AN ABSTRACT OF THE THESIS OF


Abstract approved:  

Jane V. Aldrich

Affinity labels are ligands which bind irreversibly to a receptor. This interaction gives affinity labels several advantages over conventional ligands in many types of pharmacological studies. In an effort to synthesize affinity labels based on peptide antagonists for delta (δ) opioid receptors, a general synthetic strategy was devised for the introduction of reactive functionalities (i.e. isothiocyanate and bromoacetamide) into the peptides N,N-dibenzyl leucine enkephalin and N,N-diallyl[Aib2,Aib3]leucine enkephalin (ICI-174,864). The peptide derivatives were then evaluated by radioligand binding inhibition studies using Chinese hamster ovary (CHO) cells stably transfected with δ opioid receptors. One of the analogs, N,N-dibenzyl [Phe(p-NCS)4]leucine enkephalin, had nanomolar affinity for the δ opioid receptor and exhibited wash-resistant inhibition of radioligand binding in a dose-dependent manner.

Tyr-Tic-Phe-Phe (TIPP) is a highly selective and potent antagonist for δ opioid receptors, and these attributes made this peptide an attractive candidate for affinity label development. Once again, the Boc/Fmoc orthogonal protection strategy was utilized to synthesize two potential affinity labels: [Phe(p-NCS)3]TIPP and [Phe(p-NCS)4]TIPP. In addition to high affinity and selectivity for δ opioid receptors, binding assays also revealed that both of these peptides exhibited wash-resistant inhibition of radioligand binding at low nanomolar concentrations.
The synthetic strategy used in the development of these potential affinity labels represents a general method which could be used for the synthesis of a variety of peptides with different reactive functionalities. From this synthesis, three potential affinity labels were identified: \( N,N\)-dibenzyl \([\text{Phe}(p\text{-NCS})^4]\)leucine enkephalin, \([\text{Phe}(p\text{-NCS})^3]\)TIPP, and \([\text{Phe}(p\text{-NCS})^4]\)TIPP. To our knowledge, these analogs represent the first examples of peptide-based affinity labels making use of an isothiocyanate group. These ligands represent new tools with which researchers may study \( \delta \) opioid receptor-mediated phenomena.
Doctor of Philosophy thesis of Dean Yoshimasa Maeda presented on November 24, 1997

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This thesis is dedicated to my parents, Michael and Trudy Maeda.
Chapter 1

Introduction

The alleviation of suffering has been a major medical goal for centuries. One of the most efficacious means by which analgesia is achieved is through the application of opioids. Unfortunately, the serious side effects of tolerance, physical dependence, and respiratory depression severely limits their use. It is a well documented fact that opioid effects are mediated through three major types of opioid receptors: the mu (µ), delta (δ) and kappa (κ) receptors. The opioids that exhibit high abuse potential have been shown to interact preferentially with the µ receptor (Raynor et al., 1994), and it is hoped that an analgesic that is selective for either δ or κ opioid receptors would limit the occurrence of undesired side effects. Therefore, pharmacological investigation involving the δ and κ receptors is an active area of research.

One receptor that has been implicated as a potential target for analgesia, as well as a number of other therapeutic applications, is the δ opioid receptor (Rapaka and Porreca, 1991). The discovery of δ opioid receptors was due to the judicious use of the first endogenous opioid peptides to be described, the enkephalins (Lord et al., 1977). Also, δ opioid receptors have the distinction of being the first opioid receptors to be cloned (Evans et al., 1992, Kieffer et al., 1992). Not only have the δ opioid receptors been described as being able to mediate analgesia with lessened tolerance and dependence, but they have also been implicated in a number of other ailments, including the reinforcing effects of alcohol consumption (Gianoulakis, 1996). The promise of therapeutic intervention for a number of medical problems has spurred active investigation involving the δ opioid receptor.
Despite the recent cloning of all three opioid receptor types from a variety of species and tissues (Kieffer, 1995), questions regarding δ opioid receptors still remain. Through cloning, the primary (and much of the secondary) structure of the opioid receptors are known, but the tertiary arrangement of these domains is still unknown. As a result, the amino acid residues responsible for receptor selectivity and activation are as yet undetermined. Recent pharmacological studies suggest the existence of δ receptor subtypes (Zaki et al., 1996), but to date, hybridization screening has been unable to identify these subtypes. It may be necessary to isolate and map these receptors in order to characterize these putative δ receptor subtypes. Structural information about δ opioid receptors is not the only area of current interest—the physiological consequences of δ opioid receptor activation also remains under extensive investigation.

One pharmacological tool that has been used successfully in various types of receptor studies is the affinity label. An affinity label is a ligand whose interaction with a receptor is essentially non-equilibrium in nature. Because of this interaction (which may or may not involve a covalent bond), affinity labels have many advantages over conventional ligands in a number of pharmacological experiments. If radiolabeled, an irreversible ligand could greatly aid in isolation and tryptic digest mapping, and its long-acting blockade of receptor function would help in determining δ opioid receptor-mediated physiological effects. The affinity label has also been used to determine residues near the binding site of receptors, as was done by Liu-Chen and co-workers (Chen et al., 1996) with the µ opioid receptor and its interaction with the irreversible ligand β-funaltrexamine (Ward et al., 1982).

Although there are affinity labels selective for δ opioid receptors are currently available, these ligands are either based on non-peptide antagonists (i.e. naltrindole 5'-isothiocyanate, NTII) (Portoghese et al., 1990), or peptide agonists (i.e. [D-Ala²,Leu⁵,Cys⁶]enkephalin, DALCE) (Bowen et al., 1987). In order to fully understand the binding interactions of δ opioid receptors, a wide variety of affinity labels should be developed and used.
In an earlier study, Aldrich-Lovett and Portoghese (Lovett and Portoghese, 1987) synthesized potential affinity labels for δ opioid receptors by incorporating melphalan (Mel) into N,N-dialkylated enkephalin derivatives. Mel is the nitrogen-mustard derivative of Phe, and the large steric bulk and relatively low reactivity of the N,N-bis(2-chloroethyl)aniline side chain was considered to be the reason for the lack of wash-resistant binding by the synthesized derivatives.

In order to further investigate these N,N-dialkylated enkephalins as potential affinity labels for δ opioid receptors, more reactive and less sterically demanding functional groups were planned for inclusion into the δ-selective peptide antagonists N,N-dibenzyl leucine enkephalin and ICI-174,864 (N,N-diallyl[Aib²,Aib³,Leu⁵]enkephalin). One of the specific aims of this project was to design and utilize a general synthetic methodology for the construction of peptide-based affinity labels. This was accomplished through the use of a tert-butyloxy carbonyl (Boc)/9-fluorenyl-methoxycarbonyl (Fmoc) orthogonal protection strategy. The Boc and tert-butyl based protecting groups provided semi-permanent protection for the peptides, while the Fmoc group allowed transient protection for a p-amine functionality on Phe, which was selectively deprotected and derivatized into either isothiocyanate or bromoacetamide groups. The key step in the synthesis was the selective deprotection of the Boc group in the presence of a tert-butyl ester. This deprotection was accomplished through the use of one equivalent of trimethylsilyl trifluoromethanesulphonate (Vorbruggen and Krollekiewicz, 1975). Once synthesized, the peptides were then tested under standard and wash-resistant binding conditions against Chinese hamster ovary (CHO) cells stably transfected with the mouse δ opioid receptor.

An important aspect in the development of affinity labels is the choice of the parent molecule to be derivatized. Affinity and selectivity for the receptor to be studied are important aspects to be considered. For these reasons, the highly potent and selective peptide antagonist TIPP (Tyr-Tic-Phe-Phe) (Schiller et al., 1992) was also chosen for derivatization into potential affinity labels. This tetrapeptide was only recently described,
and it represented a major advance in the field of δ opioid selective ligands. The synthesis of the TIPP-based derivatives utilized the Boc/Fmoc synthetic methodology developed in the synthesis of the enkephalin series. These potential affinity labels were synthesized with an isothiocyanate group at the para position of either Phe³ or Phe⁴, and underwent pharmacological evaluation using δ receptors expressed on CHO cells.

In this study, peptide ligands based on antagonists for δ opioid receptors were synthesized and tested. The application of the same synthetic methodology for differing peptide targets highlights the utility of this scheme for the construction of a wide variety of peptide-based affinity labels. From the binding studies, three potential affinity labels were identified: \(N,N\)-dibenzyl \([\text{Phe}(p\text{-NCS})^4]\)leucine enkephalin, and the \([\text{Phe}(p\text{-NCS})^3]\) and \([\text{Phe}(p\text{-NCS})^4]\) derivatives of TIPP. To our knowledge, these peptides represent the only examples of peptide-based affinity labels using an isothiocyanate group as their labeling moiety. In the future, these ligands might be used for the further investigation of δ opioid receptor-mediated phenomena.
Chapter 2
Literature Review: Opioid Receptors

2.1 Introduction

For centuries, people have exploited the analgesic and narcotic properties of the opium poppy, *Papaver somniferum*. The ancient Sumerians cultivated the plant for its painkilling properties, and named the opium poppy "hul gil" or "plant of joy." A major breakthrough in the understanding of opioid pharmacology came in 1806, when Sertturner isolated the major alkaloid constituent of opium responsible for much of its pharmacological properties. He named the compound morphine, after Morpheus, Greek god of sleep and dreams. While morphine is useful in the treatment of pain and other illnesses, it is often accompanied by unwanted side effects, most notably tolerance, physical dependence, and respiratory depression. In an effort to separate the analgesic properties of morphine from its undesired side effects, many synthetic and semi-synthetic analogs have been created and tested. With the numerous opiates synthesized, differences in both structure-activity relationships and opioid-mediated physiological effects prompted Portoghese (Portoghese, 1965) and Martin (Martin, 1967) to separately postulate the existence of multiple opioid receptors in the mid-1960's. This hypothesis was substantiated by subsequent experiments done by Martin and co-workers who observed the pharmacological profiles of several types of ligands in dogs chronically treated with opiates (Martin et al., 1976). Based on this study, Martin and co-workers hypothesized the existence of three separate opioid receptors, which the researchers named after the ligand used in its characterization: mu (μ, for morphine), kappa (κ, for ketocyclazocine) and sigma (σ, for compound SKF-10,047 or N-allylnormetazocine) receptors. Since that study, it has been shown that the actions of SKF-10,047 are not reversed by the opioid antagonist naltrexone (Vaupel, 1983), and therefore the σ receptor is no longer considered a "true" opioid receptor.
The isolation and characterization of the first endogenous opioid peptides allowed for the identification of another type of opioid receptor. In studies done by Lord et al. (Lord et al., 1977), these endogenous pentapeptides (the enkephalins) showed higher potency than morphine in the mouse vas deferens (MVD) smooth muscle assay, but lower potency than morphine in the guinea pig ileum (GPI) assay. The reversal in the rank order of agonist potency suggested that the enkephalins were acting at a distinct receptor in the MVD assay, and this receptor was therefore entitled the delta (δ) receptor.

Opioid receptors play a major role in the endogenous pain-controlling systems of vertebrates. In addition to its involvement in antinociceptive pathways, the opioid receptors have been implicated in a number of different biological events (Table 2.1) such as stress-induced analgesia, locomotor activity, and learning and memory (Olson et al., 1989, Olson et al., 1995). These receptors also modulate autonomic functions such as respiration, blood pressure, thermoregulation, and intestinal motility. Most recently, pharmacological research has implicated the involvement of opioid receptors with the reward pathways for alcohol usage (Gianoulakis, 1996), and in the modulation of certain immunological functions (Roy and Loh, 1996).

2.2 Endogenous mammalian opioid peptides

Opioid receptors can be demonstrated in every vertebrate and even a few invertebrate species. Since the existence of opioid receptors are widespread and have survived millennia of evolution, scientists had postulated that these receptors must function in an endogenous fashion favorable for species survival. The search for endogenous opiate-like ligands had a major breakthrough in 1975, when two laboratories independently reported that brain extracts contained substances that exhibited opioid activity. In 1975, Hughes and co-workers (Hughes et al., 1975) isolated two pentapeptides with opioid receptor activity from pig brain, which they named the enkephalins (from "encephalos," or "in the head"). Shortly after the identification of the enkephalins, a 31-amino acid fragment of β-
### Table 2.1 Physiological consequences of opioid receptor activation

<table>
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<tr>
<td>μ</td>
<td>analgesia</td>
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<tr>
<td></td>
<td>respiratory depression</td>
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<td></td>
<td>physical dependence</td>
</tr>
<tr>
<td></td>
<td>reduction of immunological function</td>
</tr>
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<td>inhibition of GI transit</td>
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<td>κ</td>
<td>analgesia</td>
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<td>feeding</td>
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<td>diuresis</td>
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<tr>
<td>δ</td>
<td>analgesia</td>
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<td>positive reinforcement for ethanol consumption</td>
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</table>

lipotropin, named β-endorphin, was found to bind to opioid receptors and exhibit potent, long-lasting analgesia (Loh et al., 1976). Dynorphin A, representative of another major class of endogenous opioid peptides, was isolated and characterized by Goldstein and co-workers in 1981 (Goldstein et al., 1981). Dynorphin A was shown to be a potent ligand for the κ class of receptors. Other endogenous mammalian peptides include α- and β-neo-endorphin (Kangawa et al., 1981, Minamino et al., 1981), which are both obtained from prodynorphin, and casomorphin (Brantl et al., 1979), obtained from the enzymatic digestion of the milk protein casein. All of these endogenous opioid peptides share the common N-terminal sequence of H-Tyr-Gly-Gly-Phe-Leu/Met (Table 2.2).

The enkephalins have been postulated as putative endogenous ligands for δ opioid receptors, while the dynorphins are considered to be endogenous ligands for κ receptors. Until very recently, the endogenous ligands for μ receptors were uncertain. Then in 1997, Zadina and co-workers (Zadina et al., 1997) described the discovery and isolation of two related tetrapeptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH₂) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH₂). The high affinity (Kᵢ = 360 pM) and selectivity (4000- and 15,000-fold preference for μ over δ and κ, respectively) make the endomorphins potential endogenous ligands for the μ receptor.

2.2.1 Biosynthesis

The differentiation of the three opioid peptide families represented by the enkephalins, endorphins and the dynorphins was justified by the cloning of their representative opioid precursors (Fig. 2.1). The primary sources of these peptides were found to be three distinct major protein precursors: pro-opiomelanocortin (POMC) (Nakanishi et al., 1979), proenkephalin A (Noda et al., 1982), and prodynorphin (Fischli et al., 1982) (also known as proenkephalin B). The discovery of pro-opiomelanocortin was of great importance in that it was the first example of a protein precursor to give rise to several important and
Table 2.2 Endogenous mammalian opioid peptides

<table>
<thead>
<tr>
<th>Precursor protein</th>
<th>Opioid Peptides</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-opiomelanocortin</td>
<td>β-endorphin</td>
<td>YGGFMTEKSEQTLPLVTFLKNAILII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KNAYKKGE</td>
</tr>
<tr>
<td></td>
<td>δ-endorphin</td>
<td>YGGFMTEKSEQTLPLVTFLKNAILII</td>
</tr>
<tr>
<td></td>
<td>(β-endorphin 1-27)</td>
<td>KNAY</td>
</tr>
<tr>
<td></td>
<td>γ-endorphin</td>
<td>YGGFMTEKSEQTLVTL</td>
</tr>
<tr>
<td></td>
<td>(β-endorphin 1-17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-endorphin</td>
<td>YGGFMTEKSEQTLV</td>
</tr>
<tr>
<td></td>
<td>(β-endorphin 1-16)</td>
<td></td>
</tr>
<tr>
<td>Proenkephalin A</td>
<td>Met-enkephalin</td>
<td>YGGFM</td>
</tr>
<tr>
<td></td>
<td>Met-enkephalin-Arg-Phe</td>
<td>YGGFMRF</td>
</tr>
<tr>
<td></td>
<td>Met-enkephalin-Arg-Gly-Leu</td>
<td>YGGFMRFGL</td>
</tr>
<tr>
<td></td>
<td>Leu-enkephalin</td>
<td>YGGFL</td>
</tr>
<tr>
<td></td>
<td>Peptide E</td>
<td>TGGFMRRVGRPEWWWMDYQKFL</td>
</tr>
<tr>
<td>Proenkephalin B</td>
<td>α-neo-endorphin</td>
<td>YGGFLRKYPK</td>
</tr>
<tr>
<td>(Prodynorphin)</td>
<td>β-neo-endorphin</td>
<td>YGGFLRKYP</td>
</tr>
<tr>
<td></td>
<td>Dynorphin A (1-17)</td>
<td>YGGFLRRIRPKLKWDENE</td>
</tr>
<tr>
<td></td>
<td>Dynorphin A (1-8)</td>
<td>YGGFLRRRI</td>
</tr>
<tr>
<td></td>
<td>Dynorphin B</td>
<td>YGGFLRRQFKVVT</td>
</tr>
<tr>
<td></td>
<td>Dynorphin B (1-29)</td>
<td>YGGFLRRQFKVVTTRSQEDPNAY</td>
</tr>
<tr>
<td></td>
<td>(Leumorphin)</td>
<td>YELFDV</td>
</tr>
</tbody>
</table>
Fig. 2.1 Diagram of the protein precursors of the opioid peptides. Melanocyte-stimulating hormone (MSH) and enkephalin (enk) are depicted as solid or shaded boxes. Cysteines and dibasic cleavage points are shown in the upper portion and biologically active peptides are shown in the lower portion of each diagram.

distinct biologically active peptides. In addition to the endorphins, pro-opiomelanocortin also provides adrenocorticotropic hormone and a family of melanocyte-stimulating hormones (MSH): α, β, and τ-MSH.

Proenkephalin was first discovered in bovine adrenal cortex, and it has subsequently been cloned from bovine and human tissues (Evans et al., 1988). It contains one copy of Leu enkephalin, four copies of Met-enkephalin, and two copies of C-terminal extended Met-enkephalin, one a heptapeptide, and the other an octapeptide.

The last of the opioid peptide precursors to be characterized was prodynorphin. It has been isolated from a variety of mammalian tissues, including brain and spinal cord, pituitary, adrenal and reproductive organs. All of the opioid peptides derived from this protein, dynorphin A and B and α- and β-neoendorphin, are C-terminal extensions of leu-enkephalin.

There are many similarities between the three opioid peptide precursors (Evans et al., 1988). They all are similar size, and have several opioid peptides in the C-terminal half of the protein. These opioid peptides are framed by basic amino acids which are thought to be targets for proteolytic cleavage by processing enzymes. A cysteine-rich N-terminal sequence is preceded by signal peptides of comparable size in all three cases. Between proenkephalin and prodynorphin, there is a high degree of homology which exceeds 50%. The precursor genes also show analogous introns and exons with respect to size and placement. All of these similarities suggest that the three genes all arise from a common ancestral gene by gene duplication.

2.2.2 Metabolism

As in the case of other biologically active peptides, the opioid peptides are quickly degraded by a number of peptidases (Marks et al., 1986). The Tyr-Gly bond is cleaved by an aminopeptidase (EC 3.4.11), and the Gly-Phe bond is susceptible to enzymatic
proteolysis by either the metalloendopeptidase EC 3.4.24.11 (enkephalinase) or angiotensin converting enzyme. An example of how important enkephalinase is to the elimination of the opioid peptides is shown by in vivo application of thiorphan, a potent inhibitor of enkephalinase. After infusion of thiorphan, analgesic properties were seen as a result of potentiated opioid activity through inhibited degradation (Garbay-Jaureguiberry et al., 1984).

2.3 Opioid receptor molecular biology

A major goal of opioid pharmacology is to develop analgesics devoid of addictive potential. It is hoped that through the exclusive activation of one receptor type, adverse side effects could perhaps be avoided. Various structure-activity relationships have been compiled for the ligands of each of the major receptor types, but ultimately, it is the receptor itself that must be characterized in order to fully understand binding and activation requirements. A major breakthrough in this area was achieved in 1992, when Evans (Evans et al., 1992) and Kieffer (Kieffer et al., 1992) simultaneously cloned the mouse δ opioid receptor (mDOR). This was achieved by incorporating cDNA libraries into COS-7 cells and then testing for radioligand binding of opioids. The clone expressing an opioid-binding protein was then isolated and characterized. During the search for novel somatostatin receptor clones, the mouse κ receptor (mKOR) was isolated and cloned by Yasuda and co-workers (Yasuda et al., 1993), which was followed by the cloning of the rat μ receptor (rMOR) by Chen and co-workers (Chen et al., 1993a) using a low stringency hybridization probe from mDOR. Since these early advancements, the major opioid receptors have been cloned from a variety of species and tissues (Satoh and Minami, 1995) (Table 2.3). High homology was seen between receptor types, and for each receptor type from different species. The conservation of amino acid residues in the receptors of differing species hints at the evolutionary importance of these receptors in mammalian development.
Table 2.3 Molecular cloning of opioid receptors

<table>
<thead>
<tr>
<th>Receptor Class</th>
<th>Species</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>mouse</td>
<td>NG108-15</td>
<td>(Evans et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>cerebrum</td>
<td>(Kieffer et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>striatum</td>
<td>(Fukuda et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SH SY5Y</td>
<td>(Knapp et al., 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Simonin et al., 1994)</td>
</tr>
<tr>
<td>μ</td>
<td>mouse</td>
<td>brain</td>
<td>(Kaufmann et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>brain</td>
<td>(Chen et al., 1993a)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>cerebrum</td>
<td>(Wang et al., 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cerebral cortex</td>
<td>(Bunzow et al., 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Wang et al., 1994)</td>
</tr>
<tr>
<td>κ</td>
<td>mouse</td>
<td>brain</td>
<td>(Yasuda et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>brain</td>
<td>(Chen et al., 1993b)</td>
</tr>
<tr>
<td></td>
<td>guinea pig</td>
<td>brain</td>
<td>(Xie et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>human</td>
<td>placenta</td>
<td>(Mansson et al., 1994)</td>
</tr>
</tbody>
</table>
The availability of all three major opioid receptors reveals similarities and differences between the opioid receptor types (Kieffer, 1995, Reisine and Bell, 1993, Satoh and Minami, 1995). The amino acid sequences of these receptors suggest the existence of seven transmembrane regions, a trait seen in the family of receptors known as G-protein coupled receptors. Regions that differ the most are the N- and C-terminals. All three receptors have the DRY sequence and Asp residues in the second and third transmembrane regions (TM2 and TM3) that are also characteristic of G-protein coupled receptors. In the N-terminus, two potential glycosylation sites have been identified, and a possible site for palmitoylation is located near the C-terminus. A proposed disulfide linkage may be achieved between two conserved Cys residues located in the first and second extracellular loops (EL1 and EL2) (see Fig. 2.2).

The availability of the cloned receptors offers an opportunity for researchers to study individual opioid receptor types with regard to pharmacological profile, structure-function analysis, cellular effector coupling, and receptor regulation. The cloned opioid receptors exhibited the expected binding affinities for various ligands based on earlier pharmacological studies, and it was confirmed that analgesics with high abuse potential have high selectivity for the μ receptor (Raynor et al., 1994) (Table 2.4). Thus, the addictive opioids morphine, fentanyl, and methadone have high affinities for the μ receptor, but little or no affinity for the δ or κ receptors.

2.3.1 Structure-function analysis of the opioid receptors

A pattern was found in the different opioid receptors in which the transmembrane helices, intracellular loops and a small portion of the C-terminus are highly homologous, but EL2 and EL3 and the N- and C-terminals are divergent (Kieffer, 1995). These observations, combined with the knowledge of how G-protein coupled receptors
Table 2.4 Binding affinities (nM) of ligands for the cloned κ-, δ-, and μ-opioid receptors

<table>
<thead>
<tr>
<th></th>
<th>κ receptor [³H]U-69,593</th>
<th>δ receptor [³H]naltrindole</th>
<th>μ receptor [³H]DAMGO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-selective compounds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynorphin A</td>
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<td>32</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>&gt;1000</td>
<td>4.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>&gt;1000</td>
<td>1.7</td>
<td>0.65</td>
</tr>
<tr>
<td>β-endorphin</td>
<td>52</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(-)-Naloxone</td>
<td>2.3</td>
<td>17</td>
<td>0.93</td>
</tr>
<tr>
<td>Levorphanol</td>
<td>6.5</td>
<td>5.0</td>
<td>0.086</td>
</tr>
<tr>
<td>Ethylketocyclazocine</td>
<td>0.40</td>
<td>101</td>
<td>3.1</td>
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<td>Etorphine</td>
<td>0.13</td>
<td>1.4</td>
<td>0.23</td>
</tr>
<tr>
<td>Pentazocine</td>
<td>7.2</td>
<td>31</td>
<td>5.7</td>
</tr>
<tr>
<td>Diprenorphine</td>
<td>0.017</td>
<td>0.23</td>
<td>0.072</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>3.9</td>
<td>149</td>
<td>1.0</td>
</tr>
<tr>
<td>Nalbuphine</td>
<td>3.9</td>
<td>&gt;1000</td>
<td>11</td>
</tr>
<tr>
<td>Nalorphine</td>
<td>1.1</td>
<td>148</td>
<td>0.97</td>
</tr>
<tr>
<td><strong>μ-selective compounds</strong></td>
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<tr>
<td>CTOP¹</td>
<td>&gt;1000</td>
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<tr>
<td>Dermorphin</td>
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<td>&gt;1000</td>
<td>0.33</td>
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<td>Methadone</td>
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<td>0.72</td>
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<td>DAMGO²</td>
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<td>2.0</td>
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<tr>
<td>PLO17³</td>
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<td>30</td>
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<td>Morphiceptin</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>56</td>
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<tr>
<td>Codeine</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>79</td>
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<tr>
<td>Fentanyl</td>
<td>255</td>
<td>&gt;1000</td>
<td>0.39</td>
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<tr>
<td>Sufentanyl</td>
<td>75</td>
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<td>0.15</td>
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<td>Lofentanil</td>
<td>5.9</td>
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<td>Naloxonazine</td>
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<td>8.6</td>
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<tr>
<td>Morphine</td>
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<td>&gt;1000</td>
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<td><strong>κ-selective compounds</strong></td>
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<td>Norbinaltorphimine</td>
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<td>U-50,488</td>
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<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>U-69,593</td>
<td>0.59</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
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<tr>
<td>ICI 204,488</td>
<td>0.71</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

1 D-Phe-cyclo[Cys-Tyr-D-Trp-Om-Thr-Pen]-Thr-NH$_2$
2 Tyr-D-Ala-Gly-NMePhe-NHCH$_2$OH
3 Tyr-Pro-NMePhe-D-Pro-NH$_2$
Table 2.4 -continued

<table>
<thead>
<tr>
<th></th>
<th>κ receptor [³H]U-69,593</th>
<th>δ receptor [³H]naltrindole</th>
<th>μ receptor [³H]DAMGO</th>
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<td><strong>δ-selective compounds</strong></td>
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</tr>
<tr>
<td>DPDPE⁴</td>
<td>&gt;1000</td>
<td>14</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>D-Ala²-Delt II</td>
<td>&gt;1000</td>
<td>3.3</td>
<td>&gt;1000</td>
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<tr>
<td>DSLET⁵</td>
<td>&gt;1000</td>
<td>4.8</td>
<td>39</td>
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<tr>
<td>BW 3734</td>
<td>17</td>
<td>0.013</td>
<td>26</td>
</tr>
<tr>
<td>DADLE⁶</td>
<td>&gt;1000</td>
<td>0.74</td>
<td>16</td>
</tr>
<tr>
<td>SIOM</td>
<td>&gt;1000</td>
<td>1.7</td>
<td>33</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>66</td>
<td>0.02</td>
<td>64</td>
</tr>
<tr>
<td>Nalriben</td>
<td>13</td>
<td>0.013</td>
<td>12</td>
</tr>
<tr>
<td>BNTX⁷</td>
<td>55</td>
<td>0.66</td>
<td>18</td>
</tr>
</tbody>
</table>


⁴Tyr-cyclo[D-Pen-Gly-Phe-D-Pen]-OH  
⁵Tyr-D-Ser-Gly-Phe-Leu-Thr  
⁶Tyr-D-Ala-Gly-Phe-D-Leu  
⁷-Benzylidenenaltrexone
Fig. 2.2 Schematic diagram of the rat \( \mu \), \( \delta \), and \( \kappa \) receptors. The opioid receptors share common characteristics with other G-protein coupled receptors, such as the seven transmembrane regions, a putative palmitoylation site near the C-terminus, and conserved Cys residues that may be involved in disulfide formation. Dark circles indicate identical residues among all three receptors; shaded circles indicate identical residues in two out of three receptors; open circles indicate unique residues for each receptor.

