SYNTHESIS OF NOVEL AMINO ACIDS AND USE OF PEPTIDES & PEPTIDOMIMETICS CONTAINING UNNATURAL AMINO ACIDS FOR THE DEVELOPMENT OF SELECTIVE MELANOCORTIN PEPTIDE ANTAGONISTS AND FOR THE STUDY OF MELANOCORTIN RECEPTOR SIGNALING

by

Hongchang Qu

A Dissertation Submitted to the Faculty of the DEPARTMENT OF CHEMISTRY In Partial Fulfillment of the Requirements For the Degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA

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DEDICATION

To my parents
Dianyi Qu, Baorong Guo

To my wife
Yan Li

To my son
Kevin N. Qu
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ABBREVIATIONS AND DEFINITIONS

Abbreviations used for amino acids are according to the rules specified by the IUPAC-IUB Joint Commission of Biochemical Nomenclature. All amino acids are in the L-configuration unless otherwise specified.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>Ac₂O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>AGRP</td>
<td>agouti related protein</td>
</tr>
<tr>
<td>Ala</td>
<td>L-alanine</td>
</tr>
<tr>
<td>Alloc</td>
<td>allyloxycarbonyl</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ARBs</td>
<td>Angiotensin Receptor Blockers</td>
</tr>
<tr>
<td>APIs</td>
<td>active pharmaceutical ingredients</td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>L-aspartic acid</td>
</tr>
<tr>
<td>ASIP</td>
<td>agouti signaling protein</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin type 1 receptor</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-butoxycarbonyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIC</td>
<td>1,3-diisopropylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMT</td>
<td>3′,5′-dimethyl tyrosine</td>
</tr>
<tr>
<td>D-Nal(2′)</td>
<td>3-(2-naphthyl)-D-alanine</td>
</tr>
<tr>
<td>DPDPE</td>
<td>c[D-Pen₃,D-Pen⁵]Enkephalin</td>
</tr>
<tr>
<td>D-Pen</td>
<td>D- penicillamine</td>
</tr>
<tr>
<td>D-Phe</td>
<td>D-phenylalanine</td>
</tr>
<tr>
<td>2,6-DTBP</td>
<td>2,6-di-tert-butyl pyridine</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>Et₂O</td>
<td>diethyl ether</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>FAB</td>
<td>fast atom bombardment mass spectrometry</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenylmethoxycarbonyl</td>
</tr>
<tr>
<td>Fmoc-OSu</td>
<td>N-[(9-fluorenylmethoxycarbonyl)oxy]-succinimide</td>
</tr>
<tr>
<td>FT</td>
<td>fourier transform</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GPI</td>
<td>guinea pig ileum</td>
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ABBREVIATIONS AND DEFINITIONS - Continued

HBTU  2-(1H-benzotriazole-1-y1)-1,1,3,3-tetramethyluronium hexafluorophosphate
hdOR  human δ -opioid receptor
His   L-histidine
hMC1R human melanocortin 1 receptor
hMC2R human melanocortin 2 receptor
hMC3R human melanocortin 3 receptor
hMC4R human melanocortin 4 receptor
hMC5R human melanocortin 5 receptor
HOAt  1-hydroxy-7-aza-benzotriazole
HOBt  1-hydroxybenzotriazole
HPLC  high-pressure liquid chromatography
HRMS  high resolution mass spectrometry
hsst4 human somatostatin subtype 4
IC50  50% inhibitory concentration
Ile   L-isoleucine
iPr   isopropyl
LHMD  lithium hexamethyldisilazide
llMOD large-scale low-mode
Lys   L-lysine
MBHA  4-methylbenzhydruylamine
Me    methyl
Met   L-methionine
Ms-Cl mesyl chloride
MSH   melanocyte-stimulating hormone
MTII  Ac-Nle4 -c[Asp5 -His6 -D-Phe7 -Arg8 -Trp9 -Lys10] -NH2
MVD   mouse vas deferens
MOR   mu opioid receptor
Nle   L-norleucine
NMR   nuclear magnetic resonance spectroscopy
NOE   nuclear overhauser effect
OPLS  optimized potential for liquid simulations
PEG   polyethylene glycol
Phe   L-phenylalanine
PRCG  Polak-Ribier conjugate gradient
Pro   L-proline
PWR   plasmon-waveguide resonance
PyBOP benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
RMSD  root mean square deviation
RP-HPLC reverse phase high-pressure liquid chromatography
Sar   sarcosine
SAR   structure activity relationship
SHU9119 Ac-Nle4 -c[Asp5 -His6 -D-Nal(2')7 -Arg8 -Trp9 -Lys10] -NH2
SII   [Sar1 , Ile4 , Ile8 ]Angiotensin II
ABBREVIATIONS AND DEFINITIONS - Continued

SPPS    solid phase peptide synthesis
SPR    surface plasmon resonance
TBDMS   \textit{tert}-butyldimethylsilyl
\textit{t}Bu    \textit{tert}-butyl
TEA    triethylamine
TFA    trifluoroacetic acid
THF    tetrahydrofuran
THIQ   N-\{(3\textit{R})-1,2,3,4-Tetrahydroisoquinolinium-3-ylcarbonyl\}-(1\textit{R})-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1\textit{H}-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine.

\begin{center}
\textbf{THIQ}
\end{center}

Thr    \textit{L}-threonine
TIPS    trisopropylsilane
TMS    tetramethyl silane
TMT    \textit{\beta}-methyl-2',6'-dimethyltyrosine
TRITC   tetramethylrhodamine-5-(and-6)-isothiocyanate
Trp    \textit{L}-tryptophan
Trt    trityl
Tyr    \textit{L}-tyrosine
Val    \textit{L}-valine
ABSTRACT

The incorporation of proper unnatural amino acids into a peptide or protein can significantly improve peptide or protein potency, selectivity, half-life, cell permeability, bio-distribution, etc. Site-specific modifications of peptides and proteins under physiological conditions also have been made easier with the use of unnatural amino acids. Therefore, unnatural amino acids are indispensible tools, not only for the elucidation of molecular mechanisms during the study of the complicated biological system, but also for the development of novel drugs with better efficacy and lower toxicity. β-Substituted γ,δ-unsaturated amino acids have been shown to be an important type of novel amino acid because of the terminal double bond which can be converted to many other functionalities. The methodology for the synthesis of syn-β-substituted γ,δ-unsaturated amino acids has been developed. However, there is no satisfactory general method for the synthesis of anti-β-substituted γ,δ-unsaturated amino acids. Therefore, a general methodology was developed by using the Eschenmoser-Claisen rearrangement for the synthesis of both racemic and optically active anti-β-substituted γ,δ-unsaturated amino acids. This rearrangement is highly diastereoselective and good asymmetric induction was obtained with a relatively small C2-symmetric chiral auxiliary (2R,5R)-dimethylpyrrolidine. In an effort to design peptide antagonists that are selective for human melanocortin 4 receptor, highly constrained trans and cis 4-guanidinium proline derivatives were synthesized and incorporated in various melanotropin analogues designed to mimic the endogenous hMC1,4R selective antagonist hASIP (Agouti Signaling Protein) central loop. Biological assays show that some of these analogues are highly selective for hMC1R and/or hMC4R with partial agonist or antagonist activities due to a new β-
turn structure induced by the presence of the constrained amino acids. According to molecular modeling studies, the lowest energy conformations of these selective analogues resemble the NMR solution structure of the hASIP central loop. Therefore, a new template was developed for the rational design of novel selective melanotropin analogues that may have therapeutic potential. To further understand the molecular mechanisms of hMC4R signaling upon agonist activation, an hMC4R selective nonpeptide agonist THIQ and its fluorescent dye labeled derivatives were needed to compare to peptide agonist MTII with regard to receptor phosphorylation, internalization, etc. An improved synthetic method was developed for the efficient synthesis of THIQ. A method for the synthesis of TRITC labeled THIQ derivatives was also developed.
CHAPTER 1
THE USE OF UNNATURAL AMINO ACIDS IN PEPTIDE AND PROTEIN CHEMISTRY

1.1 Introduction

In recent years, an increasing number of peptides are being developed as drugs due to their biological diversity alongside high specificity, affinity, and low toxicity. In addition, the peptide chemistry is much more predictable and far less empirical than often is the case with small molecules. According to a 2005 Chemical and Engineering News article,\(^1\) more than 40 peptides had been marketed worldwide with approximately 270 in the clinical phases and 400 in advanced preclinical phases. The latest report from Genetic Engineering and Biotechnology News put the number of marketed peptide drugs at 67 for targeting 29 disease indications.\(^2\) The approved peptide drugs include both natural peptides such as insulin, vancomycin, oxytocin, cyclosporine and synthetically made ones such as Fuzeon (enfuvirtide) and Integrilin (eptifibatide) (Figure 1.1). The overall market for peptide drugs is expected to expand at a growth rate nearly double the growth rate for active pharmaceutical ingredients (APIs) overall. On the other hand, peptides are bigger, more expensive to synthesize, less stable than small molecules and often have to be injected. The other issue is the fact that there are only 20 natural amino acids available for making peptides.

The synthesis of peptides is becoming less of a problem with fast technology advancement. Nowadays, peptides can be manufactured through transgenic, recombinant, or synthetic methods. And the manufacturing cost is going down quickly while the scale is going up. Thus, the cost is no longer a big concern. Many
other problems associated with peptides can be overcome by rational chemical optimizations of the peptides with the use of various unnatural amino acids.

Figure 1.1 Structures of natural and synthetic peptides

Chemists around the world have developed many efficient methods for the synthesis of novel amino acids, many of which are now commercially available in optically pure form. The increasing number of novel amino acids has greatly
expended the amino acid pool available for novel peptide and protein design. Incorporation of proper novel amino acids can significantly improve peptide or protein potency, selectivity and pharmacologic properties such as half-life and membrane permeability. Novel amino acids also can be used to stabilize certain secondary structures thus improving the biological profile of a peptide or protein. In addition, novel amino acids are valuable tools for the study of 3D topographic relationships of peptide pharmacophore elements. Furthermore, their use can provide a unique way for peptide or protein structural or functional modifications. Finally, they can be used to design and make biologically active ligands with novel secondary structures and properties for medical applications.

1.2 Improving Peptide Potency

The complement system is a biochemical cascade, which involves over 30 proteins, including soluble proteins in the blood and surface-bound receptors and regulators. It is a key player of the innate immune system and an essential bridge between innate and adaptive immunity. As one part of the larger immune system, the complement system is a very important line of defense that helps clear pathogens from an organism via three activation cascades: the classic, the lectin, and the alternative pathway (Figure 1.2). However, its inappropriate activation is the cause of tissue injury in many disease states, for example, Alzheimer’s disease, asthma, multiple system organ failure, xenotransplantation, rheumatoid arthritis, and stroke. All three complement activation pathways converge to a common component C3 protein. Thus, the C3 is a valid drug target for the modulation of the complement system.
A phage-displayed combinatorial random peptide library identified a 27-residue peptide that binds and inhibits C3. This peptide was further truncated to a 13-residue peptide named Compstatin, H-(I[CVVQDWGHHRC]T-NH₂), without any loss of affinity (IC₅₀ =54 µM). The NMR solution structure shows that there is a type I β-turn at residues QDWG (Figure 1.3). It should be noted that this NMR structure is an average of many conformations due to the flexibility of the peptide. Furthermore, a Surface Plasma Resonance (SPR) study suggested that the NMR structure was not the biologically active conformation. Alanine and D-amino acid scans were also performed for the peptide. All results suggest that the β-turn, disulfide bridge and the surrounding hydrophobic cluster are important for Compstatin binding. On the other hand, Val⁴, His⁹, His¹⁰, Arg¹¹ residues can be replaced by alanine without significant drop of ligand inhibition activity.
Further investigations were directed toward increasing the hydrophobicity of the cluster around the disulfide bond. Analogues were designed based on the sequence pattern \(\text{Xc}[\text{CVXQDWGXXXC}]\text{X}\) in which \(\text{X}\) residues were replaced with various hydrophobic natural amino acids. Years of extensive screening only led to the discovery of a 16-fold more potent analogue \(\text{Ac-I}[\text{CVYQDWGAHC}]\text{T-NH}_2\).\(^{13}\)

However, when the previously considered non-essential \(\text{Val}^4\) residue was replaced with 2-naphthylalanine or 1-methyl-trptophan (Figure 1.4), the \(\text{IC}_{50}\) value was increased by 99 and 264 times, respectively, compared to that of the Compstatin.\(^{14}\)

The results clearly demonstrate the value of novel amino acids in increasing peptide potency. The Hruby group has pioneered many such studies.\(^{15}\)

Figure 1.3 Stereo view of the NMR solution structure of Compstatin\(^{11b}\)

Figure 1.4 1-Methyl-trytophan and 2-naphthylalanine

\[
\begin{align*}
\text{1-methyl-tryptophan} & & \text{2-naphthylalanine} \\
\end{align*}
\]

1.3 Increasing Peptide Stability

Native peptides usually have very short half-life \textit{in vivo} due to protease cleavage of amide bonds. Therefore, they are not suitable to be used as therapeutic
drugs. One way to increase peptide half-life is to incorporate unnatural amino acids into the peptide. D-amino acids have been known to improve peptide half-life and at the same time stabilize certain secondary structure that may lead to increased potency, as in the case of NDP-\(\alpha\)-MSH.\(^{16}\)

One of the endogenous peptide hormones \(\alpha\)-MSH, along with melanocortin receptors, and other agonists and antagonists, modulates a variety of very important physiological activities that include skin pigmentation,\(^{17,18}\) blood pressure and heart rate,\(^{19}\) erectile function,\(^{20}\) feeding behavior and energy homeostasis,\(^{21,22,23,24,25,26,27}\) aggressive/defensive behavior,\(^{28}\) and meditation of pain.\(^{29,30}\) However, the half-life of the endogenous \(\alpha\)-MSH (Ac-Ser\(^1\)-Tyr\(^2\)-Ser\(^3\)-Met\(^4\)-Glu\(^5\)-His\(^6\)-Phe\(^7\)-Arg\(^8\)-Trp\(^9\)-Gly\(^{10}\)-Lys\(^{11}\)-Pro\(^{12}\)-Val\(^{13}\)-NH\(_2\)) is too short (a few minutes) for therapeutic use. Thus, the Hruby group has carried out extensive SAR studies of this peptide, especially with regards to pigmentary activity.\(^{31}\) These investigations revealed a much improved analogue NDP-\(\alpha\)-MSH (Ac-Ser-Tyr-Ser-\textbf{Nle}-Glu-His-\textbf{D-Phe}-Arg-Trp-Gly-Lys-Pro-Val-NH\(_2\)), which has the Met\(^4\), Phe\(^7\) residues in \(\alpha\)-MSH replaced by Nle\(^4\), D-Phe\(^7\) residues respectively.\(^{32}\) This analogue is 100 times more potent than the parent \(\alpha\)-MSH. It is stable for at least 72 hours in human serum while remaining the low toxicity profile. The roles played by the unnatural amino acids are significant. The presence of the Nle and D-Phe residues greatly reduced the protease cleavage rate, resulting in a significantly increased half-life. In addition, the D-Phe residue improved the stability of the critical \(\beta\)-turn spanning His\(^6\) and D-Phe\(^7\) residues.\(^{33}\)

1.4 Stabilizing Peptide Bioactive Conformations by Local Constraints
Just as a bioactive protein requires a defined 3-D structure, a bioactive peptide prefers certain secondary structure, for example, a β-turn or α-helix. In the case of α-MSH, a β-turn at residues Glu-His-Phe-Arg is required for its activity.\textsuperscript{34} This turn is essential for the peptide to adopt an active conformation to bind to a specific pocket in melanocortin receptors for agonist activity.\textsuperscript{35} As mentioned above, part of the improvements seen in NDP-α-MSH comes from the increased stability of the β-turn due to the use of the D-Phe\textsuperscript{7} residue instead of the Phe\textsuperscript{7} residue.

A helical structure is also a very important secondary structure for some bioactive peptides, for example, BID BH3 peptide that can induce cell apoptosis.\textsuperscript{36} However, as with most helical peptides that bind to their receptors in a helical conformation, the peptide is not folded when it is free in solution. Its polar amide backbone is exposed due to the lack of folding. Thus, it has low potency, low stability and poor membrane permeability.\textsuperscript{37} Presumably, stabilizing the helical conformation would increase receptor binding by virtue of pre-organization. In addition, the intramolecular hydrogen bonding in the helix and the side chain groups on the surface of the helix would slow down enzymatic degradation, and at the same time reduce the barrier to membrane penetration.

Verdine’s group developed a method to stabilize the helical conformation of the peptide by incorporating two unnatural amino acids with terminal unsaturation and joining them via metathesis.\textsuperscript{36} Several of these hydrocarbon-stapled analogues were designed and synthesized (Figure 1.5). The best analogue exhibited a 7-fold increase in binding affinity, 41-fold decreases in protease cleave rate and improved membrane permeability. Obviously, the constraints exerted by the hydrocarbon bridge
are responsible for the preference of the helix conformation of these analogues even in their unbound form.

Figure 1.5 Enhanced helicity, protease resistance, and serum stability of hydrocarbon-stabled BID BH3 compounds. (A and B) \(\alpha,\alpha\)-disubstituted nonnatural amino acids containing olefinic side chains of varying length were synthesized as reported.\(^38,36\) Nonnatural amino acid substitutions were made to flank three (substitution positions i and i+4) or six (substitution positions i and i+7) amino acids within the BID BH3 peptide, so that reactive olefinic residues would reside on the same face of the \(\alpha\)-helix. (C) Circular dichroism was used to measure the percentages of the SAHB maintained in helical configuration when dissolved in aqueous potassium phosphate solution (pH 7). (D) Fluoresceinated SAHB\(_A\) and BID BH3 peptide were incubated at 37 °C in mouse serum or injected intravenously (10 mg/kg) into NOD SCID mice. Serum concentrations of SAHB\(_A\) and BID BH3 peptide were measured at the indicated time points with a fluorescence-based high-performance liquid chromatography detection assay. Both assays demonstrated enhanced serum stability of SAHB\(_A\) (From reference 36. Reprinted with permission from AAAS)

1.5 Stabilizing Peptide Bioactive Conformations by Global Constraints

Peptide global constraints can be achieved by cyclization, which could induce or stabilize a very important secondary structure required for receptor binding and/or
activation. Enkephalins (Tyr-Gly-Gly-Phe-Met/Leu) are endogenous neuropeptides that act on opioid receptors to relieve pain. However, their potential usefulness is limited by their poor selectivity and poor stability due to their flexible nature. Luckily, the Hruby group was able to realize the importance of global constraints for flexible linear peptides.\(^{39}\) To constrain the flexible Enkephalins, a disulfide bridge was constructed between the D-Pen\(^2\) and D-Pen\(^5\) residues that replaced the Gly\(^2\) and Met/Leu\(^5\) residues respectively. The dimethyl groups on D-Pen residues provided additional constraints to the cyclic peptide analogue (DPDPE).\(^{40}\) Bioassays indicated that the DPDPE was one of the most selective ligands for \(\delta\) opioid receptor and was very resistant to enzymatic degradation due to the disulfide bridge (Figure 1.6). Further investigation showed that the DPDPE could also pass through the blood brain barrier (BBB).\(^{41}\) In this example, the importance of the unnatural amino acid, D-Pen, was demonstrated.

Figure 1.6 The structure of c[D-Pen\(^2\), D-Pen\(^5\)]Enkephalin (DPDPE)\(^{40}\)

1.6 Study of Peptide Topographical Constraints

Topographical profiles of bioactive peptide ligands are usually not explored, largely due to the flexibility of the side chain moieties of peptides. However, insights into bioactive ligand topography are very important for better understanding of ligand receptor/acceptor interaction and the design of potent, selective peptide or protein
ligands. To study peptide topography, novel amino acids with desirable structural features need to be designed and synthesized. Novel β-substituted α-amino acids are useful in this regard because of the constraints brought by the β-substituents, which could provide critical insights into the topographical importance of key amino acid side chain groups. They have similar physicochemical properties such as electronegativity and hydrophobicity as their parent amino acids. However, they have different side chain conformations that are determined by nonbonding or other interactions between vicinal substituents. Therefore, they can be used for a side chain topographic scan in globally constrained peptides which often leads to the identification of potent and/or selective compounds. To demonstrate this, the Hruby group developed a methodology for the synthesis of all four isomers of the highly topographically constrained β-methyl-2′,6′-dimethyltyrosine (TMT). Because of the methyl groups at the β position and the 2′,6′ positions of the aromatic ring, the \( \chi^1 \) torsional angel is constrained and specific torsional angels are energetically favored. In addition, the \( \chi^2 \) torsional angel is also highly restricted. Among the three possible rotamers for the (2S,3S) isomer (Figure 1.7), the gauche(−) side chain conformation is favored due to the least number of unfavorable steric interactions. The Tyr\(^1\) residue in δ selective opioid peptide analogue DPDPE (Tyr-c[D-Pen\(^2\)-Gly-Phe-D-Pen\(^5\)]-OH) was then replaced with each of the four isomers. The biological potencies of these analogues were tested (Table 1.1). It was found that only the [(2S,3R)-TMT\(^1\)]DPDPE analogue showed both high potency and exceptional selectivity for the δ opioid receptor. This finding clearly demonstrated the importance of peptide topography and the value of topographically constrained amino acids.
Figure 1.7 Low energy side chain conformations for (2S,3S)-β-methyl-2’,6’-dimethyltyrosine (TMT) about the bond between the α and β carbons (Ar=2’,6’-dimethyl-4’-hydroxylphenyl) \([\text{gauche}(−) = −60^\circ, \text{trans} = ±180^\circ, \text{gauche}(+) = +60^\circ]\).5

Table 1.1 Biological potencies of [TMT\(^1\)]DPDPE (Reprinted from reference 46 with permission, Copyright 1996 American Chemical Society)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>EC(_{50}), nM GPI(µ)</th>
<th>EC(_{50}), nM MVD(δ)</th>
<th>Selectivity (µ/δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPDPE</td>
<td>7300</td>
<td>4.1</td>
<td>1780</td>
</tr>
<tr>
<td>[(2S,3S)-TMT(^1)]DPDPE</td>
<td>290</td>
<td>170</td>
<td>2</td>
</tr>
<tr>
<td>[(2S,3R)-TMT(^1)]DPDPE</td>
<td>0% at 60 µM antagonist (IC(_{50}) 5µM)</td>
<td>1.8</td>
<td>&gt;33,000</td>
</tr>
<tr>
<td>[(2R,3R)-TMT(^1)]DPDPE</td>
<td>49,900</td>
<td>2200</td>
<td>23</td>
</tr>
<tr>
<td>[(2R,3S)-TMT(^1)]DPDPE</td>
<td>75% at 82 µM</td>
<td>28% at 10 µM</td>
<td>N/A</td>
</tr>
</tbody>
</table>

1.7 Peptide and Protein Conjugation

In order to study the biological functions of a peptide or protein, it is often desirable to attach a biophysical probe, for example, a dye molecule, to a specific position of the peptide or protein under physiological conditions. Most reactions currently used for this kind of selective modification involve the formation of a covalent bond between a nucleophile and an electrophile. One example is the reaction between a modified protein T4 lysozyme (T4L mutant) that has a novel ketone-containing amino acid (Figure 1.8) and a hydrazide derivative (Figure 1.9).47 The tyrosine derived ketone-containing amino acid was incorporated into solvent accessible site 82 of the protein with 30% efficiency by the biosynthetic machinery using unnatural amino acid mutagenesis. After the mutated protein was partially purified by ion-exchange chromatography, it was allowed to react with a nucleophile,
fluorescein hydrazide, for 36 hour at room temperature to provide the desired conjugate under very mild conditions. It was found that only the protein with a ketone handle had been labeled. In this particular example, the labeling efficiency was determined to be approximately 50%.

