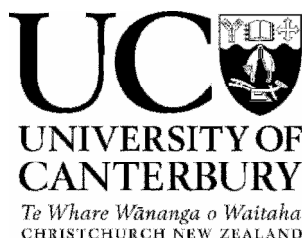# Design and synthesis of beta-strand conformationally constrained calpain inhibitors for cataract treatment via metathesis ring closure

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A thesis

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of

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Mutita Klanchantra

UC UNIVERSITY OF CANTERBURY

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CHRISTCHURCH NEW ZEALAND

University of Canterbury

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ABSTRACT

This thesis summarises the progress made in the design and synthesis of conformationally constrained β-strand peptidomimetic compounds using ring closing metathesis methodology under microwave irridation conditions. The best macrocycle were elaborated into an inhibitor for a specific protease target. Calpain was used as an example of protease targeting cataract disease.

Chapter One introduces proteases in general centring on the general context of protease inhibitor design. The significant of the β-strand 'bioactive' conformation is discussed in details in particular the exploitation of conformationally constrained to potential lock the 'bioactive' conformation.

Chapter Two illustrates *in silico* methods used to design a series of β-strand macrocycle **2.1-2.7**. The analysis of these is performed using molecular modelling software Schrodinger suite (2005). A brief discussion of ring closing metathesis methodology is also included.

Chapter Three describes the synthesis of the precursor required for RCM reactions (tripeptides dienes). Various types of allylated amino acid side chains were synthesised. The tripeptides were obtained using standard peptide coupling methodology utilising reagents such as HATU, EDC and HOAT.

Chapter Four describes the application of ring closing metathesis for the synthesis of β-strand macrocycles. The development of a new reaction conditions to optimise the ring closing metathesis reaction is discussed. In particular the effect of the use of a Lewis acid (chlorodicyclohexylborane) additive in RCM reactions is investigated.

Chapter Five discusses the mechanism of cataract formation, cataract treatment and the potential development of calpain inhibitors. One of the macrocycles synthesised in chapter 4 is elaborated into a calpain inhibitor. The in-vitro assay result of this is presented and this compound is currently undergoing *in vivo* evaluation.

ABBREVIATIONS

Asn	Aparagine
Boc	*tert*-Butoxycarbonyl
bs	broad singlet (NMR)
Cys	cysteine
d	doublet (NMR)
DCM	dichloromethane
DIPEA	diisopropylethylamine
DMF	dimethylformamide
EDC.HCl	1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide
EDTA	ethylenediaminetetraacetic acid
EtOAc	ethyl acetate
Gln	glycine
Glu	glutamic acid
Gly	glycine
h	hour (s)
HATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HIV	human immunodeficiency virus
HOAt	1-Hydroxy-7-azabenzotriazole
HOBT	1-Hydroxy-1H-benzotriazole
Hz	hertz (NMR)
IC_{50}	inhibitor concentration that decreases enzyme activity by 50%
J	coupling constant (NMR)
Leu	leucine
LRMS	low resolution mass spectrometry
min	minute (s)
mp	melting point
NMR	nuclear magnetic resonance
ppm	part per million (NMR)
q	quartet (NMR)
RCM	ring closing metathesis

rt	room temperature
Ser	serine
t	triplet (NMR)
TCE	trichloroethane
THF	tetrahydrofuran
TLC	thin layer chromatography
Tyr	tyrosine
Val	valine

ACKNOWLEDGEMENTS

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ABSTRACT

ABBRVIATIONS

ACKNOWLEDGEMENTS

1.1 PROTEASES

Proteases were initially recognized as gastric juice proteolytic enzymes that catalyse the non-specific degradation of dietary proteins.[1] These enzymes are now known to be central to many fundamental processes which determine protein synthesis, compositions, shape and size. Thus proteases are implicated in most physiological processes including cell signalling, migration and activation, fertilization, transcription, blood coagulation, morphogenesis and apoptosis. Deregulation of these proteolytic enzymes can disturb the balance of proteolytic activities and hence have been shown to engender several diseases; cataract[2,3], cancer[4,5], rheumatoid arthritis[6], bacterial and viral infections such as pneumonia[7], HIV[8,9] and neurological diseases such as Alzheimer's[10]. Consequently the ability to control protease expressions and functions is an ideal therapeutic target.

Approximately 2% of human genes encode proteolytic enzymes.[11] This large group of enzymes consists of six sub-classes which are classified according to the nature of their active site: metallo protease e.g. angiotensin-converting enzyme (ACE), serine e.g. thrombin, cysteine e.g. calpain, aspartic e.g. HIV-1 protease, threonine proteases e.g. threonine 20S proteasome and glutamic proteases.[12] These proteases catalyse sequences of the amide bond using nucleophiles; direct amide hydrolysis by water molecule (metallo and aspartic proteases) or an enzyme activated nucleophile (a nucleophilic atom from the amino acid residues) to attack the amide carbonyl atom. The thiol group of cysteine and the hydroxyl group of serine and threonine residues belong to the latter class **Scheme 1A**.
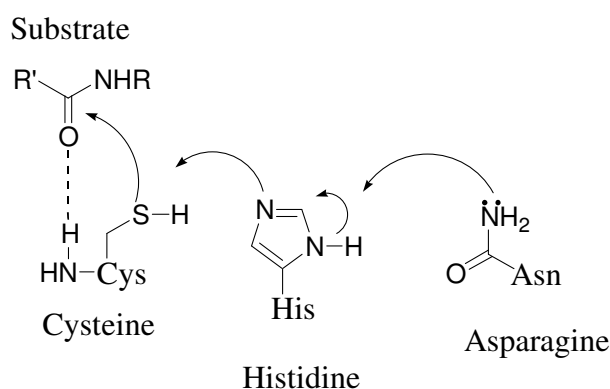
Scheme 1A: The catalytic triad of cysteine

Most proteases are sequence-specific with respect to their characters for instance; hydrophobicity/hydrophilicity and the size of enzyme sites dictate the possibility of binding between the enzyme-substrate/inhibitor.[13] This complementary binding characteristic of the enzyme-substrate/inhibitor is designated using the standard nomenclature; P3, P2, P1, P1′, P2′, P3′. This nomenclature is used to designate amino acid residues of substrate/inhibitors that bind to the corresponding sub-site of enzyme S3, S2, S1, S1′, S2′, S3′. The scissile peptide bond is P1-P1′ **Figure 1A**[14].
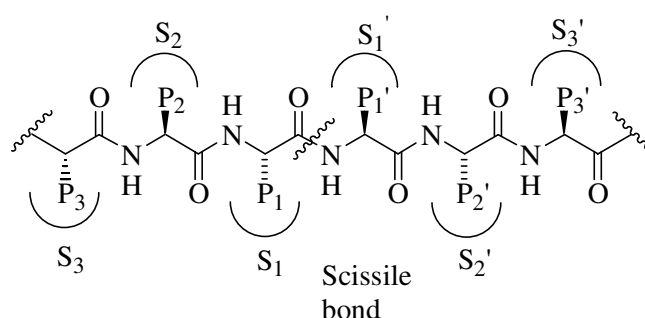
Figure 1A[14]**:** The standard nomenclature for substrate residues and their complementary enzyme binding sites [14]

Over the past decade protease drugs such as Captopril and Crixivan have shown some promising therapeutic uses towards the inhibition of angiotensin converting enzyme and HIV proteases, respectively.[13] These protease drugs have been successfully approved by the Food and Drug Administration and are commercially available. The success of protease drugs along with the fact that proteases play a vital part in disease propagation have opened up a wide-ranging field in the design of protease inhibitors.

Unfortunately designing such inhibitors is not an easy task with enormous amounts of work from the preliminary stage until the finish line. The traditional approach to protease inhibitors is to mass screen libraries of natural/synthetic products for lead compounds. This allows the establishment of structure-activity relationships (SAR) that contain a significant amount of information useful in designing protease inhibitors. However, over the past decade advances in molecular biology and protein chemistry together with advances in analytical methods such as nuclear magnetic resonance (NMR), X-ray crystallography and computer modelling programs has

allowed the elucidation of three-dimensional structures of proteases. These technologies have opened up the field of designing protease inhibitors by proving details on their active sites.[8] Once an active site has been identified a large selection of molecules can dock and interactions between enzyme-substrate/inhibitors can be investigated[8] *in silico*. A combination of these powerful techniques aids in the synthesis of protease inhibitors.

1.2 CALPAIN PROTEASE

Calpains were first isolated in 1964 (from the rat brain)[15] and were subsequently defined as an intracellular enzyme belonging to the cysteine protease class family. The name "calpain" was introduced in 1981 where 'cal' refers to the Ca^{2+} activation enzymes and 'pain' refers to the analogy between the cysteine proteases with papain.[16] The universal forms of calpains are μ-calpain and m-calpains (calpain I and II). These terms μ and m were first used in 1989 to describe the micro and millimolar concentration of Ca^{2+} required for proteases, respectively.[17] Calpains occur with their endogenous inhibitors 'calpastatins' **section 1.3.1** in almost all vertebrate cells, fungi, *Drosophila* and *Schistosoma mansoni* and as transmembrane protein in plants.[17]

1.2.1 Structure

Crystallographic structural information of calpains has become available over the last 10 years with μ and m calpains (calpain I and calpain II) being the most prominent isoforms characterized. The crystal structure solved for both expressed full length human and truncated rat m-calpain, in an absence of calcium binding, have shown them to be identical.[15] The two forms of calpain (I and II) are heterodimers consisting of large (80kDa) and small (30kDa) subunits. The large and small subunits are comprised of domains I-IV and domains V-VI respectively. The two sub units are occupied by the two catalytic sub-domains which are opposed to each other: Domain II consists of sub domains IIa(gold) and IIb(red) representing the catalytic triad Cys

105, His 262, Asn 286. Domain IV (yellow) and domain VI (orange) represent the calcium binding site 'calmodulin' like **Figure 1B**.[18]
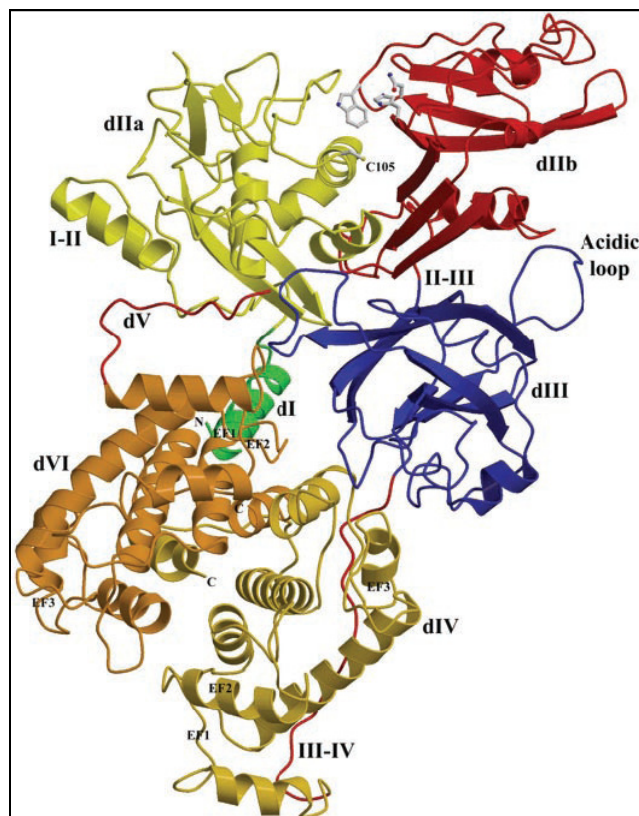
Figure 1B[18]**:** Crystallographic structure of Ca^{2+}-free human m-calpain (calpain II)

Domain I (green) is an NH_2–terminal sequence consisting of short amino acids 'anchoring helix' embedded in a surface cavity of domain VI. It has been suggested that the interaction between dI and dVI stabilizes the catalytic triad in an inactive state.[19]

Domain II is the protease domain. As previously stated this domain represents the catalytic triad (Cys, His, Asn) characteristic of cysteine proteases such as papain and cathapsins. Cysteine 105 is situated in sub domain IIa (C105 grey) whereas His and Asn (grey) are situated in sub domain IIb.[18]

Domain III (blue) is the central domain consisting of two antiparallel β-sheets. This is a C2-like domain that may possibly be involved in binding to phospholipids. The side chains of domain III consist of the solvent-exposed negatively charged acidic loop (blue). These acidic loops interact electrostatically to a positively charged basic loop on domain IIb. This electrostatic interaction is believed to have a significant role in calcium regulation of the calpain activity. [20]

Domain IV (yellow) is like domain VI (orange), these domains have sequence homology to calmodulin. The two domains have five EF-hand sequences which are involved in dimerisation of the large and small sub-units forming a heterodimer. The crystallographic evidence and the evidence from limited proteolysis illustrate that Ca^{2+} binding to the penta-EF hand domains caused a small conformational change to the overall calpain structures.[17]

Domain V: The crystal structure does not clearly indicate domain V. This domain is believed to contain the hydrophobic region; an N terminus glycine that is essential for calpain interaction with membrane which may be involved in modulation towards the calpain activity.[21]

1.2.2 Activation

The crystal structure of Ca^{2+} free human m-calpain (calpain II) **Figure 1B**[18] illustrates the active site of calpain in its inactive form (dII) and the calcium binding site (dIV and dVI). Nonetheless the mechanism of calpain is yet to be elucidated and much effort has gone into attempts to generate such mechanism. However these efforts have proven to be unsuccessful given that calpains are susceptible to autoproteolysis.[22,23] Recently Moldoveanu *et al.* successfully constructed recombinant mini-calpains (μ-calpain and m-calpain) comprising the catalytic domain II.[24] The catalytic triad is arranged in both active (**Figure 1C Left**) and non active conformations (**Figure 1C Right**).[25]
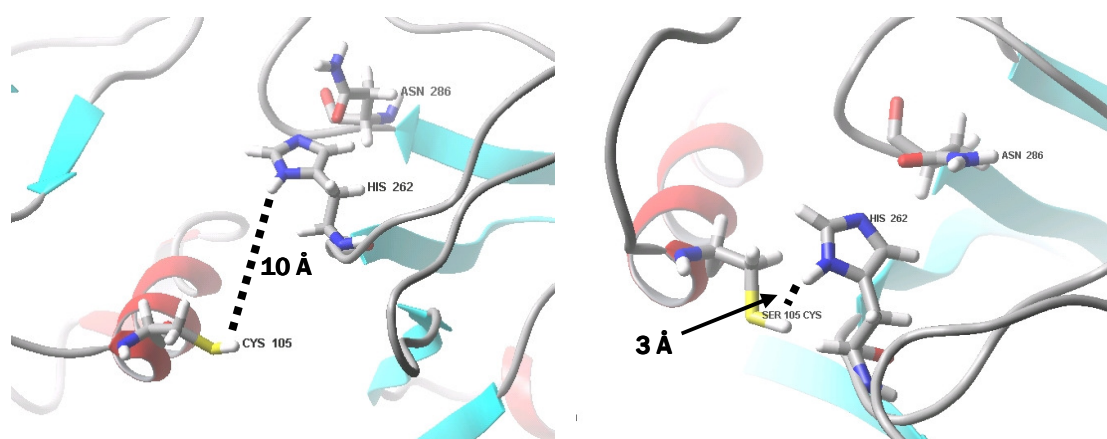
Figure 1C: Left) Crystal structure of calcium free state calpain with a significantly separated between the catalytic residues Cys 105 and His 262 by 10 A$^{\mathrm{o}}$. **Right)** Crystal structure of calcium bound calpain with the catalytic residues in close proximity of 3 A$^{\mathrm{o}}$.

The activation of Calpains is triggered by increasing the intracellular concentration of Ca^{2+} which is controlled by the endogenous inhibitor calpastatin (**section 1.3.1**). The amount of *in vitro* calcium required for the two isoforms of calpains is 5-50μM for the μ-calpain and 200-1000μM for the m-calpain.[23] The differences in requirements of Ca^{2+} are presumably due to the diversity in the acidic loop of domain dIII (an electrostatic switch) within the isoforms of calpains.[23] The acidic loop of m-Calpains consists of larger acidic residues compared to the other isoforms of calpains.[26] Thus creating a stronger charge repulsion between the calcium ions and these loops. Hence higher concentration of calcium ions in m-calpain is required to compensate these electrostatics interactions.[26, 20]

The calpain activation *in vivo* requires a much lower concentration (1μM at most) of calcium ions compared to *in vitro* to facilitate the activation of μ/m calpain. The variation in calcium sensitivity is likely due to other possible biological molecules such as protease inhibitors and phospholipids.[23,26] Nevertheless this issue still remains unclear and is yet to be resolved.

1.2.3 Function

Calpains participate in a number of cellular processes including; cytoskeletal attachments to the plasma membrane such as cell fusion of skeleton muscle myoblast and cell motility, signal transduction such as the kinases and phosphatases, degradation of enzymes required towards the progression through the cell cycle mitosis/meiosis, regulation of gene expression by cleavage of several transcription factors such as p53 and program cell death which is known as 'apoptosis'.[17]

Several calpains homologous have been reported in invertebrates.[27,28] A calpain homologous in nematodes C. *elegans* Tra-3 is involved in the sex determination cascade during early development.[15,28] Recently two of the human calpain homologous database entries Y10552 and AJ000388 have been found to be complementary to the mouse Y10656 and Y12582 entries, showing significant amino acid resemblance to Tra-3a.[15] AJ000388 (human) and Y12582 (mouse) which are localised at X-chromosomes in both species.[17,29] Consequently it is possible that calpain may be involved in sex-determination cascade in mammals.[15]

The over activation of calpains resulting from genetic or elevated levels of Ca^{2+} has also been implicated in pathological systems. Diseases such as Limb Gridle Muscular Dystrophy type 2A (LGMD2A), type II diabetes, mellitus and gastric cancer are caused by disruption in the gene Capn3a (p94), Capn10 and Capn9 (nCL4) respectively.[30, 31, 32]

Disruption of calpain function via genetic or the elevated level of Ca^{2+} is known to engender various pathological diseases such as; neurological injuries, Alzheimer's disease (AD) and Cataracts (**Chapter 5**).[17] The study of traumatic brain injuries and ischemia have illustrated the involvement of calpain activations[15,27] where the relationship between calpain and ischemia is well documented.[33, 34, 35] In the case of AD, it is believed that aggregations of β-amyloid peptide together with the build up of glutamate causes the over activation in the amount of Ca^{2+} levels which leads to the formation of AD.[27,36] Finally regarding cataract formations, it has become

increasingly clear that the over activation of calpain results in the proteolysis of the crystallins protein inducing opacification in the lens.[2] (**section 5.1**)

1.3 CALPAIN INHIBITORS

Over the past 30 years a large number of calpain inhibitors have been identified from both natural products and chemical synthesis.[37,38] Unfortunately most of these inhibitors have poor metabolic stability, low cell penetration and poor selectivity.[39] To overcome these issues the inhibitors must be modified in order to maximise their pharmaceutical potential.

1.3.1 Naturally Occurring Calpain Inhibitors

The most potent of all calpain inhibitors is the endogenous polypeptide 'Calpastatin'. Calpastatin is 120 kDa protein consisting of four repetitive inhibitory domains (A-D) which bind preferentially to separate domains of calpain.[40] Domains A and C interact with calpain domains IV and VI respectively in a Ca^{2+} dependent manner.[31] While domain B appears to inhibit calpains by binding at or near the catalytic active site (domain II).[40] Thus calpastatin selectively inhibits both of the isoforms μ-calpain and m-calpain without inhibiting other groups of cysteine proteases such as Papain and Cathepsin B. [41,17,42]

Leupeptin **1.1** is a peptidyl aldehyde class inhibitor isolated from *Streptomyces* [38]. It deactivates calpains by reversible bind to the thiol group of the active site cysteine residue of the catalytic triad. [38,42]
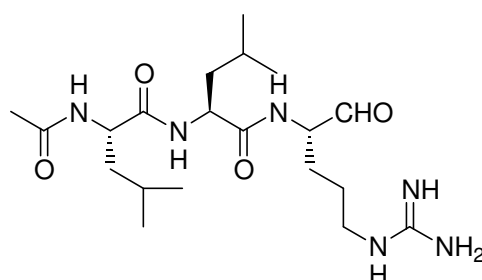
1.1

The peptidyl aldehyde class of inhibitors including Leupeptin have low selectivity towards the calpain as these inhibitors also inhibit other classes of cysteine protease such as cathepsin B and papain.[38] Furthermore, the peptidyl aldehyde inhibitors exhibit low cellular permeability, for example in the case of Leupeptin; the presence of the positively charged arginine residue is believed to be the factor that creates the poor cellular permeability.[43] Consequently this class of inhibitors is limited in its therapeutic potential.

E-64 **1.2**[43] belongs to the peptidyl epoxysuccinate class of irreversible inhibitors that are isolated from *Aspergillus japonicus*[44]. This inhibitor covalently binds to the active site cysteine of the calpain forming an irreversible sulphide linkage.
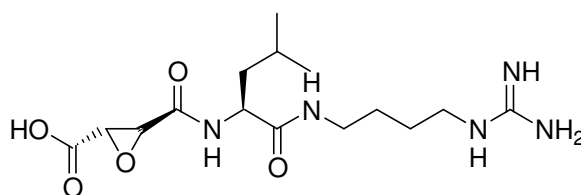
1.2

1.3.2 Chemical Synthetics Calpain Inhibitors

Simple calpain inhibitors consist of a dipeptide that interacts with S_1 and S_2 recognition sites, an aromatic capping group in the S_3 recognition site, and an electrophilic warhead that can either reversibly or irreversibly bind to the active site cysteine[23] (P_1 scissile bond) See **Figure 1A**[14].

Examples of reversible inhibitors include; aldehydes[45] (**1.3** IC_{50} 7.5 nM), α-keto carbonyl compounds such as α-ketoamides[46] (**1.4** IC_{50} 190 μM), α-ketoacids, and α-ketoesters.[47] These inhibitors contain an electrophilic warhead that forms a tetrahedral transition state intermediate during the substrate hydrolysis.[38]

1.3

1.4
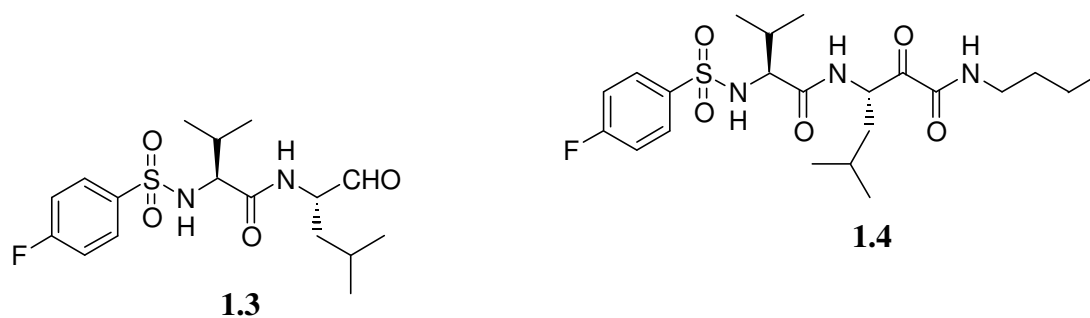
Examples of irreversible inhibitors include fluoromethylketones[48] (**1.5** IC_{50} 0.1 μM Calpain I) and peptidyl epoxides[38] (E-64 analouges) **1.2**.

1.5

P= Cbz-Leu
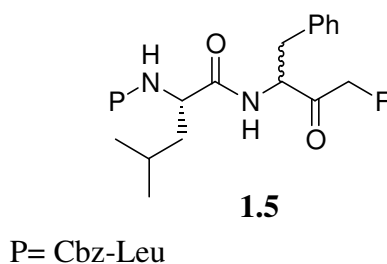
Compounds of the type **1.3**-**1.5** are typical of the vast majority of published calpain inhibitors. As illustrated by the generic structure **1.6**, these consist of a dipeptide back bone which must be able to adopt a β-strand conformation when bound into the enzyme active site. An additional recognition group and warhead are used to confer calpain inhibitory activity.

1.6
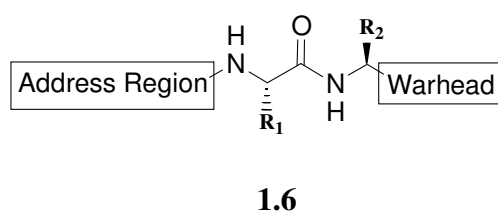
Dipeptides are flexible molecule which exist in various conformations, however only one of these conformation is responsible for its biological activity. The majority of known protease inhibitors are conformationally flexible[13,49,50,51] and have a low affinity to bind with protease. This is because the inhibitor must first spend time and energy to arrange itself into the 'bioactive' conformation to enable protease recognition.[52] As shown in **Figure 1D**[52] the maximum inhibition is dependent on the conformation selection. The random inhibitor (I_{iact}) must first form pre-equilibrium with the selected conformation (I_{act}) before the enzyme binding take places. Therefore if the inhibitor are preorganised into the desired 'bioactive' conformation the entropy loss during this pre-equilibrium stage can be minimise thus facilitating the enzyme-inhibitor binding.

Helix

$$I_{inact} \xrightleftharpoons[k_{-x}]{k_x} I_{act} \xrightleftharpoons[k_{-1}]{\overset{E\ \ k_1}{}} E.I$$

Turn
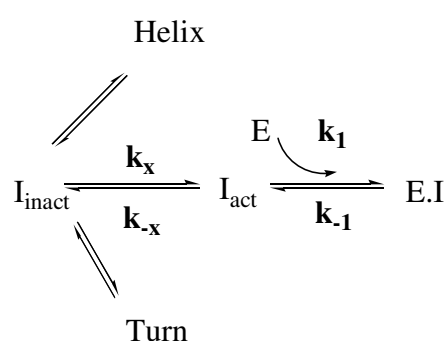
Figure1D[52]**.** Conformational equilibria and the proposed conformational selection of protease-inhibitor complex. Where $I_{inac}t/I_{act}$ = Inhibitor in an inactive/active conformation respectively.

In peptidomimetic research a key design approach is to mimic this 'bioactive' conformation by incorporating additional structural features to stabilise the desired conformation. This thesis describes such approach. **Chapter 2 and Chapter 5** detail the design and synthesis of novel conformationally constrained macrocyclic calpain inhibitors.

1.4 β-Strand 'Bioactive' CONFORMATION

Proteases have a propensity to recognise/bind polypeptide substrate via their bioactive surfaces. However once poly peptides substrate have been removed from the protease they rapidly changes their conformations in solution into random structure.[53] Theses random structures have a tendency to fold via intramolecular hydrogen bonds[52], forming a thermodynamically stable secondary structure including α helix, β-strand, β-sheet and β and γ turn.

Fairlie *et al.* have analysed over 1500 three dimensional protease crystal structures deposited in the Protein Data Bank[54] to identify the required conformation of protease classes. From this it was concluded that proteases universally bind their substrate/inhibitor in an extended β-strand conformation (**Figure 1E**[12]).
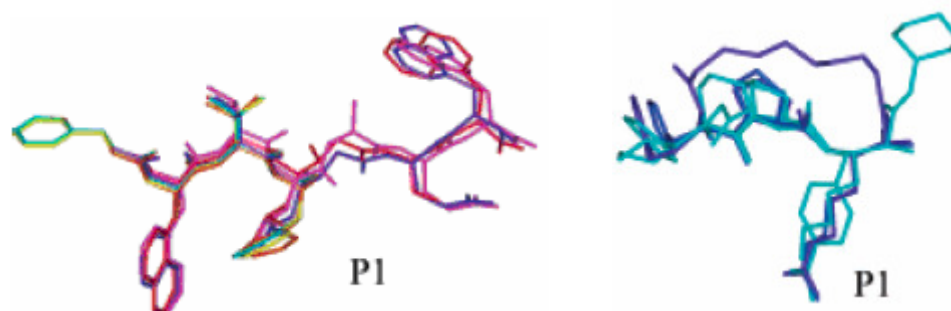
Figure1E[12]**:** An overlay of the backbone of known inhibitors bound to the proteases in the extended β-strand conformation. **Left)** substrate bound structure to mutant Feline immunodeficiency virus (FIV) retropepsin (aspartic protease). **Right)** protease-inhibitor bound structure of Trombin cyclic inhibitor PPACK (D-Phe-Pro-Arg-Chloromethyl ketone) (serine protease).

The β-strand motif consists of a short peptide sequence (<10 residue) that exists as an extended 'saw tooth' arrangement of amino acids with their side chains alternating anti-parallel to one another and the amide bonds orthogonal to the side chains (**Figure 1F a)**). The torsion angle of β-strand structure; phi (ϕ), psi(ψ), omega (ω) have optimum angles of – 120°, 120° and 180° respectively. (**Figure 1F b)**)
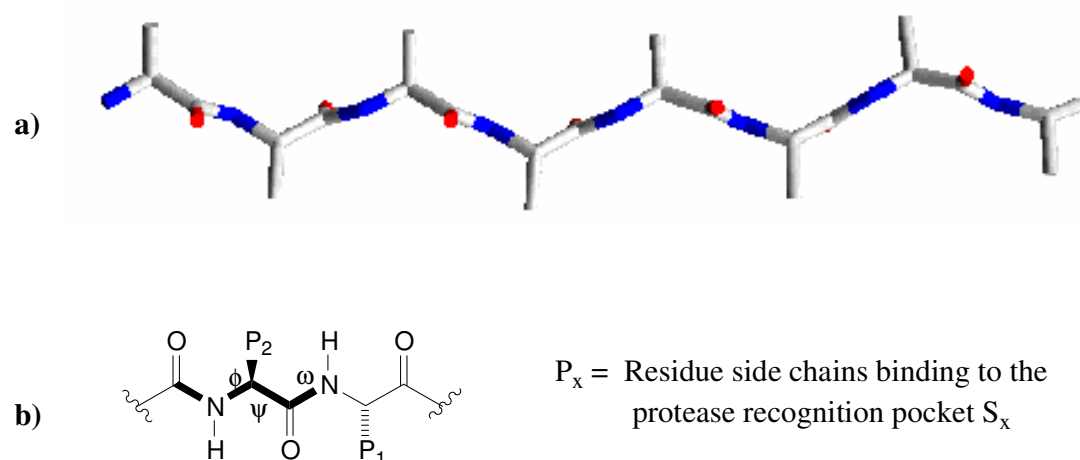
Figure 1F: a) A three dimensional β-strand conformation[54] **b)** Another view of the extended β-strand with torsional angle.

Adoption of this extended β-strand conformation means that there is a great separation between the adjacent amide bonds thus preventing intramolecular hydrogen bonding. At the same time this conformation also allows the side chain residues to have maximum exposure to the protease's active sites which in turn facilitates protease binding.[55]

The discovery that proteases bind their substrate/inhibitors universally in a 'β-strand' conformation has laid the foundation for the development of a new and general class of protease inhibitors. To be an effective protease inhibitor a molecule must adopt the β-strand 'bioactive' conformation before binding to the enzyme active site.

1.4.2 Conformationally Constrained Protease Inhibitors

Macrocyclisation is one possible approach for conformationally constraining an inhibitor into the β-strand 'bioactive' conformation. Furthermore this confers a significant entropy advantage.[56,57] In addition macrocyclisation is also expected to increase the metabolic stability and the bioavailability. Thus macrocyclic inhibitors

should exhibit more promising pharmacological properties in comparison to their acyclic analouges.[49,56,57]

The use of macrocyclisation also counteracts the cooperative nature of the enzyme inhibitor binding. If changes are made in a linear peptide inhibitor this will also induce changes in the local enzyme environment, which in turn influences the enzyme-inhibitor interactions at adjacent locations.[57] This process is commonly refer to as 'the induce fit'.[58] However macrocyclic inhibitors bind in a precise manner such that these do not induce changes in the local enzyme environment.

There are various ways to create macrocyclic peptidomimetic compounds; side chain to N-terminus cyclisation **1.7**[59], side-chain to side chain cyclisation **1.8**[59] and N to C cyclisation **1.9**.[52] Such conformationally constrained protease inhibitors have already been shown to be potential therapeutic agents.[12]
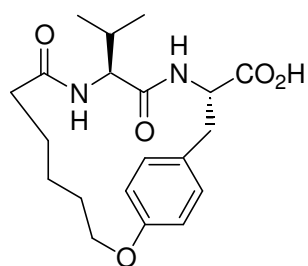
HIV-inhibitor
1.7
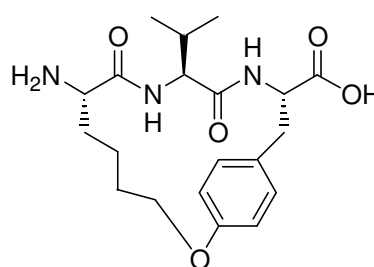
HIV-inhibitor
1.8
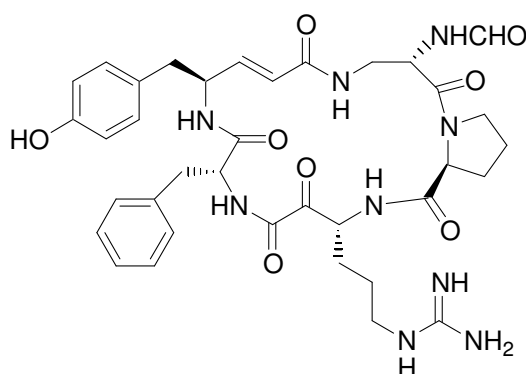
Trombin inhibitor
1.9

1.5 WORK DESCRIBED IN THIS THESIS

This thesis focuses on the design and synthesis of β-strand conformationally constrained compounds formed via side chain-side chain linkages. A virtual library of potential β-strand mimics was prepared with in the group by Steven Aitken.

This library contained a total of 272 potential macrocyclic β-strand mimics. All these underwent conformational analysis *in silico* to determine the Boltzmann weighted percentage β-strand conformation with in the conformational distribution (**Chapter 2**). The best ones of these macrocycles were selected for synthesis.

The synthesis was divided into 2 parts. Firstly tripeptide dienes were synthesise (**Chapter 3**). Next ring closing metathesis (RCM) was employed with Grubbs second generation catalyst (**2.12**) under microwave conditions (1200W) to cyclise the dienes to form the desired macrocycles (**Chapter 4**). The last part of this thesis describes elaboration of these macrocycles into calpain inhibitors (**Chapter 5**).

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2.1   DESIGNING β-STRAND INHIBITORS

A library of 272 (17 x 16) possible novel β-strand macrocyclic inhibitors consisting of 14-22 membered macrocyclic rings were created by Steven Aitken[1]. All macrocycles consist of a tripeptide backbone. The selected macrocycles **2.1-2.7** were studied *in-silico* within our group by Blair Stuart, Axel Neffe and Steve Aitken. All molecular modelling experiments were conducted with the Schrodinger suite 2005.   The macrocyclic compounds were subjected to conformational searches using Macromodel 9.0 to find both the lowest energy conformer and to create an ensemble of the low energy conformations which were within a 12kJ window of the global minimum.   The conformational searches were conducted with MCMM method and were run with a GB/SA water model, using the OPLS2001 force field, with 1000 steps for the conformational search and up to 500 iterations for the minimization of each generated structure. The minimization was performed with the default gradient convergence threshold of $\delta = 0.05$ kJ/(mol*Å). For all minimizations, the default Polak-Ribiere Conjugate Gradient method was used.[2]

The structures within the 12 kJ/mol window were analysed further (using a computer program written by Blair Stuart[2]) to determine whether these macrocycles favour β-strand conformations. The Boltzmann weighted percentage of β-strand, twist and turn for each ensemble were calculated (Note that the turn conformation resemble γ-turn). The distance between the hydrogen atom of the amide bond and the oxygen from the carbonyl at the P_2 position (shown in yellow dash for each macrocycle see **Figure 2.1**-**2.7**) was used to determine whether each individual conformer in the ensemble was a β-strand, twist or turn. As illustrated in **Figure2A**[2,3] a calculated distance of less than 3.1 Angstroms represented a β-strand and a value greater than 3.7 Angstroms represented a turn, intermediate values represented a twist.
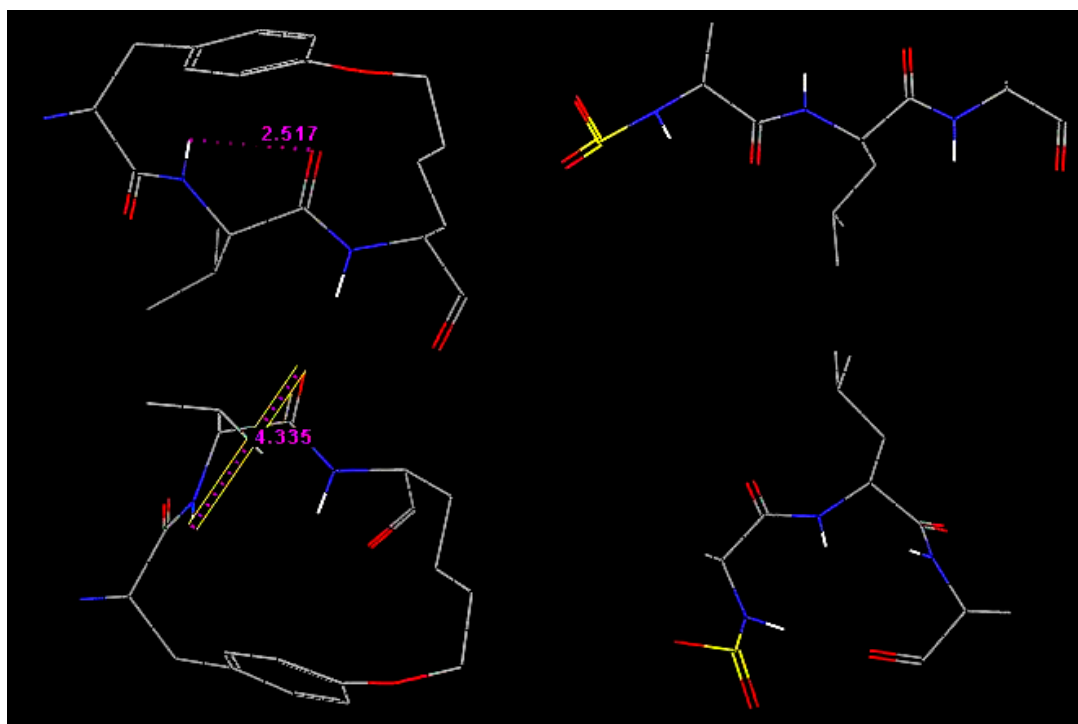
Figure 2A[2,3]**:** Distance between equivalent atoms used to distinguish between **Top**) β-
strand (<3.1 Angstroms) and **Bottom**)β-turn (>3.7), and twist (3.1<twist<3.7).