*Source:* Chan, Y.; Mestek, A.; Liu, J.; Yu, L. Molecular Cloning of a Rat \( \kappa \) Opioid Receptor Reveals Sequence Similarities to the \( \mu \) and \( \delta \) Opioid Receptors. *Biochem. J.* 1993, 295, 625-628.
communicate across the cell membrane, suggest a structure-function relationship for opioid receptors. Presumably, a binding pocket can be formed by the seven transmembrane helices with the extracellular loops discriminating between different opioid ligands, thereby affording the apparent selectivity between \( \mu \), \( \delta \), and \( \kappa \) receptors. The strong sequence homology seen in the third intracellular loop (IL3) and a small sequence in the C-terminus implies that the opioid receptors all interact with similar G-proteins. The high level of divergence in the C-terminus presents the possibility that different regulation patterns for the \( \mu \), \( \delta \), and \( \kappa \) receptors by intracellular mechanisms may be in effect (Chakrabarti et al., 1997) (Fig. 2.2).

### 2.3.2 Chimeric and site-directed mutagenesis studies

With the use of recombinant DNA, many studies have begun shedding light on the binding areas of the receptors, and on requirements for activation as well. Especially useful has been the application of chimeric receptors and site-directed mutagenesis. It was found that removal of the N-terminal regions of the opioid receptors did not adversely affect ligand binding or signal transduction (Kong et al., 1994, Wang et al., 1993). These findings suggest that putative N-glycosylation may play a role in protein targeting to the cell surface without being of crucial importance for receptor function (Kieffer, 1995). Initial studies to assess structural requirements at the receptor binding site primarily relied upon domain-swapping experiments. The resulting hybrid receptors are referred to as chimeras, after the legendary beast of Greek mythology. Chimeric studies have allowed the identification of determinants for opioid receptor selectivity.

#### 2.3.2.1 Determinants of \( \delta \)-selectivity

Comparisons of the amino acid sequences of the three major types of opioid receptors reveal that the extracellular loops and N-termini of the receptors have the highest degrees of
variability. Kong and co-workers (Kong et al., 1994) have shown that replacement of the first 78 residues of the δ receptor by those of the κ did not modify the δ-selectivity of DPDPE, naltrindole, [D-Ser², Thr⁶]enkephalin (DSLET) or deltorphin II. Meng and colleagues (Meng et al., 1996) have also demonstrated that introduction of more κ sequences (from the N-terminus up to TM3) into δ receptors barely affects δ ligand binding, suggesting that the first three transmembrane regions are little used in δ ligand recognition. These early chimeric studies therefore pointed towards the C-terminal half of the receptor as being involved in ligand recognition.

With the putative region largely responsible for δ ligand binding identified, many research groups then turned to point mutation studies to identify residues critical for ligand binding. From work done on β-adrenergic receptors (Strader et al., 1987), the Asp residue in TM3 provides important electrostatic stabilization for the cation of β-adrenergic ligands. In order to test this hypothesis for δ-opioid binding, Livingston et al. (Livingston et al., 1995) mutated Asp¹²⁸ in TM3 to Asn in the mDOR, and tested the effects of this mutation on ligand binding. The mutation abolished high affinity agonist binding to the receptor but did not affect selective antagonist binding. In a related study (Kong et al., 1993), Asp⁹⁵ located in TM2 of mDOR was mutated to Asn. It was determined that this residue is essential for the binding of specific agonists, but does not seem to play an especially important role in binding either non-specific agonists or antagonists. These two studies show that both Asp⁹⁵ and Asp¹²⁸ of the mDOR are essential for high affinity binding of selective agonists, but that only Asp¹²⁸ is needed for non-selective agonist binding to the receptor. The lack of effect of either mutation on the binding of antagonists suggests that agonists and antagonists may bind to δ receptors via distinct molecular mechanisms, possibly to different ligand binding domains. An interesting note that was not touched upon is that the selective agonists used in these studies were peptide-based, whereas the non-selective agonists and selective antagonists were non-peptide in nature. Therefore,
another possibility extrapolated from this data could be that peptides bind differently to the δ receptor than do non-peptides.

For the stabilization of the carboxy terminus of δ peptide ligands, Wang et al. (Wang et al., 1996) proposed that vicinal Arg residues located in EL3 (Arg291 and Arg292) may be responsible for anion stabilization. Another group (Varga et al., 1996) suggested that these Arg residues may act to neutralize nearby Asp residues within the receptor itself, and that it is this decrease of local negative charge that determines the selectivity of δ opioid peptides.

Charged residues are not the only important factors in ligand/receptor complex formation. Hydrophobic residues are involved in van der Waals interactions which can play a significant role in receptor binding. In a study done by Valiquette et al. (Valiquette et al., 1996) involving both chimeric and point mutation studies, it was found that residues Trp284, Val296, and Val297 of the human δ opioid receptor (hDOR) constituted a hydrophobic region which was important for normal binding of δ-selective ligands. These residues reside on EL3 of hDOR, and replacement of these residues by alanine resulted in a decrease in binding of the δ-selective ligands SNC-80, cyclo[D-Pen2,D-Pen5]enkephalin (DPDPE), deltorphin II, and naltrindole, while not affecting the binding of the non-selective ligand bremazocine. Replacement of Trp284 resulted in the largest decrease in δ binding, suggesting that this residue plays a major role in ligand recognition. In a similar study, Hjorth and co-workers (Hjorth et al., 1995) proposed that Glu297 of rKOR plays a similar role in the binding of κ ligands, and Valiquette notes that Glu297 of the κ receptor and Trp284 of the δ receptor are located at corresponding positions of the two receptors. The authors then suggest that the residue at this position (δ=Trp, κ=Glu, and μ=Lys) may represent a crucial recognition site for opioid ligands.

The crucial nature of this hydrophobic region near the end of EL3 was further emphasized by a novel "restoration of function" assay (Pepin et al., 1997). In this study, the region corresponding to EL3 of hMOR was inserted into hDOR. A library of mutants was generated in which some of the 10 amino acids in EL3 were reverted back to the
corresponding δ amino acid. The "restored" mutants all exhibited a hydrophobic region between residues 295-300, and an Arg residue at position 292.

2.3.2.2 Agonist vs. antagonist activity

In a study done by Claude and co-workers (Claude et al., 1996), chimeric studies of the opioid receptors revealed that opioid antagonists (i.e. naloxone, naltrexone, naltriben, and TIPPψ) were able to act as agonists with respect to inhibiting forskolin-stimulated adenylyl cyclase activity in chimeric opioid receptors expressed in CHO cells. By sequence analysis and back mutation, it was found that the observed agonist activity was due to the mutation of a conserved Ser to Leu in TM4. The importance of this serine residue was further demonstrated by analogous mutations created in the μ (MORS196L) and the δ (DORS177L) receptors, which resulted in imparting agonist activity to the aforementioned antagonists. The authors hypothesize that the mutation of the Ser to a non-hydrogen bonding residue results in a disruption of receptor tertiary structure, resulting in a more "permissive" receptor activation state.

2.3.2.3 Receptor down-regulation

The techniques of molecular biology have also been used in the study of opioid receptor down-regulation. It has been long known that prolonged exposure to opioids leads to decreased response to the ligand; this reduced sensitivity is thought to be due to the loss of receptors, or down-regulation. Previous studies done with other G-protein coupled receptors (i.e. adrenergic receptors) (Dohlman et al., 1991) have shown that the C-terminus is instrumental in down-regulation via the phosphorylation of certain side chains. A C-terminal truncated mDOR was cloned in CHO cells, and exhibited no change in receptor density even after prolonged (2-48 h) exposure to the δ agonist [D-Ala²,D-Leu⁵]enkephalin (DADLE) (Cvejic et al., 1996). The C-terminal truncated mutant did not exhibit any
difference in functional coupling, which confirms the observation that the C-terminus is not important for G-protein coupling. Point mutation studies of the phosphorylatable groups in the C-terminus (i.e. Ser\textsuperscript{344}, Thr\textsuperscript{352}, and Thr\textsuperscript{353}) showed that mutation of Thr\textsuperscript{353} to Ala results in a receptor type that does not down-regulate even after 48 h of agonist treatment.

2.4 Delta opioid receptor subtypes

Over the past twenty years, the opioid receptor family has been well documented, and the existence of the three major opioid receptor types is well established and accepted. In addition, pharmacological evidence for receptor subtypes for all three opioid receptors has been increasing these past few years (Mattia et al., 1991, Paul et al., 1989, Sofuoglu et al., 1991, Zukin et al., 1988). Especially compelling evidence for the existence for receptor subtypes has been compiled for the \(\delta\) opioid receptor (Zaki et al., 1996).

The first studies involving \(\delta\) opioid receptor subtypes dealt with DPDPE and the \(\delta\)-selective peptide derived from amphibian skin \([D-Ala^2,Glu^4]deltorphin\), sometimes referred to as deltorphin II. It was found that treatment with \([D-Ala^2,Cys^6]enkephalin\) (DALCE), a \(\delta\)-selective affinity label, selectively antagonizes the antinociceptive effects of DPDPE, but not that of \([D-Ala^2,Glu^4]deltorphin\) or of \(\mu\) agonists (Jiang et al., 1991). Conversely, it was also found that the \(\delta\)-selective affinity label naltrindole isothiocyanate (NTII) could selectively antagonize the effects of \([D-Ala^2,Glu^4]deltorphin\), but not DPDPE.

The hypothesis of subtypes of \(\delta\) opioid receptors was further explored by employing classic tolerance and cross-tolerance studies. It was postulated that if DPDPE and \([D-Ala^2,Glu^4]deltorphin\) were producing their supraspinal antinociception at different receptors, then antinociceptive cross-tolerance might not occur. It was found that after inducing analgesia with repeated applications of the aforementioned \(\delta\)-selective compounds and the \(\mu\)-agonist DAMGO, cross tolerance between these three compounds could not be observed (Mattia et al., 1991, Sofuoglu et al., 1991). These studies have led to the classification of the \(\delta\) opioid receptors as the \(\delta_1\) and \(\delta_2\) subtypes, a classification based on selective
antagonist studies. The δ₁ receptor subtype is defined as being activated by DPDPE, and antagonized by DALCE. The δ₂ receptor subtype is activated by [D-Ala², Glu⁴]Deltorphin, and antagonized by NTII.

Despite the existence of compelling pharmacological evidence, demonstration of δ opioid receptor subtypes in binding studies has been inconclusive so far. Negri and colleagues (Negri et al., 1991) have suggested the existence of δ subtypes in rat brain, as evidenced by biphasic inhibition of [³H][D-Ala², Glu⁴]Deltorphin binding by DPDPE. However, competition binding experiments done with [D-Ala²,Glu⁴]Deltorphin inhibition of [³H]DPDPE binding exhibited a monophasic binding pattern, suggesting receptor homogeneity. More recently, Fang and co-workers (Fang et al., 1994) have studied the binding characteristics of [³H]NTI binding in mouse brain and mouse vas deferens. Through competitive interaction with selective agonists, multiple binding sites were observed in mouse brain, but only a single binding site was evident in the vas deferens. These studies have added support for the heterogeneity of δ receptors in mouse brain and suggests the possibility that δ receptors in the mouse vas deferens may differ from those found in the brain.

With the cloning of the δ receptor the possible existence of subtypes can be examined by using antisense oligodeoxynucleotides (ODN) (Wahlestedt, 1994). Antisense ODNs are short, synthetic, single stranded DNA whose mode of action is through their hybridization to complementary sequences in the target gene or its messenger RNA; this interferes with the normal translation of the gene, resulting in a reduction, or "knock down," of the protein product. Pasternak and co-workers (Bilsky et al., 1994) examined antisense ODNs for the δ opioid receptor in mice. When given intracerebroventrically (i.c.v.), pretreatment with an antisense (but not mismatch) ODN for the δ opioid receptor blocked the antinociceptive actions of i.c.v. [D-Ala²,Glu⁴]Deltorphin, but not that of i.c.v. DPDPE. However, when the antisense (or mismatch) ODN was given intrathecally (i.t.), it was found that the antinociceptive actions of DPDPE and [D-Ala²,Glu⁴]Deltorphin were both inhibited, but
that the antinociceptive actions of the μ-agonist DAMGO was unaffected. These experiments added pharmacological evidence for receptor heterogeneity in mouse supraspinal sites, and that the analgesic activity of δ opioid agonists at the spinal level is mediated by a single δ receptor subtype, the δ2 opioid receptor.

Despite the growing amount of evidence implicating subtypes of the opioid receptors, attempts to isolate and clone the genes for the different subtypes have not yet been successful. Therefore, on the molecular level, the existence of opioid receptor subtypes has yet to be proven. The fact that extensive homologous screening for these subtypes has not proven successful has prompted researchers to speculate as to why the subtypes are so elusive. The theories for receptor diversity include alternative splicing, posttranslational modifications, a difference in associated proteins, and perhaps a difference in receptor location and ligand accessibility (compartmentalization) (Zaki et al., 1996).

There is also evidence that there are interactions between μ- and δ-receptors (Traynor and Elliott, 1993). Porreca and co-workers (Jiang et al., 1990b) have shown that at sub-analgesic doses, δ-opioid receptor agonists can modulate the efficacy of μ-opioid receptor agonists. At subanalgesic doses, DPDPE was able to potentiate the effects of i.c.v. morphine. Conversely, the agonists [D-Ala2, Met5]enkephalaminamide (DAMA) and [Met5]-enkephalin were found to inhibit the antinociceptive effects of morphine. Although these modulatory effects are also seen in opioid-induced inhibition of intestinal motility, δ-agonists do not seem to effect the development of morphine tolerance. The positive and negative modulatory effects of δ-agonists can be prevented by pretreatment with the δ antagonist ICI-174,864.

As in the case of classifying the δ1 and δ2 subtypes, irreversible antagonists were important in the study of the complexed δ receptor (δcx) and the non-complexed δ receptor (δncx) (Jiang et al., 1990a). When DALCE was given i.c.v., inhibition of δ-mediated analgesia was observed, but it did not affect the modulatory effects of DPDPE or [Met5]enkephalin on morphine-induced antinociception. In contrast, the conformationally
constrained enkephalin analog \([D-Ala^2,(2R,3S)cyclopropylPhe^4]\) enkephalin (CP-OH) is able to modulate the actions of morphine, but does not show direct analgesia.

### 2.5 Conclusions

The field of opioid pharmacology has made incredible gains towards the understanding of the actions and binding requirements of opioid receptor activation. Starting with the demonstration of stereoselective binding of opioids (Pert and Snyder, 1973, Simon et al., 1973, Terenius, 1973), through the isolation of endogenous opioids and the development of selective ligands, and culminating with the cloning of and recombinant experimentation with the opioid receptors, the pieces of the opioid puzzle are starting to fit together. Despite the overall achievements in the field, some lingering questions remain: is it possible to achieve analgesia without undesired side effects? Given the primary structures of the opioid receptors, which residues are essential for ligand binding and/or selectivity? If opioid receptor subtypes exist, are they different in amino acid composition, or by other differences, such as posttranslational modifications?

One way to help answer those questions is through the development of selective irreversible ligands (affinity labels). The affinity labels already in use have contributed greatly towards the understanding of opioid pharmacology. The recent recombinant work with opioid receptors has suggested potential differences in the binding sites of agonists and antagonists, and possibly peptides and non-peptides. A wider variety of affinity labels would help identify which residues are near the binding site by alkylation, and their irreversible nature would help in the isolation and characterization of receptors expressed in various tissues. Selective affinity labels based on antagonists may also be used for the elucidation of physiological effects.
Chapter 3
Literature Review: Delta-Selective Opioid Ligands

3.1 Introduction

While pain is an important adverse stimulus necessary for survival, for millennia humans have sought to alleviate it. For the treatment of pain, the extract of the opium poppy *Papaver somniferum* has been a mixed blessing: it is an excellent analgesic, but the serious side effects of addiction and respiratory depression precludes its therapeutic usefulness. In an effort to separate the analgesic properties of the opiate drugs from their undesired side effects, many synthetic and semi-synthetic were created and tested. These early attempts in the field of opioid medicinal chemistry did not yield a "perfect" analgesic, but many clinically useful drugs did result from these studies, such as meperidine, methadone, propoxyphene, nalorphine, and phenazocine.

Synthetic methods, screening studies, and isolation from various biological sources have resulted in a wide variety of structurally diverse opioid ligands. Selective agonists and antagonists have been instrumental in the characterization of the three major types of opioid receptors: the μ (μ), kappa (κ) and delta (δ) opioid receptors. The heterogeneity of these receptors has recently been confirmed through molecular biology (Kieffer, 1995, Reisine and Bell, 1993, Satoh and Minami, 1995). In addition, selective ligands have been used to accumulate evidence strongly suggesting the existence of receptor subtypes for all three opioid receptors (Mattia et al., 1991, Paul et al., 1989, Sofuoglu et al., 1991, Zukin et al., 1988).

The opioids with high addiction potential have been shown to interact preferentially with the μ receptor (Raynor et al., 1994). As a result, interest in the δ receptor is increasing rapidly, as research suggests that this receptor is able to produce antinociception with lesser abuse potential (Rapaka and Porreca, 1991), and it is also implicated in the reward pathways of cocaine and alcohol abuse (Gianoulakis, 1996).
3.2 Delta-selective opioid peptide agonists

In the study of heterogeneous receptors, it is important to utilize agonists and antagonists with selectivity for the receptor under question. The earliest examples of ligands with a slight preference for δ opioid receptors were the endogenous opioid peptides Leu- and Met-enkephalin (Hughes et al., 1975). These peptides were found to be more effective in inhibiting the electrically stimulated twitch of the mouse vas deferens than morphine, and were instrumental in defining the δ opioid receptor (Lord et al., 1977). Since the isolation of the enkephalins from pig brain extracts, the search for other naturally occurring opioid peptides have revealed the existence of numerous peptides from a variety of sources. An unusual source of δ-selective opioid peptides are the subcutaneous glands of the Phyllomedusa bicolor frog of South America (Erspamer, 1992). These heptapeptides are orders of magnitude more selective for the δ receptor than the endogenous opioids found in mammalian systems. This exceptional selectivity is reflected in the name for this class of opioid peptides: the deltorphins (the first reported deltorphin peptide is also referred to as dermenkephalin). The deltorphins share a common N-terminal sequence (Tyr-D-Xaa-Phe) with a class of μ-selective opioid peptides also secreted by the same species of frog, known as the dermorphins. This shared N-terminal sequence contrasts with the common sequence seen in mammalian opioid peptides, Tyr-Gly-Gly-Phe. The differences between the mammalian and amphibian opioid peptides can be seen in Table 3.1.

In the drive to synthesize peptide analogs, it should be no surprise that early attempts to develop δ-selective ligands were based on modifications to the enkephalin parent molecules, the earliest ligands known with preference for δ receptors. Enkephalin is quickly metabolized by various peptidases to inactive fragments (Hambrook et al., 1976), and therefore, one of the earliest goals of enkephalin modification was to increase metabolic
stability. Depending on the nature of the modification, the enkephalin analog could show selectivity for either $\mu$ or $\delta$ receptors (enkephalins generally have very low affinity for $\kappa$ receptors). Substitution of a D-amino acid in position 2 was one of the early modifications to enkephalins in order to increase metabolic stability. It was found that this substitution increased potency at both $\mu$ and $\delta$ receptors and is therefore found in a majority of enkephalin analogs. [D-Ala$^{2}$,D-Leu$^{5}$]enkephalin (DADLE) was an early derivative which took advantage of placing a D-amino acid in position 2 in order to increase $\delta$ selectivity (Beddell et al., 1977). These modifications to the enkephalin parent molecule were performed well before the discovery of the deltorphins, which also have a D-amino acid at the 2 position. Generally, if a hydrophilic D-amino acid (i.e. D-Ser, D-Thr) is substituted in position 2, $\delta$ selectivity is observed. Mu ligands generally prefer a more hydrophobic residue at position 2. At the C-terminus more selective $\delta$ ligands usually contain a free carboxylic acid, and lengthening the peptide chain with an amino acid such as Thr can result in increased $\delta$-selectivity. Incorporation of a D-Ser at position 2 coupled with peptide chain lengthening with Thr, led to [D-Ser$^{2}$,Thr$^{6}$]enkephalin (DSLET), which shows a slight preference for $\delta$ receptors (Gacel et al., 1988). Changing the D-Ser to a D-Thr leads to the increased $\delta$ selectivity seen for the ligand [D-Thr$^{2}$,Thr$^{6}$]enkephalin (DTLET) (Zajac et al., 1983). It was also found that increasing the steric bulk at positions 2 and 6 also helped confer increased $\delta$ selectivity to the peptides. This was done by incorporating a tBu ether into those positions. These substitutions led to [D-Ser(OtBu)$^{2}$,Thr$^{6}$]enkephalin (DSTBULET) and [D-Ser(OtBu)$^{2}$,Thr(OtBu)$^{6}$]enkephalin (BUBU) (Gacel et al., 1988). The increased steric bulk decreased affinity for the $\mu$ receptor, and in doing so, increased selectivity for the $\delta$ receptor. These modifications to enkephalin and their effect on $\delta$ receptor binding and selectivity can be seen in Table 3.2.
Table 3.1 Binding affinities of naturally occurring δ-selective opioid peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_i$ (nM)</th>
<th>𝜇</th>
<th>𝛿</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine enkephalin</td>
<td>YGGFL</td>
<td>9.43</td>
<td>2.54</td>
<td></td>
<td>3.71</td>
</tr>
<tr>
<td>Methionine enkephalin</td>
<td>YGGFM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deltorphin (or Dermenkephalin)</td>
<td>Y-DM-FHLMD-NH$_2$</td>
<td>1630</td>
<td>2.41</td>
<td></td>
<td>676</td>
</tr>
<tr>
<td>[D-Ala$^2$]deltorphin I</td>
<td>Y-DA-FDVVG-NH$_2$</td>
<td>3150</td>
<td>0.15</td>
<td></td>
<td>21000</td>
</tr>
<tr>
<td>[D-Ala$^2$]deltorphin II</td>
<td>Y-DA-FEVVG-NH$_2$</td>
<td>2450</td>
<td>0.71</td>
<td></td>
<td>3450</td>
</tr>
</tbody>
</table>

Table 3.2 Binding affinities of linear enkephalin analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_i$(nM)</th>
<th>𝜇</th>
<th>𝛿</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSLET</td>
<td>Y-DS-GFLT</td>
<td>31.0</td>
<td>4.80</td>
<td></td>
<td>6.46</td>
</tr>
<tr>
<td>DTLET</td>
<td>Y-DT-GFLT</td>
<td>23.3</td>
<td>1.35</td>
<td></td>
<td>17.3</td>
</tr>
<tr>
<td>DSTBULET</td>
<td>Y-DS(tBu)-GFLT</td>
<td>374</td>
<td>6.14</td>
<td></td>
<td>60.9</td>
</tr>
<tr>
<td>BUBU</td>
<td>Y-DS(tBu)-GFLT(tBu)</td>
<td>475</td>
<td>4.68</td>
<td></td>
<td>101</td>
</tr>
</tbody>
</table>

Conformational constraints are commonly used in peptide chemistry to limit peptide flexibility and stabilize a "bioactive" conformer in an effort to increase affinity and/or selectivity for a given receptor. These constraints can either be global (through head-to-tail or side-chain to side-chain cyclizations) or local (through incorporation of amino acid derivatives with side chain modifications). In the case of δ opioid peptides, conformational constraints were used successfully to increase δ selectivity (Table 3.3). The first examples of disulfide cyclization utilizing Cys residues at positions 2 and 5 were done by Sarantakis (Sarantakis, 1979) and Schiller (Schiller et al., 1981). These cyclic enkephalin analogs only exhibited a slight preference for δ over μ receptors, but δ selectivity increased dramatically by substituting penicillamine (Pen) residues for Cys. The cyclic pentapeptide cyclo[D-Pen\textsuperscript{2},D-Pen\textsuperscript{5}]enkephalin (DPDPE) is one of the most selective δ agonists to date (Mosberg et al., 1983). It is thought that the geminal dimethyl groups of Pen further reduces conformational flexibility of the peptide, resulting in increased selectivity. Investigation of substituents on the Phe\textsuperscript{4} residue revealed that a p-chlorine at this position resulted in increased δ selectivity (Schiller et al., 1989). In order to further limit conformational freedom, the ring size of DPDPE was contracted by deleting the Gly\textsuperscript{3} residue. The result was increased δ selectivity in the cyclic tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]-OH (JOM-13) (Mosberg et al., 1988). Unlike the DPDPE series in which δ-selectivity is maintained despite various substitutions in residues 2 and 5, any variation in JOM-13 results in greatly reduced δ affinity.
Table 3.3 Binding affinities of cyclic enkephalin analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$K_i$ (nM)</th>
<th>$K_i$ ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH (DPDPE)</td>
<td>609</td>
<td>116</td>
</tr>
<tr>
<td>Tyr-c[D-Pen-Gly-Phe-L-Pen]-OH (DPLPE)</td>
<td>3710</td>
<td>371</td>
</tr>
<tr>
<td>Tyr-c[D-Pen-Gly-pCl-Phe-D-Pen]-OH</td>
<td>901</td>
<td>574</td>
</tr>
<tr>
<td>Tyr-c[D-Cys-Phe-D-Pen]-OH (JOM-13)</td>
<td>1210</td>
<td>637</td>
</tr>
</tbody>
</table>


Fig. 3.1 Delta-selective nonpeptide agonists

Oxymorphindole (OMI) 7-Spiroindanyloxymorphone (SIOM)
3.3 Delta-selective non-peptide opioid agonists

Until very recently, all of the δ-selective agonists were peptidic in nature, but in the past few years the first nonpeptidic δ-selective opioid agonists were described (Fig. 3.1). BW373U86 was discovered through pharmacological screening, and shows a modest selectivity for δ receptors (Chang et al., 1993). In antinociceptive tests done in mice, it seems to act as a partial agonist at both δ and μ receptors. Initial tests involving BW373U86 were done with the racemate, which complicated the pharmacological profile of the compound. Therefore Rice and co-workers separated and characterized the different isomers and subjected them to opioid binding assays. It was found that isomers with the R configuration at the benzylic carbon exhibited greater affinity for δ receptors than did isomers with the S configuration. The methyl ether derivative of (+)-BW373U86 exhibited increased selectivity for the δ over μ receptor in binding assays and in smooth muscle assays (~2000-fold selectivity) (Calderon et al., 1994). The methyl ether derivative is known as SNC-80, and to date it is the most selective non-peptide agonist for the δ opioid receptor.