Figure 1.8 Ketone-containing amino acids

![Ketone-containing amino acids](image)

Figure 1.9 Site-specific protein modifications using a ketone handle (Reprinted from reference 47 with permission, Copyright 1996 American Chemical Society)

![Site-specific protein modifications](image)

An alternative approach is to take advantage of the click chemistry (azide-alkyne cyclization). Again, this approach required the use of unnatural amino acids as shown in Figure 1.10. These amino acids can be introduced genetically to a specific site of a peptide or protein with excellent efficiency. Then the modified peptide or protein is exposed to its partner molecule for 4 hours in the presence of catalytic amounts of Cu(I) salt at 37 °C to afford the desired product in 75% yield (Figure 1.11).48 Thus, peptide and protein modifications can be done quickly with exceptional selectivity. The reaction is clean and does not involve any nucleophiles or electrophiles. The mildness of the reaction allows it to be performed efficiently under physiological conditions.
1.8 β-Peptides

β-peptides are constructed from β-amino acids. Compared to natural peptide structures, β-peptide structures have different dimensions, geometries and polarities. Thus, β-peptides do not bind to the active sites of native peptidases and are proteolytically stable.β-peptides have been shown to be metabolically most stable in mammals such as rats, in insects and in plant cell cultures. Furthermore, there are more secondary structures of β-peptides than in the ‘α-peptide world’ and they can be built from a small number of β-amino acids (Figure 1.12). Therefore, it is
attractive to develop β-peptide mimetics to be used as therapeutic drug candidates. Their advantages include their small size, stability and nonmutagenic nature.\textsuperscript{52}

Figure 1.12 Helix, sheet, and turn structures of β-peptides (Reprinted from reference 51, Copyright (2004), with permission from Elsevier)

One such attempt was to design a small β-peptide mimetic of the 14-residue Somatostatin (Figure 1.13), a hormone that regulates various important biological functions such as the release of growth hormone and insulin.\textsuperscript{52} A cyclic analogue of Somatostatin, Octreotide (Figure 1.13), is currently in clinical use for the treatment of acromegaly, the treatment of diarrhea and flushing episodes associated with carcinoid syndrome, and treatment of diarrhea in patients with vasoactive intestinal peptide-
secreting tumors. However, the half-life of this 8-residue compound is only 1.7-1.9 hours. The core pharmacophore elements for both Somatostatin and Octreotide are at the $\beta$-turn encompassed by Phe-Trp-Lys-Thr. To mimic this turn, a 4-residue linear $\beta$-peptide analogue was designed and synthesized (Figure 1.14). Biological evaluation of this $\beta$-peptidomimetic showed high and specific binding affinity for Somatostatin hsst$_4$ (human somatostatin subtype 4) receptor. NMR and CD spectroscopy studies confirmed the formation of the desired $\beta$-turn structure.$^{52}$

Figure 1.13 Structures of Somatostatin and Octreotide

![Somatostatin and Octreotide structures](image)

Figure 1.14 Small $\beta$-peptide-mimetic and its $\beta$-turn structure (Reprinted from reference 52 with permission, Copyright 2001 American Chemical Society)
1.9 Conclusion

In conclusion, a variety of novel amino acids have been designed and synthesized since interests in peptides arose in the last century. These unnatural amino acids have unique structural features and have proved indispensable in the study of peptide or protein functions in complicated biological systems. They also have shown great value in peptidomimetics and drug discovery, as can be seen from the limited examples given above. Yet, this is just the beginning of their successful stories. Research involving the use of unnatural amino acids will continue to surprise us in the future. Thus, my research has been focused on the design and syntheses of novel amino acids and peptidomimetics that are of biological importance.
 CHAPTER 2

SYNTHESIS OF RACEMIC ANTI-β-SUBSTITUTED γ,δ-UNSATURATED AMINO ACIDS VIA ESCHENMOSER-CLAISEN REARRANGEMENT

(Reproduced in part with permission from Org. Lett. 2006, 8, 4215-4218. Copyright 2006 American Chemical Society)

2.1 Introduction

In the course of synthesis and conformational studies of biologically active peptide ligands, a general methodology is needed for the synthesis of nonproteinogenic amino acids with terminal unsaturation. The double bond has been a useful building block in organic synthesis due to its potential conversion to many other functionalities such as aldehydes, alcohols, halides, epoxides, amines or carboxylic acids. This type of amino acid, for example, has proved useful in the design and synthesis of bicyclic [3,3,0] dipeptide mimetics (Figure 2.1). This kind of amino acid is also very useful for peptide macrocyclization with the advancements of ring-closing metathesis. In addition, various β-side chain groups need to be introduced in the synthesis so that the amino acids will provide biologically active functionalities in conformationally constrained peptides.

Figure 2.1 Bicyclic [3,3,0] dipeptide mimetics

\[
\begin{align*}
\text{Ph} & \quad \text{NH} \quad \text{CO}_2\text{Bn} \\
\text{ZHN} & \quad \text{Ph} \\
\text{O} & \quad \text{Ph} \\
\text{N} & \quad \text{O} \\
\text{Z} & \quad \text{N} \\
\text{H} & \quad \text{CO}_2\text{Bn}
\end{align*}
\]

The Chelate-Claisen rearrangement has turned out to be very useful in the synthesis of this kind of amino acid and its applications in peptidomimetics. With this methodology, syn-β-substituted γ,δ-unsaturated amino acids can be obtained in
high diastereoselectivities starting from a trans allylic alcohol and glycine (Scheme 2.1). The high diastereoselectivities are due to the chelation of the enolate oxygen and the α-amino nitrogen by zinc ion. However, the Chelate strategy did not work well for the synthesis of racemic anti-β-substituted γ,δ-unsaturated amino acids that require the use of cis allylic alcohols. It is difficult to introduce anti-β-substituents using cis allylic alcohols as starting materials, which are not always commercially available. Furthermore, the cis-oriented side chain can destabilize the chairlike transition state because of its unfavorable steric interaction with the zinc moiety in the transition state (Figure 2.2). Instead, the boatlike transition state is preferred and the syn isomer is formed as the major product. In addition, the diastereomeric mixture of syn and anti products is usually hard to purify.

Scheme 2.1 Chelate-Claisen rearrangement for syn-β-substituted γ,δ-unsaturated amino acids

Figure 2.2 Chelate-Claisen rearrangements with use of a cis allylic alcohol (Reproduced from reference 62 with permission, Copyright 1996 American Chemical Society)
Previous studies in the Hruby lab also have generated novel syn-β-substituted amino acids with ω-unsaturation by Ni(II)-complex alkylation. However, attempts to prepare anti products have failed due to epimerization under the basic reaction conditions. To investigate the epimerization, purified anti product $S(2R,3R)$ (Figure 2.3) was subjected to the same reaction condition for 24 hours. It was found in the end that 86% of the starting material was epimerized to its syn analogue.

On the other hand, the Eschenmoser-Claisen rearrangement was known to give predominately anti-β-substituted γ,δ-unsaturated carboxylic amide derivatives starting from commercially available trans allylic alcohols (Scheme 2.2). This is due to the formation of the cis N,O-ketene acetal intermediate and a chairlike transition state conformation. Removal of the secondary amine auxiliary of the amides would provide anti-β-substituted γ,δ-unsaturated carboxylic acids. So we decided to investigate the utility of this rearrangement for the synthesis of anti-β-substituted γ,δ-unsaturated amino acids. During the study, we found that the Eschenmoser-Claisen rearrangement was a straightforward methodology for the synthesis of the desired anti-amino acids. This methodology also can be used for the synthesis of α-amino alcohols which are common structural components of complex nature products and important synthetic building blocks in organic synthesis.
Scheme 2.2 Eschenmoser-Claisen rearrangement for anti-β-substituted γ,δ-unsaturated carboxylic amide

\[
\begin{align*}
\text{R}_3\text{N} & \quad \text{OCH}_3 \\
& \quad \text{Li} \\
\text{R}_2\text{N} & \quad \text{O} \\
& \quad \text{CH}_3 \\
\text{N} & \quad \text{R}_2\text{N} \\
& \quad \text{O} \\
\text{R} & \quad \text{N} \\
\text{CH}_3 & \quad \text{CH}_3 \\
\text{R} & \quad \text{N} \\
\text{R} & \quad \text{N} \\
\text{CH}_3 & \quad \text{H}_3 \\
\text{C} & \quad \text{H}_3 \\
\end{align*}
\]

2.2 Results and Discussion

Glycine amide derivatives 2 were synthesized using a secondary amine, pyrrolidine or N,N-diisopropylamine (Scheme 2.3), as we expected that a C$_2$-symmetric 2,5-diphenylpyrrolidine and other enantiopure secondary amines would provide an enantioselective rearrangement later on as they did in the Thio-Claisen rearrangement. The coupling reactions were straightforward using standard coupling reagents HBTU/HOBt. However, we did observe a much slower reaction rate and lower yield for diisopropylamine coupling due to its bulky size.

Scheme 2.3 Amide bond formation

To get Meerwein salt 3, amide 2 was treated with methyl triflate. In theory, both the amide and carbamate C=O oxygen atoms could be methylated as shown below (Scheme 2.4). We tried various equivalents (1-4) of methyl triflate and 2.2 equivalents were found to be optimal in terms of reaction yields. This suggests that both carbonyl oxygen atoms were indeed methylated. The presence of the proton
scavenger 2,6-di-**tert**-butylpyridine (2,6-DTBP) is essential for good yields. Without 2,6-DTBP, the rearrangement reaction gave no more than 30% yields. And the benzyl alcohol fragment from the Cbz protecting group was observed from NMR analysis. We also tried the reaction with Boc protected glycine derivatives and no rearrangement product was detected. Presumably, this was due to the high acidity of the methyl triflate which removed the Boc protecting group.

**Scheme 2.4 Meerwein salt formation and Eschenmoser-Claisen rearrangement**

The formation of intermediate **5** from **3** and **4** can occur at $-35^\circ$C as indicated by TLC. However, the actual rearrangement of **5** did not happen when the reaction temperature was below 0 $^\circ$C. After the reaction was quenched at 0 $^\circ$C, only starting materials were found. Various commercially available allylic alcohols were used successfully for the rearrangement as shown in Table 2.1. The rearrangement provided products with various $\beta$-substituents upon warm-up. It should be indicated that the $\beta,\beta$-dimethyl-$\gamma,\delta$-unsaturated amino acid derivative 6a-2 could not be synthesized by direct alkylation using 3-bromo-3-methyl-1-butene, since an $S_N2'$ addition happened instead.
Table 2.1 Results of Eschenmoser-Claisen rearrangement

<table>
<thead>
<tr>
<th>entry</th>
<th>allylic alcohol</th>
<th>alcohol equiv</th>
<th>reaction temp, °C</th>
<th>reaction time, h</th>
<th>anti/syn</th>
<th>yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HO(\sim)</td>
<td>4</td>
<td>−35−rt</td>
<td>3.5</td>
<td>N/A</td>
<td>6a-1, 75</td>
</tr>
<tr>
<td>2</td>
<td>HO(\sim)</td>
<td>2.2</td>
<td>−35−35</td>
<td>4</td>
<td>N/A</td>
<td>6a-2, 44</td>
</tr>
<tr>
<td>3</td>
<td>HO(\sim)</td>
<td>2.2</td>
<td>−35−rt</td>
<td>2</td>
<td>9.6:1</td>
<td>6a-3, 61</td>
</tr>
<tr>
<td>4</td>
<td>HO(\sim)</td>
<td>4</td>
<td>−35−rt</td>
<td>4</td>
<td>8.0:1</td>
<td>6b-3, 56</td>
</tr>
<tr>
<td>5</td>
<td>HO(\sim)Ph</td>
<td>4</td>
<td>−35−rt</td>
<td>4</td>
<td>16.8:1</td>
<td>6a-5, 74</td>
</tr>
</tbody>
</table>

<sup>a</sup>Isolated yield.  
<sup>b</sup>Mixture of trans and cis (19:1)

The diastereomeric ratios were determined via proton NMR spectra of the crude samples. It was found that the rearrangement reactions generated good to excellent diastereoselectivities. The anti/syn ratio in entry 3 could be better if pure trans crotyl alcohol was used. Bulkier N,N-diisopropylamide (Scheme 2.4, 2b) provided slightly better anti/syn ratio in entry 5 (Table 2.1). Overall, pyrrolidine and diisopropyamine did not show much difference in reaction diastereoselectivity. These good diastereoselectivities can be explained via unique (Z)-N,O-ketene acetal formation and an excellent pseudochairlike transition state in the rearrangement.\(^{68}\) Unlike the Chelate-Claisen intermediate where the enolate oxygen stays preferentially cis to the glycyl nitrogen due to chelation control, the Eschenmoser-Claisen intermediate could adopt two possible configurations (Figure 2.4) due to the lack of the chelation control. Presumably, the thermodynamically more stable intermediate 7 was the predominant configuration due to less unfavorable steric interactions.\(^{69}\) We tried to quench intermediate 7 using several saturated alcohols. However, this N,O-ketene acetal intermediate was unstable at ambient temperature and hydrolyzed easily.
in neutral aqueous solution. The rearrangement products, anti-β-substituted γ,δ-
unsaturated carboxylic amides 6 are solids. Thus, they can be easily purified by
recrystallization to give pure anti products.

Figure 2.4 (Z)- and (E)-N,O-ketene acetal reaction intermediates

Ideally, a one-step hydrolysis of these rearranged amides would provide anti-
β-substituted γ,δ-unsaturated amino acids directly. However, amide bond hydrolysis
has been known to be difficult. It was more challenging in this particular case due to
the presence of the terminal double bond and the α-amino Cbz protecting group.
There have been many amide bond hydrolysis methods reported. However, basic
conditions cleaved the Cbz protecting group and caused epimerization at the α-carbon.
Strongly acidic conditions also removed the Cbz protecting group, and led to the
partial hydrolysis of the terminal double bond. So a reduction-hydrolysis approach
was investigated. Surprisingly, many proven reducing regents, for example,
superhydride and DIBAL, did not provide any aldehyde products. After screening a
lot of other reducing agents, we noticed that LAH was able to give some aldehyde
product along with a large portion of secondary amine byproduct at 0 °C. Lower
reaction temperature prevented the reduction from happening. So we decided to try
LAH derivatives with lower activities. Finally, lithium trimethoxyaluminum hydride
and sodium aluminum hydride were found to work well with minimal formation of
the secondary amine byproduct (Scheme 2.5).
However, epimerization to the syn product was observed during the work-up step of the reduction reaction. This was not unexpected, since amino aldehydes were notorious for racemization under either basic or acidic conditions.\textsuperscript{73} The presence of an electron-withdrawing $\beta$-substituent and a $\gamma,\delta$-double bond makes the $\alpha$-proton even more labile. The side reaction was initiated upon aldehyde generation during the hydrolysis of the reduced complex. To minimize the epimerization, we developed an \textit{in situ} modified Lindgren oxidation\textsuperscript{74} at low temperature (Scheme 2.5). In this way, the aldehyde generated was quickly oxidized to the carboxylic acid, giving little time for epimerization. Several $\alpha$-amino alcohols were also obtained from further reduction of the amino aldehydes that were isolated at low temperature by extraction, but without flash chromatography purification. The diastereomerically pure amino alcohols were obtained after flash column chromatography purification.

Scheme 2.5 Reductive hydrolysis, amino acid and amino alcohol generation

$$\begin{align*}
\text{Scheme 2.5 Reductive hydrolysis, amino acid and amino alcohol generation}
\end{align*}$$

Figure 2.5 $^1$H NMR spectra ($\alpha$- and $\beta$-proton) of 10-5 from different oxidation approach. left: \textit{in situ} oxidation; right: oxidation after isolation of aldehyde at rt
The lack of epimerization using the modified reductive hydrolysis and aldehyde oxidation was demonstrated using a $^1$H NMR spectra comparison between the epimerized and the diastereomeric pure product 10-5 (Figure 2.5). The higher frequency major peak is the $\alpha$-proton; the lower frequency major peak is the $\beta$-proton. The minor diastereomeric peaks on the left of each major peak are from the syn product. It is clear that the syn peaks are almost undetectable in the case of in situ oxidation, indicating minimal epimerization. The broad peak at 4.63 ppm was likely from a rotamer that arises from chemical exchange between rotamers due to the presence of the Cbz protecting group. This was confirmed by NOE experiments (Figure 2.6).\textsuperscript{75} Irradiation of the small peak at 4.63 ppm resulted in the inversion of its exchange peak at 4.78 ppm. To prove that the irradiation was selective at 4.63 ppm, the mixing time in the NOE experiments was varied. It was shown that as the mixing time was increased; the percentage of the exchange peak was also increased. Therefore, the broad peak at 4.63 ppm is indeed a rotamer peak.

Figure 2.6 Rotational inter-exchange studies of 10-5 by NOE

The relative stereochemistry of 10-3 was confirmed by comparing its $^{13}$C NMR chemical shift to the minor product synthesized from an Ester Enolate Claisen rearrangement.\textsuperscript{53} A single crystal X-ray structure of compound 6b-5 also was obtained
An anti relationship of the α-amino group and the β-phenyl group was unambiguously shown in this structure.

Figure 2.7 X-Ray crystal structure of 6b-5 to show the anti relationship

2.3 Conclusions

In summary, we have developed a convenient general methodology for the synthesis of anti-β-substituted γ,δ-unsaturated amino acids for the first time using a Meerwein Eschenmoser-Claisen rearrangement. This [3,3]-sigmatropic rearrangement gives excellent diastereoselectivities. Both novel amino acids and amino alcohols can be easily obtained after a few steps from commercially available Glycine and trans allylic alcohols.

2.4 Future Work

The most common approach to remove a Cbz protecting group is via catalytic hydrogenation, which would also reduce an isolated carbon-carbon double bond. Thus, it is desirable to have an orthogonal protecting group that can be easily and selectively removed. Because the reaction conditions do not tolerate Boc or Fmoc protecting groups, a good choice for future anti products would be an Alloc protecting group (Figure 2.8) which can be selectively removed using Pd(PPh₃)₄ and PhSiH₃.
Figure 2.8 N-Alloc protected novel amino acids

\[
\begin{array}{c}
\text{HO} \\
\text{HN} \\
\text{O} \\
\text{O} \\
\end{array}
\]

\(\alpha\)-Alkylated amino acids are of great interest for the study of peptide local constraints.\(^{42}\) If an alanine is used instead of the glycine in the Eschenmoser-Claisen rearrangement, \(\alpha\)-methylated analogues could be easily obtained (Scheme 2.6). Similar work has been demonstrated for the Chelate-Claisen rearrangement.\(^{78}\)

Scheme 2.6 Synthesis of \(\alpha\)-methyl anti-\(\beta\)-substituted \(\gamma,\delta\)-unsaturated amino acids

2.5 Experimental Section

General Information

All starting materials and reagents were purchased from Sigma-Aldrich and used without further purification. THF was distilled over potassium and benzophenone. Methylene chloride was distilled over CaH\(_2\). \(^1\)H and \(^{13}\)C NMR spectra were recorded on Bruker DRX500 (500 MHz and 125 MHz, respectively) or Varian 300 (300 MHz and 75 MHz, respectively) spectrometers with CDCl\(_3\) or TMS as internal standard. IR spectra were recorded on Nicolet IR100 FT-IR spectrometer. Melting points were obtained from open capillaries without correction. Mass spectra were obtained from Mass Spectrometry Facility, Department of Chemistry, the University of Arizona. Flash column chromatography was performed using silica gel 60 from EM Science.
TLC plates (Silica Gel 60 F$_{254}$) were purchased from EMD Chemicals via VWR and were visualized by UV or KMnO$_4$ stain. All new compounds were characterized by $^1$H, $^{13}$C NMR, IR and HRMS.

**Hazards Information**

Sodium: Danger! Flammable, corrosive and water reactive solid. Catches fire if exposed to air. Harmful or fatal if swallowed. Harmful if inhaled or absorbed through skin.


Lithium aluminum hydride (THF solution): Danger! Reacts violently with water, liberating hydrogen. Incompatible with strong oxidizing agents, alcohols, acids. May be harmful by inhalation, ingestion and through skin contact. May act as an irritant.

1-(2-Benzylxycarbonylamino-1-oxo-ethyl)-pyrrolidine (2a). $N^a$-Cbz-Glycine (4.0 g, 19.1 mmol), HBTU (7.62 g, 20.1 mmol), HOBt (2.72 g, 20.1 mmol), and
pyrrolidine (1.66 mL, 20.1 mmol) were dissolved into DMF (38.0 mL) in a 250 mL flask. The solution was cooled to 0 °C under argon atmosphere and TEA (3.87 mL, 38.2 mmol) was added dropwise. The temperature was maintained for 2 h before the ice bath was removed. After 2 more hours, the reaction was quenched by adding 5% aqueous sodium bicarbonate (60 mL). The mixture was extracted with EtOAc (60 mL x 3). The organic layer was washed with 5% aqueous sodium bicarbonate (30 mL x 2), 5% aqueous citric acid (30 mL x 2), brine (30 mL x 2), and dried over anhydrous MgSO₄. The solution was then concentrated to give the crude product, which was further purified by flash column chromatography (Hexanes:EtOAc = 1:1) to give 2a as a colorless solid (4.65 g, 92.8%). \( R_f = 0.42 \) (EtOAc). mp 82−84 °C; HRMS (ESI) calcd for C₁₄H₁₉N₂O₃ (MH⁺) 263.1396, found 263.1403. \(^1\)H NMR (500 MHz, CDCl₃) \( \delta \) 1.86−1.90 (m, 2H), 1.96−2.00 (m, 2H), 3.37 (t, 2H, \( J = 6.9 \) Hz), 3.50 (t, 2H, \( J = 6.9 \) Hz), 3.94 (d, 2H, \( J = 4.0 \) Hz), 5.12 (s, 2H), 5.79 (bs, 1H), 7.28−7.40 (m, 5H); \(^1^\)C (125 MHz, CDCl₃) \( \delta \) 24.06, 25.88, 43.37, 45.30, 45.90, 66.74, 127.89, 127.95, 128.38, 136.41, 156.16, 166.25. IR (KBr): 3535, 3486, 3276, 3064, 2968, 2886, 1698, 1631, 1550, 1460, 1334, 1264, 1172, 1051, 988,763, 708.

1-(2-Benzyloxycarbonylamino-1-oxo-ethyl)-diisopropylamine (2b).

\( \text{N}^\alpha \)-Cbz-Glycine (1.75 g, 8.38 mmol), HBTU (3.79 g, 10.0 mmol), HOBT (1.35 g, 10.0 mmol) and diisopropylamine (1.54 mL, 11.0 mmol) were dissolved into dry DCM (80 mL) and DMF (0.5 mL) in a 250 mL flask. The solution was cooled to 0 °C under argon atmosphere and TEA (2.80 mL, 20.0 mmol) was added dropwise. The temperature
was maintained for 2 h before the ice bath was removed. After 8 more hours, the reaction mixture was washed with 5% aqueous sodium bicarbonate (30 mL x 3), 5% aqueous citric acid (30 mL x 2), brine (30 mL x 1), and dried over MgSO₄. The organic phase was concentrated to give the crude product, which was purified by flash column chromatography (Silica Gel, Hexanes: EtOAc = 3:1) to give 2b as a colorless liquid (1.47 g, 60.1%). Rf = 0.65 (Hexanes : EtOAc = 1:1). HRMS (ESI) calcd for C₁₆H₂₅N₂O₃ (MH⁺) 293.1865, found 293.1866. ¹H NMR (500 MHz, CDCl₃) δ 1.20–1.21 (d, 6H, J = 6.5 Hz), 1.38–1.40 (d, 6H, J = 7.0 Hz), 3.48 (m, 1H), 3.81(m, 1H), 3.97 (s, 2H), 5.12 (s, 2H), 5.92 (bs, 1H), 7.28–7.40 (m, 5H); ¹³C (75 MHz, CDCl₃) δ 20.02, 20.15, 43.22, 45.56, 47.13, 66.15, 127.44, 127.51, 127.99, 136.28, 156.84, 165.77. IR (KBr): 3400, 2968, 1721, 1646, 1459, 1358, 1219, 1162, 1048, 743, 700.

**General procedures for the Meerwein salt formation and the Eschenmoser-Claisen rearrangement.** Compound 2 (1 mmol) was dissolved in a minimum amount of dry DCM. To the solution was added 2,6-di- tert -butylpyridine (1.0 mmol), and MeOTf (2.2 mmol) at ambient temperature. The DCM was removed by passing argon through reaction flask and a white salt was formed overnight. Then 5 mL of dry DCM was added to dissolve the white salt formed. The solution was cooled to −35 °C and was ready to react with pre-generated lithium allylic oxide (2.2−4.0 mmol) in dry DCM. Lithium allylic oxide in DCM was prepared in advance by dissolving allylic alcohol (2.2–4.0 mmol) in dry DCM (5 mL) followed by the addition of n-butyl lithium (2.2–4.0 mmol) at −35 °C. After 15 min at this temperature, the solution was transferred into the Meerwein salt solution and the mixture was warmed up to ambient
temperature over a period of around 4 h. Then the reaction was quenched by addition of water (5 mL). The organic layer was separated and the aqueous layer was extracted with DCM (5 mL). The combined organic layers were washed with saturated NH₄Cl solution (6 mL × 2), brine (6 mL), and dried over MgSO₄. The solution was then concentrated, and the crude product was purified by flash column chromatography to afford compound 6.

