

Design, Synthesis and Evaluation of Peptide-Based Affinity

Labels for Mu Opioid Receptors

By

C2009

Bhaswati Sinha

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Dissertation Committee:

Chairperson: Dr. Jane V. Aldrich

Dr. Michael F. Rafferty

Dr. Teruna J. Siahaan

Dr. Emily E. Scott

Dr. David S. Moore

Dissertation Defended _____

The Dissertation Committee for Bhaswati Sinha certifies
that this is the approved version of the following dissertation:

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Chairperson: Dr. Jane V. Aldrich

Dr. Michael F. Rafferty

Dr. Teruna J. Siahaan

Dr. Emily E. Scott

Dr. David S. Moore

Date Approved _____

Dedicated to:

My parents

Kumkum DattaChowdhury

Prithwish Chandra DattaChowdhury

My brother

Atish DattaChowdhury

My husband

Sandipan Sinha

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Abbreviations

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9-37. Amino Acids are in the L-configuration except where indicated otherwise. Additional abbreviations used in this dissertation are as follows:

Aloc: allyloxycarbonyl;

Boc: *tert*-butyloxycarbonyl;

cAMP: cyclic adenosine monophosphate;

cDNA: complementary DNA;

CHO: Chinese hamster ovary;

CNS: central nervous system;

CSU: confocal-scanning unit;

CTOP: D-Phe-*cyclo*[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH₂

Dab: 2,4-diaminobutyric acid;

DADLE: [D-Ala²,D-Leu⁵]enkephalin;

DALCE: [D-Ala²,Leu⁵,Cys⁶]enkephalin chloromethyl ketone;

DALDA: Tyr-D-Arg-Phe-Lys-NH₂

DALECK: [D-Ala²,Leu⁵]enkephalin

DAMGA: [D-Ala²,NMePhe⁴,Gly⁵]enkephalinamide;

DAMGO: [D-Ala²,MePhe⁴,glyol]enkephalin;

DAMK: Tyr-D-Ala-Gly-NMePhe-chloromethyl ketone

Dap: 2,3-diaminopropionic acid;

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene;

DCM: dichloromethane;

DIEA: *N,N*-diisopropylethylamine;

DMF: *N,N*-dimethylformamide;

Dmt: 2,6-dimethyltyrosine

DOR: δ opioid receptor

DPDPE: *cyclo*[D-Pen²,D-Pen⁵]enkephalin;

DSB: d-desthiobiotin

DSLET: [D-Ser²,Leu⁵,Thr⁶]enkephalin

Dyn A: dynorphin A;

EL: extracellular loop;

EMCCD: electron multiplier charge-coupled device;

ESI-MS: electrospray ionization mass spectrometry;

FBS: fetal bovine serum

Fmoc: 9-fluorenylmethoxycarbonyl;

GPCR: G-protein coupled receptor;

GPI: guinea pig ileum;

HOBt: 1-hydroxybenzotriazole;

HPLC: high-performance liquid chromatography;

Hyp: hydroxyproline

i.t.: intrathecal

IL: intracellular loop;

ivDde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl

JOM 6: [Tyr-*cyclo*[D-Cys-Phe-D-Pen]NH₂(Et)]

KOR: κ opioid receptor

MOR: μ opioid receptor;

MTSEA: methanethiosulfonate ethylammonium;

Mtt: 4-methyltrityl;

MVD: mouse vas deferens;

NMR: nuclear magnetic resonance;

Npys : 3-nitro-2-pyridinesulphenyl;

ORL-1: opioid-receptor like-1;

PAL: peptide amide linker;

PBS: phosphate buffered saline;

PEG: poly(ethylene glycol);

Pen: penicillamine

PS: polystyrene;

PyBOP: benzotriazole-1-yloxytripyrrolidinophosphonium

hexafluorophosphate;

RPMI: Roswell Park Memorial Institute;

SAR: structure-activity relationship;

s.c.: subcutaneous

SEM: standard error of mean;

SPPS: solid-phase peptide synthesis;

TAPS: Tyr-D-Arg-Phe-Ser-OH

TFA: trifluoroacetic acid;

Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid;

TIPP: Tyr-Tic-Phe-Phe

TIPS: triisopropylsilane;

TM: transmembrane;

Trt: trityl;

WRIB: wash-resistant inhibition of binding

Abstract

Narcotic analgesics produce pain relief generally through activation of μ opioid receptors (MOR), but the use of these analgesics is limited by their side effects, namely respiratory depression, tolerance, physical dependence and constipation. Understanding receptor-ligand interactions at the molecular level could facilitate the design of novel opioid ligands potentially with less deleterious side effects. This task is challenging since there is no crystal structure available for opioid receptors.

With the aim of understanding MOR-ligand interactions, we designed novel MOR selective peptide ligands containing a reactive affinity label group. Affinity labels that interact with the receptor in a non-equilibrium manner can provide information about specific receptor-ligand interactions. We selected two MOR selective peptides: dermorphin, an endogenous ligand present in South American frog skin, and the synthetic enkephalin analog DAMGO ([D-Ala²,NMePhe⁴,glyol]enkephalin), for developing electrophilic affinity label derivatives. We substituted D-Orn or D-Lys in position 2 (in place of D-Ala) in both dermorphin and DAMGO, and attached a bromoacetamide or an isothiocyanate group as the electrophilic functionality to the side chain amines of the D-amino acids.

For the dermorphin derivatives, we successfully identified several affinity labels with high MOR affinity ($IC_{50} = 0.1-5$ nM) and high selectivity for MOR that exhibit wash-resistant inhibition of binding to these receptors. Among these, [D-

Lys(=C=S)²]dermorphin was further modified to include a purification tag (d-desthiobiotin) and a fluorescent tag (Oregon Green or 5-carboxyrhodamine B). This multifunctional affinity label peptide was synthesized successfully using an Fmoc-solid phase synthetic strategy. Initial fluorescent microscopy studies suggest irreversible labeling of MOR expressed on SH-SY5Y cells by this multifunctional peptide, thus demonstrating the utility of the fluorescent tag.

For the DAMGO series of analogs, the bromoacetamide derivatives exhibited subnanomolar binding affinity ($IC_{50} = 0.45$ nM) to MOR. However, the isothiocyanate derivatives resulted in the formation of an unexpected cyclic O-alkyl thiocarbamate side product. This side reaction was successfully overcome by replacing the glycol in DAMGO by the glycyamide, yielding affinity label derivatives that exhibited subnanomolar affinity ($IC_{50} = 0.3-0.8$ nM) and wash-resistant inhibition of MOR binding.

These high affinity peptide-based affinity labels will be useful pharmacological tools to study MOR.

Chapter 1.
Summary of Thesis Projects

1.1 Background and Significance

Narcotic analgesics such as morphine produce pain relief mainly through activation of μ opioid receptors (MOR) which belong to the family of G-protein coupled receptors (GPCR).¹ However a plethora of side effects associated with the clinically used analgesics acting at MOR, such as respiratory depression, constipation, tolerance and physical dependence limit their therapeutic use.¹⁻³ Therefore, there is an urgent need to develop potent analgesics devoid of these severe side effects. To achieve this goal, it is of utmost importance to study the interactions of MOR selective ligands with their receptor at the molecular level.

Since the cloning of the opioid receptors in the 1990s and subsequent determination of their sequences,⁴⁻⁸ considerable advancements have been made in understanding receptor-ligand interactions at the molecular level. Information obtained from chimeric opioid receptors and receptors containing point mutations has demonstrated the complexities of ligand-receptor interactions, including differences in interactions of the same ligands with different receptors, and of different ligands with the same receptor.⁹ Through such studies, it was also found that the opioid peptides and alkaloids use common sites for binding, but their modes of interaction are different.⁹⁻¹³ Information has also been obtained on the roles of individual residues in opioid receptors from site-directed mutagenesis.⁹ For example, results from the mutation of Asp in transmembrane (TM) 2 suggested that agonists and antagonists may bind differently to this residue.^{14, 15}

In the absence of crystal structures of opioid receptors, computational models of these receptors remain an important tool for understanding structure-function relationships for these receptors.¹⁶ Homology modeling of opioid receptors based on the existing crystal structures of rhodopsin^{17, 18} is the most common approach and several groups have reported computational models of opioid receptors based on homology modeling.¹⁹⁻²³ Several reports have emerged on computational models of nonpeptide ligands binding to their receptors.²⁴⁻³¹ The rigid structures of some of these ligands make the docking studies less complicated. However, the flexible nature of opioid peptides makes the computational modeling of such ligands bound to their receptor quite challenging, and therefore reports for these compounds in the literature have been limited. There is only one example of a MOR selective peptide agonist (the tetrapeptide JOM6)³² whose computational model has been developed using structural constraints.^{33, 34} Comparisons of models of agonist-bound MOR with MOR in an inactive state³³ suggested that rotation of the side chain of Trp293 in TM6 is a major change that takes place upon agonist binding to MOR. However, a serious limitation of such homology modeling is the lack of identity between opioid receptor sequences and rhodopsin (only ~20% identity for all residues and ~29% identity in the TM regions). Therefore, homology modeling of rhodopsin and opioid receptors may generate many errors, mostly from misalignment of sequences.³⁵⁻³⁷

Although information obtained from both molecular biology techniques and computational models has provided tremendous insight into the complexities of opioid receptor-ligand interactions, these techniques suffer from potential drawbacks.

Changes in the primary sequence of receptors by site-directed mutagenesis or in chimeric receptors can affect protein secondary and / or tertiary structure, and in turn affect the interactions and affinities of various ligands for such receptors.⁹ The use of computational models of opioid receptors have inherent drawbacks associated with the low sequence homology between the template and the protein being modeled, and additional receptor specific and ligand specific experimental constraints are needed to improve the accuracy of such models.⁹ From the above discussion it is evident that there is a need to develop more direct methods to identify specific receptor-ligand interactions for opioid receptors.

Affinity labels, which are compounds that interact with their receptors in an irreversible, two-step recognition process,³⁸ can provide direct information on receptor-ligand interactions. The first step is the reversible binding of the affinity label to the receptor, followed by covalent attachment of the affinity label to the receptor, provided that the affinity label has sufficient reactivity and is properly oriented to react with an appropriate functionality on the receptor.³⁸ By identifying the attachment point of an affinity label to its receptor, direct evidence can be obtained on specific receptor-ligand interactions. Such information can then be used as an ‘anchor point’ to assess and improve existing computational models. This concept forms the central hypothesis of this research.

The objective of this research is to develop peptide-based electrophilic affinity labels selective for MOR. Since MOR is the primary opioid receptor targeted to modulate pain, it is of utmost importance to understand the molecular interactions of

MOR-selective ligands at the molecular level. Since irreversible binding of an electrophilic affinity label depends on the reactivity of the label as well as the proximity of a nearby nucleophile in the receptor, an increase in specificity can be achieved by using such labels.³⁸ We chose to design peptide-based affinity labels for two reasons. First, there is considerable evidence in the literature, based on site-directed mutagenesis of opioid receptors, suggesting different modes of binding for peptide vs nonpeptide ligands to opioid receptors.⁹⁻¹³ Since endogenous ligands for opioid receptors are peptides, it is important to explore the interactions of such peptides with their receptors and also understand the differences in their binding to receptors compared to nonpeptide ligands. Examples of peptide ligands with high affinity for MOR are the enkephalins, β -endorphin¹ and the recently identified endomorphins³⁹ the mammalian peptides and dermorphin, the only example of an endogenous MOR selective opioid peptide found in amphibian skin.⁴⁰ Furthermore, complimentary information can be obtained from studying interactions of peptides and nonpeptides with opioid receptors, and this information can be utilized in developing novel drugs targeting opioid receptors. Secondly, peptide-based affinity labels offer unique advantages over nonpeptide ligands. The polymeric nature of peptides permits easy incorporation of additional functionalities (e.g. biotin and / or a fluorescent group) which can aid in receptor isolation and characterization.

There have been very few reports of electrophilic peptide-based affinity labels selective for MOR. The only reported examples of such compounds are DAMK ([D-

Ala²,NMePhe⁴]enkephalin-1-4 chloromethyl ketone)⁴¹ and [D-Ala²,Leu(CH₂S)Npys⁵]-enkephalin (where Npys is 3-nitro-2-pyridinesulphenyl).⁴² Previous attempts in our research group to prepare affinity labels for MOR by incorporating an electrophilic functionality such as a bromoacetamide or an isothiocyanate on the para position of either Phe³ or Phe⁴ of endomorphin-2 (Tyr-Pro-Phe-PheNH₂) were unsuccessful because the modified analogs exhibited large (40- to 80-fold) decreases in MOR binding affinity compared to endomorphin-2.⁴³

The goal of the present research was to design, synthesize and evaluate electrophilic affinity labels selective for MOR. Two MOR selective ligands were chosen for further modification: dermorphin, and DAMGO, a synthetic analog of enkephalin.⁴⁴

1.2 Research Projects

1.2.1 Project 1

Discovery of Dermorphin-Based Affinity Labels with Subnanomolar Affinity for Mu Opioid Receptors (Chapter 3)

The objective of this project was to design, synthesize and evaluate the binding affinity of peptide-based electrophilic affinity labels for MOR based on dermorphin, an endogenous heptapeptide present in South American frog skin⁴⁵ exhibiting exceptionally high affinity (IC₅₀ = 0.72 nM) and selectivity (250-fold) for MOR over DOR.

Previously, the para position of Phe³ or a Phe in position 5 of dermorphin and [Lys⁷]dermorphin, was modified by introducing an electrophilic functionality such as a bromoacetamide or isothiocyanate group.⁴⁶ Modification of the ‘message’ domain (Phe³) resulted in >1000-fold decrease in MOR affinity. Introduction of a Phe residue in position 5 of dermorphin and [Lys⁷]dermorphin was well tolerated and the peptides retained nanomolar affinity for MOR, but the analogs containing the affinity label group did not exhibit wash-resistant inhibition of binding to MOR, as would be expected for an affinity label.⁴⁶

In the present study we chose an alternative location in the ‘message’ sequence, position 2, to incorporate a reactive functionality. Larger D-amino acids are tolerated at this position in peptides by MOR,⁴⁵ suggesting that introduction of an affinity label into the side chain of this residue would not interfere with the binding of these ligands to the receptor. In the present study, D-Ala at position 2 was replaced by D-Orn or D-Lys. The free amine on the side chain of these amino acids was used as a suitable handle to incorporate the electrophilic bromoacetamide or isothiocyanate functionalities (Figure 1.1). This strategy also permitted varying the length of the amino acid side chain to optimize binding of the affinity label to its receptor. For these series of analogs, [D-Orn(COCH₃)²]- and [D-Lys(COCH₃)²]dermorphin served as reversible control peptides for the respective series of compounds in the pharmacological assays.

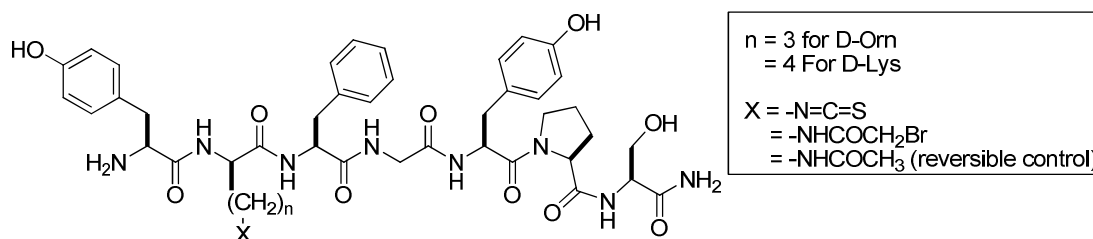


Figure 1.1. Design of potential affinity labels for MOR and the corresponding reversible control peptides based on the parent peptide dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-SerNH₂).

The new series of analogs were successfully synthesized following a solid-phase synthesis procedure. From the pharmacological assays, high affinity ligands were identified that exhibited wash-resistant inhibition of binding to MOR.⁴⁷

1.2.2 Project 2

Synthesis and Evaluation of DAMGO-Based Affinity Labels for MOR and Discovery of an Unexpected Side Reaction (Chapter 4)

Continuing our effort to design new, selective and potent peptide-based affinity labels for MOR, we chose DAMGO, a highly potent and selective agonist for MOR,⁴⁴ as a parent ligand for further modification. Based on the successful design of dermorphin-based affinity label by substituting D-Orn or D-Lys in position 2 and attaching an electrophilic functionality, i.e. an isothiocyanate or a bromoacetamide, we decided to apply the same design to develop DAMGO based-affinity labels (Figure 1.2).

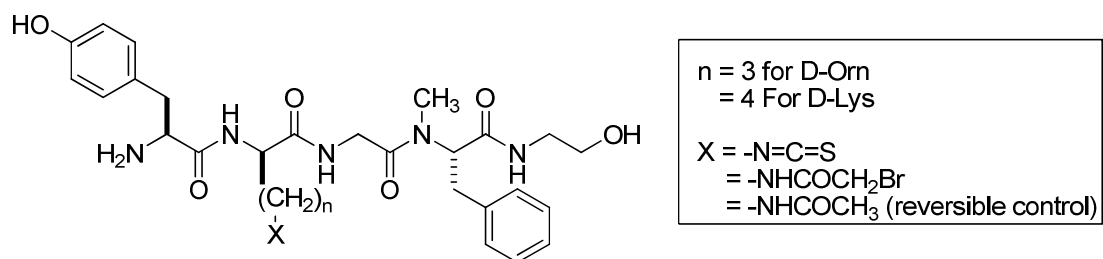


Figure 1.2. Potential affinity labels for MOR and the corresponding reversible control peptides based on the parent peptide DAMGO (Tyr-D-Ala-Gly-NMePhe-glyol)

During the attempted synthesis and purification of the isothiocyanate containing analogs of DAMGO an unexpected side reaction occurred resulting in the formation of cyclic-O-alkyl thiocarbamate derivatives (Figure 1.3). The identities of the products were determined by various techniques: (HPLC, IR, NMR and MS).⁴⁸

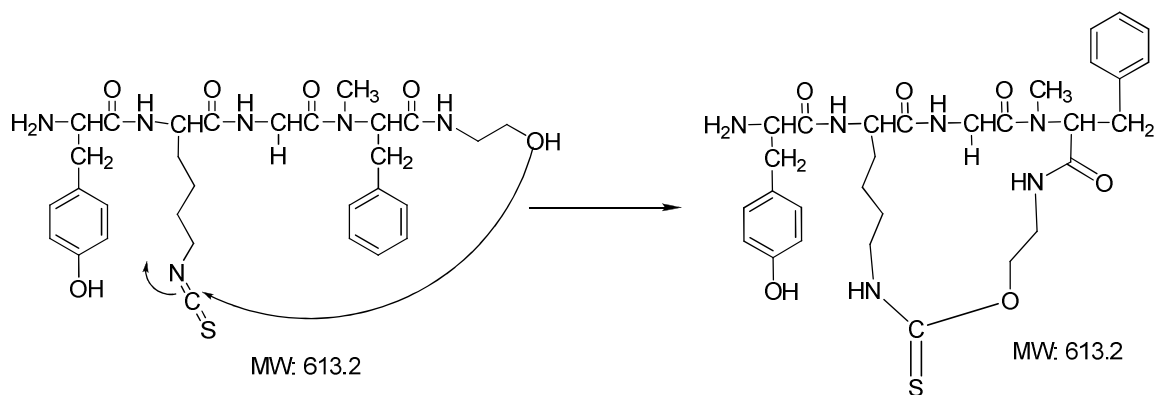


Figure 1.3. Proposed reaction for the formation of the cyclic O-alkyl thiocarbamate. Here [D-Lys(=C=S)²]DAMGO is shown as the example.

Based on the pharmacological assays analogs with high binding affinities were identified that also exhibited wash resistant inhibition of binding to MOR.

The isothiocyanate analogs of [D-Orn²] and [D-Lys²]DAMGO were modified to overcome the side reaction. This was achieved by replacing the glycol functionality by a glycylamide. Both the bromoacetamide and isothiocyanate affinity labels were

synthesized and evaluated in the glycylamide series. The affinity label functionalities were well tolerated by MOR, but most of the affinity labels in this series lost selectivity for MOR over DOR compared to the corresponding DAMGO analogs.