A different approach was taken by Portoghese and co-workers, in which they postulated a properly spaced aromatic moiety (an "address" sequence) held away from the morphinan structure (the "message" sequence) would increase δ-affinity to a non-selective opioid agonist. The non-selective agonist oxymorphone underwent indole functionalization to give the δ-selective partial agonist, oxymorphindole (OMI) (Portoghese et al., 1990). The partial success of OMI prompted the researchers to investigate the orientation of the aromatic ring in their agonist derivatives. This led to the attachment of a spiroindane ring system onto oxymorphone to give the δ₁-selective agonist, 7-spiroindanyloxymorphone (SIOM) (Portoghese et al., 1993).
3.4 Delta-selective opioid peptide antagonists

Antagonists for a specific receptor are valuable research tools for the elucidation of receptor-specific physiological effects. Such antagonists for δ opioid receptors were instrumental in ascertaining the ability of δ receptors mediate analgesia. Early work done on antagonists were based on modifying the 4,5-epoxymorphinan structure by varying the N-substituent. It was found that while certain N-substituents (like the phenethyl moiety) increase agonist activity, many other substituents, like propyl, allyl, cyclopropylmethyl, and cyclobutylmethyl, impart antagonist activity to the ligand. Following this example, the early work in synthesizing peptide-based antagonists for the δ opioid receptor concentrated on N-terminal substituents to impart antagonistic activity. This led to the synthesis of ICI-174,864, a pentapeptide enkephalin analog with diallyl functionalization at the N-terminus (Cotton et al., 1984). The glycines of enkephalin in positions 2 and 3 are replaced with Aib (2-aminoisobutyric acid) in ICI-174,864. These sterically hindered residues limit the conformational flexibility of the peptide backbone. It was also found that N,N-dibenzyl derivatives of enkephalin exhibited δ-selective antagonism (Lovett and Portoghese, 1987a).

Through either synthesis or isolation, it was found that a number of opioid peptides have a Phe residue at the 3 or 4 position, and a Tyr residue at position 1. Examples of opioid peptides with Phe at position 3 include the amphibian-derived peptides deltorphin and derrmorphin, and examples of peptides with a Phe at position 4 include the mammalian endogenous opioid peptides the enkephalins. Through the synthesis and pharmacological testing of a number of opioid peptide derivatives containing para-nitro Phe derivatives at the 3 or 4 position, it has been postulated that Phe$^3$-containing opioid peptides utilize a different aromatic binding site than do Phe$^4$-containing peptides (Schiller et al., 1983). In an effort to utilize both putative aromatic binding sites, Schiller and co-workers investigated
### Table 3.4 Activities of peptide-based antagonists for delta opioid receptors

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_c$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI-174,864</td>
<td>diallyl-Tyr-Aib-Aib-Phe-Leu-OH</td>
<td>17</td>
</tr>
<tr>
<td>(Bn)$_2$ Leu enk</td>
<td>dibenzyl-Tyr-Gly-Gly-Phe-Leu-OH</td>
<td>210</td>
</tr>
<tr>
<td>TIPP</td>
<td>Tyr-Tic-Phe-Phe-OH</td>
<td>3.0-5.9</td>
</tr>
<tr>
<td>TIPP-NH$_2$</td>
<td>Tyr-Tic-Phe-Phe-NH$_2$</td>
<td>14-18</td>
</tr>
<tr>
<td></td>
<td>Tyr-D-Tic-Phe-Phe-NH$_2$</td>
<td>(µ-agonist, IC$_{50}=37$ in GPI)</td>
</tr>
</tbody>
</table>


![Fig. 3.2 Non-peptide antagonists for delta opioid receptors](image)

**Fig. 3.2** Non-peptide antagonists for delta opioid receptors

X = NH naltrindole (NTI)
X = O naltriben (NTB)

7-benzylidenenaltrexone (BNTX)
a number of tetrapeptides containing Phe at both the 3 and 4 positions. Depending on the identity and stereochemistry of the amino acid at position 2, a number of μ- or δ-selective ligands were synthesized. When the researchers incorporated the conformationally constrained Phe analog L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) in position 2, a highly potent and selective δ antagonist Tyr-Tic-Phe-Phe (TIPP) resulted (Schiller et al., 1992).

3.5 Delta-selective non-peptide opioid antagonists

Non-peptide opioid antagonists historically have been based on the morphinan-structure, and generally exhibited low (if any) selectivity for a specific receptor type. Examples of such antagonists include naloxone and naltrexone. Portoghese and co-workers postulated that a properly placed aromatic ring on a non-selective antagonist would serve to mimic the Phe⁴ of the enkephalins, thereby imparting δ-selectivity to the ligand. This approach to ligand design resulted in a number of selective compounds, two of which showed exceptional δ-selective antagonism: naltrindole (NTI) (Portoghese et al., 1990), naltriben (NTB) (Portoghese et al., 1991) and 7-benzylidenedenaltrexone (BNTX) (Portoghese et al., 1992) (Fig. 3.2). In both of these compounds, the additional aromatic ring which imparts the δ-selectivity is held away from the naltrexone skeleton by a spacer moiety, either the pyrrole of NTI, the furan of NTB, or the ethylene bridge in BNTX. The authors suggest that the co-planar orientation of the aromatic rings of the indole-containing analogs may stabilize an antagonist state of the δ receptor. In contrast, the orthogonal arrangement of the aromatic ring in the agonist SIOM prevents such a stabilization from occurring, resulting in full agonist properties.
Fig. 3.3 Delta opioid receptor pharmacophore
3.6 Conformational analysis of δ receptor ligands

With the large number of diverse ligands (both peptidic and non-peptidic) selective for the δ receptor, many groups have turned to molecular modeling in order to identify the 3-dimensional orientation of pharmacophoric groups required for δ receptor recognition. It is a generally held belief that the minimal requirements for δ receptor binding is a protonated amine, a phenolic functionality (as in Tyr of the δ-selective opioid peptides), and an aromatic moiety (as in Phe or Phe residues of δ-selective peptides) (Fig. 3.3). In the design and synthesis of δ opioid receptor selective ligands, an understanding of the binding requirements of the δ opioid receptor must be further understood. More specifically, it is important to define the 3-dimensional spatial orientation of the important pharmacophores of δ-selective opioid peptides. The ligands used most often in conformational analyses are the δ-opioid peptides DPDPE and JOM-13, and the non-peptidic NTI and its analogs.

In the δ-selective cyclic peptide DPDPE, the constraint imposed by the internal disulfide bond serves to limit conformations available to the peptide, thereby simplifying computational procedures. Early molecular modeling work done with this peptide confirmed that the peptide backbone within the ring had a somewhat rigid structure, whereas exocyclic portions of the molecule (including the important Tyr and Phe side chains) still exhibited a wide range of conformations, which made defining a "bioactive" conformer for DPDPE difficult (Mosberg et al., 1990). Like DPDPE, the results of molecular modeling experiments with JOM-13 indicated that the Tyr and Phe side chains still exhibited a great deal of conformational freedom (Lomize et al., 1994). The researchers then turned to NMR and other experiments to analyze low energy solution conformations of these cyclic peptides.

NMR analyses of DPDPE done in H2O and DMSO had shown that there is a rapid interchange between a number of local conformations (Hruby et al., 1988), and therefore did not provide a clear representation of a single low energy conformer as the receptor bound candidate. More answers as to the conformation needed for receptor binding may be
expected from recent X-ray crystallographic studies done with DPDPE (Deschamps et al., 1996). In the X-ray structure, there were three independent molecules of DPDPE in the asymmetric unit. The conformation of the 14-membered ring and the orientation of the Phe\textsuperscript{4} side chain ($\chi_1 = \sim 60^\circ$) were essentially the same in all three molecules. Once again, the only significant conformational difference was in the orientation of the exocyclic Tyr side chain. The Tyr amide bond was trans in all conformations, but a large difference was seen in the $\psi_1$ torsion angle (-157°, -175°, +90°). X-ray studies done on JOM-13 resulted in very similar findings. The crystallographic studies done with JOM-13 revealed the presence of two distinct conformations for the 2 independent peptide molecules in the asymmetric unit. Like DPDPE, JOM-13 exhibited the greatest difference in the $\psi_1$ angle (159°, 102°); there was also a difference in the disulfide bond conformation as well (89° vs. -99°).

An extensive study done by Mosberg and co-workers (Mosberg et al., 1996, Mosberg et al., 1994a, Mosberg et al., 1994b) modified JOM-13 with amino acid analogs by introducing conformationally restricted side chains in order to investigate the spatial orientations of the phenolic and aromatic side chains of Tyr\textsuperscript{1} and Phe\textsuperscript{3}, respectively. Through the use of such analogs, the researchers had found that the Tyr\textsuperscript{1} side chain preferred the trans conformation, while the Phe\textsuperscript{3} residue preferred the gauche (-) conformation. Mosberg and co-workers (Lomize et al., 1996) then validated their work by superimposing different δ-selective ligands (both agonists and antagonists) onto their JOM-13 molecule exhibiting optimal pharmacophore placement. In their molecular modeling studies, they found that there was high spatial overlap of the major pharmacophoric groups with opioid peptides DPDPE and antagonist TIPP; and also extensive overlap with the non-peptide ligands oxymorphindole (OMI) and spiroindanyloxymorphone (SIOM). The researchers also noted that when the peptidic and non-peptidic antagonists (TIPP and naltrindole, respectively) were superimposed on JOM-13, the aromatic rings had
similar orientation, but exhibited different $\chi_2$ angles. The authors hypothesize that this
difference in the $\chi_2$ angles is a possible explanation for the different actions observed for
NTI and JOM-13 at the $\delta$ receptor (i.e. antagonist vs. agonist activity, respectively).

A peptide of interest in many conformational analyses is the tetrapeptide antagonist TIPP
(Schiller et al., 1992). It is an unusual opioid peptide in that it is completely comprised of
aromatic amino acids. Many groups have postulated as to which aromatic moiety is
responsible for the $\delta$-selectivity of this compound. Two possibilities exist: either the Tyr-
Tic dipeptide or the more commonly recognized Tyr-Xaa-Phe moiety is acting as the $\delta$
receptor pharmacophore (Fig. 3.4). The residue of Phe$^4$ is not considered essential for $\delta$
receptor recognition, because deletion of this C-terminal residue only results in a slight
(~10-fold) loss of affinity at the $\delta$ receptor (Schiller et al., 1992).

It has been reported that the dipeptide H-Tyr-Tic-NH$_2$ and the tripeptide H-Tyr-Tic-Ala-
OH have weak but measurable $\delta$ antagonist activity (Temussi et al., 1994). In order to test
whether or not the second residue of TIPP simply acts as a spacer for the aromatic Phe$^3$
residue, another study replaced the Tic residue with pipecolic acid (Pip). The resulting
tetrapeptide H-Tyr-Pip-Phe-Phe-OH was a full agonist in the MVD and GPI assay. These
observations suggest that the Tic$^2$ aromatic ring in TIPP-related $\delta$-opioid antagonists is
essential for $\delta$-receptor antagonism. Therefore it has been proposed that it is the aromatic
ring of Tic$^2$ (and not Phe$^3$) that represents the second aromatic ring needed for opioid
receptor binding and antagonist activity.

In hypothesizing which aromatic residue of TIPP is responsible for $\delta$ opioid
antagonism, an important compound for comparison is the previously mentioned $\delta$-opioid
antagonist NTI. Of interest to computational chemists is the fact that NTI is a rigid
molecule, and with the assumption that both molecules bind to the same receptor subsites,
NTI could serve as a template in molecular modeling studies comparing both $\delta$ antagonists.
Many such molecular modeling studies have been undertaken (Amodeo et al., 1996, Chew
et al., 1993, Wilkes and Schiller, 1994, Wilkes and Schiller, 1995), but due to the flexible
Fig. 3.4 A diagrammatic representation of delta opioid ligands and their pharmacophores. A. The endogenous ligand, leucine enkephalin; B. the rigid nonpeptide ligand, naltrindole (NTI); C. representation of TIPP with Tic\(^2\) as the second aromatic moiety needed for binding; D. representation of TIPP with Phe\(^3\) as the second aromatic moiety needed for binding.
nature of the peptide backbone and side chains, coupled with the added complexity of cis/trans isomerism about the peptide bond between Tyr\textsuperscript{1} and Tic\textsuperscript{2}, the results have been somewhat inconclusive. Several researchers have proposed that it is the Tic\textsuperscript{2} residue that provides the aromatic ring which corresponds to the indole of NTI.

Other research groups also have investigated the "receptor-bound" conformer of \(\delta\) opioid ligands. In an extensive molecular modeling study done by Loew and co-workers (Chao et al., 1996), they found that both agonists and antagonists can satisfy the spatial requirements needed for \(\delta\) receptor recognition.

3.7 Affinity labels for opioid receptors

Affinity labels are ligands which bind irreversibly to a receptor, and therefore are important pharmacological tools for the study of a receptor. The irreversible nature of an affinity label makes the ligand more advantageous than a reversible ligand for certain receptor studies. Affinity labels are very effective in mapping the location and distribution of specific receptor types. Selective agonists used in conjunction with affinity labels can be used in determining the relative involvement of a certain receptor in a particular opioid-induced pharmacological effect. Finally, non-equilibrium ligands are inherently long-lasting, and as such, may find use as a therapeutic agent, e.g. as a long-lasting narcotic antagonist (Takemori and Portoghese, 1985).

In order to interact with receptors in a non-equilibrium fashion, many affinity labels have electrophilic or photolabile moieties that are able to form a covalent linkage with reactive side chains found on the receptor. The success of an electrophilic affinity label is primarily due to four parameters: (1) the affinity label must exhibit high affinity for the receptor; (2) the affinity label should preferentially exhibit selectivity for the ligand; (3) the location of the electrophilic moiety on the affinity label must be able to align itself with a reactive group on receptor; and (4) the electrophilic group must be reactive enough to be
subject to attack by a receptor-bound nucleophile. If any of the above parameters are not met, the result could be an ineffective affinity label.

The affinity labeling process is thought to proceed via two distinct recognition steps (Fig. 3.5). The first recognition step involves reversible binding to the receptor (which is reflected by the affinity of the ligand for the receptor), and the second step involves the alignment of the electrophile with a compatible nucleophile on the receptor, resulting in covalent bond formation. These two separate recognition steps may lead to enhanced receptor selectivity, which is known as recognition amplification.

The nitrogen mustard derivative of oxymorphone, β-chloroxymorphamine (β-COA), is an irreversible agonist in the GPI (Caruso et al., 1980b, Caruso et al., 1979). This observation suggests that receptor occupation rather than rate of ligand-receptor dissociation is important for agonist activity in the GPI. β-COA also appears to bind irreversibly to opioid receptors in brain membranes, as exhaustive washing and eight hours of dialysis of β-COA treated membranes did not eliminate the inhibition of [3H]naltrexone binding. In mice, β-COA produces analgesia with a duration four times that of its reversible analog, oxymorphone. This β-COA-induced analgesia is inhibited by pretreatment of mice with naloxone. In addition, β-COA given i.c.v. has long-lasting antagonist effect (~ 6 days) against morphine-induced analgesia.

In the study of receptors, and especially in the elucidation of pharmacological effects, antagonists have been particularly useful. Such contributions can be seen by the use of naloxone and naltrexone as pharmacological tools in the area of opioid research. The reversible nature of such antagonists and their cross-reactivity with various opioid receptor types are inherent limitations to their use as receptor probes. Thus, there have been a number of efforts to develop highly selective antagonists of the non-equilibrium type.

The first successful antagonist affinity label synthesized was a nitrogen mustard derivative of naltrexone, β-chlornaltrexamine (β-CNA) (Portoghese et al., 1978). β-CNA proved to a potent affinity label selective for opioid receptors both in vitro and in vivo. β-
Fig. 3.5 A schematic representation illustrating the concept of recognition amplification. Although reversible binding (1º recognition) is occurring with receptor types A-C, only receptor type A is able to undergo the labeling process (2º recognition). In this case, recognition amplification is possible due to differences in the reactivity and proximity of the receptor-based nucleophiles G¹ and G².

Fig. 3.6 Affinity labels for opioid receptors
CNA irreversibly inhibits the binding of either $[^3H]naldoxone$ or $[^3H]naltrexone$ to brain membranes. This inhibition of binding withstands extensive binding and eight hours of dialysis. In the GPI and MVD, $\beta$-CNA produces irreversible antagonism that can be prevented but not reversed by treatment with naltrexone. Treatment with $\beta$-CNA blocks all opioid receptor types. In mice, $\beta$-CNA produces ultra-long antagonism (> 3 days) of morphine-induced analgesia after a single i.c.v. injection (Caruso et al., 1980b). $[^3H]\beta$-CNA has been used to isolate opioid receptor components from brain membranes (Caruso et al., 1980a). Since $\beta$-CNA is not selective between opioid receptor types, it has been used in protection studies in the presence of selective reversible ligands for membrane enrichment of a receptor type. For example, in the MVD $\beta$-CNA has been used with DSLET as the protector to irreversibly block $\mu$ and $\kappa$ receptors. Such preparations can result in a near-homogenous population of $\delta$ receptors which can then be used to assess $\delta$ activity of agonists (Ward et al., 1982). Although ligand protection is an effective way of enriching receptor types in smooth muscle preparations, the protection of selected receptor types is only as good as the selectivity of the protector. In this regard, receptor-selective affinity labels are far more advantageous.

The reason for the lack of selectivity seen for $\beta$-CNA may be due to the extremely reactive aziridinium ion generated from $\beta$-CNA. The aziridinium ion facilitates the alkylation of opioid receptors but makes the second recognition step less selective because this electrophile can react with a wide variety of nucleophiles. In an effort to develop affinity labels with increased selectivity, the nitrogen mustard of $\beta$-CNA was replaced with less reactive electrophilic moieties. One of the most selective ligands synthesized was the fumarate methyl ester derivative $\beta$-funaltrexamine ($\beta$-FNA) (Portoghese et al., 1980). It was found that $\beta$-FNA actually interacted with all three receptor types reversibly, but the irreversible process of $\beta$-FNA binding showed selectivity for $\mu$ over $\delta$ and $\kappa$ binding sites. One of the initial uses of $\beta$-FNA was to deplete the GPI of functional $\mu$ receptors, resulting in a smooth muscle assay with a near homogenous population of $\kappa$ receptors (Ward et al.,
1982). β-FNA has also been used to study the involvement of μ receptors in various opioid-induced effects, such as respiratory depression (Ward and Takemori, 1983a), decreased gastrointestinal motility (Ward and Takemori, 1983b), cardiovascular effects (Holaday and Ward, 1982), prolactin secretion (Holaday et al., 1983), spinal analgesia (Hylden and Wilcox, 1983a, Hylden and Wilcox, 1983b), and antidiuresis (Zimmerman et al., 1984). It was also instrumental in examining the phenomena of tolerance and physical dependence associated with the μ receptor. In rats, the physical dependence produced by a continuous intraperitoneal infusion of morphine was completely blocked by β-FNA (Aceto et al., 1984). When β-FNA was given intrathecally, the tolerance and dependence produced by subcutaneous morphine pellets was markedly inhibited (Delander et al., 1984). These results suggest that μ receptors in the central nervous system plays an important part in the development of dependence. In a recent study, Liu-Chen and co-workers identified a Lys residue involved in binding to β-FNA in μ opioid receptors (Chen et al., 1996).

In an effort of obtain selective affinity labels, the isothiocyanate derivatives of fentanyl (FIT), cis-(+)-3-methylfentanyl (SUPERFIT), etonitazene (BIT), and the fumaramido derivative of endoethenotetrahydrooripavine (FAO) were synthesized (Burke et al., 1984, Rice et al., 1983). In opioid binding assays using both rat brain membranes and NG 108-15 hybrid cells, FIT, SUPERFIT and FAO appear to be highly selective alkylators of δ receptors and BIT appeared to alkylate μ receptor selectively. FIT and SUPERFIT are both based on fentanyl, a μ agonist, but the incorporation of an isothiocyanate group confers irreversible δ-binding properties to the ligands. This is another example of recognition amplification. Employing NG 108-15 cells, [3H]FIT has been used to identify a protein with Mr 58,000 (Klee et al., 1982). The binding of [3H]FIT to this protein withstood extensive washing, and levorphanol (but not dextrorphan) inhibited irreversible binding.

In a recent study, Zhu et al. used SUPERFIT in combination with chimeric studies in order to determine the binding domain of the δ opioid receptor (Zhu et al., 1996). Through
the incubation of SUPERFIT with a variety of μ/δ chimeric receptors, it was found that the receptor needed the region from the beginning of the first intracellular loop to the middle of the third transmembrane region for wash-resistant binding of the affinity label.

The first reported opioid peptide-based electrophilic affinity label was the chloromethyl ketone derivative of DADLE, called DALECK (Pelton et al., 1980). The binding of this agent to opioid receptors in the receptor binding assay using [3H]DADLE, [3H]etorphine, and [3H]naloxone appeared to be irreversible. However, the agonist effects of DALECK in the GPI, MVD, and analgesic assays were all fully reversible by naloxone. Therefore, it is uncertain whether DALECK possesses selectivity or if covalent association of opioid receptors is possible with this agent in biological systems.

In another approach to develop a potential peptide affinity label, [D-Ala²,Leu⁵]enkephalin (DALA) was extended with the methyl ester of the nitrogen mustard derivative of phenylalanine melphalan (Mel) at the C-terminus. This compound, DALA-Mel-OMe, displays high affinity in displacing [3H]DALA and [3H]naloxone from brain membranes (Szucs et al., 1983). Although DALA-Mel-OMe appears to irreversibly block the binding of [3H]naloxone, the fact that pretreatment with naloxone or DALA affords only partial protection suggests that nonspecific labeling also occurred. Melphalan was also incorporated into the 4-position of N,N-dialkylated enkephalin derivatives, and these derivatives exhibited wash-resistant antagonism at high concentrations (10 μM) (Lovett and Portoghese, 1987b).

The affinity label [D-Ala²,Leu⁵,Cys⁶]enkephalin (DALCE) is believed to form a disulfide linkage with a Cys residue at or near the binding site of opioid receptors thereby causing receptor inactivation (Bowen et al., 1987). Studies by Larsen and co-workers (Larsen et al., 1981) have also suggested that in the opioid receptors there are at least two different thiol groups sensitive to N-ethylmaleimide (NEM). One of the thiols was found to be the Cys residue contained in the α-subunit of the GTP-binding protein Gι. The other was suggested to be present within the receptor protein itself. In order to further exploit
this receptor-based nucleophile, Matsueda and co-workers (Matsueda et al., 1993, Matsueda et al., 1992) have designed peptide-based affinity labels for δ and κ opioid receptors using S-(3-nitropyridinesulfenyl)cysteine [Cys(Npys)] incorporated into enkephalin and dynorphin analogs, respectively. The Npys group is stable as a protecting group for Cys, and could therefore be directly incorporated into the peptide during synthesis without any special precautions. Both [D-Ala²,Cys(Npys)⁵]leucine enkephalin and [Cys(Npys)⁸]dynorphin analogs exhibited high affinity and wash-resistant binding inhibition at δ and κ receptors, respectively.

3.8 Conclusions

In the area of opioid medicinal chemistry, a wide variety of δ-selective ligands have been either synthesized or isolated. The modifications of endogenous opioid peptides have resulted in ligands with excellent selectivity and affinity for the δ-opioid receptor. From the numerous analogs synthesized, extensive structure-activity relationships for δ-selective peptides have been compiled. In the case of non-peptidic ligands, the application of rational drug design allowed for the development of δ-selective agonists and antagonists based on oxymorphone and naltrexone, respectively. These selective ligands have made detailed examination of the δ-receptor possible and enabled researchers to define δ-receptor subtypes by virtue of analgesia induced by selective agonists and differentially blocked by selective antagonists.

In order to selectively bind to a specific opioid receptor type (or subtype), the subtle differences in structural requirements of the receptors must be understood and utilized. From the systematic synthesis and conformational analysis of constrained analogs, researchers are close to understanding the "bioactive" orientation of important pharmacophoric groups necessary for δ-receptor binding and activation.
In addition to molecular modeling and various NMR techniques, affinity labels have also been successfully used to study receptor structure and function. Due to their irreversible nature, these ligands should enable researchers to ascertain which amino acid residues are at or near the binding site based on their covalent attachment. Due to potential differences in binding modes, a wider variety of peptidic/non-peptidic-, and agonist/antagonist-based affinity labels may yield increased structural information about the binding sites of the opioid receptors. Affinity labels could also be used in the isolation and purification of the putative receptor subtypes, which have not yet been characterized by molecular biological techniques.

As the use of selective agonists and antagonists further define the physiological role of the δ receptor, potential therapeutic applications of δ-selective ligands may be uncovered. Through the use of peptidomimetics and/or peptide drug delivery systems, δ-selective therapeutic agents for the treatment of pain and other disorders may soon be realized.
Chapter 4

Synthesis and Evaluation of Enkephalin-Based Affinity Labels for Delta Opioid Receptors

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4.1 Abstract

In order to develop affinity labels for the delta (δ) opioid receptor based on peptide antagonists, the Phe⁴ residues of N,N-dibenzyl leucine enkephalin and ICI-174,864 (N,N-diallyl[Aib²,Aib³]leucine enkephalin) were substituted with either Phe(p-NCS) or Phe(p-NHCOCH₂Br). The target peptides were synthesized using Phe(p-NH₂) and a Boc/Fmoc orthogonal protection strategy which allowed for late functional group conversion of a p-amine group to the desired affinity labeling moieties within the peptides. The key step in the synthesis was the selective deprotection of a Boc group in the presence of a t-butyl ester using trimethylsilyl trifluoromethanesulfonate (TMS-OTf). Developed by our laboratory, this synthetic scheme represents a general method for the conversion of peptide substrates into potential affinity labels.