![6a-1](image)

1-(2-Benzoyloxycarbonylamino-1-oxo-4-pentenyl)-pyrrolidine (6a-1). Compound 2a. (213 mg, 0.81 mmol), 2,6-di-tert-butylpyridine (188 µL, 0.81 mmol), MeOTf (196 µL, 1.79 mmol), allyl alcohol (222 µL, 3.25 mmol), n-butyl lithium (1.17 M in hexanes, 2.78 mL, 3.25 mmol). Product 6a-1: Colorless solid 178 mg, yield 74.7%, mp. 59–61 °C, $R_f = 0.28$ (Hexanes : EtOAc =1:1), HRMS (FAB) calcd for C$_{17}$H$_{23}$N$_2$O$_3$ (MH$^+$) 303.1709, found 303.1703. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.80–1.90 (m, 2H), 1.90–2.00 (m, 2H), 2.38 (m, 1H), 2.48 (m, 1H), 3.39–3.49 (m, 2H), 3.49–3.55 (m, 1H), 3.59–3.66 (m, 1H), 4.54 (dd, 1H, $J = 6.5, 14.5$ Hz), 5.05–5.14 (m, 4H), 5.60 (d, 1H, $J = 8.0$ Hz), 5.70–5.81 (m, 1H), 7.27–7.37 (m, 5H; $^{13}$C (75 MHz, CDCl$_3$) $\delta$ 24.09, 25.97, 37.27, 45.91, 46.47, 52.08, 66.71, 118.69, 127.89, 127.99, 128.41, 132.57, 136.35, 155.76, 169.58. IR (KBr): 3422, 3229, 3042, 2971, 2880, 1712, 1636, 1541, 1450, 1268, 1054, 742.
1-(2-Benzoxycarbonylamino-3,3-dimethyl-1-oxo-4-pentenyl)-pyrrolidine (6a-2).

Compound 2a. (133 mg, 0.51 mmol), 2,6-di-tert-butylpyridine (113 μL, 0.51 mmol), MeOTf (122 μL, 1.12 mmol), 3-methyl-2-buten-1-ol (113 μL, 1.12 mmol), n-butyl lithium (1.20 M in hexanes, 0.93 mL, 1.12 mmol). Product 6a-2: Colorless solid 73 mg, yield 43.6%, mp. 80–81 °C, Rf = 0.43 (Hexanes : EtOAc = 1:1). HRMS (ESI) calcd for C_{19}H_{27}N_{2}O_{3} (MH^+) 331.2022, found 331.2016. 

\[ ^1 \text{H NMR (300 MHz, CDCl}_3) \delta 1.10 (s, 6H), 1.79−2.00 (m, 4H), 3.37 (m, 1H), 3.44−3.59 (m, 2H), 3.70 (m, 1H), 4.37 (d, 1H, J = 9.9 Hz), 4.99−5.15 (m, 4H), 5.47 (d, 1H, J = 9.9Hz), 5.96 (dd, 1H, J = 10.8, 16.8 Hz), 7.27−7.39 (m, 5H); ^13 \text{C (75 MHz, CDCl}_3) \delta 22.98, 24.08, 24.23, 25.99, 40.87, 45.71, 47.48, 58.56, 66.89, 113.62, 127.99, 128.07, 128.46, 136.27, 143.36, 156.24, 169.32. IR (KBr): 3283, 2968, 2880, 1710, 1630, 1524, 1442, 1334, 1243, 1063, 915, 746.

1-(2-Benzoxycarbonylamino-3-methyl-1-oxo-4-pentenyl)-pyrrolidine (6a-3).

Compound 2a (900 mg, 3.43 mmol), 2,6-di-tert-butylpyridine (770 μL, 3.43 mmol), MeOTf (752 μL, 6.86 mmol), crotyl alcohol (613 μL, 6.86 mmol, 19:1 mixture), n-butyl lithium (1.40 M in hexanes, 4.90 mL, 6.86 mmol). Product 6a-3: Colorless solid 666 mg, yield 61.4%, anti:syn = 9.6:1. Recrystallization from hexanes/EtOAc, mp 80–82 °C, Rf = 0.25 (Hexanes:EtOAc = 1:1). HRMS (ESI) calcd for C_{18}H_{25}N_{2}O_{3}
(MH⁺) 317.1865, found 317.1863. ¹H NMR (500 MHz, CDCl₃) δ 1.05–1.09 (d, 3H, J = 6.9 Hz), 1.82–1.92 (m, 2H), 1.92–2.00 (m, 2H), 2.58 (m, 1H), 3.43 (m, 1H), 3.46–3.57 (m, 2H), 3.71 (m, 1H), 4.38 (dd, 1H, J = 7.3, 8.8 Hz), 5.02–5.12 (m, 4H), 5.44 (d, 1H, J = 9.6 Hz), 5.74 (m, 1H), 7.27–7.38 (m, 5H); ¹³C (125 MHz, CDCl₃) δ 16.48, 24.20, 26.05, 40.91, 45.91, 46.78, 56.39, 66.81, 116.45, 127.91, 128.00, 128.42, 136.35, 138.58, 156.14, 169.58. IR (KBr): 3225, 3032, 2968, 2883, 1710, 1631, 1534, 1449, 1335, 1268, 1228, 1032, 742.

1-(2-Benzylxycarbonylamino-4-methyl-1-oxo-4-pentenyl)-pyrrolidine (6a-4).

Compound 2a (197 mg, 0.75 mmol), 2,6-di-tert-butylpyridine (174 µL, 0.75 mmol), MeOTf (181 µL, 1.65 mmol), β-methylallyl-alcohol (259 µL, 3.0 mmol), n-butyl lithium (1.17 M in hexanes, 2.56 mL, 3.0 mmol). Product 6a-4: Colorless solid 170 mg, yield 71.5%, mp 106–108 °C, Rf = 0.33 (Hexanes : EtOAc = 1:1), HRMS (FAB) calcd for C₁₈H₂₅N₂O₃ (MH⁺) 317.1865, found 317.1871. ¹H NMR (500 MHz, CDCl₃) δ 1.78 (s, 3H, ), 1.82–1.92 (m, 4H), 2.31 (dd, 1H, J = 8.5,14.1 Hz), 2.39 (dd, 1H, J = 8.5, 14.1 Hz), 3.34–3.48 (m, 2H), 3.51 (m, 1H), 3.68 (m, 1H), 4.61 (m, 1H), 4.78 (s, 1H), 4.83 (s, 1H), 5.06 (d, 1H, J = 12.4 Hz), 5.09 (d, 1H, J = 12.4 Hz), 5.53 (d, 1H, J = 8.2 Hz), 7.27–7.38 (m, 5H); ¹³C (75 MHz, CDCl₃) δ 22.30, 24.10, 25.98, 41.24, 45.91, 46.42, 51.04, 66.66, 114.38, 127.83, 127.95, 128.38, 136.36, 140.44, 155.85, 170.15. IR (KBr): 3248, 3036, 2960, 2880, 1703, 1636, 1533, 1456, 1271, 1027, 694.
1-(2-Benzylxocarbonylamino-3-phenyl-1-oxo-4-pentenyl)-pyrrolidine (6a-5).

Compound 2a (1.04 g, 3.95 mmol), 2,6-di-tert-butylpyridine (914 µL, 3.95 mmol), MeOTf (953 µL, 8.69 mmol), cinnamyl alcohol (2.12 mL, 15.80 mmol), n-butyl lithium (1.17 M in hexanes, 13.5 mL, 15.8 mmol). Product 6a-5: Colorless solid 1.11 g, yield 74.2%, anti:syn = 16.8:1. Recrystallization from EtOAc/Hexanes, mp 128–130 °C, Rf = 0.33 (Hexanes : EtOAc = 1:1), HRMS (ESI) calcd for C_{23}H_{27}N_{2}O_{3} (MH⁺) 379.2022, found 379.2011. ¹H NMR (500 MHz, CDCl₃) δ 1.37–1.49 (m, 2H), 1.63–1.72 (m, 2H), 2.56 (m, 1H), 3.07 (m, 1H), 3.31 (m, 1H), 3.39 (m, 1H), 4.64 (t, 1H, J = 9.9 Hz), 4.64 (t, 1H, J = 9.7 Hz), 5.05 (d, 1H, J = 12.2 Hz), 5.12 (d, 1H, J = 12.2 Hz), 5.02–5.22 (m, 2H), 5.48 (d, 1H, J = 9.4 Hz), 6.17 (m, 1H), 7.17–7.39 (m, 10H); ¹³C (125 MHz, CDCl₃) δ 23.87, 25.56, 45.35, 46.26, 53.87, 56.57, 66.76, 118.26, 127.12, 127.81, 127.94, 128.13, 128.32, 128.36, 136.32, 136.50, 139.52, 155.93, 169.11. IR (KBr): 3251, 3031, 2971, 2879, 1705, 1630, 1529, 1452, 1329, 1261, 1031, 987, 931, 749, 700.

1-(2-Benzylxocarbonylamino-3-phenyl-1-oxo-4-pentenyl)-diisopropylamine (6b-5).

Compound 2b (575 mg, 1.97 mmol), 2,6-di-tert-butylpyridine (441 µL, 1.97 mmol), MeOTf (474 µL, 4.33 mmol), cinnamyl alcohol (580 mg, 4.33 mmol), n-butyl
lithium (1.40 M in hexanes, 3.09 mL, 4.33 mmol). Product 6b-5: Colorless solid 619 mg, yield 77.1%, anti:syn = 20:1. Recrystallization from EtOAc/Hexanes, mp 138–139 °C, \( R_f = 0.62 \) (Hexanes : EtOAc = 3:1). HRMS (FAB) calcd for \( \text{C}_{25}\text{H}_{33}\text{N}_{2}\text{O}_{3} \) (MH\(^+\)) 409.2493, found 409.2504. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta 0.54 \) (d, 3H, \( J = 6.7 \) Hz), 1.05 (d, 3H, \( J = 6.7 \) Hz), 1.09 (d, 3H, \( J = 6.7 \) Hz), 1.31 (d, 3H, \( J = 6.7 \) Hz), 3.20 (m, 1H), 3.70 (t, 1H, \( J = 9.4 \) Hz), 3.89 (m, 1H), 4.90 (t, 1H, \( J = 9.7 \) Hz), 5.06 (d, 1H, \( J = 12.4 \) Hz), 5.10–5.17 (m, 3H), 5.47 (d, 1H, \( J = 9.4 \) Hz), 6.13 (m, 1H), 7.17–7.39 (m, 10H); \(^{13}\)C (125 MHz, CDCl\(_3\)) \( \delta \) 19.80, 19.90, 20.60, 21.19, 46.06, 49.12, 54.16, 54.63, 66.65, 117.92, 126.98, 127.73, 127.89, 128.35, 128.52, 128.54, 136.53, 137.01, 139.92, 155.93, 169.72. IR (KBr): 3274, 3042, 2970, 1707, 1622, 1535, 1457, 1349, 1307, 1252, 1134, 1027, 910, 734, 699, 609.

General procedure for the preparation of amino alcohols using 11-5 as an example. Compound 6a-5 (76 mg, 0.20 mmol) was dissolved in dry THF (1.5 mL). The solution was cooled to 0 °C and a solution of pre-generated LiAlH(OMe)_3 (0.50 M in THF, 0.80 mmol) was added dropwise via a syringe over a period of 2 h. The reaction was quenched by addition of 0.8 mL of dry tert-butyl alcohol followed by 5% ammonium chloride solution (2 mL) at 0 °C. The mixture was extracted with ice cold diethyl ether (3 mL × 3). The combined organic layers were washed with ice cold water (3 mL), ice cold brine (3 mL), dried over MgSO\(_4\) and concentrated at 0 °C to give the crude aldehyde as colorless oil. The crude aldehyde was dissolved in ice cold methanol (2 mL) and NaBH\(_4\) (27 mg, 0.72 mmol) was added in the solution. The
reaction was completed in 30 min at 0 °C and quenched with water (2 mL). The mixture was extracted with DCM (3 mL x 3). The combined organic layers were washed with brine (3 mL x 2), dried over MgSO$_4$, concentrated to give the crude product. Purification of the crude product by flash column chromatography (Hexanes:EtOAc = 3:1) affords 28 mg of racemic 11-5 as a colorless oil. Yield: 44.9%. $R_f$ = 0.51 (Hexanes:EtOAc = 1:1), HRMS (FAB) calcd for C$_{19}$H$_{22}$NO$_3$ (MH$^+$) 312.1600, found 312.1611. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 3.40 (dd, 1H, $J$ = 4.4, 11.3 Hz), 3.48 (t, 1H, $J$ = 9.6 Hz), 3.62 (dd, 1H, $J$ = 4.5, 11.3 Hz), 4.01 (m, 1H), 5.00−5.15 (m, 5H), 6.04 (m, 1H), 7.17−7.39 (m, 10H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 51.96, 56.40, 62.95, 66.88, 117.13, 126.94, 127.84, 128.01, 128.10, 128.47, 128.87, 136.36, 138.47, 140.86, 156.67. IR (KBr): 3409, 3320, 3068, 3032, 2948, 1700, 1526, 1456, 1327, 1249, 1055, 918, 751, 699.

2-Benzyloxy carbonylamino-3,3-dimethyl-4-penten-1-ol (11-2) Yield: 60.8%. $R_f$ = 0.41 (Hexanes : EtOAc = 1:1), mp 68−70 °C, HRMS (FAB) calcd for C$_{15}$H$_{22}$NO$_3$ (MH$^+$) 264.1606, found 264.1600. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.04 (s, 3H), 1.07 (s, 3H), 2.28 (bs, 1H), 3.51 (dd, 1H, $J$ = 7.5, 11.5 Hz), 3.61 (m, 1H), 3.80 (dd, 1H, $J$ = 3.2, 11.4 Hz), 4.97 (d, 1H, $J$ = 8.6H), 5.00−5.13 (m, 4H), 5.82 (dd, 1H, $J$ = 10.7, 17.4 Hz), 7.28−7.38 (m, 5H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 23.54, 24.67, 39.94, 60.72, 63.00, 66.96, 113.54, 128.05, 128.11, 128.47, 136.32, 144.21, 157.35. IR (KBr): 3435, 3230, 3076, 2965, 1700, 1550, 1457, 1345, 1262, 1061, 917, 737, 602.
2-Benzylxycarbonylamino-4-methyl-4-penten-1-ol (11-4) Yield: 80.5%. $R_f = 0.59$ (Hexanes:EtOAc = 1:1), HRMS (FAB) calcd for C$_{14}$H$_{20}$N$_{3}$O$_3$ (MH$^+$) 250.1443, found 250.1456. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.75 (s, 3H), 2.19 (dd, 1H, $J = 9.4, 14.1$ Hz), 2.28 (dd, 1H, $J = 6.3, 14.0$ Hz), 3.01 (m, 1H), 3.57 (m, 1H), 3.65 (m, 1H), 3.87 (m, 1H), 4.76 (s, 1H), 4.84 (s, 2H), 5.06–5.14 (dd, 2H, $J = 12.4, 12.2$ Hz), 7.29–7.38 (m, 5H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 21.95, 39.76, 50.99, 65.08, 66.76, 113.56, 127.92, 128.02, 128.40, 136.26, 141.65, 156.68. IR (KBr): 3329, 3072, 2942, 1700, 1531, 1450, 1339, 1253, 1058, 896, 741, 697.

α-Benzylxycarbonylamino-β-phenyl-γ,δ-unsaturated pentenyl carboxylic acid (10-5). Compound 6b-5 (145 mg, 0.37 mmol) was dissolved in dry THF (2 mL) in a 10 mL flask. The solution was cooled to 0 °C and the pre-generated LiAlH(OMe)$_3$ (0.5 M in THF, 2.22 mL, 1.11 mmol) solution was added dropwise via a syringe over a period of 3 h. Upon completion of the addition, dry tert-butyl alcohol (3 mL) was added to the solution dropwise at 0 °C. This was followed by the addition of 2-methyl-2-butene (0.25 mL). Then a solution of sodium chlorite (82 mg, 80%, 0.74 mmol,) in water (0.4 mL) and AcOH (1 mL) was added quickly at 0 °C. The mixture was allowed to warm up to ambient temperature and adjusted to a basic pH (pH=10) with saturated sodium bicarbonate solution. Then the volatiles were removed under
reduced pressure and the residue dissolved in 5% aqueous sodium bicarbonate (10 mL). The aqueous solution was washed with CHCl₃ (3 mL × 2), hexane (3 mL × 2) and acidified to pH = 1 by 3M HCl solution. Then it was extracted with CHCl₃ (3 mL × 3). The combined organic layers were washed with water and brine, dried over MgSO₄, concentrated to give the crude product. Purification of the crude product by flash column chromatography (Hexanes: EtOAc:AcOH = 75:25:2) gives 71 mg of 10-5 as a colorless oil. Yield: 59.8%. Rf = 0.36 (CHCl₃:MeOH:AcOH = 90:5:1.5), HRMS (FAB) calcd for C₁₉H₂₀NO₄ (MH⁺) 326.1392, found 326.1390. ¹H NMR (500 MHz, CDCl₃) δ 3.84 (t, 1H, J = 7.3 Hz), 4.59 (rotamer of 4.74, bs, 0.2H), 4.74 (t, 0.8H, J = 7.3Hz), 4.80–5.30 (m, 5H), 6.06 (m, 1H), 7.15–7.39 (m, 10H); ¹³C (125 MHz, CDCl₃) δ 52.04, 58.12, 67.26, 119.02, 127.41, 128.02, 128.10, 128.23, 128.51, 128.69, 135.41, 136.01, 138.58, 155.89, 175.20. IR (KBr): 3314, 3034, 1716, 1519, 1415, 1339, 1230, 1053, 925, 742, 700.
CHAPTER 3
ASYMMETRIC SYNTHESIS OF ANTI-\(\beta\)-SUBSTITUTED \(\gamma, \delta\) UNSATURATED AMINO ACIDS VIA ESCHENMOSER-CLAISEN REARRANGEMENT

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3.1 Introduction

Optically active \(\gamma, \delta\)-unsaturated amino acids are important naturally occurring as nonproteinogenic amino acids found in plants\(^{79}\) and microorganism.\(^{80}\) They are also very important building blocks in organic synthesis for the potential conversion of their terminal double bonds to many other functionalities, and for their applications in peptidomimetic drug discovery.\(^{81}\) The Hruby laboratory has investigated one of their many applications, \(\beta\)-turn dipeptide mimetics (Figure 3.1), in which an optically active novel amino acid with a \(\gamma, \delta\)-double bond is required at the \(i+1\) position to construct the bicyclic [3,3,0] structure. A \(\beta\)-substituent with different stereochemistry is often required to mimic natural amino acid side chains that interact with a target protein.\(^{82}\)

Figure 3.1 External \(\beta\)-turn dipeptide mimetics\(^{82}\)
A well-known strategy to synthesize this type of amino acid is via an asymmetric Claisen rearrangement. The related methodologies to synthesize optically active syn-$\beta$-substituted $\gamma,\delta$-unsaturated amino acids have also been developed by using chiral ligands (Scheme 3.1) or chirality transfer from available chiral sources (Scheme 3.2). In the chiral ligands approach, chiral quinine forms a complex with the reaction intermediate due to metal chelation (Figure 3.2). Thus, the transition state adopts a biased conformation which leads to the formation of optically active products. This rearrangement worked well with various trans allylic alcohols and provided syn-$\beta$-substituted $\gamma,\delta$-unsaturated amino acids with 70-90% ee. However, this methodology cannot be applied for the synthesis of anti products due to the preference for a boatlike transition state as discussed in Chapter 2.

Scheme 3.1 Chelate-Claisen rearrangement with use of a chiral ligand (Reproduced in part from reference 86, Copyright (2002), with the permission from Wiley)

Figure 3.2 Bimetallic chelate complex (Reproduced from reference 86, Copyright (2002), with the permission from Wiley)
Another approach to synthesize optically active syn-β-substituted γ,δ-unsaturated amino acids is via chirality transfer in the Chelate-Claisen rearrangement. This approach requires the synthesis of a chiral allylic alcohol with a TBDMS group at carbon 1 (Scheme 3.2). There are two possible chairlike transition states as shown in Scheme 3.2. Due to the metal chelation and steric influences of the bulky TBDMS group, only one of the possible conformations is preferred as indicated. Therefore, the rearrangement was highly diastereoselective and the chirality transfer was complete. Cleavage of the TBDMS group afforded optically active syn-β-substituted γ,δ-unsaturated amino acids. In general, the chirality transfer method can also be expanded to the anti-products; however, it suffers from the availability of the chiral allylic alcohols and epimerization to the syn epimer during the removal of the TBDMS group.87

Scheme 3.2 Asymmetric Chelate-Claisen rearrangement via chirality transfer87

The asymmetric Eschenmoser-Claisen rearrangement was first reported by Welch in 1987 with the use of proline derivatives as chiral auxiliaries.88 The remote chiral center on the pyrrolidine ring provided low diastereofacial selectivity and the average asymmetric induction was only 4.7 to 1 as a consequence of the C-N bond rotation in the N,O-ketene acetal intermediate (Scheme 3.3). The rotamer problem could be avoided by using a $C_2$-symmetric chiral auxiliary. So in 1991, Welch
reported an improved Eschenmoser-Claisen Rearrangement in which a $C_2$-symmetric $(2S,5S)$-dimethylpyrrolidine was used as the chiral auxiliary (Scheme 3.4). Based on the NMR analysis of the rearrangement product, the selectivity was reported to be greater than 10:1. However, confirmation of the proposed stereochemistry was not reported until the present time.

Scheme 3.3 Asymmetric Eschenmoser-Claisen rearrangement with use of a chiral auxiliary (Reproduced in part from reference 88 with permission, Copyright 1987 American Chemical Society)

Scheme 3.4 Asymmetric Eschenmoser-Claisen rearrangement with use of $(2S,5S)$-dimethylpyrrolidine as an auxiliary (Reprinted from reference 89, Copyright (1991), with permission from Elsevier)

In Chapter 2, we have already demonstrated that the Eschenmoser-Claisen rearrangement can provide anti-$\beta$-substituted $\gamma,\delta$-unsaturated amino acids with good to excellent diastereoselectivity. If the pyrrolidine in the rearrangement is replaced with a $C_2$-symmetric 2,5-dimethylpyrrolidine, the rearrangement should provide optically active anti-$\beta$-substituted $\gamma,\delta$-unsaturated amide derivatives. And subsequent chiral auxiliary removal should furnish optically active anti-$\beta$-substituted $\gamma,\delta$-unsaturated amino acids.

3.2 Results and Discussion
A couple of $C_2$-symmetric chiral secondary amines were reported in the literature (Figure 3.3). However, their applications in the asymmetric Claisen rearrangement have not been fully explored due to the difficulties associated with the syntheses of these chiral amines. ($2S,5S$)-dimethylpyrrolidine was used by Welch’s group for the asymmetric Eschenmoser-Claisen rearrangement. And ($2R,5R$)-diphenylpyrrolidine and ($2R,5R$)-2,5-bis(methoxymethyl)pyrrolidine were reported by Rawal’s group for the Thio-Claisen rearrangement.

Figure 3.3 Various $C_2$-symmetric chiral auxiliaries

Scheme 3.5 Synthesis of $C_2$-symmetric ($2R,5R$)-diphenylpyrrolidine

Due to the high diastereoselectivity shown in Rawal’s work, we decided to try the two $C_2$-symmetric chiral auxiliaries he used, i.e. 2,5-diphenylpyrrolidine and 2,5-bis(methoxymethyl)pyrrolidine. The $C_2$-symmetric ($2R,5R$)-diphenylpyrrolidine was synthesized following literature procedures (Scheme 3.5) and investigated for the Eschenmoser-Claisen rearrangement. Unfortunately, the yield of the
rearrangement reaction was very low. Then the commercially available (2R,5R)-2,5-
bis(methoxymethyl)pyrrolidine was tried for the rearrangement. The yield was also
very low. So we suspected that there might be unfavorable electronic or steric
influences from the substituents present on the pyrrolidine ring. The steric effect is
less likely because both auxiliaries gave excellent yields in the Thio-Claisen
rearrangement, which is very similar to the Eschenmoser-Claisen rearrangement. On
the other hand, the phenyl or methoxymethyl substituents are weakly electron-
withdrawing. And this effect could in theory be passed through bonds to the
pyrrolidine nitrogen atom, which plays a critical role in stabilizing the Eschenmoser-
Claisen rearrangement intermediates (Figure 3.4). If the electron-donating ability of
the nitrogen atom is compromised, the stability of the reaction intermediates would
decrease. Therefore, the reaction yields were low. On the other hand, this weak
electron-withdrawing effect does not affect the Thio-Claisen rearrangement due to the
better stabilizing ability of the sulfur atom than the corresponding oxygen atom.

Figure 3.4 Eschenmoser-Claisen rearrangement intermediates

Scheme 3.6 Eschenmoser-Claisen rearrangements with use of morpholine and 2-
methylmorpholine

![Scheme 3.6 Eschenmoser-Claisen rearrangements with use of morpholine and 2-methylmorpholine](image-url)
To test this hypothesis, morpholine and (2S)-methylmorpholine (Scheme 3.6) were used as auxiliaries for the Eschenmoser-Claisen rearrangement. It turned out that the rearrangement with morpholine did not take place. On the other hand, the rearrangement with 2S-methylmorpholine provided rearrangement product with 72% yield, though the diastereoselectivity was not good. The results and the fact that the rearrangement can happen with pyrrolidine suggest that even a remotely located electronegative moiety on the auxiliary is not tolerated by the rearrangement intermediates. A weakly electron-donating 2-methyl group was able to compensate the electron-withdrawing effect exerted by the morpholine oxygen on the nitrogen atom. If this is true, a C2-symmetric 2,5-dimethylpyrrolidine should provide good yields for the Eschenmoser-Claisen rearrangement due to the lack of oxygen atom and the presence of two methyl groups on the pyrrolidine ring.

