THE DESIGN AND SYNTHESIS OF IMINE-BRIDGED CYCLIC PEPTIDES AND THE SOLID PHASE SYNTHESIS OF β-TURN MIMETICS

by

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DEDICATION

To my loving husband, my soul and heart.
To Winston and Olivia, whose humor, wonderment, and faith
have sustained me.
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ABBREVIATIONS

AcOH - Acetic Acid
APA - Alanine-prolyl aldehyde
Boc-t-Butoxycarbonyl CMA - Chloroform/Methanol/Acetic Acid (90/30/10)
BOP - Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate
cPPI 1 - cyclic pentapeptide imine, [Ala-Ala-Tyr-Pro-Phe] from N^α-Fmoc protection
cPPI 2 - cyclic pentapeptide imine, [Ala-Ala-Tyr-Pro-Phe] from N^α-Boc protection
cTPI - cyclic tripeptide imine, [Tyr-Pro-Phe]
DCM - Dichloromethane
DIBAH - Diisobutyl Lithium Aluminum Hydride
DIPA - Diisopropylethylamine
DMF - Dimethylformamide
EtOAc - Ethyl Acetate
Fmoc - Fluorenylmethoxycarbonyl Hex-Hexane
HBTU - 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt - N-Hydroxybenzotriazole
LAH - Lithium Aluminum Hydride
MeOH - Methanol
NMM - N-methylmorpholine
TAA - Trialanyl aldehyde
TEA - Triethylamine
TFA - Trifluoroacetic acid
TIPS - Triisopropylsilane
Pain is perhaps the most unpleasant sensation humans experience. While pain is important in preventing further injury and as a detection mechanism for ill-health; effective relief of pain, especially chronic or neuropathic, is a critical quality of life issue. As discoveries are made regarding opioid receptor-acceptor-ligand interactions, ligands that bind specifically to a receptor subtype can modify behavior without triggering side effects. Topographical space control is an important consideration in ligand design. According to Ramachandran\(^1\), \(\alpha\)-amino acids are confined to the following low-energy conformations, \(\alpha\)-helix, \(\beta\)-sheets, extended structures, and \(\beta\)-turns. Endogenous peptides such as the enkephalins and endomorphins activate the opioid receptor subtypes, \(\delta\)- and \(\mu\)-, respectively, and are involved in the pain cascade. Research herein concerns the synthesis of \(\delta\)-opioid peptidomimetics that replace the Gly-Gly unit of Leu-enkephalins with a \(\beta\)-turn mimetic moiety. Additionally, using the bioactive nostocyclopeptolides as templates, smaller cyclized imino-bridged peptides were designed. These peptides were developed with the \(\mu\)-opioid subtype requirements in mind.
Chapter 1

INTRODUCTION OF OPIOID LIGAND SYNTHESIS

As Prof. Victor J. Hruby states in his Ralph F. Hirschmann Award address, “Since nature has chosen peptides and proteins to do the bulk of the work, that is, work involving chemistry and structures needed for living systems, in conjunction with lipids, nucleic acids, sugars, and the rest of the periodic chart, it seems reasonable to ask why and how.” Using current models to design bioactive peptides or peptidomimetics should result in ligands that bind with high specificity for targeted receptor subtypes. Subsequently, knowledge gained from these receptor-acceptor-ligand interactions can help answer “how and why.”

1.1 A brief history of drug design for opioid receptors

Opioid receptors are part of the rhodopsin G protein-coupled receptor (GPCR) superfamily, typified by seven transmembrane helices. Opioid receptors are responsible for modulating biological behaviors such as pain, feeding behavior, pigmentation, fear-flight, addiction, sexual behavior, stress response, cardiovascular function, cognition and learning, etc. There have been three opioid receptor subtypes, δ-, μ-, and κ- identified. All three receptor subtypes are expressed in the central nervous system including the medulla and spinal cord. μ and κ subtypes are expressed on the wall of the gastrointestinal tract, stomach, and proximal colon. The δ subtype is widely expressed on myenteric neurons and submucous ganglia. This current research focuses on the δ- and μ-opioid receptor subtypes.

Initial drug design for the opioid receptor system focused on the natural products isolated from plants that showed opioid receptor activity, namely morphine and its analogues. While these natural products caused desirable analgesia, they are also responsible for the undesirable side effects including tolerance and dependence, constipation, respiratory depression, and cardiotoxicity. Once opioid receptor subtypes were discovered, research focused on developing subtype-specific modified morphine analogues that would bind with high affinity
This line of research has added much to the current models of the opioid receptor subtype systems.

In 1975 endogenous opioid ligands, namely, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu-OH) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met-OH) were discovered. Enkephalins bind to the δ- and μ-receptors with high affinity, and could be used to further enhance current understanding of the receptor system. Enkephalin peptide sequences were modified systematically by addition, deletion, or replacement of key amino acids to elucidate the pharmacophore. Steric constraints were introduced to probe side chain 3-D spatial requirements. μ-selective endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) and -2 (Tyr-Pro-Phe-Phe-NH₂) were discovered in 1997 and showed high affinity for the receptor, as well. Similar systematic approaches have been applied to the endomorphins, and dynorphins (κ-selective endogenous peptides).

§ 1.2 Peptidomimetics

Peptides are short segments of amino acids linked via amide bonds (refer to Figure 1.1). Peptides are found endogenously as hormones, neurotransmitters, enzymes, immune system modulators, and growth factors among other biologically active substrates.

Using native biologically active peptides in drug design has limited viability due to the low oral availability, proteolytic breakdown, and biodegradation that leads to short duration of action that plague many, many natural peptides. One approach to overcome the issues regarding peptides has been to pursue the development of peptidomimetics. Peptidomimetics are
organic molecules, either peptide or non-peptide moieties, that affect the same biological responses upon interaction with the same receptor or acceptor. In other words the key pharmacophore of the native peptide and peptidomimetic should share common elements. Peptidomimetics are an attractive research avenue because substitution of unnatural amino acids, primarily D-amino acids, at key sites in the pharmacophore often stabilizes the receptor-ligand interaction and increases potency without affecting selectivity. Additionally, topographical space can be controlled by introducing conformational constraints into the peptidomimetic structure. Advantages to conformationally-constrained peptidomimetics include smaller size, increased binding affinity, and greater receptor selectivity than the native biologically active peptide. Peptidomimetics show greater stability against proteolytic enzymatic degradation and can penetrate membrane barriers more effectively.

§ 1.3 Topographical Space

Control of $\phi$, $\psi$, and $\chi$ angle and $\chi'$ space (Figure 1.2) has proven useful in determination of receptor model development and de novo peptide ligand design. Constraining $\chi'$ space so that
amino acids in the pharmacophore assume the preferred low energy conformations (i.e. gauche (+)-[g(+)—+60°, gauche (-)-[g(-)—060°, trans —±180°) has resulted in increased affinity and potency for peptide ligands. Considerations when undertaking de novo peptide design are that side chain groups may assume preferred conformations; constraint of χ space/angle must be compatible with backbone conformation (secondary structure); and the constraint must be consistent with binding kinetics18. In other words for success constraint of the molecule must not interfere with the ability of the ligand to bind effectively with the receptor or exist in its preferred low energy conformations.

§ 1.4 Agonists and Antagonists

Opioid ligands can be classified as agonists or antagonists. Structural differences between the two often are modest.23,24 Minor changes in atoms, bonds, or torsional angles can have a substantial effect on binding, bioactivity, potency, affinity for receptor subtype, etc.18 Agonists and antagonists bind at the same binding site with good affinity for the receptor. Agonists stabilize the receptor in its active form, while antagonists stabilize the receptor in its inactive form, and compete with agonists for the receptor site. Once bound antagonists prevent agonists from binding to the site. Current research25,26 suggests that the bound receptor-agonist complex differs structurally from the receptor-antagonist complex.3

§ 1.5 β-Turn Conformations

Understanding topographical space considerations is critical to design bioactive ligands that are potent and selective. Many bioactive ligands assume β-turn secondary structures that are essential to their bioactivity.27 A β-turn consists of a nonrepeating tetrapeptide sequence which has a distance of not less than 7Å between Cαi−1-Cαi+3 often stabilized by a hydrogen bond (see Figure 1.3). β-Turns are classified into subtypes by the geometries of the peptidic backbone of the central dipeptide (Cαi+1, Cαi+2).22
De novo peptide design encompasses β-turn peptidomimetics due to the improved bioavailability, duration of action, and oral availability found lacking in peptide analogues that elicit the same biological response. If the β-turn is stabilized by a hydrogen bond, as shown in Figure 1.3, a ten-membered ring results. Approaches to development of β-turn mimetics include mimicking the turn with comparable structures of similar ring size; replacing the ten-membered ring with hetero-bicyclic ring systems, spirolactams, azobicyclic alkanes, or cyclooctapyrroles. As noted in Figure 1.4, the geometries of the backbone substrate have been calculated, and the appropriate turn type has been assigned for these privileged structures. Endogenous peptides such as the enkephalins are conformationally flexible due to the Gly2-Gly3 unit, and numerous β-turn conformations (Type I, I', II, and III) in various media have been experimentally observed. Replacement of this Gly2-Gly3 flexible unit with a rigid, “defined,” beta-turn bicyclic moiety can assist in the elucidation of the conformation necessary for the bioactivation of the receptor-ligand complex. Replacement of the Gly2-Gly3 with the privileged [3.3.0] BTD[s] scaffold has been explored and will be discussed in Chapter 2 of this thesis.

