THE DESIGN AND SYNTHESIS OF NOVEL $\beta$-SUBSTITUTED AMINO ACIDS, BICYCLIC DIPEPTIDE MIMETICS, AND THEIR INCORPORATION INTO CHOLECYSTOKININ/OPIOID CHIMERIC PEPTIDES

by

John Maina Ndungu

A dissertation Submitted to the Faculty of the DEPARTMENT OF CHEMISTRY In Partial Fulfilment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 2004
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DEDICATION

To my parents
Peter Ndungu Maina and Lydia Muthoni Ndungu
To my grandparents
Henry Maina Gathoni and Hannah Wanjira Maina
To my brothers
Samuel Kamanga Ndungu and Daniel Muigai Ndungu
For the sacrifices they have made, their love, encouragement and support.
Ashanteni nyote
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>12</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>13</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>14</td>
</tr>
<tr>
<td>CHAPTER 1. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1 Structure-activity relationships of opioids</td>
<td>15</td>
</tr>
<tr>
<td>1.1.1 Importance of N-terminal Tyr at position 1</td>
<td>16</td>
</tr>
<tr>
<td>1.1.2 Structure-activity relationship studies at position 2</td>
<td>16</td>
</tr>
<tr>
<td>1.1.3 Structure-activity relationship studies at position 3 and 4</td>
<td>16</td>
</tr>
<tr>
<td>1.1.4 Structure-activity relationship studies at position 5</td>
<td>17</td>
</tr>
<tr>
<td>1.1.5 Cyclic analogues of enkephalins</td>
<td>17</td>
</tr>
<tr>
<td>1.2 Cholecystokinin</td>
<td>18</td>
</tr>
<tr>
<td>1.2.1 Structure-activity relationship studies for cholecystokinin</td>
<td>19</td>
</tr>
<tr>
<td>1.2.2 Cyclic analogues of CCK peptides</td>
<td>21</td>
</tr>
<tr>
<td>1.3 CCK involvement in pain modulation</td>
<td>21</td>
</tr>
<tr>
<td>1.4 Overlapping pharmacophores of opioids and cholecystokinin</td>
<td>23</td>
</tr>
<tr>
<td>1.5 CCK/opioid chimeric peptides</td>
<td>23</td>
</tr>
<tr>
<td>1.6 Peptides</td>
<td>25</td>
</tr>
<tr>
<td>1.6.1 Peptide secondary structures</td>
<td>26</td>
</tr>
<tr>
<td>1.6.2 Peptidomimetics</td>
<td>27</td>
</tr>
</tbody>
</table>
CHAPTER 2. DESIGN AND RETROSYNTHETIC ANALYSIS OF BICYCLIC DIPEPTIDE MIMETICS

2.1 Introduction ................................................................. 31
2.2 Design and retrosynthetic analysis of bicyclic dipeptide mimetics .......... 32
2.3 Synthesis of β-substituted γ,δ- and δ,ε-unsaturated amino acids ............. 34
  2.3.1 Alkylation of aspartic acid ........................................ 35
  2.3.2 Kazmaier-Claisen rearrangement reaction ............................ 39
2.4. Experimental .................................................................. 43

CHAPTER 3. SYNTHESIS OF Nle-Gly BICYCLIC DIPEPTIDE MIMETICS

3.1 Introduction ................................................................. 48
3.2 Synthesis of Nle-Gly bicyclic dipeptide mimetic from β-substituted aspartic acid ................................................................. 48
3.3 Synthesis of Nle-Gly bicyclic dipeptide mimetics via the Kazmaier-Claisen rearrangement reaction ................................................................. 50
3.4 Characterization of Nle-Gly bicyclic dipeptide mimetics ..................... 51
3.5 Experimental .................................................................. 53

CHAPTER 4. SYNTHESIS OF Nle-Asp BICYCLIC DIPEPTIDE MIMETICS

4.1 Introduction ................................................................. 59
4.2 Synthesis of analogues of proline ........................................... 61
TABLE OF CONTENTS - continued

4.3 Synthesis of bicyclic dipeptide mimetics for Nle-Asp ........................................ 64
4.4 Characterization of Nle-Asp bicyclic dipeptide mimetics .................................. 67
4.5 Synthesis of β-thiol substituted aspartic acid .................................................. 68
4.6 Experimental ..................................................................................................... 72

CHAPTER 5. SYNTHESIS OF BICYCLIC DIPEPTIDE MIMETICS FOR
Asp-Gly AND homoPhe-Gly

5.1 Introduction ..................................................................................................... 78
5.2 Synthesis of Asp-Gly bicyclic dipeptide mimetic .......................................... 78
  5.2.1 Characterization of Asp-Gly bicyclic dipeptide mimetics ......................... 81
5.3 Synthesis of a homophe-Gly bicyclic dipeptide mimetic ............................... 82
5.4 Experimental .................................................................................................. 85

CHAPTER 6. SYNTHESIS OF BICYCLIC DIPEPTIDE MIMETIC
CONTAINING CCK/OPIOID CHIMERIC PEPTIDES,
BIOLOGICAL ASSAY RESULTS, DISCUSSION AND
FUTURE WORK

6.1 Synthesis of peptides ...................................................................................... 92
6.2 Experimental .................................................................................................. 94
  6.2.1 Synthesis and purification of peptides ..................................................... 94
  6.2.2 Assay Methods ........................................................................................ 95
6.3 Biological evaluation at the CCK receptors ................................................. 99
6.4 Biological evaluation at the opioid receptors .............................................. 101
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 Discussion and conclusion</td>
<td>102</td>
</tr>
<tr>
<td>6.6 Future work</td>
<td>104</td>
</tr>
<tr>
<td>6.6.1 Structure activity relationship studies</td>
<td>104</td>
</tr>
<tr>
<td>6.6.2 Cyclized chimeric peptides</td>
<td>104</td>
</tr>
<tr>
<td>6.6.3 Synthesis of cyclized peptides using β-allyl substituted aspartic acid and ring closing metathesis</td>
<td>105</td>
</tr>
<tr>
<td>6.6.4 Synthesis of cyclized peptides using δ-allyl substituted proline analogues and ring closing metathesis</td>
<td>106</td>
</tr>
<tr>
<td>Appendix A</td>
<td>108</td>
</tr>
<tr>
<td>Appendix B</td>
<td>109</td>
</tr>
<tr>
<td>Appendix C</td>
<td>110</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>111</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>145</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>General structure of amino acid and peptide</td>
<td>25</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Definition of $\phi$, $\psi$, $\omega$, $\chi^1$, $\chi^2$ dihedral angles</td>
<td>26</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Different types of $\beta$-turn</td>
<td>27</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>External and internal $\beta$-turn mimetics</td>
<td>28</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Examples of bicyclic $\beta$-turn mimetics</td>
<td>30</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Retrosynthetic analysis of bicyclic $\beta$-turn dipeptide</td>
<td>31</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Synthetic strategy for [5,5]- and [6,5]-bicyclic dipeptide mimetics</td>
<td>32</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Design of indolizidinone bicyclic dipeptide mimetics</td>
<td>32</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Bicyclic dipeptide mimetics for homoPhe-Gly and Nle-Gly</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Retrosynthetic design for dipeptide mimetics</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Bicyclic dipeptide mimetic for Nle-Asp dipeptide unit</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Retrosynthetic analysis of a Nle-Asp bicyclic dipeptide mimetic</td>
<td>34</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Examples of $\gamma,\delta$-unsaturated amino acids</td>
<td>35</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>Silyl ketene acetals</td>
<td>36</td>
</tr>
<tr>
<td>Figure 2.10</td>
<td>Transition state for alkylation of aspartic acid</td>
<td>37</td>
</tr>
<tr>
<td>Figure 2.11</td>
<td>Chelated enolate intermediate</td>
<td>37</td>
</tr>
<tr>
<td>Figure 2.12</td>
<td>Mechanism for the formation of ortho esters</td>
<td>39</td>
</tr>
<tr>
<td>Figure 2.13</td>
<td>Oxazole and Ireland-Claisen rearrangements</td>
<td>41</td>
</tr>
<tr>
<td>Figure 2.14</td>
<td>Transition state in Kazmaier-Claisen rearrangement</td>
<td>42</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Nle-Asp bicyclic dipeptide mimetics</td>
<td>48</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>nOe data for the Nle-Gly bicyclic dipeptide mimetics</td>
<td>52</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES** - continued

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3</td>
<td>X-Ray crystal structure of a Nle-Gly bicyclic dipeptide mimetic</td>
<td>52</td>
</tr>
<tr>
<td>4.1</td>
<td>Bicyclic dipeptide mimetic for Nle-Asp dipeptide unit</td>
<td>59</td>
</tr>
<tr>
<td>4.2</td>
<td>Transition state model for the generation of proline analogues</td>
<td>63</td>
</tr>
<tr>
<td>4.3</td>
<td>X-ray crystal structure of δ-allyl substituted proline analogue</td>
<td>63</td>
</tr>
<tr>
<td>4.4</td>
<td>nOe analysis of δ-allyl substituted proline analogues</td>
<td>64</td>
</tr>
<tr>
<td>4.5</td>
<td>Optically active phosphines</td>
<td>65</td>
</tr>
<tr>
<td>4.6</td>
<td>Bicyclic dipeptide mimetic for Nle-Asp</td>
<td>67</td>
</tr>
<tr>
<td>4.7</td>
<td>nOe data for Nle-Asp bicyclic dipeptide mimetics</td>
<td>68</td>
</tr>
<tr>
<td>4.8</td>
<td>Retrosynthetic analysis for a thiol BTD for Nle-Asp</td>
<td>68</td>
</tr>
<tr>
<td>4.9</td>
<td>Mechanism for the synthesis of disulfides</td>
<td>71</td>
</tr>
<tr>
<td>5.1</td>
<td>nOe observed for Asp-Gly bicyclic dipeptide mimetics</td>
<td>82</td>
</tr>
<tr>
<td>5.2</td>
<td>X-Ray crystal structure of homoPhe-Gly bicyclic dipeptide mimetic</td>
<td>83</td>
</tr>
<tr>
<td>6.1</td>
<td>Novel BTD containing peptides</td>
<td>93</td>
</tr>
<tr>
<td>6.2</td>
<td>Structures of JMN1 and JMN2</td>
<td>104</td>
</tr>
<tr>
<td>6.3</td>
<td>Synthesis of cyclized peptides</td>
<td>106</td>
</tr>
<tr>
<td>6.4</td>
<td>Synthesis of highly constrained cyclic peptides</td>
<td>106</td>
</tr>
<tr>
<td>Scheme</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.1</td>
<td>Alkylation of aspartic acid with allyl bromide</td>
<td>38</td>
</tr>
<tr>
<td>2.2</td>
<td>Alkylation of aspartic acid with benzyl bromide</td>
<td>38</td>
</tr>
<tr>
<td>2.3</td>
<td>Alkylation of ortho ester protected aspartic acid</td>
<td>39</td>
</tr>
<tr>
<td>3.1</td>
<td>Synthesis of Nle-Gly bicyclic dipeptide mimetic</td>
<td>49</td>
</tr>
<tr>
<td>3.2</td>
<td>Hydrogenation and reduction of β-allyl substituted aspartic acid</td>
<td>50</td>
</tr>
<tr>
<td>3.3</td>
<td>Synthesis of Nle-Gly BTD via the Kazmaier-Claisen rearrangement</td>
<td>51</td>
</tr>
<tr>
<td>4.1</td>
<td>Synthesis of analogues of proline</td>
<td>60</td>
</tr>
<tr>
<td>4.2</td>
<td>Synthesis of cis-dicarboxylate proline analogues</td>
<td>62</td>
</tr>
<tr>
<td>4.3</td>
<td>Synthesis of trans-dicarboxylate proline analogues</td>
<td>63</td>
</tr>
<tr>
<td>4.4</td>
<td>Synthesis of Nle-Asp bicyclic dipeptide mimetic</td>
<td>66</td>
</tr>
<tr>
<td>4.5</td>
<td>Synthesis of Nle-Asp bicyclic dipeptide mimetic</td>
<td>67</td>
</tr>
<tr>
<td>4.6</td>
<td>Sulfenylation of aspartic acid with benzyl disulfide</td>
<td>69</td>
</tr>
<tr>
<td>4.7</td>
<td>Synthesis of Nyps protected aspartic acid</td>
<td>70</td>
</tr>
<tr>
<td>5.1</td>
<td>Synthesis of trifluoroacetyl benzotriazole</td>
<td>79</td>
</tr>
<tr>
<td>5.2</td>
<td>Synthesis of Asp-Gly bicyclic dipeptide mimetics</td>
<td>80</td>
</tr>
<tr>
<td>5.3</td>
<td>Synthesis of Asp-Gly bicyclic dipeptide mimetic</td>
<td>80</td>
</tr>
<tr>
<td>5.4</td>
<td>Synthesis of homoPhe-Gly bicyclic dipeptide mimetic</td>
<td>83</td>
</tr>
<tr>
<td>5.5</td>
<td>Synthesis of analogues of Leu-enkephalin</td>
<td>84</td>
</tr>
<tr>
<td>5.6</td>
<td>Bromination of β-benzyl homoserine</td>
<td>85</td>
</tr>
<tr>
<td>6.1</td>
<td>Protocol for the synthesis of peptides</td>
<td>93</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1 Gastrin-1 and Caerulein ................................................................. 18

Table 6.1 Biological evaluation at the CCK receptors ................................. 100

Table 6.2 Functional analysis at the opioid receptors ................................. 101

Table 6.3 Biological evaluation at the opioid receptors ................................. 102
ABSTRACT

Peptide ligands and protein receptors play critical roles in the regulation of nearly every biological system. However, peptides are characteristically highly flexible and thus identifying the basic conformational elements necessary for recognition between a peptide ligand and its receptor at the molecular level remains a formidable task. Great emphasis in peptide research has thus focused on the determination of the receptor-bound conformation adopted by bioactive peptides by synthesizing constrained analogues of the peptides. Knowledge of the three-dimensional interaction between a peptide ligand and a receptor could be invaluable in understanding bioactivity and in the design of therapeutics.

To determine the bioactive conformation of our novel chimeric peptides for the opioid and cholecystokinin receptors, constrained analogues were designed to limit the conformations that the peptides would adopt. In this regard, [5,5]- and [6,5]-bicyclic dipeptide mimetics were designed and synthesized to constrain a dipeptide unit and by extension limit the flexibility of the peptide. The bicyclic dipeptide mimetics were synthesized from precursors obtained by the β-alkylation of aspartic acid and from the Kazmaier-Claisen rearrangement reaction. A protocol for the alkylkation of aspartic acid with allyl bromide, benzyl bromide, and benzyl disulfide was developed. The bicyclic dipeptide mimetics were then introduced into the peptides whose biological activity was evaluated at both the opioid and cholecystokinin receptors. The peptides showed good binding and functional activities at the CCK receptors, but low activities at the opioid receptors.
CHAPTER 1
INTRODUCTION

The central goal of the research outlined in this thesis is the synthesis of constrained chimeric peptides that are agonists at the δ-opioid receptor and also antagonists at the cholecystokinin (CCK) receptor. The constraint would be effected by the design and syntheses of bicyclic dipeptide mimetics which would then be incorporated into the desired biologically active peptides. Structure activity relationship of the resultant peptides would aid in the elucidation of the biologically active conformation(s) of the peptides, the dream of all peptide chemists. In the introduction, a brief overview of opioid receptors and ligands, cholecystokinin, peptides, CCK/opioid chimeric peptides, and peptidomimetics is provided.

1.1 Structure-activity relationships of opioids

The enkephalin peptides, [Leu$^5$]-enkephalin (Tyr$^1$-Gly$^2$-Gly$^3$-Phe$^4$-Leu$^5$-OH) and [Met$^5$]-enkephalin (Tyr$^1$-Gly$^2$-Gly$^3$-Phe$^4$-Met$^5$-OH) were isolated by Hughes and Kosterlitz from porcine brain. The theory of multiple subtypes of opioid receptors was proposed by Martin et al.$^2$ and confirmed by Lord et al.$^3$ who demonstrated the existence of μ receptors in the guinea pig ileum (GPI) and δ receptors in the mouse vas deferens (MVD). Both Met- and Leu-enkephalin interact with both the δ and μ opioid receptors and were found to be more potent at the δ receptor than morphine based opiates which are more potent μ agonists.
1.1.1 Importance of N-terminal Tyr at position 1

Early work on the structure-activity relationships of enkephalins showed great losses of potency when Tyr\(^1\) was modified.\(^4\) Alkylation of the amino terminal group results in loss of potency at the \(\mu\) receptor and more so at the \(\delta\) receptor.\(^5\) It has been proposed that the free NH\(_2\) terminus (which is protonated at physiological pH) interacts with an anionic site on both the \(\delta\) and \(\mu\) opioid receptors.\(^6\) Addition of an amino acid at the zero position was also found to be detrimental as was replacement of Tyr\(^1\) with other amino acids.\(^10\)

1.1.2 Structure-activity relationship studies at position 2

Numerous changes at the Gly\(^2\) position have resulted in analogues with increased potency with all the successful changes involving a D-amino acid.\(^11\)-\(^13\) Hambrook et al.\(^13\) found that the first metabolic step for enkephalins was cleavage at the Tyr-Gly peptide bond. Substitution of D-amino acid at the 2-position was thus expected to increase stability but there was also an increase in potency.\(^13\) Interestingly, D-amino acids were later discovered to be naturally occurring in the amino acid sequence of the frog skin heptapeptide dermorphin, (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH\(_2\)),\(^14\) deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH\(_2\)), and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH\(_2\)).\(^15\)-\(^18\)

1.1.3 Structure-activity relationship studies at position 3 and 4

The Gly\(^3\) position in enkephalins is very intolerant to substitutions.\(^19\) On the other hand, there has been numerous successful substitutions at Phe\(^4\). Aromatic residues at position 4 are not fully required to interact with opioid receptors. The ring can be methylated, methoxylated, halogenated, nitrated, phenylated, or reduced to a cyclohexyl group.\(^3\),\(^20\)
Replacement of Phe\textsuperscript{4} with aliphatic hydrophobic amino acids leads to improved selectivity for $\mu$ receptor over $\delta$ receptor.\textsuperscript{21-23} Position 4 can also be substituted with other aromatic residues such as Trp\textsuperscript{24}, but not with Tyr.\textsuperscript{3}

1.1.4 Structure-activity relationship studies at position 5

Leu\textsuperscript{5} or Met\textsuperscript{5} can be replaced with alkyl groups, esters, or alcohols which improve potency at $\mu$ receptor while decreasing activity at $\delta$ receptors.\textsuperscript{25} The carboxyl terminal is not necessary for activity at the $\mu$ receptor. For a series of D-Ala\textsuperscript{2} analogues, replacement of the terminal carboxyl group with $-\text{NH}_2$ \textsuperscript{26} or $-\text{CONH}_2$ \textsuperscript{27} gave analogues that were more $\mu$ active than CO$_2$H terminal analogues. The C-terminal alcohol analogue FK33824 (Tyr-D-Ala-Phe-Gly-NH$_2$) was found to be more active at the $\mu$ receptor than Met-enkephalin.\textsuperscript{21}