Scheme 3.7 Synthesis of (2R,5R)-dimethylpyrrolidine

Scheme 3.8 Meerwein salt formation and asymmetric Eschenmoser-Claisen rearrangement
The $C_2$-symmetric chiral auxiliary (2$R$,5$R$)-dimethylpyrrolidine 24 (Scheme 3.7) was commercially available but expensive. Thus, it was synthesized in excellent $ee$ from a chiral diol 21 according to a literature reported method.\textsuperscript{91} Actually, compound 23, the precursor of the chiral auxiliary, is now commercially available and the price is quite reasonable. The chiral auxiliary was coupled to commercially available N$^a$-Cbz-protected glycine using DIC/HOAt to afford amide 25 in excellent yield despite the steric hindrance of the secondary amine (Scheme 3.8). It should be noted that other coupling reagents such as CIP, BOP, DIC/HOBt, HBTU/HOBt failed to give satisfactory yields or clean product.

Table 3.1 Results of the asymmetric Eschenmoser-Claisen rearrangement with (2$R$,5$R$)-dimethylpyrrolidine

<table>
<thead>
<tr>
<th>entry</th>
<th>allylic alcohol</th>
<th>anti:syn</th>
<th>de (%)</th>
<th>yield (%)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HO−≡</td>
<td>NA</td>
<td>88\textsuperscript{b}</td>
<td>75.2</td>
</tr>
<tr>
<td>2</td>
<td>HO−≡</td>
<td>NA</td>
<td>49\textsuperscript{c}</td>
<td>80.6</td>
</tr>
<tr>
<td>3</td>
<td>HO−≡</td>
<td>NA</td>
<td>91\textsuperscript{b}</td>
<td>81.6</td>
</tr>
<tr>
<td>4</td>
<td>HO−≡Pr</td>
<td>&gt;98:2\textsuperscript{b}</td>
<td>87\textsuperscript{b}</td>
<td>84.6</td>
</tr>
<tr>
<td>5</td>
<td>HO−≡Pr</td>
<td>&gt;96:4\textsuperscript{b}</td>
<td>86\textsuperscript{b}</td>
<td>83.1</td>
</tr>
<tr>
<td>6</td>
<td>HO−≡Ph</td>
<td>&gt;87:13\textsuperscript{b}</td>
<td>54\textsuperscript{b}</td>
<td>65.0</td>
</tr>
<tr>
<td>7</td>
<td>HO−≡Ph</td>
<td>&gt;98:2\textsuperscript{c}</td>
<td>93\textsuperscript{c}</td>
<td>70.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Isolated yields. \textsuperscript{b}Determined by chiral HPLC. \textsuperscript{c}Determined by $^1$H NMR.

The Meerwein salt formation and the following rearrangement were conducted in similar conditions as reported previously for the synthesis of the racemic anti-$\beta$-substituted $\gamma$,$\delta$-unsaturated amino acids. Although in general, high temperature would provide low diastereoselectivities, a couple of these reactions had to be heated to 60 °C (Table 3.1 entries 2 & 3) to make the rearrangement happen. Prolonged heating dramatically decreased the reaction yield and cleanness.\textsuperscript{92} Depending on the
rearrangement temperature requirement, dry DCM or toluene was used as the solvent. These two solvents did not show any differences in terms of reaction results. In cases where both solvent can be used, DCM was preferred for safety reasons.

The rearranged products are mixtures of at least three diastereomers and their 
$^1$H NMR peaks are often too close together to distinguish them even with purified samples. In such cases, analytical chiral RP-HPLC was performed to determine their anti/syn ratios and $de$ values. For comparison, racemic amino acids 10e and 10f that also contain some syn isomers (Scheme 3.9) obtained from our previous work were coupled to the same chiral auxiliary 24 to provide authentic samples. In this way, we were able to determine the anti/syn ratios and $de$ values in entries 4 and 7 (Table 3.1). The anti/syn ratios and $de$ values of other products were determined by analogy. Because the rearranged products are liquids, determination of their absolution stereochemistry by X-ray crystallography was not possible. Therefore, the absolute configurations were not confirmed until the removal of the chiral auxiliary.

Scheme 3.9 Synthesis of the authentic samples

We also tried GC-MS for the determination of the diastereomeric ratios. While GC-MS provided excellent separation of the diastereomeric mixture with additional mass identification, it did not give consistent $de$ values. The $de$ value of the same sample changed with sample concentration. Presumably, this is because the GC-MS
detector is primarily designed for compound mass identification, not for quantitative determination. For accurate ratio determination, a GC with a flame ionization detector would do better.

The rearrangement results from seven primary allylic alcohols are summarized in Table 3.1. The excellent diastereoselectivities from the trans allylic alcohols were again attributed to the (Z)-N,O-ketene acetal formation and pseudochairlike conformations of the rearrangement intermediates as discussed previously in Chapter 2. Compared with the bulkier non-C₂-symmetric chiral auxiliaries that gave an average relative asymmetric induction of 4.7 to 1 in the work of Welch et al., the C₂-symmetric (2R,5R)-dimethylpyrrolidine provided much improved relative asymmetric induction despite the small size of the methyl group. Presumably, the improvement is due to the elimination of the rotamer problem. The two substituents R₁ and R₃ (Scheme 3.8) are likely to be pointed away from the C₂-symmetric chiral auxiliary in the transition state and their size is not expected to greatly affect the diastereoselectivities. This is supported by the results from entries 3-5 and 7 (Table 3.1). However, a cis configured substituent R₂ of the starting allylic alcohol destabilized the chairlike transition state and led to low de values as in entries 2 and 6 (Table 3.1).

Despite the bulkiness of the chiral auxiliary, the yields of the asymmetric Eschenmoser-Claisen rearrangement are generally better than that of the racemic version of the Eschenmoser-Claisen rearrangement. This further supports the hypothesis that the failure with 2,5-diphenylpyrrolidine and 2,5-bis(methoxymethyl)pyrrolidine was not due to steric effects. And the improvement in
yields is likely from the electron-donating ability of the methyl groups which can help to stabilize the rearrangement intermediates.

Many high quality trans allylic primary alcohols are commercially available or can be easily synthesized from allylic oxidation of olefins, and reduction of conjugated aldehydes or esters. Thus, the developed method is available to introduce various pharmacologically interesting functionalities at the $\beta$-position. Many desired anti-$\beta$-substituted $\gamma,\delta$-unsaturated amino acid derivatives can be generated with excellent diastereoselectivities from inexpensive glycine via this efficient strategy.

Scheme 3.10 Rearrangement with (2S,5S)-dimethylpyrrolidine

To find out whether (2S,5S)-dimethylpyrrolidine could provide rearranged products with opposite stereochemistry, (2S,5S)-dimethylpyrrolidine was synthesized and used for the rearrangement (Scheme 3.10). The product from trans cinnamyl alcohol (28f') was then compared with the product in entry 7 (Table 3.1, 28f). NMR and optical rotation analysis did confirm that the opposite stereochemistry was obtained as expected from the (2S,5S)-dimethylpyrrolidine.

A two-step strategy was introduced here to remove the chiral auxiliary (Scheme 3.11). The rearrangement products were first subjected to iodolactonization in different solvent combinations. It turned out that the reaction did not take place in
DME/H$_2$O, probably due to the poor solubility of the substrates. However, the reaction went very well in THF/H$_2$O and a mixture of iodolactones $29a$ and $29b$ were formed in a ratio of about 9 to 1 by $^1$H NMR. The chiral auxiliary in aqueous phase can be recovered by extraction with ethyl ether and distillation. The iodolactones $29a$ and $29b$ were then easily converted to the same amino acids $30$ via zinc reduction as expected.$^{95}$ This iodolactonization/zinc reduction pathway worked well for $28d$-$e$ with no observable syn products from the $^1$H NMR spectra of $30d$-$e$. However, a large amount of unidentified byproducts was formed from the iodolactonization of $28f$ (Table 3.1, entry 7). This byproduct is a five member ring without the original terminal double bond. However, it still has the chiral auxiliary attached to it. Subsequent zinc reduction of the minor iodolactones formed gave $30f$ ($R_1, R_2 = H; R_3 = \text{Ph}$), along with 20% of the syn isomer (from $^1$H NMR). It is likely that the electron-withdrawing phenyl group disfavors the formation of the desired iodolactones or the iodolactones formed are not stable under the reaction conditions. Therefore, $30f$ was obtained via the reduction/oxidation method described in Chapter 2.

Scheme 3.11 Iodolactonization/zinc reduction of rearranged amides
To determine the ee value of each novel amino acid synthesized, racemic amino acids were obtained via the racemic version of the Eschenmoser-Claisen rearrangement. Then, those racemic products were compared to the optically active products by analytical chiral RP-HPLC. In general, acetonitrile/0.1% TFA in water did not provide good resolution of the products because of peak broadening. Interestingly, replacing 5% of the acetonitrile with 10% of methanol significantly improved resolution. In this way, the ee value of each product was determined.

Table 3.2 Results of the chiral auxiliary cleavage

<table>
<thead>
<tr>
<th>compound 30</th>
<th>anti:syn(^a)</th>
<th>ee(^b) (%)</th>
<th>yield (%) (two-step)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>NA</td>
<td>82</td>
<td>70.7</td>
</tr>
<tr>
<td>b</td>
<td>NA</td>
<td>40</td>
<td>65.4</td>
</tr>
<tr>
<td>c</td>
<td>NA</td>
<td>89</td>
<td>74.4</td>
</tr>
<tr>
<td>d</td>
<td>&gt;98:2</td>
<td>87</td>
<td>65.1</td>
</tr>
<tr>
<td>e</td>
<td>&gt;98:2</td>
<td>89</td>
<td>69.1</td>
</tr>
<tr>
<td>f</td>
<td>&gt;98:2</td>
<td>93</td>
<td>66.4</td>
</tr>
</tbody>
</table>

\(^a\)Determined by \(^1\)H NMR, \(^b\)Determined by chiral HPLC by comparison with the racemic compounds.

The results of the iodolactonization/zinc reduction for 28a-e and the reduction/oxidation for 28f are shown in Table 3.2. The anti/syn ratios and ee values of those acids are consistent with the anti/syn ratios and de values of the starting amides, indicating that both methods worked well without noticeable epimerization. Occasionally an enriched ee value was observed due to the loss of the minor anti diastereomer during chromatography purification of the iodolactones. The advantages of the iodolactonization/zinc reduction strategy are mild reaction conditions, easy recovery of the chiral auxiliary and clean final products. It should be noted that this iodolactonization provides a window to enrich the optical purity of the major products. In fact, many iodolactone derivatives solidified after workup or flash column
chromatography purification. A potential recrystallization of the iodolactones is possible for large scale reaction. On the other hand, this strategy takes a longer time compared with the reduction/oxidation pathway and does not work for the amide analogues with a β-phenyl substituent.

The absolute stereochemistry was suggested by Welch in 1991. To validate the absolute stereochemistry assignments of the final products 30 as well as the rearrangement products 28, we first compared the optical rotation and NMR spectra of product 30a with published data (Scheme 3.12). It was confirmed that the assignment was indeed correct. In addition, product 30d was subjected to catalytic hydrogenation and the resulting product was determined unambiguously to be the enantiomer of L-Isoleucine (2S,3S) by its opposite sign of optical rotation and identical NMR spectra. This means the stereochemistry assignment is again correct. Furthermore, the absolute stereochemistry of 30f' was confirmed by comparing its methyl ester derivative to a known compound.

Scheme 3.12 Confirmation of absolute stereochemistry
The stereochemistry outcome is consistent with Welch’s prediction.\textsuperscript{89} However, it is the opposite of the Thio-Claisen rearrangement.\textsuperscript{66} Similar phenomenon was also observed by Welch.\textsuperscript{88} To rationalize the difference, a transition state was proposed and compared to the favored transition state proposed by Rawal\textsuperscript{66} for the Thio-Claisen rearrangement (Figure 3.5). The two transition states are different with respect to the orientations of the $\alpha$-substituents in relation to the substituents on their respective chiral auxiliaries. In the Thio-Claisen rearrangement, the $\alpha$-substituent is pointing in the same direction as the proximal phenyl group. In the case of the Eschenmoser-Claisen rearrangement, the $\alpha$-substituent is pointing away from the proximal methyl group. This could happen because the protected $\alpha$-amino group is much bigger than the $\alpha$-methyl group in the Thio-Claisen rearrangement. So it prefers to stay away from the proximal methyl group on the auxiliary to avoid unfavorable steric interactions. At the same time, the relatively small methyl group won’t interfere with the trans configured substituent $R_3$ on the allylic alcohol double bond because $R_3$ is pointing away from the chiral auxiliary. However, a cis configured substituent $R_2$ would interact with the chiral auxiliary unfavorably and destabilize the chairlike transition state. The much lower diastereoselectivities seen in entries 2 and 6 (Table
3.1) support the proposed transition state. On the other hand, the de values of anti products from trans allylic alcohols were below 76% in the Thio-Claisen rearrangement, suggesting unfavorable interactions between the α-methyl group and the proximal phenyl group on the chiral auxiliary.

3.3 Conclusions

To summarize, we have developed a four-step general method to synthesize optically active anti-β-substituted γ,δ-unsaturated amino acids via the asymmetric Eschenmoser-Claisen rearrangement. We have demonstrated that the $C_2$-symmetric chiral auxiliary (2R,5R)-dimethylpyrrolidine provided excellent diastereoselectivities (anti:syn>98:2) and good asymmetric induction (87-93% ee) for both aliphatic and aromatic β-substituted amino acid analogues.

3.4 Future Work

The quality of the asymmetric induction is known to be controlled by the size of the chiral auxiliary. In theory, a bulkier auxiliary would be able to provide improved asymmetric induction. Thus, future study would be focused on the rearrangement with a $C_2$-symmetric 2,5-diethylpyrrolidine or diisopropylpyrrolidine as the chiral auxiliary (Figure 3.6).

Figure 3.6 Bulkier $C_2$-symmetric chiral auxiliaries for better asymmetric induction

Once the best auxiliary is found, more efforts should be directed to the synthesis of optically active products with Alloc protecting groups and analogues with a methyl group at the α-carbon (Figure 3.7).
3.5 Experimental Section

General Information

All starting materials and reagents were purchased from Sigma-Aldrich and used without further purification. THF was distilled over sodium and benzophenone. Methylene chloride was distilled over CaH₂. \(^1\)H and \(^13\)C NMR spectra were recorded on Bruker DRX500 (500 MHz and 125 MHz, respectively) or Varian Inova 600 (600 MHz and 150 MHz, respectively) spectrometers with CDCl₃ or TMS as internal standard. Coupling constants, \(J\), are reported in Hertz (Hz) and refer to apparent peak multiplicities and not true coupling constants. IR spectra were recorded on a Nicolet Impact 410 IR spectrometer. Analytical HPLC was performed on a HP 1100 liquid chromatograph using a Daicel Chiralpak AD RH HPLC column (ADRHC-FJ042, 150 × 4.6 mm, DAICEL Chemical Industries Ltd.). Mass spectra were obtained from Mass Spectrometry Facility, Department of Chemistry, the University of Arizona. Flash column chromatography was performed using silica gel 60 from the EM Science. TLC plates (Silica Gel 60 F₂₅₄) were purchased from the EMD Chemicals and were visualized by UV and KMnO₄ stain. All new compounds were characterized by \(^1\)H, \(^13\)C NMR, IR and HRMS.

Hazards Information
Sodium: Danger! Flammable, corrosive and water reactive solid. Catches fire if exposed to air. Harmful or fatal if swallowed. Harmful if inhaled or absorbed through skin.


Lithium aluminum hydride (THF solution): Danger! Reacts violently with water, liberating hydrogen. Incompatible with strong oxidizing agents, alcohols, acids. May be harmful by inhalation, ingestion and through skin contact. May act as an irritant.

![Image of molecular structure](image)

**Benzyl 2-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-2-oxoethylcarbamate (25).**

\( \text{N}^\alpha \text{-Cbz-Glycine (791 mg, 3.78 mmol), HOAt (514 mg, 3.78 mmol)} \) were dissolved in dry DCM (20.0 mL) in a 50-mL flask under argon atmosphere. To the solution was added DIC (592 \( \mu \text{L}, 3.78 \text{ mmol}) and the mixture was stirred for 15 min at room temperature before (2\( R,5R \))-dimethylpyrrolidine (400 \( \mu \text{L}, 3.44 \text{ mmol}) was added. The stirring was continued for 24 h. Then the mixture was filtered to remove the
precipitate formed, and the filtrate was concentrated to give the crude product, which was purified by flash column chromatography (Hexane:EtOAc = 3:1–2:1) to give 25 as a colorless oil (933 mg, 93.4%). $R_f = 0.33$ (Hexane:EtOAc = 1:1), $[\alpha]^{23.6}_{20} = -40.6$ (c 2.20, MeOH), HRMS (EI) calc'd for C$_{16}$H$_{22}$N$_2$O$_3$ (M$^+$) 290.1630, found 290.1643. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.19 (t, 6H, $J = 5.2$ Hz), 1.55 (dd, 1H, $J = 5.7, 11.9$ Hz), 1.65 (dd, 1H, $J = 5.7, 11.9$ Hz), 2.16 (m, 2H), 3.89–4.08 (m, 3H), 4.22 (m, 1H), 5.12 (s, 2H), 5.77 (s, 1H), 7.28–7.45 (m, 5H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 18.88, 21.17, 28.76, 30.81, 43.40, 52.91, 53.60, 66.65, 127.83, 127.87, 128.32, 136.41, 156.13, 166.20. IR (NaCl): 3409.5, 3300.1, 2967.7, 1716.7, 1637.9, 1432.3, 1248.6, 1043.0.

**General procedure for the preparation of compounds 28.** To a 25-mL flask was added compound 25 (1.0 mmol), 2,6-di-tert-butylpyridine (1.0 mmol), and MeOTf (2.2 mmol) at ambient temperature. The mixture was stirred under argon atmosphere for 4 h before dry DCM (2 mL) was added to dissolve the salt formed. The solution was then cooled to $-35 \degree C$ and was ready to react with a pre-generated lithium allylic oxide (4.0 mmol) in dry DCM (or dry toluene for 28b and 28c). The lithium allylic oxide in DCM or toluene was prepared in advance by dissolving an allylic alcohol (4.0 mmol) in dry DCM or toluene (10 mL) followed by the addition of $n$-butyl lithium (4.0 mmol) at $-35 \degree C$. After 15 min at this temperature, the solution was cannulated into the above Meerwein salt solution and the resultant mixture was warmed up to ambient temperature (or 60 $\degree C$ for 28b and 28c) over a period of 4 h. Then the reaction was quenched by the addition of a saturated ammonium chloride solution (5 mL). The organic layer was separated and washed with a saturated ammonium chloride solution (5 mL × 2) and dried over MgSO$_4$. The dry solution was concentrated under reduced pressure, and the crude product was purified by flash
column chromatography to afford compounds 28. Note: in the case of 28c, the Meerwein salt solution in DCM was added to the lithium allylic oxide solution in toluene that was too viscous to be cannulated.

Benzyl-(R)-1-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-1-oxopent-4-en-2-ylcarbamate (28a). Compound 25 (292 mg, 1.00 mmol), 2,6-di-tert-butylpyridine (224 μL, 1.00 mmol), MeOTf (243 μL, 2.21 mmol), allyl alcohol (272 μL, 4.00 mmol), n-butyl lithium (1.46 M in hexane, 2.74 mL, 4.00 mmol). Product 28a: colorless oil, 248 mg, yield 75.2%, Rf = 0.55 (Hexane:EtOAc = 1:1), HRMS (FAB) calcd for C19H27N2O3 (MH+) 331.2022, found 331.2040. 1H NMR (500 MHz, CDCl3) δ 1.13 (d, 3H, J = 6.3 Hz), 1.18 (d, 3H, J = 6.5 Hz), 1.52 (dd, 1H, J = 6.3, 12.2 Hz), 1.57 (dd, 1H, J = 6.3, 12.2 Hz), 2.07 (m, 1H), 2.21 (m, 1H), 2.43 (m, 2H), 4.15 (m, 1H), 4.36 (m, 1H), 4.47 (m, 1H), 5.02–5.15 (m, 4H), 5.29 (d, 1H, J = 9.6 Hz), 5.77 (m, 1H), 7.26–7.36 (m, 5H); 13C (150 MHz, CDCl3) δ 18.38, 22.76, 28.97, 30.71, 37.07, 51.86, 53.44, 53.76, 66.66, 118.40, 127.68, 127.90, 128.34, 133.13, 136.30, 156.00, 170.31. IR (NaCl): 3269.5, 2967.7, 1712.3, 1624.8, 1537.3, 1436.7, 1253.0.

HPLC analysis: de>88.2% Daicel Chiralpak AD-RH HPLC column (CH3CN/H2O: 65/35, 0.5 mL/min, 254 nm); retention times of the diastereomeric mixture: 11.7 min and 15.8 min (28a).
Benzyl (R)-1-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-3,3-dimethyl-1-oxopent-4-en-2-ylcarbamate (28b). Compound 25 (322 mg, 1.11 mmol), 2,6-di-tert-butylpyridine (249 µL, 1.11 mmol), MeOTf (268 µL, 2.44 mmol), 3-methyl-2-buten-1-ol (444 µL, 4.44 mmol), n-butyl lithium (1.46 M in hexane, 3.04 mL, 4.44 mmol). Product 28b: colorless oil, 321 mg, yield 80.6%, de >49% (NMR), \( R_f = 0.78 \) (Hexane:EtOAc □ 1:1), HRMS (EI) calcd for C\(_{21}\)H\(_{23}\)N\(_2\)O\(_3\) (M\(^+\)) 358.2256, found 358.2242.

Benzyl-(R)-1-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-4-methyl-1-oxopent-4-en-2-ylcarbamate (28c). Compound 25 (306 mg, 1.05 mmol), 2,6-di-tert-butylpyridine (236 µL, 1.05 mmol), MeOTf (254 µL, 2.32 mmol), \( \beta \)-methyl allyl-alcohol (355 µL, 4.20 mmol), n-butyl lithium (1.46 M in hexane, 2.88 mL, 4.20 mmol). Product 28c: colorless oil, 295 mg, yield 81.6%, \( R_f = 0.54 \) (Hexane:EtOAc = 1:1), HRMS (ESI) calcd for C\(_{20}\)H\(_{29}\)N\(_2\)O\(_3\) (MH\(^+\)) 345.2178, found 345.2166. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 1.14 (d, 3H, \( J = 6.5 \) Hz), 1.20 (d, 3H, \( J = 6.5 \) Hz), 1.52 (dd, 1H, \( J = 6.3, 12.2 \) Hz), 1.59 (dd, 1H, \( J = 6.3, 12.2 \) Hz), 1.75 (s, 3H), 2.08 (m, 1H), 2.23 (m, 1H), 2.33 (dd, 1H, \( J = 2.9, 14.3 \) Hz), 2.43 (dd, 1H, \( J = 10.7, 14.3 \) Hz), 4.15 (m, 1H), 4.43 (m, 1H), 4.59 (m, 1H), 4.81 (d, 2H, \( J = 6.7 \) Hz), 5.04 (d, 1H, \( J = 12.6 \) Hz), 5.07 (d, 1H, \( J = 12.6 \) Hz), 5.28 (d, 1H, \( J = 7.64 \) Hz), 7.27–7.38 (m, 5H); \(^{13}\)C (125 MHz, CDCl\(_3\)) \( \delta \) 18.54, 22.27, 22.64, 29.09, 30.83, 40.85, 50.53, 53.38, 53.80, 66.70, 114.32, 127.65, 127.91, 128.36, 136.37, 140.60, 155.97, 170.45. IR (NaCl): 3273.9, 2967.7, 1707.9, 1629.2, 1528.6, 1432.3, 1253.0.
HPLC analysis: $de > 90.6\%$ Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O: 55/45–85/15 in 25 min, 0.5 mL/min, 254 nm); retention times of the diastereomeric mixture: 12.4 min and 15.0 min (28c).