1.2.3 Project 3

Design, Synthesis and Evaluation of a Dermorphin-Based Multifunctional Affinity Label Probe for Mu Opioid Receptors (Chapter 5)

From the series of new dermorphin-based affinity labels, described in Project 1 above, we identified [D-Lys(CS)²]dermorphin as the lead peptide for designing a multifunctional probe with the long range goal of identifying the attachment point of this peptide to MOR. This ligand was selected due to its high affinity ($IC_{50} = 0.38$ nM), selectivity for MOR over DOR (255-fold) and wash-resistant inhibition of binding to MOR. Peptides, due to their polymeric nature, provide definite advantages over nonpeptides in receptor isolation studies. For example additional residues can be incorporated which can bear a purification tag such as biotin or d-desthiobiotin. Such a tag enables receptor enrichment via affinity purification with a streptavidin-based extraction procedure.⁴⁹⁻⁵¹ Opioid receptors are membrane proteins that are expressed at very low concentrations in different cell lines.⁵¹ Therefore affinity purification via d-desthiobiotin-streptavidin interaction would enrich the available receptor. Additionally, a fluorescent label could also be incorporated to facilitate the detection of labeled receptors. In this project, Oregon Green or 5-carboxyrhodamine was used as the fluorophore. Figure 1.4 shows the design of the multifunctional affinity label probe for MOR.

the bulky fluorescent group to the resin-bound peptide during the synthesis. The [D-Lys(CS)²]dermorphin-based multilabeled peptides containing either Oregon Green or 5-carboxyrhodamine B, along with the reversible controls, were successfully synthesized following this methodology. Preliminary microscopy experiments examining the interaction of the fluorescent affinity label peptide containing Oregon Green with MOR on SH-SY5Y cells suggest wash-resistant binding of the multifunctional affinity label dermorphin derivative, thus demonstrating the utility of this approach.

1.3 Conclusions

The peptide-based affinity labels with high MOR affinity described in this thesis will be useful pharmacological tools to study MOR and will aid in understanding MOR-ligand interactions at the molecular level.

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Chapter 2.

Literature Review

2.1 Opioid Receptors

The opioid system modulates a variety of complex physiological functions including analgesia, stress response, immunity, neuroendocrine function and cardiovascular control. This wide spectrum of neurobiological effects by the opioid system is mediated by activation of specific membrane bound receptors.¹

The term ‘opioid’ is derived from opium from which morphine, the prototypical opioid analgesic, was isolated. Although the analgesic effects of opium were known for thousands of years, opioid binding sites were first proposed by Beckett *et al.* only in the early 1950s,² followed by Portoghese *et al.* in the 1960s³ and Martin *et al.* in 1970s.⁴ The stereospecific binding of opiates to specific receptors in mammalian brain tissue was first reported around the same time in 1973.⁵⁻⁸ But it took almost two and a half decades of extensive pharmacological research since the first discovery of opiate binding sites by Beckett *et al.* to characterize the different types of opioid receptors. To date, three different types of opioid receptors have been cloned, the μ opioid receptor (MOR: μ for morphine),⁹ the κ opioid receptor (KOR: κ for ketocyclazocine),¹⁰ and the δ opioid receptor (DOR: δ for mouse vas deferens)¹⁰. A fourth opioid-like orphan receptor has been identified by homology screening which is generally referred to as the opioid-receptor-like-1 (ORL-1) receptor, although it does not bind the classical opioid receptor ligands.^{11, 12} There is also considerable pharmacological evidence for the existence of opioid receptor subtypes, particularly for subtypes of MOR, which may be products of alternative mRNA splicing,¹³

posttranslational modifications of the receptors, or homo- or hetero- receptor dimerization¹⁴ of the existing MOR, KOR, DOR and ORL-1 proteins.

2.1.1 Structure and Function of Opioid Receptors

The cloning of the μ , δ and κ opioid receptor genes in the 1990s followed by amino acid sequence comparison of the three receptors^{9, 15-18} and molecular analysis, indicate that they belong to the rhodopsin-like family of GPCRs (G-protein coupled receptors).¹⁹ GPCRs are characterized by seven putative transmembrane (TM) regions, an extracellular domain which includes the N-terminus and extracellular loops (EL), and the intracellular domain which includes the C-terminus and intracellular loops (IL)²⁰ (Figure 2.1). The highest transmembrane sequence homology among the three opioid receptors is found in TM2, TM3, and TM7.^{9, 21} A conserved Asp residue is found in both TM2 and TM3; the conserved TM3 Asp residue is thought to be essential for interaction with the protonated amine group in opioid ligands.²² The intracellular loops possess similar sequences among all three opioid receptor types. However, the extracellular loops are less conserved, particularly the second and third loops, and the highest structural diversity among opioid receptors is found in the N-terminal sequences. A few sites in the opioid receptors have been identified for possible post translational modifications. There are two possible glycosylation sites within the N-terminal region. The C-terminus and intracellular loop 3 (IL3) contain possible protein kinase C phosphorylation sites, and a possible palmitoylation site is found in the C-terminal sequence. Also, conserved

Cys residues are found in the first and second extracellular loops which are thought to be involved in a disulfide linkage.

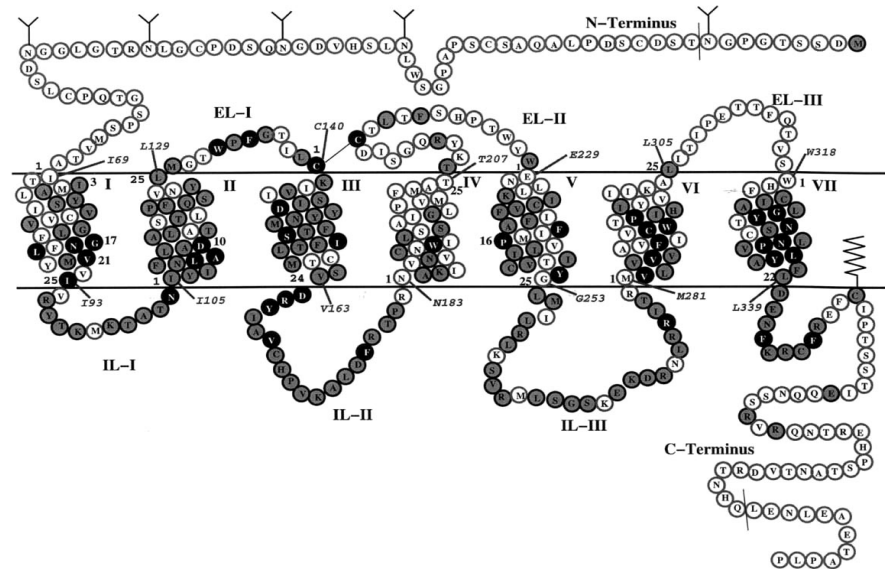


Figure 2.1.: Serpentine model of MOR²³

2.2 Mu Opioid Receptors (MOR)

Opioid analgesics (e.g. morphine), produce pain relief mainly through activation of MOR and are considered indispensable drugs for the management of pain.²⁴⁻²⁶ However, there are severe deleterious side effects associated with opioid analgesics, namely respiratory depression, addiction liability and constipation.^{27, 28} Martin *et al.* in 1976 first differentiated the pharmacological profile of MOR activation *vs.* KOR *in vivo* using morphine as the prototype agonist.^{4, 8} Administration of morphine to dogs resulted in a myriad of effects, including miosis, hypothermia, bradycardia, and analgesia and physical dependence after chronic administration.⁴ Also,

discontinuation of morphine resulted in an abstinence syndrome that could be suppressed by morphine but not by ketocyclazocaine. The latter drug has its own spectrum of actions, such as pupillary constriction, sedation and depression of flexor reflexes, which Martin attributed to activation of a separate type of receptors now referred to as KOR. These studies established the existence of different opioid receptor types.^{4, 8}

Based on radioligand binding experiments in brains of several species, the proportion of MOR is found to be 41% of the opioid receptor population in rat, 25% in guinea pig and 25% in the mouse.²⁹ A more precise and direct method to characterize regional differences in receptor distribution is autoradiography.³⁰ The most common radioligands used for autoradiography studies for MOR are [³H]DAMGO ([D-Ala²,MePhe⁴,glyol]enkephalin)³¹⁻³³ and [³H]CTOP(D-Phe-cyclo[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH₂).³⁴ Based on autoradiographic studies of MOR in rat brain, the highest densities are found in the striatum, the accessory olfactory bulb, and several areas of thalamic nuclei; in contrast, lower levels of MOR were found in the cerebral cortex and cerebellum.³⁵

The cloning of the MOR from rat brain was first reported by Chen *et al.* and Fukuda *et al.* in 1993^{9, 18} and showed 64% homology in amino acid sequences to the DOR cloned earlier by Evans *et al.* and Keiffer *et al.*^{15, 16} The expected high affinity for selective MOR ligands such as morphine and DAMGO and likewise low affinity for DOR the KOR selective ligands were observed for cloned MOR expressed on COS-7 cells.³⁶

2.2.1 Mutagenesis Studies of MOR

Since the cloning of the opioid receptors in 1990s, numerous efforts have been undertaken to investigate and identify the molecular basis of ligand recognition and selectivity for particular opioid receptor types. Generally, two approaches have been utilized to analyze receptor structures. The first approach is to design receptor chimeras where domains of one opioid receptor type have been replaced by the corresponding domain from a different opioid receptor.^{22, 37} The other approach involves site-directed mutagenesis of critical amino acid residues of a specific receptor type to investigate the effect of such changes on ligand binding to the receptor.^{38, 39} Although both of these approaches have been widely used in examining the domains of receptors involved in binding, these techniques suffer from the drawback of potential alteration of the three dimensional structure of the ligand binding region by the structural changes in primary sequences.²² Nevertheless, these techniques have been widely used over the past decade and a plethora of information has been obtained with regard to the specific domains of the opioid receptors possibly involved in binding and selectivity.

Several groups have reported the involvement of extracellular loops in the binding through constructing opioid receptor chimeras. Through such studies, it was also proposed that the binding sites for the opioid peptides and alkaloids are different.^{22, 40-43} Results obtained from constructing MOR / DOR chimeric receptors revealed the importance of extracellular loop 1 (EL1) and TM2 for the binding of MOR selective ligands.⁴¹ It was also reported that a DOR chimeric receptor which included the EL1

from MOR bound the MOR selective peptide DAMGO with high affinity, whereas the MOR receptor chimera bearing the EL1 from DOR was resulted in 100-fold lower affinity for DAMGO.⁴² The contribution of Lys108 in EL1 of DOR to ligand selectivity was demonstrated replacing Lys108 with Asn in the DOR sequence, which enabled binding of the MOR selective ligand dermorphin. However, the EL1 does not seem to play a critical role for the binding of MOR selective alkaloids.⁴⁴ An investigation of MOR / KOR chimeric receptors suggested that EL3 was critical for the high-affinity binding of DAMGO to MOR.^{17, 45} Additional studies identified four residues (Lys303, Val316, Trp318 and His319) in EL3 of MOR that may be important for recognition of DAMGO.⁴³ Also, TM6, TM7, and EL3 were found to be important for the selective binding of sufentanil to MOR over KOR.⁴⁴ The importance of EL1 and EL3 was further established with chimeric receptors between MOR and angiotensin II receptors. When EL1 and 3 from MOR were substituted with the corresponding regions of angiotensin II receptors, there were reductions in opioid receptor affinities.⁴⁶ Separate chimeric receptor studies reported by Varga *et al.*⁴⁷ and Meng *et al.*⁴⁸ it was demonstrated that Lys300 in EL3 of MOR represents a critical site for the selectivity of peptidic ligands. In addition to demonstrating the involvement of the ELs of MOR in conferring selectivity to peptidic ligands, Seki *et al.* also reported ligand-dependent selectivity. While incorporation of EL3 of MOR into a KOR chimera imparted high affinity binding for DAMGO, this result did not extend to the MOR-selective agents dermorphin and fentanyl.⁴⁶ Therefore the

molecular basis for ligand affinity and selectivity for different opioid receptors remains to be fully elucidated.

For high affinity binding to opioid receptors, the presence of protonated nitrogen in opioid ligands is required. Therefore, an aspartic or a glutamic acid residue in the binding pocket of the opioid receptors would potentially serve as a counter ion for ligand binding.²² Conflicting evidence concerning the importance of Asp147 on TM3 of MOR can be found in the literature. Although binding affinities of peptide agonists to MOR was eliminated through mutation of Asp147 to Ala in MOR, this mutation did not affect the binding of opioid antagonists diprenorphine and naloxone.²² Furthermore, mutation of Asp147 to Glu resulted in a decrease in binding for DAMGO, but not for morphine.³⁹ Therefore, it is speculated that other acidic residues, such as the conserved Asp in TM2, could act as the counter ion in agonist binding.²² Other charged amino acid residues, such as His297 of MOR, have also been implicated in the binding of opioid ligands. Thus, mutation of His297 of MOR to Ala resulted in a several fold loss in [³H]DAMGO binding to MOR.^{39, 49} Interestingly, this mutation to MOR instilled partial agonistic properties in classical opioid antagonists (e.g. naloxone).⁵⁰

One other useful approach to determine the domains of opioid receptors involved in opioid binding is through the study of irreversible ligands. Chen *et al.* demonstrated that β -funaltrexamine (β FNA), a MOR selective antagonist, labeled the Lys233 in TM5 (see details in section 2.4.2.1).^{51, 52} This result supports the proposal

of ligand binding sequences other than EL1 and EL3 for nonpeptide (opiate) antagonists.^{41-43, 45}

2.2.2 Computational Studies on MOR

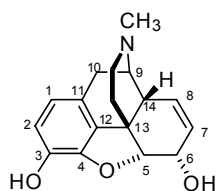
Currently, there are no high-resolution crystal structures of any of the opioid receptors. The only transmembrane receptor proteins in GPCR family whose crystal structure have been solved are rhodopsin in its dark state bound to 11-cis retinal,^{53, 54} human β_2 adrenergic receptor,^{55, 56} and recently the crystal structure of rhodopsin in its G-protein interacting conformation.⁵⁴ In the absence of crystal structures, computational models of opioid receptors are the other available option for developing structure-function relationships.⁵⁷ Homology modeling of opioid receptors based on the crystal structure of rhodopsin is the most common approach, and several applications of this approach have been reported in the literature.^{37, 58-62} Fowler and coworkers reported a homology model of the agonist bound receptor state of MOR in complex with the MOR selective cyclic peptide JOM6,⁶³ using structural constraints.^{64, 65} Comparison of models of agonist-bound MOR with MOR without a ligand⁶⁴ predicted that the rotation of side chain of Trp293 was the major change that takes place upon agonist binding to MOR, resulting in the relocation of the indole ring of Trp293 from the interface between TM6 and TM7 to the interface between transmembrane domains 3, 5, and 6. These movements of TM domains were proposed to form a π stacking interaction with the aromatic ring of Tyr¹ of JOM6. TM6 movement would then reorient the side chain of Met151, Asp147, Lys233, Lys303 and Trp318.^{64, 65} The importance of Trp293 to the activation of MOR had

been previously proposed by other groups based on mutagenesis data on rhodopsin and agonist-activated leukotriene receptors.^{66, 67}

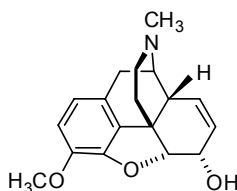
The homology model and molecular dynamics simulation of MOR in phospholipids bilayer-aqueous environment was subsequently reported by Zhang *et al.* where they demonstrated the conformational changes observed in TMs as well as EL and IL regions.⁶² They further evaluated the molecular dynamics simulation with naloxone (Figure 2.2), the universal opioid antagonist, to MOR. At least three main binding domains of naloxone were observed: a polar and aromatic domain composed of Asp147, Phe289, Trp293, Cys321 and Tyr326, possibly involved in cation- π interactions with the protonated nitrogen of naloxone; a hydrophobic domain consisting of Tyr148 from TM3, Tyr210 and Phe221 from EL2 and another hydrophobic region involving Trp318, Leu219, Ile322, Ile296 and Ile144.⁶² Based on their mutational analysis and computational study, Li and coworkers reported that mutation of Asp164 in the highly conserved Asp-Arg-Tyr (DRY) motif in TM3 to either His, Gln, Tyr or Met resulted in constitutive activation of MOR.⁶⁸ Computational modeling based on the crystal structure of rhodopsin suggests the differences in conformation resulting from the mutation are probably due to changes in the interaction between the cytoplasmic ends of TM3 and TM6 involving the conserved Arg116 in TM3 and Arg280 in TM6.⁶¹ Very recently, conformational changes in the transmembrane domains of the constitutively active Asp164Tyr MOR were reported based on identification of accessible cysteine residues within the TM domains labeled by methanethiosulfonate ethylammonium (MTSEA).⁶⁹

As discussed earlier (Chapter 1, section 1.1), the low sequence homology between MOR and rhodopsin represents a potential for significant error in homology modeling experiments.⁷⁰⁻⁷² One way to improve the accuracy of such theoretical model is by providing adequate receptor-specific (in this case opioid receptors) and ligand-specific experimental constraints. Based on the above findings there is clearly a need to develop a more direct method to examine receptor-ligand interactions and the selectivity for different opioid receptor ligands (peptide vs. non-peptide).

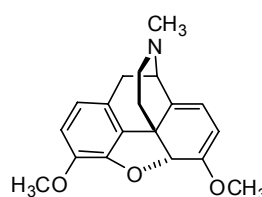
Agonists



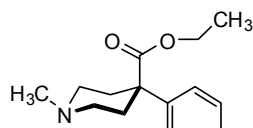
(-) Morphine



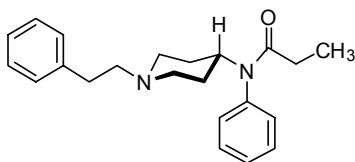
Codeine



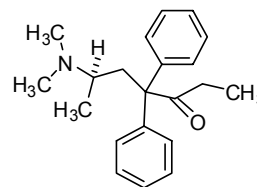
Thebaine



Mepiridine

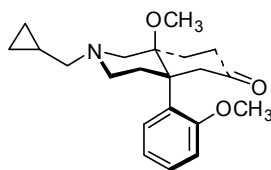


Fentanyl

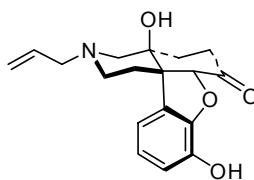


Methadone

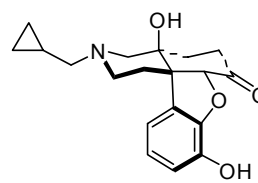
Antagonists



Cyprodime



Naloxone



Naltrexone

Figure 2.2. Morphine and morphine-derived alkaloids for MOR

2.3 Ligands for MOR

2.3.1 Small Molecule Ligands for MOR

Morphine, the prototypical MOR agonist, was first isolated from poppy seeds by Serturner in 1803. He named the compound after Morpheus, the Greek god of sleep and dreams.²⁸ But it was more than a century later that the complex structure of morphine was confirmed through total synthesis by Gates and Tschudi.^{73, 74} Later, the relative stereochemistry of morphine was established by chemical synthesis and X-ray crystallography.⁷⁵ The absolute configuration (Figure 2.2) was later proved with application of various techniques.⁷⁶ In addition to morphine, related alkaloids discovered in opium are codeine and thebaine⁷⁷ (Figure 2.2). Although thebaine is not active as an analgesic, it serves as an important synthetic intermediate for the preparation of several other potent analgesics.⁷⁸ The elucidation of the structure of morphine was followed by extensive structure-activity relationship (SAR) studies of analogs of morphine and related synthetic compounds including morphinans, benzomorphinans and phenylpiperidines. Some of the MOR selective agonists discovered from such studies include meperidine, fentanyl and methadone (Figure 2.2) which are also routinely used for the treatment of pain, alone or in combination therapy, or opiate addiction (methadone, Table 2.1).

The antagonists naltrexone and naloxone (Table 2.1 and Figure 2.2) have been extensively used to study MOR pharmacology. These compounds also exhibit significant affinity towards DOR and KOR. Naloxone is primarily used to reverse

opiate overdose whereas naltrexone is employed for treatment of narcotic addiction and alcohol dependence. The antagonist cyprodime exhibits higher selectivity for MOR over DOR and KOR.