Figure 1.3 β-turn definition
§ 1.6 External and Internal β-Turn Syntheses

The solid phase syntheses of β-turn mimetics can be categorized as internal or external paradigms (Figure 1.4a). In the internal model the solid support is internalized within a turn defined by covalent bonds. The side chains (R and R') are flexible and the available conformations of these side chains are not readily controlled or defined. However, it is easier to introduce desired amino acids in these positions due to the flexibility of the structure, making this an attractive option for development of new β-turn mimetics. External β-turn mimetics have the solid support external to the turn with the amide linkage of R'-1-R'-2 participating in the solid support.
attachment. The $\chi$ space is better defined and perhaps more predictable. The $\phi$ and $\psi$ space of the backbone conformation is constrained, as well, allowing better interpretation of the receptor-ligand complex and any resulting bioactivity. 5,5-$\beta$-turn dipeptides have been shown to have Type II geometries and are highly constrained. Leu-enkephalins have been experimentally shown to assume a type II geometry resulting in bioactivity. Replacing the Gly$^2$-Gly$^3$ with a highly constrained 5,5-BTD$^{20,21}$ should yield valuable information regarding receptor-ligand complex requirements necessary for bioactivity. Additionally, the constraint of $\phi$, $\psi$, and $\chi$ space should impart specificity for the $\delta$-opioid receptor, and increase affinity for the receptor subtype.$^{18}$

§ 1.7 $\mu$-Opioid Receptor Model

Morphine, a plant-derived $\mu$-agonist, has been the center of much research regarding opioid receptor-ligand complexes.$^3$ According to mutation studies of the opioid receptors, Asp147 in TM III is the key binding site of the cationic amine in $\mu$-selective ligands.$^{45}$ Structure-activity relationship studies of the systematic expression of chimeric receptors of $\mu$- and $\delta$-subtypes yielded insight into the pivotal domains in the transmembrane proteins thought to be responsible for activation, affinity, and potency for the receptor subtype. From these studies TM V-VII domains were proposed to be involved with morphine recognition and binding at the $\mu$-receptor.$^{44}$ The docking model of morphine to the $\mu$-opioid receptor in Figure 1.5 was elucidated via affinity labeling and molecular mechanics studies.$^{45}$

Figure 1.5 Morphine-$\mu$-Opioid Receptor Complex
Particularly significant for binding are the ionic interactions of the N-methylated amino group of the piperidine ring to Asp147 in TM III; the interaction of the phenolic ring of tyrosine and Tyr299 in TM VI; and the hydrogen bonding of the tyrosine hydroxy group to Lys303 (TM VI) and Tyr148 (TM III). One theory suggests that small ligands and peptidomimetics may be able to bind at different positions within the binding pocket that improve selectivity and potency.\(^9\) Other docking models have been proposed that illustrate this point.

\section{1.8 \(\delta\)-Opioid Receptor Model}

Biological activation of the opioid receptors requires a hydrophobic group, a cationic amine, and a phenol group.\(^{45}\) DPDPE\(^{47}\) and JOM-13\(^{48}\) are conformationally constrained potent \(\delta\)-opioid agonists. A representative docking model of the JOM-13-\(\delta\)-opioid receptor complex is shown in Figure 1.6. In this docking model the cationic N-terminal of JOM-13 interacts with Asp128 on TM III; the phenolic side chain of tyrosine interacts with the imidazole ring of His278 on TM VI, and forms \(\pi-\pi\) interactions with the phenolic ring of Tyr129 at TM III; the anionic C-terminal interacts with Lys214 on TM V; and the hydrophobic phenyl of phenylalanine interacts with a multiple number of hydrophobic amino acids on TM III and TM VII.\(^{46}\) The tyrosine moiety is critical for signal transduction at the receptor and is known as the “message” portion of the ligand. Tyrosine is essential for activation of opioid receptors. Phenylalanine is known as the “address” portion of the ligand, and is responsible for binding affinity to opioid receptors. In JOM-13 the flexible Gly\(^2\)-Gly\(^3\) spacer is replaced by the highly constrained disulfide ring, which is thought to impart JOM-13’s selectivity for the \(\delta\)-opioid receptor over the \(\mu\)- and \(\kappa\)-opioid receptors.\(^{45a, 46b}\)

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{Figure1.6.png}
\caption{JOM-13-\(\delta\)-Opioid Receptor Complex}
\end{figure}
§ 2.1 Introduction

One design approach to synthesize more selective, potent opioid agonists has been to replace the spacer Gly²-Gly³ unit of the endogenous enkephalin scaffolds with a conformationally constrained cyclic unit. Our group has investigated replacing the i+1 and i+2 amino acids of the β-turn with a constrained β-turn dipeptide scaffold to stabilize the turn. Our group has developed several different methodologies for β-turn dipeptides such as using β-substituted (R₁) γ,δ- and δ,ε-unsaturated amino acids to introduce side chain groups for [5,5]- and [6,5]-N,S-acetal bicyclic dipeptides, and the C-scaffold bicyclic system (X=CH₂) from the glutamic amino acid derivatives (Figure 2.1). To be successful the scaffold needs to a) provide sites for side chains, b) position the amide bond similar to that of Gly²-Gly³ in Leu-enkephalin, c) allow incorporation into a peptide chain as replacement for positions 2 and 3.

![Figure 2.1 Bicyclic dipeptide scaffold](image)

It is also necessary that solid phase synthesis using this scaffold must be practical. The reason for this is twofold: syntheses of enantiomerically-pure functionalized β-turn scaffolds are problematic due to low yields characteristic of multistep strategies, and it is difficult to deliver all of the possible diastereomers in solution phase synthesis. We have been able to introduce desired side chains into the β-turn mimetic using β-substituted cysteine (R₂). To overcome the diastereomer problem, our group developed successful solid phase methodologies that use
an external β-turn paradigm. This approach seemed the most reasonable as not only the φ and ψ dihedral angles on the backbone, but also, the χ1 and χ2 space of the desired side chains could be controlled. As stated in Chapter 1, it is essential to understand/control the χ torsional angles of the pharmacophore in order to bias the molecule for a particular opioid subtype, increase potency, and gain as much understanding about the binding complex as possible. From our current understanding of the δ-opioid binding pocket, requirements for receptor activation are that the ligand must have a cationic amino group, a phenolic group, and a hydrophobic group. Below in Figure 2.2 are schematic representations of JOM-13’s interaction with the δ-opioid receptor, and the proposed docking model of [3.3.0]-BTD-Leu-enkephalin with the δ-opioid receptor. JOM-13 is a potent δ-selective opioid agonist. It is the purpose of this research to use our knowledge of χ space to develop ligands equally or more potent and selective than known analogues such as JOM-13. As can be observed in both models, the message portion of the ligand is satisfied by a tyrosine, the address portion is a phenylalanine, the cationic amino group is the amino terminal of the molecule, and the spacer has been replaced by a conformationally constrained ring system.

It is important to note that the JOM-13 spacer is an eleven-membered monocyclic ring with greater flexibility than the highly constrained central bicyclic ring of [3.3.0]-BTD-Leu-enkephalin analogues. While many have reported that conformational constraint has led to increased affinity, potency, and selectivity for the receptor; disturbances in the ionic interactions with either the anionic carboxyl terminal or cationic amino terminal have significantly decreased the affinity of JOM-13 to the δ-receptor. Selective, potent binding of our ligands will greatly depend upon the abilities of these ligands to fit into the binding pocket. The [3.3.0]-BTD-Leu-enkephalin analogues may prove to be too highly constrained or too bulky, not allowing for ionic interactions of the terminals, π-π interactions of the tyrosine, etc. Binding results for MVD and GPI studies will be discussed later in the context of these docking models.
Figure 2.2 δ-Opioid Binding Pocket

JOM-13 –δ-Opioid Receptor Complex

[3.3.0]-BTD-Leu-enkephalin-δ-Opioid Receptor Complex
§ 2.2 Retrosynthetic analysis of [3.3.0]-BTD\[^{[3,3]}\]-Leu-enkephalin analogue

Our group selected an external BTD inserted peptide design because topographical space could be controlled and defined more easily.\(^{50}\) The backbone (i+1, i+2) and side chain positions remain relatively unchanged in this approach when compared with the original β-turn (Scheme 2.1).

Scheme 2.1 Restrosynthetic analysis of BTD-Leu-enkephalin analogues

This strategy requires the synthesis of two commercially unavailable protected amino acids. A β-substituted ω-aldehyde is needed for the bicyclic ring formation, and is one synthetic step
removed from the commercially available corresponding β-substituted allylic amino acid. Side chain insertion at the i+2 position can be accomplished by using a β-substituted cysteine. In the case of the [3.3.0]-BTD[2-3]-Leu-enkephalin analogues no side chains were introduced at this position, but for future generations of the BTD leu-enkephalin analogues our group has developed well defined methodologies. For total synthesis of the Leu-enkephalin analogues non-standard solid phase methodologies were developed and are illustrated in Scheme 2.2.

Scheme 2.2 BTD formation on solid phase
Firstly, simultaneous deprotection of the N^α-Fmoc and S-Fm groups is achieved by a high concentration of piperidine. Secondly, reacting the β-substituted ω-aldehyde with the amino terminal of the bound substrate forms the imine. Lastly, the N,S-thiazolidine is formed by nucleophilic attack of the cysteinyl sulfur on the imino carbon. This strategy allows for all possible chiralities and different ring sizes; reduces purification issues; and increases yields to respectable levels.

§ 2.3 Syntheses of novel amino acids

There are two commercially unavailable unnatural amino acids necessary for the synthesis of the β-turn dipeptide, namely, N^α-Fmoc-Cys-S^β-Fm and N^α-Fmoc-2-amino-4-oxo-butyric acid. In the initial design both the amino terminal and the S of Cys were protected with Fmoc, however, this proved to be untenable due to the instability of the S^β-Fmoc group, and deprotection of both groups in one step was muddled. The S^β-Fm protection was achieved by methods found in current literature (Scheme 2.3).