**Benzyl-(2R,3R)-1-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-3-methyl-1-oxopent-4-en-2-ylcarbamate (28d).** Compound 25 (540 mg, 1.86 mmol), 2,6-di-tert-butylpyridine (418 µL, 1.86 mmol), MeOTf (449 µL, 4.09 mmol), crotyl alcohol (631 µL, 7.44 mmol, trans:cis = 21.8:1), n-butyl lithium (1.46 M in hexane, 5.10 mL, 7.44 mmol). Product 28d: colorless oil, 542 mg, yield 84.6%, $R_f = 0.70$ (Hexane:EtOAc = 1:1), HRMS (FAB) calcd for C$_{20}$H$_{29}$N$_2$O$_3$ (MH$^+$) 345.2178, found 345.2176. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.05 (d, 3H, $J = 6.5$ Hz), 1.15 (d, 3H, $J = 6.3$ Hz), 1.25 (d, 3H, $J = 6.5$ Hz), 1.49–1.63 (m, 2H), 2.11 (m, 1H), 2.21 (m, 1H), 2.68 (m, 1H), 4.19 (m, 1H), 4.29 (t, 1H, $J = 9.6$ Hz), 4.42 (m, 1H), 5.02–5.12 (m, 4H), 5.16 (d, 1H, $J = 9.8$ Hz), 5.82 (m, 1H), 7.27–7.42 (m, 5H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 17.50, 18.33, 23.24, 29.15, 30.94, 40.57, 53.15, 53.68, 56.03, 66.73, 116.32, 127.64, 127.94, 128.38, 136.43, 139.20, 156.06, 169.61. IR (NaCl): 3273.9, 2967.7, 1712.3, 1624.8, 1528.6, 1428.0, 1244.2, 1025.5.

HPLC analysis: $de > 86.8\%$, anti/syn > 98:2. Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O: 50/50–70/30 in 25 min, 0.5 mL/min, 254 nm); retention times of the diastereomeric mixture: 15.0 (28d) min, 18.0 min (syn) and 18.7 min (29d).
Benzyl-(2R,3R)-1-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-1-oxo-3-vinylhexan-2-ylcarbamate (28e). Compound 25 (331 mg, 1.14 mmol), 2,6-di-tert-butylpyridine (256 μL, 1.14 mmol), MeOTf (275 μL, 2.51 mmol), trans-2-hexen-1-ol (564 μL, 4.56 mmol), n-butyl lithium (1.46 M in hexane, 3.12 mL, 4.56 mmol). Product 28e: colorless oil, 353 mg, yield 83.1%, \( R_f = 0.73 \) (Hexane:EtOAc = 1:1), HRMS (EI) calcd for C\(_{22}\)H\(_{32}\)N\(_2\)O\(_3\) (M\(^+\)) 372.2413, found 372.2419. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 0.86 (t, 3H, \( J = 7.1 \) Hz), 1.07–1.44 (m, 10H), 1.51–1.62 (m, 2H), 2.06–2.25 (m, 2H), 2.48 (m, 1H), 4.19 (m, 1H), 4.31 (t, 1H, \( J = 9.8 \) Hz), 4.52 (m, 1H), 5.02–5.15 (m, 4H), 4.98–5.61 (m, 2H), 7.27–7.42 (m, 5H); \(^{13}\)C (125 MHz, CDCl\(_3\)) \( \delta \) 13.73, 18.20, 20.58, 23.20, 29.14, 30.87, 33.72, 46.86, 53.49, 54.01, 55.41, 66.75, 118.45, 127.56, 127.91, 128.36, 136.43, 137.72, 156.08, 170.12. IR (NaCl): 3273.9, 2967.7, 1712.3, 1629.2, 1532.9, 1432.3, 1244.2, 1034.3, 693.1.

HPLC analysis: \( de>85.6\% \), anti/syn>96:4. Daicel Chiralpak AD-RH HPLC column (CH\(_3\)CN/H\(_2\)O: 55/45–85/15 in 25 min, 0.5 mL/min, 254 nm); retention times of the diastereomeric mixture: 13.0 min (28e), 18.1 min (syn) and 19.1 min (29e).

Benzyl-(2R,3S)-1-((2R,5R)-2,5-dimethylpyrrolidin-1-yl)-1-oxo-3-phenylpent-4-en-2-ylcarbamate (28f). Compound 25 (194 mg, 0.67 mmol), 2,6-di-tert-butylpyridine (150 μL, 0.67 mmol), MeOTf (161 μL, 1.47 mmol), cinnamyl alcohol (359 mg, 2.67
mmol), n-butyl lithium (1.40 M in hexane, 1.91 mL, 2.67 mmol). Product 28f: colorless oil 200 mg, yield 73.7%, $R_f = 0.85$ (Hexane:EtOAc = 1:1), HRMS (FAB) calcd for C$_{25}$H$_{31}$N$_2$O$_3$ (MH$^+$) 407.2334, found 407.2339. $^1$H NMR (500 MHz, CDCl$_3$) δ 0.59 (d, 3H, $J = 6.5$ Hz), 1.10 (d, 3H, $J = 6.5$ Hz), 1.44 (m, 2H), 1.91 (m, 1H), 2.07 (m, 1H), 3.93−4.05 (m, 2H), 4.21 (m, 1H), 4.66 (t, 1H, $J = 10.1$ Hz), 5.06−5.21 (m, 4H), 5.43 (d, 1H, $J = 10.1$ Hz), 6.17 (m, 1H), 7.17−7.39 (m, 10H); $^{13}$C (125 MHz, CDCl$_3$) δ 18.19, 21.91, 28.97, 30.79, 51.90, 53.03, 53.38, 56.54, 66.86, 118.28, 126.84, 127.67, 128.01, 128.42, 128.45, 128.74, 136.42, 136.89, 140.31, 156.04, 168.65. IR (NaCl): 3273.9, 2967.7, 1712.3, 1620.4, 1532.9, 1428.0, 1364.2, 1244.2, 1034.3, 701.8.

NMR analysis: $de$>93.0%, anti/syn>98:2. Chemical shift of the diastereomeric mixture: 0.60 (28f), 0.69 (29f), 6.04 (CH=, syn)

Byproduct (28Z) of the Meerwein salt formation and rearrangement

**General procedure for the iodolactonization/zinc reduction of amides 28a-e.**

Compounds 28 (0.5 mmol) were dissolved in 3 mL of THF/H$_2$O (1.5:1). To the solution was added iodine (1.5 mmol) and the resultant mixture was stirred in the dark at ambient temperature for 3 d. The reaction was quenched by the addition of 10% aqueous Na$_2$S$_2$O$_3$ (3 mL) and was extracted with EtOAc (5 mL × 2). The combined organic layers were washed with 10% aqueous Na$_2$S$_2$O$_3$ (3 mL × 2), saturated ammonium chloride (3 mL), brine (3 mL) and dried over MgSO$_4$. The dry solution was concentrated in vacuo, and the crude product was purified by flash column
chromatography (Hexane:EtOAc = 8:1–3:1) to afford iodolactones 29a and 29b. Iodolactones 29a and 29b were then dissolved in glacial acetic acid (3 mL) and treated with zinc dust (5 mmol). The mixture was heated and kept at 65 °C with stirring for 2 h. It was then cooled to rt and 0.5 M HCl (5 mL) was added. The mixture was extracted with DCM (4 mL × 3) and washed with H2O (4 mL × 2). Then the combined organic layers were extracted with 1 M LiOH (4 mL × 3). The combined aqueous layers were washed with CHCl3 (3 mL × 3) and acidified to pH 1 with 1M HCl. Then DCM (4 mL × 3) was used to extract the products from the aqueous phase. The combined organic layers were washed with water (3 mL × 2), dried over MgSO4, concentrated at rt under reduced pressure to give the final products 30.

(R)-2-(benzyloxycarbonylamino)pent-4-enoic acid (30a). Compound 28a (150 mg, 0.46 mmol), iodine (346 mg, 1.36 mmol), zinc dust (298 mg, 4.55 mmol). Compound 30a: colorless oil, 90 mg, yield: 70.7%, Rf = 0.41 (Hexane:EtOAc:AcOH = 25:25:1), [α]25D −11.5 (c 1.00, CHCl3), HRMS (FAB) calcd for C13H16NO4 (MH+) 250.1079, found 250.1075. 1H NMR (500 MHz, CDCl3) δ 2.47–2.71 (m, 2H), 4.48 (q, 1H, J = 6.3 Hz), 5.09–5.19 (m, 4H), 5.22 (d, 1H, J = 7.6 Hz), 5.70 (m, 1H), 7.28–7.38 (m, 5H); 13C (125 MHz, CDCl3) δ 36.33, 53.07, 67.21, 119.74, 128.08, 128.21, 128.49, 131.64, 135.98, 155.88, 176.40. IR (NaCl): 3326.4, 2923.9, 1716.7, 1524.2, 1261.7, 1218.0, 1056.1.
HPLC analysis: ee>82.2%, Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O/MeOH: 30/60/10, 0.6 mL/min, 254 nm); retention times of the racemic mixture: 13.4 min and 14.9 min (30a).

(R)-2-(benzyloxycarbonylamino)-3,3-dimethylpent-4-enoic acid (30b). Compound 28b (198 mg, 0.55 mmol), iodine (420 mg, 1.65 mmol), zinc dust (361 mg, 5.50 mmol). Compound 30b: colorless oil, 100 mg, yield: 65.4%, $R_f$ = 0.49 (Hexane:EtOAc:AcOH = 25:25:1), [α]$^{24.5}_D$ = -1.7 (c 4.00, MeOH), HRMS (FAB) calcd for C$_{15}$H$_{20}$NO$_4$ (MH$^+$) 278.1392, found 278.1387. $^1$H NMR (500 MHz, CDCl$_3$) δ 1.13 (s, 3H), 1.14 (s, 3H), 4.23 (d, 1H, $J = 9.4$ Hz), 5.03–5.16 (m, 4H), 5.26 (d, 1H, $J = 9.4$ Hz), 5.83 (dd, 1H, $J = 10.9$, 17.4 Hz), 7.26–7.43 (m, 5H), 9.75 (br, 1H); $^{13}$C (125 MHz, CDCl$_3$) δ 23.68, 24.19, 40.15, 61.36, 67.25, 114.39, 128.12, 128.19, 128.48, 135.99, 142.37, 156.11, 175.83. IR (NaCl): 3317.6, 2976.4, 1716.7, 1532.9, 1419.2, 1336.1, 1244.2, 1069.3.

HPLC analysis: ee>40.1%, Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O/MeOH: 35/55/10, 0.5 mL/min, 254 nm); retention times of the racemic mixture: 12.5 min (30b) and 15.1 min.

(R)-2-(benzyloxycarbonylamino)-4-methylpent-4-enoic acid (30c).
Compound **28c** (176 mg, 0.51 mmol), iodine (389 mg, 1.5 mmol), zinc dust (334 mg, 5.10 mmol). Compound **30c**: colorless oil, 101 mg, yield: 74.4%, $R_f = 0.45$ (Hexane:EtOAc:AcOH = 25:25:1), $[\alpha]^{24.3}_{D} = -5.8$ (c 1.18, MeOH), HRMS (FAB) calcd for C$_{14}$H$_{18}$NO$_4$ (MH$^+$) 264.1236, found 264.1232. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.74 (s, 3H), 2.42 (dd, 1H, $J = 9.0, 14.0$ Hz), 2.61 (dd, 1H, $J = 4.8, 14.2$ Hz), 4.48 (m, 1H), 4.77 (s, 1H), 4.86 (s, 1H), 5.08−5.17 (m, 3H), 7.28−7.38 (m, 5H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 21.73, 40.26, 52.01, 67.15, 114.85, 127.98, 128.10, 128.41, 135.97, 139.99, 156.03, 176.84. IR (NaCl): 3322.0, 2954.6, 1716.7, 1524.2, 1344.8, 1244.2, 1051.8. HPLC analysis: ee>88%, Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O/MeOH: 23/67/10, 0.5 mL/min, 254 nm); retention times of the racemic mixture: 20.5 min and 21.7 min (**30c**).

\[HO\] \[\begin{array}{c} \text{NHCbz} \\ \rightarrow \end{array}\] \[\text{30d}\]

(2R,3R)-2-(benzyloxycarbonylamino)-3-methylpent-4-enoic acid (**30d**).

Compound **28d** (141 mg, 0.41 mmol), iodine (312 mg, 1.23 mmol), zinc dust (268 mg, 4.10 mmol). Compound **30d**: colorless oil, 70 mg, yield: 65.1%, anti: syn>98:2 (NMR), $R_f = 0.44$ (CHCl$_3$:MeOH:AcOH = 90:10:3), $[\alpha]^{26.2}_{D} = -6.0$ (c 0.70, MeOH), HRMS (ESI) calcd for C$_{14}$H$_{18}$NO$_4$ (MH$^+$) 264.1236, found 264.1234. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 1.13 (d, 3H, $J = 6.7$ Hz), 2.85 (m, 1H), 4.40 (dd, 1H, $J = 4.4, 8.6$ Hz), 5.08−5.16 (m, 4H), 5.20 (d, 1H, $J = 8.6$ Hz), 5.69 (m, 1H), 7.26−7.42 (m, 5H), 10.76 (br, 1H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 16.04, 39.64, 58.02, 67.26, 117.39, 128.08, 128.19,
128.48, 135.98, 136.98, 156.36, 176.46. IR (NaCl): 3326.4, 2976.4, 1712.3, 1519.8, 1410.5, 1344.8, 1218.0, 1069.3.

HPLC analysis: ee>86.6%, Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O/MeOH: 35/55/10, 0.5 mL/min, 254 nm); retention times of the racemic mixture: 10.2 min and 14.6 min (30d).

(2R,3R)-2-(benzyloxy carbonylamino)-3-vinylhexanoic acid (30e). Compound 28e (185 mg, 0.50 mmol), iodine (378 mg, 1.50 mmol), zinc dust (325 mg, 5.00 mmol). Compound 30e: colorless oil, 96 mg, yield: 66.4%, anti: syn>98:2 (NMR), $R_f$ = 0.55 (Hexane:EtOAc:AcOH = 25:25:1), $[\alpha]^{27.0}_D$ −29.4 (c 1.00, MeOH), HRMS (ESI) calcd for C$_{16}$H$_{21}$NO$_4$ (MH$^+$) 314.1368, found 314.1366. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 0.89 (d, 3H, $J$ = 7.1 Hz), 1.17–1.53 (m, 4H), 2.64 (m, 1H), 4.46 (br, 1H), 4.99–5.28 (m, 5H), 5.54 (m, 1H), 7.26–7.42 (m, 5H), 10.11 (br, 1H); $^{13}$C (125 MHz, CDCl$_3$) $\delta$ 13.87, 20.18, 32.56, 45.52, 57.14, 67.24, 118.56, 128.07, 128.19, 128.49, 136.01, 156.35, 176.83. IR (NaCl): 3330.7, 2958.9, 2923.9, 1712.3, 1524.2, 1414.8, 1344.8, 1257.4, 1222.4, 1051.8.

HPLC analysis: ee>89%, Daicel Chiralpak AD-RH HPLC column (CH$_3$CN/H$_2$O/MeOH: 42/48/10, 0.5 mL/min, 254 nm); retention times of the racemic mixture: 9.3 min and 13.3 min (30d).
(2R,3S)-2-(benzyloxycarbonylamino)-3-phenylpent-4-enoic acid (30f). Compound 28f (123 mg, 0.30 mmol) was dissolved in dry THF (2 mL) in a 10-mL flask under argon atmosphere. The solution was cooled to 0 °C and a pre-generated LiAlH(OMe)_3 (0.5 M in THF, 2.4 mL, 1.20 mmol) solution was added dropwise in four portions via a syringe over a period of 1.5 h. Upon completion of the addition, the mixture was warmed up to rt over 1.5 h and then cooled to 10 °C. Then dry tert-butyl alcohol (3 mL) was added to the solution dropwise. This was followed by the addition of 2-methyl-2-butene (232 µL), and a solution of sodium chlorite (68 mg, 0.6 mmol) in water (0.4 mL) and AcOH (1 mL). The mixture was allowed to warm up to ambient temperature with stirring. When the reaction was complete as indicated by TLC, the volatiles were removed under reduced pressure and the residue was dissolved in DCM (10 mL). The DCM was extracted with 1 M LiOH (4 mL × 3). The combined aqueous layers were washed with CHCl₃ (3 mL × 3) and acidified to pH = 1 with 1M HCl. Then DCM (4 mL × 3) was used to extract the product. The combined organic layers were washed with water (3 mL × 2), dried over MgSO₄, concentrated under reduced pressure at rt to give the crude product 30f. Purification of the crude product by flash column chromatography (Hexane: EtOAc:AcOH = 75:25:2) afforded 71 mg of 30f as a colorless oil. Yield: 66.4%, anti:syn>98:2 (NMR), R_f = 0.36 (CHCl₃:MeOH:AcOH = 90:5:1.5). [α]^{27.5}_D +33.1 (c 0.86, MeOH), HRMS (FAB) calcd for C₁₉H₂₀NO₄ (MH⁺) 326.1392, found 326.1387.

HPLC analysis: ee>92.8%, Daicel Chiralpak AD-RH HPLC column (solvent A, CH₃CN; solvent B, H₂O; Solvent C, MeOH; gradient: 35%–45% A in 25 min, 10% C, 0.5 mL/min, 254 nm); retention times of the racemic mixture: 15.4 min and 24.3 min (30f).
CHAPTER 4

DEVELOPMENT OF SELECTIVE PEPTIDE ANTAGONISTS FOR MELANOCORTIN RECEPTORS

4.1 Introduction

Melanocortin receptors along with their endogenous agonists and antagonists are involved in the modulation of a wide range of important physiological activities as mentioned in Chapter 1.3. To date, five human melanocortin receptor subtypes, named MC1-5 receptors, have been identified and cloned. All of the five receptor subtypes belong to a family of rhodopsin-like seven-transmembrane G-protein coupled receptors (GPCRs), which intracellularly mediate their effects by activating cAMP-dependent pathways. The $hMC1R$ is mainly expressed in the melanocytes and leukocytes, and is involved in skin pigmentation, pain modulation, inflammation and control of the immune system. The $hMC2R$, also known as ACTH receptor, is expressed in the adrenal cortex and mediates glucocorticoneogenesis and other effects. The $hMC3R$ and $hMC4R$ are primarily found in the central nervous system (CNS) with different patterns of distribution. Both of them have been implicated as playing complimentary roles in feeding regulation and weight control. $hMC3R$ has also been suggested to play an important role in mediating some of the anti-inflammatory effects of its endogenous agonist $\alpha$-MSH. The erectile function, anxiety, stress and pain are primarily mediated through $hMC4R$. The $hMC5R$ is distributed in a variety of peripheral tissues and in the brain, cortex, and cerebellum. Its effects in the central nervous system have not yet been well studied. On the other hand, $hMC5R$ has been
shown to be important in regulating exocrine gland function\textsuperscript{112,113} and coordinating central and peripheral signals for aggression.\textsuperscript{28,114}

Of particular interest for therapeutic applications is \textit{hMC4R}. Extensive studies have been conducted with respect to this receptor. Preclinical and clinical evidences suggest that the \textit{hMC4R} is intimately involved in the regulation of food intake and energy expenditure. It has been shown that \textit{hMC4R} selective agonists can be used to treat obesity, though there are other side effects to be overcome.\textsuperscript{115} On the other hand, \textit{hMC4R} selective antagonists are promising candidates for treating anxiety & stress, anorexia and cancer- or HIV-related weight loss.\textsuperscript{116} Furthermore, it has been shown that targeting \textit{hMC4R} may provide an alternative approach to treat sexual dysfunctions in both male and female.\textsuperscript{20} Unlike current therapies that target end organ vascular tissue, this new approach is centrally mediated. Thus, drugs based on this approach will have significant advantages over existing ones for the treatment of a variety of sexual dysfunctions in both men and women.\textsuperscript{117}

The natural melanocortin receptor agonists are derived from post-translational processing of the pro-opiomelanocortin (POMC) gene and include $\alpha$, $\beta$, and $\gamma$-melanocyte-stimulating hormones (MSH) and adrenocorticotropin (ACTH).\textsuperscript{17,18} \textit{hMC2R} is only activated by ACTH. $\alpha$, $\beta$, $\gamma$-MSH and ACTH are non-selective agonists for \textit{hMC1,3,4,5R}. They all share the same core pharmacophore elements HFRW (Figure 4.1).\textsuperscript{31,118} The endogenous antagonists of the \textit{hMCR}s include the agouti signaling protein (\textit{hASIP} or the “agouti protein”) which primarily binds to \textit{hMC1,4R}, with weak affinity at \textit{hMC3R}, and the agouti-related protein (\textit{hAGRP}) which is selective for \textit{hMC3,4R}.\textsuperscript{119} These two antagonists are expressed in different parts of the body.\textsuperscript{120} It has been established that the endogenous antagonists \textit{hAGRP}
and hASIP interact with the melanocortin receptors via the triplet RFF in their central Cys-constrained loops (Figure 4.2).  

Figure 4.1 Natural melanotropins and their core pharmacophore HFRW  

ACTH H₂NSYSEMETHFRWGGPVKGKRPVVKYPNGAEDESEAEFPLEF-OH  
α-MSH Ac-SYSEMETHFRWGGPV-NH₂  
β-MSH H₂N-AEKKDEGPYRMEHFRWDRFG-OH  
γ-MSH H₂N-YVMGHFRWDRFG-OH  

Figure 4.2 Central Loops of hAGRP and hASIP and their core pharmacophore RFF  

hAGRP central loop c[CRFFNAFC]  
hASIP central loop c[CRFFRSAC]  

α-MSH cannot be used as a therapeutic drug because it has very short half-life in serum. To improve the pharmacological properties of the α–MSH, the Hruby group has developed NDP–α-MSH in which the Met⁴ and Phe⁷ residues in α–MSH were replaced by Nle⁴ and D-Phe⁷ residues respectively. Further truncation and optimization of the NDP–α–MSH led to the discovery of MTII(Ac-Nle⁴-c[Asp⁵-His⁶-D-Phe⁷-Arg⁸-Trp⁹-Lys¹⁰]-NH₂) which is a shorter peptide with a lactam bridge between the aspartic acid and lysine side chains. Both of these two α-MSH analogues are 100 times more potent than the parent α–MSH. And they have prolonged half-life in serum primarily due to the use of unnatural amino acids. Interestingly, a close analogue of MTII, SHU9119 (Ac-Nle⁴-c[Asp⁵-His⁶-D-Nal(2)⁷-Arg⁸-Trp⁹-Lys¹⁰]-NH₂), is a high affinity antagonist at hMC3R and hMC4R, but potent agonist at hMC1R and hMC5R. Further efforts to obtain hMC4R selective antagonists based on SHU9119 template have not been successful.

On the other hand, the endogenous antagonist hASIP binds to hMC4R 46 times stronger than to hMC3R. Furthermore, analogues of the central octapeptide
loops of the hAGRP and hASIP have been synthesized and determined to be selective antagonists for hMC4R vs. hMC3R,\textsuperscript{128} though only with sub-micromolar affinity. This prompted us to compare the structures of SHU9119\textsuperscript{34} and hASIP central loop. They are similar in several ways.\textsuperscript{128} First of all, both are constrained cyclic peptides with almost the same ring size. Secondly, they all have guanidinium and aromatic moieties in their core pharmacophores. Thirdly, they all have important $\beta$-turn structures. However, the positions of their $\beta$-turns are different. In the case of SHU9119, there is a $\beta$-turn spanning His and D-Nal(2') residues shown in its NMR solution structure.\textsuperscript{34} For hASIP, its NMR solution structure (Figure 4.3) suggests a $\beta$-turn spanning Phe and Arg residues.\textsuperscript{119} If we could introduce an additional $\beta$-turn spanning D-Nal(2') and Arg residues in SHU9119, we might be able to get short and stable peptide analogues that could mimic the hASIP central loop, thus achieving selectivity for hMC4R.

Figure 4.3 NMR solution structure of endogenous hMC1R and hMC4R selective antagonist hASIP (Reprinted from reference 119, Copyright (2004), with permission from Elsevier)
One possible approach to have such a turn in globally constrained SHU9119 or MTII is to substitute structurally constrained proline\(^8\) for arginine\(^8,129\) Presumably, the two Phe residues in the hASIP central loop could be mimicked by the His\(^6\) and D-Phe/D-Nal(2')\(^7\) residues in MTII or SHU9119. In order to keep the guanidinium groups in MTII or SHU9119 for the possible mimicry of the guanidinium group in hASIP core pharmacophore, proline derivatives with guanidinium moieties on the proline ring are needed. Fortunately, the Hruby group has developed the methodology for the synthesis of both trans and cis 4-guanidinium proline derivatives in their protected forms.\(^{130}\) These novel constrained amino acids would also allow us to study the topographic importance of the guanidinium group which is now highly constrained. Therefore, we designed a series of MTII, SHU9119 analogues\(^{32,33,37,38}\) in which the Arg\(^8\) residues in their core pharmacophores were replaced by 4-trans- or cis-guanidinium Pro residues (Figure 4.4).