The Boltzmann weighted percentages of conformers were calculated and these are
illustrated in the table (**Figure 2.1-2.7**) below each macrocyclic compound. The
Boltzmann weighted percentage of each macrocyclic ensemble (**compound 2.1-2.7**)
indicates that each is a potential β-strand conformational constraint.

It should be noted that the modelling pictures in this thesis are those representing the
lowest energy β-strand conformer from each ensemble rather than the global
minimum.

2.1.1   Tyrosine Based Macrocycles 2.1 and 2.2

The tyrosine based macrocycles are shown in **Figure 1 and Figure 2**. These
macrocycles consist of Tyr residues at the P_3 positions. Macrocycle **2.1** and **2.2**
contained Glu and Gly residues at the P1 positions respectively.

The Boltzmann weight percentages of the two macrocyclic ensemble conformers have shown that these tyrosine based macrocycles exhibit an excellent β-strand conformation and are two of the best β-strand macrocycle obtained from *in silico* studied in this thesis. **2.1** exhibit 96.84% β-strand and **2.2**, 90.01%

As shown in **Figure 2.2** the distance between the hydrogen atom of the amide bond and the oxygen from the carbonyl at the P_2 position is 2.18 Angstroms which clearly indicates that macrocycle **2.2** represents a β-strand conformation. Macrocycle **2.2** has also been tested for its inhibition potency against calpain. With an IC_{50} of 45nM **2.2** is a very potential calpain inhibitor. (**Chapter 5**)

Figure 2.1: Tyr-Val-Glu macrocycle

Compound 2.1

Twist %	Strand %	Turn %
2.63	96.84	0.53
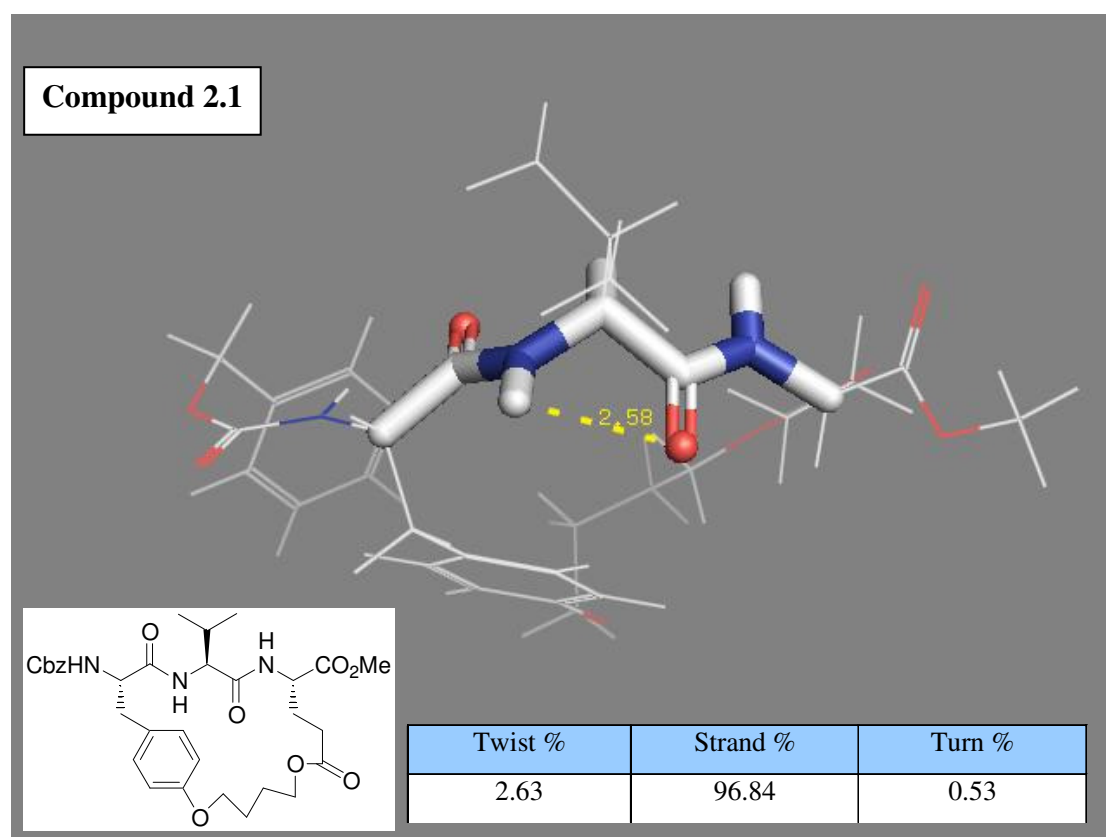
Figure 2.2: Tyr-Leu-Gly macrocycle

Twist %	Strand %	Turn %
9.99	90.01	0
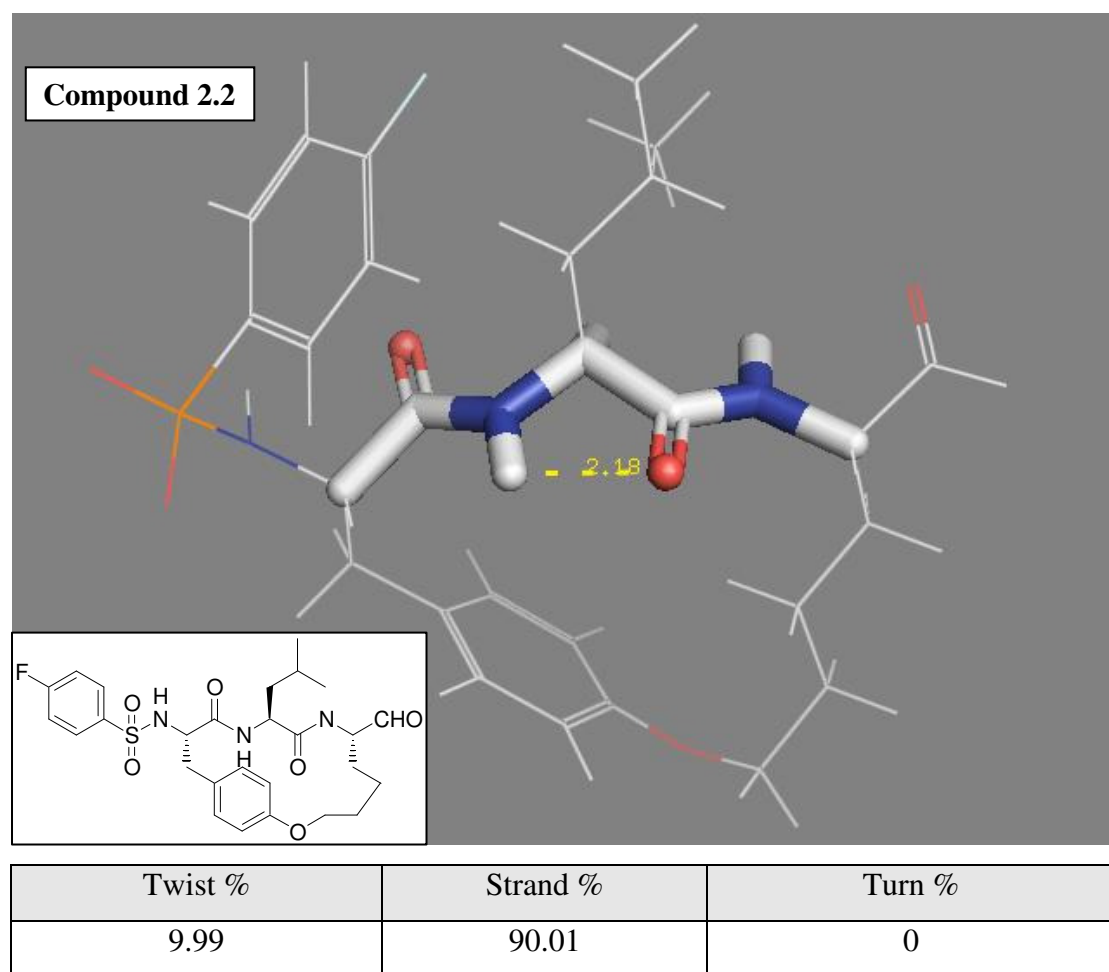
2.1.2   Glutamine based macrocycles 2.3 and 2.4

The 15-membered glutamine based macrocycles consist of Gln at the P_3 positions and Gly at the P_1 position as shown in **Figures 2.3** and **2.4**. The calculated Boltzmann weight percentages of the glutamine based macrocycle shows that the unsaturated macrocycle **2.5** exist as 59.57% β-strand conformation and macrocycle **2.4** is 39.23% β-strand.

Figure 2.3: Gln-Val-Gln macrocycle

Compound 2.3

Twist %	Strand %	Turn %
41.08	39.23	19.7

Figure 2.4:Gln-Val-Gly macrocycle

Compound 2.4

Twist %	Strand %	Turn %
31.86	59.57	8.57

2.1.3   Cysteine based Macrocycles 2.5-2.7

The cysteine based macrocycles are shown in **Figures 2.5-2.7**. These macrocycles are all contain cysteine residues at the P_1 position while the residue at the P_3 position varies. The P_3 positions of **2.5-2.7** consist of Cys, Ser and Glu residues respectively.

The calculated Boltzmann weighted distribution percentage of β-strand conformation is 7.28% for **2.5**, 55.1% for **2.6** and 50.57% for **2.7**. The Boltzmann weighted percentage of macrocycle **2.5** indicates that the Cys-Cys based macrocycle does not favoured the β-strand conformation. In comparison to Ser-Cys **2.6** and Glu-Cys **2.7**, exhibit over 50% β-strand conformers. This is postulated to be due to the influence from the large sulfur atoms which forces the geometry of macrocycle **2.5** away from the extended β-strand conformation.

<div align="center">

Figure 2.5: Cys-Val-Cys macrocycle

</div>

	Twist %	Strand %	Turn %
	0.22	7.28	92.5

Figure 2.6: Ser-Val-Cys macrocycle

Twist %	Strand %	Turn %
6.18	55.1	38.72

Figure 2.7: Glu-Val-Cys macrocycle

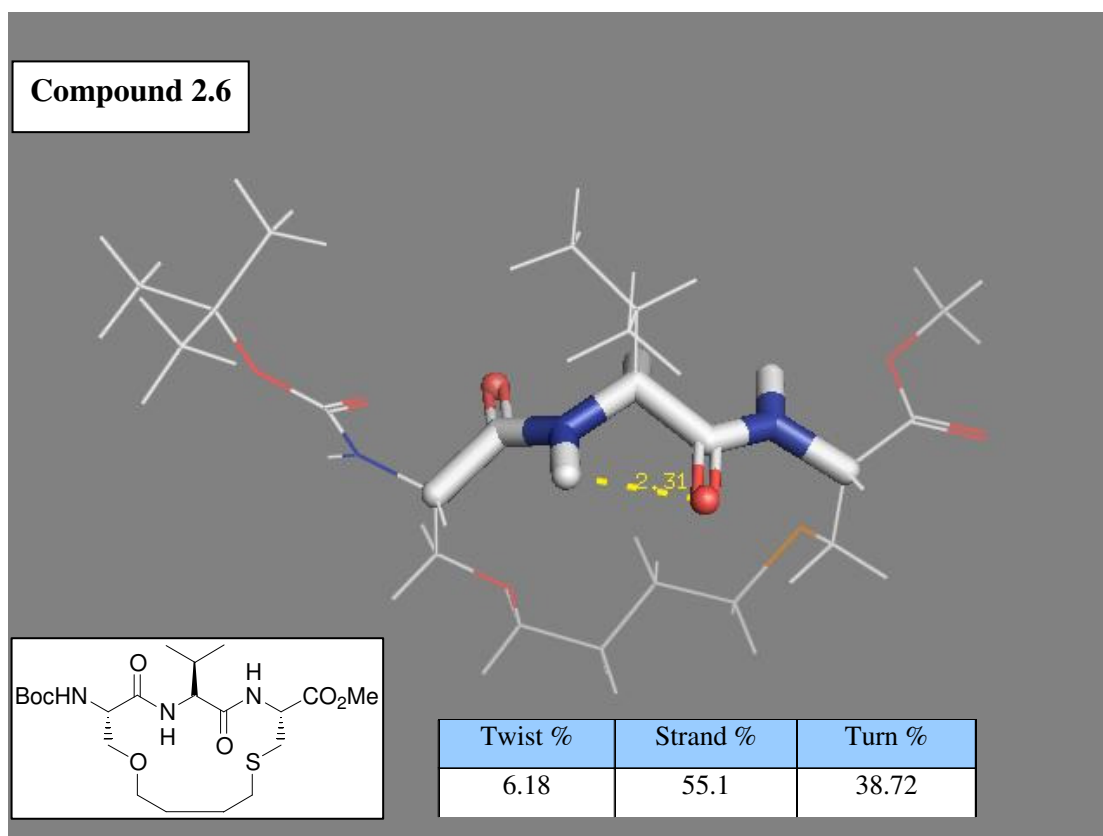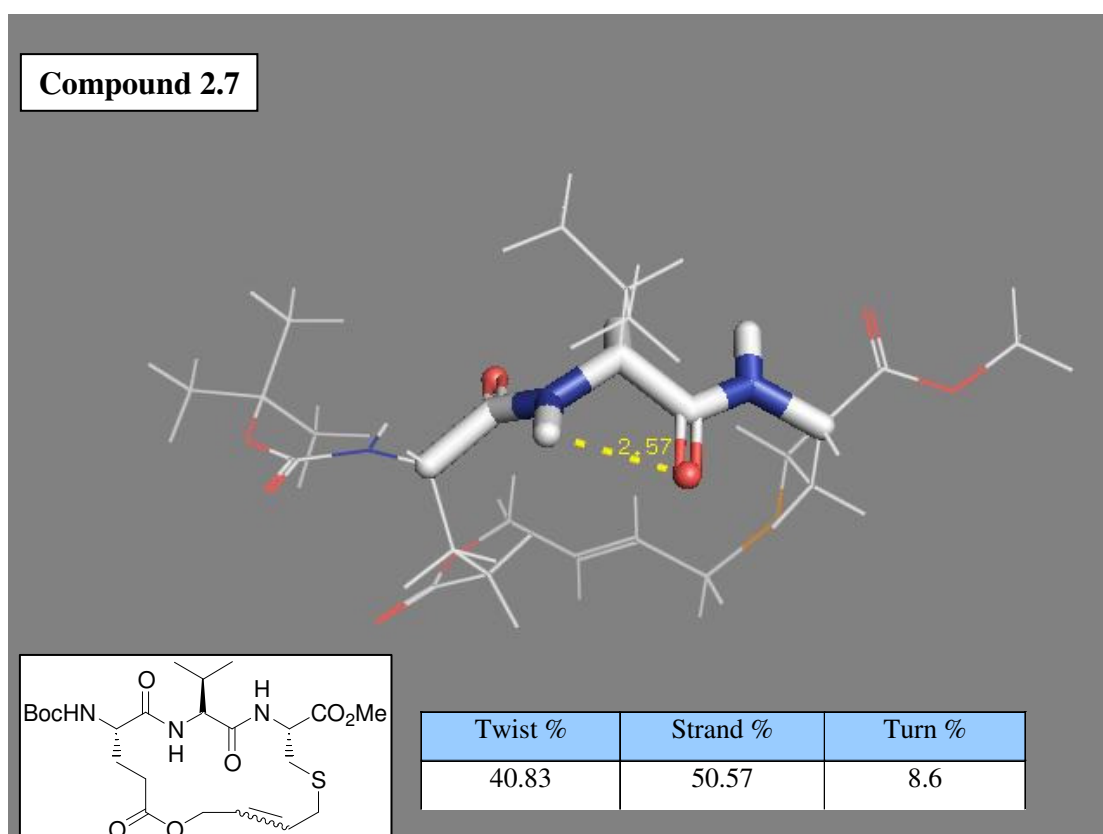| Twist %	Strand %	Turn %
40.83	50.57	8.6

This thesis details the synthesis of the potential β-strand macrocycles **2.1-2.7.** The syntheses were divided into two chapters Chapter **3** and Chapter **4**.

2.2   RING CLOSING METATHESIS VIA MICROWAVE CONDITIONS

Olefin metathesis was first discovered in the 1950's following the observation of polymerisation during the reactions of the olefins by Ziegler.[4] However it was a largely unexplored area of chemistry until the early 1990's and as such it is a relatively new reaction for many organic chemists. Therefore the use of the metathesis reaction has not been extensively investigated in many areas of organic chemistry. Nevertheless in the past decade a tremendous amount of progress has been made in the use of olefin metathesis in organic synthesis.

The etymology of the word metathesis shows that it is derived from Greek language meaning *transposition.*[5] Currently we understand the term metathesis to mean the metal-catalysed redistribution of carbon-carbon double bonds.

There are various classes of metathesis reactions such as ring-opening metathesis, ring closing metathesis, ring opening metathesis polymerization (ROM), acyclic diene metathesis polymerization (ADMP), eyene metathesis and cross metathesis (CM). However ring closing metathesis has emerged as the most prevalent type and it is a useful reaction for constructing different sizes of macrocyclic ring systems.[5,6]

2.2.1   Ring closing metathesis (RCM)

The mechanism of RCM has been studied over a number of years but in 1971 Chauvin proposed the general mechanism that is still commonly accepted. This mechanism consists of a sequence of formal [2+2] cycloadditions to give the key intermediate 'metallocyclobutane'. The metallocyclobutane is unstable and undergoes cycloreversion forming a new C=C bond and the volatile gas ethylene, as shown in

Scheme2A.[4,7]   Note that the mechanism is reversible and in order for the RCM reaction to be effective the equilibrium must be shifted into one direction i.e. to the right. Ring closing metathesis reactions are entropically driven in the forward direction because it 'cuts' one substrate into two products of which one is volatile (ethene) leaving the desired cycloalkenes to accumulate in the reaction mixture.[4]
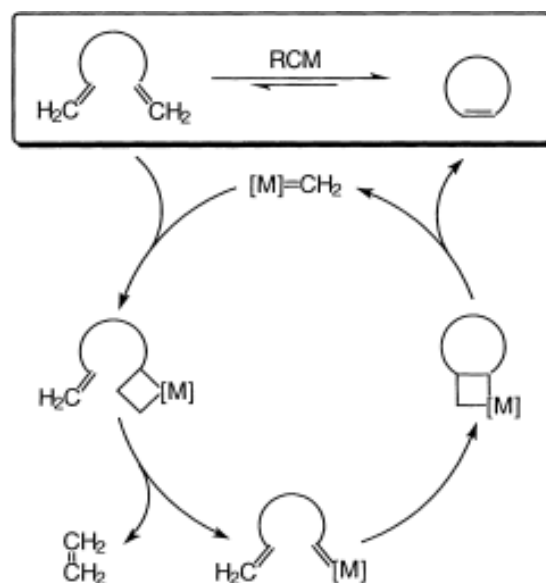

**Scheme 2A**[4]**:** Catalytic cycle of ring closing metathesis

Early olefin metathesis catalysts were poorly defined. They were multicomponent homogenous and heterogeneous systems which exhibited 'mixed' Ziegler catalyst characteristics (high activity and incompatibility with most functional groups due to a strong Lewis-acidic and alkylating character).[4]

In 1980, Schrock's tetracoordinated alkylidene species of general formula [M(=CHCMe$_2$Ph))=NAr)(OR)$_2$] was described. This was an active and well defined catalyst (M=Mo or W and Ar and R are bulky substituents)[8,9]. Catalyst **2.8** in particular is the most active and is commercially available. However the reactivity of catalyst **2.8** is offset by the fact that it is sensitive towards moisture and oxygen it must therefore be handled in rigorously dried solvents using Schlenck techniques. Nevertheless catalyst **2.8** does have an advantage in its tolerance towards certain

functional groups that inhibit ruthenium-based metathesis catalyst (typically soft ligands such as sulfur and phosphine).
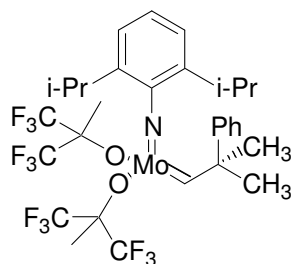
2.8

The first well defined ruthenium catalyst complex (**2.9**) was reported in 1992 by Grubbs and co-workers.[10] This catalyst has an advantage over **2.8** in that it is stable in protic solvents. The influence of the ligands on the activity of 5 coordinate 16 electrons ruthenium complexes have been extensively studied.[4,5,10] Consequently exchanging the triphenylphosphine (P(Ph)$_3$) groups with larger and more basic tricyclohexylphosphine groups (P(Cy)$_3$) (**2.10**) result in increased metathesis activity.[4]
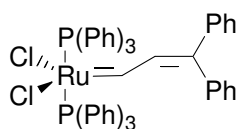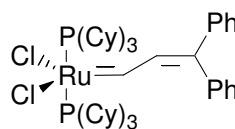
2.9   **2.10**

Further work by Grubb and his colleagues has facilitated growth in the use of RCM in organic chemistry. Grubbs first generation catalyst **2.11** and Grubbs second generation catalyst **2.12** were introduced in the late 1990's.[10] These catalysts are commercially available with **2.12** being more reactive. Although on some occasion the ruthenium catalyst may not be as active as the early transition metal complexes and Schrock's catalysts, they are more versatile in RCM application due to their improved functional group tolerance[11].
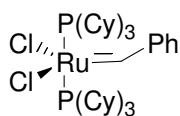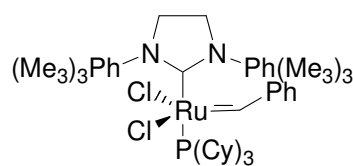
2.11   **2.12**

Indeed Grubbs second generation catalyst is now widely used in RCM reactions.[5,11] The catalyst is less sensitive to air and moisture which makes it is easier to handle. The substitution of the $P(Cy)_3$ with a more basic and sterically hindered N-heterocyclic carbene (NHC) ligand increases the dissociation rate of the second phosphine ligand, resulting in the catalytic species (a 14 electrons ruthenium intermediate) being more easily formed. This results in increased catalyst life time and higher reactivity which in turn increases the ring closing metathesis activity.

2.2.2   Microwave conditions

Microwave activation has emerged as a powerful technique to promote a variety of organic reactions.[12] RCM is generally carried out in homogenous solution at room temperature (or slightly higher) for several hours before the reaction goes to completion. The yields of some reactions can be low and occasionally the reaction does not proceed under conventional heating methods, thus optimisation of the reaction was required.

With microwave irridation as a heat source shorter reaction times can be achieved. In addition, reactions which are fail to occur using thermal heats can be achieve using microwave irridation.[13] This is because the rapid uniform core heating associated with microwaves, instead of the concentration of heat around the reaction vessels walls help reduce catalyst decomposition.[13,14,15] Apart from giving shorter reaction times, microwave irridation has also been shown to improve the yield of RCM reactions through a cleaner and more facile workup procedure.[13,15] Over the past few years investigations into a combination of solvent-free conditions and microwave activation has resulted in significant progress towards the so called 'green chemistry' eco methodology.[16] For these reasons the use of microwave irridation has contributed significantly towards metathesis chemistry.

The primary objective in this thesis is to apply and develop RCM methodology using microwave conditions for synthesis of β-strand macrocyclic compounds designed in **section 2.1**.

2.3   SYNTHESIS OF β-STRAND CONFORMATIONALLYCONSTRAINED 'BIOACTIVE' COMPOUNDS

The use of ring closing metathesis is a very attractive method for synthesising peptidomimetics research, particularly those constrained into a desired 'bioactive' conformation.

The objective of this research was to develop macrocyclic ring systems (15-22 membered rings sizes) that conformationally lock the peptide backbone into the required bioactive β-strand conformation. These constrained structures pre-organise the inhibitor/substrate into a bioactive conformation and hence reduce the entropy of binding to the enzyme/protein (**section 1.4.2**). Furthermore, β-strand backbone macrocycles are attractive targets which may be employed as generic scaffolds facilitating the synthesis of different protease inhibitors. The synthesis of different protease inhibitors can be achieved by incorporating appropriate functionality or recognition elements to the key β-strand macrocyclic templates. Consequently a novel class of protease inhibitor is attained in a pre-organised 'bioactive' conformation which in turn can effectively bind to the protease active site.

In this thesis the designed by the *in silico* studies (**2.1-2.7**) in **section 2.1** were synthesised via RCM using Grubbs second generation catalyst and microwave irridation. The general approach was to construct a suitable tripeptide diene consisting of alkenes on the amino acids side chains P_3 and P_1 **Chapter 3**. A tripeptide backbone was chosen over dipeptide because modelling demonstrated them to mimic the linear β strand saw tooth conformation, thus allowing optimum hydrogen bonding with the enzyme active site. With the tripeptide backbone in place the next step is to cyclise the two terminal diene side chains by employing the RCM procedure **(Chapter 4)** as shown in **Scheme 2B**. Finally the successfully prepared macrocyclic compound was chemically modified into a calpain inhibitor by incorporating the desired warhead at the C terminus **Chapter 5**.
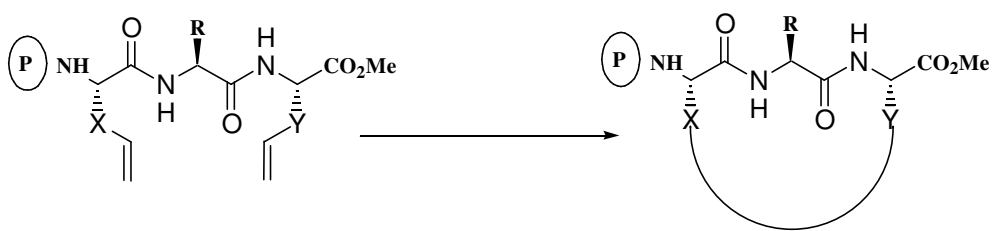
Scheme 2B: General approached of constructing macrocycle compounds

2.4:   REFERENCES FOR CHAPTER 2:

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The selected macrocycle compounds **2.1-2.7** (**sections 2.2**) were synthesised. The syntheses are divided into two chapters (**Chapter 3** and **Chapter 4**). **Chapter 3** illustrates the syntheses of β-strands tripeptide backbones **3.15, 3.21, 3.34, 3.44, 3.52, 3.58, 3.64.** These tripeptides β-strand backbones were successfully prepared by employing the general peptide coupling methods shown in **Scheme 3.0**
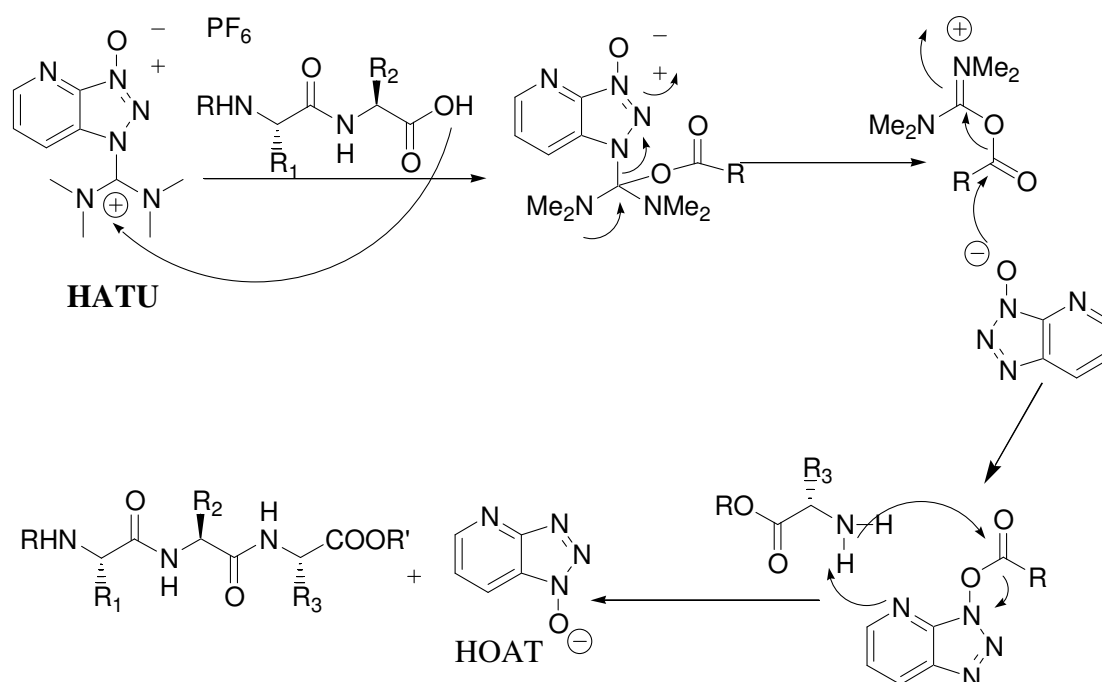
Scheme 3.0: General mechanisms of HATU coupling reactions

3.1   Synthesis of N-Boc-S-allyl-Cys-Val-S-allyl-Cys-OMe 3.15

The diene **3.15** was prepared as shown in scheme **3.1**. The amino acid **3.10** was allylated upon reaction with allyl bromide in alkaline conditions under an argon atmosphere to give N-Boc-S-allyl-Cys-OMe **3.11** in 77% yield (after silica gel-based column chromatography). The methyl ester of **3.11** was then hydrolysed by sodium hydroxide, methanol and water to give a free acid **3.12** in 83% yield. Next compound **3.12** was coupled with (S)-Val-OMe hydrochloric salts using general coupling reagent HATU in basic conditions. Purification by column chromatography gave **3.13** in 66% yield. Compound **3.13** was then treated with sodium hydroxide, methanol and water to remove the methyl ester group to give compound **3.14** in 70% yield. The dipeptide **3.14** diene was coupled to the (S)-N-allyl-Cys-OMe **3.26** (**Scheme 3.4**) using HATU coupling reagent and DIPEA to give an isolated diene **3.15** in 65% yield.
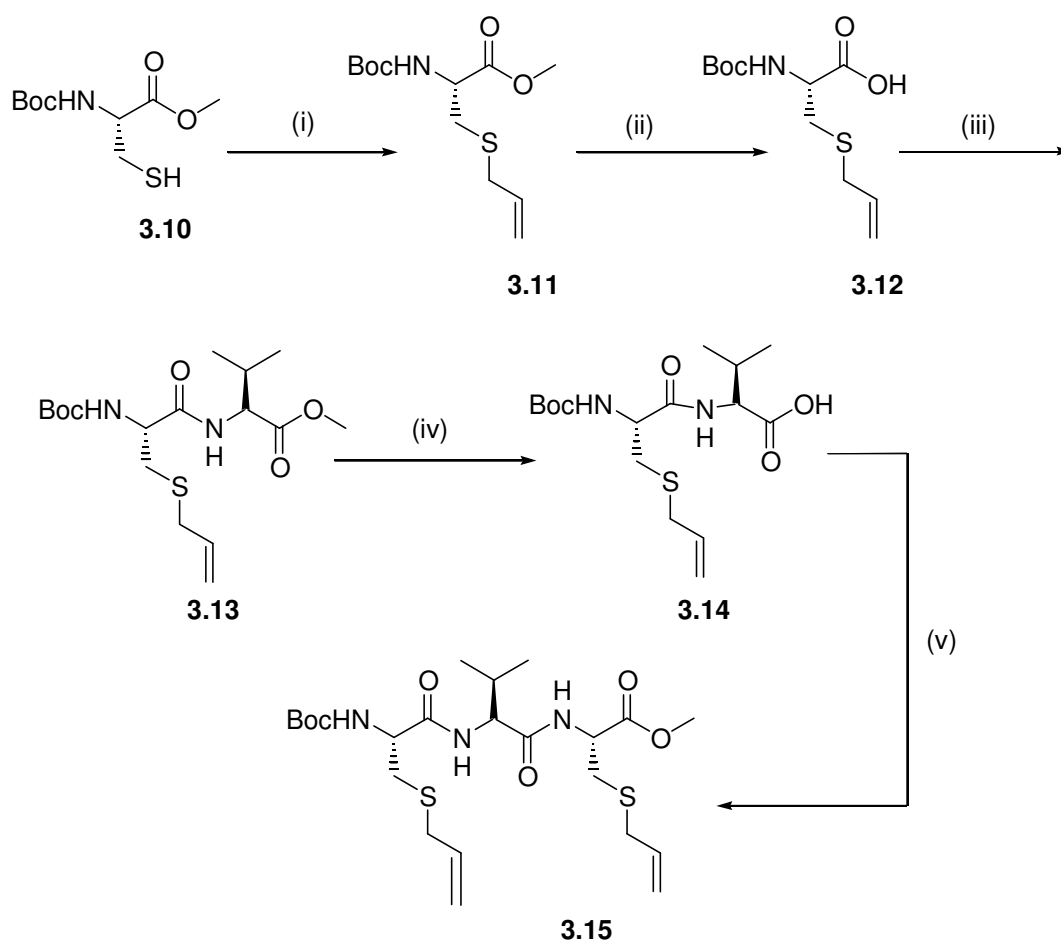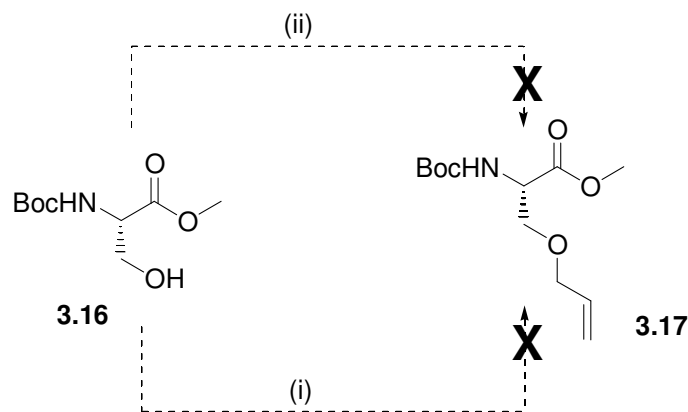
Scheme 3.1 *Reagents and conditions:* (i) allyl bromide, Et$_3$N, DCM (77%) (ii) NaOH, MeOH, H$_2$O, THF (83%), (iiii) Val-OMe.HCl, HATU, DIPEA, DMF (66%) (iv) NaOH, MeOH, H$_2$O, THF (70%) **(v)** (S) allyl-Cys-OMe **3.35**, HATU, DIPEA, DMF (65%)

3.2   Synthesis of N-Boc-O-allyl-Ser-Val-S-allyl-Cys-OMe 3.21

The synthesis of Boc-O-allyl-Ser-OMe **3.17** was attempted as shown in **Scheme 3.2**. Treatment of N-Boc-Ser-OMe **3.16** with sodium hydride as a base and allyl bromide resulted in mixtures of O-allylation, N-allylation and allylation at the methyl ester group. This may be due to the fact that the choice of sodium hydride as a base was too strong for this reaction, resulting in deprotonation as well as methyl ester hydrolysis.

The second attempt was involved treating N-Boc-Ser-OMe with allyl bromide and a weaker base (triethylamine). This resulted in returned starting material only.

Scheme 3.2 *Reagents and conditions:* (i) NaH, allyl bromide, DMF (0%) (ii) Et$_3$N, allyl bromide, DCM (0%).