Table 2.1 Opioid affinities and activity in guinea pig ileum (GPI) and mouse vas deference (MVD) of selected MOR agonists and antagonists⁷⁹

a. Agonists	K _i (nM)			K _i ratio	IC ₅₀ (nM)	
	MOR	DOR	KOR		GPI	MVD
Morphine	1.8	90	317	1/50/175	28	478
Meperidine	385	4,350	5,140	1/11/13	1,109	16,000
Fentanyl	7.0	150	470	1/21/67	0.92	26
Methadone	4.5	15	1,630	1/3.3/360	22	523
b. Antagonists	K _i (nM)			K _i ratio	K _e (nM)	
	MOR	DOR	KOR		GPI	MVD
Cyprodine	9.4	356	176	1/38/19	31	6110
Naloxone	1.8	23	4.8	1/13/2.7	1.9	12
Naltrexone	1.1	6.6	8.5	1/6.0/7.7	0.36	3.6

2.3.2 Opioid Peptides

2.3.2.1 Endogenous Opioid Peptides Interacting with MOR

The first endogenous ligands for mammalian opioid receptors discovered back in 1970s were the two pentapeptides: leucine and methionine enkephalin⁸⁰ followed by dynorphin A^{81, 82} and β -endorphin.⁸³ Since these peptides were structurally different from the alkaloid opiates, they were referred to as *opioids* to include all nonpeptides and peptides with opiate-like activity. All of these endogenous peptides share a common N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe, Table 2.2), but they differ in their C-terminal sequences and also in their preferential interaction with different opioid receptor types. Based on this observation, Goldstein proposed the common N- terminal sequence as the ‘message’ sequence required for activation of opioid receptors and the unique C-terminal sequences as ‘address’ sequences which

Table 2.2 Mammalian opioid peptides with their precursor proteins

Precursor Protein	Endogenous Peptide	Sequence
Proenkephalin	Leu-Enkephalin	Tyr-Gly-Gly-Phe-Leu
	Met-Enkephalin	Tyr-Gly-Gly-Phe-Met
	Met-Enkephalin-Arg ⁶ -Phe ⁷	Tyr-Gly-Gly-Phe-Met-Arg-Phe
	Met-Enkephalin-Arg ⁶ -Phe ⁷ -Leu ⁸	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
Prodynorphin	Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
	Dynorphin B	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	α -Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Arg-Pro-Lys
	β -Neoendorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Arg-Pro
Proopiomelanocortin	β -Endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Unknown	Endomorphin-1	Tyr-Pro-Trp-PheNH ₂
	Endomorphin-2	Tyr-Pro-Phe-PheNH ₂

provide the required affinity for a particular ligand for a particular opioid receptor type.⁸⁴ Some synthetic enkephalin analogs and the amphibian peptide dermorphin (see below)⁸⁵ interact with the MOR preferentially. Endomorphins which were relatively recently discovered by Zadina *et al.*^{86, 87} exhibit high affinity and highest selectivity for MOR among the endogenous mammalian peptides. Other enkephalin

analogs (e.g. DPDPE) as well as the deltorphin family of amphibian peptides⁸⁸ preferentially bind to DOR^{89, 90} where as dynorphin A binds preferentially to KOR.⁹¹

2.3.2.2 MOR Selective Linear Enkephalin Analogs

The enkephalin class of opioid peptides has been extensively studied since their identification.^{89, 92-95} The endogenous enkephalins show some preference for binding to DOR but are labile to degradation by a variety of proteases. Therefore, extensive

Table 2.3 Opioid receptor affinities and selectivity in the GPI and MVD of MOR selective enkephalin

Peptide	K _i (nM)		K _i Ratio	IC ₅₀ (nM)		References
	MOR	DOR	MOR/DOR	GPI	MVD	
DAMGO	1.9	345	180	4.5	33	⁷⁹
Syndyphalin-25 (Tyr-D-Met-Gly-NMePheol)	0.29 ^a	1,250 ^a	4300	0.0025	-	⁹⁶
LY 164929 (Figure 2.3)	0.6 ^a	900 ^a	1500	-	-	⁹⁷
Tyr-cyclo[D-Dab-Gly-Phe-Leu]	13.8	1158	83	14.1	81.4	⁹⁸

^aIC₅₀ values

research has been carried out to develop modified analogs with increased metabolic stability and different selectivities. As a result both MOR and DOR selective enkephalin analogs have been identified.^{89, 92, 94, 95} For example, amidation, reduction, or complete elimination of the C- terminus results in analogs with retention of MOR affinity and an appreciable increase in MOR selectivity. DAMGO (Figure 2.3), the most commonly used MOR selective peptide ligand, is an example of a reduced C-terminus with high affinity and selectivity for MOR (Table 2.3).⁹⁹ Other related MOR

selective C-terminally modified tetrapeptide analogs include syndyphalin-25 (Table 2.3),¹⁰⁰ and LY164929 (Figure 2.3)⁹⁷ that exhibit significantly higher MOR selectivity than DAMGO (Table 2.3). Also, the characteristic Tyr-Gly-Gly-Phe ‘message’ sequence’ is not an absolute requirement for interaction with MOR, as replacement of the aromatic moiety of Phe⁴ with either a cyclohexane ring¹⁰¹ or a leucine side chain^{102, 103} were found to be well tolerated.

2.3.2.3 MOR Selective Conformationally Constrained Enkephalin Analogs

Incorporation of conformationally constrained amino acids or a cyclic constraint has been a successful approach to obtain greater selectivity for one particular receptor type. The first such example of improved receptor selectivity through a cyclic

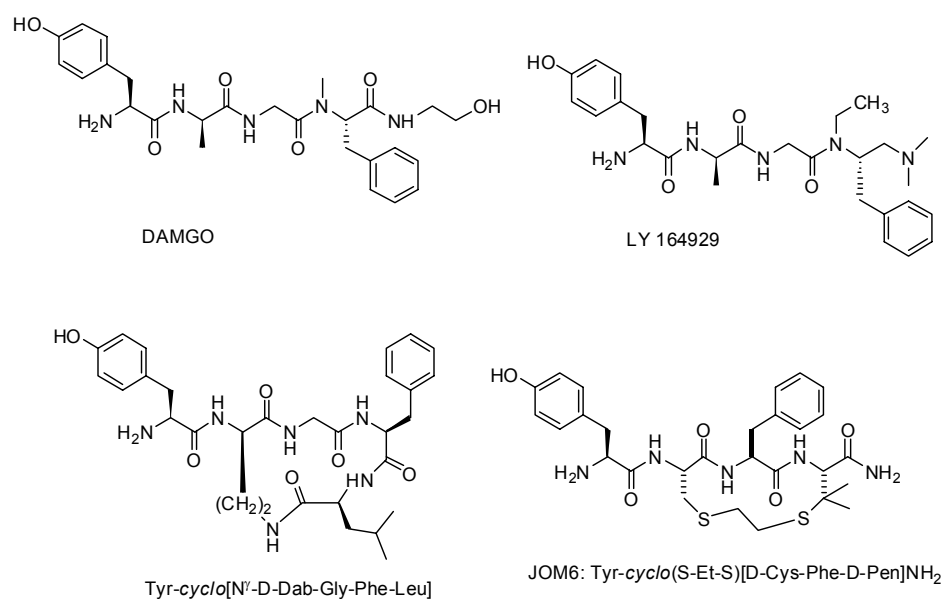


Figure 2.3. MOR selective analogs including conformationally constrained cyclic derivatives

constraint was (Tyr-*cyclo*[N^γ-D-Dab-Gly-Phe-Leu], Figure 2.3, Dab=α,γ-diaminobutyric acid), which demonstrated both high affinity and improved selectivity towards MOR.¹⁰⁴ In contrast, the acyclic linear analog [D-Dab²,Leu⁵]enkephalinamide failed to show any MOR selectivity.¹⁰⁵ Other cyclic analogs with either D-Orn or D-Lys in position 2 exhibited decreased selectivity for MOR, although these analogs show higher affinity for this receptor.⁹⁸ The restriction of conformational flexibility in Tyr-*cyclo*[N^γ-D-Dab-Gly-Phe-Leu] was also demonstrated by NMR spectroscopy^{106, 107} and computational methods,¹⁰⁶⁻¹¹⁰ however, some degree of conformational flexibility remains especially for larger ring sizes. Receptor selectivity was also achieved by incorporating constrained amino acids. One such example is the modified [Leu⁵]enkephalin analog where replacement of Tyr¹ by 2-amino-6-hydroxy-2-tetralincarboxylic acid (Hat) results in increased selectivity for MOR.¹¹¹

2.3.2.4 MOR Selective Peptides from Amphibian Skin

Amphibian skin is a rich source of a varied range of peptides which often resemble the neurotransmitter or gastrointestinal hormones of mammalian systems.¹¹² In 1981, Montecuchhi *et al.* and Brocardo *et al.* first described dermorphin, a heptapeptide isolated from the skin of the South American frog *Phyllomedusa sauvagei*,^{113, 114} and another similar peptide containing hydroxyproline (Hyp) in place of Pro⁶ from the skin of *Phyllomedusa rohdei* (Figure 2.4).¹¹⁵ Later in the 1990s, the sequences of three additional dermorphin peptides were predicted based on the cDNA library from the skin of *Phyllomedusa bicolor* (Figure 2.4).¹¹⁶

Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH ₂	dermorphin
Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-NH ₂	[Hyp ⁶]dermorphin
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-OH	[Lys ⁷ -OH]dermorphin
Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-OH	[Trp ⁴ ,Asn ⁷ -OH]dermorphin
Tyr-D-Ala-Phe-Trp-Asn-OH	[Trp ⁴ , Asn ⁷ -OH]dermorphin 1-5

Figure 2.4. Dermorphin peptides

The unique structural feature of the amphibian opioid peptides is the presence of D-Ala between the two aromatic residues Tyr¹ and Phe³, in contrast to the message sequence Tyr-Gly-Gly-Phe for all of the enkephalins and most other mammalian opioid peptides. Considerable research was performed to identify the gene responsible for the D-amino acid in these peptides. It was found that the triplet codon for L-Ala was included in the dermorphin gene^{116, 117} leading to the hypothesis that the L-Ala residue must be converted to D-Ala through posttranslational modification.¹¹⁸ Kreil *et al.* rationalized that the mechanism of epimerization should be a quantitative inversion of the chiral center at the α -carbon of alanine, as opposed to racemization by a racemases which would result in an equal quantity of L-and D-isomers.^{118, 119} However, a racemase mechanism should produce some level of detectable L-Ala dermorphin analogue, and no such isomer has been found in *Phyllomedusa* skin.^{114, 117}

2.3.2.4.1 SAR Study of Dermorphins

Based on extensive *in vitro* studies binding assays on crude or synaptosomal preparations of brain membranes and bioassays on electrically stimulated GPI and MVD, dermorphins (Table 2.4) were found to be one of the most potent and selective

Table 2.4. Affinities and MOR selectivity of natural dermorphins (amidated and acid forms) and biological activities on GPI and MVD. Taken from reference 119.

Peptide	K _i nM		K _i ratio	IC ₅₀ nM	
	MOR	DOR	DOR/MOR	GPI	MVD
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH ₂ (Dermorphin)	0.54	929	1720	1.29	16.5
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-OH	-	-	-	4.5	28.1
Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-NH ₂	0.65	1200	1846	1.6	18.1
Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Ser-OH	-	-	-	4.9	33.0
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-NH ₂	0.09	1105	>10000	1.15	13.6
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-OH	5.7	1150	201	3.82	56.3
DER-Gly-Glu-Ala-Lys-Lys-Ile-NH ₂ ^a	0.149	130	872	1.53	-
DER-Gly-Glu-Ala-Lys-Lys-Ile-Lys-Arg-NH ₂ ^a	0.002	7.2	3600	1.37	-
Tyr-D-Ala-Phe-Phe-NH ₂ (TAPP)	1.5	625	417	255	780
Tyr-D-Ala-Phe-Trp-Asn-NH ₂	0.9	480	533	5.00	73.7
Tyr-D-Ala-Phe-Trp-Tyr-Pro-Asn-NH ₂	0.32	690	2156	0.58	6.6
DAMGO	1.1	430	391	7.1	115
Morphine	11.0	500	45	150	1215

^aDER= Dermorphin

MOR agonists among the naturally occurring opioids.¹¹⁸ The primary peptide dermorphin shows 20 times higher affinity, and is 50 times more selective for MOR than morphine.¹¹⁸ [Lys⁷]dermorphin, obtained from skin of *Phyllomedusa bicolor*, exhibits 10 fold higher affinity and selectivity than DAMGO and dermorphin and is 100 times more potent in the GPI and MVD functional assays than morphine (Table 2.4). [Lys⁷]dermorphin has been further reported to differentiate between two MOR subtypes.¹²⁰

Comparisons of the MOR affinities of acid vs. amide versions of the naturally occurring dermorphins revealed that peptides with the C-terminal carboxylic acid derivatives had several-fold lower MOR affinities than that of the amidated peptides.¹²¹ The high potency of the C-terminally amidated dermorphin analogs occurs possibly as a result of suppression of the negative charge of the terminal carboxy group. Also, C-terminal amidation helps protect the analogs from possible cleavage by carboxypeptidases.¹¹⁸ Of the shorter dermorphin amide analogs, dermorphin (1-5) retains 50% and dermorphin (1-4) retains 5% of the potency in activating opioid receptors in GPI¹¹⁸ and dermorphin (1-3) was inactive.¹²² It was also found that 1-4 sequence of dermorphin is the primary end product of enzymatic degradation in rat brain.¹²² The affinities of C-terminally elongated dermorphin analogs which included residues from the precursor sequence were higher than dermorphin for MOR from rat brain, whereas in the GPI assay, the potencies were similar or slightly lower than that of dermorphin.¹²¹ Although a decrease in MOR affinity was observed with the introduction of additional residues through Glu⁹ or Ala¹⁰, probably due to the presence of acidic Glu⁹, further extension of the peptide with basic residues increases MOR affinity (Table 2.4).¹²¹ Affinity and selectivity of a dimeric derivative of dermorphin have also been investigated. Dimeric derivatives were prepared by bridging two monomers with hydrazine or diamines of various lengths.¹²³ One of these ligands, di-dermorphin, where two dermorphin molecules are linked by hydrazine, displays 5-fold greater MOR affinity and similar selectivity as dermorphin.

Extensive SAR studies with amino acid substitution in every position of dermorphin, have been reported in the literature including some of the shorter dermorphin sequences.^{85, 124-129} An alanine scan of dermorphin indicated that substitutions at positions 4, 6 and 7 were well tolerated. However, substitutions in position 1, 2, 3, or 5 resulted in large decreases in GPI potency.¹³⁰ Modifications of Gly⁴ are well tolerated, particularly in the case of tetrapeptide analogs. When Gly⁴ was replaced with sarcosine (NMeGly), in a tetrapeptide analog derived from dermorphin, an increase in opioid activity in antinociceptive assays was observed.¹³¹ Another tetrapeptide amide analog of dermorphin obtained from substituting Gly⁴ with Phe resulted in the dermorphin / enkephalin hybrid TAPP (Table 2.4) that is a potent and selective agonist at MOR.¹³² The substitutions of positions 3 and 4 of

Table 2.5. Affinity and selectivity of dermorphin analogs including tetrapeptide analogs of dermorphin modified at the 2nd position.

Peptide	K _i nM		IC ₅₀ nM	
	MOR	DOR	GPI	MVD
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH ₂	0.54	929	1.29	16.5
Tyr-Ala-Phe-Gly-Tyr-Pro-Ser-NH ₂	2171	>40000	>4000	>15000
Tyr-D-Met-Phe-Gly-Tyr-Pro-Ser-NH ₂	92.4	1340	47.0	249
Tyr-D-Pro-Phe-Gly-Tyr-Pro-Ser-NH ₂	2400	1890	>50000	>50000
Tyr-D-Arg-Phe-Gly-Tyr-Pro-Ser-NH ₂	3.9	-	92	-
Tyr-D-Arg-Phe-Gly-NH ₂	1.7	-	10.8	-
Tyr-D-Arg-Phe-Gly-OH	5.25	-	23	-
Tyr-D-Arg-Phe-Lys-NH ₂ (DALDA)	1.69	>20000	254	781
Tyr-D-Arg-Phe-Sar-OH (TAPS)	1.1	-	6.6	-

TAPP with bulky aromatic amino acids, e.g. tryptophan or naphthylalanine, are well tolerated and produce more lipophilic peptides.¹³³

Numerous substitutions of the D-Ala² residue between the two aromatic residues have been investigated. A substitution of D-Ala with the L-isomer results in a 100-

fold decrease in MOR affinity and abolishes activity in the GPI (<0.1% the potency of dermorphin, Table 2.5). Substitution of D-Ala² in dermorphin with L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), conformationally constrained analog of Phe L-Tic resulted in a DOR antagonist.¹³⁴ Similarly, substitution with D-Pro² rendered the peptide nonselective and agonist activity was lost in the GPI assay (Table 2.5).¹¹⁸ Substitution of D-Ala² with D-Met reduced binding to the MOR, whereas substitution with D-Arg resulted in analogs with either similar or in some cases increased MOR affinity and analgesic potency.^{118, 131, 135} The tetrapeptide analog TAPS (Table 2.5) was reported by Paakkari *et al.*, to be a potent antinociceptive agent and caused respiratory stimulation rather than depression that was antagonized by naloxonazine- the putative MOR₁ subtype antagonist.¹³⁶ TAPS was also shown to antagonize the respiratory depression caused by dermorphin. These results have led to the proposal that TAPS is an agonist at the MOR₁ subtype and an antagonist at the MOR₂ subtype *in vivo*.¹³⁶ Based on Schwyzer's proposal that MOR are situated in an ionic membrane compartment,¹³⁷ Schiller and coworkers hypothesized that positively charged ligands would display MOR selectivity.¹³² This hypothesis was supported by the results for the tetrapeptide dermorphin derivative with a positively charged residue Lys in position 4 which exhibited increased MOR selectivity.¹³² The [D-Arg²,Lys⁴]dermorphin analog DALDA (Table 2.5), which contains a +3 net positive charge, also demonstrated superior MOR selectivity in binding assays. Quarternization of the side chain amine of Lys⁴ in DALDA was well tolerated and the resulting analogs retained potent *in vivo* antinociceptive activity in

the mouse writhing assay after s.c. administration.¹³⁸ This antinociceptive effects were significantly reduced by the quarternized antagonist *N*-methylevallorphan. This suggest that these analogs exhibit peripheral antinociceptive activity in this assay.¹³⁸

Substitution of Tyr¹ of DALDA by Dmt [(2,6-dimethyl) tyrosine] resulted in analog with 10-fold higher affinities for both MOR and DOR¹³⁹ and 200 fold higher affinity for KOR.¹⁴⁰ It was also found that in the rat tail flick test after i.t. administration, Dmt¹[DALDA] was 220 and 3,000 times more potent than DALDA and morphine respectively.¹⁴¹

2.4 Affinity Labels

Although a considerable amount of information is available from the SAR of opioid ligands as well as from chimeric and site directed mutagenesis studies of opioid receptors, determination of the details of binding and ligand interaction with the receptor at the molecular level still remains a formidable challenge. To understand interaction of opioid receptors with their ligands at the molecular level, other approaches are needed. Affinity labels represent one such approach for probing the structure of membrane-bound proteins which cannot be readily solved by crystallography.¹⁴² Portoghese proposed that affinity labels bind to their receptors in a two-step process (Figure 2.5).¹⁴² The first reversible step depends on the relative