1.1.5 Cyclic analogues of enkephalins

Cyclization greatly decreases the available degrees of conformational freedom.\textsuperscript{28} Appropriate conformational constraints will restrict a residue or group of residues to a sufficiently small region of conformational space that when the peptide interacts with its receptor or some other acceptor molecule (enzyme, carrier protein, etc.), the conformation seen in solution will remain due to the high activation energy or free energy needed to change that conformation. Cyclic analogues have recently been employed to help elucidate the conformational requirements, in particular the topographical arrangement of the side chains, for the opioid receptors. Side chain to C-terminal and side chain to side chain cyclic lactam analogues of enkephalin have been shown to be potent and selective for the $\mu$ receptor.\textsuperscript{29-30} Potent $\delta$ opioid receptor selective cyclic enkephalin
analogue4s were developed by side chain to side chain disulfide bridges. These analogues were generally substituted with D-Cys\(^2\) and D- or L-Cys\(^5\) residues. More active analogues were obtained by substitution with Pen and/or D-pen residues at positions 2 and 5.\(^{32-35}\) Cyclic lathionine analogues have also been shown to have high potency at both \(\mu\) and \(\delta\) receptors.\(^{36-37}\)

1.2 Cholecystokinin

Cholecystokinin (CCK) is a gut-brain peptide that exerts a variety of physiological actions in the gastrointestinal tract and central nervous system through cell surface CCK receptors. It was first isolated from the porcine duodenum as a 33 amino acid peptide.\(^38\) A number of biologically active variants were subsequently described and the most abundant peptide present in the brain was shown to be the sulfated C-terminal octapeptide amide [CCK\(_{26-33}\) or CCK-8, H-Asp\(^{26}\)-Tyr(SO\(_3\)H)\(^{27}\)-Met\(^{28}\)-Gly\(^{29}\)-Trp\(^{30}\)-Met\(^{31}\)-Asp\(^{32}\)-Phe\(^{33}\)-NH\(_2\)].\(^39\) CCK-8 has been shown to be involved in numerous physiological functions such as feeding behaviour, central respiratory control, and cardiovascular tonus, vigilance states, memory processes, nociception, emotional and motivational responses. CCK is closely related to the gastrin family of peptides, and also to the naturally occurring decapteptide caerulein (CRL), which all share a common C-terminal amino acid sequence.\(^40\)

<table>
<thead>
<tr>
<th>Gastrin-1</th>
<th>pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caerulein</td>
<td>Glp-Gln-Asp-Tyr-Thr-Gly-Grp-Met-Asp-Phe-NH(_2)</td>
</tr>
</tbody>
</table>

Table 1.1 Gastrin-1 and Caerulein peptides
CCK receptors are divided into two subtypes, namely, CCK-A and CCK-B receptors (also denoted CCK-1 and CCK-2, respectively) both belonging to the class of G-protein coupled receptors characterized by seven trans membrane I domains. CCK-A receptors are located mainly in the periphery but are also found in some regions of the brain, while CCK-B receptors are predominantly located in the central nervous system (brain and spinal cord), but also are found in the stomach and vagus nerve (the longest of the cranial nerve). Studies with selective CCK receptor antagonists have revealed that although both CCK-A and CCK-B may exist in the brain, CCK-B receptors predominate in the spinal cord of rodents whereas the CCK-A receptor subtype predominates in primates. CCK-8 is the minimal sequence necessary for full activation of the CCK-A receptor, whereas for the CCK-B only the C-terminal tetrapeptide is required. Considerable interest is devoted to the pharmacology of CCK-B receptors since administration of selective agonists produces behavioral changes such as anxiety, perturbation of memory, and hyperalgesia. Dysfuctioning of CCK-B related neural pathways could also be involved in neuropsychiatric disorders. Accordingly, CCK-B antagonists have been shown to block panic attacks induced in humans by systematic administration.

1.2.1 Structure activity relationship for cholecystokinin

It has been demonstrated that replacement of both Met and Met residues by the isosteric amino acid norleucine (Nle) provides CCK analogues with virtually identical activities as CCK-8. This modification allows for an efficient synthesis of CCK analogues since side reactions associated with methionine are avoided. These analogues
should also be resistant to enzyme degradation. Replacement of Met$^{31}$ in the C-terminal tetrapeptide of CCK with Lys(NH$^6$CONHR) reversed subtype selectivity and led to the discovery of a highly potent CCK-A agonist.$^{50}$ Unsulfated analogues containing N-methylnorleucine (N-MeNle) substitutions at positions 28 and 31 were shown to have exceptional potency and selectivity for CCK-B receptors.$^{49}$

Truncation studies have shown that Trp$^{30}$ is an important residue for interaction with either the CCK-A or CCK-B receptor.$^{51-53}$ Substitution of Trp$^{30}$ with D-Trp, Nal(1), N-MeTrp, and β-methyl tryptophan resulted in compounds with less potency. The N-terminal amino group of CCK-7 is not important for cholecystokinin activity,$^{54}$ and similarly, the N-acetyl derivative of CCK-7 shows the same potency as CCK-8 in the pancreas amylase release test.$^{55}$ Structure-activity work with Ac-CCK-7 suggests that requirements for potent agonist activity includes an intact C-terminal carboxamide,$^{56}$ an aryl- or cycloalkylalanine in position 33.$^{57-58}$ Substitution of Asp$^{32}$ by alanine,$^{59}$ β-alanine,$^{60-61}$ β-aspartic acid,$^{62}$ or glutamic acid$^{61}$ leads to decreased CCK-A potency, whereas substitution by a sulfated serine, threonine, or hydroxyproline gave fully active analogues.$^{63}$ In the latter case, the hydroxyproline may act to stabilize a preferred conformation. It has also been shown that the Asp$^{32}$ plays a conformational role rather than mediating binding to the CCK-A receptor through a charge-charge interaction.$^{64}$

It has been reported that the main metabolic cleavage sites of CCK-8 is between Gly-Trp or Trp-Met dipeptide residues. In order to increase enzymatic resistance, the Trp and Met amino acids were substituted with their D enantiomers while Gly was substituted with D-
alanine. These resulted in loss of potency except for the analogue containing D-Ala which retained some activity and led to prolonged in-vivo activity.

1.2.2 Cyclic analogues of CCK peptides

CCK-8 was found by NMR to exist preferentially under folded form in aqueous solution with a proximity between Asp\(^1\) and Gly\(^4\).\(^{65}\) This property was used to synthesize cyclic peptides through amide bond (lactam bridges) formation between Asp\(^1\) or \(\alpha, \beta\)-Glu\(^1\) and Lys\(^4\) side chains to give highly potent and selective CCK-B agonists.\(^{66}\) CCK-4 was also found to adopt a folded conformation and some cyclic analogues have been synthesized. These CCK-4 analogues contain in place of Trp-Met residue dipeptide a diketopiperazine moiety resulting from a cyclization between Nle and N-substituted (D)Trp residues and coupled with a small linker to Asp-Phe-NH\(_2\).\(^{67}\)

1.3 CCK involvement in pain modulation

CCK has been found in regions of the brain known to be associated with pain modulation, for example the cortical grey matter, PAG, ventromedial thalamus and spinal dorsal horn.\(^{68}\) CCK has also been shown to co-exist with a substance P-like peptide in the spinal ganglia\(^{69}\) and in the mid brain PAG.\(^{70-71}\) Substance P has been shown to function as a neurotransmitter and/or modulator in synaptic transmission between primary afferent fibres and neurons within the spinal cord.\(^{72}\) Willets et al.\(^{73}\) demonstrated that in rat dorsal horn, a majority of neurons within the spinal cord excited by substance P could also be excited by CCK-8, suggesting a common mechanism. In addition, it has been reported that CCK-containing neurons located in the Edinger-Westphal nucleus of the PAG responds with increased firing and, on occasions, depolarization blockade to noxious
The spontaneous firing and noxious stimulation-induced firing of these neurons was suppressed by systematically injected morphine. It has been shown that administration of CCK reduces morphine analgesia which could be attributed to an independent hyperalgesic induction effect for CCK. CCK does not itself show hyperalgesia meaning it is not a pro-nociceptive effect. It has been concluded that exogenous CCK attenuates analgesia induced by morphine or release of endogenous opioids. However conflicting results emerge from these studies. Zetler, Barbaz, and Hill suggest an indirect opiate-mediated analgesic effect of CCK, whereas Faris suggests an opiate antagonistic effect of CCK. The differences could be attributed to the fact that large doses of CCK induce a 'pharmacological' analgesia whereas small doses of the peptide produces a physiological antagonism of opioid analgesia that are specific to the sulfated octapeptide form. Furthermore, endogenous CCK may be a factor in determining the magnitude of the opioid analgesic response, acting in the short-term as a negative feedback modulator of opiate action.

Studies using proglumide (4-Benzamido-N,N-dipropylglutaramic acid), a weak, non-selective, CCK antagonist, supports the observation that endogenous and exogenous CCK attenuates morphine analgesia. Proglumide was also shown to attenuate morphine tolerance and potentiated the analgesic effect of β-endorphin. It has also been demonstrated that concomitant administration of proglumide and transcript, a weak CCK antagonist, with morphine over a period of time enhances the analgesic effects of the opiate. Furthermore, proglumide and benzotript inhibited the development of morphine tolerance, but had no effect on opioid dependence. MK-329, a potent and selective
CCK-antagonist has also been shown to enhance morphine analgesia but does not block it at high doses as is the case with proglumide.\textsuperscript{68} This would suggest that tolerance may, in part, result from a progressive compensatory increase in the activity of CCK systems in response to prolonged opiate administration. This would imply that blockade of CCK receptors by CCK antagonists may reverse or prevent the development of opiate tolerance in patients.

1.4 Overlapping pharmacophores of opioids and cholecystokinin

Biophysical studies have suggested that unsulfated C-terminal CCK-7 might have biological activity at opioid receptors.\textsuperscript{87} Computational studies of CCK-7 and [Met\textsuperscript{5}]-enkephalin showed similarities in structure. The aromatic moiety of Tyr\textsuperscript{1} in [Leu\textsuperscript{5}]-enkephalin can be aligned with the Tyr\textsuperscript{29} residue of CCK-7. Similarly, the side chain of Phe\textsuperscript{4} in [Leu\textsuperscript{5}]-enkephalin can be aligned with the indole ring of Trp in CCK-7.\textsuperscript{88} Several modeling studies have suggested similarities between the proposed biologically active conformation between CCK and opioid ligands.\textsuperscript{89} Examination of these proposed bioactive conformations demonstrated topographical similarities of the surfaces of the aromatic side chain residues suggesting that, at least in part, δ-opioid receptors and CCK-B receptors have overlapping structural and topographical requirements.\textsuperscript{90}

1.5 CCK/opioid chimeric peptides

Interactions between opioid peptides and CCK-8 in regulation of pain pathways are supported by evidence from a number of laboratories.\textsuperscript{68,91-93} However, the mechanism by which CCK-8 affects nociception is not completely understood. CCK-8 has been proposed to be an anti-opioid peptide,\textsuperscript{94,95} to release endogenous opioids,\textsuperscript{96,97} and to have
intrinsic antinociceptive activity. A synthetic CCK-8 analog, SNF-9007 (Asp-Tyr-D-Phe-Gly-Trp-N-MeNle-Asp-Phe-NH₂) was found to be a potent and selective agonist at the CCK-B receptor but also had a weak affinity but robust agonist activity at the delta opioid receptor. Modelling studies of SNF-9007 showed an agreement between the receptor bound conformation of CCK-B ligands with delta opioid receptor template model based on DPDPE. Efforts were then put into synthesizing analogues of SNF-9007 that have a mixed delta and mu binding affinity and a more balanced binding affinity for the CCK-A and CCK-B receptors.

It is well known that the N-terminal tyrosine is important for potency at the opioid receptors. To improve the delta opioid receptor potency, the Asp was removed to give a nanomolar agonist at the δ opioid receptor while also retaining high selectivity at the CCK-B receptor. It has also been established that the second amino acid in opioid ligands should either be a glycine or a D-amino acid residue. When Gly was substituted for D-Phe in SNF-9007, a weak delta and mu opioid receptor agonist, and a very weak CCK agonist was obtained. When the Gly was replaced by D-Ala, bioactivity at the MVD and GPI improved slightly and interestingly, the compound showed a weak antagonist activity in the CCK assay. The analogue containing Dphe, DTrp and NmeNle had good bioactivity in all three bioassays.

Cyclic disulfide analogues with substitutions of D-Cys or D-Pen in position 2 and L-Cys, D-Cys, or D-Pen in position 5 displayed selectivity for the δ opioid receptors and antagonist activities against CCK-8 in tissue assays. Substitution of D-Trp in these cyclic disulfide analogs led to significant losses in activity. Lactam analogues obtained
by substituting L-Lys or D-Lys and Glu or D-Glu gave analogues that were agonists at \( \mu \) opioid receptor as well as antagonists at the CCK-8 receptor. The CCK-8 antagonist activity was increased when D-Trp\(^4\) was substituted while the opioid agonist activity was maintained. However, the lactam and disulfide analogues did not have any competitive binding affinities at the human CCK-A and CCK-B receptors.

### 1.6 Peptides

Proteins and peptides are formed by a series of amino acids linked together by amide bonds (Figure 1.1). They are involved in numerous physiological processes as neurotransmitters, neuromodulators, hormones, inhibitors, antibiotics, growth factors, cytokines, antigens, etc.

![Figure 1.1 General structure of amino acid and peptide](image)

Although numerous native peptides have great potential for medical applications, these peptides generally have to be modified to overcome certain problems, such as degradation by peptidases, nonselectivity for different receptors and receptor subtypes, and their limited permeation across biological membranes. Since the biological functions of peptides are determined by their chemical, structural, conformational, topographical and dynamic properties, different approaches have been designed to study these parameters. The conformation of peptides and proteins can be described by their backbone conformation and side chain conformations. The backbone conformations are
characterized by three dihedral angles $\phi$, $\psi$, and $\omega$ (Figure 1.2). The side chain conformation are characterized by the dihedral angles $\chi^1$, $\chi^2$ etc.

![Figure 1.2](image)

**Figure 1.2** Definition of $\phi$, $\psi$, $\omega$, $\chi^1$, $\chi^2$ dihedral angles

### 1.6.1 Peptide secondary structures

$\alpha$-Helixes, $\beta$-sheets, $\beta$-turns and $\gamma$-turns and extended conformations are the common secondary structures in peptides and proteins. The preference of $\beta$-turns and $\gamma$-turns, which have often been implicated as recognition elements,\textsuperscript{102-103} has led to an intense search for templates that mimic these structures.\textsuperscript{104-105} A $\beta$-turn consists of four amino
acid residues i to i + 3 leading to a reversal in the backbone direction, with amino acid residues i + 1 and i + 2 found in the two corners of the turn. A C=O···HN hydrogen bond between the i and i + 3 residues respectively, stabilizes almost all of this turns forming a 10-membered intramolecular hydrogen-bonded ring. Up to eight different subtypes and their mirror images have been reported based on the dihedral angles, $\phi$ and $\psi$, of the i + 1 and i + 2 amino acid residues (Figure 1.3). The definition of the most common $\beta$-turns (types I, II and their mirror images I’ and II’ respectively) requires that the amide bonds reside in the trans orientation and that the distance between the $\alpha$-carbon and the i and i + 3 residues be <7 Å. For a specific turn, the deviation of the dihedral angles should not be larger than 20°.

![Figure 1.3 Different types of $\beta$-turn](image)

<table>
<thead>
<tr>
<th>Turn Type</th>
<th>$\phi_i$</th>
<th>$\psi_i$</th>
<th>$\phi_{i+3}$</th>
<th>$\psi_{i+3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-60</td>
<td>-30</td>
<td>-90</td>
<td>0</td>
</tr>
<tr>
<td>I'</td>
<td>60</td>
<td>30</td>
<td>90</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>-60</td>
<td>120</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>II'</td>
<td>60</td>
<td>-120</td>
<td>-80</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>-60</td>
<td>-120</td>
<td>-80</td>
<td>0</td>
</tr>
<tr>
<td>III'</td>
<td>60</td>
<td>30</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>IV</td>
<td>-80</td>
<td>80</td>
<td>80</td>
<td>-80</td>
</tr>
<tr>
<td>IV'</td>
<td>80</td>
<td>-80</td>
<td>-80</td>
<td>80</td>
</tr>
</tbody>
</table>

1.6.2 Peptidomimetics

To elucidate the contribution of reverse-turns to the bioactive conformation of a minimal bioactive sequence, which otherwise is highly flexible, and to enhance and select for a specific profile of pharmacological activity, rigidified reverse-turn mimetics (otherwise called peptidomimetics) have been developed. As defined by Hruby;
A peptidomimetic is an organic molecule, such as a peptide analog or nonpeptide ligand, that interacts with receptor or acceptor in a similar manner as the native peptide/protein ligand to affect the same biochemical (biological) activities. In the parlance of medicinal chemistry, both peptidomimetics and native ligands/proteins should share common pharmacophore elements.

In our laboratories, there is an impetus to design and synthesize β-turn mimetics for the opioid, CCK, and melanorcotin receptors. In general, β-turn mimetics can be classified as either external or internal mimetics depending on the support, which can be either outside or inside the β-turn (Figure 1.4). These peptidomimetics are potential conformational probes enabling biological activity to be more effectively correlated with structure, and prospective therapeutics overcoming drawbacks such as proteolytic susceptibility and poor bioavailability, typical of many important bioactive peptides.

![Figure 1.4 External and internal β-turn mimetics](image)

One goal in β-turn peptidomimicry is to stabilize the relevant dihedral angles in the turn so they fall within the ranges appropriate for the sought after turn. A common strategy for β-turn peptidomimicry is to replace the i + 1 and i + 2 residues of the turn with a scaffold
to stabilize the turn. This type of scaffold best serves its purpose if it provides sites for
attaching side chain-like appendages, positions an amide bond in the central dipeptide
and possesses an amine and carboxylic acid groups so the scaffold may be incorporated
into a peptide chain in place of the putative turn residues. Mimetics where the stabilizing
hydrogen bonds in β-turns and γ-turns have been substituted with covalent bonds\textsuperscript{111-115} as
well as mimetics enhancing the reverse turn propensity of a peptide have been
developed.\textsuperscript{116-120} Residue substitution in the parent peptide for example the use of N-
methyl, dehydro-, α-methyl-, or D-amino acids to enhance the turn population has been
reported.\textsuperscript{121} There are also a variety of methods that incorporate short-range cyclizations,
which are called heterodetic cyclizations, into a peptide sequence by connecting amino
acid side chains to form amide and ester linkages, or disulfide bridges. Other cyclizations
that attach amino acid side chains to the peptide backbone have been used in the
synthesis of peptidomimetics.\textsuperscript{121-122} Several cyclic and bicyclic dipeptide analogues
intended to stabilize the peptide in a reverse turn have been reported\textsuperscript{104-105} (Figure 1.5).
Among these, the 5,5-bicyclic dipeptide mimetic i was designed as a type II β-turn
mimetic\textsuperscript{123} and the 6,5-bicyclic dipeptidomimetic BTD ii as a type II' β-turn mimetic.\textsuperscript{124-}
\textsuperscript{125} A [7,5]-bicyclic dipeptide iii has been proposed to represent many different β-turns
depending on the different chiralities on the ring and the different amino acids in the
sequence.\textsuperscript{126} An S-spirolactam iv was suggested to replace a type II β-turn, while a
spirotricycle was thought as the best mimic of a type II β-turn.\textsuperscript{124,127} Other types of
bicyclic structures have been designed as β-turn mimetics, such as azobicyclic[4.3.0]-
alkane v for type II' and cyclooctapyrrole vi for type VI β-turn\textsuperscript{128-130} and tricyclic vii type
II β-turn. Bicyclic tripeptide mimetics viii and ix were designed and synthesized for incorporation into angiotensin II. Some of the bicyclic dipeptide mimetics have also been introduced into peptides. However, it is interesting to note that most of these β-turn mimetics do not have the side chain groups found in most natural α-amino acids.