The last attempt to synthesis compound **3.17** was carried out successfully by following the literature methods.[1] N-Boc-Ser-OMe was treated with allyl ethyl carbonate (prepared *in situ* by reacting allyl alcohol, ethyl chloroformate at 0^0 C under basic conditions), allyl palladium chloride and triphenylphosphine to give N-Boc-O-allyl-Ser-OMe **3.17** in 21% yield (unoptimised) after purification by column chromatography (**Scheme 3.4**).

Allylation of **3.16** was carried out in neutral conditions to avoid deprotonation of the acidic proton from amine. Allyl ethyl carbonate, triphenylphosphine and allyl palladium (0) chloride dimmer catalyst reacted together to form the Π-allylpalladium complex (Pd$^+$). This resulted in the release of ethylate compound which removed the proton from the side chain residue. Thus generate the nucleophile that readily underwent nucleophilic attack with an electrophile (Pd$^+$ complex), resulted in the desired product **3.17** (**Scheme 3.3**). The yield obtained was relatively low compared to the literature.[1] This could be because the reaction was carried out in a larger scale and the catalytic amount of palladium chloride may need to be increased. This should be taken into account and used for future reference.
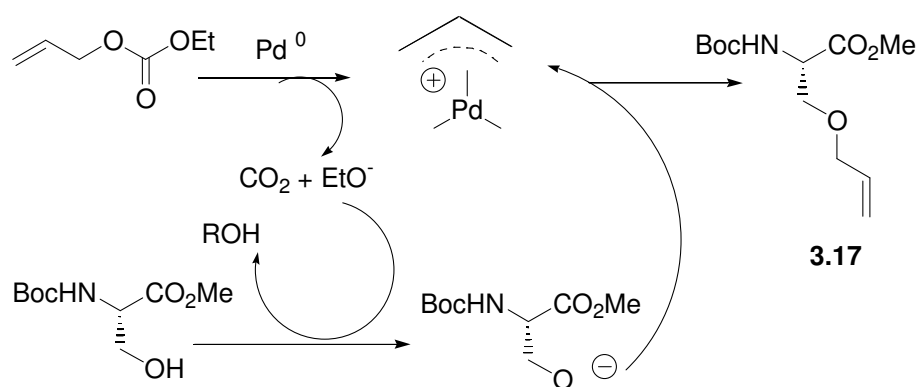
Scheme 3.3: General mechanism of O-allylation with palladium chloride [1]

Next compound **3.17** was hydrolysed by treatment with sodium hydroxide, methanol and water to give compound **3.18** (82% yield). Dipeptide **3.19** was prepared by reacting free-acid **3.18** with (S)-Val-OMe.HCl, adopting the general coupling reagent HATU and a steric hindered base DIPEA in 73% yield (after purification by column chromatography). The methyl ester of **3.19** was hydrolysed to give **3.20** (71% yield) followed by the treatment with **3.35**, HATU, DIPEA to give the desired isolated tripeptide compound **3.21** in 54% yield (**Scheme 3.4**).
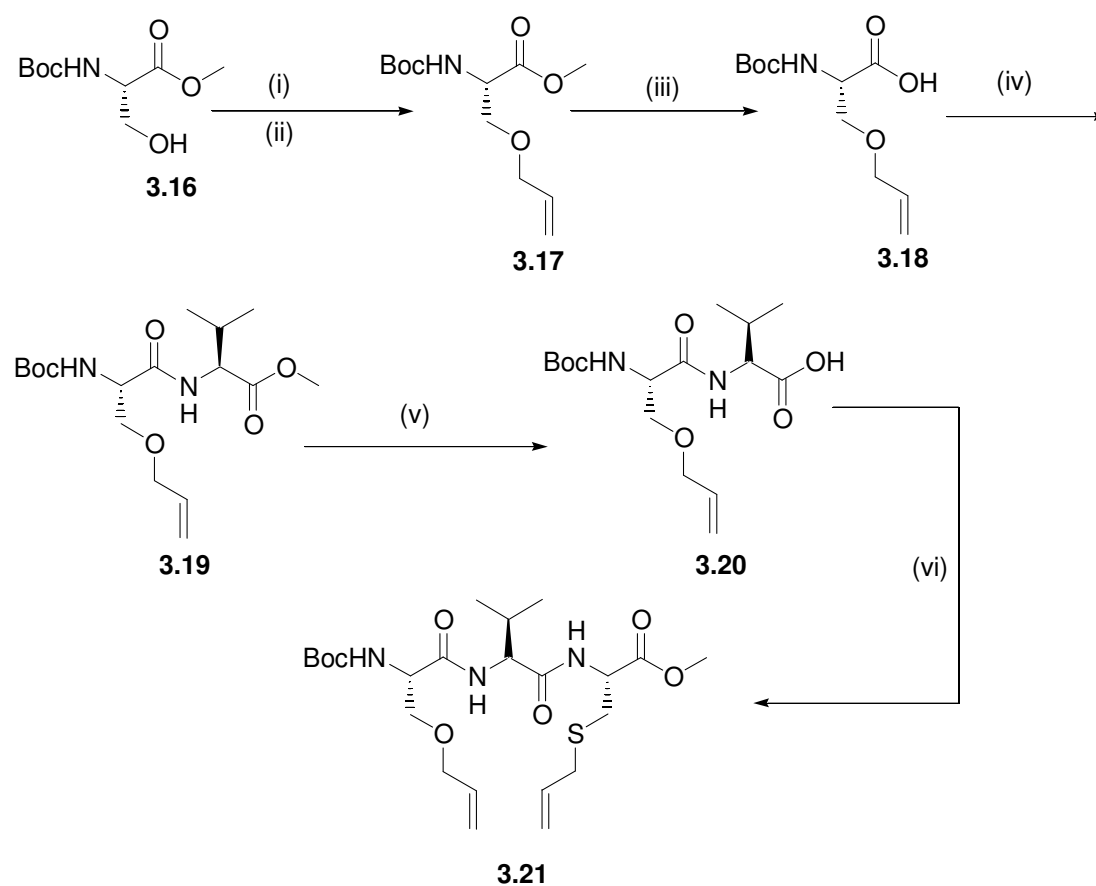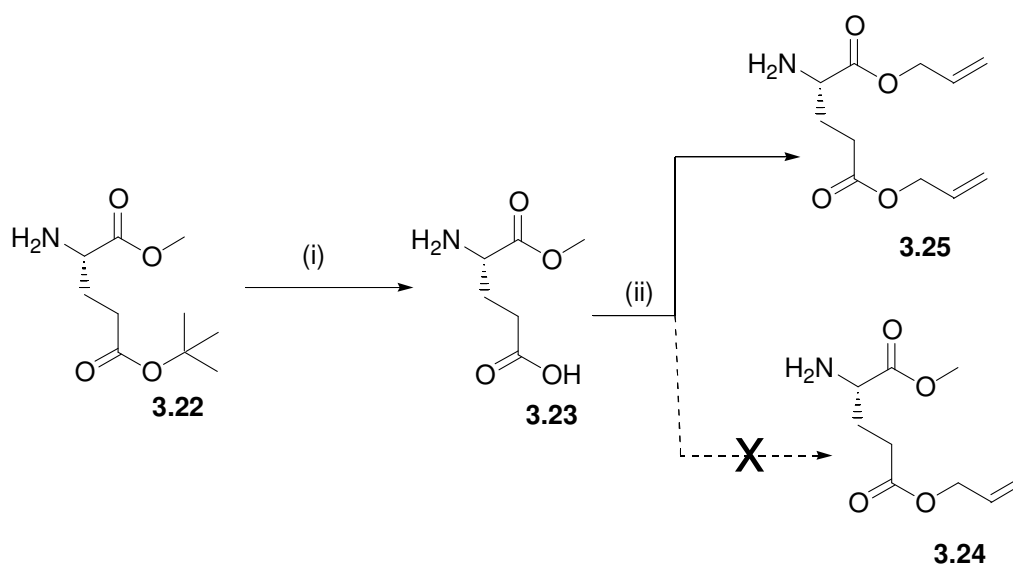
Scheme 3.4 *Reagents and conditions:* (i) allyl alcohol, ethyl chloroformate, Et₃N, ether 0⁰C (ii) allyl palladium chloride, PPh3, THF (21%) (iii) 2M NaOH$_{(aq)}$, H₂O/MeOH, THF (82%), (iv) (S)-Val-OMe.HCl, HATU, DIPEA, DMF (73%) (v) 2M NaOH$_{(aq)}$, H₂O/MeOH, THF (71%) (vi) (S)-allyl-Cys-OMe **3.35**, DIPEA, DMF (54%)

3.3 Synthesis of N- Boc-O-allyl-Glu-Val-S-ally Cys-OMe 3.34

The allylation on the side chain of L-glutamic acid was attempted as shown in **Scheme 3.5**. The t-butyl ester from (L)-H-Glu-(OtBu)OMe **3.22** was removed under acidic condition by the treatment of 2M HCl in diethyl ether to give H-Glu-OMe **3.23** 98% yield.

The attempted synthesis of H-Glu-(allyl ester) OMe **3.24** was carried out by refluxing compound **3.23** with para-toluene sulfonic acid and allyl alcohol in benzene using Dean-Stark trap.[2] Unfortunately, transesterification occurred and compound **3.25** was isolated instead of compound **3.24**.
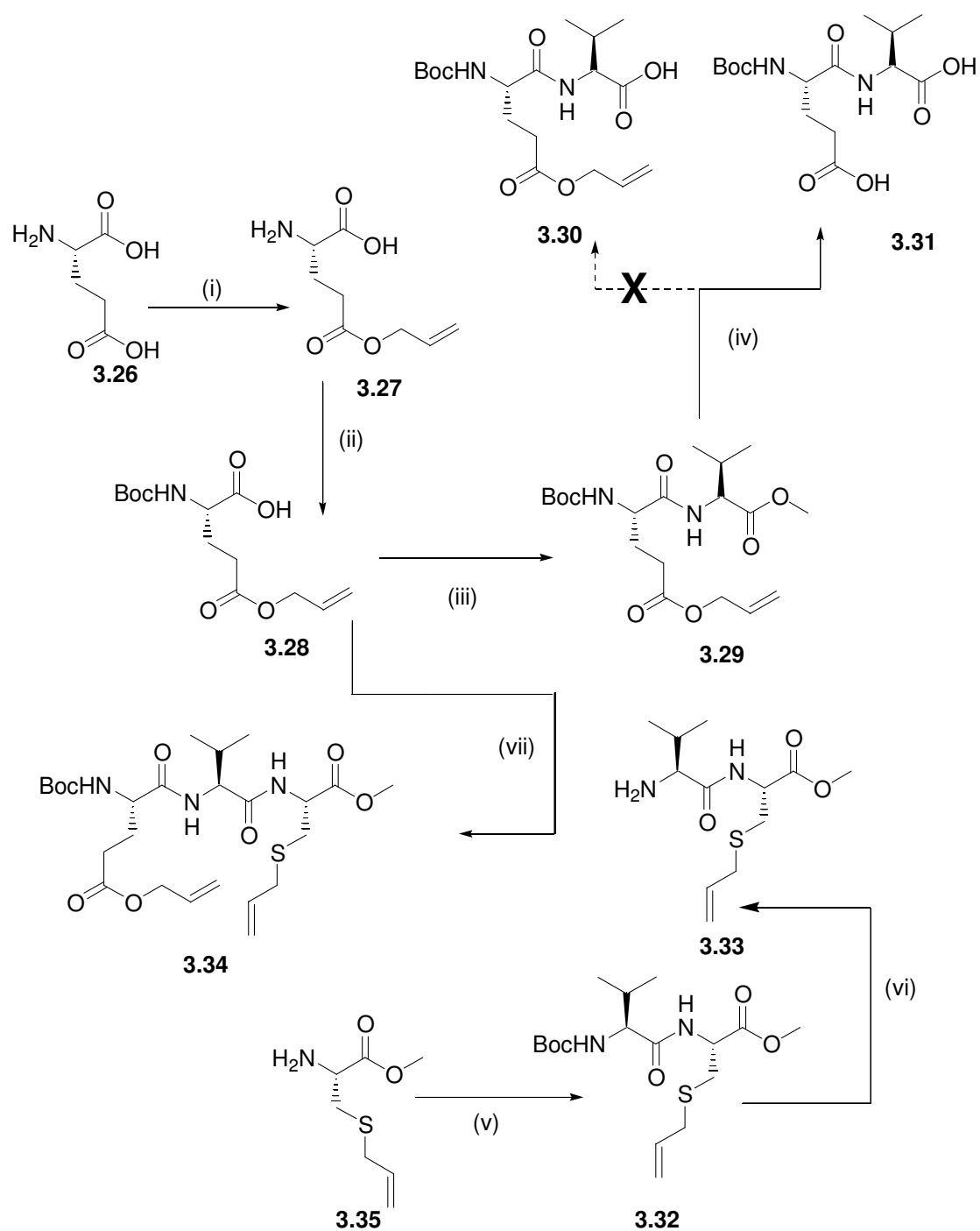

Scheme 3.5: *Reagents and conditions:* (i) 2M HCl diethyl ether (98%), (ii) para-toluene sulfonic acid, benzene, allyl alcohol, reflux 15h (15%)

A new approach was carried out attempted using a different literature method[3] and this successfully gave compound **3.27** (**Scheme 3.6**). As shown in **Scheme 3.6** (L)-glutamic acid **3.26** was selectively treated with chlorotrimethylsilane, allyl alcohol and purified by recrystallization with diethyl ether at 0° C to give the desired compound **3.27** in 77% yield. The N-terminus of **3.27** was then protected with di-tert-butyl dicarbonate under Schotten Bauman's conditions to give compound **3.28** in 80% yield. Next di-peptide **3.29** was synthesised by coupled compound **3.28** to (S)-Val-OMe.HCl with HATU under basic conditions. Purification by column chromatography gave compound **3.29** in 50% yield. Attempts to hydrolyse methyl ester **3.29** using sodium hydroxide, methanol and water to give **3.30** were unsuccessful. Characterisation of the hydrolysed compound by ^{1}H NMR (500 MHz) illustrated that both the allyl ester and the methyl ester groups had been removed.

The characterizations were carried out further by ^{13}C NMR (75 MHz) and Low Resolution Mass spectrometry. The ^{13}C NMR (75 MHz) illustrates missing peaks of the allyl group at δ 131.9, 117.9, 64.9 and the methyl ester peaks at and 51.7. The LRMS showed the presence of [ES$^+$ M + [H$^+$] found 347.2]. Consequently these characterizations suggested that the hydrolysis of dipeptide **3.29** have resulted in compound **3.31** where both allyl ester and methyl ester groups were being hydrolysed.

This problem was overcome by changing the order of the synthetic steps. In particular, **3.28** was coupled to H-Val-allyl-Cys-OMe **3.33** using HATU under basic conditions to give the desired diene **3.34** in 40% yield after purification by column chromatography. Dipeptide **3.33** was synthesised from coupling (S)-N-Boc-Val-OH.HCl to give **3.32** (88% yield after purification by column chromatography). Then Boc protecting group was removed by treatment with 4M HCl in dioxane yielding 97% of compound **3.33**.

Scheme 3.6 *Reagents and conditions:* (i) Chlorotrimethylsilane, allyl alcohol, 0° C diethyl ether (77%), (ii) Boc₂O, Et₃N, H₂O/Dioxane (80%), (iii) (S)-Val-(OMe), HATU, DIPEA, DMF, (62%) (iv) NaOH, MeOH/H₂O, THF (76%), (v) (S)-Boc-Val-OH.HCl, HATU, DIPEA, DMF (88%), (vi) MeOH, SOCl₂ (97%) (vii) Boc-Allyl-Glu-OH **3.28**, HATU, DIPEA, DMF (38%)

Several attempts were made to remove the Boc protecting group from the N-terminus of compound **3.11** (**Scheme 3.7**). The first was to use 4M HCl in dioxane. This resulted a mixture of compounds **3.36** and **3.37**. This is most likely due to traces of water in the dioxane, thus resulting in the formation of concentrated aqueous HCl. The second method attempted was the used of 2M HCl in diethyl ether resulting in the mixture of the starting material **3.11** and an ester hydrolysis. The desired compound **3.35** was achieved by retreating this mixture with thionyl chloride in methanol in 79% yield. This treatment generated HCl *in situ* which allowed the Boc protecting group to be removed while at the same time re-esterifying the acid group.
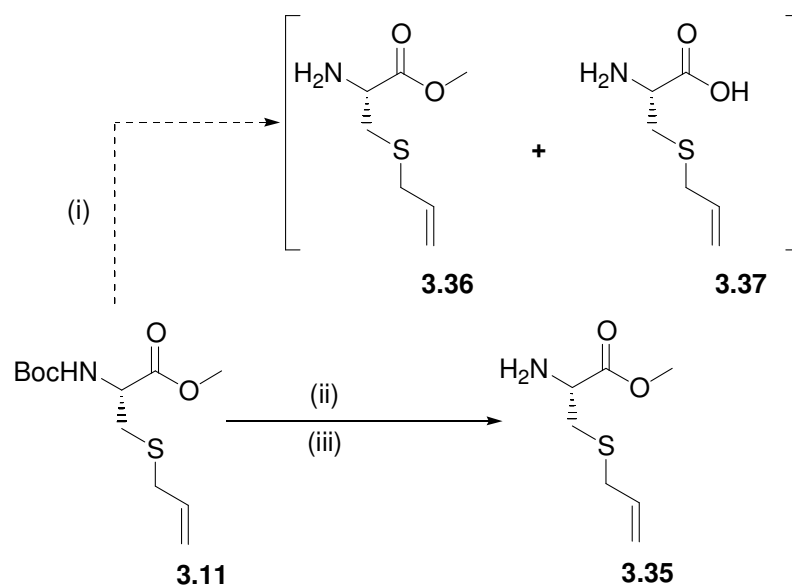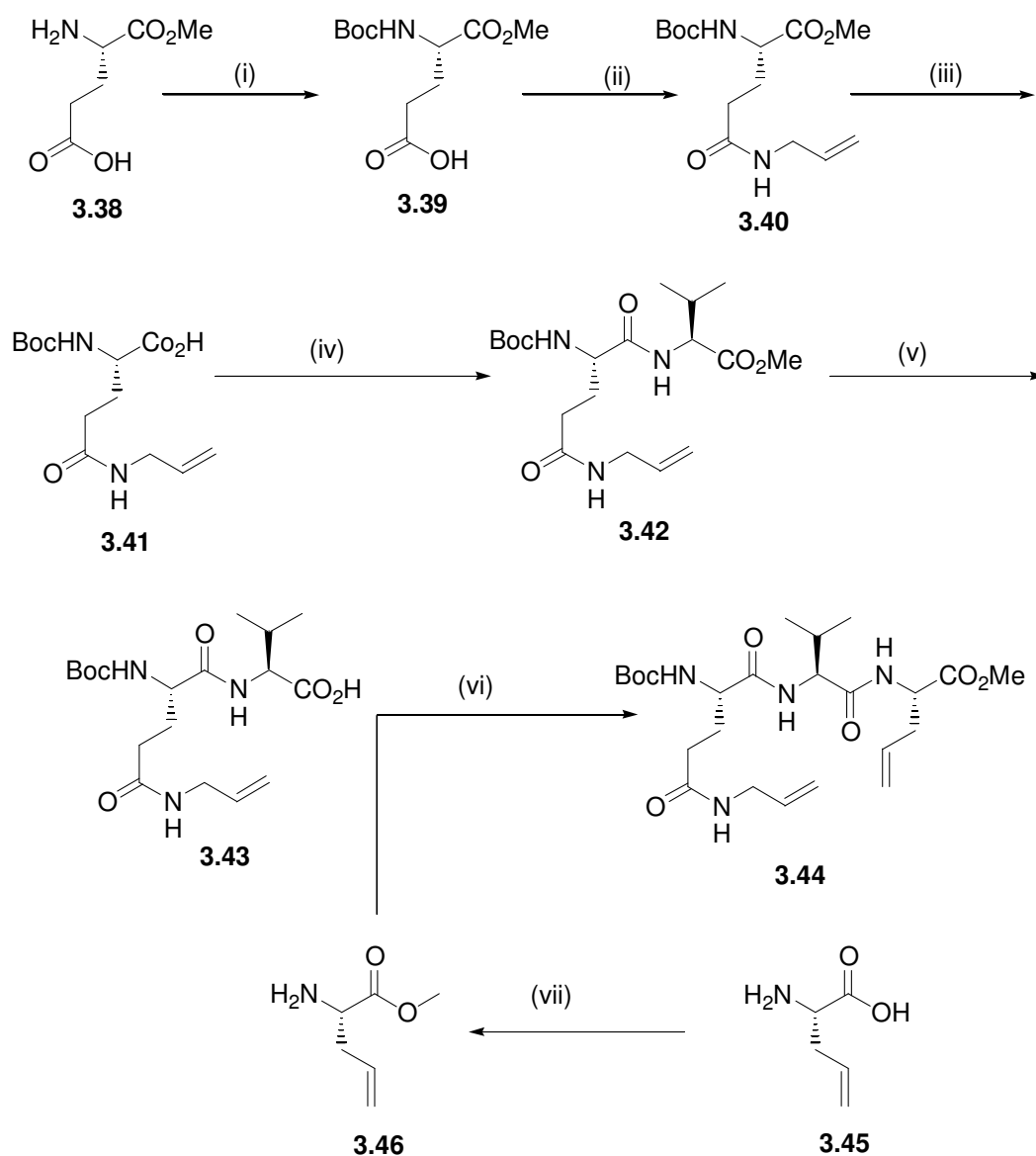

Scheme 3.7 *Reagents and conditions:* (i) 4M HCl in dioxane (ii) 2M HCl in diethyl ether (iii) MeOH, SOCl$_2$ 0°C (79%).

3.4   Synthesis of N-Boc-N-allyl-Gln-Val-allyl-Gly-OMe 3.44

The N-terminus of H-Glu-OMe **3.38** was protected on treatment of **3.38** with di-tertiary-butyl-dicarbonate under Schotten Bauman's condition to give compound **3.39** 79% yield after purification by column chromatography (**Scheme 3.8**). Compound **3.39** was then coupled to ally amine using coupling reagents EDCI, HOAT under basic conditions. Purification by column chromatography over silica gel-based gave **3.40** (55% yield). Then methyl ester of **3.40** was hydrolysed by treatment with sodium hydroxide, methanol/water to give compound **3.41** (73% yield). Di-peptide **3.42** was prepared by coupling **3.41** to (S)-H-Val-OMe.HCl using HATU and DIPEA to give 52% yield after purification by column chromatography. Subsequently **3.42** was hydrolysed with sodium hydroxide, methanol and water to give the free acid compound **3.43** (70%).

Next, the C-terminus of commercially bought (S)-allyl glycine **3.45** was protected with methyl ester group by the treatment of 10% thionyl chloride in methanol at 0^0 C to give (S)-allyl Gly- OMe **3.46** (100%). The desired tripeptide was achieved by coupling between compound **3.43** and **3.46** using standard coupling reagent HATU with hindered base DIPEA. Purification by silica gel-based column chromatography gave compound **3.44** (34% yield).

Scheme 3.8 *Reagents and conditions:* (i) Boc$_2$O, Et$_3$N, Dioxane/H$_2$O (79%)[3] (ii) allyl amine, EDC.HCl, HOAT, DIPEA, DMF (55%) (iii) NaOH, H$_2$O, THF (73%) (iv) Val-OMe.HCl, HATU, DIPEA, DMF (52%) (v) NaOH, MeOH, H$_2$O, THF (70%) (vi) (S) allyl-Gly-OMe, HATU, DIPEA, DMF (34%) (vii) MeOH, SOCl$_2$ 0°C (100%)

3.5   Synthesis of N-Cbz-N-allyl-Gln-Val-allyl-Gly-OMe 3.52

Tripeptide **3.52** was synthesised as shown in **Scheme 3.9**. The amino acid **3.48** was prepared by coupling allyl amine to N-Cbz-Glu-OMe **3.47** using standard coupling reagents EDCI and HOBT under basic conditions in 88% yield after purification by column chromatography. Treatment of **3.48** with sodium hydroxide, methanol and water gave the amino acid **3.49** in 76% yield. Next **3.49** was coupled to (S)-H-Val-OMe.HCl with the treatment of coupling reagent HATU and hindered base DIPEA to give compound **3.50** (80%) after purification by column chromatography. Compound **3.50** was then hydrolysed to give dipeptide **3.51** (41%). Coupling between dipeptide **3.51** and (S)-ally-Gly-OMe **3.46** with HATU and DIPEA resulted in an insoluble product possibly due to the present of the four amide bonds. A white solid formed during work-up in both of the EtOAC and 1M HCl aqueous layers. These white solids were collected using Buchner funnel, characterised by the ^{1}H, ^{13}C NMR and mass spectrometry giving the desired compound **3.52** in 83% yield.
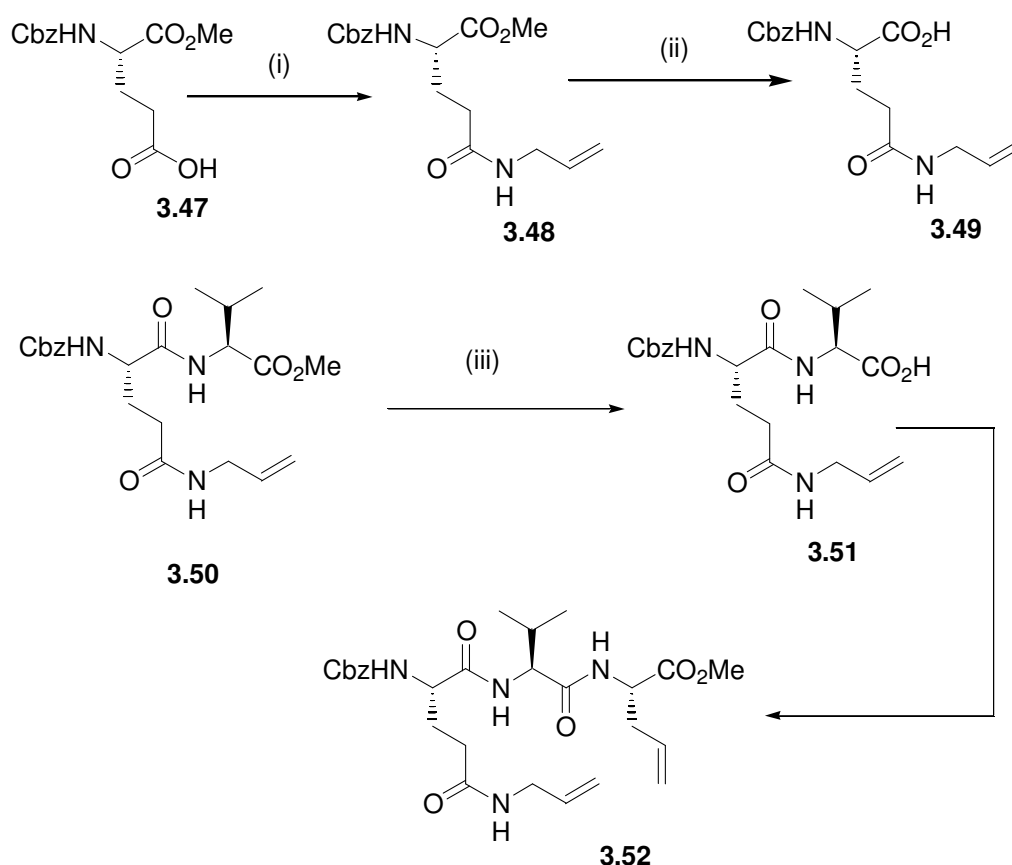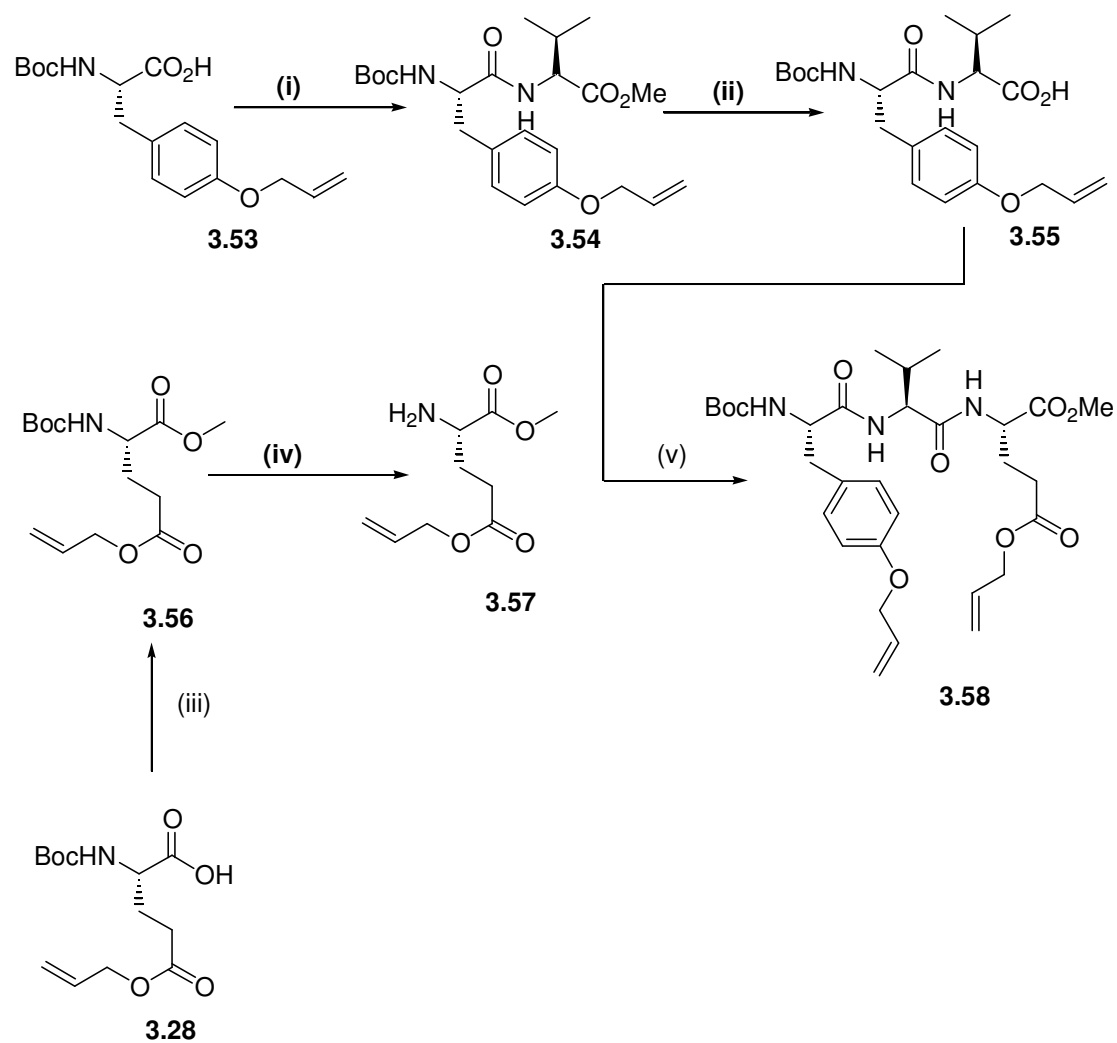
Scheme 3.9 *Reagents and conditions:* **(i))** allyl amine, EDC.HCl, HOBT, DIPEA, DMF (88%) **(ii)** NaOH, H$_2$O, THF (76%) **(iii)** Val-OMe.HCl, HATU, DIPEA, DMF (80%) **(iv)** NaOH, MeOH, H$_2$O, THF (41%) **(v)** (S)-allyl-Gly-OMe **3.46**, HATU, DIPEA, DMF (83%)

3.6   Synthesis of N-Boc-O-allyl-Tyr-Val-O-allyl-Glu-OMe 3.58

N-Boc-O-allyl-Tyr **3.53** was coupled to (S)-Val-OMe.HCl using standard coupling procedure HATU under basic conditions to give compound **3.54** (40%) **Scheme 3.10**. Treatment of compound **3.54** with sodium hydroxide, methanol and water gave compound **3.55** in 78% yield. Next compound **3.56** was prepared by treating N-Boc-O-allyl-Glu-OH **3.28** with diazomethane in diethyl ether at 0^0 C giving **3.56** in 93% yield. Then the N-terminus of **3.56** was removed by the treatment of 2M hydrochloric acid in diethyl ether giving compound **3.57** 100% yield. The desired tripeptide **3.58**

was achieved by coupling dipeptide **3.55** with **3.57** using HATU and DIPEA in an isolated 50% yield.

Scheme 3.10 *Reagents and conditions:* (i) (L)-Val-OMe.HCl, HATU, DIPEA, DMF (40%), (ii) NaOH, H₂O, THF (78%) **(iii)** CH₂N₂, Et₂O 0⁰C (60%) **(iv)** 2M HCl in diethyl ether (100%) **(v)** HATU, DIPEA, DMF (50%)

was achieved by coupling dipeptide **3.55** with **3.57** using HATU and DIPEA in an isolated 50% yield.

Scheme 3.10 *Reagents and conditions:* (i) (L)-Val-OMe.HCl, HATU, DIPEA, DMF (40%), (ii) NaOH, H_2O, THF (78%) **(iii)** CH_2N_2, Et_2O 0^0C (60%) **(iv)** 2M HCl in diethyl ether (100%) **(v)** HATU, DIPEA, DMF (50%)

3.7   Synthesis of 4-Fluoro-O-allyl-Tyr-Leu-ally Gly-OMe 3.64

The desired Tri-peptide **3.64** was synthesised as shown in **Scheme 3.11**. N-Boc-O-allyl-Tyr-OH **3.59** was coupled to (S)-Leu-OMe.HCl using the standard coupling procedure HATU under basic conditions to give dipeptide **3.60** in 59% yield (after purification by column chromatography). The 4-Fluoro-O-allyl-Tyr-Leu-OMe **3.62** was synthesised by coupling with compound **3.61** (prepared by treated compound **3.60** with 4M hydrochloric acid in 1,4-dioxane (100% yield) to 4-fluoro-benzene sulfonyl chloride under basic conditions. Purification via silica-gel based column chromatography gave **3.62** in 61% yield. This was then hydrolysed by the treatment of sodium hydroxide methanol and water to give compound **3.63** (94% yield). Next dipeptide **3.63** was coupled to (S)-allyl-Gly-OMe **3.46** using HATU and hindered base DIPEA to give the desired tripeptide **3.64** (75% yield after silica-gel based column chromatography).
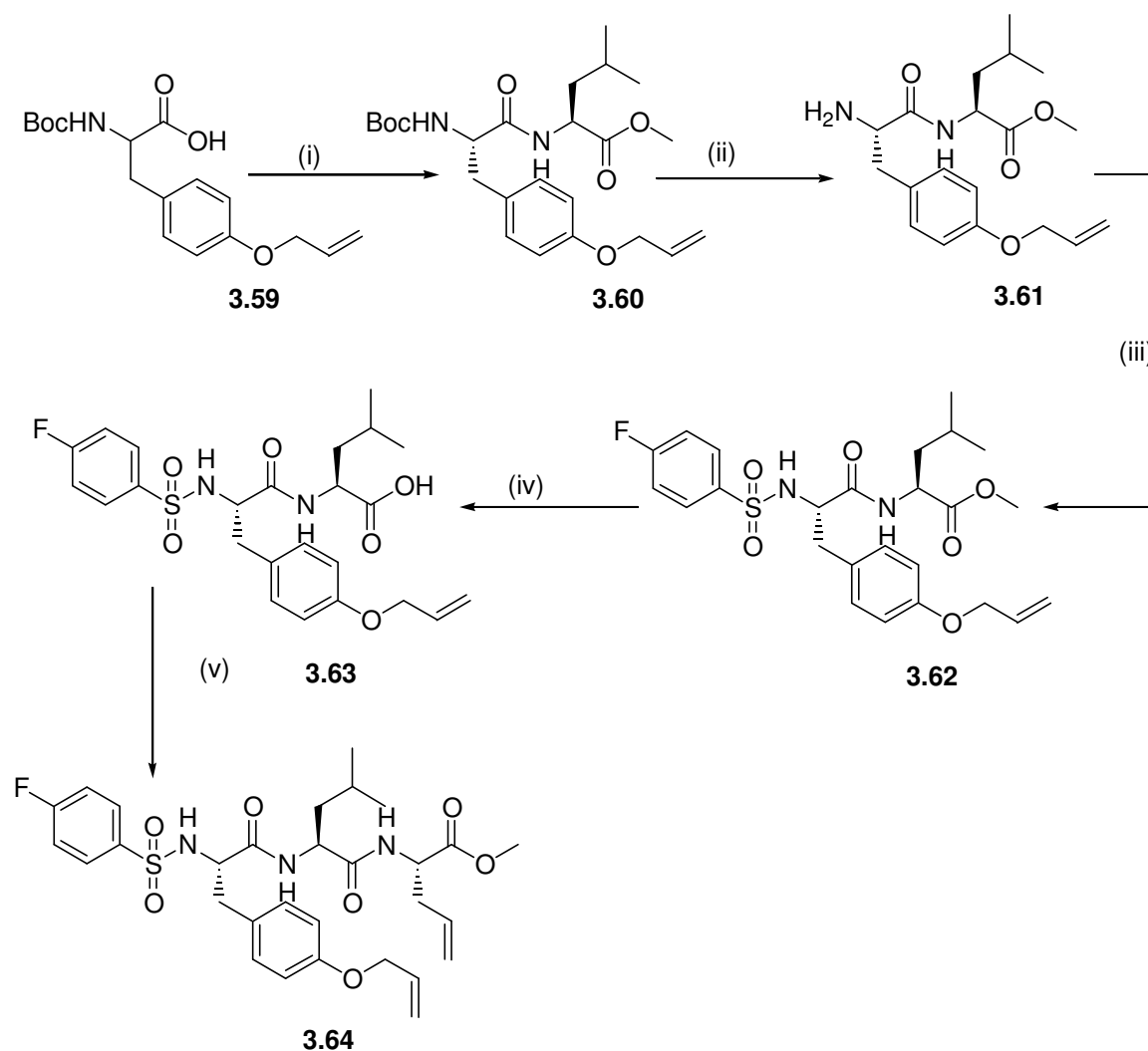
Scheme 3.11 *Reagents and conditions:* (i) (S)-Leu-OMe.HCl, HATU, DIPEA (59%), (ii) 4M.HCl in dioxane (100%), (iii) 4-Fluoro-Benzene Sulphonyl Chloride , DIPEA, DCM (61%), (iv) NaOH, MeOH/H$_2$O, THF (94%), (v) S-allyl-Gly-OMe **3.46**, HATU, DIPEA, (75%)

3.8   SUMMARY OF CHAPTER THREE

The target tri-peptide β-backbone **3.15, 3.21, 3.34, 3.44, 3.52, 3.58, 3.64** were successfully synthesised as summarised in **Table 3.1**.