An important observation to make is that while the original design of the BTD total synthesis did not purify the intermediates in order to conserve product; it was necessary to purify all of the intermediates using D-amino acids as starting materials, gel-like crude products were
obtained. This is thought to be due to possible racemization or contamination of the original D-amino acids. NMRs were taken of all of the starting materials and the D-Cys did show minor contamination in one instance, but other lots appeared "clean." The products were recrystallized in 1N HCl. The final product was purified using flash column chromatography in 72% yield. This product is insoluble in CHCl₃ and has limited solubility in MeOH, and is very soluble in DMF. Contaminants were removed using 8:1, 3:1, and 1:1 hexanes:ethyl acetate mixtures, and the final product was moved off the column with MeOH. The solvent was removed in vacuo and the product was precipitated.

2-Amino-4-oxo-butyric acid was synthesized from commercially available L-2-amino-4-pentenoic acid. The N²-Fmoc protection was completed in acetone/water (1/1, v/v) mixture and the crude product was used without purification (Scheme 2.4). The aldehyde was formed via osmylation/periodation in 76% yield crude, and was used without further purification (Scheme 2.5).

It has been previously reported that the aldehyde forms a diastereomeric lactone that is stable at room temperature, and maintains the reactivity of the γ-aldehyde.³⁰
§ 2.4 [3.3.0]-BTD^{23}-Leu-enkephalin analogues

Pre-loaded Leu-Wang resin was used as the solid substrate with a 0.68 mmol/g loading sites. The pre-loaded resin was used because loading of the first amino acid is critical, and is usually slow with incomplete esterification. The resin was swelled overnight in DCM/DMF (1/1 v/v). N$_{\alpha}$-Fmoc-Phe-OH was coupled using conventional methods and coupling was confirmed using the Kaiser test. Following deprotection with piperidine, N$_{\alpha}$-Fmoc-Cys[S$_{\beta}$-Fm] was coupled. To avoid racemization of cysteine upon coupling, a DCM/DMF mixture (1/1 v/v) was used, followed by the addition of cysteine (3 eq), HOBt-HBTU coupling reagents (3 eq each), then, DIEA (6 eq) in that order. Racemization was not observed in the (3S, 5R/S, 8S)-[3.3.0]-BTD^{23}-Leu-enkephalin analogues. Minor racemization was observed in the (3R, 5R/S, 8S)-[3.3.0]-BTD^{23}-Leu-enkephalin analogues. Deprotection of N$_{\alpha}$-Fmoc and S$_{\beta}$-Fm groups was accomplished by using a high concentration of piperidine (50% piperidine with 5% triisopropyl silane-TIPS) in DMF twice (1 x 5 min., 1 x 25 min.). TIPS was added as a scavenger to ensure reaction completion in 30 min. The Kaiser test was negative due to free sulfur reacting with the Kaiser reagent (Figure 2.3) to prevent the formation of the expected final product (resulting in Ruhemann’s purple).

![Figure 2.3 Cysteine-Ninhydrin product formation](image)

Ruhemann’s Purple

**Figure 2.3** Cysteine-Ninhydrin product formation
The Kaiser test was used at this junction in a reverse manner in that the presence of Ruhemann's purple indicated an incomplete deprotection of the $S^\beta$-Fm group.

The osmylation and periodation was completed one day previous to peptide synthesis. The product was stored overnight in a darkened hood at room temperature in an Al° covered reaction vessel. 2-amino-4-oxo-butyric acid (4 eqs.) and DIEA (2 eqs.) were dissolved in DMF and coupling was allowed to proceed under Ar° for 2 hours to form the N,S-acetal thiazolidine. Coupling reagents (1.5 eqs.), HOBT and HBTU, and DIEA (3 eqs.) were added for 45 minutes to form the second ring of the BTD. The $N^\alpha$-Fmoc group was deprotected twice (1 x 5 min., 1 x 25 min.) with 40% piperidine in DMF. A concentrated solution of piperidine was necessary to ensure complete deprotection. $N^\alpha$-Fmoc-Tyr[O-t-Bu]-OH was coupled conventionally. The pentapeptide was completely cleaved from the Wang resin with TFA/H$_2$O/TIPS (9.0/0.5/0.5) with continuous shaking for 1 hour. The peptide was filtered and washed with the TFA mixture. The TFA was evaporated in vacuo, diethyl ether was added, and the residual TFA-ether mixture was evaporated. This procedure was repeated until TFA could no longer be detected. The peptide/H$_2$O mixture was dissolved in diethyl ether and the pH was adjusted to pH 4-6 with ammonium hydroxide. The peptide was insoluble in water, but very soluble in ether, so, precipitation with ether was not possible. The peptide was concentrated and a sample was analyzed with low resolution MS. The sample was clean and the molecular ion peak [MH$^+$ = 626.26] was the only significant peak observed. The peptide was lyophilized and the isomers were separated on HPLC. The resulting diastereomeric ratio for the (3S, 5R/S, 8S)-BTD-Leu-enkephalin isomers was 24:33. Overall, the yield of crude peptide was ~38% of total product. The resulting diastereomeric ratio for the 3R,5R,8S-BTD$^{3\beta}$-Leu-enkephalin:3R,5S,8S-BTD$^{3\beta}$-Leu-enkephalin:3S,8S-BTD$^{3\beta}$-Leu-enkephalin isomers was 22:31:10. After careful consideration it is thought that the 2nd minor product results from the racemization of starting material, namely, d-allyl glycine. Unfortunately, the yields for the last isomer were too low to allow characterization. These isomers account for approximately 35% of the crude product. The total synthetic strategy and reaction conditions are listed in Scheme 2.4.
i. 25% piperidine in DMF; ii. N\textsuperscript{\alpha}-Fmoc-Phe-OH, HBTU, HOBt, DIEA; iii. Same to i; iv. N\textsuperscript{\alpha}-Fmoc-Cys\textsuperscript{S\textsuperscript{\beta}}[Fm]-OH, DCM/DMF, HBTU, HOBt, DIEA; v. 50% piperidine in DMF, 5% TIPS; vi. D-N\textsuperscript{\alpha}-Fmoc-2-amino-4-oxobutyric acid, DIEA, DMF; vii. HBTU, HOBt, DIEA; viii. 40% piperidine in DMF; ix. N\textsuperscript{\alpha}-Fmoc-Tyr[O-t-Bu]-OH, HBTU, HOBt, DIEA; x. same to i; xi. 90% TFA, 5% H\textsubscript{2}O, 5% TIPS.

Figure 2.4 Total Synthesis of (3R, 5R, 8S)- and (3R, 5S, 8S)-[3.3.0]-BTDi\textsuperscript{R3}Leu-enkephalin by N\textsuperscript{\alpha}-Fmoc based solid phase synthesis.
§ 2.5 Purification, characterization, and configuration determination, NMR, etc.

The complete synthesis of BTD\(^{2,3}\)-Leu-enkephalin diastereomeric isomers takes about 11 hours. The isolation is challenging as the retention times for the isomers are very close. The purification was performed on a C-18 reverse phase prep HPLC column using a gradient method. The gradient started at 23% (0.1% TFA to acetonitrile) and increased to 32.5% over 38 minutes. The synthetic methodology listed in Figure 2.6 was used to synthesize 4 diastereomers (3S,5S,8S)- and (3S, 5R, 8S)- and (3R,5S,8S)- and (3R, 5R, 8S)-BTD\(^{2,3}\)-Leu enkephalin. The same gradient method for purification was used to separate all 4 diastereomers.

§ 2.6 Structural and biological activity

Table 2.1 \(\delta\)- and \(\mu\)-binding activity profile for [3.3.0]-BTD\(^{2,3}\)-Leu enkephalin isomers

<table>
<thead>
<tr>
<th>Peptide Analogues</th>
<th>MVD nM IC(_{50}) ± s.e.m. or percent inhibition</th>
<th>GPI/LLMP nM IC(_{50}) ± s.e.m. or percent inhibition</th>
<th>DPDPE Agonist activity at 1 (\mu)M in the MVD</th>
<th>PL-017 Agonist activity at 1 (\mu)M in the GPI/LLMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>3S, 8S minor</td>
<td>8.9% at 50 (\mu)M</td>
<td>3.8% at 10 (\mu)M</td>
<td>6-fold shift</td>
<td>None at 10 (\mu)M</td>
</tr>
<tr>
<td>3S, 8S major</td>
<td>13±1.5 (\mu)M n=2</td>
<td>1.4% at 10 (\mu)M</td>
<td>(K_e=2.8±0.6 \mu)M n=2</td>
<td>None at 10 (\mu)M</td>
</tr>
<tr>
<td>3R, 8S minor</td>
<td>8.6 % at 10 (\mu)M</td>
<td>2.6 % at 25 (\mu)M</td>
<td>None at 10 (\mu)M</td>
<td>None at 10 (\mu)M</td>
</tr>
<tr>
<td>3R, 8S major</td>
<td>8.2 % at 10 (\mu)M</td>
<td>16.5 % at 60 (\mu)M</td>
<td>None at 10 (\mu)M</td>
<td>None at 60 (\mu)M</td>
</tr>
<tr>
<td>3S, 5S, 8R</td>
<td>14.40%</td>
<td>3.80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3S, 5R, 8R</td>
<td>10.30%</td>
<td>6.20%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R, 5S, 8R</td>
<td>15.50%</td>
<td>5.90%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3R, 5R, 8R</td>
<td>11.50%</td>
<td>4.60%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As observed, the [D,D] analogues; [3S, 8S] minor (HPLC rt 22 min)- and [3S, 8S] major (HPLC rt 24 min) are \(\delta\)-opioid selective, and show mild antagonism in competitive binding with
DPDPE. The major analogue appears to be a δ-selective partial agonist. This result is somewhat surprising in that we predicted the [3.3.0]-BTD[2,3]-Leu enkephalin isomers synthesized with L-Cys to be the most potent. A possible explanation may be that Leu-enkephalins can assume many different conformations due to the flexibility of the Gly2-Gly3 unit. Characterizations of the [3.3.0]-BTD[2,3]-Leu enkephalin isomers synthesized with L-Cys indicate the β-turns are Type II. The isomers in bold type listed in Table 2.1 are enantiomers of the aforementioned isomers and consequently are Type II. It may be that conformationally, these isomers are a better geometric fit inside the binding pocket. More work needs to be done to determine the conformation of the bridgehead hydrogen on these analogues. TOCSY followed by NOE studies should be performed on the isomers; and possible molecular modeling to determine the absolute configuration. The 3R, 8S-analogues [D,L] are not active.