Figure 1.5 Examples of bicyclic β-turn mimetics
CHAPTER 2
DESIGN AND RETROSYNTHETIC ANALYSIS FOR BICYCLIC DIPEPTIDE MIMETICS

2.1 Introduction

The development of novel β-turn mimetics has drawn significant attention in peptidomimetic design. Two reviews on the design and synthesis of bicyclic dipeptide mimetics has been provided by Lubell and Hanessian.\textsuperscript{142-143} Our group has focused on the synthesis of external β-turn mimetics in which both backbone conformation (ϕ and ψ angles) and side chain conformations (χ\textsuperscript{1} and χ\textsuperscript{2}) are constrained (Figure 2.1). In our design, n could be 0, 1, and 2 representing [5,5], [6,5] and [7,5]-bicyclic dipeptide mimetics respectively. X could be CH\textsubscript{2}, O or S making this approach more versatile from a synthetic point of view.

![Figure 2.1 Retrosynthetic analysis of bicyclic β-turn dipeptide](image)

**Figure 2.1** Retrosynthetic analysis of bicyclic β-turn dipeptide

In our group, asymmetric synthetic methodologies towards the scaffolds for [5,5]- and [6,5]-bicyclic dipeptides have been developed (Figure 2.2). The [5,5]-bicyclic dipeptides
are synthesized from \(\gamma,\delta\)-unsaturated amino acids while the syntheses of the [6,5]-analogues employs \(\delta,\varepsilon\)-unsaturated amino acids.\(^{134,144-146}\)

\[\begin{align*}
P&G&H&N\to R\to CO_2Me & + & H_2N& \to CO_2H \\
P&G&H&N\to R\to CO_2Me & + & H_2N& \to CO_2H
\end{align*}\]

\(PG = \text{protecting group}\)

**Figure 2.2** Synthetic strategy for [5,5]- and [6,5]-bicyclic dipeptide mimetics

A synthetic methodology for the synthesis of the all-carbon backbone (indolizidinone) [6,5]-bicyclic dipeptides from analogues of \(\beta, \delta\)- or \(\gamma, \delta\)-substituted prolines also has been developed (Figure 2.3).\(^{147-149}\)

**Figure 2.3** Design of indolizidinone bicyclic dipeptide mimetics

### 2.2 Design and retrosynthetic analysis of bicyclic dipeptide mimetics

In the synthesis of constrained CCK/Opioid chimeric peptides that incorporate bicyclic dipeptide mimetics two peptides; Tyr-D-Phe-Gly-Trp-Nle-Asp-Phe-NH\(_2\) and Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH\(_2\) (RSA501) were chosen as the lead compounds. A bicyclic dipeptide mimetic for homoPhe-Gly 1 was designed to be substituted for the D-Phe in Tyr-D-Phe-Gly-Trp-Nle-Asp-Phe-NH\(_2\). A bicyclic dipeptide mimetic for Nle-Gly 2 was
also designed to be substituted for Nle-Gly dipeptide unit in Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂ (Figure 2.4).

\[
\text{BocHN}^2 \text{BocHN} \quad \text{BocHN}^1 \text{BocHN}
\]

**Figure 2.4** Bicyclic dipeptide mimetics for homoPhe-Gly and Nle-Gly.

Retrosynthetically the two compounds can be obtained from β-substituted aspartic acid or β-substituted γ,δ-unsaturated amino acids (Figure 2.5).

\[
\text{R} = \text{benzyl, n-propyl}
\]

**Figure 2.5** Retrosynthetic design for dipeptide mimetics

It has been shown that CCK-4 adopts a folded conformation.⁶⁷ To introduce such a turn at the C-terminal of our target peptides a Nle-Asp bicyclic dipeptide mimetic 3 was designed to be substituted for the Nle-Asp dipeptide unit in Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂ (Figure 2.6).

\[
\text{CbzHN} \quad \text{CbzHN}
\]

**Figure 2.6** Bicyclic dipeptide mimetic for Nle-Asp dipeptide unit
This compound can be derived by elaboration of δ-allyl substituted hydroxy analogues which can be obtained from β-allyl substituted aspartic acids (Figure 2.7).

![Figure 2.7 Retrosynthetic analysis of a Nle-Asp bicyclic dipeptide mimetic](image)

As has been described above, our design is based on β-substituted γ,δ- and δ,ε-unsaturated amino acids. These can be obtained by alkylation of aspartic acid, by the Kazmaier-Claisen rearrangement and via a Nickel-II chiral auxiliary. In this chapter, a discussion on alkylation of aspartic acid and the Kazmaier-Claisen rearrangement, the methods employed in this research, are discussed.

2.3 Synthesis of β-substituted γ,δ- and δ,ε-unsaturated amino acid

The synthesis of γ,δ-unsaturated amino acids has received much attention in recent years not only because they occur naturally but also because they are used in the synthesis of complex amino acids and peptides. Examples of γ,δ-unsaturated amino acids includes the isoleucine antagonist cyclopentenylglycine x and the antibiotic furanomycin xi (Figure 2.8). Unsaturated amino acids are also important intermediates for the synthesis of functionalized amino acids and have been used as asymmetric synthetic building blocks. The terminal double bond is a precursor in organic synthesis which can be
converted to hydroxy, oxo, carboxyl, epoxy, and amino \( \alpha \)-amino acids.\(^{161}\) They have also been used in cyclization of peptides via ring closing metathesis.\(^{162}-^{165}\)

![Figure 2.8 Examples of \( \gamma,\delta \)-unsaturated amino acids](image)

2.3.1 Alkylation of aspartic acid

Alkylation of aspartic acid is a concept that has been examined in detail in the recent literature.\(^{165}-^{170}\) Treatment of aspartic acid with lithium di-isopropylamide (LDA), or lithium hexamethyldisilazide (LHMDS) in tetrahydrofuran at -78\(^\circ\)C, followed by warming to -30\(^\circ\)C, recooling to -78\(^\circ\)C and adding the electrophile results in the formation of \( \beta \)-alkylated products in 50-60\% yields. No products resulting from \( \alpha \)-deprotonation were observed. Rapoport et al.\(^{167}\) used 9-phenylfluorenyl-9-yl, a large N-protecting group to suppress \( \alpha \)-deprotonation. Diastereomeric ratios and yields for these alkylations depend on the ester identity, the reactivity of the electrophile, and the base used. Alkylation with alkyl halides gives low yields, while alkylation with bis-electrophiles gave no products. On the other hand, alkylation with activated electrophiles like allyl bromide and benzyl bromide proceeded with good yields. The diastereomeric ratios were found to depend on the ester identity at both the \( \alpha \)- and \( \beta \)-carboxyl groups. Potassium hexamethyldisilazide (KHMDs) and the corresponding lithium amide (LHMDS) were found to give opposite diastereoselectivity. Trapping studies using TMSCl showed that
the potassium and lithium enolates were different giving two distinct silyl ketene acetals \footnote{169} (Figure 2.9).

\begin{figure}[h!]
\centering
\includegraphics[width=0.8\textwidth]{silyl_ketene_acetals.png}
\caption{Silyl ketene acetals}
\end{figure}

From these results KHMDS forms an E-enolate while the lithium analogue forms a Z-enolate. It has been reported that (Z)-lithium ester enolates are significantly more reactive than the corresponding (E)-lithium ester enolates. The facial selectivity of electrophilic attack can be explained by a novel type of enolate-ester chelation that is controlled by enolate geometry. For the LHMDS/THF system, the Z-lithium enolate (Figure 2.10), in which the OM\textsuperscript{+} cannot form a cyclic chelate because of its (E)-geometry, assumes a hydrogen-in-plane conformation that is attacked opposite the bulky nitrogen. The (Z)-potassium enolate, although significantly less reactive, selectively provides the opposite stereoselectivity. This can be explained by the fact that for the (Z)-potassium enolate, the alkylation is determined by a preference for \(\alpha\)-ester chelation by the counterion of the enolate oxygen atom, giving a cyclic chelate that the electrophile attacks preferentially opposite the bulky protected nitrogen group. North \textit{et al.} has also proposed the diastereoselectivity observed is due to the formation of a chelated enolate (Figure 2.11). \footnote{170}
When the method reported by North et al. was applied to the N\textsuperscript{\textalpha}-Boc aspartic acid 4\textalpha, the reaction would not go to completion necessitating the development of a modified procedure. The protected amino acid was dissolved in THF and cooled to -42°C. LHMDS followed by HMPA were then added and the mixture stirred for 30 min before adding the electrophile. When the electrophile was allyl bromide, two products (5\textalpha and 6\textalpha) in a total yield of 57% and a ratio of 3:1 were obtained after purification by flash column chromatography (Scheme 2.1). The major isomer was found to be the (2\textS, 3\textR) isomer 5\textalpha on NMR analysis of the final bicyclic dipeptide, in agreement with literature.\textsuperscript{158-162} When 4\textbeta was subjected to the same reaction condition, a yield of 62% and a ratio of 4:1 in favor of the (2\textS, 3\textR) isomer 6\textalpha was obtained. The difference in yield and diastereomeric ratio is as a result of the different ester protection used.
Scheme 2.1 Alkylation of aspartic acid with allyl bromide

In case of alkylation with benzyl bromide, only one β-benzyl substituted aspartic acid (7a or 7b) was isolated after column chromatography (Scheme 2.2). Cbz protected aspartic acid 4c could also be alkylated to give β-benzyl substituted aspartic acid in 70% yield. However the alkylation is done at -78°C otherwise a lot of α-alkylated aspartic acid is obtained.

Scheme 2.2 Alkylation of aspartic acid with benzyl bromide

The acidity of the α-hydrogen of amino acids can be lowered by protection of the α-carboxyl group as an ortho ester. The ortho ester is stable to strongly basic reagents and is easily regenerated under mild acidic conditions. Ortho esters can be prepared from nitriles, imido esters, ortho ester exchange or directly from carboxylic acids. The ester of 3-methyl-3-hydroxymethyloxetane rearranges in the presence of BF$_3$OEt$_2$ to give bridged ortho esters (Figure 2.12). Coordination of the oxetane ester xii with lewis acid forms xiii which induces heterolysis of the dioxetane C-O bond with ester carbonyl...
participation to give zwitterions xiv that collapses to give the ortho ester xv. The 3-
methyl-3-hydroxyl oxetane ester of aspartic acid 10 was easily converted to the ortho
ester 11 in 70% yield after exposure to BF$_3$OEt$_2$. Alkylation of the ortho ester 11 with
benzyl bromide in the presence of LHMDS and HMPA gave β-alkylated aspartic acid
analogue 12 in 61% yield.

\[
\begin{align*}
\text{xii} & \xrightarrow{\text{BF}_3\text{OEt}_2} \text{xiii} & \xrightarrow{\text{LHMDS/HMPA}} \text{xiv} \\
\end{align*}
\]

**Figure 2.12** Mechanism for the formation of ortho ester

**Scheme 2.3** Alkylation of ortho ester protected aspartic acid

**2.3.2 Kazmaier-Claisen rearrangement reaction**

In 1975, Steglich et al. described the thermal Claisen rearrangement of N-benzoyl-α-
amino acid allyl esters on treatment with a dehydrating agent such as phosgene in which
a 5-allyloxazole intermediate was formed.$^{175-178}$ As a result of the fixed olefin geometry
in the oxazole ring, and a strong preference for the *chair-like* transition state, the
diastereoselectivity for these reactions is very high (Figure 2.12). However, this
methodology is limited to N-benzoyl amino acid esters (and related aromatic or
heteroaromatic N-acyl derivatives), because other common N-protecting groups like
carbamates do not allow formation of the oxazole. Barlett *et al.* investigated the Ireland-
Claisen rearrangement\textsuperscript{179} of N-acylated glycine allyl esters in detail.\textsuperscript{180} This method
offers great flexibility with respect to variation of the protecting group. In the
rearrangement of crotyl esters syn selectivity, which can be explained by the formation of
an (E)-Lithium enolate and its chelation by the anionic acylamide α-group, was observed.
However, the diastereoselectivity of the rearrangement strongly depends on solvent, base,
N-protecting group, and the substitution of the allylic ester moiety.\textsuperscript{153} Similar chelate-
bridged enolates have also been postulated in sigmatropic rearrangement of α-alkoxy-
substituted allyl esters.\textsuperscript{181-185} Corey and Lee have also described an asymmetric boron
enolate variant of the Claisen rearrangement.\textsuperscript{186} All these procedures for the Claisen
rearrangement of ester enolates of α-amino acid allyl esters make use of the Ireland
silylketene acetal variant.\textsuperscript{187} Attempts to rearrange lithiated glycine allyl esters directly
leads to decomposition of the lithium enolates, since the rearrangement occurs at high
temperatures.
To improve the stability of the enolates, Kazmaier used metal chelated enolates that also are superior to silylketene acetals, both in terms of their reactivity and selectivity. Due to the fixed enolate geometry, as a result of chelate formation, the rearrangement proceeds with high degree of diastereoselectivity, independent of the substitution pattern and the protecting groups on nitrogen. The nitro group must however be monoprotected as a Cbz, Boc or TFA since no rearrangement is observed with tertiary amino group or N-phthaloyl protected amino acids. Many different metal salts (ZnCl₂, MgCl₂, EtAlCl₂, SnCl₂, Al(OiPr)₃, CoCl₂ etc.) can be used for chelation but the best results are obtained with ZnCl₂. When E-allyl ester is used in this reaction, only the syn-product is
generated in high diastereoselectivity (95% ds), resulting from a preferential rearrangement via a chair-like transition state A (Figure 2.12). In the chair-like transition state, sterical interactions between the pseudoaxial hydrogen and the chelate complex in the boat-like transition state are avoided. When Z-allyl esters are used in this rearrangement, a diastereomeric mixture will be formed since the chair-like transition state B that is formed is competitive with a boat-like transition state C. The lower diastereoselectivity may result from the interactions of the cis-oriented side chain and the chelated enolate destabilizing the chair-like transition state.

![Transition state in Kazmaier-Claisen rearrangement](image)

**Figure 2.14** Transition state in Kazmaier-Claisen rearrangement

The Kazmaier-Claisen rearrangement described is highly diastereoselective but not enantioselective. A number of strategies have been studied for an asymmetric rearrangement.\textsuperscript{153}
2.4 Experimental

General

$^1$H and $^{13}$C NMR spectra were recorded on Bruker DRX 500 and DRX 600 spectrometers. The chemical shifts were expressed in ppm ($\delta$) downfield from tetramethylsilane (TMS) as an internal standard. X-ray crystallography was conducted at the Molecular Structure Laboratory, Department of Chemistry, University of Arizona. Mass spectrometric analysis were conducted by Mass Spectrometry Laboratory, Department of Chemistry, University of Arizona. Optical rotations were measured on a JASCO P1020 polarimeter. Column chromatography was performed with 200-400 silica gel from EM Science. Thin layer chromatography was performed with Kodak F-254 silica gel plates. Dichloromethane was distilled from CaH$_2$ and THF from potassium and benzophenone under Argon atmosphere. All chemicals were purchased from Aldrich Chemical Company and used as received while Aspartic acid was purchased from Calbiochem-Novabiochem Corporation. Melting points are uncorrected and were obtained in open capillaries. Ozone was generated from an ozone generating machine from Dr. Eugene Mash laboratories, University of Arizona.

General alkylation of aspartic acid

In a 100-mL flask, $\alpha$-methyl $\beta$-benzyl N$^{\alpha}$-tert-butoxycarbonylamino-(S)-aspartate 4a (2.3 g, 6.82 mmol) was dissolved in 10 mL THF and cooled to -42°C. Lithium bis(trimethylsilyl)amide (LHMDS), (1M solution in THF, 15.0 mL, 15.0 mmol) followed by hexamethylphosphoramide (HMPA) (0.7 mL, 4.02 mmol) were added and the mixture stirred at -42°C for 30 min before adding allyl bromide (0.8 mL, 9.24 mmol). The
mixture was stirred for a further 4 h, and 20 mL saturated aqueous NH₄Cl (20 mL) added. The mixture was warmed up to room temperature and the THF evaporated off. The product was redissolved in DCM (50 mL) and washed by saturated aqueous NH₄Cl (2 x 50 mL) and brine (60 mL). The organic layer was dried over MgSO₄, and the solvent evaporated off. After purification on a silica gel column with hexane-ethyl acetate (10:1 v/v), two colorless liquids (1.1 g of 5a and 0.4 g of 6a) were obtained in a total yield of 58%.

**Methyl 2-tert-butoxycarbonylamino-3-carbobenzyloxy-(2S, 3R)-hex-5-enoate (5a):**

\[ [\alpha]^{25}_D = +28.7^\circ \ (c = 1.52, \text{CHCl}_3); \ ^1H \text{ NMR, 500 MHz, CDCl}_3, \delta(\text{ppm}): 7.37-7.29 (5H, m), 5.84-5.69 (1H, m), 5.49 (1H, d, J = 9.92 Hz), 5.23-5.06 (4H, m), 4.60 (1H, dd, J = 3.63, 9.92 Hz), 3.56 (3H, s), 3.22-3.13 (1H, m), 2.56-2.45 (1H, m), 2.36-2.26 (1H, m), 1.44 (9H, s); \ ^13C, 125 MHz, CDCl₃, \delta(\text{ppm}): 173.3, 171.2, 155.8, 135.3, 134.1, 128.5, 128.3, 128.2, 118.2, 80.0, 67.2, 53.5, 51.9, 46.4, 32.8, 28.2; HRMS (FAB) MH⁺ calcd for 378.1917, found 378.1921.

**Methyl 2-tert-butoxycarbonylamino-3-carbobenzyloxy-(2S, 3S)-hex-5-enoate (6a):**

\[ [\alpha]^{25}_D = +25.5^\circ \ (c = 1.33, \text{CHCl}_3); \ ^1H \text{ NMR, 500 MHz, CDCl}_3, \delta(\text{ppm}): 7.39-7.31 (5H, m), 5.74-5.69 (1H, m), 5.29 (1H, d, J = 9.92 Hz), 5.21-5.13 (4H, m), 4.68 (1H, dd, J = 4.78, 8.6 Hz), 3.62 (3H, s), 2.95-2.92 (1H, m), 2.55-2.49 (1H, m), 2.28-2.25 (1H, m), 1.44 (9H, s); \ ^13C, 125 MHz, CDCl₃, \delta(\text{ppm}): 172.5, 170.5, 155.2, 135.1, 134.5, 128.6, 128.5, 117.8, 80.2, 67.5, 54.1, 52.0, 47.9, 32.2, 28.2; HRMS (FAB) MH⁺ calcd for 378.1917, found 378.1927.
The same protocol was followed for alkylation of 4b to give (2.5 g of 5b and 0.75 g of 6b) in a total yield of 58% and a ratio of 3:1 in favor of 5b.

**Methyl 2-tert-butoxycarbonylamino-3-carbobenzyloxy-(2S, 3R)-hex-5-enooate (5b):**

\[[\alpha]^{25}_D = +28.7^\circ (c = 1.52, \text{CHCl}_3)\]; \(^1\)H NMR, 500 MHz, CDCl\(_3\), \(\delta\)(ppm): 7.37-7.29 (5H, m), 5.84-5.69 (1H, m), 5.49 (1H, d, \(J = 9.92\) Hz), 5.23-5.06 (4H, m), 4.60 (1H, dd, \(J = 3.63, 9.92\) Hz), 3.56 (3H, s), 3.22-3.13 (1H, m), 2.56-2.45 (1H, m), 2.36-2.26 (1H, m), 1.44 (9H, s); \(^13\)C, 125 MHz, CDCl\(_3\), \(\delta\)(ppm): 173.3, 171.2, 155.8, 135.3, 134.1, 128.5, 128.3, 128.2, 118.2, 80.0, 67.2, 53.5, 51.9, 46.4, 32.8, 28.2; HRMS (FAB) MH\(^+\) calculated for 378.1917, found 378.1921.