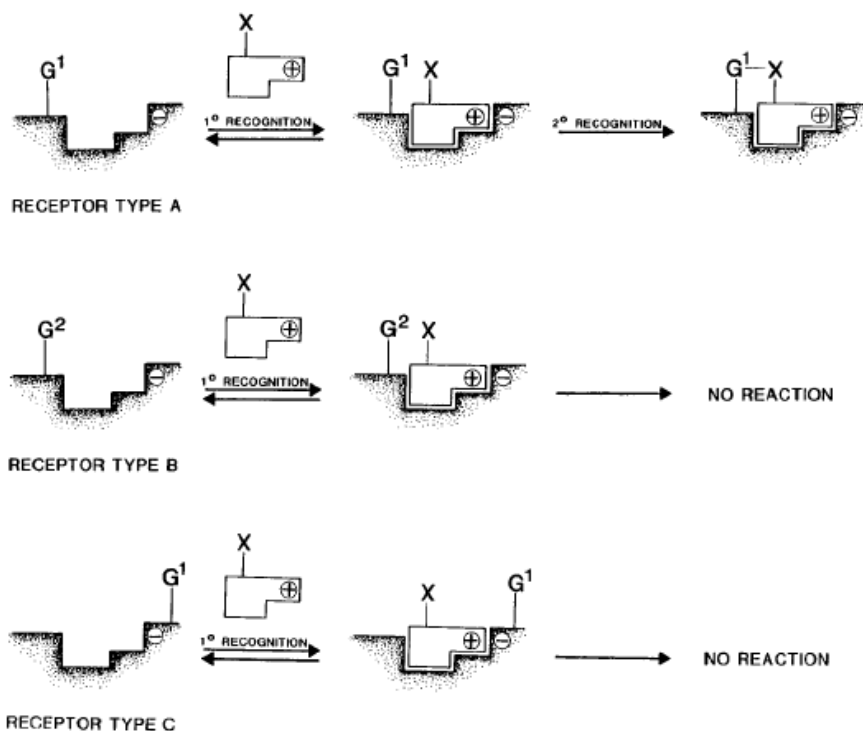


Figure 2.5. An illustration of the two steps involved in covalent binding of an affinity label to its target. Taken from reference 143

affinity of the ligand for the target site, and the second step (Figure 2.5, receptor type A) involves proper alignment of an electrophilic group on the affinity label with a compatible, receptor-based nucleophile that is in close proximity. Because of the second irreversible step, high receptor selectivity is theoretically possible, and affinity labels can detect subtle differences in receptor-ligand interactions. As indicated in Figure 2.5, in receptor type B the affinity label fails to undergo the second recognition process since it does not have sufficient reactivity to bind to the nearby nucleophile. Alternatively, if a reactive electrophile is too far away from the nucleophile on the receptor (Figure 2.5, receptor type C), then the second irreversible binding will not occur. Therefore, affinity labels undergoing irreversible binding to the receptor can be highly useful in differentiating different opioid receptor types. Site specific

interactions can be obtained by determining the attachment point of an affinity label to its receptor providing important information about the location and orientation of opioid ligands.¹⁴³

Depending upon the nature and chemical reactivity of the functionality, affinity labels can be classified as electrophilic affinity labels, which are inherently reactive, or photoaffinity labels which require photoactivation to achieve the reactive state. It should be pointed out that a ligand with extremely high affinity but without the capability of binding covalently can also be considered an affinity label; but a K_d value of not greater than 1×10^{-12} M is required.¹⁴²

2.4.1 Photoaffinity Labels

Photoaffinity labels are functionalities that can be activated by a brief exposure to light to generate a highly reactive intermediate species. Because of the high reactivity of these photolyzed intermediates, they often bind indiscriminately to nearby functionalities in the receptor binding site.¹⁴² Therefore, whether or not a photoaffinity label will bind to a particular opioid receptor type is determined primarily by its selectivity as a reversible ligand in the first recognition step. Some examples of photoreactive intermediates are the nitrene from the azido functionality and the carbene from diazo or diazirine functionalities (Figure 2.6).¹⁴⁴

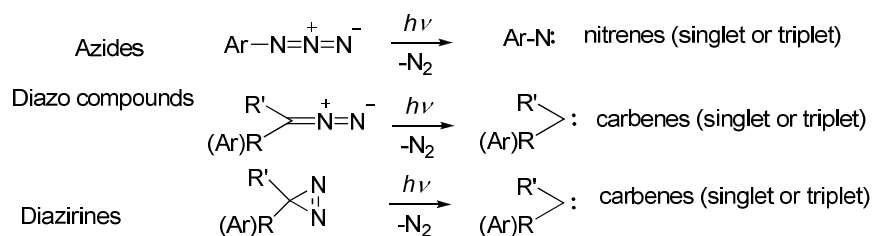


Figure 2.6. Precursors of reactive species in photoaffinity labels¹⁴⁴

The first photoaffinity label for an opioid receptor was the [^3H]norlevorphanol derivative APL (Figure 2.7), prepared by Winter and Goldstein ¹⁴⁵ APL exhibited irreversible binding when it was photolyzed in the presence of the GPI or opioid receptors in the mouse brain particulate fraction, but the binding was not effectively blocked by levorphanol suggesting extensive non-specific binding. Later, when the N-methyl quaternary derivative of APL, MAPL (Figure 2.7) was synthesized and subsequently tested on mouse brain and GPI, MAPL showed some degree of irreversible binding.¹⁴⁶ Some other examples of photoaffinity labels for opioid

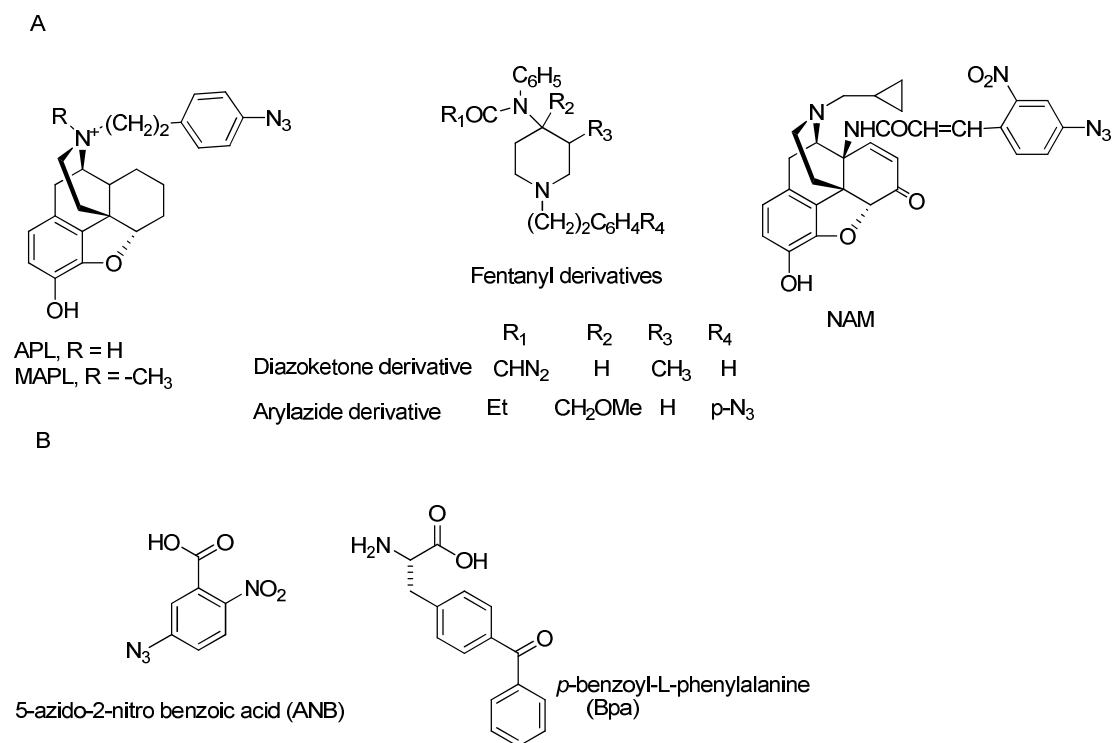


Figure 2.7. A: Photoaffinity labels for opioid receptors, B: Amino acid or acid derivative of photoaffinity labels used to characterize opioid receptors.

receptors are diazoketone and arylazide derivatives of fentanyl designed by Maryanoff and coworkers¹⁴⁷ (Figure 2.7). Later, Peers and coworkers reported the synthesis of the nitro-azido derivative of 14 β -aminomorphinone (NAM, Figure 2.7) which is a full antagonist in both the GPI and MVD preparation. Although NAM appears to bind selectively to MOR in binding studies, it is not suitable for labeling opioid receptors because of its slow irreversible binding.¹⁴⁸

There have been reports of several enkephalin-based photoaffinity label analogs with an azido group as the photoaffinity functionality (Table 2.6). Amongst these, only a few are selective for MOR. Some of the azido-containing photoaffinity labels for MOR reported are a DAMGO-based peptide (Tyr-D-Ala-Gly-Me-Phe(pN₃)-Glyol, ~ 0.3 μ M to irreversibly label 50% of MOR sites) prepared by Garbay-Jaureguiberry and coworkers,¹⁴⁹ and a photoaffinity label derivative of the somatostatin based MOR selective antagonist CTAP (D-Phe-*cyclo*[Cys-(p-N₃Phe)-D-Trp-Lys-Thr-Pen]-Thr-NH₂, IC₅₀ = 48.6 nM) reported by Landis *et al*¹⁵⁰ however, it was not reported whether this analog bound irreversibly to MOR.

Table 2.6 Enkephalin-based photoaffinity labels for opioid receptors. Taken from reference 143.

Tyr-D-Ala-Gly-Phe-Met-Tyr-NH(CH ₂) ₂ NH-C ₆ H ₄ (2-NO ₂ ,4-N ₃)
Tyr-D-Ala-Gly-Phe-Leu-NHCH(CO ₂ H)(CH ₂) ₄ NH-C ₆ H ₄ (2-NO ₂ ,4-N ₃)
Tyr-D-Ala-Gly-NH(CH ₂) ₃ NH-C ₆ H ₄ (<i>p</i> -N ₃)
Tyr-D-Ala-Gly-Phe-NH(CH ₂) ₃ NH-C ₆ H ₄ (<i>p</i> -N ₃)
Tyr-D-Ala-Gly-D / L-Phe(<i>m</i> -N ₃)-Leu-NH ₂
Tyr-D-Ala-Gly-Phe-Leu-NH(CH ₂) ₂ NH-C ₆ H ₄ (2-NO ₂ ,4-N ₃)
Tyr-D-Ala-Gly-Phe-Leu-NH(CH ₂) ₂ NHCO(CH ₂) ₂ NH-C ₆ H ₄ (2-NO ₂ ,4-N ₃)
Tyr-D-Thr-Gly-Phe(<i>p</i> -N ₃)-Leu-NH ₂
Tyr-D-Ala-Gly-MePhe(<i>p</i> -N ₃)-Gly-ol

The main disadvantage of using the azido group as a photoaffinity label for opioid receptors is that the short wavelength (~250 nm) of UV irradiation generally used to generate the reactive species can inactivate opioid receptors.¹⁵¹ In order to overcome this problem 4-azido-2-nitrobenzoic acid (ANB) or Bpa (*p*-benzoyl-phenylalanine), Figure 2.7) can be used as the photoaffinity label group. These functionalities shift the wavelength required for photolysis to longer wavelengths, which may prevent opioid receptor inactivation; this was shown by Herblin and coworkers when they reported synthesis and selective binding of a tetrapeptide morpheceptin analog containing Bpa (Tyr-Pro-Phe-Bpa-NH₂) to MOR.¹⁵² However this analog showed only modest affinity for MOR (IC₅₀ = 0.27 μM), and only 25% of the receptor was inactivated upon photolysis in the presence of 2.8 μM of this compound at a wavelength of 300-350 nm.¹⁵² The bulk of this amino acid (Bpa) may interfere with receptor-ligand interactions which could limit its incorporation to positions in the C-terminal sequence.

2.4.2 Electrophilic Affinity Labels

When the affinity label contains an electrophilic functionality, several factors may affect the selectivity of such a label: 1) receptor affinity for the ligand, 2) selectivity of ligand for the receptor 3) selectivity and chemical reactivity of the electrophile, and 4) proximity of the electrophile to a nearby nucleophile on the receptor. Since two recognition steps are involved in irreversible binding, in some cases increased receptor selectivity can be achieved, depending upon the reactivity and proximity of an appropriate nucleophile in the receptor (see Figure 2.5). Therefore, an electrophilic

affinity label can be a useful tool to selectively label one opioid receptor type among multiple types.

2.4.2.1 Small Molecule-Based Electrophilic Affinity Labels: β -Funaltrexamine and Other Analogs

The first successful electrophilic affinity label for opioid receptors was prepared by Portoghese and coworkers by incorporating a nitrogen mustard at the 6 β position of naltrexamine, resulting in β -chlornaltrexamine (β -CNA, Figure 2.8).^{153, 154} A nitrogen mustard is a highly reactive electrophile, and as such this affinity label bound irreversibly to all opioid receptor types. It should be noted that only one of the chloroethyl groups is needed for the irreversible binding.¹⁵⁵ Another example of nitrogen mustard-containing opiate analogs in the literature, such as the oxymorphone analog β -chloroxymorphanine (β -COA).^{156, 157}

In order to improve the receptor selectivity of opiate-derived affinity labels, Portoghese and co-workers prepared the β -fumaramide derivative β -funaltrexamine (β -FNA, Figure 2.8), which contains a less reactive electrophile, in place of the nitrogen mustard at the 6 β position of naltrexamine.¹⁵⁸ The resulting compound bound irreversibly to MOR where it acts an antagonist, whereas it is a reversible agonist at KOR. Whether or not β -FNA binds irreversibly to DOR remains unclear.^{159, 160} It was also shown that the configuration and orientation of the fumaramide are important for irreversible binding to MOR. Neither the 6 α analogue of β -FNA nor the 6 β -maleimide derivative with a *cis* double bond were capable of irreversible binding to MOR.¹⁶⁰

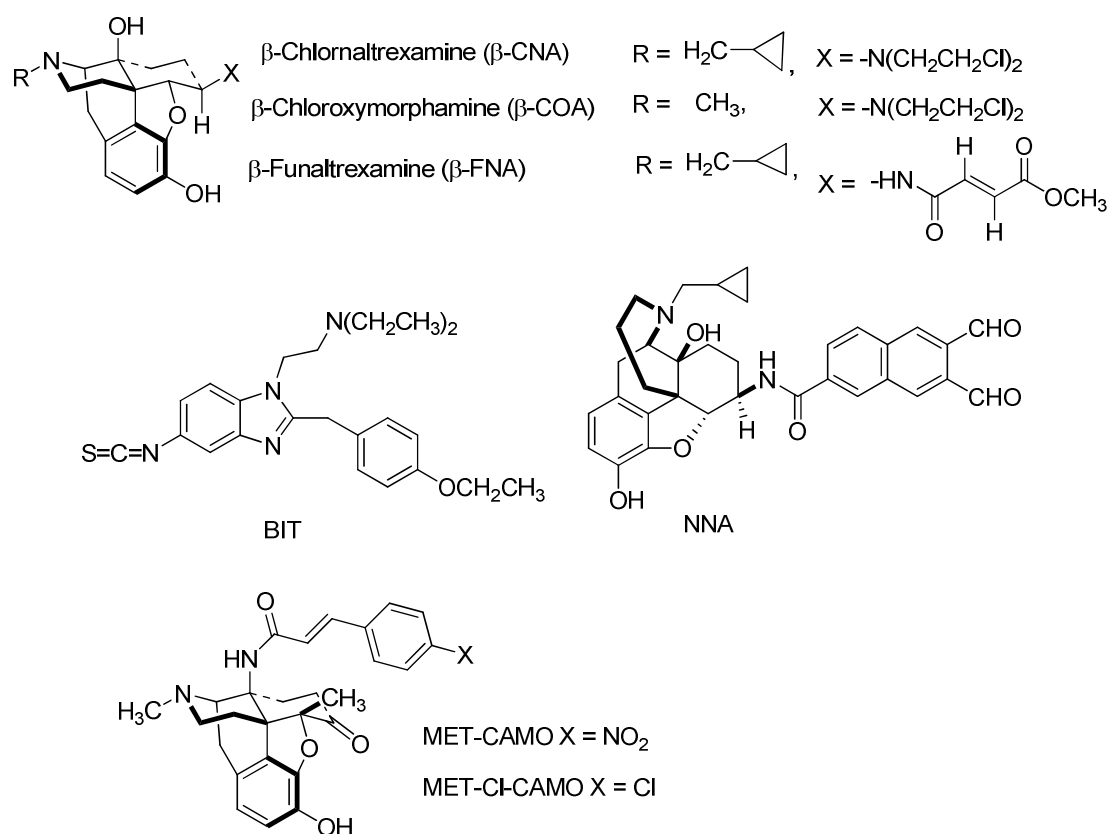


Figure 2.8. Small molecule electrophilic and reporter affinity labels for MOR

β -FNA was the first affinity label whose point of attachment to an opioid receptor was successfully determined.⁵² Liu-Chen and coworkers used molecular biology and protein isolation techniques to identify the attachment point. Initially, based on the binding of [^3H] β -FNA to MOR / KOR receptor chimeras, a region of MOR spanning from the third intracellular loop to the C-terminus was determined to be essential for irreversible binding.⁵¹ However, upon isolation and partial purification of the labeled receptor, the point of attachment was found to be in the EL2-TM5 region (Figure

activity with no agonist activity. Similarly, the *p*-chloro substituted derivative MET-Cl-CAMO (Figure 2.8) also bound irreversibly to MOR and was a long-lasting MOR selective antagonist.¹⁶⁴

Other examples of MOR selective affinity labels are hydrazone derivatives of the 6-ketone of naloxone, naltrexone and oxymphone described by Pasternak and coworkers,¹⁶⁵⁻¹⁶⁷ and the etonitazene derivative BIT designed by Rice and co-workers (Figure 2.8).^{168, 169}

Portoghese and coworkers reported the design of 'reporter affinity labels' by attaching an *o*-phthalaldehyde group to the opioid antagonists naltrexamine and 6'- and 7'- aminonaltrexamine.¹⁷⁰⁻¹⁷³ This unique approach was based on the reaction of a *o*-phthalaldehyde with an amine and thiol (from Lys and Cys side chains in the receptor), resulting in a fluorescent isoindole; detection of a fluorescence indicates that a covalent reaction has occurred. Recently, the attachment points of the reporter affinity label naltrexamine naphthalene dialdehyde derivative NNA (Figure 2.8) to MOR was determined using site-directed mutagenesis.¹⁷⁴ Lys233 and the adjacent Cys235 of TM5 were determined to be the residues involved in isoindole formation, as mutation of either of these residues prevented the generation of the fluorescent product. Although this method allows the determination of attachment points without isolating the labeled receptor, this approach is limited by the requirement of two specific nucleophilic receptor residues in close proximity to one another and the reactive functionality on the ligand.

2.4.2.2 Peptide-Based Electrophilic Affinity Labels

Although a number of nonpeptide affinity labels for opioid receptors have been reported in the literature, peptide-based affinity labels have been limited to mainly photoaffinity labels (section 2.4.1). Since endogenous ligands for opioid receptors are peptides, it is important to understand the interactions between peptides and opioid receptors at the molecular level. Peptide-based electrophilic affinity labels present a useful class of pharmacological tools which can provide valuable information regarding the binding of opioid peptides to their receptors.

The first peptide-based electrophilic affinity labels were reported by Pelton and workers in 1980.¹⁷⁵ The C-terminal chloromethyl ketone derivatives of leucine-enkephalin (Tyr-Ala-Gly-Phe-Leu-CH₂Cl) (DLECK) and its D-Ala² analog [D-Ala²,Leu⁵]enkephalin (DALECK) were prepared as potential affinity labels. Although the compounds exhibited high potency at the opioid receptors in the GPI, the analogs failed to show any irreversible activity in this tissue.¹⁷⁵ Szucs *et al.* later reinvestigated the biological effects of these compounds and reported 50% irreversible blockade of [³H]naloxone binding to opioid receptor sites in rat brain membranes by both peptides.¹⁷⁶ The tritiated derivative of DALECK was later prepared by Newman and Barnard and the preferential binding of this peptide to MOR was demonstrated. Moreover, they were able to determine the molecular weight of MOR (58 KDa) based on detection of the labeled receptor on a gel. However, the attachment point on MOR could not be determined.¹⁷⁷ There have been a few other examples of electrophilic affinity labels reported for MOR. Benyhe and coworkers

reported the chloromethyl ketone analog) of DAMGO (Tyr-D-Ala-Gly-(Me)Phe-CH₂Cl) which resulted in concentration-dependent irreversible inhibition of [³H]naloxone binding with an IC₅₀ of 1-5 μM. Shimohigashi and coworkers in 1992 reported the use of the 3-nitro-2-pyridinesulphenyl (Npys) group in a enkephalin and morphiceptin analogs. Among these, the enkephalin analog (Tyr-D-Ala-Gly-Phe-Leu(CH₂SNpys) produced irreversible inhibition of [³H]DAMGO binding in a concentration-dependent manner. The concentration required to label half of the receptors was 19 nM.¹⁷⁸

Research in our group has focused on designing and synthesizing peptidic affinity labels for MOR based on endomorphins, dermorphin or DAMGO as the parent ligand. These ligands were modified by incorporating either a bromoacetamide or an isothiocyanate functionality as the electrophile (Table 2.7). The affinities and selectivity of these peptides were evaluated in radioligand binding assays using Chinese hamster ovary (CHO) cells expressing MOR and DOR.