The target peptides were evaluated through radioligand binding inhibition experiments in Chinese hamster ovary (CHO) cells expressing δ or μ opioid receptors. Of the target peptides tested, only the N,N-dibenzyl [Phe(p-NCS)⁴]leucine enkephalin analog exhibited inhibition of radioligand binding inhibition in a wash-resistant fashion at a concentration of 1.0 μM. Therefore, this peptide analog emerges as a potential affinity label useful for further study of δ opioid receptors.
4.2 Abbreviations

Abbreviations used for amino acids are according to the rules specified by the IUPAC-IUB Joint Commission of Biochemical Nomenclature (Eur. J. Biochem. 1984, 138, 9-37). All amino acids are in the L-configuration unless otherwise specified. Additional abbreviations used are: Aib, 2-aminoisobutyric acid; BAW, butanol/acetic acid/water; Boc, tert-butyloxycarbonyl; Bpoc, 1-methyl-1-(4-biphenyl)ethoxycarbonyl; Cbz, benzylloxycarbonyl; CHO, Chinese hamster ovary; DADLE, Tyr-D-Ala-Gly-Phe-D-Leu; DALCE, Tyr-D-Ala-Gly-Phe-Leu-Cys; DAMGO, Tyr-D-Ala-Gly-MePhe-Leu-Gly-ol; DCM, dichloromethane; DIPEA, N,N-diisopropylethylamine; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; DPDPE, Tyr-cyclo[D-Pen-Gly-Phe-D-Pen]-OH; FAB-MS, fast atom bombardment mass spectrometry; FAO, fumaramidooripavine; FIT, fentanyl isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-Cl, 9-fluorenylmethyl chloroformate; GPI, guinea pig ileum; HPLC, high performance liquid chromatography; HRFAB-MS, high resolution fast atom bombardment mass spectrometry; Mel, melphalan; MVD, mouse vas deferens; NMM, N-methylmorpholine; NMR, nuclear magnetic resonance spectroscopy; NTII, naltrindole isothiocyanate; Pen, penicillamine; Ppoc, 2-triphenylphosphonioisopropoxycarbonyl; SUPERFIT, cis-(+)-3-methyl fentanyl isothiocyanate; TAEA, tris(2-aminoethyl)amine; TEA, triethylamine; Tfa, trifluoroacetyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TMS-OTf, trimethylsilyl trifluoromethanesulfonate; Tris, tris(hydroxymethyl)-aminomethane.
4.3 Introduction

Opioid-based analgesics are effective in the alleviation of pain, but many of them exhibit serious side effects, such as respiratory depression, tolerance, and physical dependence. The characterization of three heterogeneous opioid receptor types [mu (μ), kappa (κ), and delta (δ)] has raised the possibility that an opioid agonist selective for a specific receptor type may exhibit analgesic properties with lessened side effects. These receptors have been well documented both pharmacologically and at the molecular level by cloning (for reviews, see Kieffer, 1995, Reisine and Bell, 1993, Satoh and Minami, 1995).

Opioid analgesics with a high degree of abuse potential have been shown to interact preferentially with μ receptors (Raynor et al., 1994), and so considerable attention has been given to the study of δ receptors and their potential for analgesia with lessened narcotic side effects. Much has been learned about the δ opioid receptor through the use of δ-selective ligands, but the emergence of δ receptor subtypes and the problems associated with identifying receptor-specific physiological effects emphasize the continuing need for novel ligands in the study of δ opioid receptors. Affinity labels are ligands whose interaction with a receptor is considered to be essentially non-equilibrium, and they have proven to be useful pharmacological tools in the study of similar problems with various types of receptors. An affinity label most commonly incorporates an electrophilic functional group within its structure that can interact with a receptor-based nucleophile. Due to their irreversible nature, these ligands are useful for the isolation and mapping of receptors, and the resultant long-term blockade of receptor binding aids in the elucidation of physiological effects of receptor activation (Takemori and Portoghese, 1985). Affinity labels are also being used to ascertain the residues present in the binding pocket of the receptor.

Several compounds have been prepared as potential affinity labels for the δ opioid receptor. These ligands include fentanyl isothiocyanate (FIT) (Rice et al., 1983) and its chiral (+)-3-methyl derivative (SUPERFIT) (Burke et al., 1986), fumaramidooripavine (FAO) (Burke et al., 1984), and most recently, naltrindole isothiocyanate (NTII)
All of these δ-selective affinity labels are based on non-peptidic opioid ligands.

Peptide-based affinity labels are important because they could offer complimentary pharmacological information to the non-peptidic affinity labels. Two affinity labels based on peptides are [D-Ala²,Cys⁶]leucine enkephalin (DALCE) (Bowen et al., 1987) and its recently developed S-3-nitro-pyridinesulfenyl (Npys) protected derivative, [D-Ala²,Cys(Npys)⁶]leucine enkephalin (Matsueda et al., 1992). Both of these affinity labels exhibited δ-selective wash-resistant inhibition of binding, and are based on [D-Ala²]leucine enkephalin, which is an agonist at the δ opioid receptor.

In an effort to develop affinity labels based on δ-selective peptide antagonists, Aldrich-Lovett and Portoghese investigated N-terminal dialkylated enkephalins for antagonistic activity at the δ opioid receptor (Lovett and Portoghese, 1987a). In this study, it was found that N,N-dibenzyl leucine enkephalin exhibited the highest potency and selectivity for the δ opioid receptor. In a subsequent study (Lovett and Portoghese, 1987b), Phe⁴ of this pentapeptide and another δ-selective peptide antagonist, N,N-diallyl[Aib²,Aib³]leucine enkephalin (ICI-174,864) (Cotton et al., 1984), were then substituted with the nitrogen mustard analog of phenylalanine, melphalan (Mel) at the 4-position. Unfortunately, neither of the Mel-containing peptides synthesized exhibited significant wash-resistant binding inhibition in the mouse vas deferens smooth muscle assay.

The labeling process is thought to occur in two discrete steps: first, reversible binding of the affinity label to the receptor, followed by covalent linkage with the receptor. The researchers postulated that the lack of irreversible binding of the Mel-containing peptides might be attributed to potential problems in both steps. The large, sterically demanding nitrogen mustard moiety of the Mel residue may have had a deleterious effect on the reversible binding of the synthesized peptides. In addition, the relatively low reactivity of the Mel residue may have hindered the process of covalent bond formation with the receptor.
To further investigate these N,N-dialkylated enkephalins as potential affinity labels, we have incorporated more reactive and less sterically demanding functional groups at the para position of Phe\(^4\). A second goal of the syntheses of these peptide derivatives was the development of a general methodology flexible enough for the incorporation of various labeling moieties into a target peptide. The functional groups incorporated were the isothiocyanate and the bromoacetamide groups, which have shown their effectiveness in various non-peptidic affinity labels for opioid receptors, but have not been used in opioid peptides. The target peptides for this study included the p-amine, p-isothiocyanate, and the p-bromoacetamide derivatives of N,N-dibenzyl leucine enkephalin and ICI-174,864 (see Fig. 4.1). The amine-containing derivatives were prepared as reversible controls for establishing washing procedures used in the assays for wash-resistant binding inhibition. The reversible and wash-resistant assays for inhibition of radioligand binding used Chinese hamster ovary (CHO) cells stably transfected with the mouse \(\delta\) opioid receptor.

4.4 Chemistry

4.4.1 Synthetic strategy

In the synthetic scheme for these potential affinity labels, consideration of how these electrophilic groups were to be introduced had to be addressed. In the earlier study, the nitrogen mustard of Mel lent itself very well to standard conditions of peptide synthesis. It could be directly incorporated into the growing peptide chain with no special protection or reaction conditions. Unfortunately, the isothiocyanate and the bromoacetamide moieties were not expected to fare as well under the rigors of multiple peptide couplings and functional group deprotections. Therefore, a synthetic scheme was devised that would allow functional group conversion late in the peptide synthesis. p-Nitrophenylalanine was
$N,N$-dibenzyl leucine enkephalin: $R = \text{Bn}; R' = H; X = H$
1: $R = \text{Bn}; R' = H; X = \text{NH}_2$
2: $R = \text{Bn}; R' = H; X = \text{NCS}$
3: $R = \text{Bn}; R' = H; X = \text{NHCOCH}_2\text{Br}$

ICI-174,864: $R = \text{Allyl}; R' = \text{CH}_3; X = H$
4: $R = \text{Allyl}; R' = \text{CH}_3; X = \text{NH}_2$
5: $R = \text{Allyl}; R' = \text{CH}_3; X = \text{NCS}$
6: $R = \text{Allyl}; R' = \text{CH}_3; X = \text{NHCOCH}_2\text{Br}$

Fig. 4.1 Target enkephalin-based affinity labels and related peptides
chosen for the design of the peptide-based affinity labels because the nitro group can be reduced to an amine which can then transformed into a number of affinity labeling moieties, including the aforementioned isothiocyanate and bromoacetamide. However, late reduction of the nitro group to the amine was not an option in the synthesis of the target peptides, due to the N-terminal substitution by groups subject to hydrogenolysis.

An important aspect of peptide chemistry is the selective deprotection of amine and/or carboxyl groups in the presence of other protecting groups. Traditionally, acid-labile groups have been used for peptide functional group protection, primarily due to the stability peptides exhibit under acidic conditions. During the removal of acid-labile groups, selectivity arises through differences in the acid strengths needed to induce removal. The t-butyloxycarbonyl (Boc) group and t-butyl protecting groups are easily and effectively removed using trifluoroacetic acid (TFA), and therefore these t-butyl-based protecting groups were chosen as the semi-permanent protection for the peptides due to the mild conditions required for final deprotection. This then required the choice of a transient protecting group for the p-amino functionality which could be selectively deprotected to allow electrophilic functional group conversion. The protecting group in question would have to be able to be deprotected without causing appreciable loss of the tert-butyl based protecting groups. There are many "hyper acid"-labile groups for this, such as the Bpoc, Ppoc, and Ddz protecting groups, that are typically removed using acidic conditions which have little effect on Boc groups. Therefore, our laboratory initially explored the use of some of these protecting groups for these syntheses. These protecting groups are generally introduced as the azides or active esters, but unfortunately, these derivatives were not reactive enough to protect the p-amine functionality of Phe(p-NH₂). The difficulty in protection is caused by the lower nucleophilicity of the aniline-type amine due to conjugation with the aromatic ring.

We then explored possible orthogonal protection strategies to be used with Boc-type groups. One such group used in conjunction with acid-labile groups was the trifluoroacetyl
(Tfa) group. However, during the course of the synthesis it was found that this group could not be easily removed by saponification, and therefore this protection strategy was abandoned.

Another protection strategy that in the past several years has gained significant popularity in the solid phase synthesis of peptides is the use of the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group (Carpino and Han, 1972), which is typically removed through β-elimination using an organic base, most commonly piperidine. As with the Tfa group, the basic conditions for Fmoc removal provides orthogonal protection to the Boc group. The Fmoc group was readily introduced onto the p-amine functionality using 9-fluorenylmethyl chloroformate, whereas the use of the less reactive succinimide resulted in much lower yields. The p-amine of the phenylalanine could be deprotected near the end of the synthesis, so that the electrophilic functional groups would not be subjected to a lengthy synthetic process.

The target peptides share a common C-terminal dipeptide fragment, and therefore the synthetic scheme used a convergent [3 + 2] fragment condensation utilizing the C-terminal dipeptide fragment coupled to the different N-terminal fragments to yield the desired pentapeptides.

### 4.4.2 Synthesis

The synthesis of the C-terminal dipeptide (see Fig. 4.2) began with the isobutyl chloroformate-mediated coupling of Boc-Phe(p-NO2)-OH to H-Leu-OrBu as the hydrochloride salt, to give dipeptide 7 in 81% yield. Reduction of the p-nitro group by catalytic transfer hydrogenation provided the amine derivative 8 in near quantitative yield. Protection of the resulting amine by the application of 9-fluorenylmethyl chloroformate under standard conditions gave the fully protected dipeptide derivative 9 in 80% yield. The p-amine functionality was protected at the dipeptide stage rather than the amino acid
Fig. 4.2 Synthesis of the C-terminal common dipeptide fragment 10.
because the protected amino acid derivative Boc-Phe(p-NHFmoc)-OH is rather insoluble and proved difficult to purify and use in subsequent reactions.

In the key step of the synthesis, the Boc group had to be removed in the presence of a t-butyl ester. This selective deprotection was achieved using one equivalent of trimethylsilyl trifluoromethanesulfonate (TMS-OTf) in toluene (Vorbruggen and Kroliklewicz, 1975). The selective removal of the Boc group was possible because of the increased nucleophilicity of the carbamate carbonyl vs. the ester carbonyl. After the addition of the reagent, the product 10 (as the triflate salt) precipitated out of solution as a gel.

In Fig. 4.3, the assembly of the N-terminal fragment of the ICI-174,864 derivatives is outlined (Lovett and Portoghese, 1987a). The ICI-174,864 derivatives have the N-terminal tripeptide (N,N-diallyl)Tyr-Aib-Aib, (Aib = 2-aminoisobutyric acid). This α-disubstituted amino acid is difficult to couple, and attempts to couple Aib to Aib using tBuOCOC(2) can result in high levels of the isobutyl carbamate rather than peptide bond formation. Therefore, for the difficult coupling of Cbz-Aib-OH to H-Aib-OMe, the pivaloyl chloride method of peptide bond formation was used. This coupling reaction proceeded in 63% yield to give the Cbz-protected dipeptide 12. In the subsequent step when the Cbz group was removed by hydrogenolysis, care had to be taken to keep the resultant amine protonated in order to avoid spontaneous diketopiperazine formation. Hydrogenolysis of the Cbz group in the presence of one equivalent of HCl was then followed by tBuOCOC(1)-mediated coupling to Cbz-Tyr(O-tBu)-OH to give the fully protected tripeptide 14. Hydrogenolysis of the Cbz group, followed by treatment with an excess of allyl bromide and N,N-diisopropylethylamine (DIPEA) provided the diallylated tripeptide 16 in 83% yield. Saponification of the methyl ester then provided the desired N-terminal tripeptide fragment 17.

The corresponding synthesis of the dibenzylated N-terminal tripeptide fragment (see Fig. 4.4) involved similar procedures with a few exceptions: the Gly-Gly-OEt dipeptide fragment was obtained commercially as the hydrochloride salt, and derivatization of the
Fig. 4.3 Synthesis of the N-terminal tripeptide fragment 17 for ICI-174,864 derivatives
Fig. 4.4 Synthesis of the N-terminal tripeptide fragment 21 for the dibenzyl leucine enkephalin derivatives
N-terminal tripeptide used benzyl bromide/DIPEA to effect the dialklylation to tripeptide 20. Saponification of 20 led to the desired N-terminal dipeptide fragment 21 in an overall yield of 65% over 4 steps (Lovett and Portoghese, 1987a).

In Figs. 4.5 and 4.6, the N-terminal tripeptides were then coupled to the C-terminal dipeptide 10 using either iBuOCOC1 (for 21) or pivaloyl chloride (for 17) to provide pentapeptides 22 and 27, respectively. The next step involved the removal of the Fmoc group to expose the p-amine for affinity label functionalization. The Fmoc protection strategy is not very widely used in solution phase synthesis because the resulting dibenzofulvene by-product is able to react with the organic base through a Michael addition. This adduct is difficult to remove, thereby complicating product purification. Such problems are circumvented in the solid-phase synthesis of peptides by virtue of the peptide's anchorage to the insoluble support. In order to simplify the purification process of Fmoc-based peptide synthesis in solution, Carpino and co-workers developed a deprotection strategy using tris(2-aminoethyl)amine (TAEA) (Carpino et al., 1990). This reagent was used to deprotect the Fmoc group, and the resulting dibenzofulvene/TAEA adduct was then removed from the organic layer by repeated washes with pH 5.5 phosphate buffer.

Following the assembly of desired pentapeptides 23 and 28, the penultimate step in the synthetic strategy was the incorporation of the bromoacetamide and isothiocyanate functional groups. During the synthesis of the dibenzyl leucine enkephalin derivatives, functional group conversion of the amine to the bromoacetamide used bromoacetyl chloride, but extended reaction times led to the formation of the chloroacetamide 25 through halide exchange. The chloroacetamide side-product was identified by high-resolution mass spectrometry. This problem was circumvented through the use of bromoacetyl bromide. The isothiocyanates 24 and 29 were formed by reaction of the amine compounds 23 and 28 with thiophosgene, respectively. Final deprotection of the
Amine (control) synthesis:

23 → iv (72%) → 1: X = NH₂
R = OH

Isothiocyanate synthesis:

23 → v → 24: X = NCS
R = OtBu → iv (41%) → 2: X = NCS
R = OH

Chloroacetamide synthesis:

23 → vi → 25: X = NHCOCH₂Cl
R = OtBu → iv (95%) → 3a: X = NHCOCH₂Cl
R = OtBu

Bromoacetamide synthesis:

23 → vii → 26: X = NHCOCH₂Br
R = OtBu → iv (73%) → 3: X = NHCOCH₂Br
R = OH

i) iBuOCOCI, NMM; ii) 10, NMM (74%); iii) TAEA, DCM (75%); iv) TFA/10% anisole; v) thiophosgene, DIPEA (94%); vi) bromoacetyl chloride, DIPEA, overnight at r.t. (54%); vii) bromoacetyl bromide, DIPEA, 2h at 0 °C (91%)

Fig. 4.5 Synthesis of dibenzyl leucine enkephalin derivatives 1-3
**Amine (control) synthesis:**

28 \( \xrightarrow{\text{iv}} \) 4: \( X = \text{NH}_2 \)  
\( R = \text{OH} \) (78%)

**Isothiocyanate synthesis:**

28 \( \xrightarrow{\text{v}} \) 29 \( \xrightarrow{\text{iv}} \) 5: \( X = \text{NCS} \)  
\( R = \text{OH} \) (70%)

**Bromoacetamide synthesis:**

28 \( \xrightarrow{\text{vi}} \) 30 \( \xrightarrow{\text{iv}} \) 6: \( X = \text{NHCOCH}_2\text{Br} \)  
\( R = \text{OH} \) (98%)

i) Piv-Cl, TEA, DCM; ii) 10 (57%); iii) TAEA, DCM (91%); iv) TFA/10% anisole; v) thiophosgene, DIPEA (80%); vi) bromoacetyl bromide, DIPEA (58%)

**Fig. 4.6** Synthesis of ICI-174,864 derivatives 4-6
peptide derivatives was accomplished using TFA and anisole as a t-butyl cation scavenger, to yield target peptides 1-6 for pharmacological evaluation.

4.5 Pharmacological evaluation

4.5.1 Binding assays under standard conditions

These final peptides were subjected to radioligand binding assays under standard conditions using cloned δ and μ receptors expressed in Chinese hamster ovary (CHO) cells (Table 4.1). For the dibenzyl derivatives, substitution at the para position of Phe⁴ generally caused a decrease in δ-receptor binding affinity, with the notable exception of the isothiocyanate derivative, which exhibited the highest affinity of all of the peptides tested. Substitution at the para position was better tolerated by the diallyl derivatives, as the isothiocyanate and bromoacetamide-containing peptides demonstrated higher affinity for the δ receptor than the unsubstituted parent compound, ICI-174,864. Despite being the smallest substituent, the amine-containing derivatives of both N,N-dibenzyl leucine enkephalin and ICI-174,864 displayed the lowest affinity in their respective groups, indicating that steric bulk alone did not determine decreases in affinity at the δ receptor. The dipara position was better tolerated by the diallyl derivatives, as the isothiocyanate and bromoacetamide-containing peptides demonstrated higher affinity for the δ receptor than the unsubstituted parent compound, ICI-174,864. Despite being the smallest substituent, the amine-containing derivatives of both N,N-dibenzyl leucine enkephalin and ICI-174,864 displayed the lowest affinity in their respective groups, indicating that steric bulk alone did not determine decreases in affinity at the δ receptor. The different substituent effects may suggest subtle differences in the interactions between the peptide derivatives and the receptor.

In these assays, it was interesting to note that against [³H]DPDPE binding, N,N-dibenzyl leucine enkephalin exhibited a 10-fold greater affinity yet similar selectivity than
Table 4.1 Binding affinities of peptides 1 - 6 under standard conditions

<table>
<thead>
<tr>
<th>compound tested</th>
<th>IC50 ± S.E.</th>
<th>d (nM) (vs. [3H]DPDPE)</th>
<th>IC50 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPDPE</td>
<td>0.87 ± 1.2</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>DAMGO</td>
<td>ND</td>
<td>3.8 ± 1.2 (nM)</td>
<td></td>
</tr>
<tr>
<td>N,N-dibenzyl Leu enk</td>
<td>78.2 ± 1.1</td>
<td>1.6 ± 1.1</td>
<td>20</td>
</tr>
<tr>
<td>1 Bn2[Phe(p-NH2)4]Leu enk</td>
<td>486.1 ± 1.2</td>
<td>23.0 ± 1.3</td>
<td>47</td>
</tr>
<tr>
<td>2 &quot; (p-NCS) &quot;</td>
<td>34.9 ± 3.3</td>
<td>1.9 ± 1.3</td>
<td>54</td>
</tr>
<tr>
<td>3 &quot; (p-NHCH2Br) &quot;</td>
<td>149.4 ± 2.7</td>
<td>9.1 ± 1.3</td>
<td>61</td>
</tr>
<tr>
<td>3a &quot; (p-NHCH2Cl) &quot;</td>
<td>144.8 ± 3.6</td>
<td>19.0 ± 1.2</td>
<td>131</td>
</tr>
<tr>
<td>N,N-diallyl[Aib2,Aib3]Leu enk (ICI-174,864)</td>
<td>703.0 ± 1.1</td>
<td>18.9 ± 1.1</td>
<td>27</td>
</tr>
<tr>
<td>4 All2[Aib2,Aib3,Phe(p-NH2)4]Leu enk</td>
<td>1394 ± 1.1</td>
<td>87.7 ± 1.9</td>
<td>63</td>
</tr>
<tr>
<td>5 &quot; (p-NCS) &quot;</td>
<td>361.3 ± 2.8</td>
<td>33.4 ± 1.3</td>
<td>92</td>
</tr>
<tr>
<td>6 &quot; (p-NHCH2Br) &quot;</td>
<td>391.9 ± 2.8</td>
<td>&gt;100</td>
<td>&gt;255</td>
</tr>
</tbody>
</table>
All of the derivatives tested displayed a higher degree of selectivity for the δ receptor than the unsubstituted lead compounds. Between the two groups, the diallyl derivatives were more selective for the δ receptor than the dibenzyl derivatives due to greatly decreased binding to the μ receptor, but the dibenzyl compounds displayed higher affinities compared to the diallyl derivatives. Within a series, the larger acetamides were more δ-selective than either the isothiocyanates or the amines.

4.5.2 Wash-resistant binding assays

The derivatized peptides were then tested for wash-resistant binding of [3H]DPDPE binding to δ-receptors in CHO cells. The experiment involved incubation of the test compound with the CHO cell membranes for 90 min at room temperature, and then the membranes were washed via centrifugation, decanting of the buffer solution, and re-suspension in fresh buffer solution. Due to its lower affintity, the amine-containing dibenzyl peptide 1 was tested at a concentration of 3 μM, and it was found that a series of five washes was sufficient to remove most of the amine-containing compounds without an appreciable loss of protein from the CHO cell membranes. The washed CHO cell membranes then underwent radioligand binding assays. The binding assays after the washing procedure indicated that binding levels of [3H]DPDPE were close to control (untreated) binding levels (Fig.4.7). The only exception was the isothiocyanate-containing dibenzyl compound 2, which still inhibited radioligand binding by about 81% even after the washing procedures. This level of wash-resistan binding inhibition is close to that of NTII at a similar concentration. This data suggests that the isothiocyanate-containing dibenzyl compound 2 may be used as an affinity label in the study of δ opioid receptors.
Fig. 4.7 Graphical representation of the percent radioligand binding (normalized against control values) displayed by the peptide derivatives 1-6 following incubation and washing. Compound 1 was tested at 3 μM. All other compounds were tested at 1.0 μM.
4.6 Results and discussion

Utilizing a convergent [3+2] synthetic strategy, affinity labels based on the δ-selective peptide antagonists N,N-dibenzyl leucine enkephalin and ICI-174,864 were prepared. An orthogonal Boc/Fmoc protection strategy was utilized in the synthesis, and this method should prove to be a versatile method for the preparation of a wide variety of peptide-based affinity labels. Substitution at the para position of Phe of the N,N-dibenzyl enkephalin analogs generally decreased binding affinity at the δ opioid receptor, but isothiocyanate and bromoacetamide functionalization increased δ opioid receptor binding for the ICI-174,864 derivatives. This would suggest that the differences of N-terminal and backbone substitution between the N,N-dibenzyl and ICI-174,864 derivatives resulted in different interactions with δ opioid receptors.

Of the compounds tested, only the isothiocyanate-containing derivative of N,N-dibenzyl leucine enkephalin 2 exhibited wash resistant binding inhibition in CHO cell assays. At a concentration of 1 μM, it exhibited binding inhibition of [3H]DPDPE after repeated washings through centrifugation. The corresponding ICI-174,864 derivative 5 did not exhibit wash-resistant binding, despite its apparent similarity to 2. The N-terminal and backbone substitutions in 5 perhaps contributed to different spatial orientation during binding, thereby resulting in lower binding affinity and prohibiting proper alignment of the isothiocyanate with a receptor-based nucleophile.

From these observations, enkephalin analog 2 appears to be a potential affinity label selective for the δ opioid receptor. This peptide provides the first example of an isothiocyanate group used to prepare opioid peptide-based potential affinity labels. In order to confirm the irreversible nature of this compound, our laboratory is developing methods to incorporate [3H] labeling within the Phe residue. Further pharmacological studies with 2 in conjunction with other δ-selective affinity labels may provide further insight on the binding requirements and physiological roles of δ opioid receptors.
4.7 Experimental section

4.7.1 Materials

All amino acids except for Boc-Phe(p-NO₂) and Aib were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic reagents and the amino acids Boc-Phe(p-NO₂) and Aib were purchased from Aldrich Chemical Co. (Milwaukee, WI). General laboratory solvents were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ), and HPLC-grade solvents were obtained from Burdick and Jackson (Muskegon, MI).

Syntheses were monitored using thin-layer chromatography using Kieselgel 60 F254 plates of 0.20 mm thickness purchased from EM Separations (Gibbstown, NJ). Intermediates were purified by flash chromatography using 230-400 mesh, 60A silica gel also purchased from E. Merck.

NMR analyses were done on a Bruker AC300 instrument at the Department of Chemistry, Oregon State University, Corvallis, OR. The molecular weights of the amino acid derivatives and peptides were determined by fast atom bombardment (FAB) mass spectrometry in the postitive mode on a Kratos MS50RFTC in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR.