**Methyl 2-tert-butoxycarbonylamino-3-carbobenzyloxy-(2S, 3S)-hex-5-enooate (6b):**

\[[\alpha]^{25}_D = +25.5^\circ (c = 1.33, \text{CHCl}_3)\]; \(^1\)H NMR, 500 MHz, CDCl\(_3\), \(\delta\)(ppm): 7.30-7.18 (10H, m), 5.56 (1H, d, \(J = 10\) Hz), 5.06 (2H, s), 4.47 (1H, dd, \(J = 4.0, 10\) Hz), 3.58 (3H,
s), 3.42-3.39 (1H, m), 3.12-3.08 (1H, m), 2.87-2.83 (1H, m), 1.48 (9H, s); $^{13}$C, 125 MHz, CDCl$_3$, $\delta$(ppm): 172.7, 171.6, 155.7, 137.8, 135.2, 129.0, 128.9, 128.54, 128.48, 128.43, 128.40, 128.37, 128.31, 128.25, 126.7, 80.0, 66.8, 53.5, 52.4, 48.7, 34.7, 34.7, 28.2; HRMS (FAB) $\text{MH}^+$ calculated for 428.2073, found 428.2071.

**Benzyl 2-carboxycarbonylamino-3-carbobenzyloxy-4-phenyl-(25', 3/?)-butanoate (7b):** $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.30-7.18 (10H, m), 5.64 (1H, d, $J=10.0$ Hz), 5.17 (1H, d, $J=12.0$ Hz), 5.08 (1H, d, $J=12.0$ Hz), 4.45-4.42 (1H, dd, $J=4.0, 10.0$ Hz), 3.28-3.24 (1H, m), 3.06-3.02 (1H, m), 1.46 (9H, s), 1.30 (9H, s); $^{13}$C, 125 MHz, CDCl$_3$, $\delta$(ppm): 172.3, 171.3, 155.9, 138.1, 135.4, 129.1, 128.5, 128.45, 128.2, 128.1, 126.6, 81.9, 79.9, 67.1, 53.8, 49.0, 35.1, 28.3, 27.8.

**Benzyl 2-tert-butoxycarbonylamino-3-carbobenzyloxy-4-phenyl-(2S, 3R)-butanoate (7c):** $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.42-7.08 (10H, m), 5.92 (1H, d, $J=10.0$ Hz), 5.21-5.0 (4H, m), 4.49 (1H, dd, $J=3.8, 10.0$ Hz), 3.32-3.23 (1H, m), 3.04 (1H, dd, $J=7.0, 13.5$ Hz), 2.81 (1H, dd, $J=8.5, 14.0$ Hz), 1.29 (9H, s); $^{13}$C, 125 MHz, CDCl$_3$, $\delta$(ppm): 172.5, 171.4, 157.0, 138.3, 135.7, 129.6, 129.0, 128.9, 128.7, 128.6, 128.5, 127.1, 82.5, 67.7, 67.5, 54.6, 49.4, 35.5, 28.2.

**Synthesis and alkylation of ortho ester protected aspartic acid**

Boc-Asp(Bzl)-OH (4.0g, 12.4 mmol) was dissolved in a CH$_2$Cl$_2$ (40 mL) and cooled to 0°C. DCC (3.1 g, 15.0 mmol) was added and the mixture stirred for 10 min before adding 3-methyl-3-hydroxymethyloxetane (1.9 mL, 19.05 mmol) and stirring for 8 hr. DCHU was filtered off and the filtrate diluted with CH$_2$Cl$_2$ (40 mL) and washed by saturated aqueous NH$_4$Cl (2 x 40 mL) and brine (40 mL). The product was purified by column to
give the oxetane ester 10 (4.7g, 95% yield). The ester was dissolved in CH$_2$Cl$_2$ (50 mL) and BF$_3$.OEt$_2$ (70 µL, 0.55 mmol) was added and the mixture stirred at room temperature 10 hr. TEA (300 µL, 2.2 mmol) was added and the mixture stirred for 40 min. The mixture was then diluted by CH$_2$Cl$_2$ (30 mL) and washed by saturated aqueous NH$_4$Cl (2 x 40 mL) and brine (40 mL). The product was then recrystallized from ethylacetate/hexanes to give the ortho ester 11 (3.9 g, 84% yield).
CHAPTER 3

SYNTHESIS OF Nle-Gly BICYCLIC DIPEPTIDE MIMETICS

3.1 Introduction

A Nle-Gly bicyclic dipeptide mimetic was designed to be substituted for the Nle-Gly dipeptide unit in CCK-8 peptide analogues. Different templates can be used to mimic a Nle-Gly dipeptide unit. The side chain can be incorporated as part of the ring as in xvi (Figure 3.1) or outside the ring as in xvii. In order to fully study the biologically active conformation, xvi was chosen as the template since the chi-space is also constrained (Figure 3.1). This bicyclic can be obtained from β-allyl substituted aspartic acid(s) or from β-vinyl substituted norleucine, the latter being a product of the Kazmaier-Claisen rearrangement reaction.150-153

Figure 3.1 Nle-Asp bicyclic dipeptide mimetics

3.2 Synthesis of Nle-Gly bicyclic dipeptide mimetic from β-allyl substituted aspartic acid

Hydrogenation of compound 5a reduced the alkene and deprotected the benzyl ester to afford a carboxylic acid which was then subjected to reduction. Reduction of the carboxylic acid using borane and sodium perborate188 failed to give the desired product.
Utilization of a recently reported method that uses BOP, DIPEA and NaBH$_4$\textsuperscript{189} gave the desired alcohol in a yield of 53%, contaminated with some 5-membered ring lactone resulting from cyclization. Lactone formation was also found to happen during column purification while use of the crude alcohol gave low yields of the bicyclic dipeptide mimetic. Characterization of alcohol 13 could not be done due to lactone contamination.

\[ \text{BocHN} \quad \text{CO}_2\text{Bn} \quad 1. \text{H}_2, \text{Pd/C} \quad \text{CO}_2\text{Me} \quad 2. \text{BOP, DIPEA, NaBH}_4 \quad 53\% \]

\[ \text{5a} \quad \text{13} \]

\[ \text{Swern oxidation}^{190} \text{ of the alcohol afforded the aldehyde which without purification was coupled with } L-\text{Cys to form an intermediate thiazolidine. Bicyclization was then achieved by heating the reaction mixture at } 50^\circ\text{C for 3 days, followed by methylation to afford a bicyclic dipeptide mimetic for Nle-Gly 2a in 35\% yield from the alcohol 13 (Scheme 3.1). When the minor product 6a was subjected to hydrogenation followed by BOP/DIPEA/NaBH}_4 \text{ reduction, only the lactone 14 was obtained in 58\% yield (scheme 3.2). Obviously, a } \text{trans}-\text{relationship of the } \beta\text{-substituent makes this lactone thermodynamically more stable compared to the } \text{cis}-\text{analogue.} \]
Scheme 3.2 Hydrogenation and reduction of β-allyl substituted aspartic acid

3.3 Synthesis of Nle-Gly bicyclic dipeptide mimetic via the Kazmaier-Claisen rearrangement reaction

To fully investigate the stereochemical and topographical requirements of the receptors, other isomers of Nle-Gly bicyclic dipeptide mimetic were synthesized via the Kazmaier-Claisen rearrangement reaction. Coupling of Boc-Gly with commercially available trans 2-hexen-1-ol gave 17. Compound 17 was then subjected to the Kazmaier-Claisen rearrangement reaction to give racemic β-vinyl substituted Nle 18. The crude product was then subjected to ozonolysis and the resultant crude aldehyde coupled with L-Cys-OMe in a two step strategy involving thiazolidine formation and bicyclization. Two bicyclic dipeptide mimetics 2b, and 2c were isolated in a yield of 50% from 17.
Scheme 3.3 Synthesis of Nle-Gly BTD via the Kazmaier-Claisen rearrangement reaction

3.4 Characterization of Nle-Gly bicyclic dipeptide mimetics

The three Nle-Gly bicyclic dipeptide mimetics were characterized by DQF-COSY and 1D-transient nOe. The nOe's from the N\textsuperscript{\#}-Boc-products were not sufficient to assign the chiral centers since not all the nOe relationships were observable particularly for the left-hand ring. As a result, the N-trifluoroacetamide analogs (21a, 21b, and 21c) were synthesized using trifluoroacetic anhydride and pyridine\textsuperscript{192} and their 1D-transient nOe data used to assign the chiral centers. From our earlier work\textsuperscript{145-146} and also from Scheme 3.1, the 3\textit{R}-\beta-propyl substituted homoserines should give only one BTD while the 3\textit{S}-
isomers should give two BTD’s with the bridgehead-H up or down. Based on this information, the (2R, 3R)-isomer of aldehyde 19 should give only one BTD (in this case 2c), while the (2S, 3S)-isomer should give two BTD’s. However, only two products were obtained from the cyclization. Compounds 21a and 21c showed a weak nOe between H4 and H5 (1.12% and 0.83% respectively) compared to a value of 5.43% observed for 21b. This is an indication of a trans relationship for H4 and H5 in compounds 21a, and 21c and a cis relationship in 21b. A similar trans relationship is observed in 21b and 21c for H3 and H4 while a strong nOe (4.45%) indicative of a cis relationship is observed for 21a.

![Figure 3.2 nOe data for the Nle-Gly bicyclic dipeptide mimetics](image)

Additionally, the structure of compound 21c was confirmed by an X-ray crystal structure.

![Figure 3.3 X-Ray crystal structure of a Nle-Gly bicyclic dipeptide mimic](image)
3.5 Experimental

General procedure for hydrogenation of the benzyl ester and reduction of the resultant carboxylic acid

Compound 5a (2.88 g, 6.7 mmol) was dissolved in degassed methanol (40mL). Pd/C (10%) was added and the mixture was exposed to hydrogen gas for 2 h. The catalyst was filtered off through Celite and the solvent evaporated off. The crude product was dried under vacuum and then dissolved in THF (35 mL). Benzotriazol-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (3.2 g, 7.23 mmol) followed by DIPEA (1.3 mL, 7.46 mmol) were added and the mixture stirred for about 5 min until it turned clear. The solution was then cooled to 0°C and NaBH₄ (400 mg, 10.57 mmol) added slowly. The mixture was stirred for 30 min at 0°C and at room temperature for 18 h. The solvent was evaporated off and the product dissolved in ethylacetate (80 mL) and the organic phase was washed with saturated aqueous NaHCO₃ (2 x 50 mL), 5% aqueous HCl (2 x 50 mL) and brine (50 mL). The organic phase was then dried over Na₂SO₄ and the solvent evaporated off. The product was purified by column to give 1.2 g of 13 (3.71 mmol) in 55% yield. The product was then subjected to Swern oxidation. Oxalyl chloride (2.8 mL, 5.6 mmol) was dissolved in CH₂Cl₂ (40 mL). The solution was cooled to -78°C, DMSO (530 μL, 7.47 mmol) added and the solution stirred for 10 min before adding 10 1.2 g, 3.71 mmol). The mixture was stirred for 25 min at -78°C before adding DIPEA (2.6 mL, 14.93 mmol) , removing the cold bath and stirring for a further 20 min. Water (20 mL) was added, and the organic phase was separated and washed with saturated aqueous NH₄Cl (2 x 50 mL) and brine (50 mL). The organic phase was dried
over MgSO₄ and the solvent evaporated off to give 1.17 g of the crude aldehyde after
drying. The crude aldehyde was dissolved in pyridine (30 mL) containing molecular
sieves 4 Å° and the mixture stirred at room temperature for 4 h. Pyridine (60 mL) was
added and the mixture heated at 50°C for 3 days. The solvent was evaporated off and the
product dissolved in water (20 mL) which was acidified to pH = 2 using 1N aqueous
HCl. The aqueous layer was extracted with ether (4 x 20 mL) and the ether layer dried
over MgSO₄. The solvent was evaporated off and the product dried under high vacuum to
give 1.03 g of crude product. The crude product was dissolved in CH₂Cl₂ (15 mL) and
cooled to 0°C. DCC (650 mg, 3.15 mmol) was added and the mixture stirred for 10 min
before adding methanol (450 μL, 11.12 mmol) and DMAP (30 mg, 0.25 mmol) and
stirring the mixture for 8 h. The mixture was filtered and the filtrate diluted by CH₂Cl₂
(20 mL). The organic phase was washed by saturated aqueous NH₄Cl (2 x 20 mL) and
brine (30 mL). The organic phase was dried over MgSO₄ and the solvent evaporated off.
The product was then purified by column to give 470 mg of 2a as a yellow oil in 31%
yield from 5a.

**Methyl (3S, 4R, 5S, 8R)-4-benzyl-3-tert-butoxycarbonylamino-1-aza-2-oxo-6-
thiabicyclic [3.3.0]-octane-8-carboxylate (2a):** [α]²⁵^D = -88.7° (c = 0.98, CHCl₃); ^1^H
NMR, 500 MHz, CDCl₃, δ(ppm): 7.34-7.2 (5H, m), 5.04-5.0 (2H, m), 4.89(1H, d, J = 3.5
Hz), 4.69 (1H, t, J = 8.0 Hz), 3.77 (3H, s), 3.38-3.34 (IH, m), 3.25 (1H, dd, J = 4.0, 11.5
Hz), 3.04 (1H, dd, J = 5.0, 14.0 Hz), 3.02-2.94 (1H, m), 2.53(1H, m); ^1^C, 125 MHz,
CDCl₃, δ(ppm): 173.2, 169.7, 155.5, 138.1, 128.9, 128.7, 126.7, 80.5, 68.4, 57.6, 56.1,
52.9, 44.2, 36.9, 34.0, 28.3; HRMS (FAB) \text{MH}^+ \text{ calculated for } 324.1811, \text{ found 324.1818.}

The same procedure was repeated for the debenzylation and reduction of 6a to only give the lactone 14 as a white solid in 58% yield.

**3-tert-Butoxycarbonylamino-4-propyl-dihydro-furan-2-one (14):** $[\alpha]^D_{25} = -12.1^\circ (c = 1.01, \text{CHCl}_3), \text{mp} = 106-108^\circ \text{C}; ^1\text{H NMR, 500 MHz, CDCl}_3, \delta$(ppm); 5.0-4.83 (1H, br), 4.40 (1H, t, $J = 8.5$ Hz), 4.11 (1H, br), 3.86 (1H, t, $J = 10.0$ Hz), 0.94 (3H, t, $J = 7.5$ Hz); $^{13}\text{C}, 125 \text{ MHz, CDCl}_3, \delta$(ppm): 175.2, 155.5, 80.6, 70.4, 55.7, 43.0, 33.1, 28.4, 20.2, 14.1. HRMS (FAB) \text{MH}^+ \text{ calculated for } 244.1549, \text{ found 244.1559.}

**Synthesis of Nle-Gly BTD's via the Kazmaeir-Claisen rearrangement reaction**

N$_2$-Boc-Gly (10.0 g, 57.08 mmol) was dissolved in CH$_2$Cl$_2$ (120 mL) and DMF (12 mL). The solution was cooled to 0°C and DCC (15.0 g, 72.7 mmol) was added. The mixture was stirred for 10 min before adding trans 2-hexen-1-ol (8.5 mL, 72.05 mmol) and DMAP (500 mg, 4.09 mmol) and stirring the mixture at 0°C for 30 min and at room temperature for 8 h. The mixture was filtered and the filtrate washed by saturated aqueous NH$_4$Cl (2 x 60 mL) and brine (80 mL). The organic phase was dried over MgSO$_4$ and the solvent evaporated off. The product was purified by column to give 13.0 g of 17 in 89% yield. In the Kazmaeir-Claisen rearrangement reaction, 17 (12.0 g, 46.66 mmol) was dissolved in THF (500 mL). ZnCl$_2$ (0.5 M in THF, 112 mL, 56.0 mmol) was added and the mixture cooled to -78°C. Freshly prepared LDA (136.5 mmol) was slowly added and the mixture stirred for 20 min at -78°C. The cold bath was removed and the mixture stirred for 1 h. THF was evaporated off and the product dissolved in 1 N HCl
The aqueous layer was extracted with ether (4 x 50 ml). The ether layer was evaporated to 100 mL and extracted with 1 N NaOH. The NaOH layer was acidified to pH = 2 by 6 N HCl and then extracted with ether (4 x 50 mL). The ether layers were combined and evaporated to 200 mL and washed with brine (40 mL). The ether layer was then dried over MgSO₄ and the solvent evaporated off. The crude product was dried under high vacuum and then dissolved in DCM (200 mL). The mixture was cooled to -78°C and subjected to ozonolysis. Dimethyl sulfide (30 mL) was added and the mixture stirred for 70 h at room temperature. The solvent was evaporated off and the product dried under high vacuum. The dried product was dissolved in absolute ethanol (100 mL) containing 4 Å molecular sieves. L-Cysteine methyl ester hydrochloride and DIPEA were added and the mixture stirred for 10 h. The molecular sieves were filtered out and the solvent evaporated off. The product was dried under high vacuum and dissolved in CH₂Cl₂ (400 mL). The mixture was cooled to 0°C and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and DIPEA added. The mixture was stirred for 12 h and the solvent evaporated to 200 mL. The organic phase was washed by 1 N HCl (2 x 100 mL) and brine (100 mL). The solvent was dried over MgSO₄ and then evaporated off. The products were purified by column to give 4.0 g of 2b and 5.8 g of 2c in a total yield of 59% from 17.

**Methyl (3S, 4S, 5S, 8R)-4-benzyl-3-tert-butoxycarbonylamino-1-aza-2-oxo-6-thiabicyclic [3.3.0]-octane-8-carboxylate (2b):** 

\[ [\alpha]_{D}^{25} = -215.4^\circ \text{ (c} = 0.80, \text{ CHCl}_3) \]  

**1H NMR, 500 MHz, CDCl₃, δ (ppm):** 5.19 (1H, q, J = 6.0 Hz), 5.07 (1H, q, J = 4.5 Hz), 4.88 (1H, d, J = 4.5 Hz), 5.02-5.0 (1H, t, J = 8.0 Hz), 4.06 (1H, m) 3.77 (3H, s), 3.40-3.27 (2H,
m), 2.67 (1H, m), 1.84-1.79 (1H, m), 1.60-1.39 (11H, m), 0.96 (3H, t, 7.5 Hz); $^{13}$C, 125 MHz, CDCl$_3$, δ(ppm): 176.4, 170.4, 155.4, 80.3, 69.3, 59.0, 56.4, 52.8, 40.1, 35.3, 33.0, 28.3, 21.0, 14.0; HRMS (FAB) MH$^+$ calculated for 359.1641, found 359.1632.

Methyl (3R, 4R, 5S, 8R)-4-benzyl-3-tert-butoxycarbonylamino-1-aza-2-oxo-6-thiabicyclic [3.3.0]-octane-8-carboxylate (2c): $[\alpha]_{D}^{25} = -94.3^\circ$ (c = 1.60, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, δ(ppm): 5.16 (1H, q, J = 4.0 Hz), 4.95 (1H, d, J = 6.1 Hz), 4.75 (1H, d, J = 7.6 Hz), 4.42 (1H, t, J = 8.0 Hz), 3.38-3.27 (2H, m) 3.78 (3H, s), 3.38-3.27 (2H, m), 2.25-2.23 (1H, m), 1.85-1.79 (2H, m), 1.60-1.33 (11H, m), 0.96 (3H, t, 7.1 Hz). $^{13}$C, 125 MHz, CDCl$_3$, δ(ppm): 171.2, 169.5, 155.5, 80.2, 66.8, 59.4, 57.7, 53.4, 52.9, 35.0, 33.7, 28.4, 20.8, 14.1; HRMS (FAB) MH$^+$ calculated for 359.1641, found 359.1637.