In the endomorphin series of analogs, the para positions of either Phe³ or Phe⁴ was modified by incorporation of an amino functionality, which was then further derivatized to yield the bromoacetamide and isothiocyanate analogs.¹⁷⁹ Although the analogs retained selectivity for MOR, they all exhibited lower affinities than endomorphin (IC₅₀ = 4.2 nM) in binding experiments. The highest affinity analog in this series was [Phe(*p*-NHCOCH₂Br)⁴]endomorphin (IC₅₀ = 158 nM).¹⁷⁹

Table 2.7 Progress towards developing peptide-based affinity labels for MOR: analogs of endomorphin, DAMGO and dermorphin.

Parent peptide	Affinity Label Modifications	Effect on MOR Affinity (IC ₅₀) Compared to Parent Peptide
Endomorphin Tyr-Pro-Phe(<i>p</i> -X)-PheNH ₂ IC ₅₀ = 4.20 ± 0.07 nM for X = H	X = -NCS, -NHCOCH ₂ Br	Affinity decreased >1000 fold ¹⁷⁹
Tyr-Pro-Phe-Phe(<i>p</i> -X)NH ₂ IC ₅₀ = 4.20 ± 0.07 nM for X = H	X = -NCS, -NHCOCH ₂ Br	Affinity decreased 40-70 fold ¹⁷⁹
DAMGO Tyr-D-Ala-Gly-(Me)Phe(<i>p</i> -X)-Gly-ol IC ₅₀ = 0.51 ± 0.1 nM for X = H	X = -NCS, -NHCOCH ₂ Br	Affinity decreased >1000 fold ¹⁸⁰
Dermorphin Tyr-D-Ala-Phe(<i>p</i> -X)-Gly-Tyr-Pro-SerNH ₂ IC ₅₀ = 0.72 ± 0.07 nM for X = H	X = -NCS, -NHCOCH ₂ Br	Affinity decreased >1000 fold ¹⁸¹
[Phe ⁵]dermorphin Tyr-D-Ala-Phe-Gly-Phe(<i>p</i> -X)-Pro-SerNH ₂ IC ₅₀ = 1.89 ± 0.2 nM for X = H	X = -NCS, -NHCOCH ₂ Br	X = -NCS, affinity decreased >200 fold X = -NHCOCH ₂ Br, IC ₅₀ = 27.7 ± 3.5 nM Did not exhibit wash-resistant inhibition of binding ¹⁸¹
[Phe ⁵ ,Lys ⁷]dermorphin Tyr-D-Ala-Phe-Gly-Phe(<i>p</i> -X)-Pro-LysNH ₂ IC ₅₀ = 1.04 ± 0.05 nM for X = H	X = -NCS, -NHCOCH ₂ Br	X = -NCS, IC ₅₀ = 92.8 ± 17.0 X = -NHCOCH ₂ Br, IC ₅₀ = 15.1 ± 2.4 nM Did not exhibit resistant inhibition of binding to MOR ¹⁸¹

Following the same design strategy, analogs of DAMGO were also prepared by modifying the para position of NMePhe⁴. However, in every instance MOR affinity decreased over 1000 fold.¹⁸⁰

Analogues of dermorphin and [Lys⁷]dermorphin were designed by modifying both the 'message' and 'address' regions of the peptides.¹⁸¹ Introduction of an electrophile in the 'message' region (the para position of Phe³) decreased MOR affinity by >1000 fold, whereas changes in 'address' region in both dermorphin and [Lys⁷]dermorphin were well tolerated. [Phe(*p*-NHCOCH₂Br)⁵]- and [Phe(*p*-NHCOCH₂Br)⁵,Lys⁷]dermorphin showed high affinity for MOR (IC₅₀ = 27.7 and 15.1 nM, respectively). However, none of the analogs in either the dermorphin or [Lys⁷]dermorphin series showed wash-resistant inhibition of binding, and hence they were not affinity labels for MOR.¹⁸¹

2.5 Significance: Peptide vs Small Molecule-Based Affinity Labels

Since pain relief is mediated mainly through MOR, it is important to understand the interactions between MOR ligands and the receptor. The endogenous ligands of the opioid receptors are peptides, and studies of chimeric opioid receptors and site-directed mutagenesis suggest that peptide ligands may interact differently with opioid receptors than non-peptide ligands.²² Therefore, information about interactions of peptide ligands with opioid receptors is complementary to that obtained for non-peptide ligands. Moreover, information obtained from chimeric and site-directed mutagenesis studies is complicated by the potential alteration of the secondary and / or tertiary structure of receptor protein. Electrophilic affinity labels, which interact in

a two-step non-equilibrium manner, can provide direct information about ligand binding to the receptor at the molecular level. Hence, peptide-based affinity labels can be utilized as valuable pharmacological tools to provide information about the binding of these ligands to MOR.

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Chapter 3.

Discovery of Dermorphin-Based Affinity Labels with Subnanomolar Affinity for Mu Opioid Receptors

* The results described in this chapter are published in ‘Sinha, Bhaswati; Cao, Zhengyu; Murray, Thomas F.; and Aldrich, Jane V. Discovery of Dermorphin-Based Affinity Labels with Subnanomolar Affinity for Mu Opioid Receptors. J.Med. Chem. **2009**, accepted.’

* Note that each chapter has independent compound numbers.

3.1 Introduction

Narcotic analgesics produce pain relief generally through activation of μ opioid receptors (MOR), but the use of these analgesics is limited by their side effects, namely respiratory depression, tolerance, constipation and physical dependence.¹ Therefore, there is an ongoing need to develop novel analgesics with fewer side effects. Understanding receptor-ligand interactions at the molecular level can facilitate the design of novel opioid ligands. Since the cloning of the three major opioid receptors, MOR, δ opioid receptors (DOR) and κ opioid receptors (KOR), in the 1990s and determination of their sequences,^{2, 3} there have been considerable advancements in understanding opioid receptor-ligand interactions. These studies have utilized chimeric receptors (such as MOR/KOR chimeras, etc.) and site-directed mutagenesis.⁴ Although these approaches have provided considerable information regarding receptor-ligand interactions, interpreting the results can be complicated by changes in the secondary and / or tertiary structures of the protein.⁴ Also while these approaches provide information about which residues in the receptor may interact with the ligand, they often do not provide information about what portions of the ligand are involved in these interactions.

Currently, there is no high-resolution crystal structure available for any of the opioid receptors. The only transmembrane receptor proteins in the GPCR family whose crystal structures have been solved are the human β_2 adrenergic receptor,^{5, 6} bovine rhodopsin in its dark state bound to 11-cis retinal,^{7, 8} and very recently a crystal structure of rhodopsin in its G-protein interacting conformation.⁹ To date, all

of the computational models of opioid receptors were based on homology modeling using crystal structures of rhodopsin bound to retinal. Very recently Xu and coworkers have proposed several key features of monomer and homodimers forms of MOR based on the crystal structure of ligand-free opsin.¹⁰ However, the main disadvantage of these methods is the lack of amino acid sequence identity between opioid receptors and rhodopsin. In order for this method of comparative modeling to be reasonably accurate, 50% or higher identity between target and template protein is desired,⁴ but comparison of opioid receptor sequences and rhodopsin indicate only ~20% identity of all residues and ~29% identity in the TM regions.⁴ Therefore, automated homology modeling of rhodopsin and opioid receptors may generate many errors, mostly from misalignment of sequences.¹¹⁻¹³ One way to improve the accuracy of such theoretical models is by utilizing adequate receptor specific (in this case opioid receptor) experimental constraints. This can only be achieved through understanding the interactions of opioid ligands with the receptors at the molecular level.

Since pain relief is mediated mainly through MOR, it is important to understand the interactions between MOR ligands and their receptor. The endogenous ligands of the opioid receptors are peptides, and studies of chimeric opioid receptors and site-directed mutagenesis suggest that peptide ligands may interact differently with opioid receptors than non-peptide ligands.⁴ Therefore, structural information obtained from interactions of peptide ligands with opioid receptors can be complimentary to that obtained for nonpeptide ligands.

3.2 Background

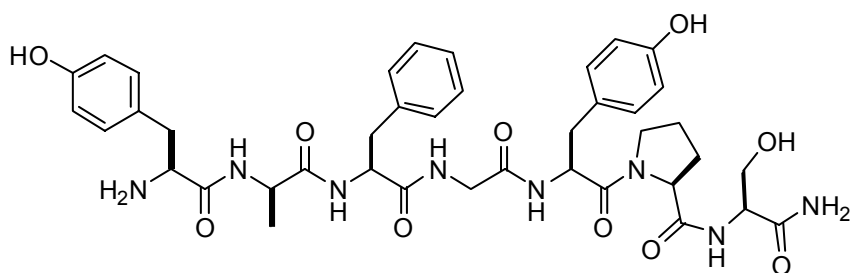
Affinity labels, which are ligands that interact with their target in a non-equilibrium manner,¹⁴ can provide detailed information about specific receptor-ligand interactions,^{15, 16} and the information obtained from affinity labels can compliment results obtained from molecular biology and computational methods. Affinity labels can be either photoaffinity or electrophilic affinity labels. Among the electrophilic affinity labels, the naltrexamine derivative β -funaltrexamine (β -FNA), a well studied affinity label for MOR, was the first electrophilic affinity label (and one of only two affinity labels¹⁷) for opioid receptors whose covalent attachment point (Lys233 in MOR) has been successfully determined.¹⁶

Although a number of nonpeptide affinity labels for opioid receptors have been reported in the literature,¹⁴ until recently peptide-based affinity labels have been limited mostly to photoaffinity labels.¹⁴ An azido-containing photoaffinity label derivative of DAMGO (Tyr-D-Ala-Gly-MePhe(*p*N₃)-Gly-ol)¹⁸ and a Bpa (*p*-benzoyl-L-phenylalanine)-containing tetrapeptide analog of morphiceptin¹⁹ are early examples of peptide-based photoaffinity labels for MOR. A disadvantage of using azido-containing photoaffinity labels is that short wavelength UV irradiation generally used to generate the reactive species can inactivate opioid receptors.²⁰ Alkylation of the receptor by electrophilic affinity labels, on the other hand, depends on the selectivity and chemical reactivity of the electrophile, and thus is not subject to the receptor inactivation that can occur with photoaffinity labels. Examples of peptide-based electrophilic affinity labels, selective for DOR, that have been reported include [D-

Ala²,Cys⁶]enkephalin (DALCE),²¹ the chloromethyl ketone of [D-Ala²,Leu⁵]enkephalin (DALECK),²² and isothiocyanate and bromoacetamide-containing TIPP (Tyr-Tic-Phe-Phe) derivatives discovered in our laboratory.^{23, 24, 25} There have been very few reports of electrophilic peptide-based affinity labels selective for MOR. The chloromethyl ketone of [Tyr-D-Ala-Gly-(NMe)Phe]enkephalin-1-4 (DAMK, IC₅₀ = 1-5 μ M for concentration dependent irreversible inhibition of [³H]naloxone binding)²⁶ and Tyr-D-Ala-Gly-Phe-Leu(CH₂S)Npys (Npys = 3-nitro-2-pyridinesulphenyl, IC₅₀ = 19 nM for concentration dependent irreversible inhibition of [³H]DAMGO binding)²⁷ are the only examples of peptide-based electrophilic affinity labels for MOR reported in the literature. Previous attempts in our group to prepare affinity labels for MOR by incorporating an electrophilic functionality such as a bromoacetamide or isothiocyanate at the para position of either Phe³ or Phe⁴ in endomorphin-2 (Tyr-Pro-Phe-PheNH₂) were unsuccessful because the modified analogs exhibited large (40- to 80-fold) decreases in MOR binding affinity compared to the parent peptide endomorphin-2.²⁸

3.3 Dermorphin and Previous Dermorphin-Based Analogs

Dermorphin (Figure 3.1), an endogenous peptide from South American frog skin,²⁹ was selected as the parent ligand for further modification in the present study. Dermorphin is a highly selective ligand with 100-fold higher affinity than morphine for MOR.²⁹ In 1981, Montecuchhi *et al.* and Brocardo *et al.* first identified dermorphin in the skin of the South American frog *Phyllomedusa sauvagei*^{30, 31} and a



Dermorphin: Tyr-D-Ala-Phe-Gly-Tyr-Pro-SerNH₂

Figure 3.1. Structure of dermorphin

dermorphin analog with hydroxyproline (Hyp) in place of Pro⁶ from the skin of *Phyllomedusa rohdei*.³² The characteristic feature of the frog skin peptides are their N-terminal tripeptide Tyr-D-aa-Phe sequence, which constitutes the ‘message’ domain³³ of these peptides (see Chapter 2.3.2.4.1 for details). This sequence is distinct from the well established tetrapeptide message sequence Tyr-Gly-Gly-Phe for the enkephalins and most of the mammalian opioid peptides.

The D-configuration at position 2 of dermorphin is critical for MOR binding and opioid activity.²⁹ Substitution of D-Ala with L-Ala results in a 100-fold decrease in MOR affinity and GPI activity.²⁹ There have been a number of reports of structure activity relationship (SAR) studies on position 2 of dermorphin. It was found that substituting D-Ala with a conformational constrained residue like D-Pro reduces the peptide’s affinity for MOR by 5000 fold.²⁹ However, replacement of D-Ala with a larger amino acid (e.g. D-Arg) is well tolerated, and D-Arg-substituted dermorphin analogs either retain, or in some cases exhibit increased, MOR affinity and analgesic potency.²⁹ Some of the [D-Arg²]dermorphin analogs are the tetrapeptide DALDA

(Tyr-D-Arg-Phe-Lys-NH₂)³⁴ and TAPS (Tyr-D-Arg-Phe-Ser-OH)³⁵ which exhibit high affinity for MOR in rat brain and potent antinociception.

C-Terminal amidation among the naturally occurring dermorphins has been found to have some beneficial effect on MOR affinity, when compared to C-terminal acid peptides.²⁹ This is thought to be due to the absence of repulsive interactions with the negative charge on the C-terminal acid derivative and also to increased resistance against carboxypeptidase-mediated cleavage.²⁹

3.3.1 Dermorphin-Based Affinity Labels

In a previous effort by our group to design potent affinity labels selective for MOR, the para position of either Phe³ in the ‘message’ sequence or a Phe in position 5 in the ‘address’ sequence of dermorphin were modified with either an isothiocyanate or a bromoacetamide functionality as a reactive electrophile.³⁶ These substitutions, however, resulted in large decreases in MOR affinity. In fact, *p*-isothiocyanate substitution on Phe³ was better tolerated by DOR, and the resulting analog exhibited higher affinity for DOR than MOR (Table 3.1). Compared to Phe³ substitution, Phe⁵-substituted analogs were generally better tolerated by both MOR and DOR (Table 3.1). Among the analogs [Phe(*p*-NHCOCH₂Br)⁵]dermorphin showed reasonable binding affinity (IC₅₀ = 27.7 nM), and hence was examined for wash-resistant inhibition of binding to MOR using [³H]DAMGO as the radioligand. Unfortunately, this ligand failed to show any evidence of irreversible binding.³⁶ In a related series of analogs, Ser⁷ of dermorphin was replaced with Lys and the

Table 3.1 Dermorphin-based potential affinity labels reported previously.³⁶

Peptide	IC ₅₀ (nM)	IC ₅₀ (nM)
	MOR	DOR
Dermorphin	0.72 ± 0.08	197 ± 28
Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH ₂		
Phe³ modifications		
$\begin{array}{c} X_1 \\ \\ \text{Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH}_2 \end{array}$		
X ₁ = NH ₂ ,	41.5 ± 7.2	>10000
X ₁ = NCS,	650 ± 720	238 ± 42
X ₁ = NHCOCH ₂ Br,	1140 ± 190	8040 ± 1050
Phe⁵ modifications		
$\begin{array}{c} X_1 \\ \\ \text{Tyr-D-Ala-Phe-Gly-Phe-Pro-Ser-NH}_2 \end{array}$		
[Phe ⁵]dermorphin	1.89 ± 0.18	218 ± 14
X ₁ = H,		
X ₁ = NH ₂	2.79 ± 0.74	212 ± 34
X ₁ = NCS	159 ± 21.8	1430 ± 280
X ₁ = NHCOCH ₂ Br	27.7 ± 3.5	373 ± 126
[Phe⁵,Lys⁷]dermorphin		
$\begin{array}{c} X_1 \\ \\ \text{Tyr-D-Ala-Phe-Gly-Phe-Pro-Lys-NH}_2 \end{array}$		
X ₁ = NH ₂	1.04 ± 0.05	476 ± 73
X ₁ = NCS	92.8 ± 17.0	5760 ± 1390
X ₁ = NHCOCH ₂ Br	15.1 ± 2.4	707 ± 142

corresponding Phe⁵-substituted analogs of [Phe⁵,Lys⁷]dermorphin showed approximately 2-fold higher MOR affinity compared to the corresponding Ser⁷ analogs, like the Ser⁷ analogs [Phe(*p*-NHCOCH₂Br)⁵,Lys⁷]dermorphin (IC₅₀ = 15.1 nM, Table 3.1) did not appear to bind irreversibly to the receptor.³⁶

3.4 Rationale for the Design of New Affinity Labels

In the present study, we chose an alternative location in the ‘message’ sequence, position 2, to incorporate a reactive functionality. Larger D-amino acids are tolerated at this position in peptides by MOR²⁹ (see section 3.3 above), suggesting that introduction of a functionality such as an affinity label into the side chain of this residue would be tolerated by the receptor. In the present study, D-Ala at position 2 was replaced by either D-Orn or D-Lys. The free amine on the side chain of these amino acids was used as a suitable handle to incorporate the electrophilic bromoacetamide or isothiocyanate functionalities (Figure 3.2). This strategy also permits varying the length of the amino acid side chain to optimize binding of the affinity label to its receptor. For this series of analogs, [D-Orn(COCH₃)²]- and [D-Lys(COCH₃)²]dermorphin served as reversible control peptides for the respective series of compounds in the pharmacological assays.