The purity of intermediates and the final peptides were determined by analysis on a Beckman model 431A high performance liquid chromatography (HPLC) system. The HPLC column used in the analyses was either a Vydac (C₄, 300 Å, 5 μ, 4.6 x 250 mm, column A) or a Zorbax Protein Plus (C₃, 300Å, 10 μ, 4.6 x 250 mm, column B) analytical column. The compounds were eluted using a linear gradient of 0 to 75% B over 50 min at a flow rate of 1.5 mL/min and detected at 214 nm; solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA.
4.7.2 Methods

4.7.2.1 Synthetic methods

Isobutyl chloroformate-mediated peptide coupling (Method I): Under an N₂ atmosphere, a 0.25 M solution of the carboxyl component (1.0 eq) in dry tetrahydrofuran (THF) was cooled to -15 °C (dry ice/MeOH) and neutralized with one equivalent of N-methylmorpholine (NMM). A stoichiometric amount of isobutyl chloroformate was then added, followed 90 sec later by a 0.5 M solution of the amine component and NMM (each 1.0 eq) in DMF. The reaction mixture was kept at -15 °C for 30 min, and then allowed to warm to room temperature. When the reaction was complete, the THF was evaporated, and the residue suspended in EtOAc. The organic layer was washed with H₂O, 5% KH₂SO₄, H₂O, 5% NaHCO₃, H₂O, and then finally with saturated NaCl. The organic layer was then dried over MgSO₄ and the EtOAc evaporated to give the crude peptide.

Final deprotection with TFA (Method II): The peptide was treated with a 10% anisole/TFA for one hour at room temperature. The TFA was then evaporated and the product precipitated with cold ether. The precipitate was washed five times with cold ether, and then dried in vacuo.

Na-tert-Butyloxy carbonyl-L-p-nitrophenylalanyl-L-leucine tert-butyl ester (7)

N-tert-Butyloxy carbonyl-L-p-nitrophenylalanine (1.18 g, 3.80 mmol) was coupled to L-leucine tert-butyl ester hydrochloride (0.85 g, 3.80 mmol) according to Method I to give 1.48 g (81%) of 7 as a white solid: R₉ (EtOAc) = 0.68; R₉ [butanol/acetic acid/water, (BAW) 3:1:1] = 0.87; HPLC (col. A) tₚ = 37.5 min; mp 161-163 °C; [α]₀ = -15.9 ° (c = 0.94, MeOH); ¹H NMR (CDCl₃) δ = 0.88 (d, 3H, J = 1.7 Hz), 0.91 (d, 3H, J = 1.4 Hz), 1.39 (s, 9H), 1.41 (s, 9H), 1.55 (m, 3H), 3.09 (dd, 2H, J = 6.7 Hz, 14.3 Hz), 4.10 (m,
2H), 5.08 (d, 1H), 6.21 (d, 1H), 7.35 (d, 2H, J = 8.7 Hz), 8.12 (d, 2H, J = 8.7 Hz); 
FAB-MS [M+H]+ 480.1 (calc. 480).

\textit{Nα-tert-Butyloxy carbonyl-L-p-aminophenylalanyl-L-leucine tert-butyl ester} (8)

Ammonium formate (1.5 g, 24.5 mmol) was added to a solution of \textit{N-tert-}
butyloxy carbonyl-L-p-nitrophenylalanyl-L-leucine tert-butyl ester 7 (1.30 g, 2.70 mmol) 
in MeOH (10 mL). Once the ammonium formate had dissolved, a suspension of 10%
Pd/C (0.26 g) in MeOH/H2O (1:1, 1 mL) was then added dropwise. After 0.5 h, the 
reaction mixture was filtered through celite, and the MeOH evaporated. The residue was 
then dissolved in EtOAc (15 mL), and the organic layer washed with H2O (10 mL) and 
saturated NaCl (10 mL), and then dried over MgSO4. The EtOAc was evaporated to yield 
dipeptide 8 as a white solid (1.15 g, 94%): Rf (EtOAc) = 0.57; Rf (EtOAc/hexane, 9:1) = 
0.52; HPLC (col. A) tR = 27.2 min; mp 158-160°C; [\alpha]D = -19.0 ° (c=1.04, MeOH); 1H
NMR (CDCl3) δ = 0.88 (d, 3H, J = 2.1Hz), 0.90 (d, 3H, J = 1.8 Hz), 1.39 (s, 9H), 1.42 
(s, 9H), 1.54 (m, 3H), 2.94 (d, 2H, J = 6.5 Hz), 4.24 (broad s, 1H), 4.41 (m, 1H), 4.95 
(broad s, 1H), 6.28 (d, 1H, J = 8.4 Hz), 6.65 (d, 2H, J = 8.4 Hz), 6.98 (d, 2H, J = 8.3 
Hz); FAB-MS [M+H]+ 450.2 (calc. 450).

\textit{Nα-tert-Butyloxy carbonyl-L-p-amino(9-fluorenylmethoxycarbonyl)
phenylalanyl-L-leucine tert-butyl ester} (9)

Ten percent Na2CO3 was added to a solution of \textit{N-tert-}
butyloxy carbonyl-L-p-aminophenyl-
alanyl-L-leucine tert-butyl ester 8 (1.10 g, 2.40 mmol) in dioxane (10 mL) to adjust the pH 
to 8. A solution of 9-fluorenylmethoxycarbonylchloroformate (0.56 g, 2.2 mmol) was added, and 
the pH was maintained using additional 10% Na2CO3; a light, white precipitate was seen to 
form immediately. The reaction was allowed to proceed overnight. The resultant 
suspension was then diluted with H2O (10 mL) and the precipitate filtered and washed with 
0.1 N HCl. The crude solid (1.53 g, 105%) was dried and recrystallized from MeOH/H2O 
to yield 1.29 g (80%) of 9 as a white solid: Rf (EtOAc) = 0.69; HPLC (col.A) tR = 43.7
min; mp 207-210 °C; [α]D = -6.2° (c = 0.45, MeOH); 1H NMR (CDCl3) δ = 0.88 (d, 3H, J = 3.3 Hz), 0.89 (d, 3H, J = 8.9 Hz), 1.40 (s, 9H), 1.41 (s, 9H), 1.51 (m, 3H), 3.01 (d, 2H, J = 6.5 Hz), 4.25 (t, 2H, J = 6.5 Hz), 4.31 (m, 1H), 4.41 (m, 1H), 4.51 (d, 2H, J = 6.6 Hz), 6.22 (d, 1H, J = 7.9 Hz), 6.56 (s, 1H), 7.12 (d, 2H, J = 8.2 Hz), 7.31 (t, 4H, J = 7.5 Hz), 7.40 (t, 2H, J = 7.3 Hz), 7.59 (d, 2H, J = 7.2 Hz), 7.76 (d, 2H, J = 7.4 Hz); FAB-MS [M+H]+ 672.3 (calc. 672).

tert-Butyl L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucinate (10)

A solution of TMS-OTf in toluene (0.358 M, 4.77 mL, 1.66 mmol) was added dropwise to a solution of N-tert-butyloxycarbonyl-L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine tert-butyl ester 9 (1.10 g, 1.70 mmol) in DCM (15 mL). After a few minutes, the product (as the triflate salt) precipitated out as a gelatinous mass. After 4.0 h, the gel was filtered and washed with additional DCM, and then dried to give 0.94 g (97%) of intermediate 10 as a white solid: Rf (EtOAc) = 0.68; HPLC (col. A.) tR = 24.3 min; [α]D = -14.5° (c = 1.00, MeOH); 1H NMR (CDCl3) δ = 0.90 (d, 6H, J = 3.3 Hz), 1.45 (s, 9H), 1.57 (m, 3H), 1.78 (broad s, 1H), 4.25 (t, 1H, J = Hz), 4.45 (m, 1H), 4.56 (d, 2H, J = Hz), 6.65 (s, 1H), 7.12 (d, 2H, J = 8.2 Hz), 7.31 (t, 4H, J = 7.5 Hz), 7.40 (t, 2H, J = 7.3 Hz), 7.59 (d, 2H, J = 7.2 Hz), 7.76 (d, 2H, J = 7.4 Hz); FAB-MS [M+H]+ 572.3 (calc. 572); Anal. calcd. for C34H42N3O5CF3SO3H: C, 58.24; H, 5.86; N, 5.82. found: C, 60.29; H, 6.25; N, 6.01.

Methyl α-aminoisobutyrate hydrochloride (11)

Thionyl chloride (0.70 mL, 9.7 mmol) was added dropwise to a cold (ice/H2O) suspension of α-aminoisobutyric acid (1.00 g, 9.7 mmol) in dry MeOH. Upon addition, the suspension slowly dissolved. The reaction mixture was then warmed to 50 °C for 1 h, followed by stirring at room temperature overnight. The MeOH was then removed in vacuo to yield 1.40 g (94%) of 11 as a white solid. A sample was then recrystallized from
Methyl α-aminoisobutyryl-α-aminoisobutyrate hydrochloride (13)

A mixture of methyl N-benzyloxy carbonyl-α-aminoisobutyryl-α-aminoisobutyrate 12 (0.93 g, 2.78 mmol), 10% Pd/C (0.23 g) and conc. HCl (0.23 mL, 2.78 mmol) in MeOH (10 mL) was hydrogenated for 45 min at 1 atm. The catalyst was then filtered through celite, and the MeOH was removed in vacuo. The resultant gummy residue was triturated with Et₂O to give 13 as a white solid (0.59 g, 89%): R₅ (CH₂Cl₂/10%MeOH) = 0.40-0.27; (BAW, 3:1:1) = 0.60; mp 174-177 °C (lit. 175.5-178.5 °C (Lovett and Portoghese, 1987a)).
1987a)); $^1$H NMR (d6-DMSO) $\delta$ = 1.07 (s, 3H), 1.40 (s, 6H), 1.48 (s, 3H), 3.51 (s, 3H), 8.25 (broad s, 3H), 8.49 (s, 2H).

**N-Benzzyloxycarbonyl-O-tert-butyl-L-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyrate methyl ester (14)**

*N*-Benzzyloxycarbonyl-O-tert-butyl-L-tyrosine (1.20 g, 3.20 mmol) was coupled to methyl $\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyrate hydrochloride 13 (0.60 g, 2.5 mmol) using Method I to give 1.34 g (98%) of 14 as an oil (1.34 g, 98%): Rf (EtOAc) = 0.50; $[\alpha]_D = +30.8^\circ$ (lit. = +31.2$^\circ$, c = 1.0, MeOH); $^1$H NMR (CDCl$_3$) $\delta$ = 1.31 (s, 9H), 1.42 (s, 3H), 1.44 (s, 3H), (s, 6H), 2.95 (t, 2H), 3.69 (s, 3H), 4.18 (q, 1H), 5.18 (s, 2H), 5.25 (broad d, 1H), 5.98 (s, 1H), 6.90 (d, 2H), 6.98 (s, 1H), 7.05 (d, 2H), 7.35 (m, 5H).

**Methyl O-tert-butyl-L-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyrate (15)**

Methyl N-benzyloxycarbonyl-O-tert-butyl-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyrate 14 (1.18 g, 2.13 mmol) was dissolved in MeOH (10 mL) and ammonium formate (0.41 g, 6.41 mmol) was then added. Once the ammonium formate had dissolved, a suspension of 10% Pd/C (0.23 g) in MeOH/H$_2$O (1:1, 1 mL) was added dropwise. After 1.0 h, the reaction mixture was filtered through celite, and the MeOH evaporated. The residue was then dissolved in EtOAc (15 mL) and the organic layer washed with H$_2$O (10 mL) and saturated NaCl (10 mL), and then dried over MgSO$_4$. The EtOAc was evaporated to yield 0.83 g (92%) of 15. A sample was recrystallized from EtOAc/hexane for analysis: HPLC (col. A) $t_R$ = 19.5 min; m.p. 119-121 $^\circ$C; $[\alpha]_D = +27.7^\circ$ (c=1.0, MeOH); $^1$H NMR (d$_6$-DMSO) $\delta$ = 1.31 (s, 9H), 1.35 (s, 6H), 1.47 (s, 6H), 2.88 (d of d, 1H), 2.91 (d of d, 1H), 3.48 (t, 1H), 3.68 (s, 3H), 6.90 (d, 2H), 7.11 (d, 2H).

**N,N-Diallyl-O-tert-butyl-L-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyrate methyl ester (16)**

Methyl O-tert-butyl-L-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyrate 15 (0.83 g, 1.9 mmol), DIPEA (1.1 mL, 5.94 mmol) and allyl bromide (1.7 mL, 20 mmol) were dissolved in benzene/MeCN (4:1, 10 mL) and stirred at room temperature overnight. The reaction
mixture was then concentrated in vacuo, and diluted with EtOAc (50 mL). The organic
layer was then washed with H₂O (30 mL), saturated NaCl, and then dried over MgSO₄.
The solvent was removed under vacuo, and the oily residue was applied to a flash silica gel
column with EtOAc/hexane (4:1) as the eluent. The collected fractions gave 0.83 g (83%)
of 16 as a slightly yellowish oil: Rₜ (CH₂Cl₂/5% MeOH) = 0.42; HPLC (col. A) tᵣ = 21.2
min; [α]D = -1.9° (c = 1.02, MeOH); FAB-MS [M+H]+ 502.0 (calc. 502).

N,N-Diallyl-0-tert-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyric
acid (17)

Methyl N,N-diallyl-0-tert-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyrate 16
(0.83 g, 1.6 mmol) was dissolved in THF (5.0 mL) and set in an ice water bath. To the
cold solution, 1 M NaOH (1.8 mL, 1.8 mmol) was added dropwise. The reaction mixture
stirred at 0 °C for 1 h, and then at room temperature for 3.5 h. The solution was
concentrated, and applied to a medium pressure C₈ column. The amount obtained from the
column was 0.62 g (72 %) of 17 as a clear glass: Rₜ (CH₂Cl₂/10% MeOH) = 0.0-0.33;
[α]D = -6.2° (c = 0.40, MeOH).

N-Benzylxocarbonyl-O-tert-butyl-L-tyrosylglycylglycine ethyl ester (18)

As described in Method I, N-benzylxocarbonyl-O-tert-butyl-L-tyrosine (0.65 g, 1.8
mmol) was coupled to ethyl glycylglycinate hydrochloride (0.35 g, 1.80 mmol) to give
0.83 g (91%) of 18 as a white solid. A sample was recrystallized from Et₂O/hexane for
analysis: Rₜ (CH₂Cl₂/5% MeOH) = 0.27; Rₜ (EtOAc) = 0.32; mp 102-104 °C (lit. 104-105
°C (Lovett and Portoghese, 1987a)); [α]D = -18.1 ° (lit. -18.5 °, c = 1.0, DMF (Lovett and
Portoghese, 1987a)).

Ethyl O-tert-butyl-L-tyrosylglycylglycinate (19)

Ammonium formate (0.31 g, 4.9 mmol) was added to a solution of N-benzylxocarbonyl-
O-tert-butyl-L-tyrosylglycylglycine ethyl ester 18 (0.84 g, 1.60 mmol) in MeOH (10 ml)
and allowed to stir until all traces of the salt dissolved. To the solution, 10% Pd/C (0.10 g)
was then added as a suspension in H₂O/MeOH (1:1, 1 mL). After 30 min, the reaction
mixture was filtered through celite, and the MeOH removed in vacuo. The residue was dissolved in EtOAc (10 mL), and the solution washed with saturated NaCl and dried over MgSO₄ to give 0.59 g (97%) of 19 as a white solid: Rₓ (CH₂Cl₂/10% MeOH) = 0.48; mp 112-113 °C (lit. 113-115 °C (Lovett and Portoghese, 1987a)); [α]D = -25.5 ° (lit. -26.0 °, c = 0.63, DMF (Lovett and Portoghese, 1987a)).

**N,N-Dibenzylo-O-tert-butyl-L-tyrosylglycylglycine ethyl ester (20)**

Benzyl bromide (1.5 mL, 12.4 mmol) was added to a solution of ethyl O-tert-butyl-L-tyrosylglycylglycinate 19 (587 mg, 1.50 mmol) in MeCN (8.0 mL). After a few minutes, DIPEA (0.8 ml, 4.6 mmol) was added. After 24 h, the MeCN was removed in vacuo, and the residue was dissolved in EtOAc (20 mL). The solution was then washed with saturated NaCl (10 mL) and dried over MgSO₄. The EtOAc was evaporated, and the residue was applied to a flash silica gel column with EtOAc/hexane (4:1) as the eluent to give 0.70 g (81%) of 20 as a clear oil: Rₓ (EtOAc) = 0.55; [α]D = -4.3° (lit. -4.4 °, c = 1.05, MeOH (Lovett and Portoghese, 1987a)).

**N,N-Dibenzylo-O-tert-butyl-L-tyrosylglycylglycine (21)**

A NaOH solution (0.25 N, 4.8 mL, 1.2 mmol) was added dropwise to a cold (ice/H₂O) solution of N,N-dibenzylo-O-tert-butyl-L-tyrosylglycylglycine ethyl ester 20 (678 mg, 1.20 mmol) in THF (5.0 mL). The reaction mixture was allowed to stir at 0 °C for 1 h, and then it was allowed to warm to room temperature and stirred for an additional 4.0 h. The reaction mixture was neutralized with 1N HCl and the solvent removed in vacuo. The residue was dissolved in EtOAc (20 mL) and washed with saturated NaCl and dried over MgSO₄ to give 0.59 g (91%) of 21 as a foam: Rₓ (DCM/MeOH, 9:1) = 0.0 - 0.25.

**N,N-Dibenzylo-O-tert-butyl-L-tyrosylglycylglycyl-L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine tert-butyl ester (22)**

The tripeptide N,N-dibenzylo-O-tert-butyl-L-tyrosylglycylglycyl-L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine tert-butyl ester 22 was coupled to L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine tert-butyl ester 10 as described by Method I, except that DMF instead of THF/DMF was used as the
solvent. The crude material was purified by flash silica gel chromatography using EtOAc as the eluent to give 0.57 g (74%) of 22 as a foam: Rf (EtOAc) = 0.43; HPLC (col. A) tR = 42.3 min; [α]D = -8.63 ° (c = 1.2, CHCl3); FAB-MS [M+H]+ 1085.7 (calc. 1085).

N,N-dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-aminophenylalanyl-L-leucine tert-butyl ester (23)

N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine tert-butyl ester 22 (269 mg, 0.25 mmol) was dissolved in DCM (2.0 mL) and tris(2- aminoethyl)amine (1.8 mL, 12.5 mmol) was then added. A white precipitate formed immediately. After 3.0 h, the solvent was removed in vacuo, and the residue was dissolved in EtOAc (30 mL). The EtOAc layer was washed with saturated NaCl, phosphate buffer at pH 5.5 (μ = 1.0, 3 x 10 mL), and saturated NaCl, and then dried over MgSO4. The solvent was removed in vacuo, and the crude residue was applied to a flash silica gel column with EtOAc as the eluent to give 160 mg (75%) of 23 as a clear glass: Rf (EtOAc) = 0.14; Rf [chloroform/methanol/acetic acid, (CMA) 95:5:1] = 0.18; Rf (BAW, 3:1:1) = 0.67; HPLC (col. A) tR = 34.0 min; FAB-MS [M+H]+ 863.5 (calc. 863).

N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-isothiocyanato-phenylalanyl-L-leucine tert-butyl ester (24)

N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-aminophenylalanyl-L-leucine tert-butyl ester 23 (37 mg, 0.04 mmol) was dissolved in DCM (0.5 mL). To the solution, DIPEA (6.5 μL, 0.08 mmol) was added, followed by thiophosgene (6.5 μL, 0.08 mmol). After 4.0 h, the solvent was removed under a stream of N2, and the crude residue was applied to silica gel flash chromatography with EtOAc as the eluent to give 38 mg (94%) of 24 as a clear glass: Rf (EtOAc) = 0.48, Rf (CMA 95:5:1) = 0.28; HPLC (col. A) tR = 41.2 min; FAB-MS [M+H]+ 905.8 (calc. 905).
N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-chloroacetamido-
phenylalanyl-L-leucine tert-butyl ester (25)

N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-aminophenylalanyl-L-leucine tert-
butyl ester 23 (77 mg, 0.09 mmol) was dissolved in DCM and chilled to 0°C in an ice
water bath. To the solution, DIPEA (31 µL, 0.18 mmol) was added, followed by
bromoacetyl chloride (11 µL, 0.14 mmol) Upon addition of the bromoacetyl chloride, a
thick white vapor formed, and the solution quickly turned a dark color. After 1.0 h at 0°C,
the solution was allowed to warm to room temperature and the reaction continued
overnight. The solvent was then removed under an N₂ stream, and the residue was applied
to silica gel flash chromatography with EtOAc as the eluent to give 49 mg (54%) of 25 as a
clear glass: HPLC (col. A) Rₜ = 37.0 min; FAB-MS [M+H]+ 941.2 (calc. 939); HRFAB-
MS (calc. for C₅₂H₆₉ClN₆O₈) 940.4787, found: 940.4778.

N,N-dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-bromoacetamido-
phenylalanyl-L-leucine tert-butyl ester (26)

N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-aminophenylalanyl-L-leucine tert-
butyl ester 23 (21 mg, 0.02 mmol) was dissolved in DCM and chilled to 0°C in an ice
water bath. To the solution, DIPEA (8.5 µL, 0.05 mmol) was added, followed by
bromoacetyl bromide (3.2 µL, 0.04 mmol) Upon addition of the bromoacetyl bromide, a
thick white vapor formed, and the solution quickly turned a dark color. After 1.0 h at 0°C,
the solution was allowed to warm to room temperature and the reaction mixture proceeded
overnight. The solvent was then removed in vacuo, and the residue was purified by silica
gel flash chromatography with EtOAc as the eluent to give 16 mg (91%) of 27 as a glass:
Rₜ (EtOAc) = 0.62; HPLC (col. A) tR = 37.8 min; FAB-MS [M+H]+ 985.4 (calc. 984);

N,N-Dibenzy1-L-tyrosylglycylglycyl-L-p-aminophenylalanyl-L-leucine (1)

N,N-Dibenzy1-O-tert-butyl-L-tyrosylglycylglycyl-L-p-aminophenylalanyl-L-leucine tert-
butyl ester 23 (20 mg, 0.02 mmol) was deprotected using Method II to yield 16 mg
81

(72%) of 1 as a white solid: HPLC (col. A) $t_R = 46.6$ min (94% purity); FAB-MS [M+H]$^+$ 751.4 (calc. 751).

\textit{N,N-Dibenzyl-L-tyrosylglycylglycyl-L-p-isothiocyanatophenylalanyl-L-leucine} (2)

\textit{N,N-Dibenzyl-O-tert-buty-L-tyrosylglycylglycyl-L-p-isothiocyanatophenylalanyl-L-leucine} tert butyl ester 24 (38 mg, 0.04) was deprotected using Method II to yield 15.4 mg (41%) of 2 as a white solid: HPLC (col. A) $t_R = 32.0$ min (91% purity); FAB-MS [M+H]$^+$ 793.3 (calc. 793).

\textit{N,N-Dibenzyl-L-tyrosylglycylglycyl-L-p-chloroacetamidophenylalanyl-L-leucine} (3a)

\textit{N,N-Dibenzyl-O-tert-buty-L-tyrosylglycylglycyl-L-p-chloroacetamidophenylalanyl-L-leucine} tert-butyl ester 25 (20 mg, 0.02) was deprotected using Method II to yield 19.1 mg (95%) of 3a as a white solid: HPLC (col. A) $t_R = 27.4$ min (87% purity); FAB-MS [M+H]$^+$ 827.4 (calc. 827).

\textit{N,N-Dibenzyl-L-tyrosylglycylglycyl-L-p-bromacetamidophenylalanyl-L-leucine} (3)

\textit{N,N-Dibenzyl-O-tert-buty-L-tyrosylglycylglycyl-L-p-bromacetamidophenylalanyl-L-leucine} tert-butyl ester 26 (20 mg, 0.02) was deprotected using Method II to yield 14.6 mg (73%) of 3 as a white solid: HPLC (col. A) $t_R = 27.8$ min (98% purity); FAB-MS [M+H]$^+$ 873.3 (calc. 872).

\textit{N,N-Diallyl-O-tert-buty-L-tyrosyl-\alpha-aminoisobutyryl-\alpha-aminoisobutyryl-L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucinetert-buty-L-leucine} tert butyl ester (27)

\textit{N,N-Diallyl-O-tert-buty-L-tyrosyl-\alpha-aminoisobutyryl-\alpha-aminoisobutyryl-L-p-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucinetert-buty-L-leucine} tert butyl ester triflate 10 (132 mg, 0.23 mmol) using pivaloyl chloride as described in the preparation of 12. Following the standard workup, the crude product was applied to
a silica gel flash column with EtOAc/hexane (4:1) as the eluent to yield 140 mg (57%) of 27 as a foam: Rf (EtOAc) = 0.61; HPLC (col. B) tR = 37.2 min; FAB-MS [M+H]+ 1041.6 (calc. 1041).

\[ N,N\text{-Diallyl-O-tert-butyl-L-tyrosyl-\(\alpha\)-aminoisobutyryl-\(\alpha\)-aminoisobutyryl-L-p-aminophenylalanin-L-leucine tert-butyl ester (28)} \]

\[ N,N\text{-Diallyl-O-tert-butyl-L-tyrosyl-\(\alpha\)-aminoisobutyryl-\(\alpha\)-aminoisobutyryl-L-p-aminophenylalanin-L-leucine tert-butyl ester 27 (221 mg, 0.21 mmol) was deprotected using TAEA as described in the preparation of 23. The crude residue was applied to flash silica gel chromatography with EtOAc as the eluent, to yield 158 mg (91%) of 28 as a clear glass: Rf (EtOAc) = 0.25; HPLC (col. B) tR = 35.0 min; FAB-MS [M+H]+ 819.6 (calc. 819). \]

\[ N,N\text{-Diallyl-O-tert-butyl-L-tyrosyl-\(\alpha\)-aminoisobutyryl-\(\alpha\)-aminoisobutyryl-L-p-isothiocyanatophenylalanin-L-leucine tert-butyl ester (29)} \]

\[ N,N\text{-Diallyl-O-tert-butyl-L-tyrosyl-\(\alpha\)-aminoisobutyryl-\(\alpha\)-aminoisobutyryl-L-p-aminophenylalanin-L-leucine tert-butyl ester 28 (15 mg, 0.02 mmol) was treated as described in the preparation of 24 to yield 13.3 mg (80%) of 20 as a clear glass: Rf (EtOAc) = 0.54; HPLC (col. B) tR = 25.0 min; FAB-MS [M+H]+ 861 (calc. 861). \]

\[ N,N\text{-Diallyl-O-tert-butyl-L-tyrosyl-\(\alpha\)-aminoisobutyryl-\(\alpha\)-aminoisobutyryl-L-p-bromoacetamidophenylalanin-L-leucine tert-butyl ester (30)} \]

\[ N,N\text{-Diallyl-O-tert-butyl-L-tyrosyl-\(\alpha\)-aminoisobutyryl-\(\alpha\)-aminoisobutyryl-L-p-aminophenylalanin-L-leucine tert-butyl ester 28 (14 mg, 0.02 mmol) was dissolved in dichloromethane and chilled to 0°C in an ice water bath. To the solution, DIPEA (6.0 \mu L, 0.03 mmol) was added, followed by bromoacetyl bromide (2.2 \mu L, 0.03 mmol). Upon addition of the bromoacetyl bromide, a thick white vapor formed, and the solution quickly turned a dark color. After 2.0 h at 0°C, the solution was allowed to warm to room temperature for 1 h. The solvent was then removed under an \text{N}_2 \text{ stream, and the residue} \]
was applied to a silica gel flash column with EtOAc as the eluent to yield 9.1 mg (58%) of 30 as a clear glass: HPLC (col. B) $t_R = 40.9$ min; FAB-MS $[M+H]^+ 941$ (calc. 940).