Table 3.1: Summary of % yield syntheses of Diene

Compounds	Yield (%)
3.15	65
3.21	54
3.34	40
3.44	30
3.52	83
3.58	50
3.64	75

The uronium salt HATU coupling reagent was chosen over the other uronium or phosphonium reagents (HBTU, HCTU, HDTU and PyAOP) due to its high yielding reactions, faster reaction times and minimisation of the loss of chiral integrity.[4] However in some coupling reaction the use of HATU reagent did not give the effective coupling yields (See **Table 3.1 - 3.4** and **3.44**). Perhaps it might be necessary to explore the use of other coupling reagents in order to enhance the results efficiency. Carpino *et al.* have illustrated that another class of peptide coupling reagents 'organophosphorous ester' (For example; phosphate esters such as DepODhbt, DepOBt or phosphinyl esters such as DtpOAt) appears to be more reactive over the other coupling reagents.[5,4] Consequently these new classes of coupling reagents are likely to be employed with the anticipation that they would offer better coupling yields whilst maintaining the chirality of the amino acid.

3.9   REFERENCES FOR CHAPTER 3:

(1)   Kazmaier, U.; Maier, S.; Zumpe, F., L *Synlett* **2000**, *11*, 1523-1535.

(2)   Xia, Z.; Smith, C. D. *J.Org.Chem* **2001**, *66*, 5241-5244.

(3)   Webster. K, L.; Maude. A, B.; M.E;, O. D.; Mehrotra. A, P.; D, G. *J.Chem.Soc.,Perkin Trans* **2001**, *1*, 1673-1695.

(4)   Albericio.F *Current Opinion in Chemical Biology* **2004**, *8*, 211-221.

(5)   Carpino.L, A.; Xia.J;; Zhang, C.; A, E.-F. *J.Org.Chem* **2004**, *69*, 62-71.

The terminal side chains of tripeptide dienes **3.15**, **3.21**, **3.34**, **3.44**, **3.52**, **3.58**, **3.64** (**Chapter 3**) under went RCM to successfully yield the ring closed macrocyclic products **2.4**, **2.5**, **2.6**, **2.7**, and **4.6**. RCM was carried out using Grubbs second generation catalyst (**2.12**) with microwave irradiation as the heat source (1200W). The use of microwave irridation is a relatively new technique for especially within our group.[1] Furthermore the application of RCM for the synthesis of conformationally constrained peptidomimetic compounds is still a largely unexplored area (**section 2.2**). Therefore one of the key objectives in this part of the work was to optimise the RCM reaction conditions via microwave irradiation to provide a standard methodology for the synthesis of conformationally constrained peptidomimetics. As described in section **1.3.2** and **1.4** peptidomimetics conformationally constrained into the 'bio-active' conformation are very attractive synthetic targets and the use of RCM is a very attractive method uses to synthesis such conformation. Therefore the development of efficient methods for their synthesis is a very valuable and worthwhile explore.

In this thesis we now report the development of new RCM reaction conditions using microwave irradiation for the cyclisation of peptide based diene precursors such as those described in **Chapter 3**.

Two different reaction conditions were developed depending on the RCM substrate. One problem which is frequently occur when ring closing is applied to peptide based dienes, is the ability of substrate able to form catalyst deactivating six-membered chelate during the catalytic cycle (**section 4.3**). The combination of microwave irradiation and addition of a Lewis acid (chlorodicyclohexylborane) is required to tackle such chelation problem. Consequently these reaction conditions then result in a productive RCM reaction. Without the addition of Lewis acid, there is no reaction occurs and the diene can be recovered from the reaction mixture or one of diene participate in chelate formation and the other reacts with a complimentary olefin to in a intermolecular cross metathesis reaction. Therefore neither the desired ring closed product is obtained nor the diene starting material recovered.

The peptide based dienes which are unable to form the chelate can be successfully ring closed without the need to add a Lewis acid. The use of microwave irradiation is still required to obtain optimal yields with more efficient time.

4.1   SYNTHESIS OF 15-MEMBERED CYSTIENE AND SERINE BASED MACROCYCLES (2.5 AND 2.6)

4.1.1   N-Boc-Cys-Val-Cys-Macrocycle-OMe (2.5)

Tripeptide **3.15** undergoes RCM using Grubbs second generation catalyst **2.12** in 1,1,2-trichloroethane under microwave conditions for 30 min to yield macrocycle **4.1** in an isolated yield of 33% (unoptimised) **Scheme 4.1.1**.

The structure of macrocycle **4.1** was elucidated by proton NMR and mass spectrometry. The proton NMR showed the absence of multiplets (corresponding to 2 x **CH=CH$_2$** protons) at 5.7ppm and multiplets at 5.2ppm (corresponding to 2 x CH=CH$_2$ protons) from the diene **3.15**. These protons are replaced by multiplets at 5.4-5.46ppm representing a new **CH=CH** bond, existing in the same regions due to the symmetrical characters of the compound.

The *E/Z* isomers of **4.1** were not separated and the compound was obtained in a mixture of *E/Z* geometric isomers. The COSY NMR displays overlap peaks of the α protons (Cys) 4.82ppm. The overlapped α protons (Cys) were coupled to two different NH at 7.07ppm and 7.35ppm thus suggested the present of such mixture isomers. These two NH peaks are exists in the ratio of 1:2. However it was not possible to identify which peaks represent the *E/Z* isomers and as such it is not possible to identify which isomer is the major isomer obtained.

Mass spectrometry confirmed the presence of desired product (LRMS [H^+] of **4.1** is 490.6 found 490.3).

Attempts to hydrogenate the resultant carbon-carbon double bond using 10% palladium on carbon with methanol as solvent, under a hydrogen atmosphere, were unsuccessful, with starting material **4.1** being re-isolated quantitatively. The reason for this was probably the lack of solubility of compound **4.1** in methanol.

The recovered starting material was resubjected to hydrogenation using a methanol/dichloromethane mixture (1:1). This achieved the desired homogenous solution and yielded the desired saturated macrocycle **2.5** in 95% isolated yield.

The structure of macrocycle **2.5** was characterised by NMR and mass spectrometry. The proton NMR displays missing **CH=CH** peak at 5.35-5.5ppm in **4.1**. These **CH=CH** peak were replaced by **CH$_2$-CH$_2$** peaks at 2.4-2.6ppm and 1.65-1.7ppm. Mass spectrometry confirmed the correct mass of **2.5** (LRMS [H^+] **2.5** $C_{21}H_{37}N_3O_6S_2$ 492.6 found 492.3).
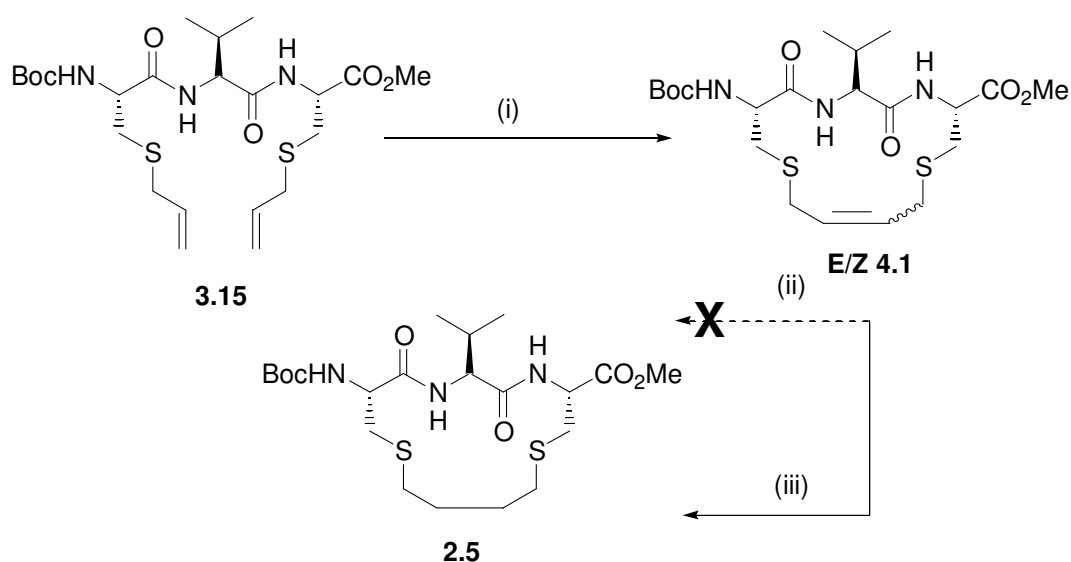
Scheme 4.1.1: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, microwave 1200W, 1,1,2-TCE 30min (33%) (ii) 10%Pd/C, MeOH, H$_2$ (0%), (iii) 10%Pd/C, (1/1) MeOH/DCM, H$_2$ (95%)

4.1.2   Synthesis of N-Boc-Ser-Cys-Val-OMe (2.6)

Tripeptide **3.21** underwent RCM using Grubbs second generation catalyst **2.12** in 1,1,2-trichloroethane under microwave conditions for 30 mins to yield macrocycle **4.2** in an isolated yield of 14% (unoptimised) **Scheme 4.1.2**.

The structure of macrocycle **4.2** was elucidated by proton NMR and mass spectrometry. The proton NMR showed the absence of multiplets corresponding to $CH=CH_2$ protons at 5.78ppm, 5.85ppm and multiplets at 5.15-5.23ppm corresponding to 2 x $CH=CH_2$ protons from the diene **3.21**.These protons are replaced by multiplets at 5.46ppm and 5.62ppm corresponding to the new $CH=CH$ protons.

The *E/Z* isomers of **4.2** were not separated and the compound was obtained in a mixture of *E/Z* geometric isomers. The proton NMR shows splitting peaks from the Boc group at 1.4-1.5ppm suggested the present of such mixture isomers. These peaks have ratio of 1:3. However it was not possible to identify which peaks represent the *E/Z* isomers and as such it is not possible to identify which isomer is the major isomer obtained.

Mass spectrometry confirmed the presence of desired product **4.2** (LRMS [H$^+$] of **4.2** is 474.6 found 474.5).

The resultant carbon-carbon double bond in **4.2** was hydrogenated using 10% palladium on carbon under a hydrogen atmosphere in (1/1) methanol/dichloromethane solvent to give the desired macrocycle **2.6** in 53% yield.

The structure of macrocycle **2.6** was characterised by NMR and mass spectrometry. The proton NMR displays missing $CH=CH$ 5.46ppm and 5.62ppm in **4.2**. The $CH=CH$ peak were replaced by CH_2-CH_2 peaks at 2.5 and 1.6ppm. Mass

spectrometry confirmed the correct mass of **2.6** (LRMS [ES$^+$ M + [H$^+$] **2.6** Calcd for C$_{21}$H$_{37}$N$_3$O$_7$S 476.6 found 476.2).
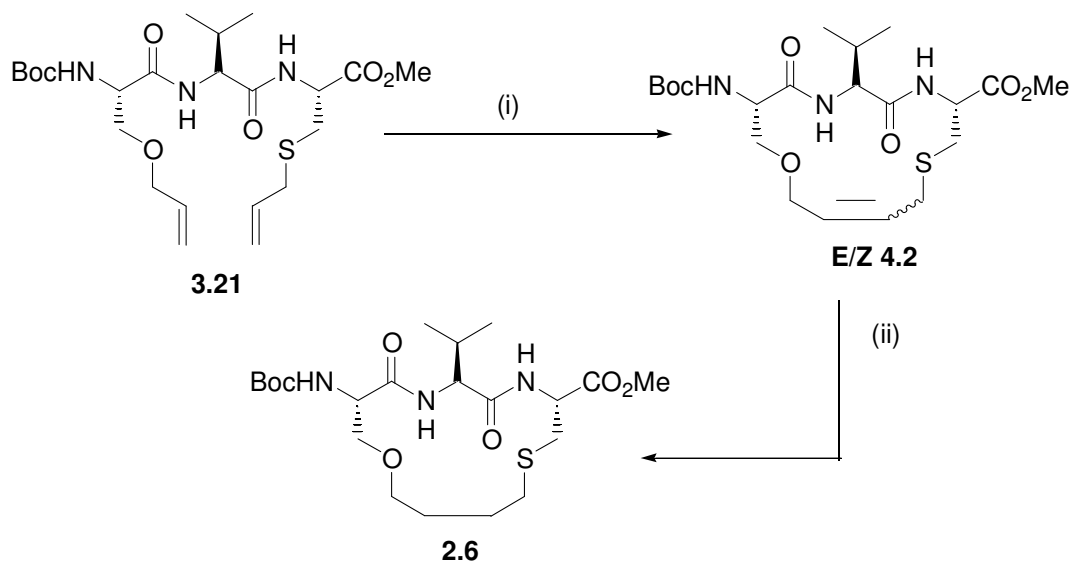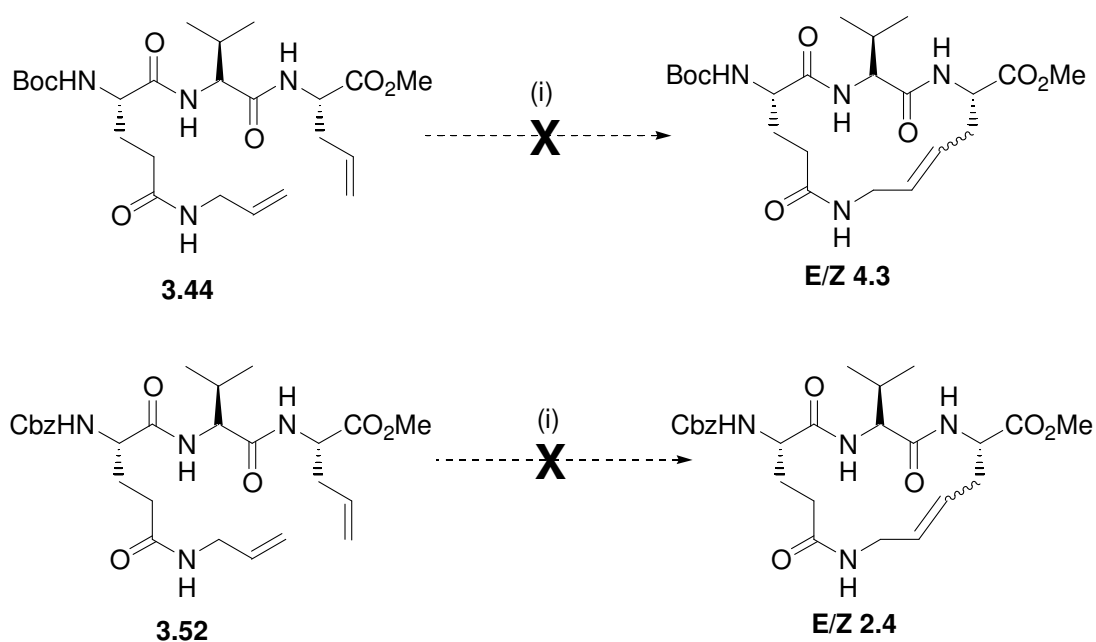

Scheme 4.1.2: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, microwave 1200W, 1,1,2-TCE (14%) (ii) 10% Pd/C, (1/1) MeOH/DCM, H$_2$ (53%)

4.2   ATTEMPTED TO SYNTHESIS 15-AND 20-MEMBERED RINGS (4.3 AND 2.4)

### 4.2.1 Attempted synthesis of N-Boc-Gln-Val-Gly-ene-Macrocycle-OMe (4.3) and Cbz-Gln-Val-Gly-ene-Macrocycle-OMe (2.4)

Attempts to synthesise 15-membered rings **4.3** and **2.4** are shown in **Scheme 4.2.1**. Tripeptide dienes **3.44** and **3.52** were treated with Grubbs second generation catalyst (**2.12**) under microwave conditions for 30 mins. However the desired macrocycles were not obtained. See the later section (**4.6**) on the effect Lewis addition on RCM for details of how this problem was overcome.

Scheme 4.2.1: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, microwave 1200W, 1,1,2-TCE 30 mins (0%)

4.2.2   Attempted synthesis of 20-membered N-Boc-Tyr-Val-Gly-ene-Macrcycle-OMe (4.5)

The attempted synthesis of the 20-membered ring **4.5** is shown in **Scheme 4.2.2**. The tripeptide diene **3.58** was treated with Grubbs second-generation catalyst **2.12** under microwave conditions for 30 mins. Once again the desired macrocycle cycle was not obtained rather started material was recovered.

3.58

E/Z 4.5
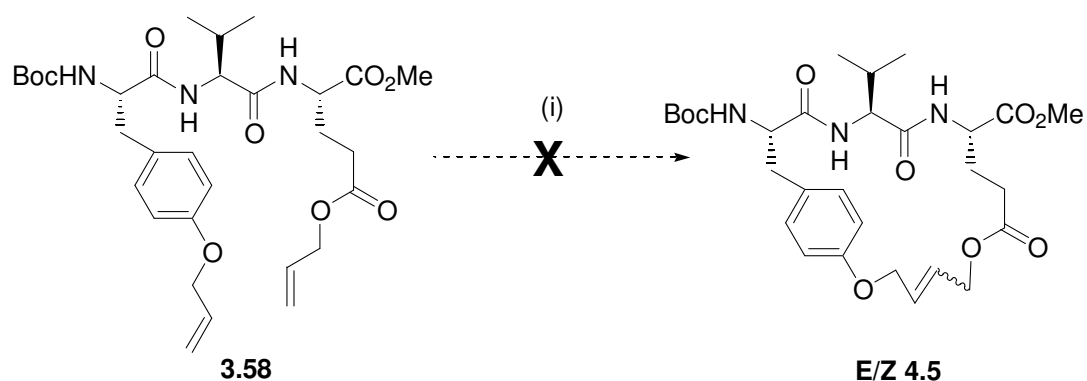
Scheme 4.2.2: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, microwave 1200W, 1,1,2-TCE 30 mins (0%)

4.3 PROPOSED SIX-MEMBERED RING CHELATION

There is a common theme found amongst the three tripeptide dienes **4.3**, **2.4**, **4.5** shown in **Schemes 4.2.1** and **4.2.2** that fail to undergo RCM. All three dienes are capable of forming a six-membered chelate complex with the catalyst during the catalytic cycle. As shown in **Scheme 4.3A** the oxygen from the carbonyl is able to form a six-membered ring with the ruthenium catalyst. The six-membered ring is thermodynamically stable and traps the ruthenium catalyst within the chelation thus preventing the next step in the RCM cycle.[2]
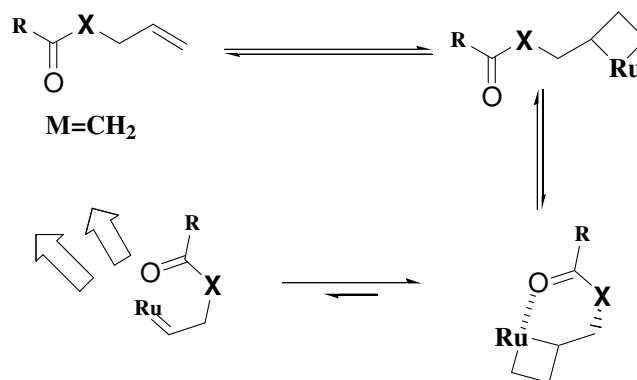
Scheme 4.3A: Formation of six-membered ring via chelation of metal and carbonyl

Rather than the desired RCM reaction this chelation forces the equilibrium driven catalytic cycle to undergo an intermolecular cross metathesis reaction between the only two metathesis active olefins (**Scheme 4.3B**).
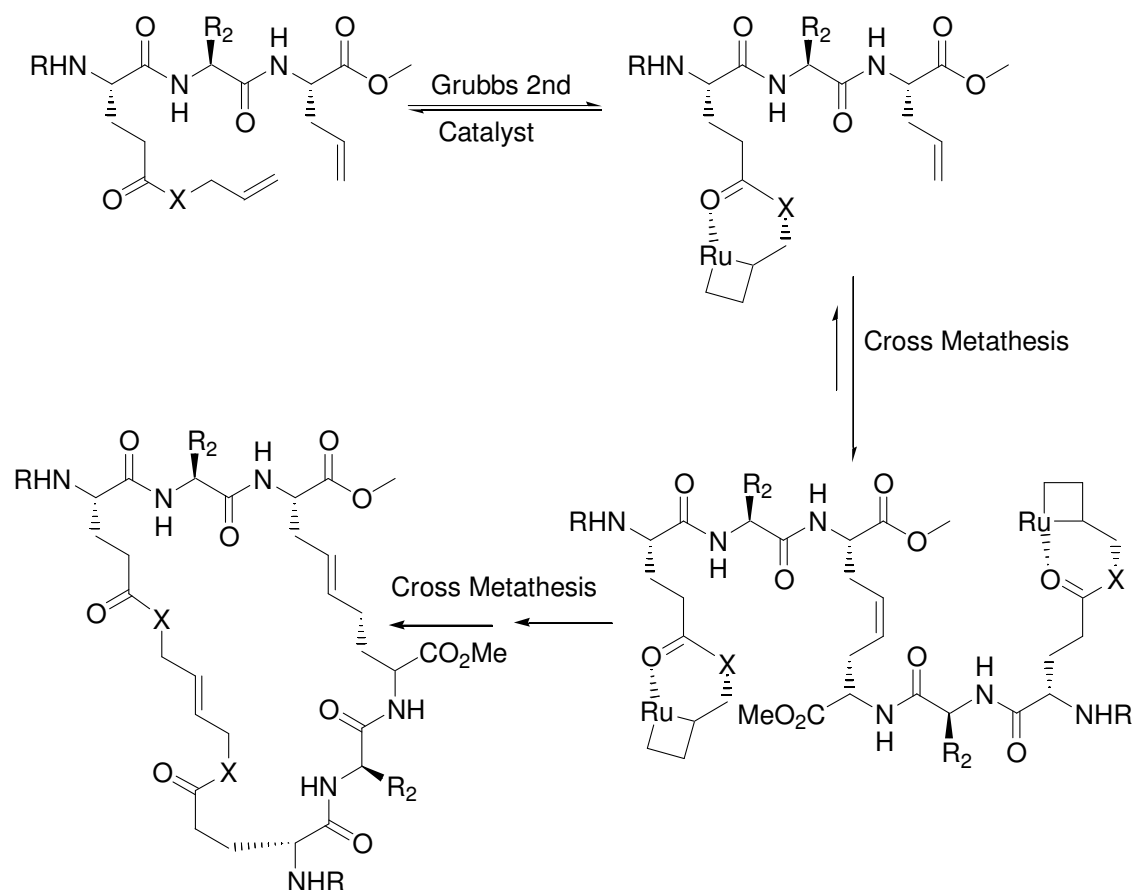

Scheme 4.3B: Intermolecular cross metathesis

The chelation between the heterocyclic atoms to Grubbs catalyst has been shown to occur in cross metathesis systems[3] where the non-productive chelation of the ruthenium carbonyl complexes was obtained.[4] This type of chelation is commonly employed in the cross metathesis to promote selective CM reactions.[3-6] However to the best of our knowledge this effect has not been recognised and applied to RCM reactions.

Several studies in the CM area have been carried out in an attempt to understand this chelation problem. In particular addition of Lewis acids such as $Ti(O^iPr)_4$[7,8,2] has been shown to suppress chelation between the carbonyl and ruthenium catalyst, thus facilitating the cleavage of this chelation. Furthermore it has been shown that the use of $Ti(O^iPr)_4$ as an additive in RCM reactions does not always give a high product yield[2]. This therefore brought us to examine a difference type of Lewis acid for our investigations. We have chosen the boron based Lewis acids rather than titanium or aluminium Lewis acids as additives in RCM reactions to facilitate cleavage of the proposed chelate complex. Our rationale for using boron based Lewis acids came from literature examples indicating that in the CM area boron based Lewis acids had significantly better product yields when compared with titanium or aluminium based Lewis acids.[9]

4.3.1 Application of the proposed six-membered ring chelation

A close examination of the structures of compounds **2.4, 4.3 and 4.5** reveals that the allyl glycine, allyl amide and allyl ester moieties are all capable of forming a six-membered chelate to Grubbs catalyst in the first step of RCM (2+2 cycloadditions) see **Scheme 4.3A**. We proposed in **section 4.3** that these types of compounds have a tendency to form the six-membered chelation to the ruthenium catalyst. Thus, addition of chlorodicyclohexylborane (Lewis acid) to the RCM reaction media would be expected to facilitate the cleavage of the ruthenium chelate, thus increased reaction yields should be observed **Scheme 4.3.1**.
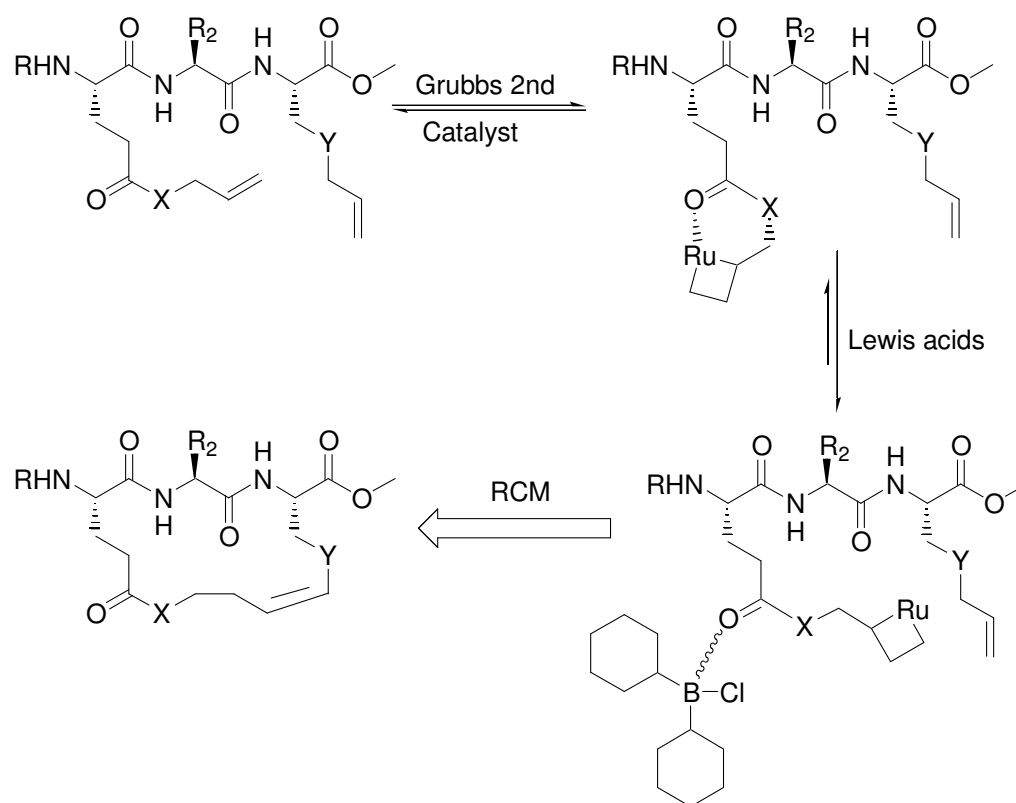
Scheme 4.3.1: Proposed mechanism of chelation of Lewis acid in RCM

The remaining sections in this chapter illustrates the application of chlorodicyclohexylborane (Lewis acid) catalyst on diene systems **3.52**, **3.58** which failed to cyclise under a standard condition earlier in **section 4.2.1** and **4.2.2** to promote productive RCM. The use of chlorodicyclohexylborane (Lewis acid) was also applied to the dienes system **3.34**, **3.64** which are capable to form six-membered ring chelation to see if improved yield could be obtained.

4.4 SYNTHESIS OF 17-MEMBERED MACROCYCLES 2.7 AND 4.6

4.4.1 Synthesis of N-Boc-Glu-Val-Cys-ene-Macrocycle-OMe (2.7)

The tripeptide diene **3.34** undergoes RCM to form a 17-membered ring as shown in **Scheme 4.4.1**. RCM was performed using two different reaction conditions, **3.34** was subjected to RCM using Grubbs second-generation catalyst **2.12** both with and without the addition of the chlorodicyclohexylborane Lewis acid. In both cases the desired macrocycle was isolated. Without the addition of the Lewis acid **2.7** was obtained in a 42% yield and with addition of the Lewis acid this was increased to 50%.

The structure was confirmed by NMR and mass spectrometry. LRMS of **2.7** gave M [H$^+$] at 516.3, while the ^{13}C NMR spectrum showed the absence of the CH=CH$_2$ at 118.5 and 117.9ppm. ^{1}H NMR spectrum also showed the absence of doublets of doublets at 5.1ppm, 5.3ppm (corresponding to (Glu) CH=**CH$_2$** protons and (Cys) CH=**CH$_2$** respectively) and multiplets at 5.7ppm corresponding to the (Glu) **CH**=CH$_2$ proton and at 5.9ppm corresponding to the (Cys) **CH**=CH$_2$ proton from the diene **3.34**. These protons are replaced by two multiplets at 5.6ppm and 5.7ppm which represents the new carbon-carbon doubled bond formed.

The *E/Z* isomer of **2.7** was not separated and the compound was obtained in a mixture of *E/Z* geometric isomers. The COSY NMR displays two different NH (Val) at 6.84ppm and 6.88ppm. which suggested the mixture of such isomers. The two NH protons exist in a ratio of 1:2. However it was not possible to identify which peaks represent the E/Z isomers and as such it is not possible to identify which isomer is the major isomer obtained.
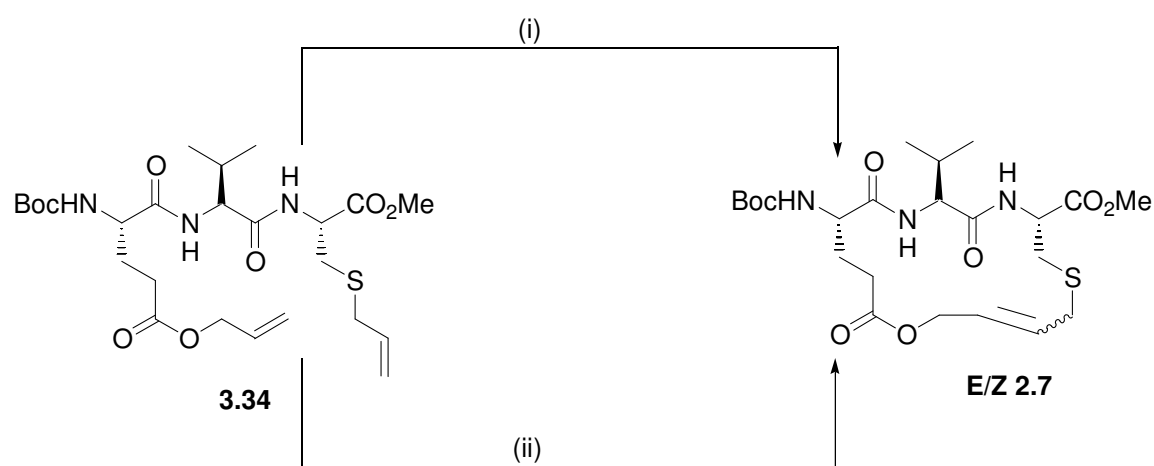
Scheme 4.4.1: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, 1,1,2-TCE, chlorodicyclohexylborane (10%) microwave 1200W, 1h (50%) (ii) Grubbs second generation catalyst **2.12**, 1,1,2-TCE, microwave 1200W, 1h (42%)

4.4.2 Synthesis of 17-membered 4-F-Ph-SO2-N-Tyr-Leu-Gly-ene-Macrocycle-OMe (4.6)

Diene **3.64** was subjected to RCM as shown in **Scheme 4.4.2**. Treatment with Grubbs second-generation catalyst **2.12** with the addition of Lewis acid (chlorodicyclohexylborane) under argon atmosphere afforded the macrocycle **4.6** in an isolated yield 65%.
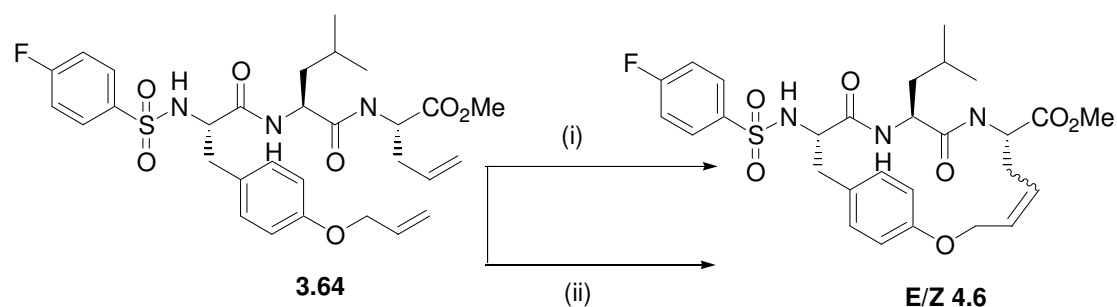
Scheme 4.4.2: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, 1,2,2-TCE, chlorodicyclohexylborane (10%) microwave 1200W, 1h (65%) (ii) Grubbs second generation catalyst **2.12**, 1,1,2-TCE, microwave 1200W, 1h (29%)

The structure of the product was confirmed by NMR and mass spectrometry. ^{1}H NMR spectrum showed the absence of doublets of doublets at 5.15ppm, 5.3ppm (corresponding to **CH=CH₂**) and the two multiplets at 5.7ppm, 6.02ppm (corresponding to CH=**CH₂**) from the diene **3.64**. The two multiplets **CH=CH₂** protons are replaced with the two multiplets at 5.4ppm and 5.8ppm representing the new carbon-carbon double bond.

The *E/Z* isomer of **4.6** was not separated and the compound was obtained in a mixture of *E/Z* geometric isomers. The proton NMR displays two different peaks of NH (Leu) at 7.58ppm and 7.65ppm suggested the present of these isomers. The two peaks are roughly exists in the ratio of 1:15. However it was unable to identify which peaks are representing the E/Z isomers or whether which isomer are the major isomers obtained.

Mass spectrometry also indicated desired product (LRMS M + [H^{+}] is 516.4 found 516.3).

RCM of diene **3.64**was also carried out in the absence of Lewis acid conditions by another group member, Joanna Duncan. The Macrocycle **4.6** was obtained in an isolated yield of 29%.