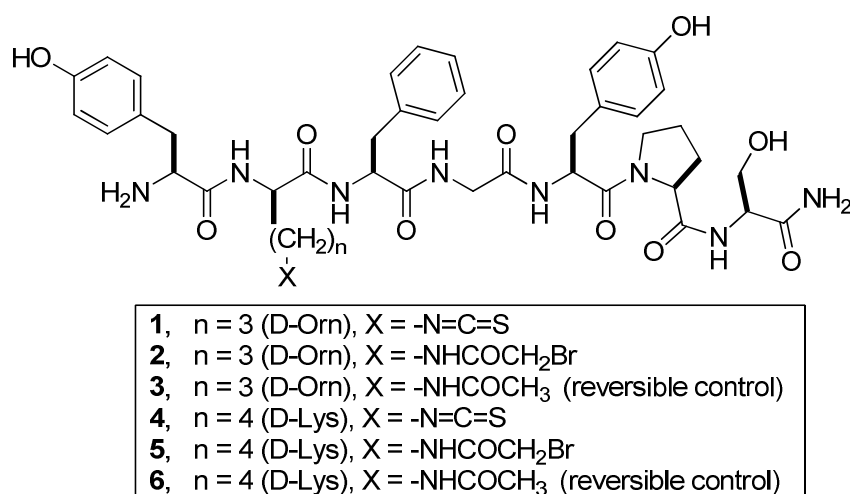


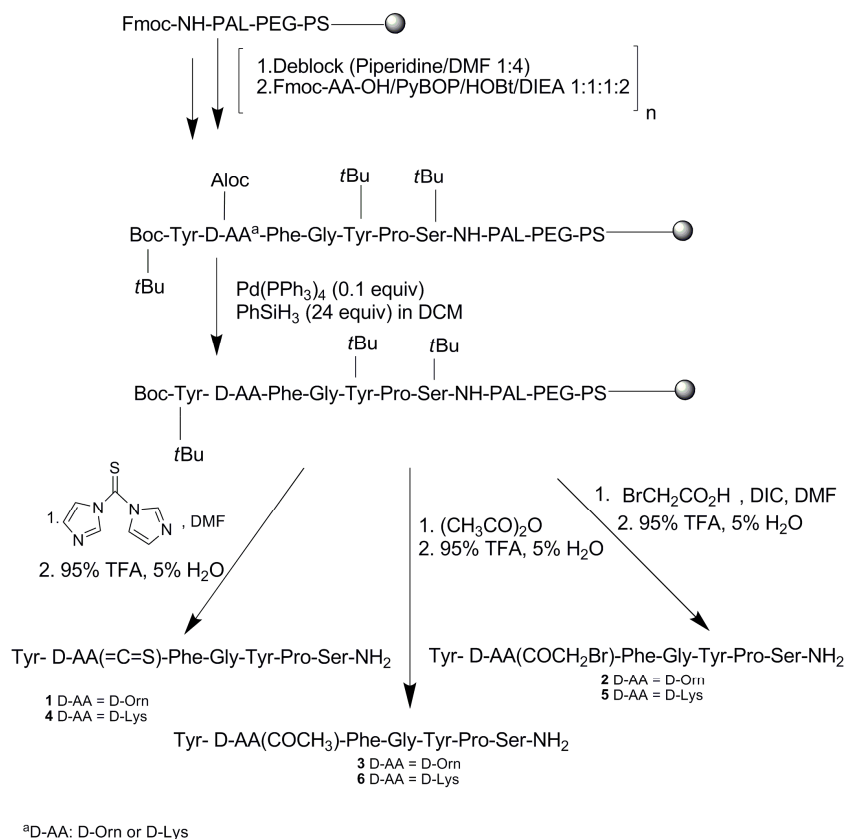
Figure 3.2. Potential affinity label derivatives for MOR and the corresponding reversible control peptides based on the parent peptide dermorphin

3.5 Results and Discussion

The potential affinity labels (**1**, **2**, **4** and **5**) and the reversible controls (**3** and **6**) were successfully prepared following a solid phase synthetic protocol developed previously in our group (Scheme 3.1).^{25, 37} Purification of the analogs was carried out by reversed phase preparative HPLC. All of the analogs (**1-6**) were obtained >97% purity as determined by analytical HPLC (Table 3.2).

Each of the peptides **1-6** displayed subnanomolar to low nanomolar affinity for MOR in in standard radioligand binding assays.³⁸ Of the analogs prepared, **1**, **2** and **4** exhibited the highest affinities for MOR (subnanomolar IC₅₀ values); their affinities were substantially higher than the corresponding Phe³-substituted analogs (IC₅₀ = 40-6050 nM).³⁶ In addition, these three potential affinity labels exhibit equal (peptide **1**)

or higher affinity (7 and 2 times higher for analogs **2** and **4**, respectively) than the



Scheme 3.1: Fmoc-based solid phase synthesis of the peptide affinity labels based on the parent peptide dermorphin

Table 3.2: Analytical data for dermorphin analogs **1-6**

Compound no.	Dermorphin Analogs	System 1 ^a t_R /Purity (%)	System 2 ^b t_R (min)/ Purity (%)	Calculated $M+H^+$	Observed $M+H^+$
1	[D-Orn(=C=S) ²]	22.7/ 100	20.86/97.6	888.3	888.3
2	[D-Orn(COCH ₂ Br) ²]	15.77/ 99.5	14.2/97.9	966.3, 968.3	966.3, 968.3
3	[D-Orn(COCH ₃) ²]	12.37/ 100.0	10.99/100	888.4	888.4
4	[D-Lys(=C=S) ²]	23.51/ 99.5	22.18/98.6	902.0	902.0
5	[D-Lys(COCH ₂ Br) ²]	16.88/ 100.0	23.69 /97.5	980.3, 982.3	980.3, 982.3
6	[D-Lys(COCH ₃) ²]	13.67/ 100.0	12.27/100	902.4	902.4

A 5-50% gradient of MeCN over 45 min at a flowrate of 1 mL / min was used.

^aSystem 1 = 0.1% trifluoroacetic acid (TFA) in aq. MeCN

^bSystem 2 = 0.09 M triethylammonium phosphate (TEAP) and MeCN

Table 3.3: Binding affinities of dermorphin derivatives for MOR and DOR^a

Dermorphin Analogs	IC ₅₀ (nM ± SEM)		^a Rel. MOR affinity	Selectivity ^b
	MOR	DOR		
1 [D-Orn(=C=S) ²]	0.81 ± 0.29	23.8 ± 2.1	0.89	29
2 [D-Orn(COCH ₂ Br) ²]	0.11 ± 0.02	342 ± 20	6.54	3110
3 [D-Orn(COCH ₃) ²]	4.25 ± 0.35	272 ± 23	0.17	64
4 [D-Lys(=C=S) ²]	0.38 ± 0.08	97.1 ± 4.9	1.89	255
5 [D-Lys(COCH ₂ Br) ²]	5.23 ± 2.31	382 ± 22	0.14	73
6 [D-Lys(COCH ₃) ²]	29.8 ± 7.6	436 ± 34	0.02	15
Dermorphin ^c	0.72 ± 0.07	197 ± 28	1.0	274

^aRelative to dermorphin (IC₅₀ MOR-dermorphin / IC₅₀ MOR-compound. [³H]DAMGO ([D-Ala²,MeNPhe⁴,glyol]enkephalin) and [³H]DPDPE (*cyclo*[D-Pen²,D-Pen⁵]enkephalin) were used as radioligands for MOR and DOR respectively.

^bIC₅₀ (DOR)/IC₅₀ (MOR). ^c From ref. 37

parent peptide dermorphin.

The two isothiocyanate-containing potential affinity labels in the two series (D-Orn and D-Lys) exhibit similar binding affinities for MOR, while the affinity of the bromoacetamide derivative **2** in the D-Orn series is 60-times higher than the corresponding D-Lys derivative **5**. Similarly, the acetylated control compound in the D-Orn series, **3**, exhibits significantly higher affinity than the corresponding control compound **6** in the D-Lys series (Table 3.3). Clearly, the lengths of the side chain in the D-Orn and D-Lys analogs as well as the identity of the attached functionality can influence binding of the dermorphin analogs to MOR. The differences in the affinities could be due to several factors, including steric and / or electronic properties that affect the interactions of the side chain with the receptor binding site.

Comparing the affinities of these peptides for DOR, the isothiocyanate derivative in the D-Orn series, **1**, exhibits unexpected high affinity for DOR, 4 times higher than the affinity of the corresponding analog **3** in the D-Lys series. The acetylated control

compounds and bromoacetamide analogs in both series (compounds **2**, **3**, **5** and **6**) show much lower affinity for DOR compared to **1** and **4**, and lower DOR affinity than the parent peptide dermorphin; no other major differences in DOR affinities was observed between the two series (Table 3.3).

Except for the isothiocyanate derivative **1**, the D-Orn series of compounds are more selective for MOR over DOR than the corresponding D-Lys compounds. The potential affinity label derivative with the highest apparent selectivity is [D-Orn(COCH₂Br)²]dermorphin, **2**, which exhibits >3000-fold difference in the IC₅₀ values for MOR over DOR, 50-fold more selective than the reversible control compound **3** and 11-fold more selective for MOR than the parent peptide dermorphin (Table 3.3). In contrast, [D-Lys(COCH₂Br)²]dermorphin, **5**, exhibits 4-fold lower selectivity for MOR compared to dermorphin due to the large decrease in MOR affinity. For the isothiocyanate derivatives, however, the trend for selectivity is reversed. The D-Orn(=C=S)² derivative **1** is 9-fold less selective for MOR than dermorphin and also 2-fold less selective than [D-Lys(=C=S)²]dermorphin, **4** (Table 3.3). The selectivities were calculated using IC₅₀ values which are a function of radioligand concentrations used; therefore comparisons of the selectivities for these peptides to those reported in other studies should be made with caution.

Since all four potential affinity labels showed subnanomolar to nanomolar affinity for MOR, they were examined to determine whether they may bind covalently to MOR. Wash resistant inhibition of [³H]DAMGO binding by these four analogs, **1**, **2**, **4** and **5**, at their IC₅₀ values was determined according to the procedure described

previously.³⁹ The acetylated derivatives **3** and **6** were included as reversible controls to verify that the washing procedure completely removed noncovalently bound compound. The washing procedure removed >80% of both reversible control peptides. In the D-Orn series, [D-Orn(=C=S)²]dermorphin (**1**) at its IC₅₀ value (0.43 nM) caused 40 ± 8% loss of [³H]DAMGO binding compared to control membranes (P<0.001) even after extensive washing (Figure 3.3), suggesting that this peptide bound covalently to a nearby nucleophile in the binding site of MOR.⁴⁰

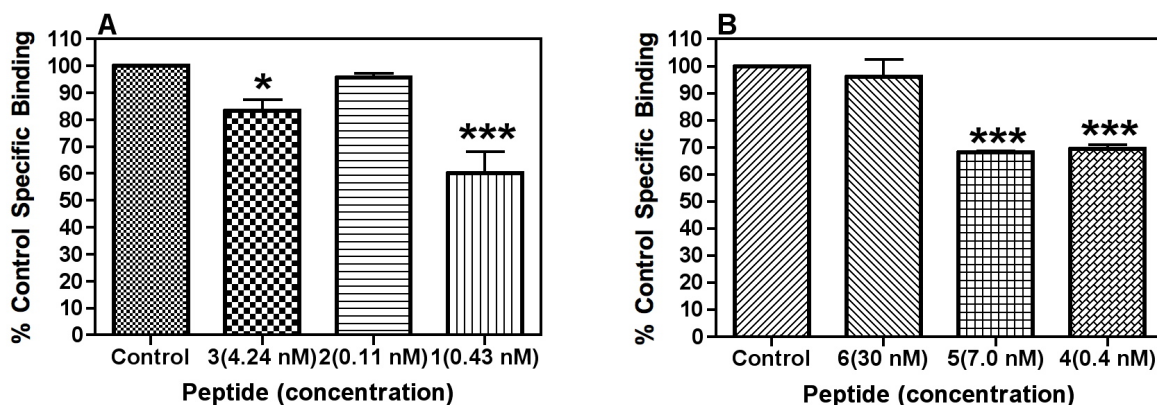


Figure 3.3.: (A) Wash-resistant inhibition of binding of [D-Orn²]dermorphin (**1-3**) and (B) [D-Lys²]dermorphin (**4-6**) derivatives. The concentration of the peptide in the incubations are indicated in parenthesis. * = p<0.05, *** = p<0.01 compared to control membranes

In contrast, although [D-Orn(COCH₂Br)²]dermorphin (**2**) shows the highest MOR affinity (IC₅₀ = 0.11 nM) of all the compounds tested, this peptide did not exhibit wash-resistant inhibition of binding to MOR at a concentration equal to its IC₅₀ of 0.11 nM (Figure 3.3), but was effectively removed by the washing procedure. However, when the wash-resistant inhibition of binding experiments were repeated at

higher concentrations (1 and 10 nM) this analog did show statistically significant concentration-dependent wash-resistant inhibition of binding ($P < 0.001$) compared to control membranes (Figure 3.4). In the D-Lys series, both the bromoacetamide and isothiocyanate derivatives exhibit statistically significant ($P < 0.001$) inhibition of [^3H]DAMGO binding after extensive washing; the inhibition of [^3H]DAMGO

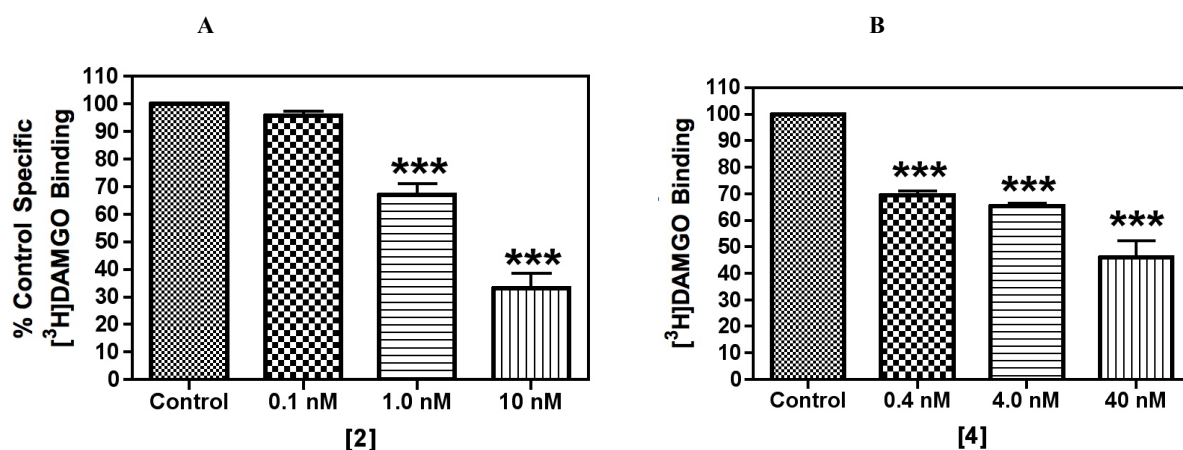


Figure 3.4: Concentration-dependent wash-resistant inhibition of binding of dermorphin analogs. A: [D-Orn(-COCH₂Br)²]dermorphin (2), B: [D-Lys(=C=S)²]dermorphin (4). ***= $p < 0.001$ compared to control

binding by compounds 4 and 5 was $31 \pm 2\%$ for both compounds (Figure 3.3). Moreover, compound 4 produced concentration-dependent wash-resistant inhibition of [^3H]DAMGO binding at higher concentrations of 4 and 40 nM ($P < 0.001$, Figure 3.4).

Time dependent wash-resistant inhibition of binding of the affinity labels 1-4 will be carried out in the near future. Since 1 exhibited considerable affinity for DOR ($\text{IC}_{50} = 24 \text{ nM}$, Table 3.3), wash-resistant inhibition of binding of 1 to DOR will also be examined.

3.6 Conclusions

In conclusion, we have successfully identified a series of dermorphin-based affinity label analogs that show exceptionally high affinity ($IC_{50} = 0.1-5$ nM) for MOR in standard binding assays. These analogs were designed by modifying position 2 of dermorphin, which is a new strategy for designing peptide-based affinity label derivatives of opioid peptides that has not been previously reported. This resulted in a substantial improvement in MOR binding affinity (between 10- to 100-fold) compared to the previous dermorphin-based analogs synthesized in our laboratory in which the para position of Phe³ or a Phe in position 5 of dermorphin or [Lys⁷]dermorphin were modified.³⁶ Three of the four potential affinity labels in the present study show subnanomolar affinity for MOR, indicating the side chains in [D-Orn(X)²]dermorphin (X = -COCH₂Br or =C=S) and [D-Lys(=C=S)²]dermorphin are well tolerated in the binding pocket of MOR. All four potential affinity labels (**1**, **2**, **4** and **5**) exhibit wash-resistant inhibition of binding to MOR. This suggests that these compounds likely bind covalently to MOR.

Comparison of the binding affinities reported for previous MOR selective affinity labels and the analogs prepared in the present study indicate that the dermorphin-based affinity labels have substantially higher MOR affinity. Previously Tyr-D-Ala-Gly-Phe-Leu(CH₂S)Npys was reported to be the highest affinity ($IC_{50} = 19$ nM in radioligand binding assays) peptide-based electrophilic affinity label for MOR, although it lacked selectivity and also showed similar affinity for DOR ($IC_{50} = 12$ nM in radioligand binding assays).²⁷ Importantly, three of these four affinity labels, [D-

Orn(=C=S)²]- (1), [D-Orn(-COCH₂Br)²]- (2) and [D-Lys(=C=S)²]dermorphin (4) appear to have higher affinity (approximately 3- to 20-fold) than the well-studied nonpeptide MOR affinity label β -FNA (IC₅₀ = 2.2 nM in radioligand binding assays).^{15, 41} With the successful identification of the peptide-based electrophilic affinity labels, the next step will be to use these affinity labels to study MOR.

3.7 Experimental

3.7.1 Solid Phase Peptide Synthesis (SPPS) of Dermorphin-Based Affinity Labels.

Materials

The PAL-PEG-PS (peptide amide linker-polyethylene glycol-polystyrene) resin and DIEA (*N,N*-diisopropylethylamine) were purchased from Applied Biosystems (Foster City, CA). 1-Hydroxybenzotriazole (HOBt) and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem (San Diego, CA). Piperidine was purchased from Aldrich Chemical Company (Milwaukee, WI), and the standard Fmoc (9-fluorenylmethoxycarbonyl) - protected L-amino acids were purchased from Novabiochem. Fmoc-D-Lys(Aloc)-OH and Fmoc-D-Orn(Aloc)-OH were purchased from Bachem (San Carlos, CA). Bromoacetic acid and 1,1'-thiocarbonyldiimidazole (TCD) were purchased from Acros Organic (New Jersey). Phosphoric acid was purchased from ICN Biomedicals

Inc. (Aurora, OH). All HPLC-grade solvents (acetonitrile, *N,N*-dimethylformamide (DMF), dichloromethane (DCM), and methanol), acetic acid, diethyl ether and triethylamine used for peptide synthesis or HPLC were purchased from Fisher Scientific Co. (St. Louis, MO). TFA was purchased from Pierce (Rockville, IL).

Synthesis of dermorphin analogs

The peptide analogs were synthesized by solid phase peptide synthesis according to methods previously developed in our laboratory.³⁷ Amino acids used for synthesis were Fmoc protected except for the N-terminal Tyr which was Boc (*tert*-butyloxycarbonyl) protected. The side chain protecting groups were *t*Bu for Ser and Tyr, and Aloc (allyloxycarbonyl) for D-Orn or D-Lys. Peptide synthesis was carried out on a high load PAL-PEG-PS resin (0.38 to 0.40 mmol/g, Scheme 3.1).³⁷ The coupling reactions for the five C-terminal residues (Phe-Gly-Tyr-Pro-Ser) were performed on a CS Bio (model CS336) automated peptide synthesizer generally for 2.5 h; the couplings generally used a 4-fold excess of amino acid with PyBOP and HOBt (4 equiv each) as the activating reagents and DIEA (8 equiv) as the base in DMF except where noted. Coupling of Fmoc-D-Lys(Aloc) and Fmoc-D-Orn(Aloc) were performed manually for 2 h using a 2-fold excess of the amino acid with PyBOP and HOBt (2 equiv each) and DIEA (4 equiv) in DMF. Completion of the reactions was confirmed by either a negative ninhydrin test for coupling to a primary amine or a negative chloranil test for coupling to a secondary amine. Fmoc deprotection was achieved using 20% piperidine in DMF (2 x 20 min); the resin was then washed with

DCM:DMF (1:1, 10 min). The N-terminal amino acid Boc-Tyr(*t*Bu)-OH was then coupled manually (4-fold excess) using PyBop, HOBt (4-fold excess each) and DIEA (8-fold excess) in DMF. After peptide chain assembly, the Alloc group was deprotected using a catalytic amount of tetrakis triphenylphosphine palladium(0) (0.1 equiv) in DCM twice for 30 min, in the presence of phenyl silane (24 equiv) as a scavenger for the allyl group.^{37, 42} After the deprotection, the resin was washed extensively following a published procedure.^{37, 43} The resin (300 mg) was then divided into three parts. One part (100 mg) was treated with a large excess (~20 equiv) of acetic anhydride in DMF (3-4 mL) for 40 min to obtain the reversible control compound. To obtain the isothiocyanate derivative, the second part of the resin (100 mg) was treated with thiocarbonyldiimidazole (TCD, 4 equiv) in a minimum amount of DMF (3 mL) for 4 h. To obtain the bromoacetamide derivative the remaining part of the resin (100 mg) was reacted with bromoacetic acid (10 equiv) and *N,N*-diisopropylcarbodiimide (DIC, 8 equiv) overnight. The bromoacetic acid was preactivated with DIC in a minimal amount of DMF (3 mL) and then this mixture was added to the resin. The completion of these reactions was confirmed by a negative ninhydrin test.