§ 2.7 Conclusions

[3.3.0]-BTD[2,3]-Leu-enkephalin analogues (Figure 2.5) were synthesized using the proposed strategy. Leu-enkephalin was chosen as the target to modify because it has preferred binding for the δ-opioid receptor, it is an endogenous ligand, and it has been studied comprehensively. Figure 2.5 [3.3.0]-BTD[2,3]-Leu-enkephalin analogues
The design rationale of these constrained enkephalins was based on the message-address concept (Figure 2.6). Tyrosine is the message portion of the molecule, and is thought to be critical for signal transduction at the binding pocket. As seen in Figure 2.2, this may be due to interactions with the phenolic moiety at TM III and the imidazole ring of His at TM VI. The BTD occupies the spacer position and due to its topographical constraint is thought to enhance selectivity and potency. Phenylalanine and leucine are the hydrophobic moieties in the address region thought to interact with hydrophobic amino acids located at TM III and TM VII. The carboxyl terminal ionically interacts with a Lys at TM VII. The cationic amino terminal interacts with an Asp at TM III. This design also satisfies the three major concerns of peptidomimetic design in that desired side chains can be added to the dipeptide; by using the external β-turn synthetic methodology, the position of the amide bond in the spacer remains in the same location; and by forming the BTD in situ, the constrained spacer is easily incorporated into the desired peptide.

Figure 2.6 Message-spacer-address of BTD
§ 2.8 Experimental section

General information and materials:

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker DRX-500 MHz NMR. The chemical shifts are $\delta$, parts per million (ppm). Tetramethylsilane (TMS) was added to the sample as a standard (0.00 ppm), CDCl$_3$ was the internal standard. For samples dissolved in DMSO, the peaks were referenced to solvent peaks at 2.49 ppm ($^1$H) and 39.5 ppm ($^{13}$C). For samples dissolved in D$_2$O, the spectra were referenced to solvent peaks at 4.67 ppm (HOD) and $^{13}$C chemical shifts were indirectly referenced to CDCl$_3$ at 7.24 ppm. $J$ coupling constants refer to apparent peak multiplicities and not true coupling constants. The Mass Spectrometry Facility located at University of Arizona’s Department of Chemistry performed all mass spec analyses on a Jeol HX-110A. 230-400 mesh silica gel (purchased from Aldrich Chemical Company) was used in flash column chromatography separations. UV active Merck silica gel 60 F$_{254}$ (0.25 mm silica gel thickness) were used for thin layer chromatography analysis. Melting points were obtained in open capillaries.

The [3.3.0]-BTD$^{23}$-Leu-enkephalins were synthesized using N$^\alpha$-Fmoc solid phase methodology under an atmosphere of Ar$^\circ$. N$^\alpha$-Fmoc-Phe-OH, N$^\alpha$-Fmoc-Tyr[OtBu]-OH, D-Cys, and N$^\alpha$-Leu-Wang Resin were purchased from Novabiochem (US). HBTU and HOBt were purchased from Quantum Biotechnologies (Montreal, Canada). L-Allylglycine, D-Cysteine, piperidine, 9-fluorenemethanol, and tosyl chloride were purchased from Aldrich (Milwaukee, WI). D-Allylglycine was purchased from Sigma (St. Louis, MO). DCM, DMF, and HPLC grade Acetonitrile were purchased from J. T. Baker (Pittsburgh, NJ). Peptides were purified on a Hewlett-Packard 1100 Series HPLC with a HP 1100 Series variable wavelength UV detector for semipreparative HPLC. The column used was a C$_{18}$ reverse phase silica column purchased from Vydac (Hesperia, CA, Cat. # 218TP1010). The purity of peptides was checked by thin layer chromatography in five different solvent systems. The peptides were developed in iodine vapor and with KMnO$_4$ oxidation. They were also
characterized by electrospray MS. In all cases, purities were greater than 95% as determined by these methods.

**Fluorenylmethyl p-toluenesulfonate:** 9-Fluorenemethanol (100 mmol) was dissolved in 100 mL CHCl₃ at 0°C. Tosyl chloride (120 mmol) was dissolved in anhydrous pyridine (200 mmol), placed in a dropping funnel, and added dropwise to the 9-fluorenemethanol solution. The resulting mixture was warmed to room temperature with stirring and reacted for 6 hours. The solution was extracted with 1N HCl (2 x 25 mL), saturated NaHCO₃ solution (2 x 25 mL), saturated NaCl solution (2 x 25 mL), and the remaining organic layer was dried with anhydrous MgSO₄. The mixture was filtered and the filtrate was concentrated *in vacuo*. The crude product was recrystallized by dissolving in CHCl₃, adding hexanes until cloudy, and allowed to stand overnight at room temperature. A pale yellow, “sparkling” crystal was filtered, washed with cold hexane, and allowed to dry over vacuum for 10 minutes. Yield 68% after recrystallization; Mp. 113-115°C.¹⁰ °H (250 MHz, CDCl₃), 2.41 (3H, s), 4.23-4.27 (3H, m), 7.27-7.29 (4H, m), 7.38 (2H, t, J=8.0 Hz), 7.52 (2H, d, J=8.0 Hz), 7.72 (2H, d, J=7 Hz), 7.75 (2H, d, J=8.0 Hz)

**9-Fluorenylmethyl-D-cysteine:** Fluorenylmethyl p-toluenesulfonate (20.6 mmol) was dissolved in anhydrous DMF at 0°C. D-Cysteine (16.5 mmol) and DIEA (49.6 mmol) were added in order. A white gelatin-like solid formed. The suspension was warmed to room temperature and allowed to react for 24 hours with stirring. EtOAc (72 mL) was added at room temperature. The solid was filtered (very gelatinous—whitish, somewhat transparent) and washed with cooled EtOAC until no UV active filtrate was detected on TLC (due to composition of solid—was very difficult to detect lack of UV activity). The crude solid (+100% yield) was recrystallized in 1N HCl. A pale yellow product was collected (pure product yield 90%). Mp.>195°C (d), °H (250 MHz, CDCl₃): 4.05-4.06 (2H, d), 4.08-4.10 (2H, s), 4.12-4.17 (1H, m) 7.31-7.38 (2H, t, J=5.5 Hz), 7.39-7.45 (4H, t, J=6 Hz), 7.61-7.65 (1H, d, J=11 Hz), 7.77-7.81 (2H, d, J=8 Hz).
**N*-9-Fluorenylmethoxycarbonyl-(S)-9-fluorenylmethyl-D-cysteine:** 9-Fluorenylmethyl-D-cysteine (11.9 mmol) was suspended in acetone: water (1:1 v:v-240 mL total) and FmocOSu (11.7 mmol). NaHCO₃ (23.8 mmol) was added and the reaction was kept at room temperature for 2 days. The product was concentrated *in vacuo*, the residue was diluted and acidified with 1N HCl to pH 2-3. The crude product was extracted with EtOAc (3 x 50 mL), the organic layers were combined and extracted with 50 mL saturated NaCl. The resulting organic layer was dried over MgSO₄ and filtered. The organic layer was concentrated *in vacuo* and the product was isolated using flash column chromatography (gradient solvent—8:1; 3:1; 1:1 (hexane:EtOAc); EtOAc; MeOH). The pure product (pale yellow) was concentrated *in vacuo* and allowed to crystallize overnight at room temperature. Yield-72%. IH (250 MHz, DMSO): 2.42-2.71 (1H, dd, J=7.0 Hz, 9.0 Hz), 2.8 (2H, d), 3.78-3.97 (4H, m), 6.87-7.10 (1OH, m), 7.33-7.44 (4H, d, J=5 Hz), 7.5-7.6 (4H, t, J=5.0 Hz); 13C (62.5 MHz, DMSO): 33.8, 36.9, 37.2, 39.7, 41.6, 59.1, 66.4, 118.4, 123.8, 123.9, 126.1, 127.0.

**N*-9-Fluorenylmethoxycarbonyl-L-allylglycine:** 4.34 mmol of L-allylglycine was suspended in 1/1 acetone/water solution (80 mL total) at 0°C. 4.25mmol Fmoc-OSu and 17.7 mmol of NaHCO₃ were added with stirring. The reaction proceeded overnight at room temperature with stirring. The reaction mixture was evaporated to dryness and the crude product was acidified to pH 2-3 with 1N HCl. The mixture was extracted with EtOAc (3 x 25 mL). The organic fractions were combined and washed with saturated brine (1 x 25 mL). The organic layer was dried over MgSO₄, filtered, and evaporated. The crude product yield was 98%. Mp: 120-122°C, 2.56-2.60 1H (250 MHz, CDCl₃): 2.63-2.72 (1H, m), 4.23 (1H, t), 4.41 (2H, d), 5.17-5.20 (2H, d), 5.41 (1H, d), 5.56 (1H, m), 7.31 (2H, t), 7.40 (3H, t), 7.55-7.59 (2H, m), 7.76 (2H, d); 13C (62.5 MHz, CDCl₃): 36.3, 47.1, 53.0, 67.2, 119.8, 123.5, 126.0, 127.1, 132.0, 141.3, 143.7, 155.0.