4.5 ATTEMPTED SYNTHESIS OF 20-MEMBERED N-Boc-Tyr-Val-Gly-ene-Macrcycle-OMe (4.5)

The second attempt to synthesise the 20-membered macrocycle **4.5** was carried out as shown in **Scheme 4.5**. On this occasion RCM of the tripeptide diene **3.58** was attempted using the Chlorodicyclohexylborane Lewis acids conditions to cyclise dienes. The diene was treated with Grubbs second generation catalyst in the presence of 10% Lewis acid (chlorodicyclohexylborane) under microwave conditions for 1h. However this still did not yield the desired macrocycle and instead starting material **3.58** was obtained. The reason for this is postulated to be that the addition of Lewis acid does not promote cleavage of the chelate to a sufficient level to favour the

intramolecular RCM. It can only be concluded that this diene forms a particularly strongly bound chelate relative to other chelate examples outlined in this thesis or there could be other factors involved.
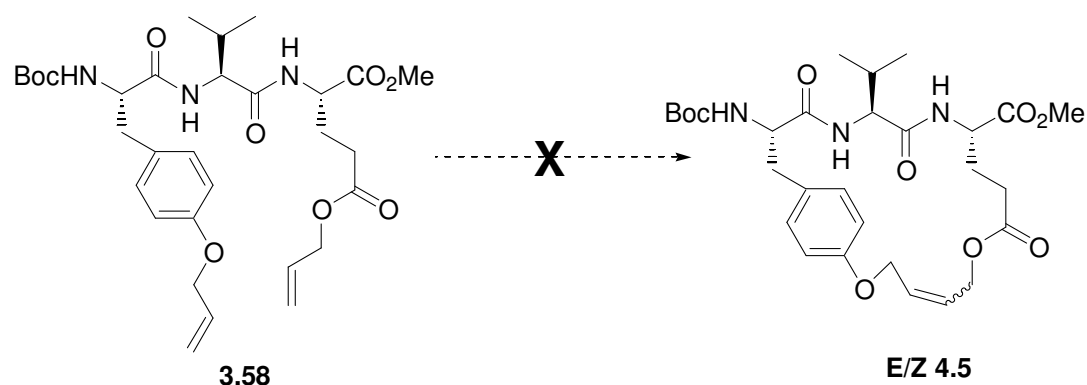
Scheme 4.5: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, chlorodicyclohexylborane (10%), 1,1,2-TCE, microwave 1200W 1h (0%)

## 4.6   SYNTHESIS OF Cbz-Gln-Val-Gly-ene-Macrocycle-OMe (2.4)

The desired macrocycle **2.4** was successfully synthesised as shown in **Scheme 4.6**. The tripeptide diene **3.52** was very polar and hence insoluble in organic solvent. A homogenous solution was not achieved in 1,1,2-TCE, however the suspension was subjected to the optimised RCM conditions. NMR of the crude reaction product indicated presence of desired product by the appearance of the missing diene peaks in **3.52**. The crude product **2.4** was not soluble in any of the following solvents; DCM, EtOAC, MeOH, THF or H$_2$O. Therefore purification using standard forward phase column chromatography was not suitable. Purification of compound **2.5** using reverse-phase chromatography was attempted. However a suitable gradient could not be achieved that showed sufficient separation of the components. Hence purification using reverse-phase chromatography could not be performed.

Another group member Kelly Anderson had previously demonstrated that purification of such polar peptides (tetrapeptide - hexapeptide) could be achieved using trituration. As such crude **2.4** was suspended in methanol, subjected to ultrasound for 15 mins

and then filtered through a grade 1 sinster under vacuum to obtain pure macrocycle **2.4** in 14% isolated yield.

The structure of **2.5** was confirmed using NMR and mass spectrometry. (LRMS M + [H$^+$] is 503.5 found 503.4). ^{1}H NMR shows the absence of multiplets at 5.75ppm and 5.03ppm (corresponding to **CH**=CH$_2$ and CH=**CH$_2$** respectively) from the diene **3.52**. These protons peaks are replaced with two multiplets at 5.23ppm and 5.5ppm representing the new carbon-carbon double bond.

The *E/Z* isomers of **2.4** were not separated and the compound was obtained as a mixture of *E/Z* geometric isomers. The COSY NMR displays two different α protons of Gln at 4.32ppm and 4.40ppm. These protons couple to overlapped NH protons at 8.4ppm thus suggested the mixture of such isomers. The two α protons exist in a ratio of 1:2. However it was not possible to identify which peaks represent the E/Z isomers and as such it is not possible to identify which isomer is the major isomer obtained.
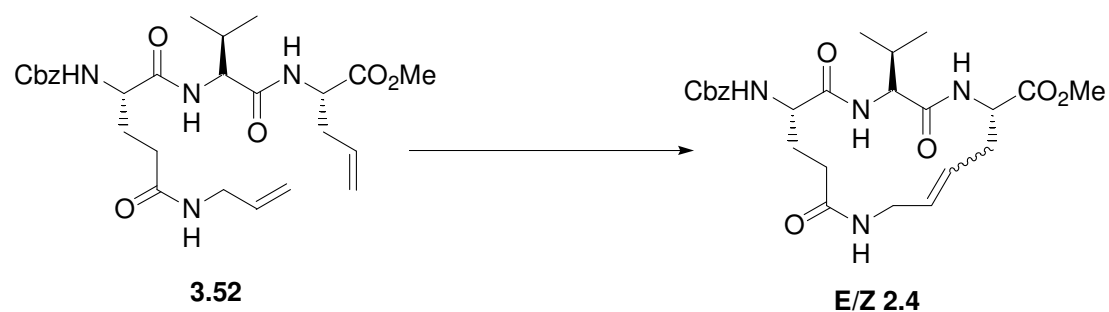
Scheme 4.6: *Reagents and Conditions:* (i) Grubbs second generation catalyst **2.12**, chlorodicyclohexylborane (10%), 1,1,2-TCE, microwave 1200W 1h (14%)

As stated above in section **4.2.1**. RCM of diene **3.52** using standard RCM microwave reaction conditions was unsuccessful. However adding 10% Lewis acid facilitates cleavage of the chelate and hence productive RCM.

4.7 SUMMARY AND FUTURE WORK OF CHAPTER 4

RCM of cysteine and serine based macrocycles **2.5** and **2.6** was achieved albeit in relatively low yields (unoptimised). The reason for low yields with these substrates is due to the fact that the 1,1,2-TCE was not anhydrous and under microwave irradiation conditions a much faster rate of catalyst decomposition is observed. Therefore it is suggested that the RCM reaction of each of these substrates be repeated using the optimised reaction conditions before publication to obtain comparable results.

The effect of using a Lewis acid additive (chlorodicyclohexylborane) on the yield of RCM is summarised in **Table 4.1**. From this it is clear that addition of the Lewis acid is beneficial. In all cases (except compound **4.5**) where the RCM reaction was performed in the presence and absence of the Lewis acid, improved isolated yields were obtained when the Lewis acid was added. Furthermore in the case of macrocycle **2.4** RCM was only achieved with added Lewis acid.

Table 4.1: Results Summary for RCM

| Compounds	% Yield (with Lewis acid)	% Yield (without Lewis acid)
N-Boc-Cys-Val-Cys-ene-Macrocycle-OMe (**2.5**)	Not performed	33%
N-Boc-Ser-Val-Val-Cys-ene-OMe (**2.6**)	Not performed	14%
N-Boc-Gln-Val-Gly-ene-Macrocycle-OMe (**2.3**)	Not performed	0%
N-Boc-Tyr-Val-Gly-ene-Macrocycle-OMe (**4.5**)	0%	0%
4-F-Ph-SO2-N-Tyr-Leu-Gly-ene-Macrocycle-OMe (**4.6**)	65%	29%
N-Boc-Glu-Val-Cys-ene-Macrocycle-OMe (**2.7**)	50%	42%
Cbz-Gln-Val-Gly-ene-Macrocycle-	14%	0%

OMe (**2.4**)

The results of this study strongly support the hypothesis that the use of Lewis acids to promote RCM in systems that contain structural features which can potentially form chelates with the ruthenium catalyst (**section 4.3)** is a valid one.

However such a strategy does not appear to be suitable for all substrates. Attempts to ring close macrocycle **4.5** with and without Lewis acid addition were not successful. This is probably because the chelate is simply too strongly "bonded" for the effect to be negated by the addition of a Lewis acid. Indeed work by another member of the group (Steven Aitken) has demonstrated that the effect of chelate formation is particularly difficult to overcome when the heteroatom presents in both of the diene (**Scheme 4.7A**) as is the case with macrocycle **4.5**
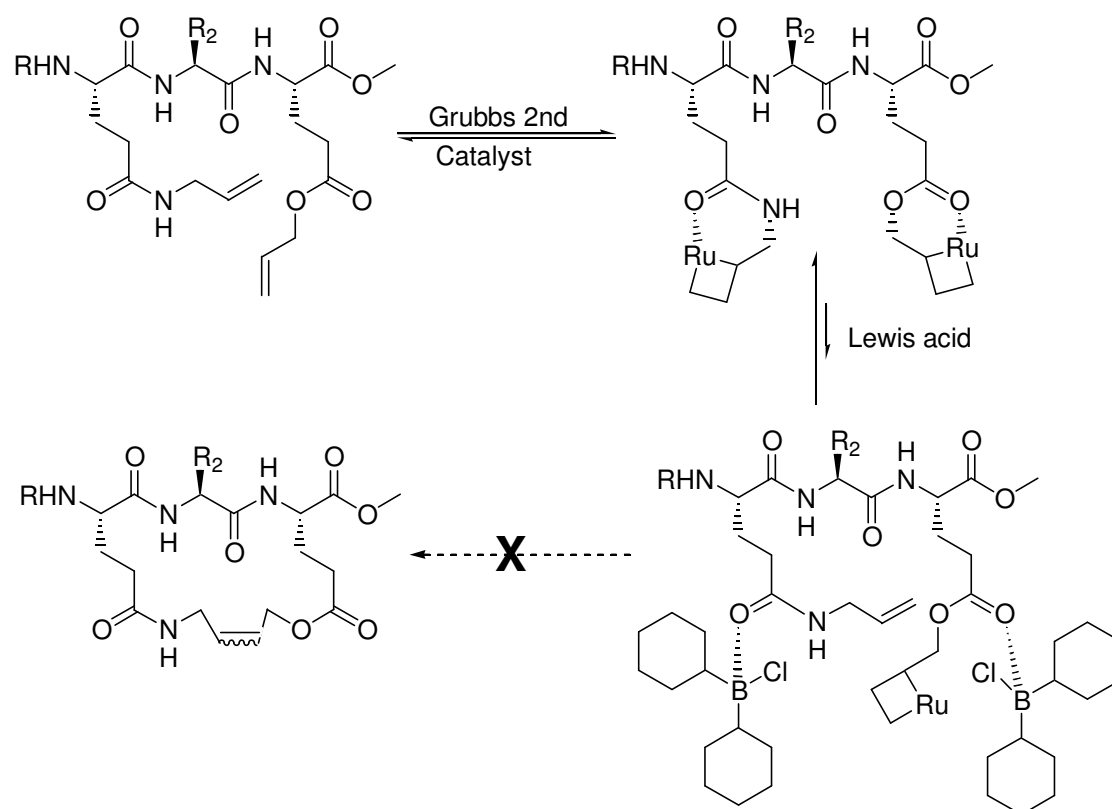
Scheme 4.7A: Formation of six-membered ring chelation where both diene consist of heteroatoms.   .

An alternative strategy to negate this problem, which would allow synthesis of macrocycles of the type **4.5**, would be to employ cross metathesis instead of RCM to synthesise the macrocycle. As shown in **Scheme 4.7B** instead of synthesising a tripeptide diene it should be possible to prepare the required dipeptide olefins, perform CM and then cyclise using an intramolecular peptide coupling to form the desired macrocycle, thus avoiding the potential chelation problems.

$R_1 = CO_2NH$
$R_2 = Val$
$R_3 = CO_2Me$
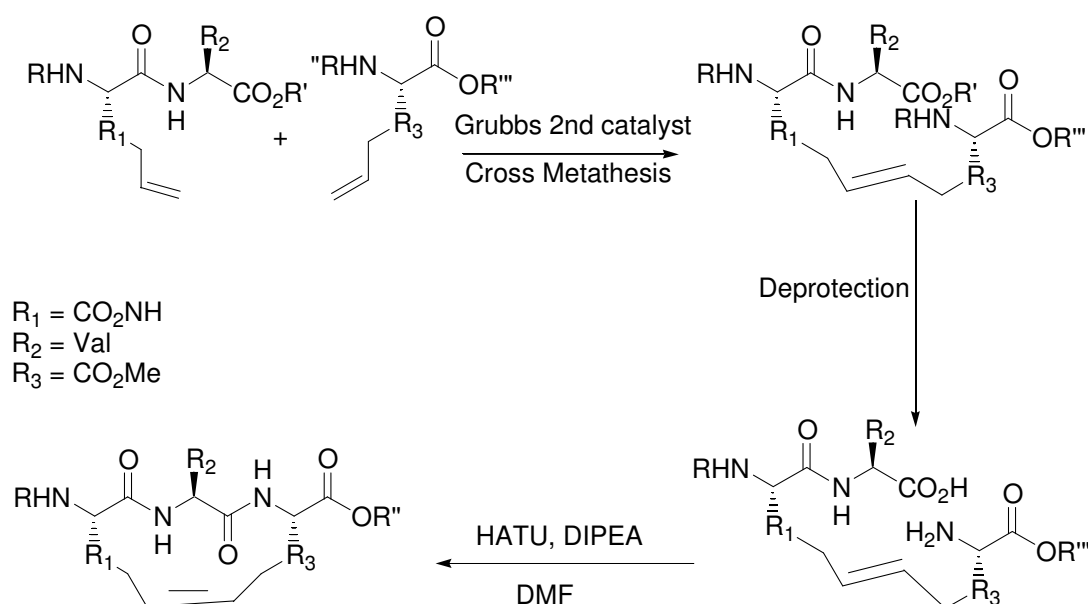
Scheme 4.7.1: Proposed cross metathesis reaction

The use of RCM chemistry is an attractive methodology for synthesising conformationally constrained peptidomimetics in a desired 'bioactive' conformation. We have successful synthesised the desired β-strand 'bioactive' macrocyclic compounds as summarised in **Table 4.1**.

4.8 REFERENCES FOR CHAPTER 4:

(1)   Aitken.S, G.; Abell.A, D. *Aust.J.Chem* **2005**, *58*, 3-13.

(2)   Kazmaier, U.; Hebach, C.; Watzke, A.; Maier, S.; Mues, H.; V, H. *Org.Biolmol.Chem* **2005**, *3*, 136-145.

(3)   Connon, S. J.; Blechert, S. *Angewandte Chemie, International Edition* **2003**, *42*, 1900-1923.

(4)   McNaughton, B. R.; Bucholtz, K. M.; Camaano-Moure, A.; and; Miller, B. J. *Organic Letters* **2005**, *7*, 733-736.

(5)   Engelhardt, F. C.; Schmitt, M. J.; Taylor, R. E. *Organic Letters* **2001**, *3*, 2209-2212.

(6)   Michaelis, S.; Blechert, S. *Organic Letters* **2005**, *7*, 5513-5516.

(7)   Fürstner, A.; Langemann, K. *Synthesis* **1997**, 792-803.

(8)   Qian Yang, W.-J. X., ; and Zhengkun Yu *Organic Letters* **2005**, *7*, 871-874.

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5.1   CALPAIN INDUCED CATARACTS

The anatomy of the eye is shown in (**Figure 5A**)[1]. It is one of the five senses that is vital for an individual. Sight is an astonishing process comprised of many parts of the eyes operationing together. Vision occurs when light enters the eyes, it is refracted by cornea and finally passes through the lens. The lens focuses this refracted light on to the retina where the light energy is converted into an electrical impulse. This electrical impulse is conducted through the optic nerves to the brain components where the image is interpreted.[2] In a normal individual the lens is totally transparent. Cataract is characterised by a disruption of this transparency.
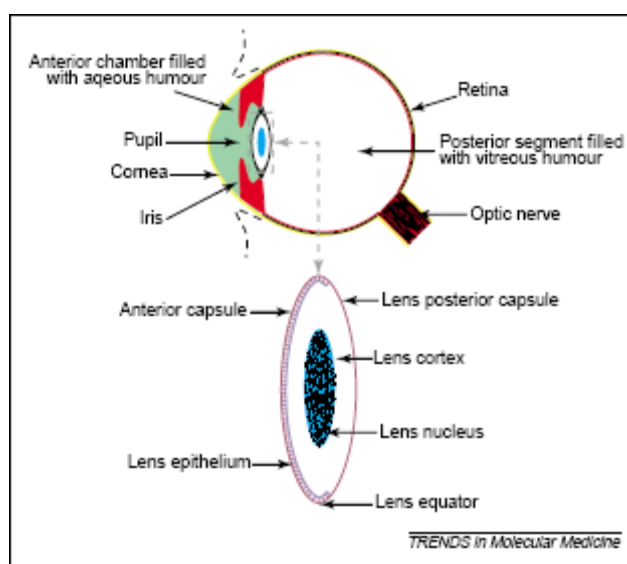
Figure 5A[1]

The lens is situated behind the iris and is contained within a cell membrane; the lens capsule. It is an ellipsoid shape and it is layered in a similar manner to that of an onion.[3] The lens is devoid of blood supply and at its centre (nucleus) it contains protein which has been present since birth.[3] During development the cells elongate and lose organelles. As a result this increases the protein content. A normal lens contains 35% (weight by weight) of protein.[4] Eighty five percent of these proteins are the water-soluble proteins; lens crystallins (α, β, γ).[4] The high concentrations of these proteins create a high degree of packing regularity thus this is what is responsible for lens transparency.[1]

Cataracts (**Figure 5B**[5]) are degenerative diseases where the lens in the eye becomes cloudy and prevents light from focusing to the retina. This results in image distortion and eventually blindness. It is the primary cause of blindness world-wide and currently the only treatment for cataracts is surgical removal.
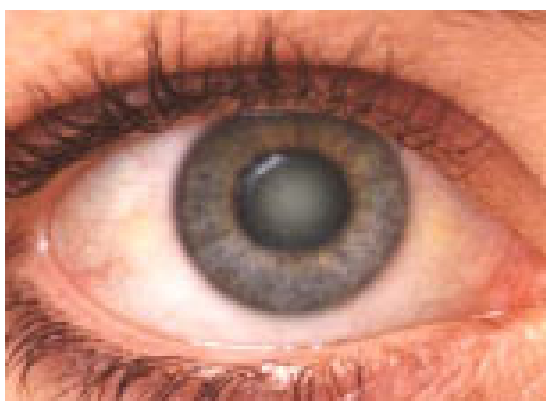

Figure 5B[5] An eye with cataracts

The two main cataracts surgeries are intraocular lens implants (IOL implants) and phaco-emulsification surgery.[4] Essentially IOL implants replace the original lens by inserting the polymer (made from polymethylmethacrylate (PMMA))[4] into the capsular bag where it is held in place by the posterior capsule.[6,7] In contrast contemporary phacoemulsification removes a cataract through a small incision made in the eye. This method is now the preferred surgical procedure. In modern phacoemulsification surgery an ultrasound probe is used to break the lens (cataract) apart without harming the capsule.[4] The use of this technology means that nowadays a much smaller incision is required. It only needs to be big enough to remove the broken fragments out of the eye via aspiration.[5,6] After removal of the fragments the foldable IOL implant is inserted through the same small incision. Once the inserted lens is inside it is unfolded to a normal IOL size and this is then held in place by the posterior capsule.[6]

Unfortunately the sad but true reality is that cataracts predominantly occur in the developing world. India alone is a home of 23.5% of the world's blind population.[8] At the present surgery still remains the only approach for cataract treatment. Whilst this is now quite safe and very effective method the cost is extremely high. It has been estimated that cataract surgery worldwide created a device market of $2.2 billion in 2000 and this was expected to increase to $2.5 billion by 2005.[9] The expensive cost of surgery, together with often long waiting periods, means that for a number of patients the disease continues to develop and within a relatively short period lead to blindness. It is therefore highly desirable that an ideal drug is developed to stop cataract formation, or at the very least slow the progression of human cataract. Consequently, the successful drug developed would improve the quality of life for people who can not afford surgery such as those in the developing world, or those who have a substantial wait before surgery can take place.

Results obtained from an enormous amount of research suggest that calpains (**section 1.2-1.3**) are implicated in cataract formations.[10,11,12] Calpain is a calcium dependent cysteine protease catalysed the breakdown of the major lens proteins; crystallins. The hypothesis is therefore, that calpain activation is induced by the elevated levels of calcium in the lens. This elevation in calcium levels is known be stimulated by a number of factors such as cellular aging, ultra violet radiation and diabetes. Thus the net result is formation of opacity in the lens and ultimately if untreated, blindness. Consequently, calpain inhibitors could provide a potential therapeutic approach for treatment of cataract. Furthermore these same calpain inhibitors could in theory also be useful to treat other diseases that believed to be cause by overactivated calpain. To date there are no calpain inhibitors approved for human use for any therapeutic indication. Thus the development of such inhibitors is an extremely worthwhile undertaking.

5.2   DESIGNED AND SYNTHESIS CALPAIN INHIBITOR VIA β-STRAND MACROCYCLE

For the past few years at Canterbury University the Abell calpain group has synthesised and tested novel calpain inhibitors. The primary objective of this research project is to discover peptidomimetic compounds that mimic β-strands and are potent calpain inhibitors (see each for discussion). It is then envisaged that the inhibitors discovered will be used to develop a pharmaceutical treatment for cataracts.

Initially our peptidomimetic design work used a potent calpain inhibitor [SJA 6017-IC$_{50}$ 7.5 nM (**1.3**)] reported by Senju pharmaceutical company[13] as a lead compound from which to design novel calpain inhibitors.

1.3
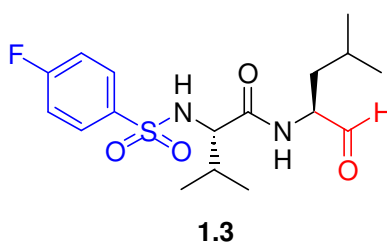
Dipeptide based inhibitors based on SJA 6017, developed in our laboratories can be divided into three segments **Figure 5C**:

1.6
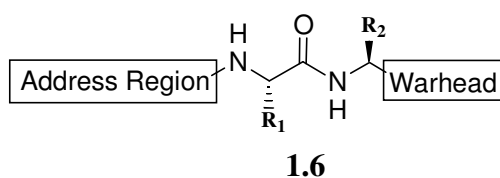
Figure 5C: Typical dipeptide calpain inhibitor

1) Address Region: Typically a hydrophobic group such as an aromatic group

2) Peptide backbone: A dipeptide that adopts a β-strand conformation.

3) Warhead: An electrophilic species which forms a covalent intermediate from the nucleophilic attack of the sulphur group of cysteine in the active site of the enzyme. Typically an aldehyde is used.

A large number of these types of peptidomimetic compound have been synthesised and tested within our group.[14,15,16] Compounds of this class have been termed as 'first generation calpain inhibitors'. The first generation compounds are flexible dipeptide molecules which exhibit variety conformations. It is postulated that there is only one conformation that is accountable for its biological activity and therefore a classical way in which medicinal chemists approach such a problem is to conformationally constrain the molecule into the 'bioactive' conformation. Furthermore it would be expected that simple dipeptides, such as the first generation calpain inhibitors, would be poor drug candidates as they have poor pharmacokinetic profiles.

Recently, the focus of our efforts has been devising a strategy that is capable of synthesising novel inhibitors conformationally constrained into the 'bioactive' conformation as discuss in this thesis. Such compounds have been termed 'second generation calpain inhibitors'. Only a small number of the macrocycles shown to be potential β-strand mimics *in-silico* has been synthesised. The reason for this is simply due to the fact that new RCM methodology was required (**section 2.2** and **Chapter 4**). RCM is a relatively new methodology in organic synthesis however work performed in **Chapter 4** of this thesis in someway contributed to the understanding of why RCM is not successful on some diene. We have now successfully devised methods to counteract the chelation between Grubbs catalyst and the carbonyl containing functional group. Consequently RCM chemistry used to form our macrocycles is now very efficient. Furthermore it is anticipated this methodology will allow successful synthesis of a variety of potential macrocyclic calpain inhibitors constrained into the 'bioactive' conformation.

5.2.1   Designing calpain inhibitor

It is envisaged that the β-strand backbone macrocycles may be employed as generic scaffolds for the synthesis of different protease inhibitors. This will be achieved by appending appropriate functionality/recognition elements to the key β-strand macrocyclic templates (**Section 1.3.2**).

An investigation of the structure activity relationships (SAR) for calpain inhibitors[17] has shown that the optimal group at the address region/P$_3$ position is the 4-Fluoro-benzene-sulfonamide and at the P$_1$ position an aldehyde warhead is required.

Consequently appending these groups onto the macrocyclic intermediate **4.6** was an attractive target as a calpain inhibitor. This was achieved in three steps; firstly **4.6** was hydrogenated using Pd/C catalysis, secondly the methyl ester was then reduced to an alcohol and finally oxidation of alcohol to an aldehyde to yield macrocycle **2.2**. Molecular modelling (**Chapter 2**) of macrocycle **2.2** suggests that this should be an excellent β-strand mimic as the Boltzmann weighted distribution shows that **2.2** is over 90% β-strand.

As shown in **Figure 5D** the macrocycle **2.2** (grey) superimposed with SJA 6017 (**1.3**) (black). From this it is clear that **2.2**accurately confine the backbone to adopt the requisite β-strand 'bioactive' conformation.
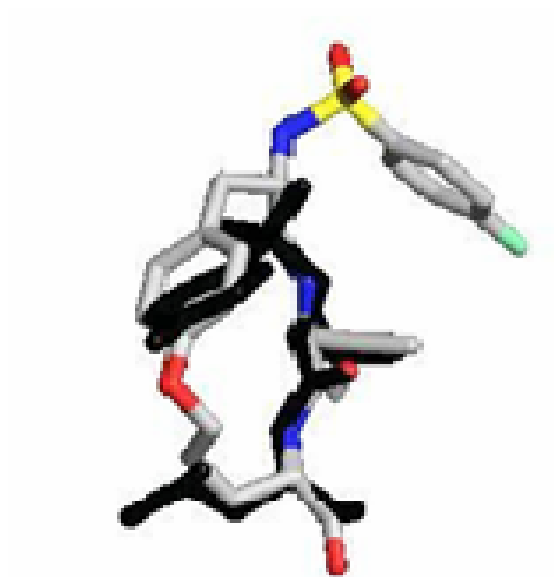
Figure 5D: Superimposition between macrocycle **2.2** (grey conformer) and SJA6017 (black conformer) and macrocyclic.

Furthermore the Induced Fit Docking protocol (by Blair Stuart and Axel Neffe) indicates that **2.2** is not only a good β-strand mimic but the docking score (binding energy calculated using GLIDE and PRIME) indicates that this will be a potent calpain inhibitor. The docking of **2.2** into the enzyme is shown in **Figure 5E.** This clearly shows that the macrocycle is bound in an also perfect β-strand conformation. **Figure 5E** also shows that aldehyde warhead is in close proximity to the active site of Cys residue (4.06Å).
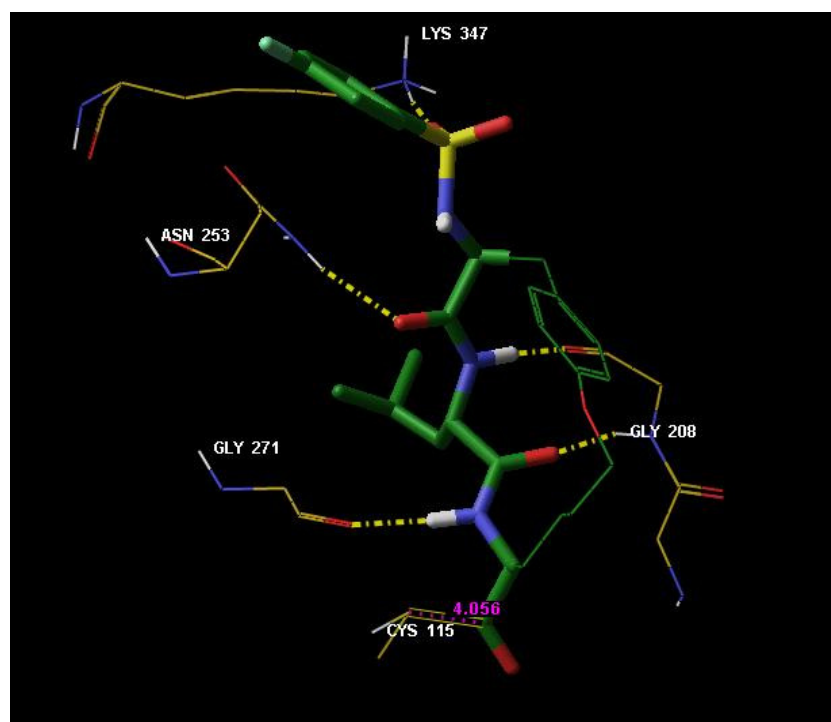
Figure 5E: Docking of **2.2** with the aldehyde warhead positions proximity to cysteine
residue (4.06Å).

5.2.2   Synthesis of Macrocycle 4-F-SO$_2$-N-Tyr-Leu-Gly-ene-Macrocycle-CHO
calpain inhibitor (2.2).

For the large scale synthesis of **2.2** for the *in-vivo* sheep trial I supplied Janna Nikkel
with a quantitative quantity of **4.6.** This was converted into the calpain inhibitor in
three steps (**Scheme 5.1**). **4.6** was hydrogenated using 10% palladium on carbon
under a hydrogen atmosphere in a methanol/dichloromethane/ethyl acetate mixture to
afford the isolated saturated macrocycle **5.1** in 95% yield. Reduction of the methyl
ester using to the alcohol **5.2** was achieved using LiALH$_4$ in THF (92%). Oxidation
using sulfur trioxide pyridine complex and DIPEA in a mixture of DCM and DMSO
afforded 2.7 in 45% yield.
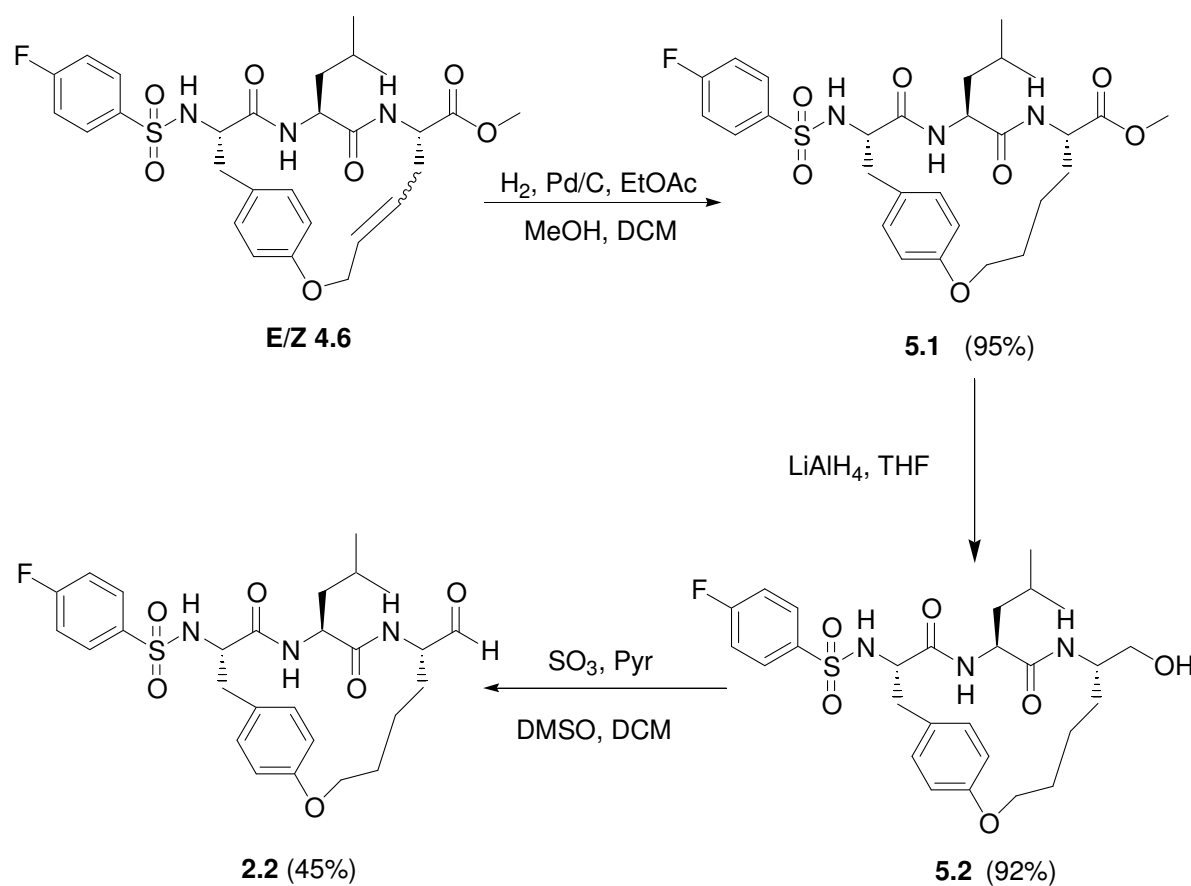
Scheme 5.1: Conversion to calpain inhibitor by Janna Nikkel

5.3   BIOLOGICAL ASSAY FOR CALPAIN INHIBITOR[18,19,20]

The inhibition constant (IC_{50}) value of **2.2** was determined using *in vitro* assay. The IC_{50} is the concentration of the inhibitor required to inhibit the activity of an enzyme by 50%. The assay employed herein is a fluorometric one. 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (BODIPY) florophore casein labelled (BODIPY-FL-casein)[18] is used a the fluorophore.

The assay was performed by Janna Nikkel at Lincoln University. The purified m-calpain obtained from sheep lung and suitable blanks (EDTA and Calcium) along with the m-calpain control solution were prepared by our collaborators at Lincoln University.

The assay was carried out in a black BMG fluorometric plate reader. A black microtiter plate was used to minimise background florescence and interference between the wells. The blanks (EDTA and Calcium), m-calpain control solution and inhibitor containing solutions were added to the well in triplicate. Once the BODIPY FL-Casein substrates were added, the well plates were immediately inserted to the BMG fluorescence reader. Fluorescence from an individual well was determined at 485 nm excitation and 538 nm emission. Fluorescence units (FU) were recorded every 1-1.5 minutes for period of time 10-20minuites.

The IC_{50} of the enzymatic inhibition activities was calculated by taking the average change in fluorescence reading (FU) obtained from the three wells together with the average sum of fluorescence obtained from the three blanks (EDTA and Calcium) as shown in **Equation 5.1**.

$$FU_{sample} = FU_{mean\ total} - FU_{(Ca\ +\ EDTA)/2}$$

Equation 5A

The percent inhibition of m-calpain for each compound was calculated using **Equation 5.2**

$$\%\ Inhibition = ((FU_{calapain\ 2} - FU_{sample})/FU_{calpain\ 2}) \times 100$$

Equation 5B

The assay data obtained were analysed using the graphical program in Microsoft Excel. Percentage inhibition vs. log of inhibitor concentration as plotted. Linear best fits were obtained and the IC_{50} values were determined by extrapolating the x-axis log value and converting back to units of concentration.

The assay results obtained from macrocycle **2.2** reveals the IC_{50} values of 45nM. This is one the most potent conformationally constrained β-strand calpain inhibitors synthesised within our group. Furthermore this conformationally constrained β-strand mimic have also shown a significant decreased in the IC_{50} values compared to the dipeptide calpain inhibitors discuss earlier in **section 1.3.2**. As such the utilisation of conformationally constrained approached does indeed optimised the inhibitors potency.

5.4   SUMMARY AND FUTURE WORK

RCM has been used to synthesise macrocyclic compounds which were designed with the aid of molecular modelling to be conformationally constrained into β-strand 'bioactive' conformation. By appending appropriate functionality on to the macrocyclic intermediate a potent calpain inhibitor (**2.2**) was obtained. The IC_{50} value of macrocycle **2.2** found to be 45 nM and this value is one of the best to date that represents a significant step forward in the design calpain inhibitors. This compound is currently undergoing *in vivo* evaluation by our collaborating group at Lincoln University

In addition to the synthesis of calpain inhibitors, it is proposed that by appending appropriate functionality to the β-strand macrocyclic intermediates, these macrocyclic templates can be modified to inhibit a wide variety of proteases.

Thus by appending the calpain specific moieties as in **2.2**, it is expected that the macrocyclic intermediates from chapter **4** (**2.3, 2.5** and **2.6**) can be elaborated into potent calpain inhibitors.