$N,N$-Diallyl-$L$-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyryl-$L$-$p$-amino-phenylalanyl-$L$-leucine (4)

$N,N$-Diallyl-$O$-tert-butyl-$L$-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyryl-$L$-$p$-amino phenylalanyl-$L$-leucine tert-butyl ester 28 (9.9 mg, 0.01 mmol) was deprotected as described in Method II to yield 8.9 mg (78%) of 4 as a white solid: HPLC (col. B) $t_R = 22.5$ min. (96% purity); FAB-MS $[M+H]^+ 707.4$ (calc. 707).

$N,N$-Diallyl-$L$-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyryl-$L$-$p$-isothiocyanatophenylalanyl-$L$-leucine (5)

$N,N$-Diallyl-$O$-tert-butyl-$L$-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyryl-$L$-$p$-isothiocyanatophenylalanyl-$L$-leucine tert-butyl ester 29 (13 mg, 0.01 mmol) was deprotected as described in Method II to yield 9.1 mg (70%) of 5 as a white solid: HPLC (col. B) $t_R = 33.3$ min (98% purity); FAB-MS $[M+H]^+ 749.3$ (calc. 749).

$N,N$-Diallyl-$L$-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyryl-$L$-$p$-bromoacetamidophenylalanyl-$L$-leucine (6)

$N,N$-Diallyl-$O$-tert-butyl-$L$-tyrosyl-$\alpha$-aminoisobutyryl-$\alpha$-aminoisobutyryl-$L$-$p$-bromoacetamidophenylalanyl-$L$-leucine tert-butyl ester 30 (9 mg, 0.01 mmol) was deprotected as described in Method II to yield 8.5 mg (98%) of 6 as a white solid: HPLC (col. B) $t_R = 40.9$ min (98% purity); FAB-MS $[M+H]^+ 829.3$ (calc. 828).

4.7.2.2 Standard binding assays

Radioligands binding assays of the potential affinity label derivatives were performed with Chinese hamster ovary (CHO) cells stably transfected with either mouse $\delta$ or rat $\mu$ opioid receptors. Cells were harvested 72 h following transfection in 50 mM Tris buffer, pH 7.4, at 4 °C and homogenized using a Dounce homogenizer. The homogenate was then centrifuged at 45,000 x g for 10 min at 4 °C. The pellet was washed twice by
resuspension and recentrifugation as in the previous step. The pellet was resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C to yield a protein concentration of 30-60 μg/mL. Incubations were performed for 90 min at 22 °C with [3H]DPDPE and [3H]DAMGO for δ and μ receptors, respectively. Binding assays were carried out in mixtures containing 100 μg membrane protein, 3 mM Mg2+, and peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) in a final volume of 2 mL 50 mM Tris buffer, pH 7.4, at 22 °C. Nonspecific binding was determined in the presence of 10 μM unlabeled DPDPE and DAMGO for the δ and μ binding assays, respectively. The reactions were terminated by rapid filtration over Whatman GF/B glass fiber filters using a Brandel M24-R cell harvester. The filters were presoaked for at least 2 h in 0.5% polyethylenimine to decrease nonspecific binding. The filter disks were then placed in minivials with 4 mL Cytoscint (ICN radiochemicals) and allowed to elute for at least 6 h before counting in a Beckman LS 6800 scintillation counter. IC50 values were then derived from nonlinear regression analysis of competition curves.

4.7.2.3 Wash-resistant binding assays

Potential affinity label derivatives for the δ opioid receptor were examined for wash-resistant inhibition of binding to opioid receptors. CHO cell membranes expressing δ receptors were incubated in the absence or presence of the enkephalin derivatives for 90 min at room temperature. The homogenates were then centrifuged at 40,000 x g for 15 min at 4 °C and the pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C. The centrifugation and resuspension steps were repeated four times as the washing protocol. After the fifth resuspension, the homogenate was recentrifuged and the final pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C. These final CHO cell membrane homogenates were then subjected to radioligand binding assays as described above. The radioligand binding to membranes treated with the enkephalin analogues were then compared to binding to untreated control membranes.
Chapter 5

Synthesis and Evaluation of Affinity Labels
Based on the Delta Opioid Antagonist, TIPP

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5.1 Abstract

In an ongoing effort to synthesize affinity labels based on δ-selective peptide antagonists, we have prepared derivatives of the tetrapeptide Tyr-Tic-Phe-Phe (TIPP) containing an isothiocyanate moiety as potential irreversible ligands. The isothiocyanate substituent was positioned at the para position of either Phe\textsuperscript{3} or Phe\textsuperscript{4} of the peptide. The synthesis was accomplished through a [2+2] convergent synthesis combining the common N-terminus Tyr-Tic to the C-terminal dipeptide fragments Phe-Phe, with a p-amine group protected as the 9-fluorenylmethoxycarbamate (Fmoc) at either position 3 or 4. This synthetic procedure was developed in our laboratory, and it allows for the late introduction of affinity labeling groups late in the synthesis for a variety of peptide substrates.

Once the derivatives were synthesized, the [(p-NCS)Phe\textsuperscript{3}]TIPP and [(p-NCS)Phe\textsuperscript{4}]TIPP derivatives (and their corresponding amine derivatives) were tested for inhibition of radioligand binding in δ and μ opioid receptors expressed in Chinese hamster ovary (CHO) cells. All of the derivatives exhibited low nanomolar affinity and excellent selectivity for the δ opioid receptor. Also, differences seen in the binding affinity profiles of these derivatives and the previously synthesized N,N-dialkylated enkephalin series suggest that neither the Phe\textsuperscript{3} and Phe\textsuperscript{4} residues in TIPP correspond to the Phe\textsuperscript{4} residue of enkephalin-like peptides at the δ and μ opioid receptors. The nature of these substituents at the para positions of these Phe residues can be further exploited to increase affinity and/or selectivity of these peptides for δ opioid receptors.

When tested for wash-resistant binding inhibition of radioligand binding, both the [Phe(p-NCS)\textsuperscript{3}]TIPP and [Phe(p-NCS)\textsuperscript{4}]TIPP were able to inhibit [\textsuperscript{3}H]DPDPE binding at the δ receptor at a low nanomolar concentration. Therefore, [Phe(p-NCS)\textsuperscript{3}]TIPP and [Phe(p-NCS)\textsuperscript{4}]TIPP represent two potential affinity labels that may prove useful in the pharmacological study of δ opioid receptors.
5.2 Abbreviations

Abbreviations used for amino acids are according to the rules specified by the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem.* 1984, 138, 9-37. All amino acids are in the L-configuration unless otherwise specified. Additional abbreviations used are: Boc, *tert*-butyloxycarbonyl; Cbz, benzylxycarbonyl; CHO, Chinese hamster ovary; DAMGO, Tyr-D-Ala-Gly-MePhe-Leu-Gly-ol; DCCI, dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DPDPE, Tyr-cyclo[D-Pen-Gly-Phe-D-Pen]-OH; FAB-MS, fast atom bombardment mass spectroscopy; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-Cl, 9-fluorenylmethyl chloroformate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; Mel, melphalan; MVD, mouse vas deferens; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance spectroscopy; NTI, naltrindole; NTII, naltrindole isothiocyanate; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; TAEA, tris(2-aminoethyl)amine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid; TIPP, Tyr-Tic-Phe-Phe; TMS-OTf, trimethylsilyl trifluoromethane-sulfonate; Tris, tris(hydroxymethyl)aminomethane.
5.3 Introduction

Affinity labels are ligands that exhibit such a high affinity for a receptor that the interaction is considered non-equilibrium. Most commonly, affinity labels interact with receptors via a covalent linkage through electrophilic attack. Due to their irreversible nature, affinity labels exhibit a number of advantages over traditional reversible ligands in various receptor studies. By forming a covalent linkage with the receptor, an affinity label can irreversibly block a receptor for in vivo studies, or if radiolabeled, enable the receptor to be purified and mapped. Affinity labels have greatly aided the study and characterization of the mu (µ), kappa (κ), and delta (δ) opioid receptors (Takemori and Portoghese, 1985). In the study of δ opioid receptors, irreversible ligands have been used to demonstrate δ-mediated analgesia, and the (+)-3R,4S enantiomer of 3-methyl fentanyl isothiocyanate (SUPERFIT) was used to purify δ receptors from NG108-15 cells to apparent homogeneity (Simonds et al., 1975). Most recently, SUPERFIT has been used in chimeric receptor binding assays to identify domains of the δ opioid receptor which are important for high affinity binding of this ligand (Zhu et al., 1996). The binding pocket of receptors are usually made up of non-contiguous amino acid residues, and a variety of affinity labels that interact with different receptor-based nucleophiles would help depict a clearer picture of the receptor binding site.

For the development of an affinity label, the parent compound should exhibit high affinity and selectivity for the receptor to be studied. In an effort to synthesize affinity labels based on peptide antagonists for the δ opioid receptor, the potent and selective tetrapeptide antagonist Tyr-Tic-Phe-Phe (TIPP) (Schiller et al., 1992) was chosen to be the parent peptide. TIPP was synthesized by Schiller and co-workers, and its development helped illustrate the differences and similarities in µ and δ receptor binding requirements. The goal of this project was to utilize a general synthetic methodology for the preparation of peptide-based affinity labels previously described by our laboratory (Chapter 4) and prepare TIPP derivatives containing an isothiocyanate moiety at the para-position of either
Fig. 5.1 Target TIPP-based affinity labels
Phe\textsuperscript{3} or Phe\textsuperscript{4} (see Fig. 5.1). These compounds were then tested for wash-resistant inhibition of radioligand binding to δ opioid receptors expressed on Chinese hamster ovary (CHO) cells.

In opioid peptides derived from amphibian skin (i.e. deltorphins, dermophins), the familiar Tyr-Gly-Gly-Phe sequence seen in endogenous mammalian opioid peptides is replaced by the tripeptide sequence Tyr-D-Xaa-Phe (Erspamer, 1992). Through the synthesis and testing of para-substituted Phe derivatives of opioid peptides (Schiller et al., 1983), it has been postulated that the Phe\textsuperscript{3} and Phe\textsuperscript{4} residues of various opioid peptides interact with different aromatic binding regions located on the opioid receptors. In an effort to utilize both of these putative aromatic binding regions, Schiller and co-workers first synthesized the tetrapeptide amide Tyr-D-Ala-Phe-Phe-NH\textsubscript{2} (Schiller et al., 1989). This peptide contains a Phe residue at both positions 3 and 4, and can be thought of as a dermorphin/enkephalin hybrid. This tetrapeptide amide was shown to be a μ-selective agonist. In subsequent studies (Schiller et al., 1992), D-Phe was substituted for the D-Ala at position 2, resulting in a fully aromatic peptide, which also exhibited μ-selective agonism. N-Methylation of Phe\textsuperscript{2} was found to have a divergent effect on the opioid activity profiles of the diastereomeric tetrapeptides. The D-N-Me-Phe\textsuperscript{2} containing analog showed a marked decrease in affinity for μ receptors, and slightly reduced affinity for the δ receptor. By comparison, the L-N-Me-Phe\textsuperscript{2} analog showed an increase in μ-receptor binding, and slightly enhanced δ-receptor affinity. Schiller and co-workers then focused on the orientation of the aromatic residue at position 2, which was evidently important for receptor selectivity. They substituted both isomers of Tic (tetrahydroisoquinoline-3-carboxylic acid) for the Phe at position 2. Tic is a conformationally constrained analog of Phe in which the aromatic ring is locked into place by a methylene bridge from the ortho position of the ring to the α-amine. The conformational constraint of Tic is such that it locks the aromatic ring of Phe into either a gauche (+) or gauche (−) conformation. It was
found that the D-Tic\(^2\) analog of TIPP was a \(\mu\)-selective agonist, whereas the L-Tic\(^2\) analog exhibited \(\delta\)-selective antagonism.

To develop TIPP into an affinity label, direct incorporation of a nitro-containing phenylalanine followed by later reduction and derivatization would be precluded because the benzylic C-N bond of Tic within a peptide has been shown to be susceptible to certain forms of hydrogenation (Majer et al., 1994). Therefore, the previously described Boc/Fmoc orthogonal protection strategy would be utilized to protect an amine at the para position of a phenylalanine, which would be deprotected at a later stage in the synthesis, followed by derivatization and final deprotection of the peptide. TIPP is an unusual opioid peptide in that it is completely comprised of aromatic amino acids, with two adjacent Phe residues at the C-terminus. It was decided to synthesize two potential affinity labels: one with an isothiocyanate at the para-position of Phe\(^3\), the other with an isothiocyanate on Phe\(^4\). The bromoacetamides were not considered for development due to the adverse effects on binding affinity seen in the previous enkephalin series (see Chapter 5). As an antagonist for the \(\delta\) opioid receptor, TIPP is more selective and potent than \(N,N\)-dibenzyl leucine enkephalin, and it was hoped that through derivatization, the increased selectivity and affinity of TIPP would result in more potent affinity labels for \(\delta\) opioid receptors.

5.4 Chemistry

A [2+2] convergent synthesis was envisioned in which C-terminal dipeptides containing an Fmoc-protected anilide amine at either Phe\(^3\) or Phe\(^4\) would be coupled to the common Boc-protected N-terminal sequence of Tyr-Tic. The N-terminal dipeptide was initially synthesized as the methyl ester, but saponification of the methyl ester required long reaction times and the reaction yields were somewhat low (50-60%). The possibility for racemization of the C-terminal aromatic residue under such prolonged basic conditions prompted us to utilize the phenyl ester as the carboxyl protecting group. As early as 1959, the phenyl ester was suggested as a carboxyl protecting group, but it was initially found to
be unsatisfactory due to excessive racemization of the peptide substrate upon saponification attempts. Kenner and Seely (Galpin et al., 1979, Kenner and Seely, 1972) then serendipitously discovered that basic peroxide was an effective and mild method to remove the phenyl ester protecting group. The modified synthesis is depicted in Fig. 5.2. The synthesis of the N-protected Tyr-Tic fragment began with the esterification of Boc-Tic with DCC and phenol to provide the fully protected amino acid 5. The Boc group was then selectively removed using 30% TFA in DCM to provide the carboxyl-protected Tic derivative 6 in 69% yield.

Due to the secondary nature of the α-amino of Tic, difficulties in coupling the Tyr residue to Tic due to increased steric hindrance were expected. In order to overcome these difficulties, the coupling reagent PyBOP (Coste et al., 1990) was used. PyBOP has been shown to be very effective in the coupling of N-methyl and other sterically hindered amino acids. The protected phenyl ester 6 was coupled to Boc-Tyr(OtBu)-OH using PyBOP, and the phenyl ester of the resultant dipeptide 7 was saponified with excess hydrogen peroxide and one equivalent of NaOH in dioxane/H₂O to give the desired N-terminal dipeptide 8 in 72% yield.

Synthesis of both C-terminal dipeptides were very similar, as can be seen in Figs. 5.3 and 5.4. The synthesis of H-Phe(p-NHFmoc)-Phe-OtBu 12 will be discussed. In the synthesis of the Phe³-substituted derivatives, Boc-Phe(p-NO₂)-OH was coupled to H-Phe-OtBu using isobutyl chloroformate, which provided dipeptide 9 in 91% yield. The coupling was followed by reduction of the nitro group by catalytic transfer hydrogenation (Anwar and Spatola, 1980), and the resulting amine 10 then protected using 9-fluorenylmethyl chloroformate, to give the fully protected dipeptide 11. In order to selectively remove the Boc group in the presence of a t-butyl ester, one equivalent of trimethylsilyl trifluoromethanesulfonate (TMS-OTf) (Marsmann and Horn, 1972, Vorbruggen and Krolikiewicz, 1975) , which was used successfully in the previous
i) DCC, phenol, DCM (86%); ii) 40% TFA/DCM (69%); iii) Boc-Tyr(OtBu)-OH, PyBOP, DIPEA, DCM (90%); iv) H₂O₂, 1M NaOH, dioxane (72%)

Fig. 5.2 Synthesis of N-terminal common dipeptide fragment 8
Fig. 5.3 Synthesis of the C-terminal dipeptide fragment 12 for 3-substituted TIPP derivatives

i) iBuOCOC1, NMM; ii) HCl-Phe-OtBu, NMM (91%); iii) NH4HCO2, 10% Pd/C (97%); iv) Fmoc-Cl, 10% NaHCO3, dioxane (89%); v) TMS-OTf, toluene (75%)
i) isobutylene, cat. H$_2$SO$_4$, dioxane; ii) Boc-Phe-OH, tBuOCOC$_1$, NMM (93%); iii) NH$_4$HCO$_2$, 10% Pd/C (97%); iv) Fmoc-Cl, 10% NaHCO$_3$, dioxane (95%); v) TMS-OTf, toluene (80%)

Fig. 5.4 Synthesis of the C-terminal dipeptide fragment 17 for 4-substituted TIPP derivatives
syntheses of the enkephalin analogs (see Chapter 4), was utilized once again to provide the desired C-terminal dipeptide fragment 12 in 75% yield.

The common N-terminal dipeptide fragment was then coupled to the Phe<sup>3</sup>- and Phe<sup>4</sup>-substituted C-terminal dipeptides through the use of PyBOP. The final syntheses of the target compounds can be seen in Figs. 5.5 and 5.6. The Fmoc group was then removed by treatment of the peptides with piperidine. After purification of the resultant amines by silica gel column chromatography, thiophosgene treatment provided the corresponding isothiocyanate derivatives. The peptides were then fully deprotected using TFA containing anisole as a scavenger.

5.5 Pharmacological evaluation

5.5.1 Binding assays under standard conditions

The amine- and isothiocyanate-substituted derivatives were then tested for affinity in radioligand binding assays using cloned δ- and μ-receptors expressed in CHO cells vs. [<sup>3</sup>H]DPDPE and [<sup>3</sup>H]DAMGO, respectively (Table 5.1). In general, substitution on the aromatic rings of the Phe residues resulted in a slight drop in affinity for the δ receptor, but slightly increased affinity at the μ receptor. Despite the overall decrease in selectivity for δ over μ receptors, all of the derivatives exhibited low nanomolar affinity and excellent selectivity for δ receptors, which was expected from the pharmacological profile of the parent peptide TIPP.

Despite overall similarities in binding affinities for δ and μ receptors, binding differences as a result of amine and isothiocyanate substitution at the para position of Phe<sup>3</sup> and Phe<sup>4</sup> could be observed. The [Phe(Φ-NCS)<sup>3</sup>]TIPP derivative exhibited a slight drop in affinity at the δ receptor but also resulted in an increase of affinity for the μ opioid receptor. This is in contrast with the [Phe(Φ-NH<sub>2</sub>)<sup>3</sup>]TIPP derivative, which resulted in a sharp
Fig. 5.5 Synthesis of Phe$_3$-substituted TIPP derivatives 1 and 2

i) 12, PyBOP, DIPEA (88%); ii) piperidine, DMF (99%); iii) TFA/10% anisole; iv) thiophosgene, DIPEA (95%)
Fig. 5.6 Synthesis of the Phe$^4$-substituted TIPP derivatives 3 and 4

i) 17, PyBOP, DIPEA (87%); ii) piperidine, DMF (84%); iii) TFA/10% anisole; iv) thiophosgene, DIPEA (95%)
Table 5.1 Binding affinities of TIPP derivatives 1-4 under standard conditions

<table>
<thead>
<tr>
<th>compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>vs. [³H]DPDPE (nM)</th>
<th>vs. [³H]DAMGO (µM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ratio (µ/δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIPP</td>
<td>5.8 ± 1.2</td>
<td>22.7 ± 15</td>
<td></td>
<td>3900</td>
</tr>
<tr>
<td>[Phe(p-NH₂)&lt;sub&gt;3&lt;/sub&gt;]TIPP 1</td>
<td>87.7 ± 15.3</td>
<td>&gt;100</td>
<td></td>
<td>&gt;1140</td>
</tr>
<tr>
<td>[Phe(p-NCS)&lt;sub&gt;3&lt;/sub&gt;]TIPP 2</td>
<td>12.4 ± 2.4</td>
<td>7.2</td>
<td></td>
<td>580</td>
</tr>
<tr>
<td>[Phe(p-NH₂)&lt;sub&gt;4&lt;/sub&gt;]TIPP 3</td>
<td>11.8 ± 2.5</td>
<td>1.59 ± 0.3</td>
<td></td>
<td>135</td>
</tr>
<tr>
<td>[Phe(p-NCS)&lt;sub&gt;4&lt;/sub&gt;]TIPP 4</td>
<td>5.4 ± 1.3</td>
<td>6.7</td>
<td></td>
<td>1200</td>
</tr>
</tbody>
</table>
decrease in \(\mu\) receptor affinity, suggesting that an amine at this position is not well tolerated for binding at the \(\mu\) receptor. The \([\text{Phe}(p-\text{NH}_2)^4]\) and \([\text{Phe}(p-\text{NCS})^4]\)TIPP derivatives exhibited similar \(\delta\)-receptor affinity to the parent compound, but both substitutions at the para position of Phe\(^4\) resulted in an increase of \(\mu\)-receptor binding affinity.

5.5.2 Wash-resistant binding assays

The peptides were then tested for wash-resistant inhibition of binding through pre-incubation of the test compounds with the CHO cells, followed by a washing procedure, and then subjected to a standard radioligand binding assay. The washing procedure consisted of centrifugation, decanting of the supernatant, followed by re-suspension of the CHO cells in fresh buffer. This cycle was repeated five times, which was found to be sufficient enough to remove most of the amine-containing control peptides without sacrificing large amounts of receptor protein. Due to their highly lipophilic nature, low concentrations of the TIPP derivatives were tested. Peptides 1, 2, 3, and 4 were tested for wash-resistant inhibition of radioligand binding at concentrations of 60, 10, 8, and 9 nM, respectively. For comparison, the \(\delta\)-selective affinity label NTII was tested at a concentration of 1 \(\mu\)M.

In the wash-resistant binding assays (Fig. 4.7), the Phe\(^3\) isothiocyanate-containing compound exhibited excellent wash-resistant binding inhibition of radioligand binding. Due to the large variance seen in the wash-resistant assays for the Phe\(^4\) compounds, additional tests must be done in order to ascertain its labeling capabilities. These compounds were tested at a concentration near their IC\(_{50}\)'s as determined by the reversible binding assays, and so the highest effective inhibition of the radioligand should be approximately 50%. The \([\text{Phe}(p-\text{NCS})^3]\)TIPP derivative exhibited inhibition at a much lower concentration than the previously synthesized \(N,N\)-dibenzyl[\text{Phe}(p-\text{NCS})^4]\)leucine enkephalin, which suggests that the increased affinity of the TIPP derivatives facilitated the primary binding step of the labeling process, resulting in a more potent affinity label.
Fig. 5.7 Graphical representation of the percent radioligand binding (normalized against control values) displayed by the peptide derivatives 1-4 following incubation and washing. Compounds 1, 2, 3, 4 and NTII were tested at concentrations of 60 nM, 10 nM, 8 nM, 9 nM, and 1 μM, respectively.
5.6 Results and discussion

In continuing our efforts to synthesize affinity labels for the δ opioid receptor based on peptide antagonists, we have once again used a Boc/Fmoc orthogonal protection strategy which highlights its overall versatility for the introduction of affinity labeling moieties into a variety of peptide substrates. The key step in the synthesis was the selective removal of a Boc protecting group in the presence of a t-butyl ester. This selective deprotection was accomplished through the use of one equivalent of TMS-OTf in toluene. Difficulty in saponifying a methyl ester was encountered during the synthesis of the common N-terminal dipeptide fragment, prompting us to use the phenyl ester for carboxylic acid protection, which is an effective and often overlooked method in peptide chemistry.

The peptides synthesized were the [Phe(p-NH₂)³], [Phe(p-NCS)³], [Phe(p-NH₂)⁴] and [Phe(p-NCS)⁴] derivatives of TIPP, which were then tested in binding assays using cloned δ receptors. At the δ receptor, the substitutions at the para position on either Phe seem to be well tolerated, as evidenced by the low nanomolar affinity and selectivity of the peptide derivatives. When tested for wash-resistant inhibition of radioligand binding, [Phe(p-NCS)³]TIPP 2 exhibited wash-resistant inhibiton of radioligand binding. If this peptide is truly interacting with the receptor in a non-equilibrium fashion, then this ligand may provide an important tool with which to study the binding pocket of δ opioid receptors.

It is difficult to tell whether or not the corresponding isothiocyanate 4 is inhibiting radioligand binding in a wash-resistant fashion. The high lipophilicity of these compounds and the instability of the isothiocyanate group during storage in aqueous solutions made testing these derivatives difficult, and further tests must be done in order to determine the effectiveness of compound 4 as a potential affinity label.

The binding pocket of G-protein coupled receptors are oftentimes made up of non-contiguous segments of amino acids, which are located on the transmembrane regions and/or different regions of the extracellular loops. Through their irreversible nature, affinity labels can be used to determine the regions which are near the binding site of the
receptor. A recent study had shown that the δ-selective non-peptide affinity label SUPERFIT required the region consisting of the beginning of the first intracellular loop to the middle of the third transmembrane region of the δ opioid receptor for reversible and irreversible binding (Zhu et al., 1996). In another study, Liu-Chen and co-workers (Chen et al., 1996) had determined that the μ-selective non-peptide affinity label β-FNA interacted with a Lys residue located near the beginning of the fifth transmembrane region (Lys^{233}). These studies have shown the utility of affinity labels in the determination of important residues located at or near the binding site locus.

Many questions have been raised about the interaction of the δ opioid receptor and its ligands, most specifically concerning the binding of the tetrapeptide TIPP. The TIPP tetrapeptide is considered an unusual opioid peptide in that it is completely comprised of aromatic amino acids. It is generally believed that the requirements for opioid receptor recognition are a protonated amine, a phenol ring, and an aromatic ring. In the case of the TIPP molecule, there has been much speculation as to which aromatic residue fulfills the requirement of the aromatic pharmacophore. From molecular modeling studies, it has been speculated that either Tic² or Phe³ fulfill this requirement (Chao et al., 1996, Wilkes and Schiller, 1995).