**N*-9-Fluorenylmethoxycarbonyl-D-allylglycine:** Crude product (Yield-99%) ¹H and ¹³C spectra are identical to its isomer.
General procedure for 2-N^α-Fmoc-4-oxo-butyric acid: N^α-FmocL-allylglycine (1 eq) was dissolved in THF/H2O (2/1, 4 mL/mmol). A 100-mL flask was covered with Al° foil and the hood lights were turned off. Osmium tetroxide (5% mmol) was added to the flask at room temperature with stirring. After 5 minutes sodium periodate (2.5 eq) was added over a 15-minute period. The reaction was stirred for 4 hours at room temperature. The mixture was filtered and concentrated in vacuo. The residue was dissolved in EtOAc and saturated ammonium chloride solution. The mixture was extracted with EtOAc (2 x 10mL/mmol). The organic fractions were combined, washed with saturated sodium chloride, dried over MgSO₄, and filtered. The solution was concentrated in vacuo and used in the peptide synthesis without further purification.

N^α-Fmoc solid phase synthesis methodology for [3.3.0]-BTD[2,3]-Leu-enkephalin: All isomers were synthesized manually under a constant stream of Ar°. N^α-amino acids were added in the following manner:

1. Deprotect protecting group [25% piperidine-(5 min, wash with DMF, 25 min)]

2. Wash peptide [DMF, 3x, 1.5 min; DCM, 3x, 1.5 min]

3. Couple amino acid [Amino acid-3 eqs., HBTU-3 eqs., HOBt-3 eqs., DIEA-6 eqs.—60 min total under Ar°]

4. Wash peptide [DMF, 3x, 1.5 min; DCM, 3x, 1.5 min]

N^α-Fmoc-Leu-Wang resin (100-200 mesh, 0.68 mmol/g active sites) was swollen in DMF/DCM (1/1) overnight—the SPPS was flooded with Ar°, then capped. The resin was washed and the N^α-Fmoc-Leu-resin was deprotected. N^α-Fmoc-Phe-OH was coupled under typical conditions with a total reaction time of 70 min. Washing the peptide followed coupling. Complete coupling was confirmed via the Kaiser test. The dipeptide was deprotected, washed, and coupled with N^α-Fmoc-d-Cys[Fm] dissolved in DCM./DMF.
(1/1—to prevent racemization) using conventional methods. The deprotection of the N\textsuperscript{\alpha}-Fmoc and S\textsuperscript{\beta}-Fm groups required a 50% piperidine and 5% TIPS in DMF solution (5 min, washed with DMF, 25 min). The peptide was washed and coupling was confirmed via a Kaiser test that, as expected, was negative. N,S-Thiazolidine formation was achieved by coupling a solution of 2-N\textsuperscript{\alpha}-Fmoc-4-oxo-butyric acid in 10 mL of DMF and 2 eqs. of DIEA with stirring for 2 hours followed by washing of the peptide. Lactamization was accomplished by stirring the peptide for 45 min in a solution of HBTU (1.5 eqs.), HOBt (1.5 eqs.), and DIEA (3 eqs.) in DMF. The peptide was washed and deprotected by 40% piperidine in DMF (5 min, washed with DMF, 25 min). Complete coupling was confirmed with the Kaiser test yielding a positive result. N\textsuperscript{\alpha}-Fmoc-Tyr[O-t-Bu]-OH was coupled completely as confirmed by the Kaiser test. The peptide was washed.

**General procedure for cleavage and deprotection of peptides.** The peptide was cleaved in the following manner: Addition of the peptide-bound resin and cleavage cocktail [TFA/TIPS, H\textsubscript{2}O (9.0/0.5/0.5)] into a 20 mL plastic syringe equipped with a fritted disc, followed by constant shaking for 60 min using a mechanical shaker. The solution was filtered through the frit. The resin was washed with TFA (4-5 mL), and solution was filtered. The volume of TFA was reduced to 4-5 mL by blowing a stream of Ar\textsuperscript{\circ} over the filtrate. The residue was neutralized to pH 4-6 with ammonium hydroxide. The crude peptide was diluted with 150 mL diethyl ether, but was more soluble in the ether than in the water fraction. Consequently, the peptide did not precipitate out of solution as expected. The diethyl ether was rotovapped off, the aqueous layer was frozen in -78°C bath (acetone/dry ice), and the residue was lyophilized.

**General procedure for purification of peptides:** The solid resulting after lyophilization was dissolved in a mixture of HPLC grade acetonitrile, 0.1% TFA in water, and minimal methanol to about 10 mg crude/mL solution. Insoluble impurities were removed by passing the mixture through a syringe filter (Gelman Laboratory, Acrodisc, 25 mm syringe filter with 1\muM glass fiber filter membrane). The yield and diasteromeric ratio were determined on the HPLC equipped with a C\textsubscript{18} reverse phase column by using a 23-32.5% gradient of acetonitrile in 38
min at 218 nm. The peptide was purified on a semi-preparative HPLC with C$_{18}$ reverse phase column using the above conditions and methods. Flow rate was 9 mL/min.

(3S, 8S)-[3.3.0]-BTD$^{[a,b]}$ -Leu-enkephalin (minor product): High res MS: $m/\zeta$ 626.2448; $^1$H NMR 500 MHz (D$_2$O): 1.52 (2H, m), 2.0 (m, 1H), 2.4 (m, 1H), 2.75 (2H, m), 3.00 (1H, m), 3.10 (2H, m), 3.40 (t, 1H), 4.10 (1H, t), 4.35 (d, 1H), 4.56 (m, 1H), 4.60 (1H, m), 4.90 (1H, m), 6.80 (2H, d), 7.11 (2H, d), 7.25-7.29 (2H, m), 7.35 (1H, m), 7.45 (2H, t)

(3S, 8S)-[3.3.0]-BTD$^{[a,b]}$ -Leu-enkephalin (major product): High res MS: $m/\zeta$ 626.2448; $^1$H NMR 600 MHz (D$_2$O): 1.52 (2H, m), 2.0 (m, 1H), 2.4 (m, 1H), 2.75 (1H, t), 3.00 (2H, d), 3.10 (2H, m), 3.40 (t, 1H), 4.10 (1H, t), 4.35 (d, 1H), 4.56 (m, 1H), 4.60 (1H, m), 4.90 (1H, m), 6.80 (2H, d), 7.11 (2H, d), 7.25-7.29 (2H, m), 7.35 (1H, m), 7.45 (2H, t)

(3R, 8S)-[3.3.0]-BTD$^{[a,b]}$ -Leu-enkephalin (minor product): High res MS: $m/\zeta$ 626.2447; $^1$H NMR 500 MHz (D$_2$O): 1.52 (2H, m), 2.0 (m, 1H), 2.4 (m, 1H), 2.75 (1H, t), 3.00 (2H, d), 3.10 (2H, m), 3.40 (t, 1H), 4.10 (1H, t), 4.35 (m, 1H), 4.56 (m, 2H), 4.90 (1H, t), 6.80 (2H, d), 7.11 (2H, d), 7.25-7.29 (2H, m), 7.35 (1H, m), 7.45 (2H, t)

(3R, 8S)-[3.3.0]-BTD$^{[a,b]}$ -Leu-enkephalin (major product): High res MS: $m/\zeta$ 626.2447; $^1$H NMR 500 MHz (D$_2$O): 1.52 (2H, m), 1.75 (m, 1H), 2.4 (d, 1H), 2.6 (1H, d), 2.80 (2H, m), 2.90 (1H, m), 3.00 (m, 2H), 3.10 (1H, m), 3.50 (m, 1H), 4.00 (1H, m), 4.10 (1H, t), 4.25 (1H, m), 4.90 (1H, m), 6.80 (2H, d), 7.11 (2H, d), 7.25-7.29 (2H, m), 7.35 (1H, m), 7.45 (2H, t)
§ 3.1 Introduction

![Chemical Structure of Nostocyclopeptides]

Nostocyclopeptides are natural products isolated from the terrestrial cyanobacterium Nostoc sp. ATCC53789. These cyanobacteria are isolated from lichen found in Scotland. Nostoc species are the only known cryptophycin-producing cyanobacteria that have been identified. Cryptophycins have been implicated in apoptosis of various cancers including prostate cancer cell lines. The biological activities of the nostocyclopeptides have yet to be determined. However, the imino-bridge in the nostocyclopeptides A1 and A2 is interesting particularly because this linkage has not been reported in any other natural cyclic peptide. The nostocyclopeptide biosynthetic genes cluster has been isolated by Drs. Richard Moore and Bradley Moore. This gene cluster is responsible for the biosynthesis of the nostocyclopeptides including the aldehyde formation and subsequent enzymatic cyclization via imine formation. The cluster has seven open reading frames including a C-terminal reductase.
domain that is predicted to catalyze the aldehyde formation and cyclization. In order to probe this gene cluster, Dr. Moore requested that our group provide him with peptidic imines.

Upon closer inspection of the nostocyclopeptide A2, it is notable that the imino-bridge is flanked by a phenylalanine and a tyrosine, which presupposes a possible \( \mu \)-opioid receptor interaction. In addition the macrocycle contains an unnatural amino acid, 4-methyl proline, and a D-glutamine moiety, both \( \beta \)-turn inducing amino acids. Initially, a cyclic tripeptide was chosen for the model study due to cost, time, and control of chemistry considerations. L-Tyrosine, L-phenylalanine, and L-proline were chosen as the amino acids for the cyclic tripeptide because they could serve two purposes: provide a good template for methodology development of the imine formation; and provide a good candidate for a \( \mu \)-selective opioid agonist.