3.7.2 Cleavage from Resin

Each part of the resin was then cleaved by treating with 95% TFA and 5% water (total volume 1.5 mL) for 2 h, followed by filtration. For the acetylated reversible controls, the peptide cleaved from the resin was diluted with 3-4 mL of 10% aqueous acetic acid and then back extracted with ether (2 x 2 mL). The aqueous layer was

collected and lyophilized to give the crude peptide. For the affinity label peptides, extraction with ether was not carried out. Instead, the TFA solution was concentrated under nitrogen, diluted with 10% acetic acid (3-4 mL) and lyophilized to give the crude products.

3.7.3 Purification and Analysis

The crude peptides were purified by preparative reversed-phase HPLC (Shimadzu LC-6AD system equipped with a Shimadzu SPD-10AVP detector) on a Vydac C₁₈ column (10 μ , 300 Å, 22 x 250 mm). For the affinity label analogs (**1**, **2**, **4** and **5**) the purified fractions were immediately lyophilized to avoid potential degradation of the affinity labels by water acting as a nucleophile. For purification, a linear gradient of 5-50% aqueous MeCN containing 0.1% TFA over 45 min at a flow rate of 20 mL/min was used. The purification was monitored at 214 nm and 280 nm.

The purity of the final peptides was verified by analytical HPLC (Shimadzu LC-10ATVP system equipped with a Shimadzu SPD-10AVP detector) with a Vydac C₁₈ column (5 μ , 300Å, 4.6 x 50 mm). The purity was evaluated in two solvent systems. A linear gradient of 5-50% solvent B at a flow rate of 1 mL/min was used; the eluents were monitored at 214 and 280 nm. For system 1, solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in MeCN. For system 2, solvent A was 0.09 M TEAP at pH 2.5 and solvent B was MeCN.⁴⁴ Molecular weights of the pure peptides were verified by ESI-MS mass spectroscopy (Table 3.2) using a Waters LCT Premier time of flight mass spectrometer.

3.7.4 Pharmacological Assays

The purified peptides were examined for affinity for MOR and DOR following standard radioligand binding assays using [^3H]DAMGO and [^3H]DPDPE, respectively, as the radioligands. Based on the binding affinities and selectivity obtained from initial radioligand binding experiments, compounds **1-6** were then further evaluated for wash-resistant inhibition of binding of [^3H]DAMGO to MOR.

3.7.4.1 Radioligand Binding Assays

Radioligand binding assays for compounds **1-6** were performed using membranes derived from Chinese hamster ovary (CHO) cells stably expressing MOR and DOR. CHO cells were harvested at confluency in 25 mM Tris buffer (pH 7.4) and centrifuged at 40,000g for 25 min. The pellets were resuspended using 25 mM Tris buffer, pH 7.4. This procedure was repeated 3 times. Incubations were carried out in triplicate with varying concentrations of peptides (0.1 nM to 10 μM) for 90 min at room temperature using 1 nM [^3H]DAMGO and 0.15 nM [^3H]DPDPE as radioligands for MOR and DOR, respectively. Binding assays were performed in the presence of protease inhibitor cocktail (10 μM bestatin, 3 μM captopril and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+} . To determine the nonspecific binding, 10 μM of unlabeled DAMGO and DPDPE was used for MOR and DOR, respectively. The reactions were stopped by filtration using a 48 or 96-well Brandel cell harvester. The filters were incubated with scintillation cocktail (9.5 mL) with shaking for at least 6 h. The radioactivity was then measured by scintillation counting for 5 min.

3.7.4.2 Wash-Resistant Inhibition of Binding Assays²³

CHO cells stably expressing MOR were homogenized in 25 mM Tris buffer (30 mL), pH 7.4, using a Dounce glass tissue homogenizer (Pestle A, 10-12 times) and centrifuged at 40,000 g for 25 min. The pellets were resuspended using 25 mM Tris buffer, pH 7.4, and this procedure of centrifugation-resuspension was repeated for a total of 3 times. The CHO membrane homogenates (9.9 mL at 200 µg/mL protein) were preincubated in borosilicate glass tubes in the presence or absence of 100 µL of experimental peptides (**1-6**) at the final concentrations indicated in Figures 3.3 and 3.4. Each tube was gently inverted 3 times every 15 min. After 90 min incubation, the homogenates were centrifuged at 40,000 g for 15 min. The pellets were resuspended in 25 mM Tris (8 mL) using a teflon / glass homogenizer. For each sample a separate homogenizer was used to avoid cross-contamination. The centrifugation and resuspension steps were repeated for a total of 5 washes. After the fifth centrifugation, the pellets were homogenized in 25 mM Tris buffer, pH 7.4 (12 mL). The radioligand binding assay was then performed as described above. The results are expressed as percentage of control membranes that were preincubated in the absence of compounds (Figures 3.3 and 3.4).

3.8 Bibliography

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Chapter 4.

**Synthesis and Evaluation of DAMGO-Based Affinity Labels for
MOR and Discovery of an Unexpected Side Reaction**

* Note that each chapter has independent compound numbers.

4.1 Introduction

There are three main classes of opioid receptors: μ , δ and κ , which belong to the superfamily of G-protein coupled receptors (GPCR).¹ Among these, μ opioid receptors (MOR) have been the main target of opioid analgesics.² Although the clinically important opioid analgesic agents, e.g. morphine, methadone, fentanyl and related drugs, produce pain relief through activation of MOR, these agents are also associated with severe side effects, namely respiratory depression, constipation, tolerance and physical dependence.¹

In order to develop potent analgesics with less severe side effects it is imperative to understand the interaction of opioid ligands with their receptors at the molecular level. Morphine, the prototypical MOR agonist, and the related analogs are non peptide in nature, but the endogenous ligands for MOR, which were identified after successful characterization of multiple opioid receptors in the 1970s, were found to be peptides.^{3, 4} The endogenous mammalian opioid peptides for MOR are the enkephalins, β -endorphin,⁵ Met-enkephalin-Arg-Phe,⁶ endomorphin-I and endomorphin-II.^{7, 8} Since different ligands (peptide vs nonpeptide) may interact differently with receptors,⁹ information obtained from the interactions of peptides with receptors can be complimentary to that obtained about the interactions of non-peptidic ligands.

Currently, there is no high-resolution crystal structure available for any of the opioid receptors. The only transmembrane receptor proteins in the GPCR family

whose crystal structures have been solved are the human β_2 adrenergic receptor,^{10, 11} rhodopsin in its dark state bound to 11-cis retinal,¹² and very recently the crystal structure of rhodopsin in its G-protein interacting conformation.¹³ To date, all of the computational models of opioid receptors were based on homology modeling starting from crystal structure of rhodopsin bound to retinal. However, the main disadvantage of this method is the lack of sequence homology between amino acid sequences in opioid receptors and rhodopsin. Therefore, homology modeling of opioid receptors based on rhodopsin may generate a number of errors, mostly from misalignment of sequences.¹⁴⁻¹⁶ One way to improve the accuracy of such theoretical models is by utilizing receptor specific (in this case opioid receptor) experimental constraints.

Since the cloning of the three major opioid receptors in the 1990s and determination of their sequences,^{17, 18} there have been considerable advancements in understanding opioid receptor-ligand interactions. The study of chimeric receptors and receptors containing point mutations has revealed the complexity of receptor-ligand interactions, including differences between the interactions of the same ligand with different receptors as well as differences in interactions of different ligands with the same receptors.⁹ These data should be interpreted with caution, as changes in the primary sequence of a receptor could have significant effects on the secondary and / or tertiary structure of the receptor protein which in turn can affect the affinities of various ligands.⁹ Use of a more direct pharmacological approach could avoid this potential problem.

Affinity labels, compounds that bind to their receptors in a non-equilibrium manner, can provide specific information based on the attachment point of the ligands to their receptors¹⁹ which can then be used as ‘anchor points’ to evaluate and improve current computational models for receptor-ligand interactions. Therefore, affinity labels can be utilized as complimentary pharmacological tools to existing molecular biology techniques and computational models. An affinity label, as described in chapter 2, consists of two different structural elements: an affinity core that binds to the binding site in the receptor, and a reactive group (i.e. an electrophilic group or photoaffinity label) to bind covalently to the receptor. In the first step, the affinity label binds to the receptor in a reversible manner. In the second step, depending upon the reactivity of the affinity label and its proximity to a nearby functionality on the receptor, the affinity label may undergo irreversible binding to the receptor. This second step can provide additional selectivity to the ligand for a given receptor.¹⁹ Among electrophiles, Michael acceptors, halomethylketones and isothiocyanate have been commonly used.¹⁹ A number of non-peptide based affinity labels for opioid receptors have been reported in the literature. Among these β -FNA (β -funaltrexamine), a fumarate methyl ester derivative of naltrexone, has been one of the most extensively used affinity label derivative for opioid receptors.²⁰ This is the first of only two affinity labels whose point of attachment to any opioid receptor (Lys233 in TM 5) has been identified.^{21, 22} Portoghese and co-workers recently identified the attachment points of a reporter affinity label, an analog of naltrexamine containing a fluorogenic naphthalene dialdehyde moiety, to Lys233 and Cys235 of MOR.²³

As mentioned earlier, because of potential differences in the interactions of peptide and non-peptides with opioid receptors,⁹ complimentary information can be obtained from peptide-based affinity labels. However, peptide-based affinity labels have mostly been limited to photoaffinity labels.¹⁹ Early examples of peptide-based photoaffinity labels for MOR are an azido containing analog of DAMGO (Tyr-D-Ala-Gly-MePhe(*p*N₃)-Gly-ol)²⁴ and a tetrapeptide analog of morpheceptin containing Bpa (*p*-benzoyl-L-phenylalanine).²⁵ However, a disadvantage of using azido containing photoaffinity labels is that the short wavelength UV irradiation generally used to generate the reactive species can inactivate opioid receptors.²⁶ Alkylation of the receptor by electrophilic affinity labels, on the other hand, depends on the selectivity and chemical reactivity of the electrophile, and the receptors are not subjected to the photoinactivation that can occur with photoaffinity labels. Examples of peptide-based electrophilic affinity labels, selective for δ opioid receptor (DOR), that have been reported include [D-Ala²,Cys⁶]enkephalin (DALCE),²⁷ the chloromethyl ketone of [D-Ala²,Leu⁵]enkephalin (DALECK),²⁸ and isothiocyanate and bromoacetamide-containing TIPP (Tyr-Tic-Phe-Phe) derivatives discovered in our laboratory.²⁹⁻³¹ There have been very few reports of electrophilic peptide-based affinity labels selective for MOR. The chloromethyl ketone of Tyr-D-Ala-Gly-(NMe)Phe (DAMK, IC₅₀ = 1-5 μ M for dose-dependent irreversible inhibition of [³H]naloxone binding)³² and Tyr-D-Ala-Gly-Phe-Leu(CH₂SNpys) (Npys = 3-nitro-2-pyridinesulphenyl, IC₅₀ = 19 nM for irreversible inhibition of [³H]DAMGO

binding)³³ are the only examples of peptide-based electrophilic affinity labels for MOR reported in the literature.

4.2 DAMGO-Based Affinity Labels: Design Strategy

Continuing our effort to design new, selective and potent peptide-based affinity labels for MOR, we chose DAMGO, a highly potent and selective agonist for MOR,³⁴ as a parent ligand for further modification (Figure 4.1). DAMGO is 200-fold selective for MOR over DOR and shows negligible affinity for kappa opioid receptors (KOR).³⁴ DAMGO was identified from a series of analogs based on the

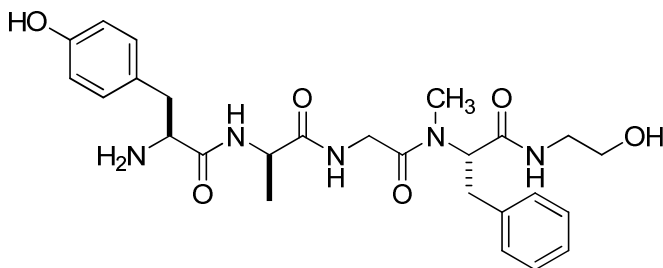


Figure 4.1. DAMGO (Tyr-D-Ala-Gly-NMePhe glyol)

tetrapeptide Tyr-D-Ala-Gly-MePhe-OH³⁴ which was identified earlier by modification of enkephalin (Tyr-Gly-Gly-Phe-Leu(Met)-OH).³⁴ The substitution of a D-amino acid in position 2 of the enkephalin resulted in enzymatically stable analogs.³⁵ Because of its high affinity and selectivity for MOR, DAMGO is routinely used to characterize MOR and thus represents an attractive lead ligand to design affinity labels. There has been only one report of a photoaffinity label derivative of DAMGO in the literature. Tyr-D-Ala-Gly-NMePhe(*p*-N₃)glyol is a DAMGO-based

photoaffinity label with similar selectivity as DAMGO for MOR but with lower affinity than the parent compound ($K_i = 25 \text{ nM}$).²⁴ A higher concentration of the photoaffinity probe ($0.3 \text{ }\mu\text{M}$) was required to label 50% of the MOR population, making the labeling experiments very challenging.²⁴ Moreover, the UV wavelength (254 nm) used for photoactivation of the receptor bound ligand could potentially inactivate the opioid receptor.²⁶

As an alternative strategy, electrophilic affinity labels were designed by incorporating either a bromoacetamide or an isothiocyanate functionality at the *para* position of NMePhe⁴ by another member in our research group (Figure 4.2). However

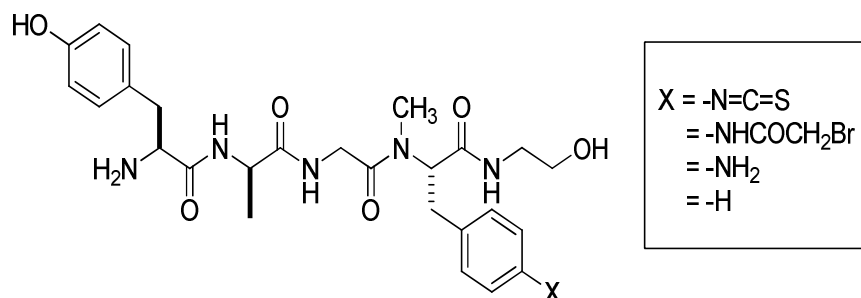


Figure 4.2. Previously designed DAMGO-based affinity labels

the modified analogs exhibited >1000-fold decrease in MOR affinity, and thus this modification was not tolerated by MOR at all (Table 4.1).³⁶

Table 4.1 Binding affinity of previously prepared DAMGO affinity labels.

DAMGO Analogs	MOR IC ₅₀ ± SEM (nM)
DAMGO	0.51 ± 0.1
[N-MePhe(<i>p</i> -NH ₂) ⁴]DAMGO	160 ± 35
[N-MePhe(<i>p</i> -NCS) ⁴]DAMGO	2490 ± 1070
[N-MePhe(<i>p</i> -NHCOCH ₂ Br) ⁴]DAMGO	1210 ± 52

4.3.1 Loading of Fmoc-Gly-ol onto the DHP-HM Resin and Synthesis of DAMGO Analogs

To introduce the glyol functionality into the proposed DAMGO derivatives, the first step of the synthesis (Figure 4.4) involved loading of Fmoc-Glyol onto the DHP-HM resin in the presence of pyridinium *p*-toluene sulfonate (PPTS) in dichloroethane

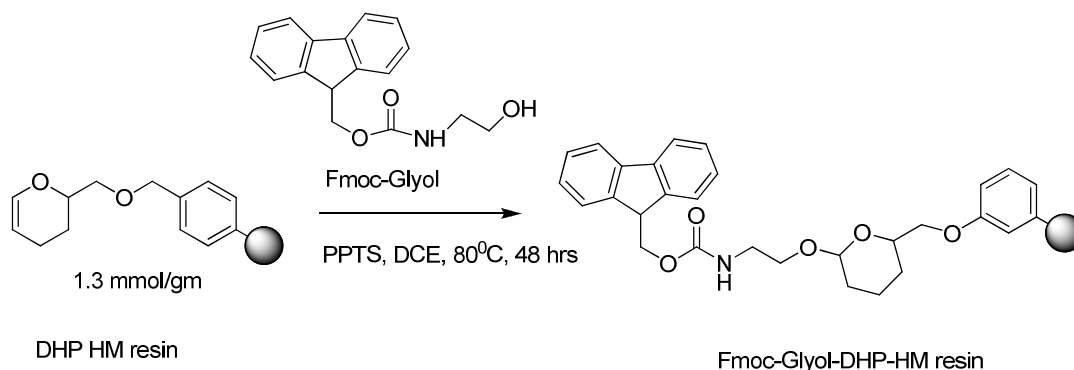
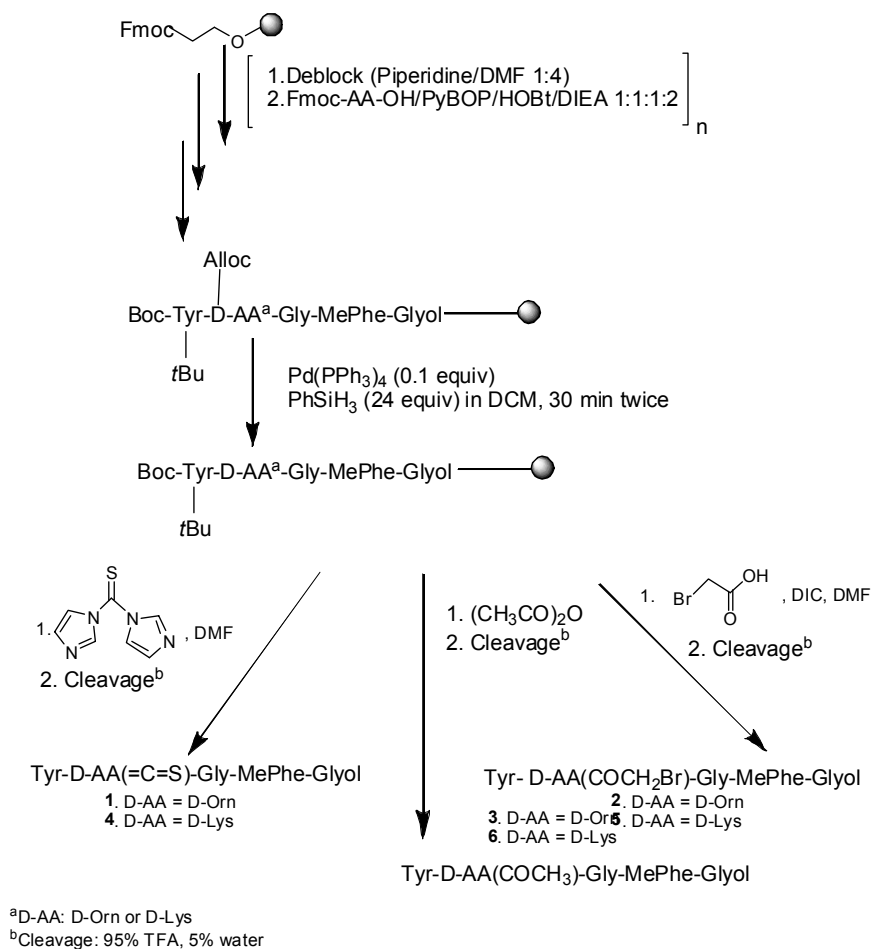


Figure 4.4. Loading of Fmoc-Glyol onto the DHP-HM resin.

(DCE).³⁸ The percentage loading was determined by Fmoc quantitation which is based on measuring the absorbance of the dibenzofulvene adduct formed by deprotection of the Fmoc group from the resin following treatment with piperidine. The loading was 91%, which is within acceptable limits.

