO₃.Pyr 358 mg (2.251 mmol, 4 equiv.) was dissolved in DMSO (3 mL) and heated before addition to the reaction mixture. The mixture was stirred at room temperature for 2 hr and partitioned between EtOAc (10 mL) and HCl (10 mL; 1N). The aqueous phase was washed twice with EtOAc (10 mL). The combined organic phases were washed with saturated brine (10 mL) and dried with anhydrous MgSO₄ and the solvent was removed *in-vacuo*. Flash chromatography on silica (EtOAc, $R_f = 0.61$) gave 5-formyl pyrrole 2-carboxylate L-valine L-methioninol 49 mg (0.1386 mmol, 25%) as a yellow solid. m.p. 72°C; IR ν_{\max} (KBr) 1734 (CHO), 1718 (CHO), 1665 (CONH), 1655 (CONH); ¹H NMR ((CD₃)₂SO) (300 MHz) $\delta_H = 12.82$ (1H, s, NH of Pyro), 9.74 (1H, s, CHO of Met), 9.54 (1H, s, CHO of Pyro), 8.72 (1H, d, $J = 9.0$ Hz, NHCH), 8.50 (1H, d, $J = 8.4$ Hz, NHCH), 7.09 (1H, d, $J = 1.8$ Hz, CH of Pyro),

7.07(1H, d, $J = 1.8$ Hz, $\underline{\text{C}}\underline{\text{H}}$ of Pyo), 4.50 (1H, t, $J = 7.5$ Hz, $\text{NH}\underline{\text{C}}\underline{\text{H}}$ of Val), 4.36-4.27 (1H, m, $\text{NH}\underline{\text{C}}\underline{\text{H}}$ of Met) 2.70-2.50 (2H, m, $\text{S}\underline{\text{C}}\underline{\text{H}}_2$ of Met), 2.26-2.09 (5H, m, $\text{S}\underline{\text{C}}\underline{\text{H}}_3$ of Met, $\underline{\text{C}}\underline{\text{H}}(\text{CH}_3)_2$ of Val) and $\text{CH}\underline{\text{C}}\underline{\text{H}}_2\text{CH}_2$ of Met), 1.95-1.80 ($\text{CH}\underline{\text{C}}\underline{\text{H}}_2\text{CH}_2$ of Met), 1.06 (3H, d, $J = 4.2$ Hz, $\underline{\text{C}}\underline{\text{H}}_3$ of Val), 1.04 (3H, d, $J = 4.2$ Hz, $\underline{\text{C}}\underline{\text{H}}_3$ of Val); ^{13}C NMR ($(\text{CD}_3)_2\text{SO}$) $\delta_{\text{C}} = 200.6$ ($\underline{\text{C}}\underline{\text{H}}\text{O}$ of Met), 181.3 ($\underline{\text{C}}\underline{\text{H}}\text{O}$ of Pyo), 171.7 ($\underline{\text{C}}\underline{\text{O}}\text{NH}$ of Val), 159.4 ($\underline{\text{C}}\underline{\text{O}}\text{NH}$ of Pyo), 134.2, 132.3, 118.3, 113.8, 58.2, 57.5, 30.3, 29.5, 27.5, 19.4 ($\underline{\text{C}}\underline{\text{H}}_3$ of Val), 18.6 ($\underline{\text{C}}\underline{\text{H}}_3$ of Val), 14.5 ($\text{S}\underline{\text{C}}\underline{\text{H}}_3$ of Met); (HRMS) (CH_3CN) calcd. for $\text{C}_{16}\text{H}_{24}\text{N}_3\text{O}_4\text{S}$ $[\text{M} + \text{H}]^+$: 354.1488, found 354.1485.

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