A cyclic tripeptide is a substituted nine-membered ring. Medium size rings are somewhat difficult to form due to ring strain, most notably transannular strain. A potential problem with imine formation is that the imino-bridge will “rigidfy” the ring, and that may introduce more strain by restricting the conformational freedom of the ring. However, I believed that Pro would promote ring formation due to the \( \beta \)-turn and cis-conformation that Pro provides. Further, I felt that the phenyl rings of Tyr and Phe might sterically limit access to the imine reducing reactivity of the site.

c[Tyr-Pro-Phe] is interesting because upon first look it is similar to endomorphin-2, Tyr-Pro-Phe-Phe-NH\(_2\), an endogenous selective \( \mu \)-agonist. Designing selective and potent \( \mu \)-opioid ligands is important in analgesia as it has been reported that co-administration of a peripherally selective opioid antagonist and a \( \mu \)-selective agonist reduces side effects such as constipation.\(^{72}\)

As stated earlier, opioid receptors require a cationic amino-region (usually a terminal amino group), a phenolic ring (provided by Tyr in this example), and a hydrophobic region (provided by Phe in this example).\(^3\) Introduction of conformational constraints into the key pharmacophore has increased ligand selectivity, binding affinity, and potency for a receptor subtype.\(^{11}\) Although the cyclic tripeptide imine (cTPI) is lacking a cationic amino group, other
conformationally constrained endomorphin mimetics (Figure 3.2) lacking a cationic amino group have shown good μ selectivity (greater than 100-fold) over δ- and κ-opioid receptors. Additionally, the mimetic elicits analgesia similar to morphine.73

Figure 3.2 Endomorphin mimetic lacking cationic amino group.

Further, it has been reported that small ligands may be able to bind at different positions in the binding pocket of the μ-opioid receptor.73 A medium size ring may prove valuable in further elucidating the variety of the receptor binding pocket. The opioid receptors are G-protein coupled receptors that have seven transmembrane domains (Figure 3.3).74 In the docking models of the μ-opioid receptor ligand interactions at the TM III, TM V-VII domains are the most important.43, 44 The design of the cTPI should be able to interact effectively with the binding pocket.
One final benefit of methodology development for peptide imine synthesis is the development of solid phase synthetic strategies for the intermediate aldehydes. Peptide aldehydes are useful in our group as intermediates for the BTD formation, but have also shown inhibitory effects of serine and cysteine proteases (i.e. trypsin, plasmin, papain); prohormone convertases; aspartyl proteases, and caspases.

§ 3.2 Retrosynthetic analysis of Nostocyclopeptide

For reasons stated in the introduction c-[Tyr-Pro-Phe] imine was selected as the desired target for methodology development on the road to total synthesis of the nostocyclopeptides. Nostocyclopeptide A2 was selected as the target for total synthesis because phenylalanine was thought to provide greater benefit to the model study than leucine via possible stabilization of the imino-bridge. In the retrosynthetic analysis of nostocyclopeptide A2 the model study used the three L-amino acids highlighted in Scheme 3.1 with the cyclization occurring head to tail between the C- and N-terminals. The aldehyde was formed by reduction of a Weinreb amide, followed by acid workup. The resulting ring is a medium size, 9-membered ring with a putative β-turn due to the presence of proline. Note that 4-methylproline was not used in the model study, as the desire was to simplify conformational issues, especially since transannular strain is a concern in rings of this size. Our group has well-established methodology to introduce an alkyl group at that position in proline. The chemistry was first tested in solution phase in order to establish reduction time and workup protocol for the Weinreb amide to
aldehyde synthesis. The synthesis was then moved to solid phase using a Weinreb AM resin as the solid substrate.

Scheme 3.1 Retrosynthetic analysis of nostocyclopeptide for pilot study
§ 3.3 Weinreb Amide route to peptide aldehyde synthesis

Several options were available for the synthesis of the intermediate aldehyde: oxidation of N-protected amino alcohols\(^{90, 81, 82}\); reduction of amino acids\(^{80}\); or reduction of esters\(^{84}\). The most important problem posed by these strategies is purification. Epimerization often resulted from column chromatography purification using silica gel.\(^ {85}\) This led researchers to use the crude product contaminated with alcohol and starting material, which resulted in reduced yields and byproduct formation. Peptide aldehyde formation via reduction of a Weinreb amide has been shown to improve yields and purity.\(^ {86}\) Weinreb amides have also been used as linkers for solid supports making it possible to use solid phase chemistry to synthesize peptide aldehydes.\(^ {87, 88}\) This is a particularly attractive avenue as solid phase chemistry generally increases yields and reduces impurities in multistep synthetic strategies. Advantages of Weinreb amide chemistry are as follows: they are selective in their reactions with organometallic reagents such as LAH; little or no over-reduction has been reported presumably due to the five-membered intermediate (Figure 3.4) formed upon addition of the organometallic species (LAH, DIBAH, etc.); the intermediate is remarkably stable and affords high yields of relatively pure aldehydes upon treatment with acid.\(^ {87}\)

![Figure 3.4 Intermediate formed during Weinreb amide reduction](image)

Formation of the Weinreb amide in solution was achieved by the reaction of the amino acid with pivaloyl chloride to form the acid chloride, followed by addition of N,O-dimethoxyhydroxylamine chloride to form the Weinreb amide (Scheme 3.2).
Scheme 3.2 Synthesis of phenylalaninal in solution

On solid phase the N\textsuperscript{α}-Fmoc-protected N,O-dimethoxyhydroxylamine is attached as a linker to the polymer matrix (Figure 3.5). As always, coupling of the first amino acid to the linker is the critical step.

Figure 3.5 Structure of Weinreb amide AM Resin
§ 3.4 Model Study in Solution Phase

The original approach in solution phase was to synthesize the linear tripeptide by first coupling N,O-dimethoxyhydroxylamine to Boc-Phe-OH with BOP and TEA (Scheme 3.3). The reaction was followed with TLC using CMA (chloroform/MeOH/acetic acid 90/10/3) as the mobile phase. Coupling completion was confirmed by spraying ninhydrin solution on TLC plate. The TLC was developed by heating to 120°C for 60 sec. A sample was taken for each step and submitted for low resolution MS.

Scheme 3.3 Solution phase synthesis of tripeptide aldehyde
The synthesis of the Weinreb amide and coupling of all amino acids proceeded in reasonable yields - 72% for the amide and 59% yield for the tripeptide amide. However, reduction with LiAlH₄ proved problematic. To prevent epimerization the synthesis needs to be robust and the purity relatively high, so that column chromatography is unnecessary. MS revealed that the Weinreb amide was not deprotected in appreciable yields and the synthesis was fairly dirty. After a few more attempts the aldehyde formation was observed with Jones reagent (Cr VI-orange to Cr IV-green). The MS indicated that BOP reagent was still present even after multiple washings, and subsequently seemed to be causing oligomerization.

It was my desire to optimize the aldehyde formation in solution and apply the reaction conditions to solid phase. Since I was moving the synthesis to solid phase, I decided to use only N⁵-Boc-protected Phe abandoning the total synthesis of the cTPI in solution, which should make optimization of the reaction conditions for aldehyde formation simpler. I also pursued another synthetic strategy for the generation of the Weinreb amide (Scheme 3.4).

\[
\begin{align*}
\text{NMM-Cls} & \xrightarrow{10 \text{ minutes, Ar}} \text{NMM-Cl (ppt)} \\
\text{Cl-HN(Me)OMe} & \xrightarrow{50\% \text{TFA/DCM}} \\
\end{align*}
\]

Scheme 3.4 Synthesis of Phenylalanyl aldehyde
The beauty of this approach is that yields are quantitative for the formation of acid chloride and Weinreb amide. The NMM-Cl precipitates out and can be filtered easily reducing impurity issues. Reaction was monitored for 30, 45, 60 min with Jones reagent and TLC. After 45 min the orange to green change in Jones reagent indicated formation of aldehyde. The change was slow, however after 65 min, the change was immediate. IR confirmed formation of the aldehyde with a peak at 1734.22 cm\(^{-1}\). TLC indicated increased product yield, but what looked like alcohol was also present due to over reduction of the aldehyde. More optimization was needed. Attempted optimizations of LAH reduction in solution phase several more times by varying the temperature conditions and reaction time. Jones reagent and MS confirmed formation of aldehyde, however, there was also Weinreb amide and alcohol present.

Scheme 3.5 Synthesis of cTPI
The final step in the formation of cTPI was attempted by working up the aldehyde and concentrating it \textit{in vacuo}, then adding the residue to anhydrous benzene in a Dean Stark trap. The imine was continuously extracted until all of the water was removed via azeotropic distillation. The desired amine did not form, but rather, what looked like an oligomerization occurred. Since solid phase synthesis of cTPI was going to present issues and time was of an essence, I decided to move the synthesis over to solid phase.