**General procedure for N$^\alpha$-trifluoroacetamide protection**

The N$^\alpha$-Boc group was deprotected with TFA (20%) and the resultant compounds dissolved in CH$_2$Cl$_2$ and treated with 2.0 eq trifluoroacetic anhydride and 3 eq pyridine to give the N$^\alpha$-trifluoroacetamide analogues.

Methyl (3S, 4R, 5S, 8R)-4-benzyl-3-[2,2,2-trifluoroacetylamino]-1-aza-2-oxo-6-thiabicyclic [3.3.0]-octane-8-carboxylate (21a): white solid, mp = 119-121 °C, $[\alpha]_{D}^{25} = -151.9^\circ$ (c = 0.58, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, δ(ppm): 7.48 (1H, d, J = 7.0 Hz), 5.08 (1H, q, J = 4.0, 8.0 Hz), 4.96 (1H, d, J = 5.0 Hz), 4.89 (1H, t, J = 8.0 Hz), 3.84 (3H, s), 3.53-3.41 (2H, m), 2.71-2.69 (2H, m), 1.50-1.38 (4H, m), 0.97 (3H, t, J = 7.5 Hz); $^{13}$C, 125 MHz, CDCl$_3$, δ(ppm): 171.3, 170.4, 158.1 (q, J = 37.7 Hz), 116.1 (q, J = 286.2 Hz),
Methyl (3S, 4S, 5S, 8R)-4-benzyl-3-[2,2,2-trifluoroacetylamino]-1-aza-2-oxo-6-thiabicyclic [3.3.0]-octane-8-carboxylate (21b): white solid, mp = 109-111 °C, $[\alpha]^{25}_D = -259.0^\circ$ (c = 0.69, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.37 (1H, d, J = 8.0 Hz), 5.21 (1H, d, J = 6.5 Hz), 5.01 (1H, q, J = 5.0 Hz, 8.5 Hz), 4.41 (1H, t, J = 9.5 Hz), 3.80 (3H, s), 3.47-3.29 (IH, m), 2.77-2.74 (1H, m), 1.78-1.62 (2H, m), 1.45-1.39 (2H, m), 0.96 (3H, t, J = 7.5 Hz); $^{13}$C $\delta$(ppm): 174.6, 170.3, 157.6 (q, J = 37.6 Hz), 115.6 (q, J = 286.2 Hz), 69.3, 59.0, 55.4, 53.0, 39.6, 35.4, 32.9, 20.9, 14.0; HRMS (FAB) MH$^+$ calculated for 355.0939, found 355.0933.

Methyl (3R, 4R, 5S, 8R)-4-benzyl-3-[2,2,2-trifluoroacetylamino]-1-aza-2-oxo-6-thiabicyclic [3.3.0]-octane-8-carboxylate (21c): white solid, mp = 145-147 °C $[\alpha]^{25}_D = -144.8^\circ$ (c = 0.54, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.21 (1H, d, J = 7.5 Hz), 5.13 (1H, q, J = 3.5, 7.0 Hz), 4.81 (1H, d, J = 7.5 Hz), 4.77 (1H, t, J = 8.5 Hz), 3.79 (3H, s), 3.42-3.33 (2H, m), 2.42-2.38 (2H, m), 1.81-1.75 (1H, m), 1.68-1.63 (1H, m), 1.45-1.39 (2H, m), 0.96 (3H, t, J = 7.5 Hz); $^{13}$C, 125 MHz, CDCl$_3$, $\delta$(ppm): 169.3, 169.1, 157.7 (q, J = 37.7 Hz), 115.6 (q, J = 286 Hz), 67.0, 58.3, 57.7, 53.1, 52.5, 35.4, 33.5, 20.7, 14.0; HRMS (FAB) MH$^+$ calculated for 355.0939, found 355.0933.
CHAPTER 4
SYNTHESIS OF Nle-Asp BICYCLIC DIPEPTIDE MIMETICS

4.1 Introduction

To introduce a turn at the C-terminal of our target peptides, a Nle-Asp bicyclic dipeptide mimetic was designed to be substituted for the Nle-Asp dipeptide unit in Tyr-Nle-Gly-Trp-Nle-Asp-Phe-NH₂.

![Bicyclic dipeptide mimetic for Nle-Asp dipeptide unit](image)

Synthesis of indolizidinone type compounds through dehydroamino acid intermediates has been reported by a number of groups. Revesz employed the Schollkopf bislactam ether methodology to synthesize the indolizidinone scaffold without any side chain appendages. Lubell assembled all the isomers of the indolizidinone skeleton by a Claisen condensation of two identical glutamic acid derivatives. Kahn followed a similar approach to synthesize one stereoisomer of the indolizidinone. In our group, synthetic methodologies have been developed to introduce side chains on both sides of the ring, starting from analogues of pyroglutamic acid. Based on this work, the synthesis of a bicyclic dipeptide mimetic for Nle-Asp starting from aspartic acid was designed (Figure 2.7). Oxidation of carbamate protected δ,ε-unsaturated amino acids leads to the formation of hemiaminals instead of the free aldehyde. The hemiaminal can be converted to 2,4-pyrrolidinedicarboxylate (PDC), an inhibitor of glutamate uptake.
L-glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. In this role, glutamate binds to a number of different proteins, transport systems, and enzymes. In addition, abnormal levels of glutamate have been linked to neurological disorders, including ischemia, anoxia, hypoglycemia, epilepsy, and Huntingtons, Parkinsons, and Alzheimers diseases. As a result, conformationally restricted glutamate analogues were synthesized to study the interactions between synthetic amino acids and the proteins to which glutamate binds. The L-trans isomer of PDC gave the most potent and selective inhibitor of glutamate uptake.

Scheme 4.1 Synthesis of analogues of proline.

Methylation of the hemiaminals, generation of N-acyliminium ion and addition of vinyl cuprates gives 5-vinylproline analogues (Scheme 4.1). Ethynyl groups can also be introduced at the 5-position. The hemiaminals also can be allylated to give analogues of proline that are substituted with an allyl group at C-5. Elaboration of both the allyl
or the vinylic group can afford dehydroaminoacids which on asymmetric hydrogenation and lactam cyclization would give indolizidinone analogues.

4.2 Synthesis of analogues of proline

When \(5b\) was subjected to ozonolysis, only the cyclized hemiaminal \(22\) was obtained. The hemiaminal was then reacted with BF\(_3\).OEt\(_2\) and allyl trimethyl silane to give compounds \(24a\) and \(24b\) in low yield (Scheme 4.2). The yield was improved by methylating the hydroxyl group and the resultant compound \(23a\) reacted with BF\(_3\).OEt\(_2\) and allyl trimethyl silane at -78°C \(\rightarrow\) RT to give compounds \(24a\) and \(24b\) in 48% yield and a ratio of 1:1. When BF\(_3\).OEt\(_2\) and allyl trimethyl silane were added at -78°C and the reaction warmed to room temperature, the two products were formed in equal amounts. North et al. have reported similar reaction in which the hemiaminal was reacted with TMS-CN or trimethyl silane.\(^{170}\) The yields for the reactions were however low, typically 20%. 
Scheme 4.2 Synthesis of cis-dicarboxylate proline analogues

Multzer et al. have shown that neighbouring group participation of the alpha carboxyl group in xviii (Scheme 4.1) leads to stereoselective formation of the cis isomer. This stereoselectivity is attributed to the facial preference with which allylsilane attacks the cyclic acyliminium intermediate generated by BF₃ catalyzed Ome-elimination from aminal xviii. The mechanistic pathway would involve the formation of a BF₃ complex with the ester in which the fluoride would acquire sufficient nucleophilicity to attack the trimethylsilyl group and, hence, facilitate the concomitant allyl transfer to the iminium function (Figure 4.2).
Figure 4.2 Transition state model for the generation of proline analogues

The structure of compound 24a was proved by X-ray Crystal structure (Figure 4.3).

Figure 4.3 X-ray crystal structure of δ-allyl substituted proline analogue

When the minor isomer 6b was subjected to osmylation and allylation, product 24c was obtained in 51 % yield (Scheme 4.3).

Scheme 4.3 Synthesis of trans-dicarboxylate proline analogue
The three δ-allyl substituted proline analogues (24a, 24b, and 24c) were further characterized by NOE experiments. The stereochemistry of compound 24c was confirmed from the nOe data of the final bicyclic dipeptide mimetic.

![Diagram of compounds 24a, 24b, and 24c]

**Figure 4.4** nOe analysis of δ-allyl substituted proline analogues

### 4.3 Synthesis of bicyclic dipeptide mimetics for Nle-Asp.

Compound 24a was subjected to ozonolysis and the resultant aldehyde 25a subjected to a Horner-Emmons olefination\(^{208}\) to give the dehydroamino acid 26a in 58% yield. When Osmylation was used for the oxidation of 24a, followed by olefination, 71% yield of the dehydroamino acid was obtained. Attempts to cyclize 26a failed possibly due to the E-stereochemistry of the alkene that keeps the ester functionality away from the amine. Asymmetric homogeneous hydrogenation\(^{209-210}\) of 26a using Burks catalyst [Rh(I) (COD) (S,S)-Et-DuPHOS]\(^{211}\) gave 27a in high yields. The catalyst high asymmetric induction is attributed to two factors. First, the substrate olefin coordinates in a bidentate manner, with the amide oxygen acting as the second ligand, to form two diastereomeric olefin complexes.\(^{204}\) The two diastereomers undergo reaction at substantially different rates and they equilibrate rapidly with the rate determining step being the oxidative addition of hydrogen to the metal olefin complex. The minor diastereomer undergoes this reaction at a rate \(10^3\) greater than the other and provided the two diastereomers can equilibrate
rapidly, high asymmetric induction is achieved. However, high hydrogen pressure and lower temperatures decrease the enantioselectivity since both interfere with this equilibration. For the diphosphine catalysts such as DIOP, DIPAMP, CHIRAPHOS, BPPM and BINAP, asymmetric induction is primarily effective with Z-acetamidoacrylates. The E-isomer undergoes E-Z isomerization that often compromises the enantiselectivity. In contrast, related catalyst containing the phosphole ligands DUPHOS or BPE ligands reduce both E and Z acetamidoacrylates with equally high enantioselectivity, and, for a given catalyst configuration, the same absolute stereochemistry is obtained regardless of olefin geometry.

**Figure 4.5** Optically active phosphines

Asymmetric hydrogenation was followed by deprotection of the Boc group, then the compound was dissolved in pyridine and heated at 50°C for 4 days to give the indolizidinone 3a in 50% yield from 24a.
Scheme 4.4 Synthesis of Nle-Asp bicyclic dipeptide mimetic

Oxidation of compound 24b by ozonolysis followed by Horner-Emmons olefination gave the desired compound 25b in a yield of 19%. An unidentified compound was also formed in a similar yield. The reaction was optimized by changing the protocol to oxidation by osmylation followed by olefination to give the desired dehydroamino acid in 70% yield. From these results it is clear osmylation gives better results in the oxidation of the proline analogues. Compound 25b was then subjected to asymmetric hydrogenation, N\textsuperscript{\textalpha}-Boc deprotection and cyclization to give the desired bicyclic compound 3b in 65% yield.
Scheme 4.5 Synthesis of Nle-Asp bicyclic dipeptide mimic

Compounds 24c was subjected to the same reaction conditions in Scheme 4.6 to give the bicyclic dipeptide mimetics 3c (Figure 4.4).

Figure 4.6 Bicyclic dipeptide mimic for Nle-Asp

4.4 Characterization of Nle-Asp bicyclic dipeptide mimetics

The three Nle-Asp bicyclic dipeptide mimetics were characterized by 1H NMR, DQF COSY and 2D transient nOe. The three bicyclic dipeptide mimetics were then characterized by nOe measurements. A strong nOe value was observed for H³ and H⁶ in compounds 3b and 3c (4.2% and 4.0% respectively) signifying a cis relationship between the two hydrogens. For compound 3a, the nOe value between H³ and H⁶ was relatively...
low (1.9%) due to the *trans* relationship between the hydrogens. The *cis* relationship between H₈ and H₉ in 3a and 3b resulted in a strong nOe value of 4.8% compared with the weak value of 1.9% observed for compound 3c.

![Figure 4.7 nOe data for Nle-Asp bicyclic dipeptide mimetics](image)

**4.5 Synthesis of β-thiol substituted aspartic acid**

A Nle-Asp bicyclic dipeptide mimetic can also be synthesized from β-vinyl substituted Nle (products of Kazmaier-Claisen rearrangement) and β-thiol substituted aspartic acid (Figure 4.3). β-Thiol substituted aspartic acid can be synthesized by the β-sulfenylation of aspartic acid with sulfur electrophiles. Sulfenylation has been reported by Enders and Klumpen in which case di-toluoyl disulfide and methyl disulfide were used as electrophiles.²¹³

![Figure 4.8 Retrosynthetic analysis for a thiol BTD for Nle-Asp](image)
When the Cbz protected aspartic acid 28 was subjected to sulfenylation with benzyl disulfide at -42°C, α-alkylated products were preferentially formed. The desired product 29 was obtained when the reaction was carried out at -78°C.

Scheme 4.6 Sulfenylation of aspartic acid with benzyl disulfide

Further manipulation of 29 to get the free amino acid failed. Sodium in liquid ammonia reduction\(^ {214} \) gave products that could not be identified while no reaction was observed with Pd/C in liquid ammonia or transfer hydrogenation.\(^ {215} \) To circumvent the problem described above, attempts to sulfenylate Boc protected aspartic acid were undertaken. Matsueda and coworkers have reported the synthesis of 3-nitro-2-pyridinesulfenyl (Nyps) halides that react with amino, hydroxyl and thiol functions to form sulfenamides, sulfenates, and mixed disulfides, respectively.\(^ {216-218} \)
Scheme 4.7 Synthesis of Nyps protected aspartic acid

The Nyps group can also serve as a protecting group and in activation during peptide synthesis. This group is easily removed under neutral conditions using tertiary phosphine and water, but it is sufficiently resistant to acids such as TFA, HCl, and HF. We consequently designed sulfenation of aspartic acid with (p-MeOC₆H₄CH₂)₂S which would give a p-Methoxybenzyl protected thiol 31 that is easier to convert to Nyps disulfide 32 (Scheme 4.8). (p-MeOC₆H₄CH₂)₂S was synthesized in large scale using a method reported in literature. It is well established that DMSO in combination with a variety of acidic co-reagents can be used for the conversion of thiols to disulfides. However DMSO has a low oxidizing power, a problem that can be circumvented by prior treatment of DMSO with oxophilic co-reagents under acidic conditions. 1,1,1,3,3,3-Hexamethyldisilazane (HMDS), a weak but stable oxophilic base was found to effect the conversion of thiols to disulfides in high yields. Although the precise role of HMDS is
not known, it has been postulated that HMDS reacts with the first molecule of the thiol to give a reactive species \textit{xxv} and trimethylsilyl amine (Scheme 4.5). Intermediate \textit{xxv} in turn reacts with the second thiol molecule in the presence of trimethylsilylamine with concomitant release of NH$_3$ to afford the corresponding oxonium intermediate \textit{xxvi}. Intermediate \textit{xxvi} collapses to form the disulfide along with the evolution of dimethylsulfide and hexamethyldisiloxane as by-products. Using this methodology, 4-methoxy-$\alpha$-toluenethiol was converted to the disulfide in the presence of 1.2 eq of HMDS in 76% yield.

\[
\begin{align*}
\text{Me}_3\text{Si-N-SiMe}_3 + \text{DMSO} & \rightarrow \text{RSH} \\
\text{OSiMe}_3 + \text{Me}_3\text{SiNH}_2 & \rightarrow \text{NH}_3 \\
\text{Me}_3\text{SiNH}_2 & \rightarrow \text{Me}_3\text{SiO}_\oplus + \text{SR} + \text{CH}_3
\end{align*}
\]

\textit{xxv}

\[
\begin{align*}
\text{Me}_3\text{SiO}_\oplus + \text{OSiMe}_3 & \rightarrow \text{RS-SR} \\
\text{Me}_3\text{O-SiMe}_3 & \rightarrow \text{Me-S-Me}
\end{align*}
\]

\textit{xxvi}

\textbf{Figure 4.9} Mechanism for the synthesis of disulfides

All attempts of effecting the sulfenation of N$\alpha$-Boc-protected aspartic acid with (p-MeOC$_6$H$_4$CH$_2$)$_2$S and benzyl disulfide failed with only the starting material being recovered. Though the actual reason for the failure of this reaction is not clear, solubility of the disulfides at the low temperatures was found to be low.
4.6 Experimental

General procedure for the synthesis of 5-allyl substituted proline analogues

In 500-mL flask, 5b was dissolved in dichloromethane (300 mL and cooled to -78°C. The solution was exposed to O₃ until a persistent blue color appeared. Dimethylsulfide (50 mL) was added and the mixture stirred at room temperature for 60 hr. The solvent was evaporated off and the product dried under high vacuum. The dried product was dissolved in methanol (100 mL) and para-toluene sulfonic acid (220 mg, 1.16 mmol) added. The mixture was stirred at room temperature for 12 hr and the solvent evaporated off. The product was redissolved in ethyl acetate (150 mL) and the organic layer washed by aqueous NH₄Cl (2 x 50 mL) and brine (50 mL). The organic phase was dried over MgSO₄ and the solvent evaporated. The product was dried under high vacuum to give 8.16 g of crude product, a 91.5 % yield. The crude product was dissolved in ether (120 mL) and cooled to -78°C. BF₃OEt₂ (8.0 mL, 63.13 mmol) and allyl trimethyl silane (10.0 mL, 62.92 mmol) were added and the mixture stirred at -78°C for 20 min. The cold bath was removed and the mixture stirred for a further 40 min before cooling to -78°C and slowly adding concentrated aqueous NaHCO₃. The solution was slowly warmed to room temperature and the aqueous phase separated. The organic phase was washed with concentrated aqueous NaHCO₃ (2 x 50 mL) and brine (50 mL). The organic phase was dried over MgSO₄ and the solvent evaporated off. The product was purified by column to give 2.45 g of a white solid 24a and 2.59 g of a yellowish liquid 24b in a total yield of 55%. When the reaction was repeated with 6b, only one 5-allyl proline analogue 24c was obtained in 50% yield.
Benzyl (2S, 3R, 5R)-5-allyl-1-(tert-butoxycarbonyl)-3-methoxycarbonyl proline (24a): $\left[\alpha\right]_{D}^{25} = -0.016^\circ$ (c = 0.62, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.44-7.27 (5H, br), 5.81-5.67 (1H, m), 5.19-4.99 (4H, m), 4.59 (0.4H, d, $J$ = 8.0 Hz), 4.50 (0.6H, d, $J$ = 8.5 Hz), 4.22-4.14 (0.6H, td, $J$ = 3.0, 9.0 Hz), 4.10-4.02 (0.4H, td, $J$ = 3.0, 9.0 Hz), 3.54 (2H, s), 3.46 (1H, s), 3.42-3.28 (1H, m), 2.63-2.55 (0.6H, br), 2.54-2.40 (1.4H, m), 2.20-2.11 (1H, m), 1.91 (1H, q, $J$ = 6.5 Hz), 1.46 (3.6H, s), 1.32 (5.4H, s); $^{13}$C, 125 MHz, CDCl$_3$, $\delta$(ppm): 170.63, 170.54, 170.40, 170.1, 154.1, 153.3, 135.42, 135.19, 134.48, 134.37, 128.72, 128.51, 128.40, 128.36, 128.12, 117.87, 117.78, 80.51, 80.43, 67.18, 67.07, 61.87, 61.66, 56.86, 56.79, 52.02, 51.97, 45.1, 44.1, 39.3, 38.4, 31.0, 30.0, 28.3, 28.1; HRMS (FAB) $M^+$ calculated for 404.2073, found 404.2066.