In the case of other opioid peptides, it was also questioned whether or not the Phe³ of select opioid peptides interacted with the receptor in an identical fashion as the Phe⁴ residue of the endogenous mammalian peptides. Through analysis of the binding affinities of Phe(p-NO₂) derivatives, Schiller and co-workers (Schiller et al., 1983) had concluded that the Phe³ residue of morphiceptin and dermorphin may bind to a site different from that interacting with the Phe⁴ side chain of enkephalins at the μ and δ receptors. It was thought that the binding affinities of TIPP and its Phe³- and Phe⁴-substituted derivatives compared with the binding data of the N,N-dialkylated enkephalins substituted at Phe⁴ may offer some similar insight as to the receptor binding requirements for the TIPP-like peptide antagonists.
In Table 5.2, the IC50 ratios of the substituted derivatives of the enkephalin- and TIPP-based peptides are presented. These ratio values are comparisons between the IC50 of the substituted derivative with respect to the IC50 of the unsubstituted parent compound. The earlier study done with N,N-dialkylated enkephalin analogs showed that identical substitution at the para position of Phe4 resulted in similar changes in δ and μ opioid receptor affinity. With respect to the underivatized parent compounds (i.e. N,N-dibenzyl leucine enkephalin and ICI-174,864), substitution of an amine at the para position in both peptides decreases affinity at both δ and μ receptors, whereas substitution of an isothiocyanate increases affinity at the δ receptor with similar affinity at the μ receptor. Therefore, analogous substitution resulted in similar changes in affinity for the δ and μ receptors, which is to be expected if both peptides are using the same binding subsite for the Phe4 aromatic rings.

Analysis of the IC50 ratios of the TIPP derivatives suggests that Phe3 and Phe4 of TIPP may bind to different subsites on the μ receptor than does the Phe4 residue of the enkephalin-based series, but the same conclusion could not be made with respect to the δ opioid receptor. At the μ receptor, incorporation of an amine at Phe3 of TIPP resulted in a large decrease in affinity, while incorporation of an isothiocyanate resulted in a slight increase in μ affinity. These binding results contrast with the decrease in μ affinity exhibited by both the amine- and isothiocyanate-containing enkephalin derivatives at the μ receptor. Likewise, the Phe4-substituted derivatives of TIPP also exhibited different binding characteristics than the enkephalin-based series at the μ receptor. An amine moiety at Phe4 resulted in a large increase of affinity at the μ receptor, which is the only example where incorporation of an amine resulted in an increase in μ binding. The corresponding isothiocyanate resulted in a slight increase in μ receptor binding. The unique pharmacological profile exhibited by the Phe4-substituted TIPP derivatives strongly suggests that the Phe4 aromatic ring does not utilize the same μ receptor binding subsite as the Phe4 aromatic moiety of enkephalin-based analogs.
Table 5.2 IC\textsubscript{50} ratios of enkephalin- and TIPP-based derivatives. IC\textsubscript{50} ratio = IC\textsubscript{50} of the substituted analog divided by the IC\textsubscript{50} of the parent compound.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} ratio</th>
<th>δ</th>
<th>μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>N,N-dibenzyl[(p-NH\textsubscript{2})Phe\textsuperscript{4}]leucine enkephalin</td>
<td></td>
<td>6.2</td>
<td>14.4</td>
</tr>
<tr>
<td>&quot;    &quot;</td>
<td></td>
<td>0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>N,N-diallyl[Aib\textsuperscript{2},Aib\textsuperscript{3},(p-NH\textsubscript{2})Phe\textsuperscript{4}]leucine enkephalin</td>
<td></td>
<td>2.0</td>
<td>4.6</td>
</tr>
<tr>
<td>&quot;    &quot;</td>
<td></td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>[(p-NH\textsubscript{2})Phe\textsuperscript{3}]TIPP</td>
<td></td>
<td>15.1</td>
<td>101</td>
</tr>
<tr>
<td>[(p-NCS)Phe\textsuperscript{3}]TIPP</td>
<td></td>
<td>2.1</td>
<td>0.3</td>
</tr>
<tr>
<td>[(p-NH\textsubscript{2})Phe\textsuperscript{4}]TIPP</td>
<td></td>
<td>2.0</td>
<td>0.07</td>
</tr>
<tr>
<td>[(p-NCS)Phe\textsuperscript{4}]TIPP</td>
<td></td>
<td>0.9</td>
<td>0.3</td>
</tr>
</tbody>
</table>
The differences seen in the \( \mu \) receptor IC\(_{50} \) ratio's were not seen in the binding data obtained from \( \delta \) receptor binding. In contrast with the enkephalin-based series, a slight decrease in affinity was observed when an isothiocyanate group was placed on the Phe\(^3 \) residue of TIPP, but the difference was not enough to suggest the use of a different aromatic binding subsite.

Despite the lack of distinct differences in the IC\(_{50} \) binding ratios for binding to \( \delta \) opioid receptors, experimental data obtained from other research laboratories have suggested that the Phe\(^3 \) and Phe\(^4 \) residues of TIPP do not correspond to the aromatic residue needed for \( \delta \) opioid receptor binding. Truncation studies have shown that Tyr-Tic-Phe (Schiller et al., 1992) and Tyr-Tic (Temussi et al., 1994) are able to bind to \( \delta \) opioid receptors. This would indicate that Tic\(^2 \) provides the second aromatic moiety for \( \delta \) opioid receptor binding, and Phe\(^3 \) and Phe\(^4 \) may be interacting with auxiliary binding subsites.

In our assays, \([\text{Phe}(p\text{-NCS})^3]\text{TIPP}\) showed wash-resistant inhibition of radioligand binding. At a concentration of 10 nM, this peptide was able to inhibit radioligand binding by 57\% following a wash procedure. The corresponding isothiocyanate derivative \([\text{Phe}(p\text{-NCS})^4]\text{TIPP}\) also showed some measure of wash-resistant inhibition of radioligand binding, but additional tests are needed to obtain more consistent results. The possibility that the Phe\(^3 \) and Phe\(^4 \) residues of TIPP might interact with different binding subsites could mean that the affinity labels based on TIPP may be interacting with different receptor-based nucleophiles. Further pharmacological studies with these affinity labels may offer additional information about residues at or near the binding site of the \( \delta \) opioid receptor.

5.7 Experimental section

5.7.1 Materials

All amino acids except for Boc-Phe\((p\text{-NO}_2)\) and Tic were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic reagents and the amino acids Boc-Phe\((p\text{-NO}_2)\)
and Tic were purchased from Aldrich Chemical Co (Milwaukee, WI). General lab solvents were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ), and HPLC-grade solvents were obtained from Burdick and Jackson (Muskegon, MI).

Syntheses were monitored using thin-layer chromatography using Kieselgel 60 F254 plates of 0.20 mm thickness purchased from EM Separations (Gibbstown, NJ). Intermediates were purified by flash chromatography using 230–400 mesh, 60A silica gel also purchased from E. Merck.

NMR analyses were done on an Bruker AC300 instrument at the Department of Chemistry, Oregon State University, Corvallis, OR. The molecular weights of the amino acid derivatives and peptides were determined by fast atom bombardment (FAB) mass spectrometry in the postitive mode on a Kratos MS50RFTC in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR.

The purity of intermediates and the final peptides were determined by analysis on a Beckman System Gold high performance liquid chromatography (HPLC) system consisting of a model 126 solvent module, model 168 detector, and model 507 autosampler. The HPLC column used was a Vydac analytical column (C\text{18}, 300 Å, 5 μ, 4.6 x 250 mm) equipped with a guard cartridge. The compounds were eluted using a linear gradient of 25 to 100% B over 50 min at a flow rate of 1.5 mL/min and detected at 214 nm; solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA.

5.7.2 Methods

5.7.2.1 Synthetic methods

Isobutyl chloroformate-mediated peptide coupling (Method I): Under an N\text{2} atmosphere, a 0.25 M solution of the carboxyl component (1.0 eq) in dry THF was cooled to -15 °C (dry ice/MeOH) and neutralized with one equivalent of N-methylmorpholine. A
stochiometric amount of isobutyl chloroformate is then added, followed 90 sec later by a
cold 0.5 M solution of the amine component and N-methylmorpholine (each 1.0 eq) in
DMF. The reaction mixture is kept at -15 °C for 30 min, and then allowed to warm to
room temperature. After completion of the reaction, the THF was evaporated, and the
residue suspended in EtOAc. The organic layer was washed with H2O, 5% KHSO4, H2O,
5% NaHCO3, H2O, and finally with saturated NaCl. The organic layer was then dried
over MgSO4 and the EtOAc evaporated to give the crude peptide.

Final deprotection with TFA (Method II): The peptide was treated with a 10%
anisole/TFA for one hour at room temperature. The TFA was then evaporated and the
product precipitated with cold ether. The precipitate was washed five times with cold ether,
and then dried in vacuo.

tert-Butyloxy carbonyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (2.6 g, 9.4 mmol)
and phenol (0.88 g, 9.4 mmol) were dissolved in EtOAc (20 mL) and cooled to -15 °C in a
dry ice/MeOH bath. To the chilled solution, dicyclohexylcarbodiimide (DCCI, 2.13 g,
10.3 mmol) suspended in EtOAc (5 mL) was added, and the reaction mixture was allowed
to warm to room temperature and stirred overnight. Following the addition of a few drops
of glacial acetic acid, the dicyclohexylurea reaction by-product was filtered off, and the
filtrate was washed with 5% NaHCO3, H2O, 0.1 N HCl, H2O, and saturated NaCl. The
organic layer was then concentrated in vacuo and passed through a plug of silica gel with
30% hexane/EtOAc as the eluent. The filtrate was then collected and evaporated to give
2.86 g (86%) of 5 as crystals which formed spontaneously during the evaporation process:
Rf (EtOAc) = 0.65; HPLC tR = 25.7 min; mp 75-78 °C; 1H NMR (CDCl3) δ = 1.51 (s, 9H),
1.55 (s, 9H), 3.31 (d, 2H, J = 5.3 Hz), 3.33 (dd, 2H, J = 5, 10 Hz), 4.63
Phenyl L-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (6)

tert-Butyloxy carbonyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl phenyl ester 5 (1.00 g, 2.8 mmol) was dissolved in dichloromethane (DCM, 5 mL) and trifluoroacetic acid (TFA, 3 mL) added dropwise. Vigorous bubbling was seen, which ceased after approximately 10 min. After a total reaction time of 0.5 h, the DCM and TFA were removed in vacuo with repeated dilutions of ethyl ether (5 x 5 mL aliquots). At the final dilution, white crystals spontaneously began to form. These crystals were filtered and washed with additional ethyl ether to yield 0.72 g (69%) of 6 as the TFA salt: Rf (EtOAc) = 0.44; HPLC tR = 20.8 min; mp (dec.) 106 °C; [α]D = -56.4° (c = 1.32, DMSO); 1H NMR (CDCl3) δ = 2.68 (broad s, 1H), 3.27 (dd, 2H, J = 10 Hz, 5 Hz), 4.08 (dd, 1 H, J = 4.9, 10 Hz), 4.24 (d, 2H, J = 4.6 Hz), 7.17 (m, 5H), 7.30 (m, 2H), 7.46 (m, 2H); FAB-MS [M+H]+ 254 (calc. 254).

tert-Butyloxy carbonyl-L-O-tert-butyl-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl phenyl ester (7)

tert-Butyloxy carbonyl-L-O-tert-butyl-tyrosine (0.33 g, 1.0 mmol), L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid 6 (0.30 g, 1 mmol) and benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP, 0.42 g, 1.0 mmol) were dissolved in DCM (5 mL) at room temperature. After a few minutes, N,N-diisopropylethylamine (DIPEA, 420 μL, 3 mmol) was added dropwise. After reacting overnight, the reaction mixture was diluted with EtOAc (15 mL) and washed with H2O, 5% KHSO4, H2O, 5% NaHCO3, H2O, and then saturated NaCl. The organic layer was dried over MgSO4, concentrated in vacuo and passed over a silica gel plug with EtOAc as the eluent. The filtrate was then evaporated to give 0.42 g (90%) of 7 as a whitish foam: Rf (EtOAc) = 0.69; HPLC tR = 47.5 min; mp 110-113 °C; [α]D = -5.2 ° (c = 1.24, toluene); FAB-MS
[M+H]+ 573 (calc. 573). Anal.: calcd for C$_{34}$H$_{40}$N$_{2}$O$_{6}$: C, 71.31%; H, 7.04%; N, 4.89%. Found: C, 71.09; H, 7.00; N, 4.94.

tert-Butyloxycarbonyl-L-0-tert-butyl-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (8)

tert-Butyloxycarbonyl-L-O-tert-butyl-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl phenyl ester 7 (0.40 g, 0.69 mmol) was dissolved in dioxane (5 mL). 30% H$_2$O$_2$ (785 µL) was added, followed by 1 N NaOH (700 µL). The reaction was allowed to proceed overnight. The reaction mixture was then chilled in an ice bath, and the solution neutralized using 1 N HCl. The solution was concentrated in vacuo, and dissolved in EtOAc. The organic layer was washed with saturated Na$_2$SO$_3$, saturated NaCl, and dried over MgSO$_4$. The EtOAc was removed in vacuo, which yielded 0.25 g (72%) of 8 as a clear glass: R$_f$ (EtOAc) = 0.21-0.40; HPLC t$_R$ = 38.0 min; [α]$_D$ = -53.8° (c = 1.19, MeCN); FAB-MS [M-H]$^-$ 495 (calc. 495).


tert-Butyloxycarbonyl-L-4-nitrophenyalanyl-L-phenylalaninetert-butyl ester (9)

tert-Butyloxycarbonyl-L-4-nitrophenylalanine (1.40 g, 4.50 mmol) was coupled to L-phenylalanine tert-butyl ester hydrochloride (1.08 g, 4.20 mmol) as described by Method I to yield a white solid (1.95 g, 91%). This was recrystallized from hot MeOH to give 1.64 g (76%) of 9 as a white solid: R$_f$ (EtOAc) = 0.59; (EtOAc/hexane, 4:1) = 0.51; [chloroform/methanol/acetic acid (CMA), 85:10:5] = 0.80; HPLC t$_R$ = 26.2 min; mp 125-127 °C; [α]$_D$ = -1.60° (c = 1.23, MeOH); $^1$H NMR (CDCl$_3$) δ = 1.36 (s, 9H), 1.39 (s, 9H), 3.02 (d, 2H, J = 6.0 Hz), 3.08 (overlapping signal, 1H), 3.16 (d of d, 1H, J = 6.8 Hz, J = 17 Hz), 4.35 (m, 1H), 4.62 (q, 1H, J = 6.1 Hz), 4.95 (broad s, 1H), 6.18 (broad d, 1H), 7.07 (m, 2H), 7.21 (m, 3H), 7.32 (d, 2H, J = 8.6 Hz), 8.09 (d, 2H, J = 8.7 Hz); FAB-MS [M+H]$^+$ = 514.3 (calc. 514).
tert-Butyloxy carbonyl-L-4-aminophenylalanyl-L-phenylalanine tert-butyl ester (10)

tert-Butyloxy carbonyl-L-4-nitrophenylalanyl-L-phenylalanine tert butyl ester 9 (1.02 g, 2.00 mmol) and ammonium formate (1.13 g, 18.0 mmol) was dissolved in MeOH (15 mL). Once the ammonium formate had dissolved, 10% Pd/C (0.10 g) was added as a suspension in MeOH/H2O (1:1, 1 mL). The reaction mixture was allowed to stir for 0.5 h, and filtered through Celite and the MeOH removed in vacuo. The residue was dissolved in EtOAc (10 mL) and the organic layer washed with H2O, and saturated NaCl. The EtOAc layer was dried over MgSO4 and evaporated. The resulting white solid (10) weighed 0.93 g (97%): Rf (EtOAc) = 0.42; HPLC tR = 14.5 min; [α]D = -4.1° (c = 1.28, MeOH); FAB-MS [M+H]+ 484.1 (calc. 484).

tert-Butyloxy carbonyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalanine tert-butyl ester (11)

tert-Butyloxy carbonyl-L-4-aminophenylalanyl-L-phenylalanine tert-butyl ester 10 (0.48 g, 1.0 mmol) was dissolved in dioxane (7 mL). 9-Fluorenylmethyl chloroformate (0.25 g, 1.0 mmol) was then added as a solution in dioxane (1 mL) and 10% Na2CO3 was added dropwise in order to keep the pH at 8. A white precipitate formed upon the addition of the Na2CO3 solution. Once the pH of the suspension stabilized, the reaction mixture was allowed to proceed overnight. The white precipitate was then filtered, washed with cold 10% citric acid, and dried. The resulting white solid weighed 0.56 g (89%): Rf (EtOAc) = 0.70; (EtOAc/hexane, 2:3) = 0.29; mp 173-174 °C; [α]D = 9.9° (c = 0.45, DMSO); 1H NMR (CDCl3) δ = 1.33 (s, 9H), 1.39 (s, 9H), 3.00 (m, 4H), 4.25 (t, 1H, J = 6.6 Hz), 4.29 (overlapping signal, 1H), 4.51 (d, 2H, J = 6.7 Hz), 4.62 (q, 1H), 5.11 (broad s, 1H), 6.32 (broad d, 1H), 6.67 (s, 1H), 7.04 (m, 4H), 7.21 (m, 2H), 7.29 (t, 4H, J = 8.0 Hz), 7.39 (t, 2H, J = 7.3 Hz), 7.59 (d, 2H, J = 7.4 Hz), 7.74 (d, 1H, J = 8.1 Hz), 7.75 (d, 2H, J = 7.4 Hz); FAB-MS [M+H]+ = 706 (calc. 706).
**tert-Butyl L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalanate (12)**

**tert-Butyloxycarbonyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalanine tert-butyl ester** 11 (66 mg, 0.09 mmol) was dissolved in DCM (0.5 mL). To this solution, TMS-OTf (0.967 M solution in toluene, 100 µL, 0.09 mmol) was added dropwise via syringe. Unlike previous deprotections with this reagent, the product did not precipitate out of solution as the triflate salt, and so the workup was modified as follows: after 3 h, the reaction mixture was diluted with EtOAc (10 mL) and the organic layer was washed with 5% NaHCO₃, H₂O, and saturated NaCl. The organic layer was then dried over MgSO₄ and concentrated in vacuo. The residue was then applied to silica gel flash chromatography with EtOAc as the eluent to give 45 mg (75%) of 12 as a whitish foam:

1H NMR (CDCl₃) δ = 1.38 (s, 9H), 2.54 (d of d, 1H, J = 13.8 Hz, J = 4.5 Hz), 3.04 (d, 2H, J = 6.3 Hz), 3.09 (partially obscured d of d, 1H, J = 3.7 Hz), 3.53 (d of d, 1H, J = 9.3 Hz, J = 3.9 Hz), 4.25 (t, 1H, J = 6.7 Hz), 4.51 (d, 2H, J = 6.6 Hz), 4.74 (q, 1H, J = 6.3 Hz), 6.84 (broad s, 1H), 7.08 (m, 4H), 7.21 (m, 2H), 7.29 (t, 4H, J = 8.0 Hz), 7.39 (t, 2H, J = 7.3 Hz), 7.59 (d, 2H, J = 7.4 Hz), 7.74 (d, 1H, J = 8.1 Hz), 7.75 (d, 2H, J = 7.4 Hz); FAB-MS [M+H]+ (-tBu) = 550.6 (calc. 550).

**tert-Butyloxycarbonyl-L-phenylalanyl-L-4-nitrophenylalaninetert-butyl ester** (14)

**tert-Butyloxycarbonyl-L-phenylalanine** (1.19 g, 4.50 mmol) was coupled to L-4-nitrophenylalanine tert-butyl ester (1.16 g, 4.50 mmol) as described for Method I to yield 2.15 g (93%) as a white solid. The crude product was then recrystallized from hot MeOH to give 1.69 (73%) of 14 as a white solid: Rf (EtOAc) = 0.64; HPLC: tR = 25.7 min; mp 147-149 °C; [α]D = -15.3° (c = 1.16, MeOH); 1H NMR (CDCl₃) δ = 1.34 (s, 9H), 1.39 (s, 9H), 3.01 (d of d, 2H, J = 3.3 Hz, J = 6.8 Hz), 3.12 (t, 2H, J = 6.2 Hz), 4.29 (q, 1H, J = 6.3 Hz), 4.62 (q, 1H, J = 6.5 Hz), 4.88 (broad s, 1H), 6.37 (broad d, 1H) 7.23 (m, 7H), 8.07 (d, 2H, J = 8.7 Hz); FAB-MS [M+H]+ = 514.3 (calc. 514).
**tert-Butyloxycarbonyl-L-phenylalanyl-L-4-aminophenylalanine tert-butyl ester** (15)

*tert*-Butyloxycarbonyl-L-phenylalanyl-L-4-nitrophenylalanine tert-butyl ester 14 (1.02 g, 2.00 mmol) was reduced through catalytic transfer hydrogenation as described for the preparation of 10 to yield 0.94 g (97%) of 15 as a white solid: $R_f$ (EtOAc) = 0.48; HPLC $t_R = 13.5$ min; mp 166-167 °C; $[\alpha]_D = -4.8^\circ$ (c = 1.31, MeOH); FAB-MS [M+H]$^+$ = 484.1 (calc. 484).

**tert-Butyloxycarbonyl-L-phenylalanyl-L-4-amino(9-fluorenylmethoxy carbonyl) phenylalanine tert-butyl ester** (16)

*tert*-Butyloxycarbonyl-L-phenylalanyl-L-4-aminophenylalanine tert-butyl ester 15 (0.48 g, 1.0 mmol) was protected as the Fmoc carbamate as described for the preparation of 11 to yield 0.74 g (95%) of 16 as a white solid: $R_f$ (EtOAc) = 0.28; HPLC $t_R = 26.2$ min; mp 113-115 °C; $[\alpha]_D = -5.3^\circ$ (c = 1.19, MeOH); FAB-MS [M+H]$^+$ = 706.3 (calc. 706).

**tert-Butyl L-phenylalanyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanate** (17)

*tert*-Butyloxycarbonyl-L-phenylalanyl-L-4-aminophenylalanine tert-butyl ester 15 (96 mg, 0.14 mmol) was selectively deprotected using TMS-OTf as described for the preparation of 12 to yield 70 mg (80%) of 17 as a foam: $R_f$ (EtOAc/hexane, 4:1) = 0.10 - 0.21; FAB-MS [M+H]$^+$ = 606 (calc. 606).

**tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydro isoquinoline-3-carboxyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalanine tert-butyl ester** (18)

*tert*-Butyloxycarbonyl-L-0-tert-butyl-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalanine tert-butyl ester 16 (61 mg, 0.12 mmol), tert-butyl L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalanate 12 (93 mg, 0.12 mmol), and PyBOP (64 mg, 0.12 mmol) was dissolved in DMF (1.0 mL) at room temperature. After a few minutes, DIPEA (64 µl, 0.36 mmol) was added dropwise to the reaction mixture. Once the addition was complete,
the reaction was allowed to proceed overnight. The DMF was then removed in vacuo, and
the residue redissolved in EtOAc (10 mL). The organic layer was then washed with 5%
KH2SO4, H2O, 5% NaHCO3, H2O, and saturated NaCl, and dried over MgSO4. The
organic layer was concentrated, and the resulting oil was applied to a silica gel flash column
with DCM/hexane (4:1) as the eluent to give 117 mg (88%) of 18 as a whitish foam: Rf
(EtOAc) = 0.67; HPLC tR = 39.7 min; FAB-MS [M+Na]+ = 1106 (calc. 1106). Anal.: calcd. for C65H73N5O10.1/2 H2O: C, 71.44%; H, 6.79%, N, 6.46%. found: C, 71.44; H,
6.85; N, 6.38.

**tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-4-aminophenylalanyl-L-phenylalanine tert-butyl ester (19)**

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-
L-4-amino(9-fluorenylmethoxycarbonyl)-phenylalanyl-L-phenylalanine tert -butyl ester 18
(70 mg, 0.063 mmol) was dissolved in DMF (1.0 mL) at room temperature. To the
solution, piperidine (6.0 µL) was then added. The reaction was allowed to proceed for 1.5
h, then the reaction mixture was concentrated under vacuo, and the residue applied to silica
gel flash chromatography with EtOAc/hexane (3:1) as the eluent. The amount obtained
from the column was 54 mg (99%) of 19 as a foam: Rf (EtOAc/hexane, 3:1) = 0.47;
HPLC tR = 25.3 min; FAB-MS [M+H]+ 862 (calc. 862).

**tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-4-isothiocyanatophenylalanyl-L-phenylalanine tert -butyl ester (20)**

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-
L-4-aminophenylalanyl-L-phenylalanine tert -butyl ester 19 (14 mg, 0.016 mmol) was
dissolved in DCM (1.0 ml) at room temperature. To the solution, thiophosgene (1.9 µL,
0.024 mmol) was added, followed by slow addition of DIPEA (7.0 mL, 0.04 mmol). The
reaction was allowed to proceed for 1.5 h, then the reaction mixture was concentrated and
applied to silica gel flash chromatography with EtOAc/hexane (4:1) as the eluent. The amount obtained from the column was 13.9 mg (95%) of 20 as a clear glass: HPLC $t_R = 39.0$ min; FAB-MS [M+H]+ 904.2 (calc. 904).

L-Tyrosyl-L-1,2,3,4-tetrahydro-isoquinoline-3-carboxyl-L-4-amino-phenylalanyl-L-phenylalanine (1)

Tert-butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-4-aminophenylalanyl-L-phenylalanine tert-butyl ester 19 (14 mg, 0.016 mmol) was deprotected as described in Method II to yield 11 mg (80%) as a white solid: HPLC (0 to 75% B over 50 min) $t_R = 25.5$ min (96% purity); FAB-MS [M+H]+ 650 (calc. 650).

L-Tyrosyl-L-1,2,3,4-tetrahydro-isoquinoline-3-carboxyl-L-4-isothiocyanatophenylalanyl-L-phenylalanine (2)

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydro-isoquinoline-3-carboxyl-L-4-isothiocyanatophenylalanyl-L-phenylalanine tert-butyl ester 20 (11 mg, 0.010 mmol) was deprotected as described by Method II to yield 7 mg (65%) of peptide 2 as a white solid: HPLC (0 to 75% B over 50 min) $t_R = 32.5$ min (96% purity); FAB-MS [M+H]+ 692 (calc. 692).