The macrocyclic templates prepared in this thesis also provide both the methodology and synthetic route with the attempt to validate the generic β-strand protease inhibitor design strategy. An example of such approached is shown in **Scheme 5.2.**

Incorporating the required additional recognition moieties known to confer HIV-1 protease inhibition to one our macrocycles should result in the synthesis of a potent HIV-1 protease inhibitor

HIV-Protease Inhibitor
Aspartic protease

1) (S)-Boc-Phe-epoxide
2) HCl, dioxane
3) 2-Quinoline-Val-OH, HATU

Beta-strand
Key intermediate
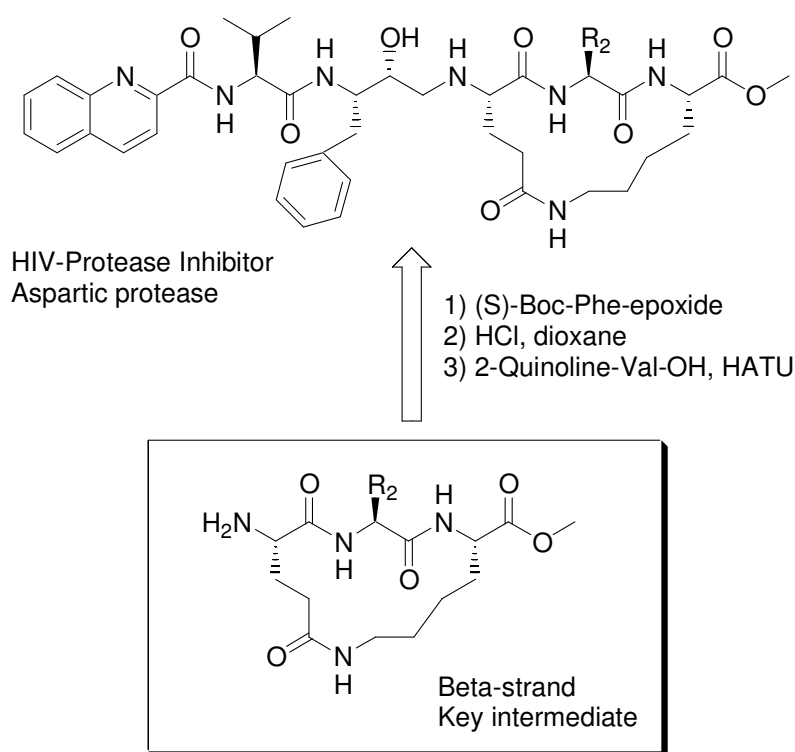
Scheme 5.2: Proposed of possible Aspartic protease inhibitor-HIV inhibitor

The successful synthesis of **2.2** and its potent IC_{50} value against calpain makes a compelling case that the combination of a β-strand conformational constraint strategy together with the use of molecular modelling to design β-strand mimics is a methodology which could well confer the synthesis of generic protease inhibitor templates.

The key reaction in which allowed the efficient synthesis of **2.2** was without doubt is the RCM step.   The significant success of this synthesis is due to our new methodology of combining a Lewis acid additive (chlorodicyclohxyl borane) and microwave irradiation (1200W).   Once more this is a very significant result and one which will probably find extensive use particularly in the field of conformationally constrained peptidomimetics.

5.5 REFERENCES FOR CHAPTER 5:

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6.1   GERNERAL METHODS AND EXPERIMENTAL PROCEDURES

General Experimental Methods

Unless otherwise indicated all experiments were performed in oven-dried glassware. Solvents and reagents used in reactions were purified according to literature procedures.[1] Dichloromethane and 1,1,2 Trichloroethane were distilled from calcium hydride. Tetrahydrofuran was distilled from sodium and benzophenone. Dimethylformamide was obtained dry from Aldrich and stored under argon. Petroleum ether describes a mixture of hexanes in the boiling point range 50-70°C.

Thin-Layer Chromatography (TLC)

Analytical thin layer chromatography was conducted on plastic backed Macherey-Nagel SIL G/UV254 plates. Visualisation was by ultraviolet light and/or potassium permanganate solution and/or PMA.

Flash Column Chromatography

Flash chromatography was performed on Merk silica 60 using a Buchi sepacore automated flash chromatography system. All eluting solvents were distilled before use.

Nuclear Magnetic Resonance

Proton (^{1}H) detected NMR spectrums were obtained either on a Varian UNITY 300 spectrometer at 300 MHz or a Varian INOVA 500 spectrometer at 500 MHz. All carbon (^{13}C) detected NMR spectrums were obtained on a Varian XL300 spectrometer operating at 75 MHz. Spectra were obtained at 23°C. Chemical shifts were reported in parts per million (ppm) on the δ scale.

Mass Spectrometry

Mass spectra were carried out by Bruce Clark (University of Canterbury) using the Micromass LCT mass spectrometer. The spectrums were processed via MassLynx 3.5 Global editions.

Melting Point (mp)

Melting points were taken on an electrothemal apparatus without being corrected.

General Procedure A: Peptide Coupling Using HATU

To a stirring solution of the acid (1equiv) in dry DMF at room temperature was added HATU (1.1 equiv). After 5 min, the amine (2 equiv) was added followed by DIPEA (4 equiv) and the solution was continued to stir for 18hr. The DMF was removed by diluting with a large amount of ethyl acetate and partition with 1M HCl. The organic phase was washed sequentially with 1M HCl $_{(aq)}$, brine and dried over magnesium sulphate. The solvent was removed *in vacuo* to yield the crude peptide compound which can be purified by flash column chromatography on Merk silica gel 60.

General Procedure B: Peptide Coupling Using EDC/HOAT

To a stirring solution of the acid (1equiv) in dry DMF at room temperature was added EDC (1.1 equiv), HOAt (1.1 equiv). After 5 min, the amine (2 equiv) was added followed by DIPEA (4 equiv) and the solution was continued to stir for 18hr. The DMF was removed by diluting with a large amount of ethyl acetate and partition with 1M HCl $_{(aq)}$. The organic phase was washed sequentially with 1M HCl $_{(aq)}$, brine and dried over magnesium sulphate. The solvent was removed *in vacuo* to yield the crude peptide compound which can be purified by flash column chromatography on Merk silica gel 60.

General Procedure C: Methyl Ester Hydrolysis

A solution of 2M sodium hydroxide (2.5 equiv) was added to a solution of methyl ester (1 equiv) in THF. The minimum amount of H_2O/MeOH was added to obtain a homogenous solution. The mixture was stir for 15hr and the solvent was removed under reduced pressure. The residue was partitioned between ethyl acetate and 10% citric acid and the aqueous phase was extracted twice more with ethyl acetate. The combined organic extracts were washed with 2x brine, dried over magnesium sulphate and filtered. The solvent was removed *in vacuo* to yield the crude acid.

General Procedure D: Ring Closing Metathesis I

The Diene was dissolved in 1,1,2-Trichlorethane (0.01M). To this a portion (10%mol) of Grubbs second generation catalyst was added. The solution was heated at reflux in the microwave (1200 W) for 10 minutes. Two further additions of Grubb's second generation catalyst (**2.12**) (2x10%) were added, after each the reaction mixture was subjected to 10 minutes heating in the microwave. The resulted solution was concentrated *in vacuo* to yield crude product which was purified via silica gel-based column chromatography.

General Procedure E: Ring Closing Metathesis I

The Diene was dissolved in 1,1,2-Trichlorethane (0.01M) under argon atmosphere. To this a portion (10%mol) of Grubbs second generation catalyst (**2.12**) was added. The solution was heated at reflux in the microwave (1200 W) for 20 minutes. Two further additions of Grubb's second generation catalyst (2x10%) were added, after each the reaction mixture was subjected to 20 minutes heating in the microwave. The resulted solution was concentrated *in vacuo* to yield crude product which was purified via silica gel-based column chromatography.

General Procedure F: Ring Closing Metathesis III (Lewis Acid)

The Diene was dissolved in 1,1,2-Trichlorethane (0.01M) under argon atmosphere. To this a portion (10%mol) of Grubbs second generation catalyst (**2.12**) and (10% mol) chlorodicyclohexylborane Lewis acid were added. The solution was heated at reflux in the microwave (1200 W) for 20 minutes. Two further additions of Grubb's second generation catalyst (2x10%) were added, after each the reaction mixture was subjected to 20 minutes heating in the microwave. The resulted solution was concentrated *in vacuo* to yield crude product which was purified via silica gel-based column chromatography.

6.2   EXPERIMENTAL DESCRIBED FROM CHAPTER 3

Synthesis N-Boc-S-allyl-Cys-OMe (3.11)
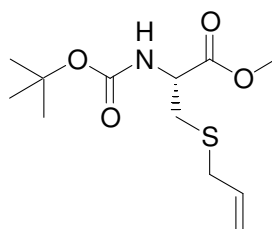
3.11

To a stirred solution of N-Boc-L-Cys-OMe **3.10** (5.50g, 23.4mmol) in dry DCM (30mL) under argon atmosphere was added allyl bromide (2.5mL, 25mmol) and triethylamine (1.4mL, 25mmol). The white precipitation was formed and the resulting solution was stirred for 15hr. The solvent was removed *in vacuo* and the residue was partition between ethyl acetate (25mL) and 1M HCl $_{(aq)}$ (15mL). The organic phase was washed with brine (2 x 15mL), dried over MgSO$_4$ and filtered through fluted filter paper. Removal of solvent gave white residue which was purified by column chromatography R$_f$ = 0.26 (1/6 (EtOAc / (50/70) Petroleum ether)). The column was eluting with gradient of (50/70) Petroleum ether) to (1/5 (EtOAc / (50/70) Petroleum ether)) to give a white solid compound **3.11** (4.9g, 18mmol, 77%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 5.64 (dt, 1H, J=7.4Hz, J=15.4Hz, 1H, **CH**=CH$_2$), 5.36 (d, 1H, J=7.1Hz, N**H**), 5.02 (dd, 2H, J=7.0Hz, J=8.7Hz, CH=**CH**$_2$), 4.39 (d,1H, J=5.5Hz, α **CH**), 3.64 (s, 3H, OC**H**$_3$), 3.02 (m, 2H, SC**H**$_2$), 2.77 (ddd, 2H, J=4.6Hz, J=13.9Hz, J=18.9Hz, **CH**$_2$S), 1.34 (s, 9H, C(C**H**$_3$)$_3$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 171.5, 155.4, 133.5, 117.6, 80.2, 53.0, 52.3, 34.9, 32.6, 28.2;

LRMS [ES[+] M + [H^{+}] Calcd for C$_{12}$H$_{21}$NO$_4$S 276.3 found 276.2].

Synthesis N-Boc-S-allyl-Cys-OH (3.12)

3.12
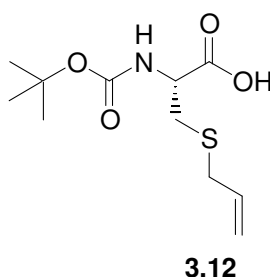
N-Boc-S-allyl-Cys-OMe **3.11** (4.9g, 18mol) was hydrolysed according to general procedure **C** to give oily compound **3.12** (3.86g, 15mmol, 83%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 5.73 (dt, 1H, J=7.3Hz, J=16.9Hz, **CH**=CH$_2$), 5.40 (d, 1H, J=7.7Hz, N**H**), 5.11 (dd, 2H, J=7.0Hz, J=8.7Hz, CH=C**H**$_2$), 4.52 (d, 1H, J=6.8Hz, α C**H**), 3.13 (d, 2H, J=7.2Hz, SC**H**$_2$), 2.91 (ddd, 2H, J=5.0Hz, J=14.1Hz, J=19.6Hz, C**H**$_2$S), 1.43 (s, 9H, C(C**H**$_3$)$_3$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 175.1, 155.3, 133.5, 117.8, 80.4, 52.9, 35.0, 32.4, 28.2;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{11}$H$_{19}$NO$_4$S 262.3 found 262.1]

Synthesis N-Boc-S-allyl-Cys-Val-OMe (3.13)

3.13
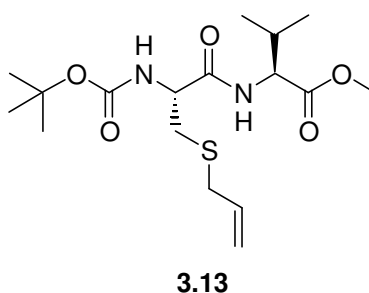
N-Boc-S-allyl-Cys-OH **3.12** (3.76g, 14mmol) was coupled to (S)-Val-OMe.HCl (4.8g, 29mmol) according to general procedure **A** gave a crude white residue R$_f$ = 0.36 (1/3 EtOAc / (50/70) petroleum ether). Purification was achieved by column chromatography, eluting with a gradient of (50/70) Petroleum ether) to (1/1 (EtOAc / (50/70) Petroleum ether)) to give a white solid compound **3.13** (3.6g, 9.6mmol, 66%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 6.87 (bs, 1H, Val(N**H**)), 5.78 (tdd, 1H, J=7.2Hz, J=9.9Hz, J=17.1Hz, **CH**=CH$_2$), 5.34 (bs, 1H, Boc(N**H**)), 5.15 (dd, 2H, J=13.4Hz, J=26.4Hz, CH=**CH$_2$**), 4.51 (dd, 1H, J=4.9Hz, J=8.7Hz, Val α (**CH**)), 4.25 (d, 1H, J=6.5Hz, Cys α **CH**), 3.72 (s, 3H, OC**H$_3$**), 3.17 (dd, 2H, J=4.3Hz, J=6.7Hz, SC**H$_2$**), 2.83 (ddd, 2H, J=6.3Hz, J=13.9Hz, J=47.0Hz, C**H$_2$**S), 2.17 (m, 1H, Val(C**H**)), 1.45 (s, 9H, C(C**H$_3$**)$_3$), 0.92 (dd, 6H, J=6.9Hz, J=12.2Hz, Val(C**H$_3$**)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 171.9, 170.6, 155.4, 133.8, 117.9, 80.4, 57.3, 53.8, 52.6, 35.1, 32.7, 31.2, 28.2, 18.9, 17.7;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{17}$H$_{30}$N$_2$O$_5$S 375.5 found 375.3];

mp = 140-145 ^{0}C

Synthesis N-Boc-S-allyl-Cys-Val-OH (3.14)

3.14
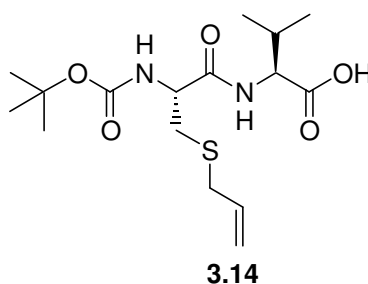
N-Boc-S-allyl-Cys-Val-OMe **3.13** (2.8g, 7.5mmol) was hydrolysed following general procedure **C** to give white gluey compound **3.14** (1.88g, 5.2mol, 70%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.04 (bs, 1H, Val(N**H**)), 5.77 (m, 1H, C**H**=CH$_2$), 5.45 (bs, 1H, Boc(N**H**)), 5.16 (ddd, 2H, J=7.6Hz, J=7.6Hz , J=18.0Hz, CH=C**H$_2$**), 4.54 (dd, 1H, J=4.6Hz, J=8.5Hz, Val α (**CH**)), 4.31 (d, 1H, J=6.9Hz Cys α (**CH**)), 3.16 (d, 2H, J=6.9Hz, SC**H$_2$**), 2.84 (ddd, 2H, J=6.6Hz, J=13.1Hz, J=18.7Hz, C**H$_2$**S), 2.26 (m,1H, (Val(C**H**)), 1.45 (s, 9H, C(C**H$_3$**)$_3$), 0.97 (dd, 6H, J=6.6Hz, J=14.1Hz, Val(C**H$_3$**)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 174.6, 171.6, 155.7, 133.7, 117.9, 80.2, 57.2, 53.8, 35.1, 32.6, 30.9, 28.2, 18.9, 17.6;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{16}$H$_{28}$N$_2$O$_5$S 361.5 found 361.3].

Synthesis N-Boc-S-allyl-Cys-Val-S-ally-Cys-OMe (3.15)
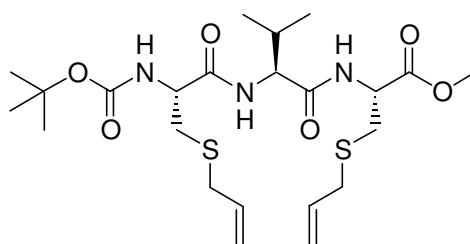
3.15

N-Boc-S-allyl-Cys-Val-OH **3.14** (0.94g, 2.6mmol) was coupled to S-allyl-Cys-OMe **3.35** (0.91g, 5.2mmol) according to general procedure **A** gave white residue $R_f = 0.28$ (1/2 EtOAc / (50/70) petroleum ether). Purification was achieved by column chromatography, eluting with gradient of (50/70) Petroleum ether) to 100% EtOAc to give a white solid compound **3.15** (0.86g, 1.7mmol, 65%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.01 (d, 1H, J=7.1Hz, Val (**NH**)), 6.92 (d, 1H, J=8.7Hz, N**H**), 5.73 (m, 2H, **CH**=CH$_2$), 5.46 (d, 1H, J=7.4Hz, Boc (N**H**)), 5.11 (m, 4H, CH=C**H**$_2$), 4.74 (dd, 1H, J=5.8Hz, J=13.4Hz, Cys α (C**H**)), 4.39 (m, 1H, Val α (C**H**)), 4.28 (d, 1H, J=5.4Hz, Cys α (C**H**)), 3.73 (s, 3H, OC**H**$_3$), 3.10 (ddd, 4H, J=6.9Hz, J=16.7Hz, J=20.6Hz, 2(SC**H**$_2$)), 2.85 (m, 4H, 2(C**H**$_2$S)), 2.17 (m, 1H, Val(C**H**)), 1.43 (s, 9H, C(C**H**$_3$)$_3$), 0.94 (m, 6H, Val(C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 170.9, 170.7, 170.5, 155.7, 133.7, 133.5, 117.9, 117.9, 80.1, 58.4, 53.9, 52.5, 51.5, 35.1, 34.9, 32.3, 30.9, 30.7, 28.2, 19.1, 17.8;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{23}$H$_{39}$N$_3$O$_6$S$_2$ 518.7 found 518.3].

Synthesis pf N-Boc-O-allyl-Ser-OMe (3.17)
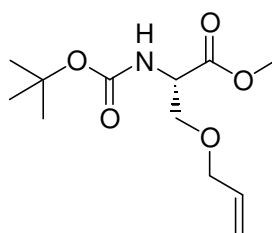
3.17

Allyl ethyl carbonate was made *in situ*. To a stirring solution of allyl alcohol (1.97mL, 29mmol) and ethyl chloroformate (3.12mL, 32.7mmol) at 0^0C in diethyl ether (20mL) was added dropwised triethylamine (4.57mL, 32.8mmol). The solution was leaved to stir for 20min and resulted white precipitate formation which was removed by filtering through Buchner funnel. The filtrate was collected and the ether solvent was removed by purged with nitrogen for 1-2h. Next the resulted solution was dissolved in THF (4mL). To this a solution of allyl palladium chloride dimmer (0.083g, 0.23mmol) and triphenylphosphine (0.3g, 1mmol) in THF (3mL) was added. The solution mixtures were added to a stirring solution of N-Boc-Ser-OMe (5.0g, 2.3mmol) in THF (20mL). The solution was leaved to stir for 18h and the solvent was removed *in vacuo* resulted crude yellow oily compound R_f = 0.27 (1/6 EtOAc / (50/70) petroleum ether). Purification was achieved by column chromatography, eluting with gradient of (50/70) Petroleum ether) to 1/1 EtOAc / (50/70) petroleum ether) to give an oily yellow compound **3.17** (1.25g, 4.8mmol, 21%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 5.64 (ddd, 1H, J=5.5Hz, J=10.7Hz, J=21.9Hz, C**H**=CH$_2$), 5.37 (d, 1H, J=8.6Hz, N**H**), 5.01 (dd, 2H, J=13.6Hz, J=34.6Hz, CH=C**H**$_2$), 4.23 (m, 1H, α C**H**), 3.78 (t, 2H, J=6.2Hz, OC**H**$_2$), 3.65 (dd, 1H, J=3.2Hz, J=9.4Hz, C**H**$_2$O), 3.55 (s, 3H, OC**H**$_3$), 3.46 (dd, 1H, J=3.4Hz, J=9.5Hz, C**H**$_2$O), 1.26 (s, 9H, C(C**H**$_3$)$_3$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 170.8, 155.2, 133.9, 116.9, 79.4, 71.8, 69.6, 53.7, 52.0, 28.0;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{12}$H$_{21}$NO$_5$ 260.3 found 260.3].

Synthesis of N-Boc-O-allyl-Ser-OH (3.18)

3.18
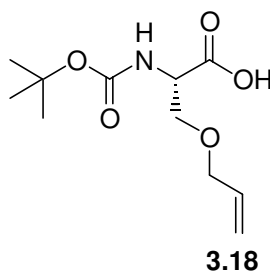
N-Boc-O-allyl-Ser-OMe **3.17** (0.9g, 3.48mmol) was hydrolysed according to general procedure **C** gave clear oily compound **3.18** (0.7g, 2.85mmol, 82%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 5.79 (qd, 1H, J=5.5Hz, J=10.6Hz, **CH**=CH$_2$), 5.47 (d, 1H, J=8.5Hz, N**H**), 5.16 (dd, 2H, J=13.8Hz, J=36.6Hz, CH=**CH$_2$**), 4.42 (m, 1H, α **CH**), 3.95 (d, 2H, J=5.3Hz, O**CH$_2$**), 3.85 (dd, 1H, J=2.3Hz, J=9.4Hz, **CH$_2$**O) 3.63 (dd, 1H, J=3.3Hz, J=9.6Hz, **CH$_2$**O), 1.39 (s, 9H, C(**CH$_3$**)$_3$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 174.8, 155.6, 133.9, 117.4, 80.2, 72.2, 69.6, 53.7, 28.2;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{11}$H$_{19}$NO$_5$ 246.2 found 246.1].

Synthesis of N-Boc-O-allyl-Ser-Val-OMe (3.19)

3.19
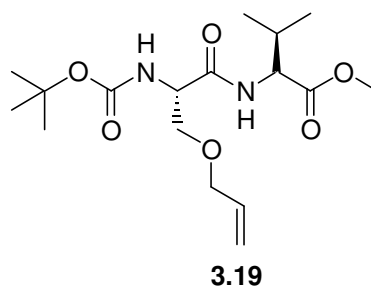
N-Boc-O-allyl-Ser-OH **3.18** (0.63g, 2.57mmol) was coupled to (S)-Val-OMe.HCl (0.86g, 5.14mmol) using general procedure **A** gave crude oily compound R$_f$ = 0.25 (1/3 EtOAc / (50/70) petroleum ether). Purification was achieved by column chromatography, eluting with gradient of (50/70) Petroleum ether) to 100% EtOAc to give white solid compound **3.19** (0.67, 1.87mmol, 73%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.06 (d, 1H, J=5.6Hz, Val (N**H**)), 5.69 (m, 1H, **CH**=CH$_2$), 5.50 (d, 1H, J=5.6Hz, Ser (N**H**)), 5.03 (dd, 2H, J=13.1Hz, J=48.6Hz, CH=**CH$_2$**), 4.35 (m, 1H , Ser α **CH**), 4.14 (s, 1H, Val α **CH**), 3.84 (d, 2H, J=4.4Hz, O**CH$_2$**), 3.64 (dd, 1H, J=4.3Hz, J=9.3Hz, **CH$_2$**O), 3.53 (s, 3H, O**CH$_3$**), 3.39 (dd, 1H, J=10.6Hz, J=17.0Hz, **CH$_2$**O), 1.98 (m, 1H, Val (**CH**)), 1.27 (s, 9H, C(**CH$_3$**)$_3$), 0.73 (m, 6H, Val (**CH$_3$**)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 171.7, 170.2, 155.5, 133.9, 117.1, 79.8, 71.9, 69.5, 56.9, 53.7, 51.7, 30.9 28.0, 18.7, 17.4;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{17}$H$_{30}$N$_2$O$_6$ 359.4 found 359.3].

Synthesis of N-Boc-O-allyl-Ser-Val-OH (3.20)

3.20
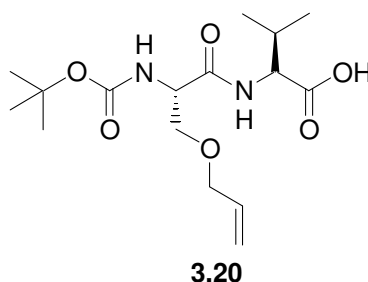
N-Boc-O-allyl-Ser-Val-OMe (0.89g, 2.48mmol) was hydrolysed by general procedure **C** gave compound **3.20** (0.61g, 1.77mmol, 71%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.01 (d, 1H, J=5.0Hz, Val (N**H**)), 5.83 (tt, 1H, J=5.4Hz, J=15.7Hz, C**H**=CH$_2$), 5.60 (d, 1H, J=38.8Hz Ser (N**H**)), 5.20 (dd, 2H, J=13.1Hz, J=41.5Hz, CH=C**H**$_2$), 4.56 (m, 1H, Val α C**H**), 4.35 (d, 1H, J=34.9Hz, Ser α C**H**),   3.99 (d, 2H, J=4.3Hz, C**H**$_2$), 3.79-3.53 (m, 2H, C**H**$_2$O), 2.22 (m, 1H, Val C**H**), 1.42 (s, 9H, C(C**H**$_3$)$_3$), 0.91 (m, 6H, (C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 174.7, 170.6, 155.8, 133.8, 117.6, 80.2, 72.2, 69.5, 57.1, 56.9, 31.0, 28.2, 18.9, 17.4;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{16}$H$_{28}$N$_2$O$_6$ 345.4 found 345.3].

Synthesis of N-Boc-O-allyl-Ser-Val-S-allyl-Cys-OMe (3.21)

3.21
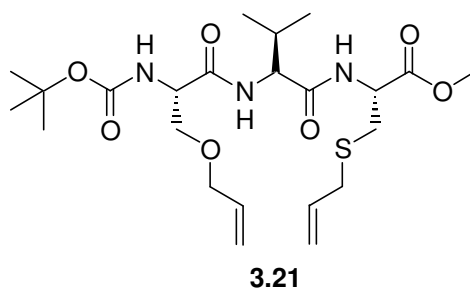
N-Boc-O-allyl-Ser-Val-OH **3.20** (0.61g, 1.77mmol) was coupled to (S)-allyl-Cys-OMe **3.35** (0.62g, 3.54mmol) following general procedure **A** gave oily orange compound R$_f$ = 0.5 (1/1 EtOAc / (50/70) petroleum ether). Purification was achieved by flash column chromatography, eluting with gradient of (50/70) Petroleum ether) to 100% EtOAc to give white solid compound **3.21** (0.48, 0.96mmol, 54%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 5.88 (m, 1H, Ser C**H**=CH$_2$), 5.75 (qd, 1H, J=7.1Hz, J=10.0Hz, Cys C**H**=CH$_2$), 5.15-5.23(m, 4H, 2(CH=C**H**$_2$)), 4.60 (m, 1H, Cys α C**H**), 4.32 (m, 2H, (Val α C**H**), (Ser α C**H**)), 4.00 (t, 2H, J=6.1Hz, OC**H**$_2$), 3.71 (s, 3H, OC**H**$_3$), 3.65 (m, 2H, C**H**$_2$O), 3.14 (d, 2H, J=5.0Hz, SC**H**$_2$), 2.93-2.77 (m, 2H C**H**$_2$S), 2.11 (td, 1H, J=6.3Hz, J=13.6Hz, Val C**H**), 1.44 (s, 9H, C(C**H**$_3$)$_3$), 0.95 (m, 6H, (C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 171.8, 171.4, 171.0, 155.5, 134.3, 133.9, 116.8, 116.4, 79.9, 71.8, 69.4, 58.2, 55.0, 52.1, 51.6, 34.3, 34.3, 31.2, 27.4, 18.5, 18.4;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{23}$H$_{39}$N$_3$O$_7$ S 502.4 found 502.3].

Synthesis of N-Glu-OMe (3.23)
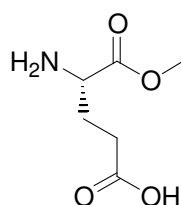
3.23

L-Glu-(OtBu)-OMe (1.0g, 4.8mmol) was dissolved in 2M HCl in diethyl ether (20mL). The resulting solution was leaved to stir at room temperature for 15hr. The solvent was removed *in vacuo* to give white crude product compound (0.8g, 4.73mmol, 98%):

^{1}H NMR (300 MHz) (CD$_3$OD) δ 4.05 (t, 1H, J= 5.8Hz, α C**H**), 3.75 (s, 3H, OC**H**$_3$), 2.4 (m, 2H, C**H**$_2$), 1.98 (m, 2H, C**H**$_2$)

Synthesis of N-O-allyl-Glu-O-allyl (3.25)
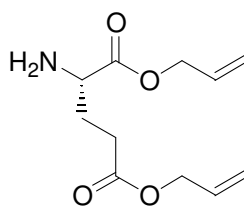
3.25

To a stirring solution of compound **3.23** (0.76g, 4.76mmmol) in benzene (50mL) and allyl alcohol (2mL) was added para toluene sulfonic acid (0.9 g, 4.8mmol). The mixture was refluxed using Dean-Stark trap for 15h.[2] The solvent was removed *in vacuo* and the residue was partition with ethyl acetate (30mL) and 1M NaOH (aq) (20mL). The organic phase was washed with brine (2 x 15mL), dried over $MgSO_4$ and filtered. The solvent was removed in *vacuo* to give crude orange compound. Purification by flash chromatography over silica gel using EtOAc/ (50/70) petroleum ether (2/1) as eluent afforded **3.25** (0.16g, 0.7mmol, 15%):

^{1}H NMR (300 MHz) ($CDCl_3$) δ 5.85 (m, 2H, 2($CH=CH_2$)), 5.35 (m, 4H, 2($CH_2=CH$)), 4.62 (dd, 4H, J=HZ, OCH_2), 3.57, (dd, 1**H**, J=3.17Hz, J=5.15 Hz, α C**H**), 2.47 (m, 2H, γ C**H**$_2$), 2.14 (m, 1H, β C**H**$_2$), 1.94 (m, 1H, β C**H**$_2$);

LRMS [ES^+ M + [H^+] Calcd for $C_{11}H_{17}NO_4$ 228.2 found 228.2].

Synthesis of O-allyl-Glu-OH (3.27)

3.27
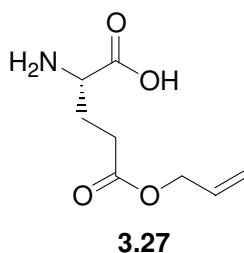
To a stirred suspension of L-Glu-OH (5g, 34mmol) in dry ally alcohol (120mL) was added dropwised chlorotrimethylsilane (10mL).[3] The reaction was leaved to stir under nitrogen atmosphere for 15hr. Recrystalization at 0 [0]C with diethyl ether give white precipitate product **3.27** which was collected under suction using Buchner funnel (4.8g, 26mmol, 77%);

^{1}H NMR (500 MHz) (CD_3OD) δ 5.95 (ddd, 1H, J=5.7Hz, J=10.9Hz, J=22.1Hz, C**H**=CH$_2$), 5.27 (ddd, 2H, J=1.2Hz, J=13.8Hz, J=11.5Hz, C**H**$_2$=CH), 4.61 (d, 2H, J=5.7Hz, OC**H**$_2$), 4.05 (t, 1H, J=6.7Hz, α C**H**), 2.63 (m, 2H, γ C**H**$_2$), 2.21 (m, 2H, β C**H**$_2$);

^{13}C NMR (75 MHz) (CD_3OD) δ 171.9, 170.0, 132.2, 117.2, 65.2, 51.8, 29.2, 25.3;

LRMS [ES^+ M + [H^+] Calcd for $C_8H_{13}NO_4$ 188.2 found 188.2]; mp = 123-130 [0]C

Synthesis of N-Boc-O-allyl-Glu-OH (3.28)

3.28
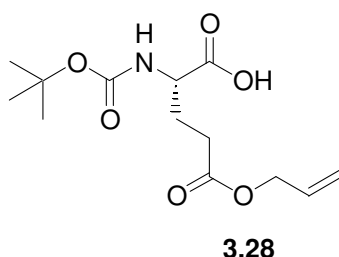
To a stirring solution of compound **3.27** (4.75g, 25.4mmol), di-tert-butyl dicarbonate (8.1g, 37.2mmol) in dioxane (30mL) and water (30mL) was added dropwised triethylamine (14mL, 96mmol). [3] The solution was leave to stir at rt. for 15h and the solvent was removed in *vacuo*. The residue was partition with ethyl acetate (20mL) and 10% citric acid (15mL). The aqueous phase was re-extracted with ethyl acetate (2 x 20mL). The organic phase was washed with brine (2 x 10mL), dried over $MgSO_4$ and filtered. The solvent was removed in *vacuo* to give yellow oily compound **3.28** (5.8g, 20mmol, 80%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 5.83 (ddd, 1H, J=5.8Hz, J=11.0Hz, J=16.3Hz, **CH**=CH$_2$), 5.27 (ddd, 2H, J=1.3Hz, J=13.9Hz, J=11.6Hz, **CH$_2$**=CH), 4.51 (d, 2H, J=5.6Hz, OC**H$_2$**), 4.12 (t, 1H, J=6.7Hz, α **CH**), 2.41 (m, 2H, γ **CH$_2$**), 2.16-1.93 (m, 2H, β **CH$_2$**), 1.36 (s, 9H, (CH$_3$)$_3$);

LRMS [ES$^+$ M + [H$^+$] Calcd for $C_{13}H_{21}NO_6$ 288.3 found 288.2].

Synthesis of N-Boc-O-allyl-Glu-Val-OMe (3.29)

3.29
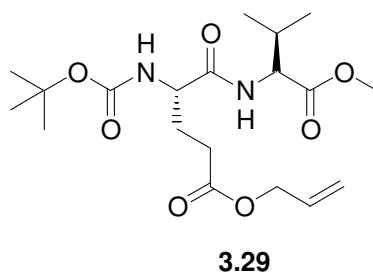
N-Boc-O-allyl-Glu-OH **3.28** (2.0g, 7.0mmol) and (S)-Val-OMe.HCl (2.3g, 13.7mmol) was coupled via general procedure **A**. This yields a pale yellow crude product R_f = 0.38 (1/2 EtOAc/ (50/70) petroleum ether). Purification was achieved by column chromatography, eluting with gradient of 1/3 (EtOAc/ (50/70) petroleum ether) to 1/1 (EtOAc/ (50/70) petroleum ether) gave compound **3.29** (1.7g, 4.2mmol, 62%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 6.97 (d, 1H, J=8.0Hz, Val (N**H**)), 5.80 (ddd, 1H, J=5.7Hz, J=10.9Hz, J=22.7Hz, C**H**=CH$_2$), 5.52 (d, 1H, J=7.8Hz, Glu (N**H**)), 5.16 (ddd, 2H, J=1.2Hz, J=13.9Hz, J=11.5Hz, CH=C**H**$_2$), 4.48 (d, 2H, J=5.7Hz, OC**H**$_2$), 4.39 (dd, 1H, J=5.1Hz, J=8.6Hz, α Val (C**H**)), 4.17 (d, 1H, J=6.3Hz, α Glu (C**H**)), 3.62 (s, 3H, OC**H**$_3$), 2.39 (m, 2H, γ C**H**$_2$), 2.06 (m, 1H, Val (C**H**)), 2.06 (m, 1H, β C**H**$_2$), 1.86 (qd, 1H, J=7.6Hz, J=14.8Hz, β C**H**$_2$), 1.32 (s, 9H, (C**H**$_3$)$_3$), 0.81 (dd, 6H, J=6.9Hz, J=13.6Hz, Val (C**H**$_3$)$_2$);

^{13}C-NMR (75 MHz) δ 172.5, 171.7, 170.7, 155.5, 131.9, 117.9, 79.4, 64.9, 57.0, 53.3, 51.7, 30.6, 30.1, 28.0, 27.4, 18.7, 17.5;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{19}$H$_{32}$N$_2$O$_7$ 401.4 found 401.3].

Synthesis of N-Boc-Glu(OH)-Val-OH (3.31)
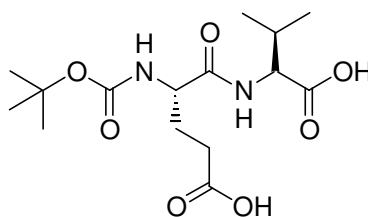
3.31

N-Boc-O-allyl-Glu-Val-OMe **3.29** (1.27g, 3.17mmol) was hydrolysed according to procedure **C** to give crude white solid product **3.31** (0.87g, 2.4mmol, 76%);

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.56 (d, 1H, J=8.4Hz, Val (N**H**)), 5.79 (d, 1H, J=8.8Hz, Glu (N**H**)), 4.52 (dd, 1H, J=4.8Hz, J=8.4Hz, α C**H**), 4.47 (dd, 1H, J=7.7Hz, J=15.4Hz, α C**H**), 2.45 (m, 2H, γ C**H**$_2$), 2.24 (qd, 1H, J=6.7Hz, J=13.1Hz, Val (C**H**)), 2.01-1.91 (m, 2H, β C**H**$_2$), 1.41 (s, 9H, C(C**H**$_3$)$_3$), 0.94 (dd, 6H, J=6.7Hz, J=14.0Hz, Val(C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) δ 176.6, 174.9, 172.7, 156.2, 80.6, 57.3, 53.1, 30.6, 29.6, 28.2, 27.3, 18.9, 17.7;

LRMS [ES$^+$ M + [H$^+$] Calcd for $C_{15}H_{26}N_2O_7$ 347.4 found 347.2].