Benzyl (2S, 3R, 5S)-5-allyl-1-(tert-butoxycarbonyl)-3-methoxycarbonyl proline (24b): $\left[\alpha\right]_{D}^{25} = +0.17^\circ$ (c = 1.39, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.44-7.28 (5H, br), 5.80-5.65 (1H, br), 5.22-4.96 (4H, m), 4.83 (0.4H, d, $J$ = 6.5 Hz), 4.68 (0.6H, d, $J$ = 8.5 Hz), 3.90-3.71 (1H, br), 3.63-3.41 (3H, br), 3.25-3.12 (1H, br), 3.09-2.97 (0.6H, br), 2.87-2.76 (0.4H, br), 2.36-2.11 (3H, m); $^{13}$C, 125 MHz, CDCl$_3$, $\delta$(ppm): 170.7, 170.3, 153.9, 153.2, 135.3, 134.4, 128.6, 128.45, 128.38, 128.2, 117.3, 80.4, 67.0, 61.7, 61.4, 57.5, 52.0, 45.4, 44.9, 39.7, 38.4, 33.4, 32.7, 28.3, 28.2; HRMS (FAB) $M^+$ calculated for 404.2073, found 404.2061.

Benzyl (2S, 3S, 5S)-5-allyl-1-(tert-butoxycarbonyl)-3-methoxycarbonyl proline (24c): $\left[\alpha\right]_{D}^{25} = 0.1165^\circ$ (c = 1.05, CHCl$_3$); $^1$H NMR, 500 MHz, CDCl$_3$, $\delta$(ppm): 7.40-7.30 (5H, br), 5.84-5.69 (1H, br), 5.36-4.98 (4H, m), 4.72-4.65 (0.3H, br), 4.57 (0.5H, d, $J$ = 6.5 Hz), 4.07 (0.65H, br), 3.97 (0.35H, br), 3.67 (3H, s), 3.25-3.13 (1H, br), 2.76-2.66
(0.6H, br), 2.63-2.53 (0.4H, br), 2.32-2.13 (2H, br), 2.10-1.96 (1H, br), 1.55-1.28 (9H, br); $^{13}$C, 125 MHz, CDCl$_3$, δ(ppm): 172.1, 171.6, 171.3, 170.6, 153.8, 153.2, 135.6, 135.3, 134.6, 134.4, 134.3, 128.4, 128.3, 128.2, 117.0, 80.4, 77.2, 66.8, 62.3, 57.5, 52.3, 46.5, 45.6, 39.1, 38.2, 33.4, 32.5, 28.3, 28.0; HRMS (FAB) MH$^+$ calculated for 404.2073, found 404.2063.

**General procedure for the synthesis of dehydroamino acids**

Compound **24a** (1.83 g, 4.54 mmol) was dissolved in THF (60 mL)/H$_2$O (30 mL) mixture. OsO$_4$ (60 mg, 0.24 mmol), was added and the mixture stirred at room temperature for 10 min before adding NaIO$_4$ (2.7 g, 12.62 mmol) and stirring the mixture for a further 4 hr. The mixture was filtered and the THF evaporated. Ethyl acetate (50 mL) was added and the aqueous layer separated. The organic phase was washed with saturated NH$_4$Cl (2 x 30 mL) and brine (30 mL). The organic phase was dried over MgSO$_4$ and the solvent evaporated off. The crude product was dried under high vacuum and used in the next step without purification. In another 100-mL flask, N-(benzyloxy carbonyl)-α-phosphonoglycine trimethyl ester (1.7g, 5.13 mmol) was dissolved in CH$_2$Cl$_2$ (30 mL). DBU (750 μL, 5.02 mmol) was added and the mixture stirred for 10 min. The crude product from above, dissolved in CH$_2$Cl$_2$ (10 mL) was added and the mixture stirred at room temperature for 8 hr. The CH$_2$Cl$_2$ layer was diluted to 60 mL and washed with aqueous 1 N HCl (2 x 30 mL) and brine (30 mL). The organic phase was dried over MgSO$_4$ and evaporated off. The product was then purified by column to give 1.91g of yellowish liquid **26a** in 71% yield.
General method for the synthesis of Nle-Asp bicyclic dipeptide mimetics

The dehydroamino acid 26a (2.4g, 3.92 mmol) was dissolved in degassed methanol (50 mL). [Rh-(S,S)-EtDuPHOS]-OTf (6 mg, 0.008 mmol) was added and the mixture exposed to hydrogen at 70 psi for 20 hr. The solvent was evaporated and the product purified by passing it through a short silica column using ethyl acetate. The solvent was evaporated and the product, obtained in quantitative yield, dried under high vacuum. The product was then dissolved in CH2Cl2 (30 mL) and cooled to 0°C. TFA (10 ml) was added and the mixture stirred at 0°C for 10 min and at room temperature for 40 min. The solvent was evaporated off and the product dissolved in ethyl acetate (50 mL). The organic layer was washed with NaHCO3 (2 x 30 mL) and brine (30 mL). The organic phase was dried over MgSO4 and the solvent evaporated off. The crude product was dried under high vacuum and dissolved in pyridine (250 mL). The mixture was then heated at 50°C for 4 days. The solvent was then evaporated off and the product dissolved in ethyl acetate (50 mL). The organic phase was washed by aqueous 1 N HCl (2 x 20 mL) and brine (30 mL). The product was purified by column to give 1.0 g of 3a in 53 % yield.

(3S, 6R, 8R, 9S) Benzyl 2-oxo-3-N-(benzyloxycarbonyl)amino-8-methylester-1-azabicyclo[4.3.0]nonane-9-carboxylate (3a): $[\alpha]^{25}_D = -15.9^\circ$ (c = 0.6, CHCl3); $^1$H NMR, 500 MHz, CDCl3, $\delta$(ppm): 7.58-7.22 (11H, m), 6.5 (1H, br), 5.11 (4H, d, $J = 8.5$ Hz), 4.04 (1H, d, $J = 7.0$ Hz), 3.78-3.68 (3H, m), 3.46 (3H, s), 3.34-3.26 (1H, m), 2.38-2.22 (3H, m), 1.81-1.71 (1H, m); $^{13}$C, 125 MHz, CDCl3, $\delta$(ppm): 172.7, 171.8, 164.9, 154, 136.1, 135.2, 131.4, 128.58, 128.54, 128.48, 128.44, 128.17, 128.14, 128.03, 67.23,
67.17, 62.5, 57.3, 52.3, 51.8, 47.3, 35.0; HRMS (FAB) MH^+ calculated for 481.1975, found 481.1958.

(3S, 6S, 8R, 9S) Benzyl 2-oxo-3-N-(benzyloxycarbonyl)amino-8-methylester-1-azabicyclo[4.3.0]nonane-9-carboxylate (3b): [α]^25_D = +5.2° (c = 0.6, CHCl₃); ^1H NMR, 500 MHz, CDCl₃, δ(ppm): 7.40-7.32 (10H, m), 5.79 (2H, d, J = 5.5 Hz), 5.13 (4H, s), 4.78 (1H, d, J = 8.5 Hz), 4.23-4.15 (1H, m), 3.83-3.71 (1H, m), 3.48 (3H, s), 3.35-3.26 (1H, m), 2.56-2.45 (1H, m), 2.38 (1H, p, J = 6.28 Hz), 2.24-2.05 (2H, m), 1.83-1.71 (1H, m), 1.68-1.56 (1H, m); ^13C, 125 MHz, CDCl₃, δ(pppm): 169.9, 169.0, 156.0, 136.4, 135.0, 128.6, 128.5, 128.4, 128.02, 127.94, 67.6, 66.8, 60.1, 55.7, 52.1, 50.2, 45.7, 34.9, 26.91, 26.67; HRMS (FAB) MH^+ calculated for 481.1975, found 481.1995.

(3S, 6S, 8S, 9S) Benzyl 2-oxo-3-N-(benzyloxycarbonyl)amino-8-methylester-1-azabicyclo[4.3.0]nonane-9-carboxylate (3c): [α]^25_D = +34.9° (c = 1.2, CHCl₃); ^1H NMR, 500 MHz, CDCl₃, δ(ppm): 7.41-7.27 (10H, m), 5.77 (1H, d, J = 4.5 Hz), 5.18 (2H, q, J = 12.0 Hz), 5.11 (2H, s), 4.88 (1H, s), 4.26-4.14 (1H, br), 3.90-3.77 (1H, br), 3.73 (3H, s), 3.12 (1H, d, J = 7.5 Hz), 2.58-2.42 (2H, m), 2.14-2.01 (1H, br), 1.91-1.81 (1H, m), 1.70-1.58 (2H, m); ^13C, 125 MHz, CDCl₃, δ(pppm): 171.8, 170.0, 168.8, 156.0, 136.4, 135.1, 128.6, 128.5, 128.4, 128.2, 128.01, 127.96, 67.5, 66.7, 61.0, 55.1, 52.7, 50.3, 46.1, 34.9, 26.9, 26.6; HRMS (FAB) MH^+ calculated for 481.1975, found 481.1958.

Synthesis of p-methoxybenzyl disulfide

4-methoxy-α-toluene thiol (10.0 mL, 71.8 mmol) was dissolved in CH₃CN (90 mL) and dimethyl sulfoxide (16.0 mL, 225.4 mmol) added. To the solution was added hexamethyldisilazane (18.0 mL, 85.3 mmol) and the mixture stirred for an overnight.
Aqueous NaOH (10%, 70.0 mL) was added and the organic phase evaporated off. The product was then dissolved in CH$_2$Cl$_2$ (50.0 mL) washed by 10% NaOH (2 x 30 mL) and water ( 2 x 30 mL). The organic phase was dried over MgSO$_4$ and the solvent evaporated off. The product was then recrystallized from ethyl acetate/hexanes to give 7.84 g of product in 71.3% yield.
CHAPTER 5
SYNTHESIS OF BICYCLIC DIPEPTIDE MIMETICS FOR Asp-Gly AND homoPhe-Gly

5.1 Introduction
The dipeptide Asp-Phe is found at the C-terminal of CCK-peptides. Deltorphin I (Tyr-D-Phe-Asp-Val-Val-Gly-NH₂) also has a central Asp-Val dipeptide. As was described in Chapter 3, our protocol for the synthesis of bicyclic dipeptide mimetics employs δ,e- and γ,δ-unsaturated amino acids and cysteine or its analogues. β-Phenyl substituted cysteine has been synthesized in our group and if coupled with β-allyl substituted aspartic acid would give a bicyclic dipeptide mimetic for Asp-Phe. In this chapter, the protocol for the synthesis of an Asp-Phe bicyclic dipeptide mimetic and other aspartic acid-containing bicyclic mimetics is developed by synthesizing a bicyclic dipeptide mimetic for Asp-Gly.

5.2.1 Synthesis of Asp-Gly bicyclic dipeptide mimetic
It has been established that the oxidation of the carbamate protected β-allyl amino acids leads to a 5-membered cyclic hemiaminal instead of the free aldehyde. This problem can be avoided by protecting the amino group as a trifluoroacetamide or by bisprotection. Introduction of a second Boc group was unsuccessful due to steric hinderance of β-substitution. A number of methods for protection of an amino group as a trifluoroacetamide were attempted. Deprotection of the Nα-Boc group followed by coupling with ethyl trifluoroacetamide gave the product in non-reproducible and variable yields (25-45%) while use of trifluoroacetic anhydride led to the formation of
a mixture of products. A recently reported method that uses (trifluoroacetyl)
benzotriazole 35,224 was found to be more efficient. This reagent is easily synthesized in
large scale from benzotriazole and trifluoroacetic acid in THF (Scheme 5.1).

Scheme 5.1 Synthesis of trifluoroacetyl benzotriazole

When 5a was treated with TFA followed by reaction with 35, the N'-trifluoroacetyl
amino acid 36a was obtained in good yield (Scheme 5.2). Compound 36a was then
subjected to oxidation with Osmium tetraoxide and NaIO4 to afford aldehyde 37a. In a
one pot reaction, the aldehyde was coupled with L-cysteine to form two bicyclic
dipeptide mimetics 38a and 38b, via the formation of an N,S-thiazolidine, bicyclization
and methylation.
Scheme 5.2 Synthesis of Asp-Gly bicyclic dipeptide mimetics

Subjecting the minor isomer 6a to the same set of reaction conditions afforded only one bicyclic dipeptide 38c (Scheme 5.3).

Scheme 5.3 Synthesis of Asp-Gly bicyclic dipeptide mimic
5.2.2 Characterization of Asp-Gly bicyclic dipeptide mimetics

The $^1$H-spectra were assigned by DQF-COSY while the stereochemistry of the three bicyclics was assigned by 1D transient nOe experiments (Figure 5.1). A lot of nOe data was obtained for compound 38c and 38a but there were more overlaps for compound 38b. In all the isomers, H$_3$ was well resolved and was irradiated to determine the stereochemistry at C-4 and C-6. In 38a and 38b, a weak nOe indicative of a trans relationship was observed for H$_3$ and H$_4$. In 38c, a cis relationship between H$_3$ and H$_4$ is proved by the strong nOe value. A cis relationship for the bridge-head H$_6$ with H$_3$ in 38a and 38c is confirmed by the strong nOe’s (1.50 % and 2.52 % respectively) in 38a and 38c. In comparison, 38b shows an nOe value of 0.20 % that would be as a result of a trans relationship. The coupling constants could not be used to assign the stereochemistries due to overlap of the critical hydrogens.

The formation of two products from the (2S,3R) isomer 5a but only one product from the (2S,3S) isomer 6a agrees with earlier work done in our labs. However, the stereochemical outcome of the bridge head-H in the three bicyclic compounds is “unusual” in that the favored product would have to be formed via the less thermodynamically stable cis-relationship in the formation of thiazolidine. This may indicate an extra effect of the 4-benzyl carboxylate group other than steric hinderance in the formation of the thiazolidine ring. The possible pathway in this cascade three-bond formation process is not known.
**Figure 5.1** NOE observed for Asp-Gly bicyclic dipeptide mimetics.

5.3 Synthesis of a homoPhe-Gly bicyclic dipeptide mimetic

When 7a was subjected to hydrogenation and BOP, DIPEA and NaBH₄ reduction, the alcohol 39, contaminated by the lactone, was obtained in 53% yield. The alcohol was then subjected to the Swern oxidation. The crude aldehyde was then coupled with L-cysteine under the same set of reaction conditions as described in Scheme 5.2 to give 1 in 42% yield.
Scheme 5.4 Synthesis of homoPhe-Gly bicyclic dipeptide mimetic.

The stereochemistry of compound 1 was confirmed from its X-ray crystal structure (Figure 5.2).

Figure 5.2 X-Ray crystal structure of homoPhe-Gly bicyclic dipeptide mimetic

 Compound 1 was consequently used in the synthesis of analogues of Leu-enkephalin as illustrated in Scheme 5.5.
Scheme 5.5 Synthesis of analogues of Leu-enkephalin

Different attempts were made to manipulate the β-benzyl substituted aspartic acid. Deprotection of the β-carboxyl group in 7c followed by reduction afforded the alcohol in 70% yield with no lactone formation. This may be explained by the the bulky protection groups that hinder cyclization. Attempts to mesylate the alcohol\textsuperscript{225} led to formation of the lactone, a side reaction which was more severe when PB\textsubscript{r}\textsubscript{3}\textsuperscript{226} was used. To lower the acidity of the reaction medium, carbon tetrabromide and phosphine\textsuperscript{227} were used. The bromide 45 was obtained in excellent yield. However reaction of the bromide with NaCN to give the nitrile failed while deprotection of the Cbz and Benzyl esters with hydrogenation also failed.
Scheme 5.6 Bromination of β-benzyl substitutted homoserine

5.4 Experimental

**General procedure for Nα-deprotection and reprotction as a trifluoro acetamide**

In a 50-mL flask, 5a (690 mg, 1.83 mmol) was dissolved in DCM (10 mL) and cooled to 0°C. TFA (2.5 mL) was added and the mixture stirred at 0°C for 10 min and at room temperature for 40 min. The solvents were then evaporated off and the residue dried under high vacuum. The product was dissolved in 8 mL THF to which was then added 35 (470 mg, 2.18 mmol) and TEA (510 µL, 3.66 mmol) and the mixture stirred for 4 h. THF was evaporated off and the product redissolved in EtOAc (30 mL) and washed by saturated aqueous NH₄Cl (2 x 20 mL) and brine 20 mL. The organic layer was then dried over MgSO₄, and the solvent evaporated off. After column purification using hexanes-
ethyl acetate (4:1 v/v), 520 mg (76% yield) of 36a was obtained as a yellow liquid. The same procedure was employed in the synthesis of 36b (yellow liquid).

**Methyl 2-(2,2,2-trifluoro-acetylamino)-3-carbobenzyloxy-(2S, 3R)-hex-5-enoate (36a):** 
\[[\alpha]_D^{25} = +39.1^\circ \text{ (c = 1.31, CHCl}_3)\]; \(^1\)H NMR, 500 MHz, CDCl\(_3\), \(\delta\) (ppm): 7.27 (1H, d, \(J = 9.15 \text{ Hz}\)), 7.40-7.32 (5H, m), 5.76-5.68 (1H, m), 5.18-5.08 (4H, m), 4.87 (1H, dd, \(J = 3.24, 9.53 \text{ Hz}\)) 3.67 (3H, s), 3.36-3.32 (1H, m), 2.57-2.51 (1H, m), 2.29-2.23 (1H, m); \(^13\)C, 125 MHz, CDCl\(_3\), \(\delta\) (ppm): 173.0, 169.5, 157.4 (q, \(J = 37.6 \text{ Hz}\)), 134.8, 132.9, 128.7, 128.4, 119.2, 115.7 (q, \(J = 286 \text{ Hz}\)), 67.4, 53.0, 51.7, 45.6, 33.0; HRMS (FAB) MH\(^+\) calcd for 374.1215, found 374.1214.

**Methyl 2-(2,2,2-trifluoro-acetylamino)-3-carbobenzyloxy-(2S, 3S)-hex-5-enoate (36b):** 
\[[\alpha]_D^{25} = +22.9^\circ \text{ (c = 1.52, CHCl}_3)\]; \(^1\)H NMR, 500 MHz, CDCl\(_3\), \(\delta\) (ppm): 7.39-7.34 (5H, m), 7.18 (1H, d, \(J = 7.25 \text{ Hz}\)), 5.83-5.74 (1H, m), 5.18-5.11 (4H, m), 4.88 (1H, dd, \(J = 4.0, 8.2 \text{ Hz}\)), 3.74 (3H, s), 3.06-3.02 (1H, m), 2.69-2.63 (1H, m), 2.49-2.43 (1H, m); \(^13\)C, 125 MHz, CDCl\(_3\), \(\delta\) (ppm): 171.3, 169.2, 156.6 (q, \(J = 37.9 \text{ Hz}\)), 135.0, 133.8, 128.7, 128.6, 128.5, 118.7, 115.5 (q, \(J = 285.8 \text{ Hz}\)), 67.5, 53.0, 52.5, 47.1, 32.5; HRMS (FAB) MH\(^+\) calcd for 374.1215, found 374.1225.