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydro-isoquinoline-3-carboxyl-L-phenylalanyl-L-4-amino(9-fluorenylmethoxy-carbonyl)-phenylalanine tert-butyl ester (21)

tert-Butyloxycarbonyl-L-O-tert-butyl-tyrosyl-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-amino(9-fluorenylmethoxycarbonyl)-phenylalanate 17 (140 mg, 0.19 mmol) using PyBOP (136 mg, 0.26 mmol) as described by the preparation of 18 to give 174 mg (87 %) of 21 as a whitish foam: HPLC $t_R = 39.9$ min; FAB-MS [M+H]+ 1085 (calc. 1084). Anal.: calcd. for C$_{65}$H$_{73}$N$_5$O$_{10}$H$_2$O: C, 70.82%; H, 6.86%; N, 6.35%. found: C, 70.86; H, 6.76; N, 6.39.
tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-aminophenylalanine tert-butyl ester (22)

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-amino(9-fluorenylmethoxycarbonyl)-phenylalanine tert -butyl ester (21) (100 mg, 0.09 mmol) was treated with piperidine (200 µl) as described for the preparation of 19 to yield 67 mg (84%) of 22 as a foam: HPLC $t_R = 24.3$ min; FAB-MS [M+H]$^+$ 862 (calc. 862).

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-isothiocyanato-phenylalanine tert -butyl ester (23)

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-aminophenylalanine tert -butyl ester 22 (14 mg, 0.016 mmol) was treated with thiophosgene (1.9 µl, 0.024 mmol) as described in the preparation of 20 to yield 13.9 mg (95%) of 23 as a clear glass: HPLC $t_R = 38.8$ min; FAB-MS [M+H]$^+$ 905 (calc. 905).

L-Tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-aminophenylalanine (3)

Tert-butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-aminophenylalanine tert butyl ester 22 (17 mg, 0.02 mmol) was deprotected as described in Method II to give 13 mg (78%) of 3 as a white solid: HPLC (0 to 75% B over 50 min.) $t_R = 29.3$ min (94% purity); FAB-MS [M+H]$^+$ 650 (calc. 650).

L-Tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-isothiocyanatophenylalanine (4)

tert-Butyloxycarbonyl-O-tert-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl-L-phenylalanyl-L-4-isothiocyanatophenylalanine tert -butyl ester 23 (10 mg, 0.011 mmol) was deprotected as described by Method II to yield 6 mg of 4 (70%) as a white solid:
HPLC (0 to 75% B over 50 min.) \( t_R = 32.3 \text{ min} \) (97% purity); FAB-MS \([M+H]^+\) 692 (calc. 692).

5.7.2.2 Standard binding assays

Radioligands binding assays of the potential affinity label derivatives were performed with Chinese hamster ovary (CHO) cells stably transfected with either mouse \( \delta \) or rat \( \mu \) opioid receptors. Cells were harvested 72 h following transfection in 50 mM Tris buffer, pH 7.4, at 4 \( ^\circ C \) and homogenized using a Dounce homogenizer. The homogenate was then centrifuged at 45,000 \( \times g \) for 10 min at 4 \( ^\circ C \). The pellet was washed twice by resuspension and recentrifugation as in the previous step. The pellet was resuspended in 50 mM Tris buffer, pH 7.4, at 4 \( ^\circ C \) to yield a protein concentration of 30-60 \( \mu g/mL \).

Incubations were performed for 90 min at 22 \( ^\circ C \) with \([^3H]DPDPE\) and \([^3H]DAMGO\) for \( \delta \) and \( \mu \) receptors, respectively. Binding assays were carried out in mixtures containing 100 \( \mu g \) membrane protein, 3 mM Mg\( ^{2+} \), and peptidase inhibitors (10 \( \mu M \) bestatin, 30 \( \mu M \) captopril, and 50 \( \mu M \) L-leucyl-L-leucine) in a final volume of 2 mL 50 mM Tris buffer, pH 7.4, at 22 \( ^\circ C \). Nonspecific binding was determined in the presence of 10 \( \mu M \) unlabeled DPDPE and DAMGO for the \( \delta \) and \( \mu \) binding assays, respectively. The reactions were terminated by rapid filtration over Whatman GF/B glass fiber filters using a Brandel M24-R cell harvester. The filters were presoaked for at least 2 h in 0.5% polyethyleneimine to decrease nonspecific binding. The filter disks were then placed in minivials with 4 mL Cytoscint (ICN radiochemicals) and allowed to elute for at least 6 h before counting in a Beckman LS 6800 scintillation counter. IC\( _{50} \) values were then derived from nonlinear regression analysis of competition curves.

5.7.2.3 Wash-resistant binding assays

Potential affinity label derivatives for the \( \delta \) opioid receptor were examined for wash-resistant binding inhibition of opioid receptors. CHO cell membranes expressing \( \delta \)
receptors were incubated in the absence or presence of the TIPP derivatives for 90 min at room temperature. The homogenates were then centrifuged at 40,000 x g for 15 min at 4 °C and the pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C. The centrifugation and resuspension steps were repeated four times as the washing protocol. After the fifth resuspension, the homogenate is recentrifuged and the final pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C. These final CHO cell membrane homogenates were then subjected to radioligand binding assays as described above. The radioligand binding to membranes treated with the TIPP analogues were then compared to binding to untreated control membranes.
Chapter 6

Towards the Synthesis of Affinity Labels Based on 2-Substituted Derivatives of the Delta Opioid Antagonist, TIPP: Nitration of L-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic Acid

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6.1 Introduction

Affinity labels (irreversible ligands) are important pharmacological tools used in the study of receptors. In the area of opioid pharmacology, affinity labels have been used in the isolation and mapping of the various opioid receptors (Takemori and Portoghese, 1985). In addition, affinity labels based on antagonists can also be used in determining the physiological effects of opioid receptor activation, which is an area of research still under active investigation. The potential irreversible ligand should ideally exhibit both high affinity and selectivity for the receptor to be investigated. In order to develop affinity labels for the delta (δ) opioid receptor, the tetrapeptide Tyr-Tic-Phe-Phe (TIPP) (Schiller et al., 1992) was an ideal candidate for affinity label derivatization, due to its high selectivity and affinity for the δ receptor, as well as its lack of intrinsic activity. In our previous efforts to synthesize affinity labels based on TIPP (chapter 5), we had found that introduction of an isothiocyanate group at the para position of either Phe3 or Phe4 was well tolerated. Both derivatives exhibited wash-resistant binding inhibition of [3H]DPDPE binding to cloned δ receptors at low nanomolar concentrations. In an effort to further explore affinity labels based on TIPP, we investigated the possibility of introducing an isothiocyanate group onto the aromatic ring of the constrained Phe analog Tic at position 2 of the peptide.

It is generally believed that the prerequisites for δ opioid receptor binding are a protonated amine, a phenolic moiety, and a second aromatic moiety. Due to its fully aromatic character, there has been much speculation as to which aromatic amino acid of TIPP fulfills the requirement of the second aromatic ring (see Chapter 3). Antagonist activity at the δ-receptor has been reported for the Tyr-Tic-Ala tripeptide and the Tyr-Tic dipeptide fragments of TIPP (Temussi et al., 1994), which illustrates the importance of the Tic2 residue over Phe3. Furthermore, substitution of pipecolic acid for Tic at the 2-position resulted in a complete loss of δ-selective antagonism. These experiments strongly suggest that the Tic residue at position 2 is responsible for the antagonist activity of TIPP.
The significance of the Tic\textsuperscript{2} residue has also been emphasized by molecular modeling studies comparing TIPP to the rigid, non-peptide δ-selective antagonist naltrindole (NTI). This antagonist was developed by Portoghese and co-workers using the "message-address" concept of ligand design (Portoghese et al., 1988). It is postulated that the addition of an indole moiety to the naltrexone skeleton provided the second aromatic group needed to complete the pharmacophore for δ-selective binding. NTI is a potent and selective antagonist for the δ opioid receptor, and various computational studies have shown that the Tic\textsuperscript{2} residue of TIPP corresponds to the indole aromatic region of NTI (Amodeo et al., 1996, Chew et al., 1993, Wilkes and Schiller, 1994, Wilkes and Schiller, 1995).

In a subsequent experiment done by Portoghese and co-workers (Portoghese et al., 1990), NTI has been developed into a very potent affinity label for the δ-opioid receptor by incorporation of an isothiocyanate group at the 5' position of the indole aromatic ring. If the Tic residue mimics the indole aromatic moiety of NTI, then it is possible that introduction of an isothiocyanate group onto the Tic residue of TIPP may also result in an effective affinity label like naltrindole 5'-isothiocyanate (NTII).

In the earlier syntheses, the isothiocyanate was introduced via a synthetic scheme developed by our laboratory utilizing Phe(p-NO\textsubscript{2}). The nitro group was reduced to an amine and protected as the Fmoc carbamate, which was deprotected and derivatized to the isothiocyanate at a later step in the synthesis. In this case, because the isothiocyanate is to be on the Tic residue, a Tic(NO\textsubscript{2}) derivative was needed.

6.2 Chemistry

The Tic(7-NO\textsubscript{2}) derivative was reported to have been prepared via Pictet-Spengler cyclization of Phe(p-NO\textsubscript{2}) (Miyake et al., 1984). The Pictet-Spengler reaction (Figure 6.1) has proven to be a very effective method in preparing tetrahydroisoquinolines from Phe (Hayashi et al., 1983). It involves condensation of formaldehyde and the α-amine to
Fig. 6.1 Pictet-Spengler cyclization of Phe to Tic
the Schiff base, followed by acid catalyzed electrophilic attack of the aromatic ring by the imine. In our attempts, standard Pictet-Spengler reaction conditions were insufficient to promote cyclization of Phe(p-NO₂) to Tic(7-NO₂). The strong electron withdrawing effect of the nitro group was enough to preclude electrophilic attack of the aromatic ring by the imine.

As an alternative route to Tic(7-NO₂), direct nitration of optically active Tic was then considered. Although generation of mixed nitration products was a possibility, we reasoned that the desired regioisomer could be obtained as the major product through examination of the substitution pattern of Tic (Fig. 6.2). Alkyl groups are ortho/para directing groups in electrophilic substitution of aromatic rings. The 5- and 8-positions directly adjacent to the methylenes of Tic were not expected to undergo extensive nitration due to steric hindrance. Of the two positions left for nitration, the electronegativity of the methyl amine substituent should disfavor carbocation formation between positions 1 and 8, therefore nitration would be directed towards the 7-position of the aromatic ring.

The reaction of Tic with HNO₃/H₂SO₄ resulted in one major product evident by HPLC. Mass spectroscopy identified the molecular ion of the Tic(NO₂) derivative, and the molecular formula was confirmed through elemental analysis. ¹H NMR indicated that nitration occurred at either positions 6 or 7, as evidenced by the apparent ABX coupling pattern in the aromatic region. But the specific regiochemistry of the nitration reaction was still undetermined. Differential nOe NMR spectroscopy was used to selectively irradiate the aromatic proton singlet (Figure 6.3) at δ = 4.81. When the singlet was irradiated, the benzylic protons at C-1 (δ = 2.10, q, J = 16 Hz) were perturbed. This suggested that these protons are near to the isolated aromatic proton, thereby implicating nitration at the 7-position.

It was then decided that the peptide would be constructed in a linear fashion, for the sake of synthetic simplicity (Fig. 6.4). The Tic derivative was protected as the Fmoc carbamate, then coupled to Phe-Phe-OtBu dipeptide via PyBOP (Coste et al., 1990).
Fig. 6.2 Expected regiochemistry of nitration onto Tic.
Fig. 6.3 Differential nOe spectroscopy of Tic(NO₂). The $^1$H NMR spectra of Tic(NO₂) in 37% DCl in D₂O is shown below, the effects of irradiation at the aromatic singlet proton are shown in the above spectra. The benzylic protons ($\delta \approx 3.5$) are perturbed, indicating close proximity to the proton being irradiated.
Fig. 6.4 Synthetic scheme towards 2-substituted affinity labels based on TIPP.

i) HNO₃/H₂SO₄, 0 °C, (53%); ii) Fmoc-Cl, 10% Na₂CO₃, (53%); iii) PyBOP, DIPEA (82% total mass).
condensation. Upon the work up of the reaction, it was found that there were two major products as evidenced by TLC. The two major products were separated by silica gel chromatography. The earlier eluting product (R_f = 0.68) represented 31% of the expected yield, while the slower eluting product (R_f = 0.50) was 51%. Mass spectral analyses showed that both of the compounds exhibited the expected molecular ion peak and very similar fragmentation patterns as would be expected for Fmoc-Tic(7-NO2)-Phe-Phe-OtBu (Fig. 6.5). NMR analyses of the major components proved to be difficult due to extensive line broadening and overlap, but both spectra were very similar.

6.3 Discussion and future studies

In order to introduce reactive functionalities onto the Tic^2 residue of the δ-selective tetrapeptide antagonist TIPP, a nitrated Tic derivative was prepared. The nitration was accomplished through treatment of Tic with HNO_3 and H_2SO_4. The homogeneity of the reaction was confirmed by HPLC, the molecular formula of the nitrated Tic derivative was confirmed through mass spectral and elemental analyses, and the regiochemistry of the reaction was examined through ^1H NMR.

However, the coupling of Fmoc-Tic(7-NO2)-OH to H-Phe-Phe-OtBu resulted in two major products as evidenced by TLC. Upon mass spectral analyses, both products exhibited the expected molecular peak, and displayed identical fragmentation patterns.

The most probable explanation for the two major products at the tripeptide stage is due to racemization of Tic(7-NO2), resulting in diastereomer formation. In order to check the chiral integrity of Tic(7-NO2), the amino acid was examined through derivatization with Marfey's reagent (Marfey, 1984). HPLC analysis of the derivatives indicated that the product of the nitration reaction was predominantly comprised of one isomer of Tic(7-NO2) (Fig. 6.6).
Fig. 6.5 Mass spectral data for products resulting from the Fmoc-Tic(7-NO₂) to Phe-Phe-OtBu coupling step. Above spectrum (A) is of the earlier eluting product, $R_f$ (EtOAc) = 0.68; lower spectrum (B) is of the later eluting product, $R_f$ (EtOAc) = 0.50.
Fig. 6.6 Marfey derivatization and HPLC analysis of I) L-Tic(NO₂); and II) blank Marfey's reagent.
Future work will involve further analysis of the problems in the syntheses. If racemization is occurring, it could be happening at either of two steps: the Fmoc protection of Tic(7-NO₂), or the coupling of Fmoc-Tic(7-NO₂) to Phe-Phe-OtBu. Deprotection of Fmoc-Tic(7-NO₂) followed by Marfey derivatization would indicate if extensive racemization is occurring during amine protection. Problems during Fmoc protection could then perhaps be circumvented by using a milder base for neutralization, or through the use of a different protection strategy. If the coupling reaction proves to be the problem step, then use of a different coupling reagent may help to alleviate the problem.

Once the questions surrounding this synthesis are answered, then the target isothiocyanate containing TIPP analog can be prepared. Evaluation of this peptide may help in identifying the role of the Tic aromatic ring in δ selective binding, and may provide another affinity label for the study of δ opioid receptors.

6.4 Experimental section

6.4.1 Materials

Synthetic reagents and amino acids were purchased from Aldrich Chemical Co (Milwaukee, WI). General laboratory solvents were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ), and HPLC-grade solvents were obtained from Burdick and Jackson (Muskegon, MI).

Syntheses were monitored using thin-layer chromatography using Kieselgel 60 F254 plates of 0.20 mm thickness purchased from EM Separations (Gibbstown, NJ). Intermediates were purified by flash chromatography using 230-400 mesh, 60Å silica gel also purchased from E. Merck.

NMR analyses were done on an Bruker AC300 instrument at the Department of Chemistry, Oregon State University, Corvallis, OR. The molecular weights of the amino acid derivatives and peptides were determined by fast atom bombardment (FAB) mass
spectrometry in the postitive mode on a Kratos MS50RFTC in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR.

The purity of intermediates and the derivatization of Tic(7-NO₂) by Marfey's reagent were determined by analysis on a Beckman System Gold high performance liquid chromatography (HPLC) system consisting of a model 126 solvent module, model 168 diode array detector, and model 507 autosampler. The HPLC column used was a Vydac analytical column (C₁₈, 300 Å, 5 μ, 4.6 x 250 mm) equipped with a guard cartridge. The compounds were eluted using a linear gradient of 0 to 75% B over 50 min at a flow rate of 1.5 mL/min and detected at 214 nm; solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA.

6.4.2 Synthetic methods

L-1,2,3,4-Tetrahydroisoquinoline-7-nitro-3-carboxylic acid
L-Tic (0.45 g, 2.5 mmol) was dissolved in concentrated H₂SO₄ (2 mL) and cooled in an ice/H₂O bath. 10 N HNO₃ (0.43 mL, 4.35 mmol) was then added dropwise, and the solution was then allowed to warm to room temperature and stirred for 24 h. The solution was then warmed to 50 °C for 2 h, then cooled in an ice/H₂O bath and neutralized with NaOH. The white precipitate was then collected and recrystallized from H₂O to give 0.29 g (53%) as a white amorphous solid: HPLC tR = ; [α]D²⁵ (c = 1.0, 3N HCl) = -126.7°; ¹H NMR (37% DCl/D₂O) δ = 0.94 (dd, 1H, J = 11 Hz, 17 Hz), 1.13 (dd, 1H, J = 5 Hz, 17 Hz), 2.07 (m, 1H), 2.10 (q, 2H, J = 16), 4.81 (s, 1H), 5.08 (d, 2H, J = 8.3 Hz), 5.63 (d, 2H, J = 8.3); FAB-MS [M+H]+ 223 (calc. 223).

Derivatization of 1,2,3,4-tetrahydroisoquinoline-7-nitro-3-carboxylic acid with Marfey's reagent
1,2,3,4-Tetrahydroisoquinoline-7-nitro-3-carboxylic acid (1.1 mg, 5 μmoles) and Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (1.9 mg, 7 μmoles) were dissolved in acetone
(200 µL) and 1.0 M NaHCO₃ (40 µL). The solution was then warmed to 40 °C for 1 h, then neutralized with 2 M HCl (20 µL). The resulting solution was then subjected to HPLC analysis and monitored at 340 nm.

**N-9-Fluorenylmethoxycarbonyl-1,2,3,4-tetrahydroisoquinoline-7-nitro-3-carboxylic acid**

L-1,2,3,4-Tetrahydroisoquinoline-7-nitro-3-carboxylic acid (0.20 g, 0.90 mmol) was suspended in 10% Na₂CO₃ (2.4 mL) and dioxane (1.4 mL). The suspension was warmed to 50 °C to dissolve the starting material. The resulting solution was cooled in an ice/H₂O bath, and Fmoc-Cl (0.23 g, 0.90 mmol) was then added in small portions over 15 min, then left overnight. The reaction mixture was then acidified to Congo using 4N HCl, and the white precipitate was collected, washed with H₂O, and dried to yield 0.21 g (53%) as a white solid: Rₓ (BAW) = 0.84; HPLC tᵣ = 21.7 min; [α]D⁰ = +63.2 ° (c = 1.2, CHCl₃); FAB-MS [M+H]+ 445 (calc. 445).

**N-9-Fluorenylmethoxycarbonyl-L-1,2,3,4-tetrahydroisoquinoline-7-nitro-3-carboxyl-L-phenylalanine tert-butyl ester**

N-9-Fluorenylmethoxycarbonyl-L-1,2,3,4-tetrahydroisoquinoline-7-nitro-3-carboxylic acid (0.10 g, 0.23 mmol) and L-phenylalanine tert-butyl ester (0.82 g, 0.23 mmol) was dissolved in DCM (1 mL). PyBOP (0.12 g, 0.23 mmol) was then added to the solution, followed by the dropwise addition of DIPEA (78 µL, 0.46 mmol). The reaction was then allowed to proceed overnight. The reaction mixture was applied directly to flash chromatography with EtOAc/hexane (1:1) as the eluent. The column yielded 55 mg (31%) of an early eluting product (A) as a glass; and later fractions yielded 94 mg (51%) of a later eluting product (B), which also appeared as a glass: Rₓ (EtOAc/hexane 1:1) compound A = 0.68; compound B = 0.50; FAB-MS [M+H]+ compounds A and B: 795 (calc. 795).
Potential affinity label derivatives of $\delta$ selective opioid peptide antagonists were prepared for future use as pharmacological probes for $\delta$ opioid receptors. These peptide analogs were assembled using an orthogonal tert-butyloxy carbonyl (Boc)/9-fluorenylmethoxycarbonyl (Fmoc) protection strategy, a versatile method which should prove useful for the preparation of a wide variety of peptide-based affinity labels. The key step in this synthetic scheme was the selective removal of the Boc group in the presence of a tert-butyl ester. This selective deprotection was accomplished through the use of one equivalent of trimethylsilyl trifluoromethanesulfonate (TMS-OTf) in toluene. The orthogonal nature of the synthesis allows for the selective removal of the Fmoc group, freeing an anilide amine for conversion to the reactive isothiocyanate or bromoacetamide groups.

In our first study, the peptides chosen for derivatization were the $\delta$ peptide antagonists $N,N$-dibenzyl leucine enkephalin (Lovett and Portoghese, 1987) and $N,N$-diallyl[Aib$^2$,Aib$^3$]leucine enkephalin (ICI-174,864) (Cotton et al., 1984). Instead of glycines at positions 2 and 3, ICI-174,864 has the highly hindered 2-aminoisobutyric acid (Aib) at these positions. A [3+2] convergent synthesis was used, in which the two N-terminal tripeptides were coupled to a common C-terminal dipeptide fragment. Isothiocyanate and bromoacetamide groups were incorporated into these peptides, but it was found that extended reaction times using bromoacetyl chloride for acylation gave rise to a chloroacetamide due to halide exchange. Also synthesized were the amine-containing compounds as reversible controls for use in the wash-resistant binding assays. These pentapeptides were then tested for inhibition of radioligand binding using Chinese hamster ovary (CHO) cells stably transfected with mouse $\delta$ opioid receptors.
From the binding assays done under standard conditions, these enkephalin derivatives all exhibited nanomolar to micromolar affinity for δ opioid receptors, and in general, substitution at the para position of Phe⁴ led to decreased affinity but increased selectivity for δ receptors. Surprisingly, the parent peptides exhibited a switch in rank-order potency when compared to previous studies done on the mouse vas deferens (MVD) smooth muscle assay [Lovett, 1987 #10]. This discrepancy points to possible differences in δ receptors in the MVD and CHO cells, which may indicate subtype differences.

Of the five potential affinity labels tested, only N,N-dibenzyl [Phe(p-NCS)⁴]leucine enkephalin (JVA-602) exhibited wash-resistant inhibition of radioligand binding in the CHO cell assays. The corresponding N,N-diallyl [Aib²,Aib³,Phe(p-NCS)⁴]leucine enkephalin analog (JVA-606) did not exhibit wash-resistant inhibition, despite its apparent similarity to JVA-602. After pretreatment and washing, JVA-602 still exhibited only 60% of control radioligand binding at a test concentration of 0.1 μM, and this inhibition increased to 81% when a test concentration of 1 μM was used.

In order to develop other potential affinity labels for δ opioid receptors, and test the versatility of our synthetic scheme, the tetrapeptide antagonist Tyr-Tic-Phe-Phe (TIPP) was selected for derivatization. At its 2-position, TIPP has the constrained phenylalanine analog Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), and it is more potent and selective for δ opioid receptors than the parent enkephalin derivatives. It was anticipated that these attributes would result in a more effective affinity label after functionalization.

The two phenylalanines of TIPP allowed introduction of an isothiocyanate group at the para positions of either Phe³ or Phe⁴, and so a [2+2] convergent synthesis was planned in which Tyr-Tic was coupled to either Phe(p-NHFmoc)-Phe-OrBu or Phe-Phe(p-NHFmoc)-OrBu. The selective deprotection using TMS-OTf was once again used, which allowed the assembly of the isothiocyanate- and amine-containing TIPP derivatives.

The CHO cell binding assays indicated that the amine- and isothiocyanate-containing derivatives of TIPP still retained low nanomolar affinity and good selectivity for δ opioid
receptors. Due to their highly lipophilic nature, the TIPP analogs were tested at a very low concentrations in the wash-resistant binding assays, but both isothiocyanate-containing compounds were still able to inhibit radioligand binding after the wash procedures. The peptide [Phe(p-NCS)\(^3\)]TIPP (JVA-611) was tested at a concentration of only 10 nM, and it was able to inhibit binding to 45% of control, and [Phe(p-NCS)\(^4\)]TIPP (JVA-613) inhibited binding to only 30% of control at a test concentration of only 9 nM.

In an effort to further explore synthesizing affinity labels based on TIPP, we investigated the possibility of introducing an isothiocyanate group onto the aromatic ring of the constrained Phe analog Tic at position 2 of the peptide. An isothiocyanate group at the 2-position of TIPP may help to explain the significance of the Tic aromatic residue in \(\delta\)-selective binding. To introduce an isothiocyanate at this position, a nitrated Tic derivative was needed, which was prepared via direct nitration of Tic using HNO\(_3\)/H\(_2\)SO\(_4\). However, upon coupling of this Tic derivative onto Phe-Phe-OtBu, two major products were visible by thin layer chromatography. Both products exhibited the expected molecular ion peak and fragmentation pattern consistent with the desired tripeptide. The reason for two products is uncertain at this time; their appearance may be due to loss of chiral integrity of the Tic(7-NO\(_2\)) derivative, resulting in diastereomer formation. Once the reason for the second product is identified and corrected, the synthesis of the potential affinity label [Tic(7-NCS)\(^2\)]TIPP can resume, and evaluation of this peptide might be used in the further characterization of \(\delta\) opioid receptors.

From this study, three potential affinity labels have been identified. They are peptides containing a Phe(p-NCS) derivative, which are able to still inhibit radioligand binding to CHO cells even after extensive washing. These peptides (JVA-602, 611, 613) will be subjected to further pharmacological tests to validate whether or not their interactions with \(\delta\) opioid receptors involve covalent binding. Our laboratory is also investigating means to synthesize these peptides in radiolabeled form, in order to make them more useful in \(\delta\) receptor isolation and domain-mapping experiments.
To our knowledge, JVA-602, 611, and 613 represent the only examples of affinity labels which use based on δ-selective peptide antagonists. These peptides, used in conjunction with other agonist- and non-peptide-based affinity labels, may help to further understand the binding requirements and physiological actions of δ opioid receptors.
Bibliography


Jiang, Q.; Takemori, A. E.; Sultana, M.; Portoghese, P. S.; Bowen, W. D. Differential Antagonism of Opioid δ Antinociception by [D-Ala²-Leu⁵,Cys⁶]enkephalin and 5'-


Kieffer, B. L. Recent Advances in Molecular Recognition and Signal Transduction of Active Peptides: Receptors for Opioid Peptides. *Cellular and Molecular Neurobiology* 1995, 15, 615-635.


Mattia, A.; Vanderah, T.; Mosberg, H. I.; Porreca, F. Lack of Antinociceptive Cross-
tolerance Between [D-Pen\textsuperscript{2},D-Pen\textsuperscript{5}]enkephalin and [D-Ala\textsuperscript{2}]deltorphin II in Mice: Evidence for Delta Receptor Sub-types. *J. Pharmacol. Exp. Ther.* 1991, 258, 583-587.