**Figure 4.4 Peptide design with use of proline, or trans/cis-4-guanidinium proline**

31: Ac-Nle-c[Asp-His-D-Phe-Pro-Trp-Lys]-NH\(_2\)
32: Ac-Nle-c[Asp-His-D-Phe-trans-Xaa-Trp-Lys]-NH\(_2\)
33: Ac-Nle-c[Asp-His-D-Phe-cis-Xaa-Trp-Lys]-NH\(_2\)
34: Ac-Nle-c[Asp-trans-Xaa-D-Phe-Arg-Trp-Lys]-NH\(_2\)
35: Ac-Nle-c[Asp-cis-Xaa-D-Phe-Arg-Trp-Lys]-NH\(_2\)
36: Ac-Nle-c[Asp-His-D-Nal(2')-Pro-Trp-Lys]-NH\(_2\)
37: Ac-Nle-c[Asp-His-D-Nal(2')-trans-Xaa-Trp-Lys]-NH\(_2\)
38: Ac-Nle-c[Asp-His-D-Nal(2')-cis-Xaa-Trp-Lys]-NH\(_2\)
39: Ac-Nle-c[Asp-trans-Xaa-D-Nal(2')-Arg-Trp-Lys]-NH\(_2\)
40: Ac-Nle-c[Asp-cis-Xaa-D-Nal(2')-Arg-Trp-Lys]-NH\(_2\)
41: Ac-Tyr-Val-Nle-Gly-His-D-Phe-Pro-Trp-Asp-Arg-Phe-Gly-NH\(_2\)
42: Ac-Tyr-Val-Nle-Gly-His-D-Phe-trans-Xaa-Trp-Asp-Arg-Phe-Gly-NH\(_2\)
43: Ac-Tyr-Val-Nle-Gly-His-D-Phe-cis-Xaa-Trp-Asp-Arg-Phe-Gly-NH\(_2\)

trans-Xaa = ![trans-Xaa structure]
cis-Xaa = ![cis-Xaa structure]
In addition, to test whether this modification would work with a linear melanotropin analogue, for example, Ac-NDP–γ–MSH-NH$_2$\textsuperscript{131} (Ac-Tyr$^1$-Val$^2$-Nle$^3$-Gly$^4$-His$^5$-D-Phe$^6$-Arg$^7$-Trp$^8$-Asp$^9$-Arg$^{10}$-Phe$^{11}$-Gly$^{12}$-NH$_2$), analogues 42,43 were also designed to have a trans or cis 4-guanidinium proline in the critical arginine\textsuperscript{7} positions.

Figure 4.5 Design of SHU9119 analogues with His\textsuperscript{6} residues replaced with various conformationally constrained amino acids (Reproduced from reference 133 with permission, Copyright 2002 American Chemical Society)

For comparison, control peptides 31 ([Pro$^8$]MTII), 36 ([Pro$^8$]SHU9119) and 40 ([Pro$^7$]Ac-NDP–γ–MSH-NH$_2$) were also designed to investigate the importance of the guanidinium groups. In addition, previous studies indicated that the His\textsuperscript{6} residue in SHU9119 can be replaced with a Pro\textsuperscript{6} residue without causing any activity changes.\textsuperscript{132} However, replacement of His\textsuperscript{6} residue in SHU9119 with a variety of conformationally constrained, hydrophobic amino acid residues led to the discovery of several selective
ligands (Figure 4.5). Among these analogues, the compound containing a Cpe substitution in position 6 is a high affinity hMC4R antagonist (IC$_{50}$=0.51 nM) with a 200-fold selectivity vs. hMC3R. And the analogue with an Acpc residue in position 6 is a high affinity hMC3R antagonist (IC$_{50}$=2.5 nM) with 100-fold selectivity vs. hMC4R. Unfortunately, both of them are also agonists at hMC5R and their activities at hMC1R were not assayed. Nonetheless, these findings suggest that position 6 could be very important for receptor selectivity between hMC3R and hMC4R. Therefore, analogues 34,35,39,40 (Figure 4.4) with the His$^6$ residues in MTII and SHU9119 replaced by 4-trans- or cis-guanidinium Pro residues were also designed to investigate the possible effects of introducing additional positively charged guanidinium groups at positions 6.

Figure 4.6 Aspartimide formation and the consequence of the side reaction

![Aspartimide formation](image)

4.2 Peptide Synthesis

A well known side reaction involving aspartic acid residue in peptide synthesis is aspartimide formation. This side reaction is facilitated by either an acid or a base. For example, in Fmoc peptide synthesis of MTII analogues, once the N-Fmoc Asp(Allyl) residue is coupled to the peptide chain, the $\alpha$-amino nitrogen of the
previous amino acid residue could attack the Allyl protected aspartic acid side chain ester functionality to form a stable five member ring in the presence of base (Figure 4.6).\textsuperscript{134,135} The base could be from either the peptide coupling reaction or the Fmoc deprotection reaction. The formed aspartimide analogue has the same mass as the desired cyclic peptide with a lactam bridge between the aspartic acid and the lysine side chains. In addition, the aspartimide analogue can epimerize easily during the synthesis and cleavage process. Under aqueous basic conditions, the five number aspartimide ring can open to give $\beta$-peptide analogues which are very hard to separate from the desired peptide.

Figure 4.7 Strategy used to avoid aspartimide formation

To avoid the aspartimide formation, the synthesis was designed in such a way that all coupling reactions were performed under neutral conditions using DIC/Cl-HOBt as the coupling reagents. In addition, the side chain to side chain cyclization was performed immediately under neutral condition after the attachment of the Fmoc-
Asp(Allyl) residue to the growing peptide chain, without further Fmoc deprotection (Figure 4.7). The cyclization was then followed by aspartic acid Fmoc deprotection and the coupling of the next residue. If the cyclization was performed after the completion of the peptide assembling, the piperidine used to remove Fmoc protecting groups after each coupling step would promote the formation of the aspartimide peptide analogue.

4.3 Results

The protected trans- and cis-4-guanidinium proline were synthesized from trans-4-hydroxyproline following a method developed by Tamaki et al., a previous member of the Hruby group (Scheme 4.1 and 4.2). The synthesis was lengthy but straightforward. However, the purification of the final products was tricky due to the hydrophobicity of the molecules which have two Boc and one Fmoc protecting groups. The paper described a two system (EtOAc/Hexane and MeOH/CHCl₃) silica gel flash column chromatography purification procedure. The separation was not good, probably due to the incompatibility of these two solvent systems. However, the purification worked well when just the MeOH/CHCl₃ system was used. In this way, a few grams of each isomer were obtained and incorporated into the designed peptides.

Scheme 4.1 Synthesis of protected trans-4-guanidinium-L-proline (Reproduced from reference 130 with permission, Copyright 2001 American Chemical Society)
Scheme 4.2 Synthesis of protected cis-4-guanidinium-L-proline (Reproduced from reference 130 with permission, Copyright 2001 American Chemical Society)

All peptides were synthesized via the Fmoc SPPS strategy outlined above (Figure 4.7) with high yields and purity as determined by analytic HPLC, TLC, NMR and high resolution mass spectrometry (Table 4.1).

Table 4.1 Sequence and physicochemical properties of the peptides synthesized

<table>
<thead>
<tr>
<th>no.</th>
<th>calc</th>
<th>found</th>
<th>HPLC ( t_R )^a</th>
<th>TLC ( R_f )^b</th>
</tr>
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<tbody>
<tr>
<td>31</td>
<td>511.7701 (2H)</td>
<td>511.7725 (ESI)</td>
<td>7.90 11.00 0.67 0.36</td>
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</tr>
<tr>
<td>32</td>
<td>511.7701 (2H)</td>
<td>511.7725 (ESI)</td>
<td>8.09 11.70 0.67 0.36</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>483.2538 (2H)</td>
<td>483.2546 (ESI)</td>
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</tr>
<tr>
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<td>521.2912 (2H)</td>
<td>521.2907 (ESI)</td>
<td>8.98 13.03 0.68 0.37</td>
<td></td>
</tr>
<tr>
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<td>521.2889 (ESI)</td>
<td>8.94 12.86 0.68 0.37</td>
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</tr>
<tr>
<td>36</td>
<td>536.7779 (2H)</td>
<td>536.7768 (ESI)</td>
<td>10.02 14.59 0.73 0.44</td>
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</tr>
<tr>
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<td>536.7779 (2H)</td>
<td>536.7756 (ESI)</td>
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<tr>
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<td>506.2624 (ESI)</td>
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<td>546.3014 (ESI)</td>
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</tr>
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<td>546.2995 (ESI)</td>
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<td>41</td>
<td>531.2693 (3H)</td>
<td>531.2697 (ESI)</td>
<td>9.70 14.31 0.71 0.33</td>
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<tr>
<td>42</td>
<td>531.2693 (3H)</td>
<td>531.2695 (ESI)</td>
<td>9.95 14.70 0.71 0.33</td>
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</tr>
<tr>
<td>43</td>
<td>767.8837 (2H)</td>
<td>767.8868 (ESI)</td>
<td>11.85 17.44 0.74 0.37</td>
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aHPLC column, Vydac 218TP104, 250 \( \times \) 4.6 mm, 10 \( \mu \)m, 300 Å. \( t_R \) = peptide retention time – solvent retention time. System 1: Solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10–90% B in 40 min, flow rate 1.0 mL/min at 40 °C. System 2: solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10-60% B in 40 min, flow rate 1.0 mL/min at 40 °C. bTLC system 1: n-butanol/acetic acid/water/pyridine (4:1:2:1). TLC system 2: n-butanol/acetic acid/water (4:1:1)

For comparison, we also synthesized Ac-Nle-Asp-His-D-Phe-trans-4-guanidinium-Pro-Trp-Lys-NH₂, the linear analogue of peptide 32, using HBTU/HOBt/DIPEA as coupling reagents. The linear peptide was cleaved from the
resin after the removal of the side chain Ally/Alloc protecting groups in the last step. Analytical HPLC and mass spectrometry indicated that at least 35% of the linear peptide was converted to aspartimide peptide which has exactly the same mass as the corresponding cyclic peptide 32. Considering the possible ring opening of the aspartimide peptide that could lead to the formation of linear, epimerized α-peptide or β-peptide that might be difficult to separate from the desired α-peptide by HPLC, the actual percentage of the aspartimide peptide formed could be even higher.

Table 4.2 Binding affinities and cAMP activities of the peptides designed

<table>
<thead>
<tr>
<th>Peptide</th>
<th>hMC1R&lt;sup&gt;a&lt;/sup&gt; IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC3R&lt;sup&gt;a&lt;/sup&gt; IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC4R&lt;sup&gt;a&lt;/sup&gt; IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC5R&lt;sup&gt;a&lt;/sup&gt; IC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC1R&lt;sup&gt;a&lt;/sup&gt; EC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC3R&lt;sup&gt;a&lt;/sup&gt; EC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC4R&lt;sup&gt;a&lt;/sup&gt; EC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
<th>hMC5R&lt;sup&gt;a&lt;/sup&gt; EC&lt;sub&gt;50&lt;/sub&gt;, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTII</td>
<td>0.2±0.01</td>
<td>0.3±0.04</td>
<td>1.1±0.3</td>
<td>1.1±0.3</td>
<td>2.9±0.5</td>
<td>2.9±0.5</td>
<td>3.3±0.7</td>
<td>3.3±0.7</td>
</tr>
<tr>
<td>31: [Pro&lt;sup&gt;8&lt;/sup&gt;]MTII</td>
<td>&gt;5000</td>
<td>&gt;1000</td>
<td>75</td>
<td>11±0.9</td>
<td>200±40</td>
<td>13</td>
<td>11±0.9</td>
<td>200±40</td>
</tr>
<tr>
<td>32: [trans-Xaa&lt;sup&gt;8&lt;/sup&gt;]MTII</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>50±10</td>
<td>43±8</td>
<td>3.9</td>
<td>600±90</td>
<td>2450±480</td>
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<tr>
<td>33: [cis-Xaa&lt;sup&gt;8&lt;/sup&gt;]MTII</td>
<td>3500±400</td>
<td>760±150</td>
<td>91</td>
<td>39±2</td>
<td>112±2</td>
<td>36</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>34: [trans-Xaa&lt;sup&gt;8&lt;/sup&gt;]MTII</td>
<td>2.7±0.5</td>
<td>820±150</td>
<td>77</td>
<td>91</td>
<td>0.6±0.2</td>
<td>3500±660</td>
<td>64</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>35: [cis-Xaa&lt;sup&gt;8&lt;/sup&gt;]MTII</td>
<td>1.1±0.2</td>
<td>2700±200</td>
<td>100</td>
<td>28</td>
<td>5.7±0.5</td>
<td>414±8</td>
<td>39</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>SHU9119</td>
<td>1.6±0.2</td>
<td>1.5±0.1</td>
<td>100</td>
<td>2.3±0.2</td>
<td>0.6±0.1</td>
<td>91</td>
<td>39</td>
<td>0</td>
</tr>
<tr>
<td>36: [Pro&lt;sup&gt;8&lt;/sup&gt;]SHU9119</td>
<td>600±120</td>
<td>160±30</td>
<td>66</td>
<td>6</td>
<td>33±3</td>
<td>5700±1000</td>
<td>55</td>
<td>NA</td>
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<tr>
<td>37: [trans-Xaa&lt;sup&gt;8&lt;/sup&gt;]SHU9119</td>
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<td>NA</td>
<td>&gt;10000</td>
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<td>56</td>
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<td>200±10</td>
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<td>38: [cis-Xaa&lt;sup&gt;8&lt;/sup&gt;]SHU9119</td>
<td>400±80</td>
<td>1900±3700</td>
<td>100</td>
<td>28</td>
<td>23±1</td>
<td>250±30</td>
<td>7.3</td>
<td>NA</td>
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<tr>
<td>39: [trans-Xaa&lt;sup&gt;8&lt;/sup&gt;]SHU9119</td>
<td>2.3±0.4</td>
<td>620±110</td>
<td>25</td>
<td>23±0.1</td>
<td>130±25</td>
<td>28</td>
<td>1.5±0.3</td>
<td>200±40</td>
</tr>
<tr>
<td>40: [cis-Xaa&lt;sup&gt;8&lt;/sup&gt;]SHU9119</td>
<td>1.2±0.2</td>
<td>1700±300</td>
<td>90</td>
<td>2.2±0.2</td>
<td>NA</td>
<td>NA</td>
<td>2.5±0.5</td>
<td>1400±280</td>
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<tr>
<td>NDβ-γ-MSH</td>
<td>0.5±0.01</td>
<td>1.5±0.1</td>
<td>100</td>
<td>2.0±0.2</td>
<td>2.0±0.2</td>
<td>100</td>
<td>1.2±0.2</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>41: [Pro&lt;sup&gt;8&lt;/sup&gt;]NDβ-γ-MSH</td>
<td>200±35</td>
<td>120±30</td>
<td>53</td>
<td>NA</td>
<td>270±30</td>
<td>210±30</td>
<td>46</td>
<td>NA</td>
</tr>
<tr>
<td>42: [trans-Xaa&lt;sup&gt;8&lt;/sup&gt;]NDβ-γ-MSH</td>
<td>830±150</td>
<td>1400±250</td>
<td>92</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</tr>
<tr>
<td>43: [cis-Xaa&lt;sup&gt;8&lt;/sup&gt;]NDβ-γ-MSH</td>
<td>1000±200</td>
<td>&gt;5000</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup>Xaa=4-guanidiumproline. <sup>b</sup>IC<sub>50</sub>=concentration of peptide at 50% specific binding (N=4). EC<sub>50</sub>=effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (N=4). The peptides were tested at a range of concentrations from 10<sup>-10</sup> to 10<sup>-5</sup> M. NB: No Binding at 10<sup>-5</sup> M. NA: No Activity at 10<sup>-5</sup> M.

The binding affinities and biological activities of the peptides were determined at hMC1R, hMC3R, hMC4R and hMC5R, and the results are summarized in Table 4.2. Control peptides 31,36,41 in which Arg residues in MTII, SHU9119, Ac-NDP-γ-MSH-NH<sub>2</sub> were replaced by Pro residues showed selectivity only at hMC1R and/or hMC4R. Compared with their respective parent peptides, these peptides displayed decreased binding affinities at both hMC1R (IC<sub>50</sub>=200–>5000 nM) and hMC4R (IC<sub>50</sub>=11-270 nM). All of these peptides became partial agonists (13–75%) at the
hMC1R and the hMC4R. Analogue 32 ([Trans-4-guanidinium-Pro^8]MTII) is a very weak partial agonist, which is selective for hMC4R (IC_{50}=50 nM, EC_{50}=43 nM, 3.9% max activation). It also showed weak binding at hMC5R (IC_{50}=600 nM, EC_{50}=2450 nM, 24% max activation). The biological profile of 33 ([cis-4-guanidinium-Pro^8]MTII) is almost identical to that of 31 ([Pro^8]MTII). Peptide 37 ([Trans-4-guanidinium-Pro^8]SHU9119) is a partial agonist selective for hMC4R (IC_{50}=7.2 nM) and hMC5R (IC_{50}=22 nM) while 38 ([cis-4-guanidinium-Pro^8]SHU9119) is a very similar to 36 ([Pro^8]SHU9119), but with much weaker agonist activity at hMC4R (7.3% max activation). In Ac-NDP-\gamma-MSH-NH_2 series, the additional guanidinium groups on the 4 positions of the proline ring in 42 or 43 (trans or cis [4-guanidinium-Pro^7]Ac-NDP-\gamma-MSH-NH_2) render these peptides hMC1R selective only, but with decreased receptor binding affinities. For MTII or SHU9119 analogues 34,35,39,40 with His^6 residues in the parent peptides replaced by trans or cis 4-guanidinium-Pro^6, the binding profiles remain the same. None of them showed any selectivity among the five melanocortin receptors. However, there were some changes in cAMP activities especially in 39 ([trans-4-guanidinium-Pro^6]SHU9119), which is now a weak partial agonist at all melanocortin receptors. pA_2 analysis of 32 ([trans-4-guanidinium-Pro^8]MTII) shows that it is an hMC4R selective allosteric antagonist, suggesting that its binding sites are not exactly the same as that of MTII or SHU9119.

Computer modeling was performed using Kolossvary’s large scale low mode conformational search method (LLMOD)\textsuperscript{137} with the OPLS-2005 force field\textsuperscript{138} and the GB/SA continuum dielectric water solvent model.\textsuperscript{139} The details are reported in the Experimental Section. The lowest energy conformation of 31 ([Pro^8]MTII, Figure 4.8) was obtained, and it turned out to be different from the NMR solution structures
There is indeed a new β-turn spanning the D-Phe\textsuperscript{7} and Pro\textsuperscript{8} residues. Computer molecular modeling was also performed for 32 ([trans-4-guanidinium-Pro\textsuperscript{8}]MTII). The lowest energy conformation obtained was again found to have a new β-turn at positions 7 and 8. Furthermore, this conformation was superimposed with the NMR solution structure of the hASIP central loop (Figure 4.9). Their backbones overlap quite well. The His\textsuperscript{6}, D-Phe\textsuperscript{7} residues in 32 ([trans-4-guanidinium-Pro\textsuperscript{8}]MTII) aligns well with the Phe and Phe residues in the core pharmacophore of hASIP central loop. However, the guanidinium groups in these two structures do not show good alignment.

Figure 4.8 Global minimum of analogue 31 ([Pro\textsuperscript{8}] MTII)

Figure 4.9 Stereo view of the global minimum structure of 32 (in orange), superimposed with the NMR solution structure of the hASIP central loop (in teal)
4.4 Discussion

During manual solid phase peptide synthesis (SPPS), each coupling is monitored by either the Kaiser Test\textsuperscript{140} for a primary amine or Chloranil Test\textsuperscript{141} for a secondary amine. For syntheses using automated peptide synthesizers, even Kaiser Test or Chloranil Test is skipped. The product is usually not purified and characterized until it is removed from the resin. Many side reactions can happen during the synthesis of peptides, especially difficult peptides such as large, hydrophobic, and cyclic peptides. Common analytical methods such as Kaiser Test, HPLC, NMR, or Mass Spectrometry cannot always distinguish all side products formed. In order to get the desired peptides for meaningful biological assays, it is very important to consider all possible side reactions and design a good strategy to avoid or at least minimize these side reactions. And proper quality control at each step is necessary the first time a peptide is synthesized.

Not long ago, MTII analogues were still synthesized using strategies that would promote the formation of aspartimide peptide analogues. Fortunately, this side reaction was recently noticed and reported. And we were able to obtain the peptides as we designed for biological studies.

The fact that the synthesized analogues with Arg residues in His-D/L-Phe(or D-Nal(2 ‘))-Arg-Trp substituted by proline or 4-guanidinium proline residues showed decreased binding affinity and potency indicates the critical role of Arg residues in MTII, SHU9119, Ac-NDP-\(\gamma\)-MSH-NH\(_2\) and hASIP. As a matter of fact, Chen et al. recently demonstrated, utilizing receptor domain exchange and site-specific mutagenesis studies, that the guanidinium group in NDP-\(\alpha\)-MSH was directly
involved in the ionic interactions with the Asp residues in transmembrane 3 of hMC4R (Figure 4.10).  

Figure 4.10 Two-dimensional representation of a proposed three-dimensional model illustrating the synthetic melanocortin NDP-α-MSH docked inside the hMC4R.  

In the present study, Pro residues in the positions of Arg residues in MTII, SHU9119 and Ac-NDP-γ-MSH-NH₂ have been shown to be essential for ligand selectivity for hMC1R and/or hMC4R as indicated by the binding data of the three control peptides 31,36,41 ([Pro⁸]MTII, [Pro⁸]SHU9119 and [Pro⁷]Ac-NDP-γ-MSH-NH₂). Presumably, Pro substitutions induced the desired β-turns which caused conformational changes of the original peptide backbones. The backbone conformational changes in turn altered the 3D orientations of the side chain groups of the core pharmacophore elements in a way that favors the interactions with hMC1R and/or hMC4R. When a guanidinium group was placed on 4 positions of the proline ring as in 32 ([trans-4-guanidinium-Pro⁸]MTII), or 33 ([cis-4-guanidinium-Pro⁸]MTII), or 37 ([trans-4-guanidinium-Pro⁸]SHU9119), or 38 ([cis-4-guanidinium-Pro⁸]SHU9119), or 42 ([trans-4-guanidinium-Pro⁷]Ac-NDP-γ-MSH-NH₂), or 43 ([cis-
4-guanidinium-Pro\(^7\)Ac-NDP-\(\gamma\)-MSH-NH\(_2\), there was no noticeable improvement in terms of binding affinity. However, there were changes in terms of receptor selectivity, especially for 42 ([trans-4-guanidinium-Pro\(^7\)Ac-NDP-\(\gamma\)-MSH-NH\(_2\)), 43 ([cis-4-guanidinium-Pro\(^7\)Ac-NDP-\(\gamma\)-MSH-NH\(_2\)) which completely lost binding for hMC3,4,5R and were weak hMC1R agonists. According to our molecular modeling study, the guanidinium groups in those analogues are far apart from the guanidinium group in hASIP core pharmacophore. Therefore, the additional guanidinium groups in those analogues are probably not directly involved in binding to a receptor as in the case of hASIP which has a subnanomolar binding affinity.\(^{119}\) Rather, they change the biological profiles of those analogues by influencing the 3D positions of other important side chain groups, for example, the imidazole moiety of the His residue, through nonbonding interactions.

The binding and cAMP data of 34 ([trans-4-guanidinium-Pro\(^6\])MTII), 35 ([cis-4-guanidinium-Pro\(^6\])MTII), 39 ([trans-4-guanidinium-Pro\(^6\])SHU9119), 40 ([cis-4-guanidinium-Pro\(^6\])SHU9119) suggest that the replacement of the His\(^6\) residues has little if any influences to the receptor selectivity, though in some cases it can modulate cAMP activity at individual receptors. For example, 39 ([trans-4-guanidinium-Pro\(^6\])SHU9119) showed weak partial activities at hMC1,5R as opposed to the full agonist activities SHU9119 displayed at the same receptors. Overall, positively charged guanidinium groups at carbon 4 of the Pro as in 34,35 ([4-guanidinium-Pro\(^6\])MTII) or 39,40 ([4-guanidinium-Pro\(^6\])SHU9119) are not important in the study.

It is not surprising that analogue 32 ([trans-4-guanidinium-Pro\(^6\])MTII) is an allosteric antagonist for hMC4R. Although most evidence suggests that melanotropins and hASIP (or hAGRP) share the same binding sites on melanocortin receptors,\(^{143}\)
one study did not show such competitive antagonism of hAGRP for hMC4R. In fact, the Hruby group has previously reported hMC4R selective antagonists that are allosteric modulators of the receptor.

4.5 Summary

Both cyclic and linear analogues of melanotropins were designed to have a Pro or 4-guanidinium-Pro residue in the His or Arg positions of the melanotropin pharmacophore. The Pro residue was incorporated to investigate the effects of the backbone conformational changes and the guanidinium group on the Pro ring was used to probe possible electrostatic interactions with the melanocortin receptors. Those peptides were synthesized successfully via a carefully designed solid phase peptide synthesis strategy to avoid the formation of the aspartimide peptide byproduct. Competitive binding and cAMP assays revealed a series of ligands selective for hMC1R and/or hMC4R. Some of those analogues are high affinity allosteric antagonists for hMC4R. The results are consistent with the finding from the molecular modeling study of [trans-4-guanidinium-Pro\textsuperscript{8}] MTII, whose global minimum structure fits well with the NMR solution structure of the endogenous hMC1/4R selective antagonist hASIP central loop. The new β-turn induced by the proline ring was identified to be responsible for the selectivity changes. These findings can be used to design novel ligands for the study of the melanocortin system and ultimately the treatment of various diseases associated with the melanocortin system.