**General procedure for oxidation, thiazolidine formation and bicyclization**

In a 25-mL flask, 36a (330 mg, 0.88 mmol) was dissolved in THF (10 mL) and water (5 mL). OsO\(_4\) (11 mg, 0.043 mmol) was added and the mixture stirred for 5 min before slowly adding NaIO\(_4\) (526 mg, 2.46 mmol) and stirring the mixture for a further 4 h. The mixture was then filtered, THF evaporated off and EtOAc (30 mL) added. The organic phase was extracted with saturated aqueous NH\(_4\)Cl (2 x 20 mL), brine (20 mL) and then
dried over MgSO₄. The solvent was evaporated off and the product dried under high vacuum. The crude aldehyde was redissolved in 3 mL pyridine to which was added preactivated molecular sieves, 4Å° (450 mg). L-Cys (35 mg, 0.29 mmol) was added and the mixture stirred at RT for 4 h. Pyridine (5 mL) was then added and the mixture stirred at 50°C for 4 days. The solvent was then evaporated off and the product dissolved in water. The aqueous layer was acidified to pH = 2 by 1 N HCl and extracted by ether (4 x 5 mL). The organic phase was then dried over MgSO₄ and the solvent evaporated off. The product was redissolved in DCM (3 mL) and treated with excess diazomethane. The solution was stirred for 30 min and the solvents evaporated off. Two compounds (33a, 65 mg; 38b, 140 mg) were obtained as yellow liquids after separation on a silica gel column using hexane-ethyl acetate (2:1 v/v), in 42% total yield. When the reaction was repeated with compound 36b, only one product, 38c, was obtained as a yellow liquid in 48% yield.

**Methyl [3S, 4R, 6S, 9R]-1-Aza-3[2,2,2-trifluoroacetylamino]-4-carboxbenzoyloxy-2-oxo-7-thiabicyclic[4.3.0]-nonane-9-carboxylate (38a):** [α]²⁵_D = -24.8° (c = 1.06, CHCl₃); ¹H NMR, 500 MHz, CDCl₃, δ (ppm): 7.49 (1H, d, J = 5.5 Hz), 7.36-7.34 (5H, m), 5.24 (1H, d, J = 12.0 Hz), 5.1 (1H, d, J = 12.0 Hz), 5.09 (1H, dd, J = 5.0, 10.0 Hz), 4.98 (1H, d, J = 7.0 Hz), 4.89 (1H, dd, J = 6.5, 8.5 Hz), 3.77 (3H, s), 3.42 (1H, dd, J = 6.5, 12.0 Hz), 3.27 (1H, d, J = 12.0 Hz), 3.08-3.04 (1H, m), 2.40-2.36 (1H, m); ¹³C, 125 MHz, CDCl₃, δ (ppm): 172.7, 169.0, 165.6, 157.4 (q, J = 37.74 Hz), 135.0, 128.64, 128.61, 128.6, 115.5 (q, J = 286 Hz), 68.1, 62.5, 59.4, 53.1, 51.2, 44.8, 33.3, 32.9; HRMS (FAB) MH⁺ calcd for 461.0994, found 461.0998.
Methyl [3S, 4R, 6S, 9R]-1-Aza-3[2,2,2-trifluoroacetylamino]-4-carbobenzyloxy-2-oxo-7-thiabicyclic[4.3.0]-nonane-9-carboxylate (38b): \([\alpha]_{D}^{25} = -157.9^\circ\) (c = 0.57, CHCl₃); \(^1H\) NMR, 500 MHz, CDCl₃, δ(ppm): 7.39-7.32 (5H, m), 7.11 (d, J = 5.0 Hz), 5.20-5.10 (3H, m), 5.03 (1H, dd, J = 2.5, 7.0 Hz), 4.55 (1H, dd, J = 5.0, 7.0 Hz), 3.78 (3H, s), 3.38-3.31 (2H, m), 3.21 (1H, dd, J = 3.0, 7.5 Hz), 2.63 (1H, m, dt, J = 2.5, 8.5 Hz), 2.11 (1H, dt, J = 2.5, 8.5 Hz), 1.36-1.33 (1H, m); \(^13C\), 125 MHz, CDCl₃, δ(ppm): 170.6, 169.8, 163.7, 157.4 (q, J = 37.6 Hz), 134.9, 128.71, 128.7, 128.4, 115.5 (q, J = 286.2 Hz), 67.8, 61.1, 60.7, 53.0, 52.2, 43.5, 31.7, 31.6; HRMS (FAB) MH⁺ calcd for 461.0994, found 461.0988.

Methyl [3S, 4R, 6S, 9R]-1-Aza-3[2,2,2-trifluoroacetylamino]-4-carbobenzyloxy-2-oxo-7-thiabicyclic[4.3.0]-nonane-9-carboxylate (38c): \([\alpha]_{D}^{25} = -11.6^\circ\) (c = 0.34, CHCl₃); \(^1H\) NMR, 500 MHz, CDCl₃, δ(ppm): 7.63 (1H, d, J = 5.0 Hz), 7.38-7.32 (5H, m), 6.35 (1H, d, J = 12.5 Hz), 6.25 (1H, d, J = 12.0 Hz), 5.0 (1H, d, J = 6.5 Hz), 4.96 (1H, dd, J = 4.5, 11.0 Hz), 4.67 (1H, t, J = 7.0 Hz), 3.74 (3H, s), 3.72-3.67 (1H, m), 3.42 (1H, dd, J = 3.0, 12.5 Hz), 3.33 (1H, d, J = 12.0 Hz), 2.70-2.64 (1H, m), 2.48-2.43 (1H, m); \(^13C\), 125 MHz, CDCl₃, δ(ppm): 171.1, 168.9, 165.0, 157.1 (q, J = 37.70 Hz), 135.0, 128.63, 128.57, 128.5, 115.4 (q, J = 285.7 Hz), 67.5, 61.6, 59.0, 53.0, 49.6, 41.5, 33.8, 31.7; HRMS (FAB) MH⁺ calcd for 461.0994, found 461.0998.

General procedure for hydrogenation of the benzyl ester and reduction of the resultant carboxylic acid

Compound 7a (2.88 g, 6.7 mmol) was dissolved in degassed methanol (40mL). Pd/C (70 mg, 10%) was added and the mixture was exposed to hydrogen gas for 2 h. The catalyst
was filtered off through Celite and the solvent evaporated off. The crude product was
dried under vacuum and then dissolved in THF (35 mL). Benzotriazol-yl-oxy-
tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (3.2 g, 7.23 mmol)
followed by DIPEA (1.3 mL, 7.46 mmol) were added and the mixture stirred for about 5
min until it turned clear. The solution was then cooled to 0°C and NaBH₄ (400 mg, 10.57
mmol) added slowly. The mixture was stirred for 30 min at 0°C and at room temperature
for 18 h. The solvent was evaporated off and the product dissolved in ethylacetate (80
mL) and the organic phase was washed with saturated aqueous NaHCO₃ (2 x 50 mL), 5%
aqueous HCl (2 x 50 mL) and brine (50 mL). The organic phase was then dried over
Na₂SO₄ and the solvent evaporated off. The product was purified by column to give 1.2 g
of 39 (3.71 mmol) in 55% yield.

**Methyl 3-benzyl-2-tert-butoxycarbonylamino-4-hydroxy-(2S, 3R)-butanoate (39):**

\[ \left[ \alpha \right]_{D}^{25} = +35.3^\circ \ (c = 0.58, \ CHCl_3); \ \ ^1H \ NMR, 500 MHz, \ CDCl_3, \ \delta (ppm); \ ^1H \ 5 \ 7.29-7.20 \ (5H, m), \ 5.59 \ (1H, d, \ J = 9.0 Hz), \ 4.42 \ (1H, t, \ J = 5.5 Hz), \ 3.72 \ (1H, s), \ 3.64-3.50 \ (2H, m), \ 2.78-2.73 \ (2H, m), \ 2.28-2.13 \ (2H, m); \ ^13C \ 125 MHz, \ CDCl_3, \ \delta (ppm): \ 173.1, 156.1, 139.3, \ 129.1, \ 128.5, \ 126.3, \ 80.1, \ 61.2, \ 55.4, \ 52.3, \ 44.7, \ 34.4, \ 28.3; \ \text{HRMS (FAB)} \ \text{MH}^+ \ \text{calculated for 324.1811, found 324.1818.}

**Synthesis of homophe-Gly bicyclic dipeptide mimetic**

Oxalyl chloride (2.8 mL, 5.6 mmol) was dissolved in CH₂Cl₂ (40 mL). The solution was
cooled to -78°C, DMSO (530 µL, 7.47 mmol) added and the solution stirred for 10 min
before adding 39 (1.2 g, 3.71 mmol). The mixture was stirred for 25 min at -78°C before
adding DIPEA (2.6 mL, 14.93 mmol), removing the cold bath and stirring for a further 20
min. Water (20 mL) was added, and the organic phase was separated and washed with saturated aqueous NH₄Cl (2 x 50 mL) and brine (50 mL). The organic phase was dried over MgSO₄ and the solvent evaporated off to give 1.17 g of the crude aldehyde after drying. The crude aldehyde was dissolved in pyridine (30 mL) containing molecular sieves 4 Å° and the mixture stirred at room temperature for 4 h. Pyridine (60 mL) was added and the mixture heated at 50°C for 3 days. The solvent was evaporated off and the product dissolved in water (20 mL) which was acidified to pH = 2 using 1N aqueous HCl. The aqueous layer was extracted with ether (4 x 20 mL) and the ether layer dried over MgSO₄. The solvent was evaporated off and the product dried under high vacuum to give 1.03 g of crude product. The crude product was dissolved in CH₂Cl₂ (15 mL) and cooled to 0°C. DCC (650 mg, 3.15 mmol) was added and the mixture stirred for 10 min before adding methanol (450 µL, 11.12 mmol) and DMAP (30 mg, 0.25 mmol) and stirring the mixture for 8 h. The mixture was filtered and the filtrate diluted by CH₂Cl₂ (20 mL). The organic phase was washed by saturated aqueous NH₄Cl (2 x 20 mL) and brine (30 mL). The organic phase was dried over MgSO₄ and the solvent evaporated off. The product was then purified by column to give 470 mg of 2 as a yellow oil in 31% yield from 34%.

Methyl (3S, 4R, 5S, 8R)-4-benzyl-3-tert-butoxycarbonylamino-1-aza-2-oxo-6-thiabicyclic [3.3.0]-octane-8-carboxylate (2): [α]D²⁵ = -88.7° (c = 0.98, CHCl₃); ¹H NMR, 500 MHz, CDCl₃, δ(ppm); 7.34-7.2 (5H, m), 5.04-5.0 (2H, m), 4.89 (1H, d, J = 3.5Hz), 4.69 (1H, t, J = 8.0Hz), 3.77 (3H, s), 3.38-3.34 (1H, m), 3.25 (1H, dd, J = 4.0, 11.5Hz), 3.04 (1H, dd, J = 5.0, 14Hz), 3.02-2.94 (1H, m), 2.53 (1H, m); ¹³C, 125 MHz,
CDCl₃, δ(ppm): 173.2, 169.7, 155.5, 138.1, 128.9, 128.7, 126.7, 80.5, 68.4, 57.6, 56.1, 52.9, 44.2, 36.9, 34.0, 28.3; HRMS (FAB) MH⁺ calculated for 324.1811, found 324.1818.

**General procedure for the synthesis of diazo methane**

A mixture of ether (1 mL/mmol) and KOH (40% wt, 0.3 mL/mmol) was added to a 20-mL vial and cooled down to 0°C in ice-water bath. N-nitrosomethyl urea (1.0 eq) was added in small portions and the mixture vigorously stirred. After all the urea was added and dissolved the vial contents were chilled to -78°C to freeze the water and the ether phase separated into a 20-mL vial containing a crystal of solid KOH. The solution was dried in the refrigerator for 2.5 hr before use.
CHAPTER 6
SYNTHESIS OF BICYCLIC DIPEPTIDE MIMETIC CONTAINING CCK/OPIOID CHIMERIC PEPTIDES, BIOLOGICAL ASSAY RESULTS, CONCLUSION AND FUTURE WORK

6.1 Synthesis of peptides
All the peptides were synthesized using the N\textsuperscript{\textalpha}-Fmoc/t-butyl chemistry and Rink-amide AM resin. The N\textsuperscript{\textalpha}-Boc protected BTD’s were first hydrolyzed using LiOH/H\textsubscript{2}O/MeOH system. The Boc group was then removed using TFA (30%) in CH\textsubscript{2}Cl\textsubscript{2}. The amino group was then Fmoc protected and the crude BTD used in peptide synthesis. For bicyclic dipeptide 3b, the benzyl carboxylate and carbobenzyloxyamino groups were deprotected by hydrogenation. The amino terminal was then Fmoc protected, and the product used in peptide synthesis without purification. The protocol used for the synthesis of the peptides is illustrated in Scheme 6.1. In case of peptide JMN8 the peptide was synthesized and cleaved from the solid support using the same protocol. The crude product was then dissolved in a H\textsubscript{2}O/MeOH system and 2.0 eq. of LiOH added. The mixture was stirred for 30 min and MeOH evaporated. The crude product was then obtained by lyophilization. Low yields were obtained during the synthesis of JMN7 while all the other peptides were obtained in reasonable yields.
Scheme 6.1 Protocol for the synthesis of peptides

The peptides that were synthesized are shown below:

Figure 6.1 Novel BTD containing peptides
6.2 Experimental

6.2.1 Synthesis and purification of peptides

Rink amide resin (0.5g) was swelled in DMF in a course sintered filter fitted vessel for an overnight. The resin was deprotected with piperidine (20% v/v in DMF) first for 5 min and then for 20 min. The resin was then washed with DMF (3 times) and DCM (3 times). The first amino acid was then coupled to the resin using HBTU, HOBT, and DIPEA. In all the couplings, 3 equivalents of each reagent (amino acid, HBTU, HOBT, and DIPEA) were used. The coupling was allowed to proceed for 1 hr before coupling the next amino acid. This protocol was followed until the last amino acid was coupled and N-terminal deprotected. The completeness of the coupling was monitored by a negative Kaiser test. The resin was then dried and transferred to a 20-mL borosilicate scintillation vial. The peptide was cleaved from the resin and other side chain protecting groups removed using a cleavage cocktail (10 mL per gram of resin). The cleavage cocktail was made of 95% TFA, 2.5% TIS and 2.5% water. The cleavage was allowed to proceed for 2 hr before the resin was filtered through a cotton plugged glass pipette. The resin was then washed with an additional TFA (2-3 mL). The TFA-peptide solution was then transferred to a propylethylene conical centrifuge tube and the solvent gently evaporated to about 3 mL using argon. The peptide was then precipitated by addition of ether (10 mL). The precipitate was isolated by centrifugation. The organic layer was then decanted and the precipitate washed two more times with ether. The product was then dried in air to give 50-90% crude peptide.
The crude peptides were purified using a Vydac C18 semi-preparative column. The crude peptides were dissolved in a mixture of aqueous 0.1% TFA, MeOH and acetonitrile, with the percentage of MeOH and acetonitrile not exceeding 20%. The solution was then filtered through a 0.45 micron cellulose acetate filter (Aerodisc). The loading used was 10 mg crude product per injection and the gradient depended on peptide. The purified fractions were combined and the acetonitrile removed by rotary evaporation. The pure peptide was then obtained by lyophilization.

6.2.2 Assay Methods

(The assays were performed in the department of pharmacology, University of Arizona)

**In vitro isolated tissue bioassays for opioid agonist**

**Mouse Isolated Vas Deferens** (performed by Peg Davis in Dr. Frank Porreca’s laboratory)

Male ICR mice under ether anesthesia were sacrificed by cervical dislocation and the *vasa deferentia* removed. The tissues were tied to gold chains with suture silk and mounted between platinum wire electrodes in 20 mL organ baths at a tension of 0.5 g and bathed in oxygenated (95% O₂, 5% CO₂) magnesium free Kreb’s buffer at 37°C. They were then stimulated electrically (0.1 Hz, single pulses, 2.0 msec duration) at supramaximal voltage. Following an equilibrium period, the compounds were added to the bath in volumes of 14-60 μL until maximum inhibition was reached. Response to an IC₅₀ dose of DPDPE (10 nM) was measured to determine tissue integrity before testing.
Guinea Pig Isolated Ileum (performed by Peg Davis in Dr. Frank Porreca’s laboratory)

Male Hartley guinea pigs under ether anesthesia were killed by decapitation and a non-terminal portion of the ileum removed. The longitudinal muscle with myenteric plexus (LMMP) was carefully separated from the circular muscle and cut into strips as described in literature. These tissues were tied to gold chains with suture silk and mounted between platinum wire electrodes in 20 mL organ baths at a tension of 1.0 g containing 37°C oxygenated (95% O₂, 5% CO₂) Kreb’s buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.19 mM KH₂PO₄, 1.18 mM MgSO₄ 25 mM NaHCO₃, and 11.48 mM glucose), and allowed to equilibrate for 15 min. The tissues were stimulated electronically (0.1 Hz, 0.4 msec duration) at supramaximal voltage. Following an equilibration period, compounds were cumulatively added to the bath in volumes of 15-60 μL aliquots until maximum inhibition was reached. Response to an IC₅₀ dose of PL-017 (100 nM) was measured to determine tissue integrity before analogue testing.

Antagonism test (performed by Peg Davis in Dr. Frank Porreca’s laboratory)

Drugs were tested for their antagonist activity by adding an IC₅₀ dose of PL-017 in the GPI or DPDPE in the MVD to the bath after the final 1.0 μL dose of the test drugs. Blunting of the agonist effect of DPDPE or PL-017 indicated antagonist activity of the test compound.

CCK studies in the guinea pig isolated ileum (performed by Peg Davis in Dr. Frank Porreca’s laboratory)

Male Hartley guinea pigs under ether anesthesia were killed by decapitation and a non-terminal portion of the ileum removed. The longitudinal muscle with myenteric plexus...
(LMMP) was carefully separated from the circular muscle and cut into strips as described in literature. These tissues were tied to gold chains with suture silk and mounted between platinum wire electrodes in 20 mL organ baths at a tension of 1.0 g containing 37°C oxygenated (95% O₂, 5% CO₂) Kreb’s bicarbonate buffer. The tissues were stimulated electronically (0.1 Hz, 0.4 msec duration) at supramaximal voltage to stabilize base-line force and tissue health. Response to an IC₅₀ dose of PL-017 (100 nM) was measured to determine tissue integrity before analogue testing. Following an equilibration period the tissues were challenged with KCl (67 mM) to determine initial maximal muscle contractility.

An initial non-cumulative CCK-8 dose response curve was constructed using concentrations from 1 to 100 nM. The test compound was added to the bath in concentrations from 1 to 100 nM. If no agonist activity was observed after 3 min, a dose of CCK-8 dose response was added to determine the test compound’s antagonist activity until a complete CCK-8 dose-response curve was reconstructed using a dose of antagonist which would seem to cause a three fold shift rightward. Tissues were again challenged with KCl to determine tissue changes during the assay. After thorough washing, electrical stimulation was again applied, tissue resiliency tested with 100 nM PL-017, and an opioid dose response curve was constructed with the test compound.

For CCK studies, contraction height was calculated as a percentage of the maximal KCl contraction and calculated as an A₅₀. For opioid studies, percentage inhibition was calculated using the average tissue contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of agonist.
IC$_{50}$ and E$_{max}$ estimates were determined by computerized nonlinear least-squares analysis (MINSQ, Micromath).

$[^{35}S]$GTP-$\gamma$-S-binding assays (performed by Dr Shou-ma in Dr. Josephine Lai’s laboratory)

Cells expressing hDOR (or rMOR for mu-receptor studies) were incubated with increasing concentrations of the test compounds in the presence of 0.1 nM were incubated with increasing $[^{35}S]$GTP-$\gamma$-S (1000-1500 Ci/mmol, NEN, Boston, MA) in assay buffer (total volume of 1 mL, duplicate samples) as a measure of agonist-mediated G protein activation. After incubation (90 min, 30°C), the reaction was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by four washes with ice-cold 25 mM Tris/120 mM NaCl, pH 7.4. Filters were pretreated with assay buffer prior to filtration to reduce nonspecific binding. Bound reactivity was measured by liquid scintillation spectrophotometry after an overnight extraction with EcoLite (ICN, Biomedicals, Costa Mesa, CA) scintillation cocktail.