\section*{§ 3.5 Solid phase synthesis of cTPI}

Employing the N\textsuperscript{\alpha}-Fmoc-protected Weinreb AM resin to build the peptide yields a peptidic aldehyde upon cleavage (reduction of the amide) from the resin. The first coupling is critical as the linker is a hindered secondary amine that sometimes proves difficult.\textsuperscript{39} This resin is stable to N\textsuperscript{\alpha}-Boc- and N\textsuperscript{\alpha}-Fmoc-protected amino acids. HBTU, HOBt, and DIEA were used as coupling reagents and the tripeptide was synthesized using Fmoc-protected amino acids. The Kaiser test was used to confirm complete coupling when primary amines were employed and the chloranil test for secondary amines. Fmoc-Phe-OH was coupled, followed by Fmoc-Pro-OH and Fmoc-Tyr [OtBu]-OH using conventional SPPS. The resin was cleaved using 8-10 eq. LAH at -20°C for 45 minutes. Workup included quenching with KHSO\textsubscript{4} and Na\textsuperscript{+}, K\textsuperscript{+} tartrate, and extraction with EtOAc, the fractions were collected and washed with brine, the residue was dried with MgSO\textsubscript{4}, anhydrous, filtered, and concentrated \textit{in vacuo}. The residue was dried over vacuum for 2 hours. The reduction was followed by TLC, the spots were developed by spraying with ninhydrin solution and heated for 60 sec at 120°C. The presence of a free amino terminal would yield a highly colored purplish spot. The TLC showed that the mixture did not appear to have any free amine remaining. The yield of the crude was 43%. The ESIMS revealed that the desired product was formed in trace amounts, over reduced products were formed, while the major products appeared to be a mixture of aldol-type products. In addition to these products a trace amount of diketopiperazine was formed (Figure 3.6). This will be discussed later.
To optimize the LAH reduction and subsequently improve yields cleavage times were varied at 30 min, 1 hr, 2 hr, 4 hr, and 6 hr under the same reaction conditions (-78°C for the cleavage followed by continuous extraction in benzene). The organic residue was extracted in workup and concentrated in vacuo. Both the organic layer and the water layer for each experiment were submitted for MS analysis. At 30 mins the organic layer had a base peak that indicated dkp formation, other strong peaks were polymers of the dkp[ESIMS: \( m/z \) 338.2; 663.1; 1000.0; 1325.9]. The desired imine was formed in trace amounts, but not enough to separate and further characterize. The aqueous layer was completely devoid of any product from the reaction and showed only the polypropylene glycol envelope that resulted from the storage container. The ether layer at 1 hour has the imine peak (albeit, it is over reduced), and evidence that the unreacted aldehyde is still present. Again, yields were too low to isolate. The aqueous layer had a very small product peak. Otherwise, nothing important was observed. The fractions at 2 hours were completely devoid of any recognizable product, but a polymer was present. The organic fraction at 4 hours has a trace amount of product observed but the base peak and other significant peaks were very polymer-like. The aqueous layer was the same as previously. At six hours the dkp had been formed in significant amounts with the oligomerization of dkp accounting for the other peaks. From this data it was determined that the best timeframe for reduction was between 30 min and 1 hour. Future experiments were cleaved for 45 min.
The next attempt resulted in 3.2% yield, and was subjected for MS/HPLC analysis to determine what the impurities were in an effort to optimize the strategy. The major fraction was the over reduced imine (Figure 3.7). However, the desired imine was made in good proportion to the other impurities. The sample is still relatively dirty with only a minimal amount of dkp was observed. An NMR was performed on the crude sample in CDCl₃. Low concentration and sample impurity made it difficult to make a conclusive statement except to say that there is a doublets of doublets at δ 4.5-5.4 ppm (integrates to 2Hs), which corresponds to vinylic hydrogens present in mixture, which seems to corroborate the formation of the two imines.

![Figure 3.7 Alcoholic imine formed by LAH over reduction](image)

§ 3.6 Synthesis of c[PPI] 1

At this point it seemed that the transannulation strain in the ring and the constrain of the imine might make the ring too stressed and synthesis of this adduct might naturally result in low yields. Molecular modeling might be shed some light on the energies and strain of the
molecule, and could assist in providing a new direction. c[TPI] and c[PPi] 1 (Figure 3.8) were modeled and energy studies were performed for the following adducts: linear, hemiaminal, and imine adducts.

![Diagram of molecule](image)

Figure 3.8 c[PPi] 1

The energy differences between imine and linear adducts is approximately 250 kcal/mole for c[TPI]. The energy differences between the hemiaminal adduct and the imine is about 140 kcal/mole. Although it is dicey to use low energy conformations of the models to predict synthesis, in light of the difficulty in cyclizing the ring it seems restricting the ring to this extent is not favorable, and perhaps the yields are appropriate. Energy differences for the c[PPi] 1 molecule are not as great. This may be attributed to greater flexibility in the ring as can be inferred from the rmsd information. Rmsd for c[TPI] is 0.38Å—not a great deal of conformational differences between the hemiaminal and the imine. Rmsd for c[TPI] is 2.09Å—much greater conformational differences between the hemiaminal and the imine. This flexibility of the c[PPi] 1 should invoke easier ring formation. Another very real issue is that several c[TPI] experiments indicated the possibility that the hemiaminal is being formed, but the imine is not being formed. After consultation with Dr. Robin Polt it was suggested that continuous extraction to form the imine might not be the most efficacious strategy. Rather, introducing a drying agent such as TiCl₄ or molecular sieves might be the more effective strategy. At this point it seemed reasonable to make a pentapeptide imine.
As mentioned earlier, Fmoc-N\textsuperscript{α}-protection is not preferred in SPPS using Weinreb amide resin due to the instability of the Fmoc group in LAH.\textsuperscript{89} The terminal Tyr group was protected with Boc- in one synthesis and Fmoc- in another synthesis to test if the Fmoc might be effectively reducing yields by interfering with cleavage from the resin.

§ 3.7 Acetylation of TAA (Trianyl aldehyde) and APA (Alanyl prolyl aldehyde)

In an effort to absolutely define the appropriate cleavage conditions and subsequently increase yields a new strategy was employed. N\textsuperscript{α}-Fmoc-Ala-OH would replace various amino acids and the N-terminal would be acetylated. One problem with LAH reduction on the resin is that the Fmoc group is unstable to LAH. So, trying to keep the peptide protected during cleavage in an effort to eliminate any oligomerization and focus completely on the aldehyde formation proved unsuccessful. Capping the N-terminal with an acetyl group allowed for manipulation of reaction conditions to see if the coupling and cleaving were successful. LAH reduction was allowed to proceed for 45 min at \(-78^\circ\) C. The TAA was analyzed by ESIMS. The yield was doubled to 12%, but was the over reduced product for TAA. The APA was observed in ESIMS- \( m/\ell\): 284.3. This result suggests that the cleavage or the coupling protocol needs to be revised.
§ 3.8 Conclusions

The original synthetic strategy was too constrained and I believe the transannulation strain was too great to allow for facile imine formation. In addition the coupling of the first amino acid may not be effectively occurring. Other coupling reagents such as HOAt and DIC should be used to see if the first coupling is being completed. From all indications (chloranil, ninhydrin tests) the coupling is complete, but ~12% yield with the acetylation studies suggests that these tests are not conclusive. In addition I reported several times the product as analyzed by ESIMS was the over-reduced product. Because of yield and purity issues it was not feasible to take NMRs of all these samples, so this conclusion is questionable. Another issue may be the acidity of the hemiaminal. Because the imine is not a stabilized Schiff's base, I presume that the formation of the imine is not all that favorable. I used continuous extraction with benzene in a Dean Stark trap. Dr. Polt suggested that this method may be too harsh for the formation of the imine. As can be witnessed via ESIMS, oligomerization was occurring in most samples. A drying agent should be employed in the future, and there are several protocols to determine which agent would work best here. Additionally, the instability of the N\textsuperscript{\textalpha}-Fmoc group and the t-Bu ester protection of Tyr to LAH reduction, I believe has caused major problems with the optimization of the reduction step. The evidence here is that all samples submitted for ESIMS were t-Bu ester protected, and the results revealed both t-Bu ester protected and Tyr-OH products present. Different side chain protection should be explored. Lastly, changing to a larger ring size is definitely the way to go here. There needs to be more work done to select
the appropriate amino acids, side chain protection, and N\textsuperscript{\alpha}\textsuperscript{-}protection to be successful in imine formation. One last comment I have regarding this research is that other reducing agents in the Li family such as DIBAH were not used because reported yields of the peptide aldehydes were much lower, and not as pure. I felt at the time that the yields were already too low to afford this change. In retrospect I believe that several different reducing agents might have been tried in comparison.

§ 3.9 Experimental section

General information and materials:

\textsuperscript{\textbf{1}H and \textsuperscript{\textbf{13}C} NMR spectra were recorded on a Bruker AM-250 250 MHz or Bruker DRX-500 MHz NMR. The chemical shifts are \(\delta\), parts per million (ppm). Tetramethylsilane (TMS) was added to the sample as a standard (0.00 ppm), CDCl\textsubscript{3} was the internal standard. For samples dissolved in D\textsubscript{2}O, the spectra were referenced to solvent peaks at 4.67 ppm (HOD) and \textsuperscript{\textbf{13}C} chemical shifts were indirectly referenced to CDCl\textsubscript{3} at 7.24 ppm. J coupling constants refer to apparent peak multiplicities and not true coupling constants. The Mass Spectrometry Facility located at University of Arizona's Department of Chemistry performed all mass spec analyses on a Jeol HX-110A (high resolution); Finnigan LCQ HPLC/MS (ESIMS); or Bruker Reflex-III MALDI/TOF (FAB+). 230-400 mesh silica gel (purchased from Aldrich Chemical Company) was used in flash column chromatography separations. UV active Merck silica gel 60 F\textsubscript{254} (0.25 mm silica gel thickness) were used for thin layer chromatography analysis. Melting points were obtained in open capillaries. IRs were taken on a Nicolet FTIR.