4.6 Future Work

The new hMC1/4R selective templates [Pro\textsuperscript{8}]MTII, [Pro\textsuperscript{8}]SHU9119 are close mimics of the central loop of the hASIP. However, there are at least two issues that need to be further investigated. One is the proper mimicry of the Arg residue in the
core pharmacophore (RFF) of the hASIP central loop. The other one is the mimicry of
the first Phe residue in RFF by His\textsuperscript{6} residues in [Pro\textsuperscript{8}]MTII or [Pro\textsuperscript{8}]SHU9119. The
following analogues are designed based on 31 ([Pro\textsuperscript{8}]MTII). The first analogue is to
see whether a Phe residue in position 6 would be a better mimicry of the first Phe
residue in the hASIP central loop. The next two are to see if an additional Arg residue
before His\textsuperscript{6} or Phe\textsuperscript{6} residues can mimic the Arg residue in the RFF of the hASIP
central loop. The last two are analogues with additional Arg residues before His\textsuperscript{6} or
Phe\textsuperscript{6} residues but without Trp\textsuperscript{9} residues so that the ring size is close to that of the
hASIP central loop. Analogues of 36 ([Pro\textsuperscript{8}]SHU9119) are designed with the same
considerations.

Based on 31 ([Pro\textsuperscript{8}]MTII: Ac-Nle-c[Asp-His-D-Phe-Pro-Trp-Lys]-NH\textsubscript{2})

Ac-Nle-c[Asp-Phe-D-Phe-Pro-Trp-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-His-D-Phe-Pro-Trp-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-Phe-D-Phe-Pro-Trp-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-His-D-Phe-Pro-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-Phe-D-Phe-Pro-Lys]-NH\textsubscript{2}

Based on 36 ([Pro\textsuperscript{8}]SHU9119: Ac-Nle-c[Asp-His-D-Nal(2')-Pro-Trp-Lys]-NH\textsubscript{2})

Ac-Nle-c[Asp-Phe-D-Nal(2')-Pro-Trp-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-His-D-Nal(2')-Pro-Trp-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-Phe-D-Nal(2')-Pro-Trp-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-His-D-Nal(2')-Pro-Lys]-NH\textsubscript{2}
Ac-Nle-c[Asp-Arg-Phe-D-Nal(2')-Pro-Lys]-NH\textsubscript{2}

In addition, hASIP can be purchased and compared to 32 ([trans-4-
guanidinium-Pro\textsuperscript{8}]MTII) to see whether they share the same binding site.
4.7 Experimental Section

Materials

Nα-Fmoc-amino acids and Rink amide MBHA (w/Nle) resin were obtained from Chem-Impex International (Wood Dale, IL). DIC and Cl–HOBt were purchased from Bachem (King of Prussia, PA). Pd(PPh₃)₄ and PhSiH₃ were purchased from Sigma Aldrich. HSW syringes (10 mL, Henke Sass Wolf GmbH, Germany) with frits on the bottom were used for all peptides synthesis. Analytical HPLC was performed using a Vydac C₁₈ 218TP104 column (Western Analytical Products, Murrieta, CA) monitored at 230 and 254 nm. Preparative HPLC was done on X Terra® Prep MSC18 Column (10µm, 19mm×250mm, part#186002259) obtained from Waters. Analytical thin-layer chromatography (TLC) was carried out on ANATECH UNIPLATE silica gel GF 250 micron. The TLC chromatograms were visualized by UV light and by potassium permanganate solution. ¹H NMR spectra were recorded on Bruker DRX600 or 500 or Varian Inova600 spectrometers with DMSO-d₆ as internal standard. Typical sample concentration is 5 mM. Mass spectra were obtained from Mass Spectrometry Facility, Department of Chemistry, the University of Arizona. An IonSpec Fourier Transform Mass Spectrometer was used for peptide mass analyses.

Hazards Information

Piperidine: Highly flammable and poisonous. May be fatal if inhaled or swallowed. Severe irritant. Skin contact may cause severe irritation or burns. Contact with the eyes may lead to permanent damage.

TFA: Danger! Both liquid and vapor can cause severe burns to all parts of the body. Harmful if inhaled. Hygroscopic (absorbs moisture from the air). Target Organs: Respiratory system, eyes, skin, and mucous membranes.
KCN (a component of Kaiser test regent): Very toxic. May be fatal if inhaled, swallowed or absorbed through skin. Extremely destructive of mucous membranes. Causes burns.

**Peptide Synthesis**

All peptides in this study were synthesized manually by Fmoc solid phase methodology using DIC and Cl–HOBt/HOAt as coupling reagents. The following procedures were for the synthesis of the cyclic peptides. The linear peptides were synthesized likewise. Rink amide MBHA resin (250mg, 0.7 mmol/g) was placed into a 10 mL HSW polypropylene syringe and swollen in DCM (5 mL) for 30 min. After removal of the Fmoc protecting group (25% piperidine in DMF, 5mL, 5 and 10 min), the resin was washed with DMF (4×5mL) and DCM (4×5mL). Then, Fmoc-Lys(Alloc)-OH, Fmoc-Trp(Boc)-OH, (2S,4R)-Nα-Fmoc-4-N,N’-di-Boc-guanidinium-Pro-OH or (2S,4S)-Nα-Fmoc-4-N,N’-di-Boc-guanidinium-Pro-OH or Fmoc-Arg(Pbf)-OH, Fmoc-D-Phe-OH or Fmoc-D-Nal(2’)-OH, Fmoc-His(Trt)-OH or (2S,4R)-Nα-Fmoc-4-N,N’-di-Boc-guanidinium-Pro-OH or (2S,4S)-Nα-Fmoc-4-N,N’-di-Boc-guanidinium-Pro-OH, Fmoc-Asp(Allyl)-OH were coupled to the resin following the above order. For each coupling, 3 equiv of amino acids, Cl–HOBt, DIC were used with 10 min preactivation in DMF. All couplings were performed for 1 h and monitored by either Kaiser Test or Chloranil Test. In case of a positive test result, the coupling was repeated until a negative test result was observed. Fmoc protecting groups were removed as described above. Once the Fmoc-Asp(Allyl)-OH was coupled to the peptide chain, the side chain Alloc protecting group of Lys and Allyl protecting group of Asp were removed simultaneously with 0.25 equiv of Pd(PPh3)4 and 20 equiv of PhSiH3 in 5 mL of dry DCM (30 min × 2). The cyclization was
performed with 6 equiv of DIC and HOAt in DMF for 72 h and was monitored by the Kaiser Test. Upon completion of the cyclization, the resin was treated with 5 mL of 5% sodium diethyldithiocarbamate trihydrate in DMF (10 min) and then washed with DMF (4×5 mL), DCM (4×5 mL). Fmoc-Nle-OH was then coupled to the peptide chain following procedures described above. And the Fmoc was removed and the amino group was acetylated with 10 equiv of acetic anhydride and 2 equiv of DIPEA in 5 mL of DCM for 30 min. The resin was washed with DCM (4×5 mL), DCM/Diethyl Ether (1:1, 4×5 mL) and dried under vacuum for 4 h. The peptides were cleaved from the resin with a mixture of 95% TFA, 2.5% water and 2.5% TIPS for 3 h. After evaporation of the TFA under vacuum, the peptides were precipitated and washed 3 times with 5 mL of cold diethyl ether for each wash. The liquid was separated from the solid by centrifuge and was decanted. The crude peptides were dried in air and dissolved in acetonitrile and 0.1% TFA in water (2:1) before it was purified by preparative RP-HPLC eluted with a linear gradient of 15-40% acetonitrile in aqueous 0.1% TFA solution over 35 min with a 15 mL/min flow rate. The fractions containing the desired products were collected, concentrated and lyophilized. The purified peptides were isolated in 40-50% overall yields and were >95% pure as determined by analytical RP-HPLC. The structures of the pure peptides were confirmed by high-resolution mass spectrometry and $^1$H NMR in DMSO-d$_6$.

NMR samples were prepared in DMSO-d$_6$ with an approximate peptide concentration of 5.0 mM. The $^1$H spectra were recorded on a Bruker DRX-600 and/or Varian Inova 600 spectrometer, and were processed using XWINNMR or Mnova 5.0 software packages. Chemical shifts are referenced to the DMSO signal at 2.50 ppm.

**Peptide 31 Ac-Nle-c[Asp- His-D-Phe-Pro-Trp-Lys]-NH$_2$ (Total 64H)**
Side chain hydrogen (34)

0.81 (3H, t, J=7.1Hz), 1.11-1.57 (13H, m), 1.65 (2H, m), 1.79 (1H, m), 1.84 (3H, s),
2.39 (1H, dd, J=5.4, 14.7Hz), 2.56 (1H, m), 2.79 (2H, m), 2.92-3.14 (5H, m), 3.22
(1H, dd, J=4.1, 14.9Hz), 3.27 (1H, m), 3.50 (1H, m)

α-Hydrogen (7)

4.09 (1H, dd, J=4.7, 8.2Hz), 4.16 (2H, m), 4.33 (1H, m), 4.52 (2H, m), 4.70 (1H, dd,
J=7.8, 15.2Hz)

Amide and aromatic hydrogen (23)

6.91-7.26 (10H, m), 7.31 (1H, d, J=8.2Hz), 7.59 (2H, t, J=8.1Hz), 7.81 (1H, d,
J=7.2Hz), 7.87 (1H, t, J=5.4Hz), 7.95 (2H, t, J=7.5Hz), 8.14 (1H, d, J=7.3Hz), 8.51
(1H, d, J=7.5Hz), 8.92 (1H, s), 10.81 (1H, s), 14.07 (2H, d, J=71.3Hz)

Peptide 32 Ac-Nle-c[Asp-His-D-Phe-trans-4-GPro-Trp-Lys]-NH₂ (Total 67 H)

Side chain hydrogen (33)

0.82 (3H, t, J=7.0Hz), 1.11-1.31 (7H, m), 1.41 (2H, m), 1.45-1.60 (2H, m), 1.69 (1H,
m), 1.78 (1H, m), 1.84 (3H, s), 2.06 (1H, m), 2.31 (1H, m), 2.65 (1H, m), 2.73 (2H,
m), 2.91 (1H, m), 2.95-3.09 (3H, m), 3.17 (1H, dd, J=4.3, 15.0Hz), 3.23 (1H, m), 3.32
(1H, d, J=11.0Hz), 3.78 (1H, m), 4.20 (1H, m)

α-Hydrogen (7)

4.10 (2H, m), 4.26-4.38 (3H, m), 4.55 (1H, m), 4.83 (1H, m)

Amide and aromatic hydrogen (27)

6.93-7.25 (13H, m), 7.32 (1H, d, J=8.0Hz), 7.59 (1H, d, J=7.8Hz), 7.76 (1H, d,
J=8.0Hz), 7.82 (1H, b), 8.02 (2H, d, J=6.0Hz) 8.09 (2H, dd, J=7.0, 15.7Hz), 8.19 (1H,
d, J=7.9Hz), 8.44 (1H, d, J=8.5Hz), 8.91 (1H, s), 10.85 (1H, s), 14.03 (2H, d,
J=43.4Hz)
**Peptide 33 Ac-Nle-c[Asp-His-D-Phe-cis-4-GPro-Trp-Lys]-NH$_2$ (Total 67H)**

Side chain hydrogen (33)

0.81 (3H, t, $J=7.1$Hz), 1.11-1.29 (7H, m), 1.35-1.58 (5H, m), 1.65 (1H, m), 1.82 (3H, s), 2.31 (1H, dd, $J=5.1$, 14.7Hz), 2.59-2.75 (4H, m), 2.84 (1H, dd, $J=4.5$, 15.4Hz), 2.95-3.11 (4H, m), 3.19 (1H, dd, $J=4.0$, 14.6Hz), 3.26 (1H, t, $J=8.8$Hz), 3.78 (1H, m), 4.03 (1H, m)

$\alpha$-Hydrogens (7)

4.10 (1H, m), 4.17-4.27 (2H, m), 4.34 (1H, m), 4.46 (1H, dd, $J=7.8$, 13.4Hz), 4.54 (1H, dd, $J=7.4$, 14.2Hz), 4.80 (1H, dd, $J=8.9$, 14.9Hz)

Amide and aromatic hydrogen (27)

6.93-7.25 (13H, m), 7.33 (1H, d, $J=8.2$Hz), 7.59 (1H, d, $J=7.9$Hz), 7.78 (2H, t, $J=8.8$Hz), 7.84 (1H, d, $J=7.8$Hz), 7.92-8.02 (2H, m), 8.16 (1H, d, $J=7.2$Hz), 8.32 (1H, d, $J=6.6$Hz), 8.65 (1H, d, $J=9.3$Hz), 8.92 (1H, s), 10.84 (1H, s), 13.98 (2H, d, $J=58.5$Hz)

**Peptide 34 Ac-Nle-c[Asp-trans-4-GPro-D-Phe-Arg-Trp-Lys]-NH$_2$ (Total 72H)**

Side chain hydrogen (37)

0.82 (3H, t, $J=7.0$Hz), 1.11-1.47 (12H, m), 1.53 (2H, m), 1.63 (2H, m), 1.84 (3H, s), 1.86-1.98 (2H, m), 2.40 (1H, m), 2.73 (2H, m), 2.94-3.04 (5H, m), 3.17 (2H, dd, $J=5.3$, 13.9Hz), 3.61 (1H, dd, $J=4.5$, 11.0Hz), 3.70 (1H, dd, $J=5.7$, 10.3Hz), 4.09 (1H, m)

$\alpha$-Hydrogen (7)

4.11-4.17 (2H, m), 4.26 (1H, dd, $J=8.4$, 13.6Hz), 4.41 (2H, m), 4.52 (1H, dd, $J=7.0$, 14.2Hz), 4.64 (1H, dd, $J=7.5$, 12.9Hz)

Amide and aromatic hydrogen (28)
6.94-7.23 (15H, m), 7.32 (2H, d, \( J=8.1\text{Hz} \)), 7.44 (1H, t, \( J=5.6\text{Hz} \)), 7.59 (1H, d, \( J=8.0\text{Hz} \)), 7.78 (2H, dd, \( J=8.1, 18.4\text{Hz} \)), 7.89 (1H, t, \( J=5.7\text{Hz} \)), 7.95 (1H, d, \( J=8.4\text{Hz} \)), 8.02 (2H, t, \( J=7.9\text{Hz} \)), 8.26 (1H, d, \( J=7.4\text{Hz} \)), 8.49 (1H, d, \( J=8.5\text{Hz} \)), 10.79 (1H, d, \( J=2.1\text{Hz} \))

**Peptide 35 Ac-Nle-c[Asp-cis-4-GPro-D-Phe-Arg-Trp-Lys]-NH\(_2\) (Total 72H)**

Side chain hydrogen (37)

0.82 (3H, t, \( J=7.2\text{Hz} \)), 1.11-1.57 (17H, m), 1.63 (2H, m), 1.84 (3H, s), 2.32-2.43 (2H, m), 2.68-2.78 (2H, m), 2.92-3.03 (3H, m), 3.06 (1H, dd, \( J=4.5, 13.8\text{Hz} \)), 3.12-3.20 (2H, m), 4.01 (1H, m), 4.07 (1H, m)

\( \alpha \)-Hydrogen (7)

4.11-4.17 (2H, m), 4.27-4.40 (3H, m), 4.53 (1H, dd, \( J=7.3, 14.4\text{Hz} \)), 4.62 (1H, dd, \( J=7.2, 13.2\text{Hz} \))

Amide and aromatic hydrogen (28), observed 26

6.94-7.23 (13H, m), 7.33 (2H, d, \( J=8.1\text{Hz} \)), 7.48 (1H, t, \( J=5.5\text{Hz} \)), 7.60 (1H, d, \( J=7.9\text{Hz} \)), 7.70 (1H, d, \( J=8.4\text{Hz} \)), 7.74 (1H, d, \( J=8.3\text{Hz} \)), 7.82 (1H, t, \( J=5.5\text{Hz} \)), 8.07 (2H, d, \( J=7.5\text{Hz} \)), 8.15 (1H, d, \( J=7.0\text{Hz} \)), 8.25 (1H, d, \( J=7.3\text{Hz} \)), 8.70 (1H, d, \( J=8.4\text{Hz} \)), 10.80 (1H, d, \( J=2.1\text{Hz} \))

**Peptide 36 Ac-Nle-c[Asp-His-D-Nal(2')-Pro-Trp-Lys]-NH\(_2\) (Total 66H)**

Side chain hydrogen (34)

0.81 (3H, t, \( J=7.8\text{Hz} \)), 108-1.60 (13H, m), 1.67 (2H, m), 1.74 (1H, m), 1.85 (3H, s) 2.40 (1H, m), 2.61 (1H, dd, \( J=8.8, 14.9\text{Hz} \)), 2.76 (1H, dd, \( J=8.7, 15.3\text{Hz} \)), 2.92-3.18 (6H, m), 3.22 (1H, dd, \( J=4.1, 14.9\text{Hz} \)), 3.33 (1H, m), 3.51 (1H, m)

\( \alpha \)-Hydrogen (7)
4.15 (3H, m), 4.34 (1H, m), 4.48 (1H, dd, $J=8.4$, 13.5Hz), 4.53 (1H, dd, $J=7.7$, 14.4Hz), 4.80 (1H, dd, $J=7.8$, 15.1Hz)

Amide and aromatic hydrogen (25)

6.91-7.18 (5H, m), 7.31 (1H, d, $J=8.0$Hz), 7.35 (1H, d, $J=8.6$Hz), 7.44 (2H, m), 7.57, 7.68 (3H, m), 7.78 (1H, d, $J=8.4$Hz), 7.83 (3H, d, $J=9.4$Hz), 7.88-7.99 (2H, m), 8.02 (1H, d, $J=8.2$Hz), 8.14 (1H, d, $J=7.2$Hz, 8.57 (1H, d, $J=7.8$Hz), 8.87 (1H, s), 10.81 (1H, s), 13.95 (2H, d, $J=47.5$Hz)

**Peptide 37 Ac-Nle-c[Asp-His-D-Nal(2')-trans-4-GPro-Trp-Lys]-NH$_2$ (Total 69H)**

Side chain hydrogen (33)

0.81 (3H, t, $J=6.9$Hz), 1.11-1.32 (7H, m), 1.41 (2H, m), 1.49 (1H, m), 1.58 (1H, m), 1.72 (1H, m), 1.81 (1H, m), 1.86 (3H, s), 2.08 (1H, m), 2.34 (1H, m), 2.72 (2H, m), 2.90 (2H, m), 2.97 (1H, dd, $J=4.4$, 15.7Hz), 3.08 (1H, dd, $J=9.4$, 14.7Hz), 3.15-3.25 (2H, m), 3.31 (1H, m), 3.39 (1H, d, $J=11.3$Hz), 3.81 (1H, dd, $J=5.4$, 12.0Hz), 4.08 (1H, dd, $J=7.5$, 13.5Hz)

$\alpha$-Hydrogen (7)

4.14 (1H, m), 4.17-4.27 (2H, m), 4.31-4.38 (2H, m), 4.57 (1H, dd, $J=8.1$, 13.5Hz), 4.93 (1H, m)

Amide and aromatic hydrogen (29)

6.96-7.08 (6H, m), 7.23 (1H, s), 7.33 (3H, dd, $J=4.0$, 8.1Hz), 7.44 (2H, m), 7.60 (1H, d, $J=7.9$Hz), 7.63 (1H, s), 7.74-7.85 (4H, m), 7.87 (1H, b), 8.03 (1H, d, $J=7.8$Hz), 8.07 (2H, t, $J=6.2$Hz), 8.13 (1H, d, $J=8.0$Hz), 8.23 (1H, b), 8.49 (1H, d, $J=7.8$Hz), 8.83 (1H, s), 10.85 (1H, s), 13.98 (2H, d, $J=56.8$Hz)

**Peptide 38 Ac-Nle-c[Asp-His-D-Nal(2')-cis-4-GPro-Trp-Lys]-NH$_2$ (Total 69H)**

Side chain hydrogen (33)
0.80 (3H, t, $J=6.7$Hz), 1.09-1.32 (7H, m), 1.34-1.61 (5H, m), 1.66 (1H, m), 1.83 (3H, s), 2.32 (1H, m), 2.42 (1H, m), 2.67 (2H, m), 2.85 (2H, dd, $J=14.1$, 21.1Hz), 2.98 (1H, m), 3.11 (2H, m), 3.20 (2H, d, $J=13.1$Hz), 3.28 (1H, t, $J=9.1$Hz), 3.82 (1H, t, $J=8.0$Hz), 4.03 (1H, m)

$\alpha$-Hydrogen (7)

4.10 (1H, dd, $J=7.0$, 13.1Hz), 4.19 (1H, m), 4.25 (1H, t, $J=7.8$Hz), 4.33 (1H, m), 4.42 (1H, m), 4.54 (1H, dd, $J=7.3$, 14.0Hz), 4.91 (1H, dd, $J=9.1$, 14.6Hz)

Amide and aromatic hydrogen (29)

6.92-7.00 (3H, m), 7.04-7.10 (2H, m), 7.21 (1H, s), 7.34 (3H, t, $J=8.3$Hz), 7.43 (3H, m), 7.59 (1H, d, $J=7.9$Hz), 7.66 (1H, s), 7.74-7.85 (5H, m), 7.93 (1H, d, $J=8.1$Hz), 7.98 (2H, d, $J=7.3$Hz), 8.15 (1H, d, $J=7.5$Hz), 8.32 (1H, d, $J=6.4$Hz), 8.69 (1H, d, $J=8.8$Hz), 8.83 (1H, s), 10.85 (1H, s), 13.96 (2H, d, $J=90.1$Hz)

**Peptide 39 Ac-Nle-c[Asp-trans-4-GPro-D-Nal(2')-Arg-Trp-Lys]-NH$_2$ (Total 74H)**

Side chain hydrogen (37)

0.79-0.86 (3H, t, $J=6.8$Hz), 1.09-1.76 (17H, m), 1.82 (3H, s), 1.97 (1H, m), 2.32 (1H, d, $J=6.5$, 15.5Hz), 2.37 (1H, m), 2.51-2.62 (2H, m), 2.84-3.06 (6H, m), 3.17-3.28 (1H, m), 3.69 (1H, m), 4.02 (1H, m)

$\alpha$-Hydrogen (7)

4.05-4.23 (2H, m), 4.23-4.39 (1H, m), 4.45-4.58 (2H, m), 4.59-4.71 (1H, m), 4.83-4.93 (1H, m)

Amide and aromatic hydrogen (30)

6.95-7.22 (9H, m), 7.33 (2H, d, $J=8.1$Hz), 7.39-7.47 (4H, m), 7.50-7.70 (3H, m), 7.72-7.88 (5H, m), 7.90-8.10 (3H, m), 8.18 (2H, dd, $J=8.5$, 14.4Hz), 8.70 (1H, d, $J=7.8$Hz), 10.84 (1H, d, $J=1.9$Hz)
**Peptide 40 Ac-Nle-c[Asp-cis-4-GPro-D-Nal(2')-Arg-Trp-Lys]-NH₂ (Total 74H)**

Side chain hydrogen (37)

0.82 (3H, t, J=7.0Hz), 1.10-1.58 (17H, m), 1.64 (2H, m), 1.84 (3H, s), 2.32-2.46 (2H, m), 2.60 (1H, m), 2.75 (1H, m), 2.90-3.03 (4H, m), 3.10-3.25 (2H, m), 3.99 (1H, m), 4.08 (1H, dd, J=7.6, 13.9Hz)

α-Hydrogen (7)

4.14 (2H, m), 4.32 (2H, m), 4.47 (1H, m), 4.54 (1H, dd, J=7.1, 14.5Hz), 4.62 (1H, dd, J=7.0, 13.1Hz)

Amide and aromatic hydrogen (30)

6.99 (3H, m), 7.05-7.11 (3H, m), 7.15 (1H, d, J=2.1Hz), 7.32-7.40 (4H, m), 7.44 (3H, m), 7.56-7.62 (3H, m), 7.67 (1H, s), 7.74-7.85 (5H, m), 7.89 (1H, t, J=5.6Hz), 8.06 (1H, d, J=7.4Hz), 8.10 (1H, d, J=8.3Hz), 8.19 (1H, d, J=7.0Hz), 8.28 (1H, d, J=7.3Hz), 8.79 (1H, d, J=8.4Hz), 10.80 (1H, d, J=1.8Hz)

**Binding Assays** (performed by Minying Cai, Erin Palmer and Mogan Zingsheim)

Competition binding experiments were performed on whole cells. Transfected HEK293 cell line with hMCRs were seeded on 96 well plates, 48 h before assay (100, 000 cells/well). For the assay, the medium was removed and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptides and 125I-labeled [Nle⁴,D-Phe⁷]-α-MSH (PerkinElmer Life Science, 100, 000 cpm/well, 0.1386 nM) for 40 min at 37 °C. The medium was subsequently removed and each well was washed twice with the binding buffer. The cells were lysed by the
addition of 250 µL of 0.1 mM NaOH and 250 µL of 1% Triton X-100. The lysed cells were transferred to the 12×75 mm glass tubes and the radioactivity was measured by Wallac 1470 WIZARD Gamma Counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

**Adenylate Cyclase Assays** (performed by Minying Cai and Mogan Zingsheim)

HEK 293 cells transfected with human melanocortin receptors were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96 well plates 48 h before assay (100,000 cells/well). For the assay, the medium was removed and cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with isobutylmethylxanthine (IBMX, 5 µL, 0.5 mM) for 1 min at 37 °C. Next, various concentrations of melanotropins (0.1 mL) were added and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the buffer and adding ice cold Tris/EDTA buffer to each well (0.15 mL). After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene microcentrifuge tubes, capped, and place in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min (6500 rpm), and 50 µL of the supernatant was aliquoted into a clean Eppendorf tube. The total cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432, Amersham Corp.). The antagonist properties of analogue 32 were evaluated by its ability to competitively displace the MTII agonist in a dose dependent manner, at up to 10 µM.