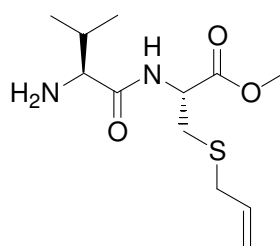## Synthesis of N-Boc-Val-S-allyl-Cys-OMe (3.32)
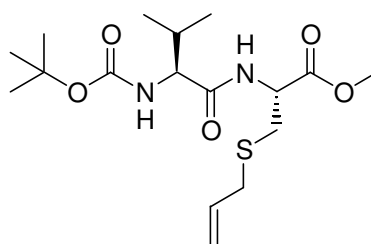
3.32

Compound **3.35** (1.4g, 8mmol) was coupled to (S)-Boc-Val-H.HCl (3.4g, 16mmol) following the general procedure **A** gave pale yellow crude product R_f = 0.30 (1/3 EtOAC / (50/70) petroleum ether). Purification was achieved was achieved by column chromatography, eluting with gradient (50/70) petroleum ether /1:2 (EtOAC / (50/70) petroleum ether) give creamy white solid compound **3.32** (2.7g, 7mmol, 88%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 6.67 (d, 1H, J=6.7Hz, N**H**), 5.72 (m, 1H, **CH**=CH$_2$), 5.10 (m, 2H, **CH$_2$**=CH) 4.77 (dd, 1H, J=5.5Hz, J=12.9Hz, α **CH**), 3.98 (dd, 1H, J=5.5Hz, J=6.6Hz, α **CH**), 3.75 (s, 3H, OC**H$_3$**), 3.10 (m, 2H, SC**H$_2$**), 2.91 (ddd, 1H, J=5.4Hz, J=14.0Hz, J=32.1Hz, **CH$_2$**S), 2.14 (dd, 1H, J=6.8Hz, J=13.1Hz, Val (**CH**)), 1.44 (s, 9H, (C**H$_3$**)$_3$), 0.95 (dd, 6H, J=6.8Hz, J=22.1Hz, Val(C**H$_3$**)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 171.4, 170.9, 155.7, 133.5, 117.9, 79.8, 59.5, 52.5, 51.5, 35.0, 32.3, 30.9, 28.2, 19.1, 17.6;

LRMS [ES$^+$ M + [H$^+$] Calcd for $C_{17}H_{30}N_2O_5S$ 375.5 found 375.3].

Synthesis of H-Val-S-allyl-OMe (3.33)

3.33

N-Boc-Val-S-allyl-Cys-OMe **3.32** (1.1g, 3mmol) was dissolved in 4M HCl in dioxane (20mL) and the resulting solution was leaved to stir for 18h at room temperature. The solvent was removed *in vacuo* to yield crude compound **3.33** (0.8g, 2.9mmol, 97%); ^{1}H NMR (500 MHz (CD$_3$OD) δ 5.79 (tdd, 1H, J=7.2Hz, J=10.0Hz, J=17.1Hz, C**H**=CH$_2$), 5.14 (m, 2H, 2H, C**H**$_2$=CH), 4.63 (dd, 1H, J=5.1Hz, J=8.6Hz, α C**H**), 3.76 (d, 1H, J=5.4Hz, α C**H**),3.73 (s, 3H, OC**H**$_3$), 3.18 (m, 2H, SC**H**$_2$), 2.99 (dd, 1H, J=5.1Hz, J=13.9Hz, β C**H**$_2$), 2.81 (dd, 1H, J=8.6Hz, J=13.9Hz, β C**H**$_2$), 2.23 (m, 1H, Val (C**H**)), 1.08 (m, 6H, Val (C**H**$_3$)$_2$);
LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{12}$H$_{22}$N$_2$O$_3$S 275.1 found 275.1].

Synthesis of N-Boc-O-allyl-Glu-Val-S-allyl-Cys-OMe (3.34)
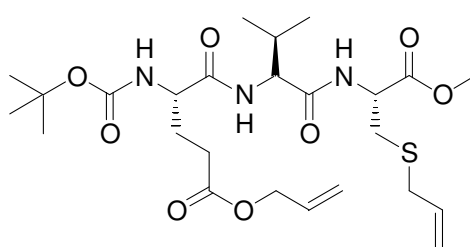
3.34

H-Val-S-allyl-OMe **3.33** (0.8g, 2.9mmol) was coupled to N-Boc-O-allyl-Glu-OH **3.28** (0.4g, 1.4mmol) by the coupling general procedure **A** to give a crude yellow product R$_f$ = 0.29 (1/1 EtOAC/- (50/70) petroleum ether). Purification was achieved by column chromatography, eluting with gradient 1/3 (EtOAC/- (50/70) petroleum ether) to 1/2 (EtOAC/- (50/70) petroleum ether) gave creamy white solid compound **3.34** (0.60g, 1.2mmol, 38%):

^{1}H -NMR (500 MHz) (CDCl$_3$) δ 6.97 (d, 1H, J=7.3Hz, Cys (N**H**)), 6.91 (d, 1H, J=8.2Hz, Val (N**H**)), 5.89 (m, 1H, Glu (C**H**=CH$_2$)), 5.72 (m, 1H, Cys (C**H**=CH$_2$)), 5.42 (d, 1H, J=7.9Hz, Glu (N**H**)),5.28 (ddd, 2H, J=5.9Hz, J=18.6Hz, J=35.9Hz, Glu (C**H**$_2$=CH)), 5.09 (dd, 2H, J=5.8Hz, J=10.3Hz, Cys (C**H**$_2$=CH)), 4.74 (m, 1H, α C**H**), 4.57 (d, 1H J=5.7Hz, OC**H**$_2$), 4.35 (m, 1H, α C**H**), 4.19 (d, 1H, J=5.8Hz, α C**H**), 3.73 (s, 3H, OC**H**$_3$), 3.09 (m, 2H, SC**H**$_2$), 2.87 (ddd, 2H, J=5.5Hz, J=13.9Hz, J=20.1Hz, C**H**$_2$S), 2.47 (m, 2H, γ C**H**$_2$), 2.14 (m, 1H, Val (C**H**)), 2.14 (m, 1H, β C**H**$_2$), 1.94 (dt, 1H, J=7.2Hz, J=14.5Hz, β C**H**$_2$), 1.41 (s, 9H, (C**H**$_3$)$_3$), 0.91 (m,6H, Val (C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 173.4, 171.6, 170.9, 170.7, 155.7, 133.5, 131.6, 118.5, 117.9, 80.1, 65.4, 58.3, 53.2, 52.5, 51.4, 34.9, 32.3, 30.7, 30.5, 28.2, 27.8, 19.1, 17.6;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{25}$H$_{41}$N$_3$O$_8$S 544.6 found 544.3].

Synthesis of S-allyl-Cys-OMe (3.35)

3.35
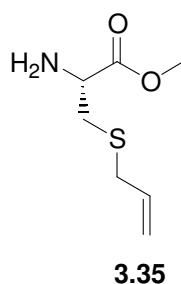
Compound **3.11** (3.5g, 0.012mol) was dissolved in 4M HCl in dioxane (25mL). The reaction was leaved to stir at room temperature for 16h. The solution was then concentrated *in vacuo* to yield white solid. This white solid was dissolved in MeOH (30mL) at 0 ^{0}C and was added dropwised thionyl chloride (3mL). The resulting solution was leaved to stir at room temperature for 20h. The solvent was removed *in vacuo* to give pale yellow compound **3.35** (1.7g, 0.097mol, 79%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 5.81 (tdd, 1H, J=7.2Hz, J=10.0Hz, J=17.1Hz, C**H**=CH$_2$), 5.19 (dd, 2H, J=13.5Hz, J=17.0Hz, C**H**$_2$=CH), 4.27 (dd, 1H, J=4.4Hz, J=7.8Hz, α C**H**), 3.85 (s, 3H, OC**H**$_3$), 3.22 (m, 2H, SC**H**$_2$), 3.10 (dd, 1H, J=4.5Hz, J=14.8Hz, β C**H**), 2.94 (dd, 1H, J=8.0Hz, J=14.8Hz, β C**H**);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 168.3, 133.3, 117.4, 52.6, 51.9, 34.1, 29.9;

LRMS [ES$^+$ M +[H$^+$] Calcd for C$_7$H$_{13}$NO$_2$S 176.2 found 176.1]

Synthesis of N-Boc-Glu-OMe (3.39)

3.39
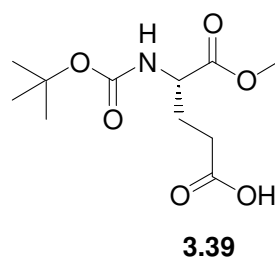
L-H-Glu-OMe (2.7g, 17mmol) and di-tert-butyl dicarbonate (4.4g, 20mmol) were dissolved in dioxane (25mL) and water (25mL). Triethylamine (6mL) was added dropwised and the solution was leaved to stir at room temperature for 15h. The solution was evaporated under reduced pressure. The residue was partition between EtOAc (50mL) and 10% citric acid (25mL). The aqueous phase was re-extracted with EtOAc (2 x 20mL). All the organic phases were combined, washed with brine (2 x 15mL), dried over $MgSO_4$ and the solvent was removed *in vacuo* to give clear oily compound **3.39** (3.5 g, 13.4mmol, 79%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 4.27 (dt, 1H, J=4.8Hz, J=8.6Hz, α **CH**), 3.72 (s, 3H, OC**H**$_3$), 2.35 (t, 2H, J=7.4Hz, γ **CH**$_2$) , 2.11 (td, 1H, J=6.8Hz, J=12.6Hz, β **CH**$_2$) 1.89 (td, 1H, J=8.0Hz, J=22.7Hz, β **CH**$_2$), 1.42 (s, 9H, C(C**H**$_3$)$_3$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 174.2, 173.4, 155.0, 80.1, 52.8, 51.8, 30.1, 28.2, 27.5; LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{11}$H$_{19}$NO$_6$ 262.3 found 262.2]

Synthesis of N-Boc-N-allyl-Gln-OMe (3.40)

3.40
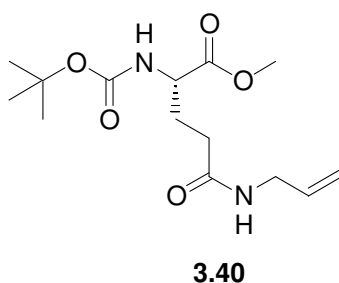
N-Boc-Glu-OMe **3.39** (4.2g, 16mmol) was coupled to ally amine (2.03g, 35.6mmol) according to general procedure **B** to give an oily yellow residue crude product R_f = 0.45 (2/1EtOAc-(50/70) petroleum ether). Purification was achieved using flash chromatography, eluting with a gradient of 1/1 (EtOAc / (50/70) petroleum ether to 100% (EtOAc) to yield purify compound **3.40** (2.64g, 8.8mmol, 55%); ^{1}H NMR (500 MHz) (CDCl$_3$) δ 6.18 (s, 1H, N**H**), 5.82 (ddd, 1H, J=5.4Hz, J=10.6Hz, J=16.5Hz, C**H**=CH$_2$), 5.34 (d, 1H, J=6.4Hz, Boc(N**H**)), 5.14 (dd, 2H, J=13.7Hz, J=33.2Hz CH=C**H**$_2$), 4.27 (dt, 1H, J=4.8Hz, J=8.6Hz, α C**H**), 3.86 (t, 2H, J=4.7Hz, CONHC**H**$_2$), 3.72 (s, 3H, OC**H**$_3$), 2.30 (t, 2H, J=7.4Hz, γ C**H**$_2$) , 2.11 (td, 1H, J=6.8Hz, J=12.6Hz, β C**H**$_2$) 1.89 (td, 1H, J=8.0Hz, J=22.7Hz, β C**H**$_2$), 1.42 (s, 9H, C(C**H**$_3$)$_3$);
^{13}C NMR (75 MHz) (CDCl$_3$) δ 172.7, 171.1, 155.7, 134.1, 116.2, 79.9, 60.3, 53.0, 52.33, 41.9, 32.4, 28.2;
LRMS [ES M [H$^+$] Calcd for C$_{14}$H24N$_2$O$_5$ 301.3 found 301.2.]

Synthesis of N-Boc-N-ally-Gln-OH (3.41)

3.41
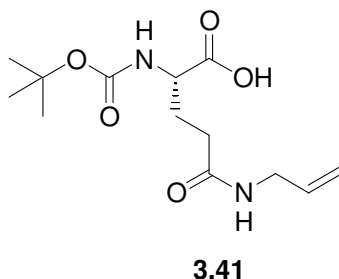
N-Boc-N-allyl-Gln-OMe **3.40** (3.31g, 10.5mol) was hydrolysed using general procedure **C** gave a clear oily crude compound **3.41** (2.2g, 7.7mmol, 73%):
^{1}H NMR (500 MHz) (DMSO) δ 7.94 (t, 1H, J=5.3Hz, N**H**), 7.05 (d, 1H, J=8.0Hz, Boc (N**H**)), 5.75 (ddd, 1H, J=5.0Hz, J=10.2Hz, J=15.9Hz, C**H**=CH$_2$), 5.07 (dd, 2H, J=13.6Hz, J=34.9Hz, CH=C**H**$_2$), 3.83 (dt, 1H, J=4.8Hz, J=8.6Hz, α C**H**), 3.65 (t, 2H, J=5.0Hz, CONHC**H**$_2$), 2.18 (t, 2H, J=7.6Hz, γ C**H**$_2$), 1.89(m, 1H, β C**H**$_2$), 1.71 (dt, 1H, J=7.4Hz, J=16.1Hz, β C**H**$_2$), 1.36 (s, 9H, C(C**H**$_3$)$_3$));
^{13}C NMR (75 MHz) (DMSO) δ 173.9, 171.3, 155.7, 135.4, 114.9, 78.0, 59.8, 53.1, 31.7, 28.2, 26.7;
LRMS [ES M + [H$^+$] Calcd for C$_{13}$H$_{22}$N$_2$O$_5$ 287.3 found 287.2].

Synthesis of N-Boc-allyl-Gln-Val-OMe (3.42)

3.42
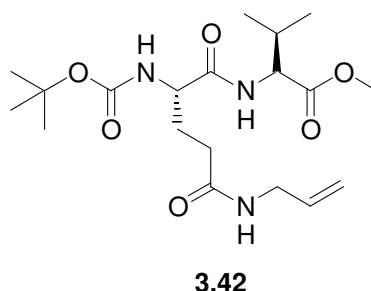
N-Boc-ally-Gln-OH **3.41** (1.1g, 3.85mmol) was coupled to Val(OMe).HCl (1.3g, 7.7mmol) according to general procedure **A** gave white solid crude product R_f = 0.27 (2/1EtOAc-(50/70) petroleum ether). Purification was achieved using flash chromatography, eluting with a gradient of 1/1 (EtOAc / (50/70) petroleum ether to 100% EtOAc) to yield purified compound **3.42** (0.8g, 2mmol, 52%):

^{1}H NMR (500 MHz) (DMSO) δ 7.95 (d, 1H, J=5.7Hz, Val (N**H**)), 7.93 (d, 1H, J=6.7Hz, N**H**), 6.88 (d, 1H, J=8.2Hz, BOC (N**H**)), 5.76 (ddd, 1H, J=5.2Hz, J=10.4Hz, J=22.3Hz, **CH**=CH$_2$), 5.05 (ddd, 2H, J=1.5Hz, J=13.7Hz, J=11.6Hz, CH=**CH**$_2$), 4.17 (dd, 1H, J=6.3Hz, J=8.1Hz, α Val (C**H**)), 4.02 (t, 1H, J=7.1Hz, α Gln (C**H**)), 3.65 (t, 2H, J=5.4 CONH**CH**$_2$), 3.61 (s, 3H, OC**H**$_3$), 2.15 (m, 2H, γ **CH**$_2$), 2.02 (m, 1H, Val (C**H**)), 1.83-1.67 (m, 2H, β **CH**$_2$), 1.35 (s, 9H, C(C**H**$_3$)$_3$)), 0.83 (m, 6H, Val(C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (DMSO) δ 172.2, 171.9, 171.5, 155.3, 135.4, 114.9, 78.1, 59.8, 57.2, 53.9, 51.8, 40.8, 31.8, 30.1, 28.2, 18.9, 18.1;

LRMS [ES M+ [H$^+$] Calcd for C$_{19}$H$_{33}$N$_3$O$_6$ 400.4 found 400.3].

Synthesis of N-Boc-N-allyl-Gln-Val-OH (3.43)

3.43
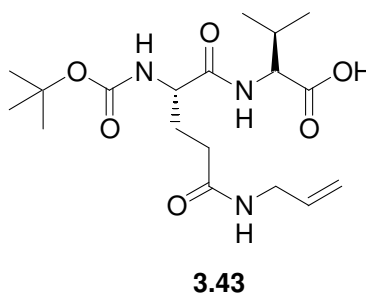
N-Boc-N-allyl-Gln-OMe **3.42** (2.2g, 5.6mol) was hydrolysed according to general procedure **C** to give a clear oily compound **3.37** (1.5g, 3.9mmol, 70%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 5.83 (ddd, 1H, J=5.5Hz, J=10.6Hz, J=22.5Hz, **CH**=CH$_2$), 5.13 (ddd, 2H, J=1.4Hz, J=13.7Hz, J=11.6Hz, CH=**CH$_2$**), 4.33 (d, 1H, J=5.3Hz, α Val (**CH**)), 4.10 (m, 1H, α Gln (**CH**)), 3.79 (d, 2H, J=1.2Hz, CONH**CH$_2$**), 2.33 (t, 2H, J=7.2Hz, γ **CH$_2$**), 2.19 (m, 1H, Val(**CH**)), 2.06 (dt, 1H, J=7.0Hz, J=13.9Hz, β **CH$_2$**), 1.87 (dt, 1H, J=7.9Hz, J=15.5Hz, β **CH$_2$**), 1.43 (s, 9H, C(**CH$_3$**)$_3$), 0.97 (m, 6H, Val(**CH$_3$**)$_2$);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 173.5, 173.3, 170.1, 156.4, 134.1, 114.9, 79.3, 57.5, 53.9, 41.4, 31.8, 30.5, 27.8, 27.3, 18.2, 16.8;

LRMS [ES M+ [H$^+$] Calcd for C$_{18}$H$_{31}$N$_3$O$_6$ 386.4 found 386.3].

Synthesis of N-Boc-allyl-Gln-Val-ally-Gly-OMe (3.44)

3.44
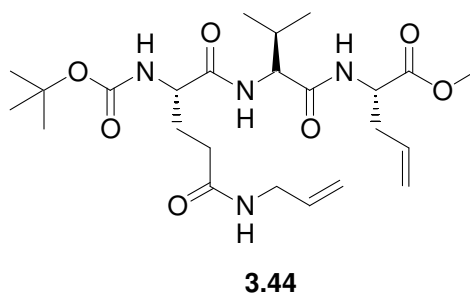
N-Boc-N-ally-Gln-Val-allyl-Gly-OME **3.43** (0.26g, 0.68mmol) was coupled with (S)-allyl-Gly-OMe **3.46**(0.17g, 1.35mmol) following general procedure **A** gave pale red residue R$_f$ = 0.5 (100% EtOAc). Purification was achieved using flash chromatography, eluting with a gradient of 1/1 (EtOAc / (50/70) petroleum ether to 100% (EtOAc) to yield white solid compound **3.44** (0.1g, 0.20mmol, 30%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 5.80 (m, 2H, **CH**=CH$_2$), 5.12 (m, 2H, CH=**CH$_2$**), 4.45 (m, 1H, α Gly (**CH**)), 4.24 (m, 1H, α Val (**CH**)), 4.07 (dd, 1H, J=4.5Hz, J=8.9Hz, α Gln (**CH**)), 3.79 (t, 2H, J=4.5Hz, CONH**CH$_2$**), 3.69 (s, 3H, O**CH$_3$**), 2.53 (m, 2H, Gly (**CH$_2$**)), 2.29 (t, 2H, J=7.6Hz, β Gln **CH$_2$**)), 2.03 (m, 2H, Val (**CH**), β Gln (**CH**)), 1.86 (m, 1H, β Gln (**CH**)), 1.43 (s, 9H, C(**CH$_3$**)$_3$), 0.95 (m, 6H, Val(**CH$_3$**)$_2$));

^{13}C NMR (75 MHz) (CD$_3$OD) 173.5, 172.7, 171.8, 171.4, 156.7, 133.6, 132.1, 117.9, 114.5, 79.8, 58.2, 53.9, 52.8, 51.7, 41.3, 35.5, 31.9, 31.2, 27.9, 27.2, 18.2, 17.3; LRMS [ES M+ [H$^+$] Calcd for C$_{24}$H$_{40}$N$_4$O$_7$ 497.6 found 496.4].

Synthesis of ally-Gly-OMe (3.46)
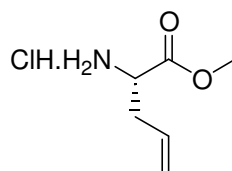
3.46

(S)-allyl-Gly-OH (1.00g, 8.69 mmol) was suspended in methanol (30 mL). This was cooled to 0°C and SOCl$_2$ (5.0 mL) was added portionwise. The solution was stirred at 0°C for 1hour and then at room temperature for 18 hours before being concentrated *in-vacuo* to yield a white solid compound **3.40** 1.36g,8.24mmol, 95%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 5.78 (dddd, 1H J=7.2Hz, J=7.2Hz, J=7.1Hz, J=10.0Hz, **CH=**CH$_2$), 5.28 (dd, 2H, J=13.1Hz, J=13.1Hz, CH=**CH$_2$**), 4.16 4.15 (dd, 1H, J=5.4Hz, J=7.0Hz, α **CH**), 3.84 (s, 3H, O**CH$_3$**), 2.68 (m, 2H, **CH$_2$**);
^{13}C NMR (75 MHz) (CD$_3$OD) δ 169.1, 130.3, 120.3, 52.1, 47.1, 34.4;
LRMS [ES M+ [H$^+$] Calcd for C$_6$H$_{11}$NO$_2$ 130.2 found 130.1].

Synthesis of N-Cbz-N-allyl-Gln-OMe (3.48)
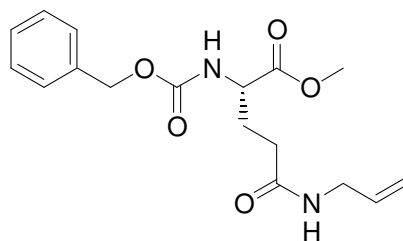
3.48

N-Cbz-Glu-OMe (5.0g, 17mmol), was coupled to ally amine (1.94g, 34mmol) following general method **B** to give clear oily crude compound R_f = 0.32 (2/1 (EtOAc / (50/70) Petroleum ether)). Purification was achieved by column chromatography, eluting with gradient (50/70) petroleum ether)-100% EtOAc to give white solid compound **3.48** (5g, 15mmol, 88%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 7.31 (m, 5H, Ar,) 5.80 (ddd, 1H, J=5.5Hz, J=10.6Hz, J=22.4Hz, **CH**=CH$_2$), 5.12 (m, 4H, **CH$_2$**=CH, Z- **CH$_2$**), 4.20 (dd, 1H, J=5.0Hz, J=9.2Hz, α **CH**), 3.77 (d, 2H, J=4.7Hz, CONH**CH$_2$**), 3.70 (s, 3H, O**CH$_3$**), 2.31 (t, 2H, J=7.5Hz, γ **CH$_2$**), 2.15 (td, 1H, J=7.6Hz, J=13.0Hz, β **CH$_2$**), 1.93 (ddd, 1H, J=7.4Hz, J=14.5Hz, J=16.3Hz, β **CH$_2$**);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 173.1, 172.7, 157.2, 136.7, 134.1, 128.1, 127.7, 127.4, 117.2, 66.3, 53.6, 51.2, 41.2, 31.7, 27.1;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{17}$H$_{22}$N$_2$O$_5$ 335.4 found 335.3].

Synthesis of N-Cbz-N-allyl-Gln-OH (3.49)

3.49
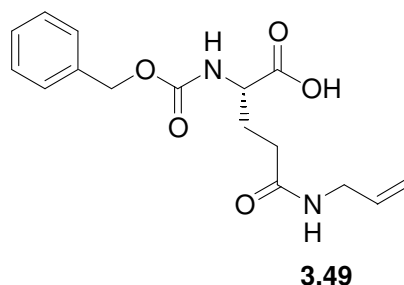
N-Cbz-N-allyl-Gln-OMe **3.48** (5g, 15mmol) was hydrolysed following the general procedure **C** to give oily yellow compound **3.49** (3.7g, 11.4mmol, 76%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 7.31 (m, 5H, Ar,) 5.80 (ddd, 1H, J=5.5Hz, J=10.6Hz, J=22.4Hz, **CH**=CH$_2$), 5.12 (m, 4H, **CH$_2$**=CH, Z- **CH$_2$**), 4.20 (m, 1H, α **CH**), 3.77 (d, 2H, J=4.7Hz, CONH**CH$_2$**), 2.31 (t, 2H, J=7.5Hz, γ **CH$_2$**), 2.15 (m, 1H, β **CH$_2$**), 1.93 (m, 1H, β **CH$_2$**);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 172.3, 171.6, 155.6, 135.1, 132.4, 126.4, 126.3, 126.0, 115.6, 64.6, 51.9, 39.2, 30.2, 25.7; LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{16}$H$_{20}$N$_2$O$_5$ 321.3 found 321.2]
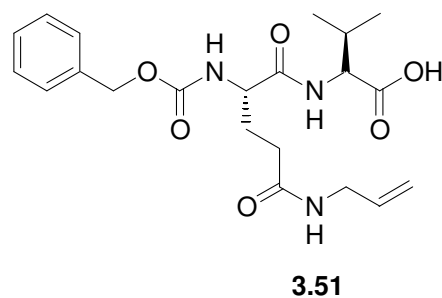
Synthesis of N-Cbz-N-allyl-Gln-Val-OMe (3.50)

3.50
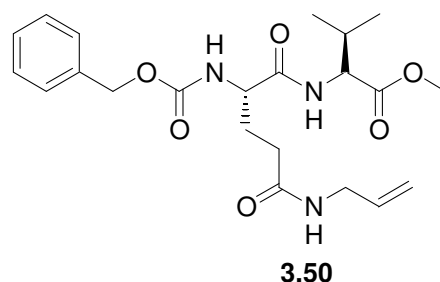
N-Cbz-N-allyl-Gln-OH **3.49** (3.66g, 11.4mmol) was coupled to (S)-Val-OMe.HCl (3.83g, 23mmol) according to the general procedure **A** method gave crude product R_f = 0.43. Purification was achieved by column chromatography, eluting with gradient 1/3 (ethyl acetate/ (50/70) petroleum ether) – 2/1 (ethyl acetate/ (50/70) petroleum ether) over 16min to give compound **3.50** (4g, 9.2mmol, 80%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 7.31 (tdd, 5H, J=6.84Hz, J=9.28Hz, J=6.35, Ar), 5.80 (ddd, 1H, J=5.38Hz, J=10.6Hz, J=16.4Hz, **CH**=CH$_2$), 5.12 (m, 4H, **CH$_2$**=CH, Z-**CH$_2$**), 4.35 (m, 1H, Val α **CH**), 4.23 (dd, 1H, J=5.86Hz, J=8.31Hz, Gln α **CH**), 4.0-3.77 (m, 3H, CONH**CH$_2$**,O**CH$_3$**), 2.56 (m, 2H, γ **CH$_2$**), 2.2-2 (m, 2H, Val (**CH**), β **CH$_2$**), 1.93 (m, 1H, β **CH$_2$**);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 173.4, 173.2, 172.3, 157.3, 136.0, 134.0, 128.2, 127.7, 127.5, 117.1, 66.4, 57.9, 54.3, 51.3, 41.5, 31.6, 30.4, 27.9, 18.1, 17.3;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{22}$H$_{31}$N$_3$O$_6$ 434.5 found 434.3]

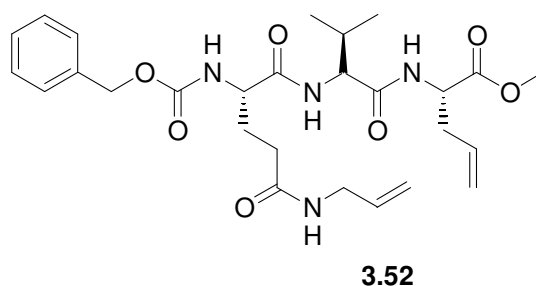**Synthesis of N-Cbz-N-allyl-Gln-Val-OH (3.51)**

3.51

N-Cbz-N-allyl-Gln-Val-OMe **3.51** (4g, 9.2mmol) was hydrolysed according to general procedure **C** to give a white solid compound **3.51** (1.6g, 3.8mmol, 41%):

^{1}H NMR (500 MHz) (CD$_3$OD) δ 7.31 (m, 5H, Ar), 5.80 (ddd, 1H, J=5.5Hz, J=10.6Hz, J=22.4Hz, **CH=CH$_2$**), 5.12 (m, 4H, **CH$_2$**=CH, Z-**CH$_2$**), 4.35 (m, 1H, Val α **CH**), 4.20 (dd, 1H, J=5.0Hz, J=9.2Hz, Gln α **CH**), 3.77 (d, 2H, CONH**CH$_2$**), 2.31 (t, 2H, J=7.5Hz, γ **CH$_2$**), 2.20 (m, 1H, Val (**CH**)), 2.15-1.93 (m, 2H, β **CH$_2$**), 0.91 (m, 6H, Val (**CH$_3$**)$_2$);

^{13}C NMR (75 MHz) (CD$_3$OD) δ 177.3, 177.2, 176.7, 161.4, 140.1, 137.6, 132.2, 131.7, 131.5, 119.5, 70.6, 61.6, 61.5, 45.5, 35.8, 34.2, 32.2, 22.3, 20.9;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{21}$H$_{29}$N$_3$O$_6$ 420.5 found 420.3].

## Synthesis of N-Cbz-N-allyl-Gln-Val- allyl-Gly -OMe (3.52)

3.52

To a stirring solution of N-Cbz-N-allyl-Gln-Val-OH **3.51** (1.53g, 3.65mmol), (S)-allyl-Gly-OMe **3.46** (1.2g, 7.3mmol) and HATU (1.53g, 4.0mmol) in DMF (30mL) was added DIPEA (2.5mL, 14.6mmol). The orange solution was leaved to stir at room temperature for 15h before being partition with EtOAC and 1M HCl$_{(aq)}$. The partition resulted in a white solid formation between the two layers which was collected using Buchner funnel gave compound **3.52** (1.6g, 3.0mmol, 83% yield):

^{1}H NMR (500 MHz) (DMSO) δ 7.31 (m, 5H, Ar), 5.80 (m, 2H, J=5.5Hz, J=10.6Hz, J=22.4Hz, 2(**CH=CH$_2$**)), 5.12 (m, 6H, 2(**CH$_2$**=CH), Z-**CH$_2$**), 4.37 (m, 1H, Gly α **CH**), 4.20 (t, 1H, J=5Hz, Gly α **CH**), 4.02 (dd, 1H, J=5.0Hz, J=9.2Hz, Gln α **CH**), 3.62 (d, 2H, CONH**CH$_2$**), 3.59 (s, 3H, O**CH3**), 2.39, (m, 2H, Gly (**CH$_2$**)), 2.18 (m, 2H, γ **CH$_2$**), 1.98 (m, 1H, Val(**CH**)), 1.89-1.75 (m, 2H, β **CH$_2$**), 0.91 (m, 6H, Val (**CH$_3$**)$_2$);

^{13}C NMR (75 MHz) (DMSO) δ 171.7, 171.5, 171.1, 170.5, 155.9, 136.5, 135.3, 133.6, 128.4, 127.8, 127.6, 117.9, 114.9, 65.4, 61.6, 57.0, 54.4, 51.9, 51.7, 34.9, 31.9, 30.9, 28.1, 19.0, 17.8;

LRMS [ES$^+$ M + [H$^+$] Calcd for $C_{27}H_{38}N_4O_7$ 531.4 found 531.3].
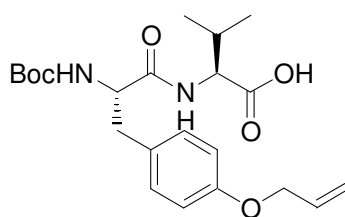
Synthesis of N-Boc-O-allyl-Tyr-Val-OMe (3.54)
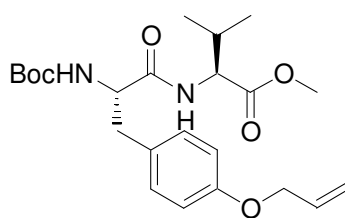
3.54

N-Boc-O-allyl-Tyr (5g, 16.3mmol) was coupled to (s)-Val-OMe.HCl (5.4g, 32.5mmol) according to general procedure **B**. The crude product was purified using column chromatography R$_f$ value = 0.25 in 1/5 EtOAc/ (50/70) Pet ether, eluting with a gradient of (50/70) petroleum ether to 100% EtOAc to yield a white solid compound **3.54** (2.8g, 6.52mmol, 40%):

^{1}H NMR (500 in CDCl$_3$) δ 7.09 (2H, d J=6.5Hz, Ar-**H**), 6.80 (2H, d J=6.4Hz, Ar-**H**), 6.47 (1H, bs, Val (N**H**)), 5.97-6.05 (1H, m, C**H**=CH$_2$), 5.30 (2H, ddd J=1.1Hz, J=13.9Hz, J=11.3Hz, CH=C**H$_2$**), 5.12 (1H, bs, Tyr (N**H**)), 4.47-4.49 (2H, m, OC**H$_2$**CH=CH$_2$), 4.43 (1H, dd J=6.3Hz, J=7.3Hz, Tyr α C**H**), 4.30-4.34 (1H, m, Val α (C**H**)), 3.66 (3H, s, OC**H$_3$**), 2.98 (2H, d J=3.4Hz, CHC**H$_2$**Ph), 2.04-2.11 (1H, m, CHC**H**(CH$_3$)$_2$), 1.39 (9H, s, (C(C**H$_3$**)$_3$)), 0.83 (6H, dd J=6.7Hz, J=14.6Hz, Val (C**H$_3$**)$_2$);

^{13}C NMR (75 MHz in CDCl$_3$) 174.7, 171.9, 157.5, 133.2, 130.3, 128.6, 117.6, 114.8, 68.7, 57.2, 37.0, 31.0, 28.2, 18.8, 17.6;

LRMS [ES$^+$ M + [H$^+$] Calcd for $C_{23}H_{34}N_2O_6$ 435.3 found 435.3]

Synthesis of N-Boc-O-allyl-Tyr-Val-OH (3.55)

3.55

N-Boc-O-allyl-Tyr-Val-OMe **3.54** (2.5g, 5.75mmol) was hydrolysed using general procedure **C** to give white solid compound **3.55** (1.88g, 4.48mmol, 78%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.10 (d, 2H, J=8.4Hz, 2H Ar), 6.82 (d, 2H, J=8.2Hz, 2H Ar), 6.70 (d, 1H, J=5.8Hz, N**H** (Val)), 6.03 (m, 1H, C**H**=CH$_2$), 5.32 (dd, 2H, J=14.3Hz, J=60.7Hz, CH=C**H**$_2$), 4.97 (d, 1H, J=7.7Hz, N**H** (Tyr)), 4.56 (d, 1H, J=5.8Hz, α C**H**(Val)), 4.49 (dd, 2H, J=6.8Hz, J=12.3Hz, O-C**H**$_2$), 4.39 (d, 1H, J=4.3Hz,α C**H** (Tyr)), 3.08 (m, 2H, C**H**$_2$), 2.19 (qd, 1H, J=6.7Hz, J=13.7Hz, C**H**(Val)), 1.39 (s, 9H, (C(C**H**$_3$)$_3$)), 0.90 (dd, 6H, J=6.8Hz, J=13.3Hz, Val (C**H**$_3$)$_2$);

^{13}C NMR (75MHz) (CDCl$_3$) δ 174.9, 171.8, 157.5, 155.8, 133.2, 130.3, 128.5, 117.5, 114.7, 80.3, 68.7, 57.1, 55.8, 37.0, 30.9, 28.1, 18.8, 17.5;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{22}$H$_{32}$N$_2$O$_6$S 421.4 found 421.3].