Phosphoinoside (PI) hydrolysis assays (performed by Dr. Balaz Hargattai in Dr. Josephine Lai’s laboratory)

Transfected cells were incubated with 0.5 mL IMDM 0.2 μM [$^3$H]myoiositol (final concentration) for 20-22 hrs at 37°C in the presence of humidified air (95% O$_2$, 5% CO$_2$). After the removal of media, the cells were incubated further for 1 hr at 37°C. After removal of the media solution, 0.5 mL of IMDM was added and then LiCl stock solution to make a final concentration of 10 mM. Following a 10 min equilibration time, the test compound was added and the cells were incubated for 1 hr at 37°C in the presence of
humidified air (95% O₂, 5% CO₂). The cells were placed in ice and the test compound and media were removed. The reactions were terminated with the addition of 0.5 mL cold methanol. The cells were scraped and transferred to a chloroform/water mixture (1 mL/0.5 mL). The sample was centrifuged at 2100 rpm for 10-15 min at 4°C. 0.9 mL aliquots of the supernatant containing the water soluble [³H]-ionositol phosphates was diluted with 2 mL of H₂O, which was purified into anion exchange columns (CAG1-X8, 100-200 mesh Bio-Rad laboratory). Following the loading of the sample, the columns were washed with 5 mL water to remove [³H]-ionositol precursor, followed by 5 mL of 5 mM sodium tetraborate/60 mM sodium formate and 0.1 M formic acid mixture into scintillation vials. 9 mL of Aquamix was added to each vial and radioactivity was measured by scintillation spectrometry.

**Analysis**

Percentage inhibition was calculated using the average tissue contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of agonist. IC₅₀ and Eₘₐₓ estimates were determined by computerized nonlinear least-squares analysis (MINSQ, Micromath).

**6.3 Biological evaluation at the CCK receptors**

In the PI hydrolysis assay at CCK-A and CCK-B receptors (Table 6.1), peptides JMN₁, 2, 5, and 6, showed potent activity at the CCK-A receptors and no activity at the CCK-B receptors. Substitution of L-Trp for D-Trp in JMN₆ to give JMN₇ led to 80 fold loss in biological activity at CCK-A. JMN₈ on the other hand was not potent at both the CCK-
A and CCK-B receptors. The six peptides also showed no agonist activity at CCK-A in the presence of naloxone (Table 6.2).

In the competitive binding assays at the cloned human CCK-A and CCK-B receptors (Table 6.3), JMN5 and 6 had good binding affinities while JMN1 and 2 had moderate binding affinities. Substitution of L-Trp for D-Trp in JMN6 to give JMN7 led to loss of affinity at both the CCK receptors. JMN8 had no binding affinity at both receptors.

Table 6.1 Biological evaluation at the CCK receptors

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC50 (nM) hCCK-A</th>
<th>EC50 (nM) hCCK-B</th>
<th>[125I] CCK8 hCCK-A</th>
<th>[125I] CCK8 hCCK-B</th>
<th>Functional analysis</th>
<th>Binding affinity (ki, nM)</th>
<th>CCK-A agonist activity</th>
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<tbody>
<tr>
<td>SNF9007</td>
<td>n/d</td>
<td>n/d</td>
<td>3270</td>
<td>2.1</td>
<td>no response</td>
<td>3270</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>RSA501</td>
<td>790</td>
<td>3100</td>
<td>140</td>
<td>14</td>
<td>no response</td>
<td>14</td>
<td>none</td>
</tr>
<tr>
<td>JMN1</td>
<td>no response</td>
<td>4.1</td>
<td>740</td>
<td>70</td>
<td>no response</td>
<td>740</td>
<td>none</td>
</tr>
<tr>
<td>JMN2</td>
<td>no response</td>
<td>6.9</td>
<td>1200</td>
<td>100</td>
<td>no response</td>
<td>1200</td>
<td>none</td>
</tr>
<tr>
<td>JMN5</td>
<td>no response</td>
<td>2.3</td>
<td>2400</td>
<td>16</td>
<td>no response</td>
<td>2400</td>
<td>none</td>
</tr>
<tr>
<td>JMN6</td>
<td>no response</td>
<td>1.9</td>
<td>810</td>
<td>11</td>
<td>no response</td>
<td>810</td>
<td>none</td>
</tr>
<tr>
<td>JMN7</td>
<td>no response</td>
<td>160</td>
<td>1800</td>
<td>1400</td>
<td>no response</td>
<td>1800</td>
<td>none</td>
</tr>
<tr>
<td>JMN8</td>
<td>no response</td>
<td>no response</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>no response</td>
<td>&gt;10,000</td>
<td>none</td>
</tr>
</tbody>
</table>

*a*Phosphoinositide (PI) hydrolysis assay in hCCK-A and hCCK-B receptors in HEK cell lines. *b*Competition against [125I] CCK8 (sulfated) in hCCK-A and hCCK-B receptors in HEK cell lines in the presence of naloxone. *c*Contraction of isolated tissue relative to initial contraction with KCl in the presence of naloxone in GPI/LMMP. n/d, not determined.
Table 6.2 Functional analysis at the opioid receptors

<table>
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<tr>
<th></th>
<th>MVD</th>
<th>GPI/LMMP</th>
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<tr>
<td>RSA501</td>
<td>17.0%</td>
<td>n/d</td>
</tr>
<tr>
<td>JMN1</td>
<td>28.5%</td>
<td>none</td>
</tr>
<tr>
<td>JMN2</td>
<td>18.0%</td>
<td>none</td>
</tr>
<tr>
<td>JMN3</td>
<td>18.7%</td>
<td>none</td>
</tr>
<tr>
<td>JMN4</td>
<td>1.0%</td>
<td>none</td>
</tr>
<tr>
<td>JMN5</td>
<td>12.5%</td>
<td>none</td>
</tr>
<tr>
<td>JMN6</td>
<td>18.0%</td>
<td>none</td>
</tr>
<tr>
<td>JMN7</td>
<td>2.0%</td>
<td>none</td>
</tr>
<tr>
<td>JMN8</td>
<td>5.6%</td>
<td>n/d</td>
</tr>
</tbody>
</table>

|^| antagonist activity^a |
|-----------------------|
| DPDPE antagonism      |
| agonist activity^a    |
| PL-017 antagonism     |

<table>
<thead>
<tr>
<th>^</th>
<th>RSA501</th>
<th>JMN1</th>
<th>JMN2</th>
<th>JMN3</th>
<th>JMN4</th>
<th>JMN5</th>
<th>JMN6</th>
<th>JMN7</th>
<th>JMN8</th>
</tr>
</thead>
<tbody>
<tr>
<td>agonist activity^a</td>
<td>17.0%</td>
<td>28.5%</td>
<td>18.0%</td>
<td>18.7%</td>
<td>1.0%</td>
<td>12.5%</td>
<td>18.0%</td>
<td>2.0%</td>
<td>5.6%</td>
</tr>
<tr>
<td>DPDPE antagonism</td>
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<td>none</td>
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<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>n/d</td>
</tr>
<tr>
<td>agonist activity^a</td>
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<td>9.4%</td>
<td>7.2%</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td>none</td>
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<td>none</td>
</tr>
<tr>
<td>PL-017 antagonism</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

^a% inhibition of muscle contraction at 1 μM at electrically stimulated isolated tissues. n/d, not determined.

6.4 Biological evaluation at the opioid receptors

In the competitive binding assays at cloned opioid receptors, all the peptides except JMN2 showed micromolar binding affinities (Table 6.3). JMN2 had a slightly high affinity at both the mu and delta receptors. In the GTP-γ-S assay, JMN1, 2 and 5 had no activity. JMN7 showed moderate activity at both the receptors which could result from the low binding affinity. The peptides also showed no agonist or antagonist properties at both the receptors (Table 6.2).

At the MVD, all the peptides showed low agonist activity but no antagonist properties. However, this low value may be attributed to the low binding affinities. At the GPI/LMMP, JMN1, 2, 5, 6, and 7 showed low agonist properties while the rest were inactive. Interestingly, JMN7 shows low agonist activity at both the MVD and GPI/LMMP compared to the GTP binding assay.
Table 6.3 Biological evaluation at the opioid receptors

<table>
<thead>
<tr>
<th>Drug</th>
<th>( EC_{50} ) (nM)</th>
<th>( EC_{50} ) (nM)</th>
<th>[^{35}S]GTP-( \gamma )-S binding assay</th>
<th>[^{3}H]DPDPE</th>
<th>[^{3}H]DAMGO</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNF9007</td>
<td>n/d</td>
<td>n/d</td>
<td>250</td>
<td>5200</td>
<td></td>
</tr>
<tr>
<td>RSA501</td>
<td>1000</td>
<td>1800</td>
<td>74</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td>JMN1</td>
<td>n/d</td>
<td>n/d</td>
<td>1850</td>
<td>9280</td>
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<tr>
<td>JMN2</td>
<td>no response</td>
<td>no response</td>
<td>337</td>
<td>225</td>
<td></td>
</tr>
<tr>
<td>JMN5</td>
<td>no response</td>
<td>no response</td>
<td>1720</td>
<td>3470</td>
<td></td>
</tr>
<tr>
<td>JMN6</td>
<td>no response</td>
<td>no response</td>
<td>1410</td>
<td>8610</td>
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<tr>
<td>JMN7</td>
<td>460</td>
<td>775</td>
<td>2750</td>
<td>8110</td>
<td></td>
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<tr>
<td>JMN8</td>
<td>no response</td>
<td>876</td>
<td>1480</td>
<td>3120</td>
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\[^{a}\]\[^{35}S\]GTP-\( \gamma \)-S binding assay. \[^{b}\]Competitive assay against radiolabelled \[^{3}H\]DPDPE at hDOR and \[^{3}H\]DAMGO at rMOR. hDOR and rMOR were expressed from CHO cell lines.

6.5 Discussion and Conclusion

Alkylation of aspartic acid provided a route to access different bicyclic dipeptide mimetics for SAR studies on CCK/opioid chimeric peptides. Both \( \beta \)-allyl and \( \beta \)-benzyl substituted aspartic acids were employed in the synthesis of bicyclic dipeptide mimetics. The bicyclic dipeptide mimetics were then introduced into peptides which were tested at both the CCK and opioid receptors. JMN1-7 showed good activity at the CCK-B receptor which may be attributed to the C-terminal tetrapeptide, a CCK pharmacophore. This will corroborate the observation that the tetrapeptide (CCK-4) is the minimal sequence required for activity at CCK-B receptors. Inactivity of JMN8 at the CCK receptors may thus be as a result of interference with this tetrapeptide. However, unlike in most other CCK peptides where substitution of D-Trp leads to antagonistic properties, JMN7 still retained its agonist properties. There was however a considerable loss of affinity at both CCK receptors on substituting D-Trp for Trp in JMN6. The peptides were
all inactive at the CCK-B receptor and the lack of the sulfate group on Tyr may be a partial reason. JMN5 was 150 fold more selective for the CCK-B receptor over CCK-A receptor. Nle5 has been shown to provide a balanced activity between CCK-A and CCK-B receptors in related peptides but this does not seem to apply in the series of peptides that were synthesized. Substitution of NMeNle for Nle5 would thus be relevant to explore its effect on activity at the CCK receptors. NMeNle5 in combination with D-Trp also has been shown to have activities at both the opioid and CCK receptors. Further work would also be required to synthesize peptides that have antagonistic properties at the CCK receptors. This would involve SAR studies on the six peptides particularly for the C-terminal tetrapeptide.

The peptides were all found to have weak activities at the opioid receptors. Compared to JMN1 and JMN3, JMN2 has a better binding affinity at the opioid receptor which contradicts the observation that R-chirality at the second amino acid (Figure 6.2) is important for opioid activity. Except for JMN7 which had D-Trp in the sequence, all the other peptides were inactive at the opioid receptors. It would thus be important to substitute D-Trp into the other analogues to study its significance in opioid activity. JMN8 retains some opioid activity even though it was basically inactive at the CCK receptors. This would further compliment the argument that the C-terminal tetrapeptide is a CCK pharmacophore with Trp overlapping between the two receptors.
6.6 Future work

6.6.1 Structure activity relationship studies

Based on the results that were obtained, structure activity studies of the peptides would be worthwhile in an attempt to develop ligands that are antagonists and have balanced activity at CCK receptors. This would involve the substitution of Trp with D-Trp or other biologically equivalent amino acids like Nal. The Nle\(^5\) may also be substituted with NMeNle and D-Nle. JMN7 which shows activity at the opioid receptors could also be modified by introducing NMeNle or D-Nle in place of Nle. Multiple substitutions could also be investigated even though it was shown not to lead to additive change in activity.

6.6.2 Cyclized chimeric peptides

The synthesis of cyclized peptides using ring closing metathesis reaction (RCM) could offer a new path towards constrained peptides. A number of strategies for the synthesis of ring closing metathesis cyclized peptides have been reported.\(^{228-231}\) The solid phase ring closing metathesis reaction applying Grubbs catalyst has been used for the tethering of preformed secondary structures in peptides such as \(\beta\)-turns\(^ {232}\) and \(\alpha\)-helices.\(^ {233}\) RCM has also been applied in the synthesis of a rigidified protein-derived homodetic 10mer peptide epitope.\(^ {234}\) Research has shown that cyclization is not affected by ring size, experimental
conditions, stereochemical orientations, but is more affected by inability to form appropriate secondary structures. All published synthesis of peptide cycles with more than five amino acids contain proline or N-alkyl residues within their sequence so as to effect formation of the appropriate secondary structure. Reversible backbone protection, for example use of Mutter’s pseudoproline Ser(ψMeMe‘pro), can also be used to overcome the difficulties in cyclization.

To synthesize other constrained analogues of our desired peptides, we propose the synthesis of two classes of CCK/Opioid chimeric peptides cyclized on solid support by ring closing metathesis reaction.

6.6.3 Synthesis of cyclized peptides using β-allyl substituted aspartic acid and ring closing metathesis

In the first case β-allyl substituted aspartic acid would be substituted for Asp in the peptide Tyr‘-D-Nle‘-Gly3-Trp4-Nle5-Asp6-Phe7. Nle2 and Gly3 would then be alternately substituted with 2-amino-4-pentenoic acid to set the conditions necessary for ring closing metathesis. The olefin that results after metathesis can also be reduced to give more analogues of these peptides. However, the indole ring of Trp4 residue is unstable to reduction and may thus be substituted by the biologically equivalent amino acids. Nle5 may also have to be substituted with Pro to facilitate the formation of secondary structures. Cyclization at D-Nle2 position would give a 15 membered ring peptide while cyclization at Gly3 position would give a 18 membered ring peptide. Compared to disulfide bond and lactam cyclization that has been used so far, this protocol provides more constrain at the Asp6 position.
6.6.4 Synthesis of cyclized peptides using \(\delta\)-allyl substituted proline analogues and ring closing metathesis

In the second approach, Nle\(^2\) and Gly\(^1\) would be alternately substituted with 2-amino-4-pentenoic acid while Asp\(^6\) will be substituted with \(\delta\)-allyl substituted proline analogues (Scheme 6.3). In this case the proline analogues may favor the formation of the secondary structure. To synthesize saturated analogues, Trp\(^4\) will be substituted with biologically equivalent analogues.

Figure 6.3 Synthesis of cyclized peptides

Figure 6.4 Synthesis of highly constrained cyclic peptides.
These approaches may lead to ligands with good biological activities since the pharmacophore in the peptide is maintained. This may also provide a strategy to the synthesis of cyclized analogues to compliment disulfide and lactam cyclizations.
Appendix A

R\textsubscript{f} Values for the CCK/opioid chimeric peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tbody>
<tr>
<td>JMN1</td>
<td>0.75</td>
<td>0.84</td>
<td>0.57</td>
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<tr>
<td>JMN2</td>
<td>0.67</td>
<td>0.75</td>
<td>0.43</td>
</tr>
<tr>
<td>JMN3</td>
<td>0.44</td>
<td>0.63</td>
<td>0.28</td>
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<tr>
<td>JMN4</td>
<td>0.47</td>
<td>0.63</td>
<td>0.31</td>
</tr>
<tr>
<td>JMN5</td>
<td>0.72</td>
<td>0.76</td>
<td>0.51</td>
</tr>
<tr>
<td>JMN6</td>
<td>0.73</td>
<td>0.80</td>
<td>0.57</td>
</tr>
<tr>
<td>JMN7</td>
<td>0.73</td>
<td>0.78</td>
<td>0.55</td>
</tr>
<tr>
<td>JMN8</td>
<td>0.52</td>
<td>0.76</td>
<td>0.40</td>
</tr>
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</table>

\textsuperscript{a}R\textsubscript{f} values on thin layer chromatograms of silica gel in the following solvents: (A) 1-butanol/water/acetic acid (4:1:1), (B) chloroform/methanol/water (4:4:1), (C) methanol/ethylacetate/hexanes/water (6:4:4:1)
Appendix B

Retention times for the CCK/opioid chimeric peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HPLC Retention timea</th>
<th>k'</th>
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<td>JMN1</td>
<td>22.9</td>
<td>5.5</td>
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<td>JMN3</td>
<td>14.3</td>
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<td>JMN4</td>
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</tr>
<tr>
<td>JMN7</td>
<td>23.7</td>
<td>6.1</td>
</tr>
<tr>
<td>JMN8</td>
<td>17.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

aHPLC retention time; $k' = [(\text{peptide retention time} - \text{solvent retention time})/\text{solvent retention time}]$ in a solvent of 10% ACN in 0.1 TFA and a gradient of 10-90% ACN over 40 min. An analytical Microsorb-MV C18 5μm 100A column was used with a flow rate of 1 mL/min.
Appendix C

High resolution mass spectrometry for the CCK/opioid chimeric peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>molecular formula</th>
<th>HR-MS</th>
<th>Calculated</th>
<th>Observed</th>
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<tbody>
<tr>
<td>JMN1</td>
<td>C₄₉H₆₁N₉O₁₀S</td>
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<td>968.4348</td>
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<tr>
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<td>C₄₉H₆₁N₉O₁₀S</td>
<td></td>
<td>968.4340</td>
<td>968.4348</td>
</tr>
<tr>
<td>JMN3</td>
<td>C₂₅H₂₈N₄O₆S</td>
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[2,3]-wittig rearrangement of 13-membered diallylic ethers as key reaction.


Abbreviations

Abbreviations are used for amino acids and designation of peptides follows the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in J. Biol. Chem 1972, 247, 977-983. Additional abbreviations are used as follows:

Boc \( \text{tert-butoxycarbonyl} \)
BOP \( \text{Benzotriazole-1-yl-oxo-tris-(dimethylamino)} \)-phosphonium hexafluorophosphate
Fmoc \( \text{9-fluorenylmethoxycarbonyl} \)
tBu \( \text{tert-butyl} \)
DMF \( \text{N,N-dimethyl formamide} \)
DBU \( \text{1,8-Diazabicyclo[5.4.0]undec-7-ene} \)
HBTU \( \text{2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl hexafluorophosphate} \)
HOBt \( \text{N-hydroxylbenzotriazole} \)
RP-HPLC \( \text{reversed-phase high performance liquid chromatography} \)
TFA \( \text{trifluoroacetic acid} \)
TIS \( \text{triisopropyl silane} \)
TLC \( \text{thin layer chromatography} \)