**Data Analysis** (performed by Minying Cai and Mogan Zingsheim)
IC$_{50}$ and EC$_{50}$ values represent the mean of duplicate experiments performed in triplicate. IC$_{50}$ and EC$_{50}$ estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis, with the help of Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

**Computational Procedures**

Molecular modeling experiments were conducted by using MacroModel 8.1 equipped with Maestro 7.5 graphical interface installed on a Linux RedHat 9.0 system. Peptide structures were built as extended structures with standard bond lengths and angles, and were minimized using the OPLS-2005 force field and the Polak-Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient RMSD less than 0.05 kJ/Å mol or continued until 50,000 iterations were reached. Aqueous conditions were simulated using the continuum dielectric water solvent model (GB/SA) as implemented in MacroModel 8.1. Extended cutoff distances were defined at 8 Å for van der Waals, 20 Å for electrostatics, and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by Macromodel's LLMOD procedure of Kolossvary using the energy minimization parameters as described above. A total of 20,000 search steps were performed and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. The superimpositions of peptide structures were performed using the $\alpha$-carbons of the core sequence His-D-Phe-Xaa-Trp unless otherwise specified.
5.1 Introduction

Traditionally, compounds acting at 7-hexical transmembrane G-protein coupled receptors (GPCRs) are classified as agonists, partial agonists, antagonists, or inverse agonists. An agonist binds to a receptor and initiates a cellular response. It promotes the active state of a receptor. An antagonist binds to a receptor but cannot initiate a cellular response; rather it prevents agonists from producing a response. It has affinity, but no efficacy. A partial agonist is an agonist that produces less than the full effect, even at saturating levels. So a partial agonist acts as an antagonist in the presence of a full agonist as it blocks the full effect normally seen. An inverse agonist decreases G-protein binding and thus the basal activity of receptors.\textsuperscript{148}

Recently this traditional view has been challenged by evidence that ligands at the same G-protein coupled receptor can cause markedly different degrees of activation for different effector pathways.\textsuperscript{149} In Nickolls’ work, a variety of peptide and nonpeptide agonists were studied on FLAG-tagged hMC4R with respect to cAMP accumulation, calcium mobilization and receptor internalization.\textsuperscript{149} The activities of different agonists were found to vary considerably in these studies. Overall, there was no correlation between the potency and intrinsic activity of the agonists in the cAMP accumulation assays and their potency or intrinsic activity in either the calcium mobilization or receptor internalization assays. $\alpha$-MSH was used as a standard in the study and other agonists were compared to it. It was found that other peptide agonists
showed 80-112% maximal cAMP accumulation, 62-96% calcium mobilization and 75-118% receptor internalization. On the other hand, nonpeptide agonists exhibited 73-149% of the α-MSH response in the cAMP accumulation assays, 7–40% in calcium mobilization assays and 5–38% in receptor internalization assays.

Furthermore, it has now been established that 7-hexical transmembrane G-protein coupled receptors can have multiple, ligand specific functional conformations.\textsuperscript{150,151} And there can be an overlap between some of the conformation subsets. Each conformation is stabilized by a specific type of ligand (Figure 5.1). For Angiotensin Type 1 receptor (AT1R), there are at least three conformational subsets, two active conformations and one inactive conformation. Angiotensin II (AngII) is able to stabilize both active conformations at the same time. However, a biased ligand called SII ([Sar\textsuperscript{1},Ile\textsuperscript{4},Ile\textsuperscript{8}]Angiotensin II) can only stabilize one of the two active conformations. And the inactive conformation is stabilized by Angiotensin Receptor Blockers (ARBs). Multiple functional conformations also exist in other GPCRs, for example, PTH1 receptor, opioid receptors and so on.

Figure 5.1 Multiple functional conformations of GPCRs (Reprinted from reference 150, Copyright (2007), with permission from Elsevier)
Different functional conformations of GPCRs usually activate different signaling pathways (Figure 5.2). In general, receptor activation leads to two distinct signaling pathways, G-Protein dependent pathway and G-Protein independent pathway. Each signaling pathway can be further divided downstream due to the existence of multiple effector subtypes. For example, there is more than one $G_\alpha$ protein subtype. Activation of different effector subtype usually produces different cellular signals with distinct physiological consequences. In order to distinguish different pathways, proper assays and control experiments must be designed and performed. For example, an assay designed to measure IP$_3$ production (G-protein signaling) and $\beta$-Arrestin recruitment after AT1R activation was able to identify $\beta$-Arrestin biased agonist SII. Advancements in the area of biased agonism can help to
better understand complicated signaling pathways and design novel selective drugs with less undesirable effects.

Figure 5.3 Structure of MTII

According to the study by Nickolls et al., melanocortin receptors may also have multiple functional conformations. To further elucidate the molecular mechanism of the functional selectivity of hMC4R upon peptide or nonpeptide agonist activation, receptor phosphorylation studies were designed. Presumably, different agonist treatment of the receptor would cause different conformational changes and hence different phosphorylation patterns, which can be revealed by mass spectrometric analysis by using a purified protein sample after MTII (Figure 5.3) or THIQ (Scheme 5.1, \(N-[(3R)-1,2,3,4-Tetrahydroisoquinolinium-3-ylcarbonyl]-(1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine\).) treatment. THIQ is a nonpeptide hMC4R selective agonist, which was developed by Merck in 2002. This compound was developed as an MTII mimetics. It is very potent at hMC4R with IC\(_{50}\)=1.2 nM and EC\(_{50}\)=2.1 nM. Its selectivity for hMC4R is 1184-fold vs. hMC3R and 350-fold vs. hMC5R. However, the synthetic methodology described in the original paper was lengthy and the detailed reaction conditions were not available. Thus, a simple and efficient synthetic
route needs to be developed. In addition, fluorescent dye labeled THIQ derivatives were also needed for future cell imaging studies.

Scheme 5.1 Literature reported scheme for the synthesis of hMC4R selective nonpeptide agonist THIQ (1R,3R) (Reproduced from reference 153 with permission. Copyright 2002 American Chemical Society)

5.2 Results and Discussion

An extensive literature search resulted in the discovery of a commercially available intermediate 44 (Scheme 5.2) with the cyclohexyl moiety in place. Next, the carboxylic acid functional group in the molecule needs to be converted to a hydroxyl functional group. In general, there are three ways to accomplish this. One way is to do a direct reduction using highly reactive lithium aluminum hydride (LAH)\textsuperscript{154}. But the Boc protecting group may also be removed under the same conditions.\textsuperscript{155} A second way is illustrated in the original synthesis of THIQ developed by Merck (Scheme 5.1). The carboxylic acid functionality was first converted to ester functionality with the concurrent loss of the Boc protecting group. Then the ester was reduced to alcohol by
LAH in THF. Finally the Boc protecting group was reinstalled to protect the secondary amine present in the molecule.

Scheme 5.2 New scheme for the synthesis of THIQ

A third way is to use borane, which was reported in 1973 for the conversion of carboxylic acids to alcohols under very mild conditions and in the presence of many other functional groups such as ester, nitro, halogen, keto, nitrile, etc. BH$_3$/Me$_2$S was used for the reduction and the reaction was found to be very selective, clean and efficient. Thus, the alcohol product 45 was obtained in high purity and excellent yield. The following mesylation of 45 was straightforward and the reaction gave 46 in quantitative yield. The next step was a substitution type reaction. We anticipated the activation energy of the reaction to be high due to the steric hindrance of both reactants. So we tried different temperatures and it turned out that the reaction proceeded well at 130 °C. We were able to obtain the key intermediate 47 in three easy steps. The rest of the reactions were standard solution phase amide bond formation. After a series of deprotection and coupling reactions, the final product 49 (THIQ) was obtained, purified by RP-HPLC and characterized.
To label the THIQ with tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) for future cell imaging studies, a linker was needed so that the dye molecule wouldn’t interfere with the conformation and function of the THIQ. One such linker was a commercially available 7-amino-heptanoic acid. For the synthesis, THIQ was first coupled in solution phase to the Boc-7-amino-heptanoic acid via its N-terminal (Scheme 5.3). Then the Boc protecting group was removed by TFA. Finally the dye molecule was attached to the linker in the dark and the resulting product 51 was purified by preparative RP-HPLC and characterized by mass spectrometry. The choice of solvent was found to be critical for the last reaction. The reaction worked well in DMF, but not in water. Presumably, this was due to the poor solubility of the 50 (THIQ-Linker molecule) in water.

Scheme 5.3 5(6)-TRITC labeled THIQ with 7-amino-heptanoic acid as a linker

Another type of linker is Mini-PEG which is hydrophilic and nontoxic (Scheme 5.4). The use of such a link may help with the water solubility of the labeled
molecule. Boc protected Mini-PEG is also commercially available. The synthesis of the 53 was conducted in similar fashion as described above for 51.

Scheme 5.4 5(6)-TRITC labeled THIQ with Mini-PEG as a linker

5.3 Conclusions

hMC4R selective nonpeptide agonist THIQ and its fluorescent dye labeled derivatives were successfully synthesized via an improved synthetic methodology. This new methodology is efficient and easy to follow.

5.4 Future Work

PWR (Plasmon-waveguide resonance) is a highly sensitive bioanalytical method that can be used to examine protein conformational as well as mass changes. It has been demonstrated to be a valuable tool for the direct study of opioid receptor conformational changes upon different agonists binding. Future studies can be focused on the study of the conformational changes of hMC4R upon activation by MTII or THIQ by using PWR.

5.5 Experimental Section
**General Information**

All starting materials and reagents were purchased from Sigma-Aldrich and used without further purification. THF was distilled over sodium and benzophenone. Methylene chloride was distilled over CaH$_2$. $^1$H and $^{13}$C NMR spectra were recorded on Bruker DRX500 (500 MHz and 125 MHz, respectively) or Varian 300 (300 MHz and 75 MHz, respectively) spectrometers with CDCl$_3$ or TMS as internal standard. Preparative RP-HPLC was performed on a HP 1100 liquid chromatograph using a Vydac (218TP1010, 25 × 1 cm, 12 µm) column. Mass spectra were obtained from Mass Spectrometry Facility, Department of Chemistry, the University of Arizona. Flash column chromatography was performed using silica gel 60 from the EM Science. TLC plates (Silica Gel 60 F$_{254}$) were purchased from the EMD Chemicals and were visualized by UV and KMnO$_4$ stain.

**Hazards Information**

*Sodium:* Danger! Flammable, corrosive and water reactive solid. Catches fire if exposed to air. Harmful or fatal if swallowed. Harmful if inhaled or absorbed through skin.

*TFA:* Danger! Both liquid and vapor can cause severe burns to all parts of the body. Harmful if inhaled. Hygroscopic (absorbs moisture from the air). Target Organs: Respiratory system, eyes, skin, and mucous membranes.
tert-Butyl 4-cyclohexyl-4-(hydroxymethyl)piperidine-1-carboxylate (compound 45). 1.0 mmol of commercially available N-Boc-4-cyclohexylpiperidine-4-carboxylic acid 44 was dissolved in 10 mL of dry THF and the solution was cooled to 0 °C. To the solution, 6 equiv of borane dimethylsulfide was added dropwise. The resulting mixture was stirred for 12 h at room temperature. Then the reaction was quenched by slow addition of saturated aqueous NH₄Cl solution (5 mL). The product was extracted with ethyl ether (10 mL x 3) and washed with saturated aqueous NaHCO₃ solution (10 mL x 2) and brine. The ethyl ether layer was dried and evaporated to give crude 45, which was further purified by flash chromatography (Hexane:EtOAc = 8:1). Yield: 94%. R_f = 0.37 (Hexane:EtOAc = 3:1). HRMS (FAB) calcd for C₁₇H₃₂NO₃ (MH⁺) 298.2382, found 298.2390. 1H NMR (300 MHz, CDCl₃) δ 1.00–1.28 (m, 6H), 1.30–1.58 (m, 13H), 1.60–1.81 (m, 6H), 3.12–3.24 (m, 2H), 3.44–3.62 (m, 4H). 13C (75 MHz, CDCl₃) δ 26.66, 27.04, 27.29, 28.41, 28.96, 37.15, 39.50, 42.41, 64.05, 79.21, 155.04.

Boc
\[\begin{array}{c}
\text{OMs} \\
\end{array}\]

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tert-Butyl 4-cyclohexyl-4-((methylsulfonyloxy)methyl)piperidine-1-carboxylate (compound 46). 1.0 mmol of 45 and 2 mmol of TEA was dissolved in 10 mL of dry THF. The solution was cooled to 0 °C before 1.2 mmol of methanesulfonyl chloride was added slowly with good agitation. The resulting mixture was warmed to room temperature and the stirring was continued for 2 h. Then 6 mL of saturated aqueous
NaHCO₃ solution was added and the product was extracted with ethyl acetate (10 mL x 3). The combined organic layer was washed with brine, dried and concentrated to give compound 46 in 98% yield. \( R_f = 0.78 \) (Hexane:EtOAc = 1:1). The product was used for the next step without further purification. HRMS (FAB) calcd for C₁₈H₃₄NO₅S (MH⁺) 376.2158, found 376.2155. \(^1\)H NMR (300 MHz, CDCl₃) \( \delta \) 1.00–1.28 (m, 6H), 1.30–1.82 (m, 19H), 3.00 (s, 3H), 3.20–3.30 (m, 2H), 3.44–3.60 (m, 2H), 4.18 (s, 2H).

\[ \text{NBoc} \]

**tert-Butyl 4-((1H-1,2,4-triazol-1-yl)methyl)-4-cyclohexylpiperidine-1-carboxylate (compound 47).** 1.0 mmol of 46 and 2 mmol of sodium 1,2,4-triazeole was added to 10 mL of dry DMF. The solution was heated to 130 °C and kept for 24 h. Then the solution was cooled to room temperature and diluted with 50 mL of water. The mixture was extracted with ethyl acetate three times. The combine organic phases were washed with brine and dried over anhydrous MgSO₄. Removal of ethyl acetate afforded crude compound 47, which was further purified by flash chromatography (Hexane:EtOAc = 3:1 – 1:1). Yield: 90%. \( R_f = 0.25 \) (Hexane:EtOAc = 1:1). HRMS (ESI) calcd for C₁₉H₃₃N₄O₂ (MH⁺) 349.2604, found 349.2592. \(^1\)H NMR (300 MHz, CDCl₃) \( \delta \) 0.96–1.54 (m, 17H), 1.60–1.82 (m, 7H), 3.40 (t, 4H, \( J = 6.3Hz \)), 4.18 (s, 2H), 7.93 (s, 1H), 8.04 (s, 1H). \(^{13}\)C (125 MHz, CDCl₃) \( \delta \) 26.29, 26.41, 27.00, 28.39, 29.64, 37.83, 40.29, 52.42, 79.47, 144.09, 151.08, 154.84.
(R)-tert-butyl 1-((1H-1,2,4-triazol-1-yl)methyl)-4-cyclohexylpiperidin-1-yl)-3-(4-chlorophenyl)-1-oxopropan-2-ylcarbamate (compound 48). 1.0 mmol of compound 47 was dissolved in 5 mL of 50% TFA in DCM. The solution was stirred at room temperature for 1 h before the volatiles were completely evaporated under reduced pressure. Then the residue was dissolved in a mixture of DCM and DMF (DCM, 2 mL; DMF, 2 mL). And 1 mmol of Boc-D-Phe(pCl)-OH, 8 equiv of DIPEA, 1.05 equiv of HBTU and HOBT was added to the above mixture. The reaction was kept for 1 h before 10 mL of 5% aqueous NaHCO₃ was added. The product was extracted with ethyl acetate and washed with 5% aqueous NaHCO₃ (2 times), 5% aqueous citric acid (2 times), water, brine and dried over MgSO₄. Evaporation of the solvent gave crude compound 48, which was used in the next step without further purification. HRMS (FAB) calcd for C₂₈H₄₁ClN₅O₃ (MH⁺) 530.2898, found 530.2891.

¹H NMR (500 MHz, CDCl₃) δ 0.80–1.00 (m, 2H), 1.02–1.50 (m, 16H), 1.60–1.72 (m, 4H), 1.76–1.88(m, 2H), 2.85–3.00 (m, 2H), 3.10 (m, 1H), 3.30–3.80 (m, 3H), 4.09 (s, 1H), 4.15 (s, 1H), 4.80 (m, 1H), 7.08–7.30 (m, 5H), 7.89 (s, 1H), 7.96 (s, 1H).
THIQ (49): N-[(3R)-1,2,3,4-Tetrahydroisoquinolinium-3-ylcarbonyl](1R)-1-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1H-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine. Boc-D-Tic-OH was coupled to compound 48 in the same way as described above. After work-up, the Boc protecting group of the precursor of THIQ was removed in 50% TFA in DCM to give crude THIQ, which was further purified by RP-HPLC to give pure THIQ (49). HRMS (FAB MH⁺): calcd for C₃₃H₄₂ClN₆O₂ 589.3058, found: 589.3049. Rf = 0.63 (CHCl₃:MeOH = 8:1), [α]²⁴_D = +12.19° (c=1.0, MeOH). ^1H NMR (500 MHz, CDCl₃) δ 0.80–0.98 (m, 2H), 1.05–1.45 (m, 8H), 1.52–1.71 (m, 4H), 1.75–1.90 (s, 2H), 2.80–3.50 (m, 8H), 4.09 (s, 1H), 4.15 (s, 1H), 4.38–4.51 (m, 3H), 4.96–5.10 (m, 1H), 6.85–7.30 (m, 8H), 7.91 (s, 1.52H), 8.00 (s, 0.48), 8.17 (s, 0.39H), 8.40 (s, 0.61H).
5(6)-TRITC labeled THIQ with 7-amino-heptanoic acid as a linker (compound 51). 18 mg (0.0306 mmol) of 49 (THIQ) was coupled with 8.3 mg (0.0338 mmol) of 7-(Boc-amino)-heptanoic acid using 1.1 equiv of HBTU/HOBt, 2 equiv of NMM in 1.0 mL of DMF. Upon completion of the coupling, 10 mL of ethyl acetate was added to the reaction and the resulting mixture was washed with 5% aqueous sodium bicarbonate (5 mL x 2), followed by 5% aqueous citric acid (5 mL x 2) and bring (5 mL x 1). The organic layer was dried over anhydrous MgSO₄ and the volatiles were removed under vacuum. Then to the residue was added 2 mL of 50% TFA in DCM. Volatiles were removed completely under vacuum after 1 h. The residue was then dissolved in 1.0 mL of DMF and was added 20 mg (0.045 mmol) of the tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) in 2 mL of DMF and 5 equiv of NMM. The mixture was stirred for 24 h in dark at room temperature. RP-HPLC purification afforded compound 51 in 42.3% yield from THIQ. MH⁺(ESI) calcd for C₆₅H₇₆ClN₁₀O₆S, 1159.5359, found 1159.5411. HPLC retention time: 13.6 min (CH₃CN/H₂O: 40/60–90/10 in 20 min, 4.0 mL/min, 254 nm).

5(6)-TRITC labeled THIQ with Mini-PEG as a linker (compound 53).

24 mg (0.0406 mmol) of 49 (THIQ) was coupled with 11.8 mg (0.0447 mmol) of Boc-Mini-PEG using 1.1 equiv of HBTU/HOBt, 2 equiv of NMM in 1.0 mL of DMF.
Upon completion of the coupling, 10 mL of ethyl acetate was added to the reaction and the resulting mixture was washed with 5% aqueous sodium bicarbonate (5 mL x 2), followed by 5% aqueous citric acid (5 mL x 2) and brine (5 mL x 1). The organic layer was dried over anhydrous MgSO$_4$ and the volatiles were removed under vacuum. Then to the residue was added 2 mL of 50% TFA in DCM. The volatiles were removed completely under vacuum after 1 h. The residue was dissolved in 1.0 mL of DMF and was added 20 mg (0.045 mmol) of the tetramethylrhodamine-5-(and-6)-isothiocyanate (5(6)-TRITC) in 2 mL of DMF and 5 equiv of NMM. The mixture was stirred for 24 h in dark at room temperature. RP-HPLC purification afforded compound 53 in 27.2% yield from THIQ. MH$^+$ (ESI) calcd for C$_{64}$H$_{74}$ClN$_{10}$O$_8$S 1177.5100, found 1177.5111. HPLC retention time: 18.6 min (CH$_3$CN/H$_2$O: 20/80–90/10 in 25 min, 4.0 mL/min, 254 nm).
APPENDIX

$^1$H and $^{13}$C NMR Spectra of New Compounds

![NMR Spectra of New Compounds](image)
Chapter 3
[Chemical structures and spectra images]
Chapter 4

Peptide 31 Ac-Nle-c[Asp-His-D-Phe-Pro-Trp-Lys]-NH$_2$
Peptide 32 Ac-Nle-c[Asp-His-D-Phe-trans-4-GPro-Trp-Lys]-NH$_2$

Peptide 32 (with increased spectrum window)
Peptide 33 Ac-Nle-c[Asp-His-D-Phe-cis-4-GPro-Trp-Lys]-NH₂

Peptide 33 (with increased spectrum window)
Peptide 34 \text{Ac-Nle-c[Asp-trans-4-GPro-D-Phe-Arg-Trp-Lys]-NH}_2
Peptide 35 Ac-Nle-c[Asp-cis-4-GPro-D-Phe-Arg-Trp-Lys]-NH₂
Peptide 36 Ac-Nle-c[Asp-His-D-Nal(2')-Pro-Trp-Lys]-NH$_2$
Peptide 37 Ac-Nle-c[Asp-His-D-Nal(2')-trans-4-GPro-Trp-Lys]-NH₂

Peptide 37 (with increased spectrum window)
Peptide 38 Ac-Nle-c[Asp-His-D-Nal(2')-cis-4-GPro-Trp-Lys]-NH$_2$
Peptide 39 Ac-Nle-c[Asp-trans-4-GPro-D-Nal(2')-Arg-Trp-Lys]-NH$_2$
Peptide 40 Ac-Nle-c[Asp-cis-4-GPro-D-Nal(2')-Arg-Trp-Lys]-NH$_2$
<table>
<thead>
<tr>
<th>Compound</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>NHBoc</td>
</tr>
</tbody>
</table>

**NMR Data**

- **Solvent**: DMSO-d$_6$
- **Resonance Values**
  - 1.2 ppm
  - 1.5 ppm
  - 1.8 ppm
  - 2.0 ppm
  - 2.3 ppm
  - 2.5 ppm
  - 3.0 ppm
  - 3.5 ppm
  - 4.0 ppm
  - 4.5 ppm
  - 5.0 ppm
  - 5.5 ppm
  - 6.0 ppm
  - 6.5 ppm

**Chemical Shifts**

- 1H (1.2 ppm) 1.5 ppm 1.8 ppm 2.0 ppm 2.3 ppm 2.5 ppm 3.0 ppm 3.5 ppm 4.0 ppm 4.5 ppm 5.0 ppm 5.5 ppm 6.0 ppm 6.5 ppm

**Additional Information**

- **Temperature**: 298 K
- **Field**: 400 MHz
- **Resolution**: 0.02 ppm
- **Precision**: 0.001 ppm
- **Sensitivity**: 1000 ppm

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**Notes**

- Fine-tuned for maximum clarity and readability.
REFERENCES


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(a) Morimoto, Y; Takaishi, M; Kinoshita, T; Sakaguchi, K; and Shibata, K. Synthesis and absolute configuration of lactone II isolated from Streptomyces sp. Go
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92 A major byproduct (usually <5% yield) in the rearrangement was isolated by flash column chromatography. The compound was identified as 28Z (see experimental section) by NMR and mass spectrometry.

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112 Van der Kraan, M.; Adan, R. A.; Entwistle, M. L.; Gispen, W. H.; Burbach, J. P.; and Tatro, J. B. Expression of melanocortin-5 receptor in secretory epithelia supports


136 The Mitsunobu reaction gives a mixture of tran- and cis- 4-bromo-substituted analogue which can be separated by flash column chromatography.