The peptide imines were synthesized using Fmoc solid phase methodology under an atmosphere of Ar\textsuperscript{o}. N\textsuperscript{\alpha}-Fmoc-Phe-OH, N\textsuperscript{\alpha}-Fmoc-Tyr[OtBu]-OH, N\textsuperscript{\alpha}-Fmoc-Pro-OH, and N\textsuperscript{\alpha}-Weinreb AM Resin were purchased from Novabiochem (US). HBTU and HOBt were purchased from Quantum Biotechnologies (Montreal, Canada). N\textsubscript{\alpha}-
Dimethoxyhydroxylamine, piperidine, LAH in Et₂O, and LAH in THF were purchased from Aldrich (Milwaukee, WI). DCM, DMF, THF, and HPLC grade Acetonitrile were purchased from J. T. Baker (Pittsburgh, NJ). Peptides were analyzed on a Hewlett-Packard 1100 Series HPLC with a HP 1100 Series variable wavelength UV detector for semipreparative HPLC. The column used was a C₁₈ reverse phase silica column purchased from Vydac (Hesperia, CA, Cat. # 218TP1010). The purity of peptides was checked by thin layer chromatography in two solvent systems, CMA (chloroform/MeOH/AcOH 90/10/3) and EtOAc. The peptides were developed in iodine vapor and with KMnO₄ oxidation. They were also characterized by electrospray MS and FAB+ MS.

**Phenylalanine Weinreb amide:** 1.89 mmol (1 eq) of Boc-N°'-Phe-OH were dissolved in DCM and added to a 100 mL round bottom flask. The contents were cooled to -15°C (ethylene glycol and dry ice) and 1 eq. of pivaloyl chloride and 1 eq of NMM were added to the flask with constant stirring under Ar. The reaction time was 10 minutes. The flask was temporarily warmed to nearly rt and the ppt. was filtered. After lowering the flask temperature to -15°C N₂O-hydroxylamine (1 eq) dissolved in DCM (with a few drops of TEA to increase solubility) was added to the flask. The reaction was followed with TLC. When reaction was complete, the organic layer was extracted with 3 x 30 mL 3N HCl; 1 x 30 mL NH₄Cl; 3 x 30 mL NaHCO₃; the organic fractions were collected and washed 3 x 30 mL brine; 2 x 30 mL H₂O. The residue was dried with MgSO₄, filtered and concentrated in vacuo. Yield-72% Observed in ESIMS m/z 209.1 [MH+J (deprotected); 308.9 (Boc-N°'-protected); 331 [MNa+] Ph

**Phenylalanyl aldehyde:** The Boc-N°-Phenylalanyl amide was deprotected with 50% TFA/DCM at 0°C. The reaction was followed with TLC/ninhydrin. Once the deprotection was complete (about 30 min), the amide was reduced with LAH/Et₂O (1M). The reaction conditions were: 5 eq. of LAH/Et₂O were added to the reaction flask at 0°C and allowed to react for 45 min. The reaction periodically tested for aldehyde formation using Jones reagent, and also followed with TLC/ninhydrin. Yield-72% crude. Presence of aldehyde confirmed by ESIMS m/z 150.0
**cTPI in solution phase:** The Boc-N°-Phe amide was synthesized by reacting 1 eq. of Boc-N°-Phe-OH with N,O-Dimethoxyhydroxylamine (1 eq) dissolved in TEA, 1 eq of BOP was added to facilitate complete coupling. The Boc group was deprotected with 50% TFA/DCM at 0°C for 30 min. The reaction was followed with TLC. When reaction was complete, TFA was rotovapped off, toluene was added to the flask and rotovapped until no residual TFA was detected. The residue was dried with MgSO4, filtered and concentrated in vacuo. 1.2 eq of Boc-Pro-OH and 1.2 eq of HOBt were dissolved in DMF and added to the flask at 0°C. NMM (<2eq) was added with stirring under Ar°. The reaction proceeded at 0°C for 30 min., then, at warmed to room temperature and continued for 2-3 hours. Reaction was followed by TLC (CMA mobile phase). When reaction was complete the residue was dissolved in 50 mL EtOAc. The organic layer was extracted in the following manner: 2 x 50 mL EtOAc; 2 x 50 mL 5% NaHCO3; 2 x 50 mL 5% citric acid; the organic layer was washed with 1 x 50 ml brine, and 1 x 50 mL H2O. The layer was dried over MgSO4, filtered, concentrated in vacuo. These procedures were repeated for the coupling of Boc-N°-Tyr-[OtBu]-OH. The amide was deprotected accordingly. Yield 34% The sample was submitted for ESIMS m/z 469.7 (Y-P-F-N(OMe)Me)

**General procedures for solid phase synthesis:** Fmoc solid phase synthesis methodology for peptidyl imines: All imines were synthesized manually under a constant stream of Ar°. N°-amino acids were added in the following manner:

1. Deprotect Weinreb amide resin (1 eq) using 25% piperidine (5 min, wash with DMF, 25 min).

2. Couple AA1 (3 eq) to resin (3 eq HOBt, 3 eq HBTU, 6 eq DIEA for 1 hour, followed by Kaiser test)

3. Wash 3 x 10 mL DMF, 3 x 10 mL DCM (30 sec each wash)

4. Deprotect protecting group [25% piperidine-(5 min, wash with DMF, 25 min)]
5. Couple remaining amino acids in same manner [Amino acid-3 eqs., HBTU-3 eqs., 
HOBt-3 eqs., DIEA-6 eqs.—60 min total under Ar°]

6. Wash peptide [DMF, 3x, 1.5 min; DCM, 3x, 1.5 min]

7. Deprotect Fmoc-N°-Tyr-[OtBu]~ and wash peptide. Dry over Ar° for 10-15 min.

8. Add peptide-resin to round bottom flask

N°-Fmoc-Weinreb amide resin (100-200 mesh, 0.89 mmol/g active sites) was swollen in 
DMF—the SPPS was flooded with Ar°, then capped. The resin was washed deprotected. N°-
Fmoc-Phe-OH was coupled under typical conditions with a total reaction time of 60 min. 
Washing the peptide followed coupling. Complete coupling was confirmed via the Kaiser test. 
The N°-Fmoc-Phe-resin was deprotected, washed, and coupled with N°-Fmoc-Pro-OH 
dissolved in 10 mL DMF using conventional methods. The N°-Fmoc was deprotected and 
coupled with N°-Fmoc-Tyr [OtBu]-OH dissolved in 10 mL DMF using conventional 
methods. The peptide was washed and coupling was confirmed via a Kaiser test. The 
peptide-resin was dried over Ar° for 10-15 min. Yield-4%. m/z 447.1 (OtBu ester protection 
on Tyr); HPLC —CI 8 reverse phase column retention time: 29.62 min; 394 (Over-reduced and 
ester imine)-36.13 min

**Synthesis of c-PPI 1:** The linear peptide was synthesize according to conventional Fmoc-
protocol. m/z: Base peak 606.4; linear aldehyde 524.5; cyclic imine in trace amount 506.6

**Synthesis of cPPI 2:** The linear peptide was synthesized according to conventional Fmoc 
protocol save the terminal Gly, which was Boc-protected. The Boc-group was deprotected 
using 50% TFA/DCM for 30 min under Ar° with constant gentle stirring. The peptide was 
cleaved accordingly.

**General procedure for cleavage and deprotection of peptides.** The resin was moved to a 
250 mL round bottom flask and 100 mL of Et₂O were added. The flask was cooled to -15°C
and a septum was placed over the flask. Argon was flushed through the flask for a few minutes, then, 8-10 eq of LAH/Et₂O (1M) were added. The reaction was stirred constantly for 45 minutes. Then sat'd. KHSO₄ (1 mL) and Na+, K+ tartrate (1 mL) were added to the flask to quench the reaction. The mixture was stirred and warmed to rt for 30 min. The resin and LiAl₄O₅ ppt. were filtered and washed with DCM. The filtrate was dried with MgSO₄, filtered, and evaporated to dryness.
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APPENDIX A

1. New methodology for synthesis of bicyclic β-turn dipeptides (BTD) on solid phase.

APPENDIX B

$^1$H and $^{13}$C NMR Spectra and MS DATA
Current Data Parameters
NAME  DaniceSS24
EXPNO  1
PROCNO  1

F2 – Acquisition Parameters
Date  20040314
Time  17.32
INSTROM  spec
PRIORITY  5 mm Nalorac H
PULPROC  1g
TD  16384
SOLVENT  CDC13
NS  16
DN  4
SMEA  7977.407 Hz
FIDQS  0.456222 Hz
AQ  1.0912244 sec
RG  456.1
SW  66.600 usec
DE  6.00 usec
TE  298.6 K
D1  3.000000 sec
MCREST  0.000000 sec
MCWRK  0.01500000 sec

F2 – Processing parameters
SI  16384
SF  600.1300133 MHz
WDW  EM
SSB  0
LB  0.20 Hz
GB  0
PC  1.00

[Chemical Shifts and Spectra]
Danice Andrus RS25
Standard 1D 1H Spectrum
Nalorac 3-Res. 3-axis Gradient Probe

7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
1.296 0.623 0.680 0.524 0.771 0.433 0.374 0.373 1.053 0.858 0.445 0.453 0.762 1.582 3.157
Danice Andrus
RS 22
Standard 1D 1H Spectrum
Nalorac 3-Res. 3-axis Gradient Probe
Fmoc-D-Cys[Fm]-OH

\(^1\text{H NMR}\)

\(^{13}\text{C NMR}\)

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**Fmoc-D-Cys[Fm]-OH**

\(^1\text{H NMR}\)

\(^{13}\text{C NMR}\)
D-Cys[Fm]-OH

\(^1\)H NMR

\[^{13}\)C NMR
Fmoc-oxo-Butyric Acid

$^1$H NMR

$^{13}$C NMR