Synthesis of N-Boc-O-allyl-Glu-OMe (3.56)
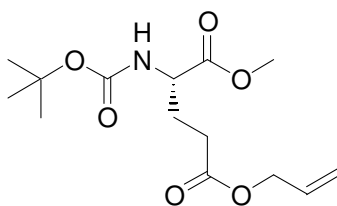
3.56

To a stirred solution of N-Boc-O-allyl-Glu-OH **3.28** (5.8g, 20mmol) in diethyl ether (40mL) at 0^{0}C was added dropwised excess diazomethane. The solution was leaved to stir at 0^{0}C for 1h and the excess diazomethane was purges with argon for15-20min. The solution was removed *in vacuo* to give clear oily yellow crude compound R$_f$ = 0.3

(1/3 (EtOAc/(50/70) Pet ether)). Purification was achieved using flash chromatography, eluting with a gradient of 1/5 (EtOAc/ (50/70) Pet ether to 100% (EtOAc) to yield purify compound **3.56** (3.6g, 12mmol, 60%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 5.89 (ddd, 1H, J=5.7Hz, J=10.4Hz, J=22.9Hz, C**H**=CH$_2$), 5.26 (ddd, 2H, J=1.3Hz, J=13.8Hz, J=11.6Hz, CH=C**H**$_2$), 5.12 (d, 1H, J=7.6Hz, N**H**) 4.57 (d, 2H, J=5.8Hz, O-C**H**$_2$), 4.33 (d, 1H, J=5.1Hz, α C**H**), 3.73 (s, 3H, C**H**$_3$O), 2.42 (m, 2H, γ C**H**$_2$), 2.18 (dt, 1H, J=7.1Hz, J=13.6Hz, β C**H**$_2$),1.94 (dt, 1H, J=8.3Hz, J=14.6Hz, β C**H**$_2$), 1.42 (s, 9H, C(C**H**$_3$)$_3$);

^{13}C NMR (75 MHz) δ 173.0, 172.7, 155.7, 132.4, 118.8, 80.1, 65.7, 53.2, 52.8, 30.6, 28.6, 28.1;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{14}$H$_{23}$NO$_6$ 302.3 found 302.1]

Synthesis of O-allyl-Glu-OMe (3.57)
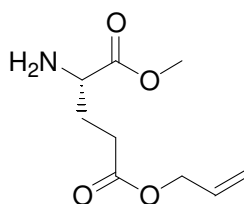
3.57

N-Boc-O-allyl-Glu-OMe **3.56**(2.9g, 12mmol) was dissolved in 2M HCl in diethyl ether (12mL). The resulting reaction was leaved to stir for 18h and the solvent was removed *in vacuo* to give yellow solid compound **3.57** (2.4g, 12mmol, 100%):

^{1}H NMR (500 MHz) (DMSO) δ 8.72 (bs, 2H, N**H**), 5.89 (ddd, 1H, J=5.5Hz, J=10.6Hz, J=22.5Hz, C**H**=CH$_2$), 5.24 (dd, 2H, J=14.1Hz, J=43.9Hz, CH=C**H**$_2$), 4.54 (d, 1H, J=5.4Hz, OC**H**$_2$), 4.02 (d, 1H, J=4.6Hz, α C**H**), 3.71 (s, 3H, OC**H**$_3$), 2.56 (m, 2H, γ C**H**$_2$), 2.06 (m, 2H, β C**H**$_2$);

^{13}C NMR (75 MHz) δ 171.4, 169.5, 132.6, 117.9, 64.6, 52.8, 51.1, 29.0, 25.1;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_9$H$_{15}$NO$_4$ 202.2 found 202.1].

Synthesis of N-Boc-O-allyl-Tyr-Val-O-ally-Glu-OMe (3.58)
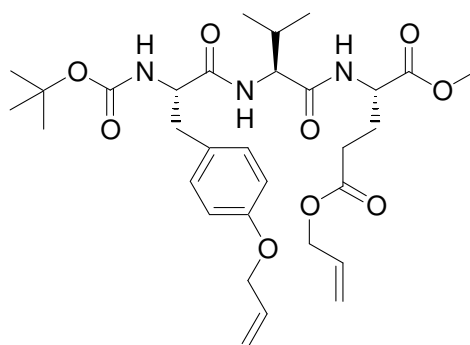

3.58

N-Boc-O-allyl-Tyr-Val-OH **3.55** (1g, 2.4mmol) was coupled to O-allyl-Glu-OMe **3.57** (0.9g, 4.6mmol) using general procedure **A** to give a yellow crude compound R_f = 0.5 (1/1 (EtOAc / (50/70) Pet ether). Purification was achieved using flash chromatography, eluting with a gradient of 1/3 (EtOAc / (50/70) Pet ether to 100% (EtOAc) to yield a white solid compound **3.58** (0.7g, 1.2mmol, 50%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.10 (d, 2H, J=8.5Hz, **CH$_2$** Ar), 6.87 (d, 2H, J=6.8Hz, Val (N**H**)), 6.83 (d, 2H, J=8.5Hz, **CH$_2$** Ar), 6.58 (d, 1H, J=8.3Hz, Glu (N**H**)), 6.03 (ddd, 1H, J=5.3Hz, J=10.5Hz, J=22.5Hz, Tyr (**CH**= CH$_2$)), 5.89 (ddd, 1H, J=5.9Hz, J=11.2Hz, J=16.3Hz, Glu (**CH**=CH$_2$)), 5.38 (dd, 2H, J=16.3Hz, J=32.1Hz, Tyr (CH=**CH$_2$**)), 5.24 (dd, 2H, J=10.1Hz, J=23.6Hz, Glu (CH=**CH$_2$**)), 5.02 (d, 1H, J=5.0Hz, Tyr (N**H**)), 4.57 (d, 2H, J=5.7Hz, Glu (**CH$_2$**CH=CH$_2$)), 4.53 (d, 1H, J=5.2Hz, Glu α (**CH**)), 4.50 (d, 2H, J=5.3Hz, Tyr (**CH$_2$**CH=CH$_2$)), 4.32 (d, 1H, J=4.5Hz, Tyr α (**CH**)), 4.25 (dd, 1H, J=6.4Hz, J=8.0Hz, Val α (**CH**)), 3.73 (s, 3H, O**CH$_3$**), 3.01 (m, 2H, Tyr β (**CH$_2$**)), 2.42 (m, 2H, γ **CH$_2$**), 2.20 (td, 1H, J=7.5Hz, J=12.9Hz, β **CH$_2$**), 2.14 (m, 1H, Val (**CH**)), 2.02 (dt, 1H, J=7.8Hz, J=14.5Hz, β **CH$_2$**), 1.39 (s, 9H, C(**CH$_3$**)$_3$), 0.88 (dd, 6H, J=6.8Hz, J=17.0Hz, Val (**CH$_3$**)$_2$);

^{13}C NMR (75MHz) (CDCl$_3$) δ 172.5, 171.8, 171.4, 170.7, 157.5, 155.7, 133.2, 131.9, 130.2, 128.5, 118.4, 117.6, 114.7, 80.1, 68.7, 65.4, 58.4, 55.8, 52.4, 51.7, 37.3, 30.7, 30.1, 28.2, 26.7, 18.9, 17.7;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{31}$H$_{45}$N$_3$O$_9$ 604.3 found 604.3];

mp= 83-87 ^{0}C.

Synthesis of N-Boc-O-allyl-Tyr-Leu-OMe (3.60)

3.60
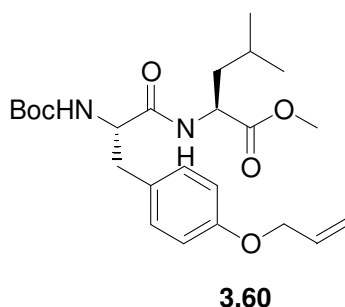
N-Boc-O-allyl-Tyr-H (18.6g, 60.5mmol), HATU (25.3g, 67mmol) and Leu-OMe **3.59** (22g, 121mmol) were dissolved in anhydrous DMF (100mL) at 0^0C. To this DIPEA (42.2mL, 242mmol) was added. The yellow solution was stirred at room temperature, for 18h. The reaction mixture was partitioned between ethyl acetate and 1M HCl$_{(aq)}$. The organic phase was then washed sequentially with 1M HCl$_{(aq)}$ and brine before being dried (MgSO$_4$), filtered and concentrated *in vacuo* to give yellow crude product R$_f$ = 0.33 (1/3 (EtOAc / (50/70) Petroleum ether)). Purification was achieved by flash column chromatography. The column was eluting with a gradient of 1/5 (EtOAc/ (50/70) Petroleum ether) to 100% EtOAc to give a white solid compound **3.60** (16g, 35.7mmol, 59%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.02 (d, 2H, J=8.1 Hz, Ar), 6.77 (d, 1H, J=5.4Hz, N**H**), 6.73 (d, 2H, J=8.0, Ar), 5.94 (dq, 1H, J=4.9Hz, J=10.2Hz, C**H**=CH$_2$), 5.30 (d, 2H, J=17.2Hz, CH=C**H**$_2$), 5.17 (d, 1H, J=10.5Hz, N**H**), 4.49 (m, 1H, Tyr α (C**H**)), 4.5 (d, 2H, 4.2 Hz OC**H**$_2$), 4.34 (d, 1H, J=4.7Hz Leu α (C**H**)), 3.61(s, 3H, OC**H**$_3$) 2.90 (ddd, 2H, J=6.6Hz, J=13.7Hz, J=20.9Hz C**H**$_2$-Ar), 1.51 (m, 2H, C**H**$_2$-CH-(CH$_3$)$_2$) 1.43 (dd, 1H, J=9.2Hz, J=17.7Hz, CH$_2$-C**H**-(CH$_3$)$_2$), 1.32 (s, 9H, C(C**H**)$_3$) 0.81 (m, 6H, Leu (C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 174.1, 172.8, 171.3, 157.3, 155.3, 133.2, 130.3, 128.8, 117.2, 114.5, 79.6, 68.5, 55.5, 52.0, 50.0, 41.1, 37.3, 28.1, 24.5, 22.6, 21.7;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{24}$H$_{36}$N$_2$O$_6$ 449.6 found 449.6].

Synthesis of O-allyl-Tyr-Leu-OMe (3.61)

3.61
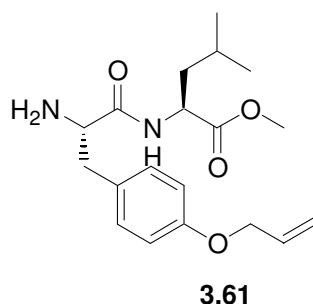
To N-Boc-O-allyl-Tyr-Leu-OMe **3.60** (15.7g, 35mmol) was added 4M HCl in 1,4-dioxane (80mL). This was stirred at room temperature for 16 hours and the solution was then concentrated *in-vacuo* to yield a white solid **3.61** (12.2 g, 100%):

^{1}H NMR (500 MHz in CDCl$_3$) 8.28 (bs, 2H, N**H$_2$**), 7.67 (d, 1H, J=6.4Hz Leu N**H**), 7.27 (d, 2H, J=8.4Hz, Tyr Ar-**H**), 6.82 (d, 2H, J=8.4Hz, Tyr Ar-**H**), 6.00 (ddd, 1H, J=5.2Hz, J=10.5Hz, J=22.2Hz, **CH**=CH$_2$), 5.31 (dd, 2H, J=14.5Hz, J=59.7Hz, CH=**CH$_2$**), 4.60 (1H, m, Tyr α **CH**), 4.44 (d, 2H, J=5.2Hz, O**CH$_2$**), 4.32 (m, 1H, Leu **CH**), 3.63 (s, 3H, O**CH$_3$**), 3.36 (dd, 1H, J=4.3Hz, J=13.9Hz Tyr **CH$_2$**), 3.20 (1H, dd J=8.2Hz, J=14.0Hz, Tyr **CH$_2$**), 1.56 (m, 3H, Leu **CH$_2$**CH and Leu CH$_2$CH), 0.83 (m, 6H, Leu C(**CH$_3$**)$_2$);

^{13}C NMR (75 MHz in CDCl$_3$). 172.4, 168.2, 157.9, 133.1, 131.0, 126.2, 117.5, 114.9, 68.6, 67.0, 54.6, 52.2, 51.4, 40.1, 36.2, 24.5, 22.5, 21.9;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{19}$H$_{28}$N$_2$O$_4$ 349.5 found 349.4].

Synthesis of 4-F-Ph-SO2-N-Tyr-O-Allyl-Leu-OMe (3.62)

3.62
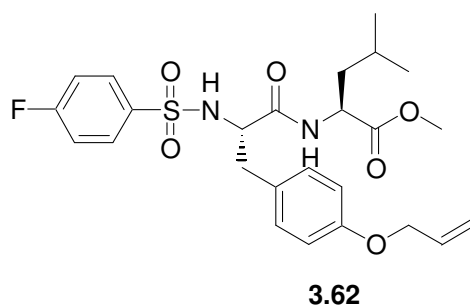
Tyr-O-allyl-Leu-OMe.HCl **3.61** (15.7g, 45mmol) and 4-fluoro-benzene sulfonyl chloride (7.97g, 40.9mmol) were dissolved in anhydrous DCM (100mL). To this

DIPEA (15.7mL, 90mmol) was added. This was stirred at room temperature for 18 hours before being concentrated *in-vacuo*. The residue was then partitioned between EtOAC and 1M $HCl_{(aq)}$. The organic phase was then washed sequentially with 1M $HCl_{(aq)}$ and brine before being dried ($MgSO_4$), filtered and concentrated *in-vacuo*. $R_f =$ 0.42 (1/2 (EtOAc / (50/70) Petroleum ether)). Purification was achieved using flash chromatography, eluting with gradient of (50/70) Pet ether to 1/1 (EtOAc / (50/70) Pet ether to yield a dark yellow compound **3.62** (12.57g, 24.9mmol, 61%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.69 (2H, dd J=5.0Hz, J=8.9Hz, Ar-**H** (4-F-Ph)), 7.08 (2H, t, J=8.6Hz, Ar-**H** (4-F-Ph)), 6.91 (2H, d J=8.6Hz, Ar-**H** (Tyr)), 6.73 (2H, d J=8.6Hz, Ar-**H** (Tyr)), 6.42 (1H, d J=8.2Hz, Leu (N**H**)), 6.05 (1H, ddd J=5.3Hz, J=10.6Hz, J=22.6Hz, **CH**=CH$_2$), 5.45 (1H, d, J=7.93Hz, Tyr (N**H**)), 5.40 (2H, ddd J=15.46Hz, J=46.39Hz, J=10.3Hz, CH=C**H**$_2$), 4.49 (3H, m, O-C**H**$_2$, and α C**H** (Leu)), 3.86 (1H, dd, J=6.8Hz, J=13.8Hz α C**H** (Tyr)), 3.71 (3H, s, O-C**H**$_3$), 2.92 (2H, m, C**H**$_2$ (Tyr)), 1.55 (1H, m,C**H**CH$_2$ Leu), 1.42 (1H, m,CHC**H**$_2$Leu), 0.88 (6H, d, J=6.2Hz Leu (C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 172.5, 169.9, 166.6, 163.2, 157.7, 135.2, 135.1, 133.0, 130.2, 129.8, 129.7, 127.2, 117.7, 116.3, 116.0, 114.8, 68.6, 57.9, 52.3, 50.9, 41.3, 37.7, 24.6, 22.5, 21.8;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{25}$H$_{31}$FN$_2$O$_6$S 507.3 found 507.2]

## Synthesis of 4-F-Ph-SO2-N-Tyr-O-Allyl-Leu-OH (3.63)

3.63
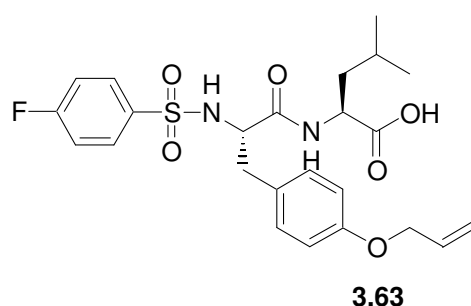
N-4-F-Ph-SO$_2$-O-allyl-Tyr-Leu-OMe **3.62** (11.96, 23.7mmol) was hydrolysed according to general procedure **B** to yield a yellow compound **3.63** (11g, 22.3mmol, 94%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.69 (2H, dd J=4.75Hz, J=8.32Hz, Ar-**H** (4-F-Ph)), 7.08 (2H, t, J=8.72Hz, Ar-**H** (4-F-Ph)), 6.97 (1H, d J=8.32Hz, Leu (N**H**)), 6.91 (2H, d J=8.33Hz, Ar-**H** (Tyr)), 6.73 (2H, d J=8.33Hz, Ar-**H** (Tyr)), 6.05 (1H, ddd J=5.17Hz, J=10.3Hz, J=15.86Hz, C**H**=CH$_2$), 5.45 (1H, d, J=7.93Hz, Tyr (N**H**)), 5.40 (2H, ddd J=17.05Hz, J=42.82Hz, J=10.7Hz, CH=C**H**$_2$), 4.45 (1H, dd, J=8.32Hz, J=13.5Hz α C**H** (Leu)), 4.42 (2H, d, J=4.36Hz, OC**H**$_2$),3.95 (1H, dd, J=7.93Hz, J=13.48Hz α C**H** (Tyr)), 3.71 (3H, s, OC**H**$_3$), 2.92 (2H, m, C**H**$_2$ (Tyr)), 1.55 (1H, m,C**H**CH$_2$ Leu), 1.42 (1H, m,CHC**H**$_2$Leu), 0.88 (6H, d, J=6.2Hz Leu (C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 175.7, 170.9, 166.6, 163.2, 157.7, 135.1, 135.0,133.0, 130.2, 129.8, 129.7, 127.1, 117.7, 116.3, 116.0, 114.8, 68.6, 58.0, 51.0, 40.8, 37.5, 24.6, 22.7, 21.6;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{24}$H$_{29}$FN$_2$O$_6$S 492.5 found 492.2].

Synthesis of 4-F-Ph-SO2-N-Tyr-O-Allyl-Leu-Gly-allyl-OMe (3.64)
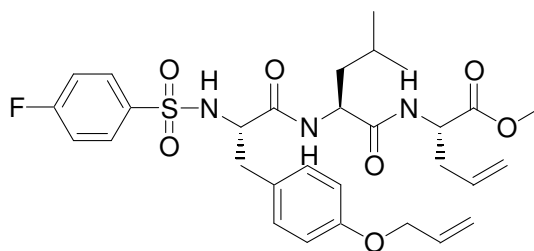
3.64

4-F-Ph-SO2-N-Tyr-O-Allyl-Leu-H **3.64** (3.3g, 6.6mmol) was coupled with (s)-allyl-Gly-OMe **3.46** (2.2g, 13mmol) following general procedure **A** to give a crude orange compound R$_f$ = 0.38 (1/1 (EtOAc / (50/70) Pet ether)). Purification was achieved using flash chromatography, eluting with gradient of (50/70) Petroleum ether to 2/1 (EtOAc / (50/70) Pet ether) to yield a white solid compound **3.64** (3g, 4.96mmol, 75%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.60 (dd, 2H, J=5.0Hz, J=8.3Hz, Ar-**H** (4-F-Ph)), 7.04 (t, 2H, J=8.3Hz Ar-**H** (4-F-Ph)), 6.98 (d, 1H, J=8.0Hz, Gly (N**H**)), 6.90 (d, 1H, J=8.4Hz, Leu (N**H**)), 6.83 (d, 2H, J=8.3Hz Ar-**H** (Tyr)), 6.69 (d, 2H, J=8.2Hz, Ar-**H** (Tyr)), 6.04 (ddd, 1H, J=5.7Hz, J=10.6Hz, J=22.4Hz, Tyr **CH**=CH$_2$), 5.70 (tdd, 1H, J=7.2Hz, J=10.0Hz, J=17.1Hz, Gly **CH**=CH$_2$), 5.41 (d, 1H, J=17.3Hz, Tyr (N**H**)), 5.32 (dd, 2H, J=8.3Hz, J=14.7Hz, Tyr **CH**$_2$=CH), 5.11 (t, 2H, J=12.9Hz, Gly **CH**$_2$=CH), 4.61 (dd, 1H, J=7.4Hz, J=13.1Hz, Gly α **CH**), 4.46 (m, 3H, m, O**CH**$_2$, and Leu α **CH**), 3.77 (m, 1H, Tyr α **CH**), 3.73 (3H, s, O**CH**$_3$), 3.03 (dd, 1H, J=4.6Hz, J=14.3Hz, β **CH**(Tyr)), 2.70 (dd, 1H, J=9.0Hz, J=14.2Hz, Tyr β **CH**), 2.52 (2H, m, Gly (**CH**$_2$)), 1.72 (1H, m, Leu **CH**CH$_2$), 1.49 (2H, m, Leu CH**CH**$_2$), 0.91 (6H, d, J=6.2Hz Leu (**CH**$_3$)$_2$););

^{13}C NMR (75 MHz) (CDCl$_3$) δ 171.7, 171.5, 170.4, 167.3, 163.7, 157.7, 134.4, 134.3, 133.0, 132.3, 130.2, 129.9, 129.8, 127.2, 118.9, 117.7, 116.3, 116.0, 114.8, 68.6, 58.4, 52.2, 51.9, 51.9, 41.0, 37.5, 36.1, 24.6, 22.8, 21.9;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{30}$H$_{38}$FN$_3$O$_7$S 604.2 found 604.3].

6.3   EXPERIMENTAL DESCRIBED FROM CHAPTER 4

### Synthesis of N-Boc-Cys-Val-Cys-ene-Macrocycle-OMe (4.1)
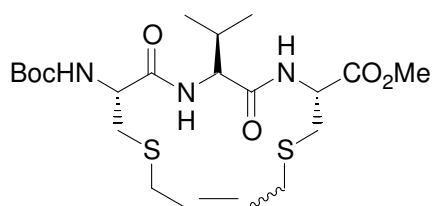
4.1

RCM of N-Boc-S-allyl-Cys-Val-S-ally-Cys-OMe **3.15** (0.94g, 1.8mmol) was carried out following general procedure **D** to give black solid crude product R$_f$ = 0.23 (1/1 (EtOAc / (50/70) Pet ether)). Purification was achieved using flash chromatography, eluting with gradient of (50/70) Petroleum ether to 100% EtOAc to yield a brown solid compound **4.1** (0.3g, 0.6mmol, 33%) mixtures of E/Z isomers:

^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.30 (d, 1H, J=8.0Hz, N**H**) 7.17 (d, 1H, J=7.8Hz, N**H**), 7.35 (d, 1H, N**H**) 7.07 (d, 1H, J=7.0Hz, N**H**), 6.88 (d, 1H, J=7.4Hz, N**H**), 5.71(d, 1H, J=7.6Hz, N**H**), 5.46 (m, 2H, 2(C**H**=CH$_2$)), 4.85 (m, 1H, α C**H**), 4.41 (dd, 1H, J=7.4Hz, J=15.2Hz, Val α (C**H**)), 4.28 (m, 1H, α C**H**), 3.78 (s, 3H, OC**H**$_3$), 2.8-3.2 (m, 8H, 2(CH=C**H**$_2$) and 2(SC**H**$_2$)), 2.10 (m, 1H, Val C**H**), 1.43 (s, 9H, C(C**H**$_3$)$_3$), 0.96 (m, 6H, C(C**H**$_3$)$_2$);

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{21}$H$_{35}$N$_3$O$_6$S$_2$ 490.6 found 490.3].

Synthesis of N-Boc-Cys-Val-Cys-Macrocycle-OMe (2.5)

2.5
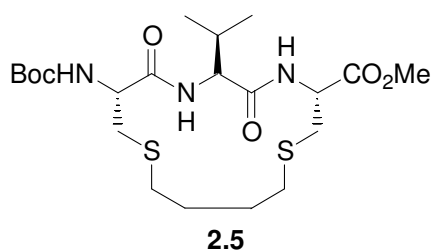
N-Boc-S-allyl-Cys-Val-S-ally-Cys-ene-Macrocycle-OMe **4.1** was dissolved in methanol (20mL) and dichloromethane (20mL). The solution was de-gas and 10% palladium on carbon was added. This was subjected to hydrogenation while stirring for 18 hours. The mixture was then filtered through celite and concentrated *in vacuo* to yield brown solid compound **2.5** (0.26g, 0.53mmol, 95%):

^{1}H NMR (500 MHz) (CDCl$_3$) 7.03 (d, 1H, J=6.2Hz, Cys (N**H**)), 6.84 (d, 1H, J=7.4Hz, Val (N**H**)), 5.53 (d, 1H, J=6.7Hz, Cys (N**H**)), 4.88 (m, 1H, Cys α (C**H**)), 4.43 (m, 1H, Val α (C**H**)), 4.29 (m, 1H, Cys α (C**H**)), 3.78 (s, 3H, OC**H**$_3$), 2.8-3.2 (m, 4H, 2(C**H**$_2$S), 2.4-2.6 (m, 4H, SC**H**$_2$CH$_2$CH$_2$C**H**$_2$S), 2.15 (m, 1H, Val(C**H**)), 1.65-1.7 (m, 4H, SCH$_2$C**H**$_2$C**H**$_2$CH$_2$S), 1.45 (s, 9H, C(C**H**$_3$)$_3$), 0.96 (m, 6H, Val C(C**H**$_3$)$_2$);

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{21}$H$_{37}$N$_3$O$_6$S$_2$ 492.6 found 492.3.

Synthesis of N-Boc-Ser-Val-Cys-ene-Macrocycle-OMe (4.2)
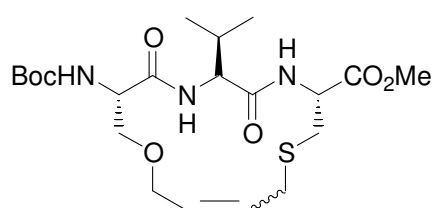
4.2

RCM of N-Boc-O-allyl-Ser-Val-S-ally-Cys-OMe **3.21** (0.48g, 0.96mmol) was carried out following general procedure **D** to give black solid crude product R_f = 0.27 (1/1 (EtOAc / (50/70) Pet ether)). Purification was achieved using flash chromatography, eluting with gradient of (50/70) Petroleum ether to 100% EtOAc to yield a brown solid compound **4.2** (0.06g, 0.13mmol, 14%) mixtures of E/Z isomers:

^{1}H-NMR (500 MHz) (CDCl$_3$) δ 6.89 (d, 1H, J=8.2Hz, N**H**), 6.58 (d, 1H, J=8.8Hz, N**H**), 5.62 (m, 3H, N**H**, 2(C**H**=CH$_2$)), 5.46 (m, 1H, (C**H**=CH$_2$)), 4.73 (m, 1H, α **CH**), 4.29 (m, 1H, α **CH**), 4.20(m, 1H, α **CH**), 3.78-3.9 (m,5H, OC**H**$_2$ and OC**H**$_3$), 3.68-3.75 (m,2H, C**H**$_2$O), 3.2(m, 2H, SC**H**$_2$), 2.85 (m, 2H, C**H**$_2$S), 2.13 (m, 1H, Val(C**H**)), 1.45-1.5 (s, 9H, C(C**H**$_3$)$_3$), 0.96 (m, 6H, Val C(C**H**$_3$)$_2$);

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{21}$H$_{35}$N$_3$O$_7$S$_2$ 474.6 found 474.5].

Synthesis of N-Boc-Ser-Val-Cys-Macrocycle-OMe (2.6)
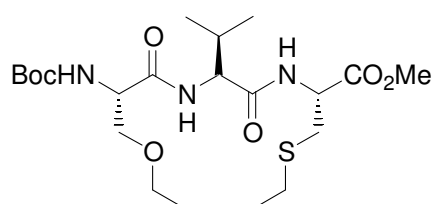
2.6

N-Boc-O-allyl-Ser-Val-S-ally-Cys-ene-Macrocycle-OMe **4.2** was dissolved in methanol (15mL) and dichloromethane (15mL). The solution was de-gas and 10% palladium on carbon was added. This was subjected to hydrogenation while stirring for 18 hours. The mixture was then filtered through celite and concentrated *in vacuo* to yield brown solid compound **2.6** (0.12mg, 0.24mmol, 53%):

^{1}H NMR (500 MHz) (CDCl$_3$) δ 6.86 (d, 1H, J=14.9Hz, N**H**), 6.56 (d, 1H, J=10.1Hz, N**H**), 5.43 (m, 1H, N**H**), 4.78 (m, 1H, Cys α C**H**), 4.5 (m, 1H, Val α C**H**), 4.02 (m, 1H, Ser α C**H**), 3.79 (s, 3H, OC**H$_3$**), 3.5-3.7 (m, 2H, C**H$_2$**O), 3.35-3.6 (m, 2H, OC**H$_2$**), 2.9-3.2 (m,2H, C**H$_2$**S), 2.48 (m, 2H, SC**H$_2$**), 2.08 (m, 1H, Val (C**H**)), 1.58-1.79 (m, 4H, OCH$_2$C**H$_2$**C**H$_2$**CH$_2$S), 1.45 (s, 9H, C(C**H$_3$**)$_3$), 0.96 (m, 6H, Val C(C**H$_3$**)$_2$);
LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{21}$H$_{37}$N$_3$O$_7$S 476.6 found 476.2].

**Synthesis of N-Boc-Glu-Val-Cys-ene-Macrocycle-OMe (2.7)**
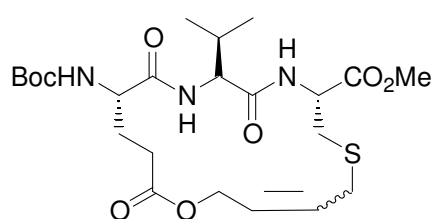
E/Z 2.7

Compound **3.34** (0.4g, 0.74mmol), (0.2g, 0.37mmol) undergo RCM via general procedures **F** and **E** respectively R$_f$ = 0.6 (2/1 (EtOAc / (50/70) Petroleum ether)). Purification was achieved using flash chromatography, eluting with gradient of (50/70) Pet ether to 100% EtOAC to yield brown compound **2.7** (0.19g, 0.37 mmol, 50% conditions **F**) and (0.08mg, 0.155mmol, 42% conditions **E**). The product was obtained as mixture of E/Z isomers:
^{1}H NMR (500 MHz) (CDCl$_3$) δ 7.34 (d, 1H, J=8.9Hz, Cys (N**H**)), 6.88 (d, 1H, J=8.88 Hz, Val (N**H**)) 6.84 (d, 1H, J=9.1Hz, Val (N**H**)), 5.70 (ddd, 1H, J=5.7Hz, J=12.8Hz, J=17.5Hz, Glu (C**H**=CH$_2$)), 5.61 (ddd, 1H, J=5.6Hz, J=8.8Hz, J=14.7Hz, Cys (C**H**=CH$_2$)), 5.53 (d, 1H, J=8.2Hz Glu (N**H**)), 4.74 (m, 1H, OC**H$_2$**), 4.57 (m, 1H, Cys α C**H**), 4.39 (m, 2H, Val α C**H** and Glu α C**H**), 4.29 (m, 1H, OC**H$_2$**), 3.78 (s, 3H, OC**H$_3$**), 3.25 (dd, 1H, J=9.0Hz, J=13.9Hz, SC**H**), 3.07 (ddd, 2H, J=5.5Hz, J=14.2Hz, J=19.6Hz, SC**H**), 2.66 (ddd, 1H, J=6.8Hz, J=13.7Hz, J=18.0Hz , C**H$_2$**S), 2.45 (m, 2H, γ C**H$_2$**), 2.23 (m, 1H, Val (C**H**), 1.98 (m, 2H β C**H$_2$**), 1.45 (s, 9H, C(C**H$_3$**)$_3$), 0.96 (m, 6H, Val C(C**H$_3$**)$_2$);

^{13}C NMR (75 MHz) (CDCl$_3$) δ 172.1, 171.5, 171.4, 170.4, 155.3, 130.4, 128.1, 79.8, 64.0, 58.3, 53.2, 52.6, 51.4, 32.2, 31.3, 30.8, 30.0, 28.2, 27.7, 18.9, 18.1;

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{23}$H$_{37}$N$_3$O$_8$S 516.5 found 516.3].

Synthesis of 4-F-Ph-SO2-N-Tyr-Leu-Gly-ene-Macrocycle-OMe (4.6)
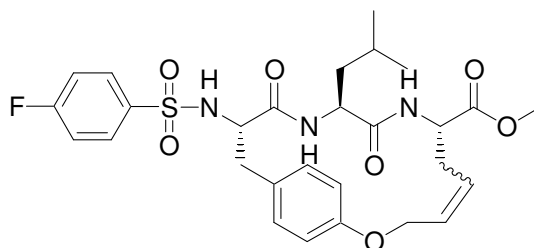

E/Z 4.6

RCM 4-F-Ph-SO2-N-Tyr-O-Allyl-Leu-Gly-allyl-OMe **3.64** (2.95g, 4.9mmol) (1.18g, was achieved via general procedure **F** R$_f$ = 0.3 (1/1 (EtOAc / (50/70) Pet ether)). Purification was achieved using flash chromatography eluting with a gradient of (50/70) Pet ether to 100 % EtOAc to yield a brown solid **4.6** (0.92g, 1.6mmol, 65% condition **F**). The product was obtained as a mixture of E/Z isomers:

^{1}H NMR (500 MHz in DMSO) δ 8.18 (1H, d J=7.6Hz, Phe (N**H**)), 8.08 (1H, d J=8.3Hz, Gly (N**H**)), 7.89 (2H, dd J=6.9Hz, J=13.6Hz, Ar-**H** (4-F-Ph)), 7.58 (1H, d J=7.5Hz, Leu (N**H**)), 7.34 (2H, t J=8.0Hz, Ar-**H** (4-F-Ph)), 6.90 (2H, d J=7.5Hz, Ar-**H** (Tyr)), 6.64 (2H, d J=7.7Hz, Ar-**H** (Tyr)), 5.54 (1H, m, OCH$_2$CH**C**H), 5.40 (1H, m, OCH$_2$CH**C**H), 4.62 (2H, m, OC**H$_2$**CHCH), 4.25 (2H, m, Tyr α **CH**) and Gly α **CH**), 3.80 (m, Leu α **CH**), 3.54 (3H, s, CO$_2$C**H$_3$**), 2.65 (2H, m, Tyr (C**H$_2$**)), 2.19 (2H, m, OCH$_2$CHCHC**H$_2$**), 1.19 (2H, m, CHC**H$_2$** Leu), 1.01 (1H, m, C**H**CH$_2$ Leu), 0.70 (6H, m, Leu (C**H$_3$**)$_2$);

LRMS [ES$^+$ M + [H$^+$] Calcd for C$_{28}$H$_{34}$FN$_3$O$_7$S 575.6 found 575.7].

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Synthesis of N-Cbz-Gln-Val-Gly–ene-Macrocycle-OMe (2.4)
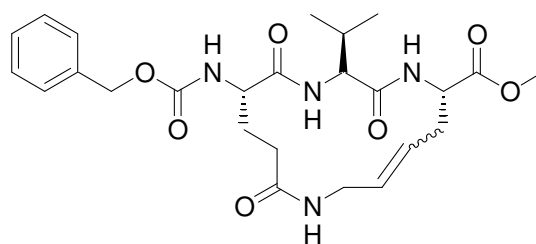
E/Z 2.4

RCM 4-F-Ph-SO2-N-Tyr-O-Allyl-Leu-Gly-allyl-OMe **3.52** (0.79, 1.3mmol) and was achieved via general procedure **F**. The crude compound was sonicated in methanol solvent for 10min and the resulting solid was collected via Buchner funnel (Pyrex 5) to gave grey compound **2.4**   (0.09g, 0.18mmol, 14%). The product was obtained as a mixture of E/Z isomers:

^{1}H NMR (500 MHz) (DMSO) δ 8.32 (d, 1H, J=8.5Hz, Gly (N**H**)), 8.12 (d, 1H, J=9.0Hz, Glu (N**H**)), 7.75 (d, 1H, J=9.4Hz, Val (N**H**)), 7.55 (dd, 1H, J=4.7Hz, J=6.4Hz, CON**H**CH$_2$), 7.28 (m, 5H, Cbz), 5.47 (m, 1H, Gln (C**H**=CH$_2$)), 5.28 (m, 1H, Gly (C**H**=CH$_2$)), 4.99 (d, 2H, J=10.7Hz, OC**H**$_2$), 4.4 (m, 1H, Gly α C**H**), 4.32 (m, 1H, Gly α C**H**), 4.21 (m, 1H, Val α C**H**), 4.06 (m, 1H, Glu α C**H**), 3.81 (m, 1H, CONHC**H**$_2$), 3.60 (s, 3H, OC**H**$_3$), 3.23 (m, 1H, CONHC**H**$_2$), 2.18-2.35 (m, 4H, Gly C**H**$_2$, Gln γ C**H**$_2$), 1.7-1.9 (m, 3H, Val (C**H**), Gln β C**H**$_2$), 0.96 (m, 6H, Val C(C**H**$_3$)$_2$);

^{13}C NMR (75 MHz) (DMSO) δ 171.7, 171.5, 171.0, 170.6, 155.7, 137.1, 131.0, 129.6, 128.4, 127.8, 124.8, 65.4, 58.1, 53.2, 52.0, 51.0, 41.5, 32.5, 30.9, 29.9, 27.2, 18.9, 18.6;

LRMS [ES^{+} M + [H^{+}] Calcd for C$_{25}$H$_{36}$N$_4$O$_7$ 503.5 found 503.4].

6.4   REFERENCES FOR CHAPTER 6:

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(2)   Xia, Z.; Smith, C. D. *J.Org.Chem* 2001, *66*, 5241-5244.

(3)   Webster. K, L.; Maude. A, B.; M.E;, O. D.; Mehrotra. A, P.; D, G. *J.Chem.Soc.,Perkin Trans* 2001, *1*, 1673-1695.