With the Fmoc-Glyol loaded DHP-HM resin in hand, the syntheses of the proposed DAMGO derivatives (Scheme 4.1) were then carried out according to the solid phase peptide synthesis protocol described in Chapter 3 (section 3.7.1).



Scheme 4.1 Synthetic scheme for DAMGO analogs.

4.3.2 Side Reaction: Formation of Cyclic O-Alkyl Thiocarbamates

Although the synthesis and purification of the acetamide and bromoacetamide derivatives **2**, **3**, **5** and **6** proceeded smoothly (Table 4.2), analyses of the isothiocyanate containing DAMGO analogs revealed a side reaction. The analysis of purified fractions from the attempted synthesis of **4**, [D-Lys(=C=S)²]DAMGO, by HPLC and ESI-mass spectrometry revealed an unexpected result: two pure fractions

had the same molecular weight ([D-Lys(=C=S)²]DAMGO, 613.2), but the fractions differed in their HPLC retention times by ~8 min (Table 4.3 and Figure 4.5). A similar patterns in HPLC and mass spectra were seen in the case of the attempted synthesis of [D-Orn(=C=S)²]DAMGO, **1**. Therefore, we investigated the side reaction by characterizing fractions A and B of **4**.

Table 4.2 Analytical data for DAMGO analogs **2**, **3**, **5** and **6**

Compound no.	DAMGO Analogs	System 1 ^a t _R /Purity(%)	System 2 ^b t _{R (min)} / Purity (%)	Calculated M+H ⁺	Observed M+H ⁺
2	[D-Orn(COCH ₂ Br) ²]-	14.05/ 98.4	10.03/ 98.0	677.2	677.2, 679.2 ^c
3	[D-Orn(COCH ₃) ²]-	8.85/ 100.0	6.88/ 98.0	599.3	599.3
5	[D-Lys(COCH ₂ Br) ²]-	13.14/ 97.4	11.15/ 97.0	691.2	691.2, 693.2 ^c
6	[D-Lys(COCH ₃) ²]-	10.04/ 97.8	8.35/ 85.3	613.7	613.3

5-50% gradient of MeCN over 45 min at a flowrate of 1 mL/min were used.

^aSystem 1 = 0.1% TFA in aq. MeCN

^bSystem 2 = 0.09 M TEAP and MeCN

^cDue to isotopes of Br

Table 4.3 HPLC retention times and molecular weights of two pure fractions (A and B) obtained from the attempted synthesis of [D-Lys(=C=S)²]DAMGO, **4**

Fraction	System 1 ^a (t _R)	Observed M+H ⁺
A	8.72	613.2
B	15.35	613.2

^aSystem 1 = 0.1% TFA in aq. MeCN

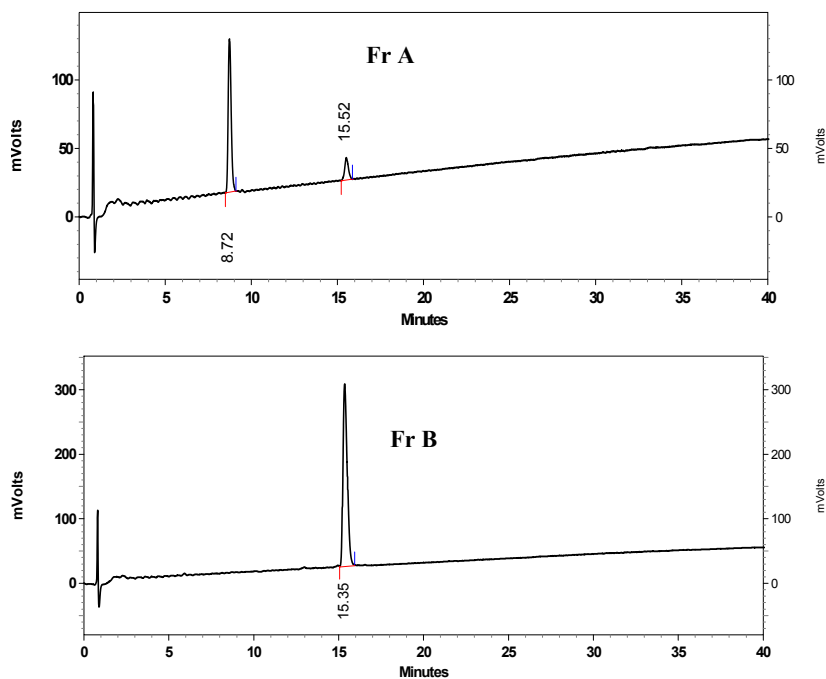
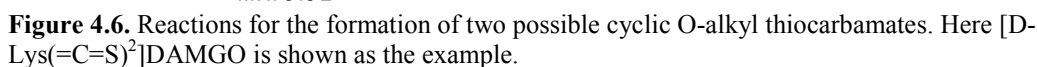


Figure 4.5. HPLC spectra of fractions A (top) and B (bottom) obtained during purification of the products obtained from the attempted synthesis of [D-Lys(=C=S)²]DAMGO, **4**, in a 15-50% gradient of MeCN containing 0.1% TFA over 45 min at a flowrate of 1 mL/min.

When the fractions were investigated the following day, the MS/MS of both fractions were identical, suggesting that one of the samples could have degraded to yield the second product. Subsequent analytical HPLC using the same gradient as above showed a major peak at 8.72 min for both fractions, indicating that fraction B had converted to A. Based on these results, it was proposed that the linear [D-Lys(=C=S)²]DAMGO reacted to yield a cyclic analog.

Based on the hypothesis mentioned above, the following possible side products were proposed (Figure 4.6):³⁹



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cyclic thiocarbamate. Based on this reaction (a) seems to be the one which is more likely to have occurred. To identify the compounds, the peptides were characterized by Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopy.³⁹

4.3.2.2 Characterization of the Side Products: FTIR of Fr A and Fr B

If either of the proposed reactions (Figure 4.6) occurs, then one of the fractions A or B would show IR stretching for the isothiocyanate functionality and the other would not because of the formation of the cyclic product (either **I** or **II**, Figure 4.6). Figure 4.7 shows the IR spectra of fractions A and B, analyzed as KBr pellets. The IR spectra of fraction A did not show any bands characteristic of an isothiocyanate functionality (around 2100-2200 cm^{-1}), whereas fraction B has a distinct band around 2189 cm^{-1} (Figure 4.7) which is characteristic of the isothiocyanate functionality. These results are consistent with our hypothesis that a thiocarbamate cyclic product was formed which was isolated as fraction A.³⁹

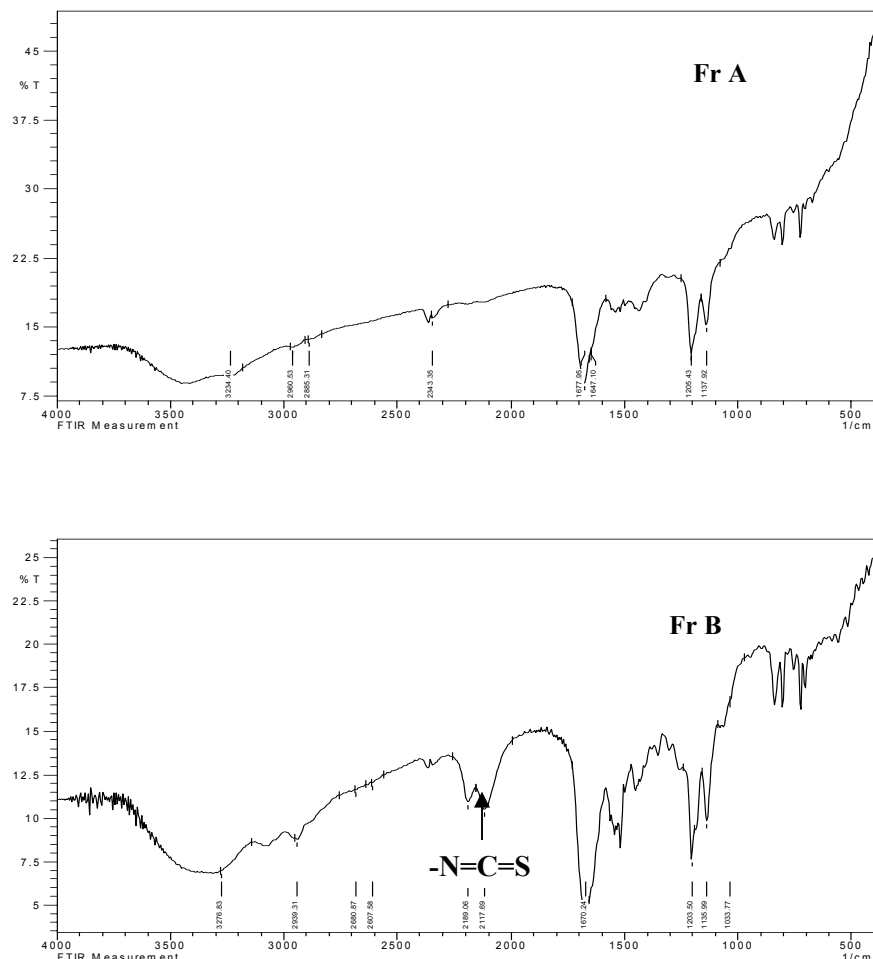


Figure 4.7. IR spectra of fractions A (top) and B (bottom) obtained from the attempted synthesis of $[\text{D-Lys(=C=S)}^2]\text{DAMGO}$, **4**. Fraction B shows isothiocyanate stretching at around 2100-2190 cm^{-1} (indicated by arrow), whereas fraction A does not.

4.3.2.3 Characterization of the Products by Proton NMR

Next, we used NMR to determine which of the two proposed products (Figure 4.6.) were formed from the cyclization reaction. Table 4.4 shows the chemical shifts of relevant functional groups. As can be seen from the table, both fractions showed a singlet at 9.35 ppm corresponding to the phenol functionality of the N-terminal Tyr.

This result rules out the possibility of reaction (b) (Figure 4.6) since there would be no Tyr phenol proton in this potential side product. Fraction A did not show any peak

Table 4.4 ^1H NMR data of fractions A and B obtained from the attempted synthesis of [D-Lys(=C=S) 2]DAMGO, **4**

Fraction	δ (ppm)	Corresponding Functional Group ^a
A	No broad peak around 3.4	No glycol (OH)
	9.35 (singlet)	Tyr phenol
B	3.4 (broad)	Glycol
	9.35 (singlet)	Tyr phenol

^aexchanged upon treatment with D₂O

for the glycol hydroxyl group (around 3.4 ppm), suggesting that this functionality was not involved in the side reaction; and hence the identity of the cyclic O-alkyl thiocarbamate in fraction A is structure **I** (Figure 4.7). Fraction B showed a broad band around 3.4 ppm (Table 4.4), characteristic of an aliphatic hydroxyl group, i.e. from the glycol functionality, indicating that fraction B is the desired linear isothiocyanate containing peptide. As expected for hydroxyl groups, the peaks at 3.4 and 9.35 were exchangeable with D₂O.

4.3.3 Radioligand Binding and Wash-Resistant Inhibition of Binding Assays

Since compounds **1** and **4** resulted in unstable side products, these two compounds were not subjected to radioligand binding assays. The other four DAMGO analogs **2**, **3**, **5** and **6** were tested for their binding affinity to both MOR and DOR, stably expressed on CHO cells, following the standard procedure. These results are shown in Table 4.5

Table 4.5: Binding affinities of DAMGO derivatives for MOR and DOR under standard assay conditions

DAMGO Analogs	IC ₅₀ (nM ± SEM)		IC ₅₀ ratio (DOR/MOR)
	MOR	DOR	
2 [D-Orn(COCH ₂ Br) ²]	0.45 ± 0.06	33.1 ± 0.9	73
3 [D-Orn(COCH ₃) ²]	0.58 ± 0.11	102 ± 6	176
5 [D-Lys(COCH ₂ Br) ²]	0.45 ± 0.25	103 ± 1	229
6 [D-Lys(COCH ₃) ²]	1.13 ± 0.22	268 ± 28	237
DAMGO	0.51 ± 0.01	281 ± 20	550

Compounds **2**, **3**, **5** and **6** exhibited very high affinity towards MOR in the range of 0.4 - 1 nM (Table 4.5). The binding affinities of all of the analogs for MOR were comparable to that of DAMGO (Table 4.5). Therefore, modification by incorporating derivatives of either D-Orn or D-Lys to DAMGO derivatives were well tolerated by MOR. As far as selectivity is concerned, compounds **3**, **5**, and **6** showed an IC₅₀ ratio of >100-fold, whereas the bromoacetamide analog **2**, [D-Orn(COCH₂Br)²]DAMGO, showed somewhat higher affinity for DOR and therefore lower selectivity for MOR. However, the bromoacetamide analog in the D-Lys series, compound **5**, exhibited high selectivity for MOR over DOR (237 fold, Table 4.5). Thus, the difference in side chain length in D-Lys vs. D-Orn (one less methylene group in D-Orn) influenced the binding affinity for DOR.

Compounds **2**, **3**, **5** and the **6** were also examined for wash-resistant inhibition of [³H]DAMGO binding to MOR according to the established procedure (see Chapter 3). The washing procedure effectively removed control compound **3** in the D-Orn² series, (Figure 4.8). As seen in Figure 4.8, compound **2** exhibited >40% inhibition of

[³H]DAMGO binding to MOR compared to untreated control membranes. This suggests that compound **2** may interact with MOR in a non-equilibrium manner, and

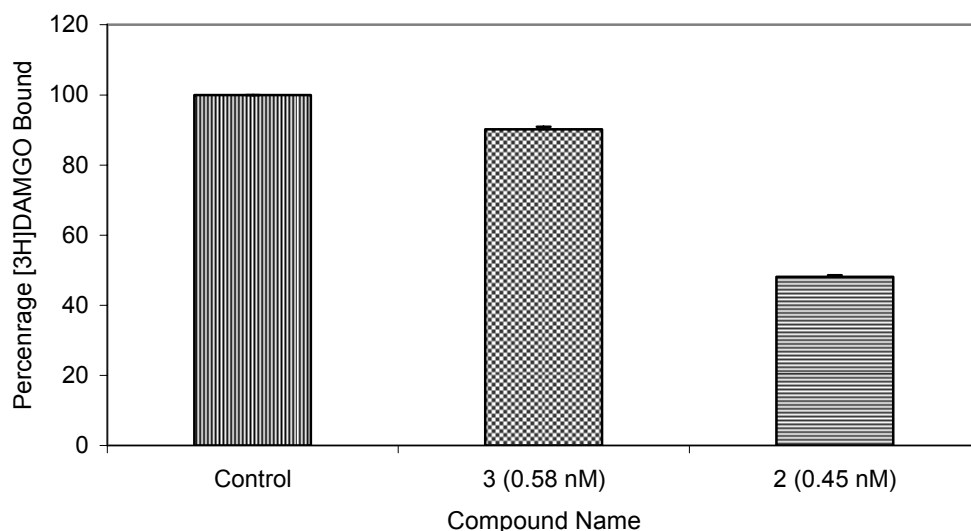


Figure 4.8. Wash-resistant inhibition of binding of [D-Orn²]DAMGO analogs **2** and **3**. The membranes were incubated with the peptides at the concentrations indicated in parentheses.

thus be an affinity label for MOR. However, the corresponding analog **5** in the D-Lys series [D-Lys(COCH₂Br)²]DAMGO, **5**, was removed by washing (Figure 4.9). Therefore, **5** may not appear to be affinity label for MOR. It is important to note that the small difference in the side chain of the amino acid at the 2nd position (D-Orn in the case of **2** and D-Lys in the case of **5**) could have a significant effect on covalent binding to opioid receptors. The discovery of this new DAMGO-based electrophilic affinity label **2** demonstrated a successful strategy for developing peptide-based affinity labels through fine tuning the position of the affinity label to facilitate alkylation at the binding site in MOR.

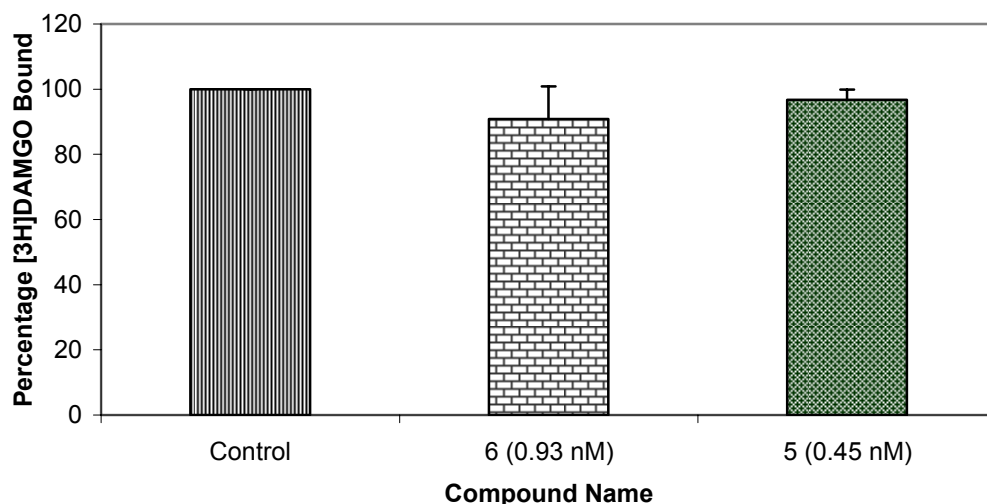


Figure 4.9. Wash-resistant inhibition of binding by [D-Lys²]DAMGO analogs: **5** and **6**

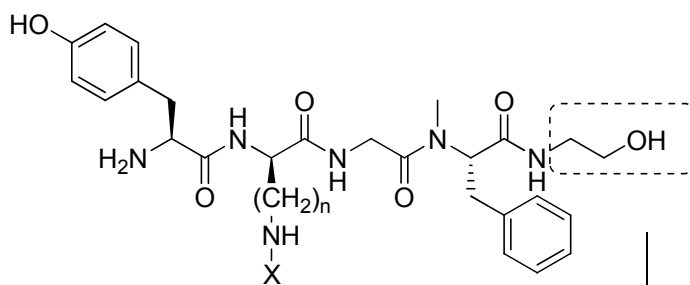
4.3.4: Overcoming the Side Reaction: Design and Synthesis of DAMGA ([D-Ala²,N-MePhe⁴,Gly⁵]enkephalinamide) Analogs.

Since the DAMGO-based isothiocyanate analogs [D-Orn(=C=S)²]DAMGO, **2**, and [D-Lys(=C=S)²]DAMGO, **4**, resulted in the formation of cyclic O-alkyl thiocarbamate derivatives, these isothiocyanate analogs were not tested for binding to MOR. As discussed earlier, characterization of the linear peptide and cyclic side product revealed that the C-terminal glycol functionality participated in the formation of the cyclic product. Therefore, in order to overcome the side reaction, we modified the C-terminal glycol functionality.

The C-terminal glycol functionality was replaced by the glycyl amide functionality which can not participate in the cyclization. The bromoacetamide and the reversible acetylated control compounds for both [D-Orn²]- and [D-Lys²]DAMGA were also

prepared, as indicated in Figure 4.10, to compare their affinities for MOR with the previously prepared DAMGO analogs.

DAMGO Analogs



DAMGA Analogs

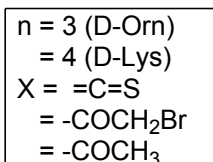
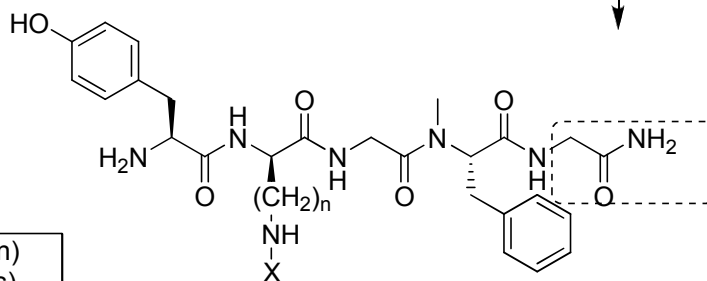
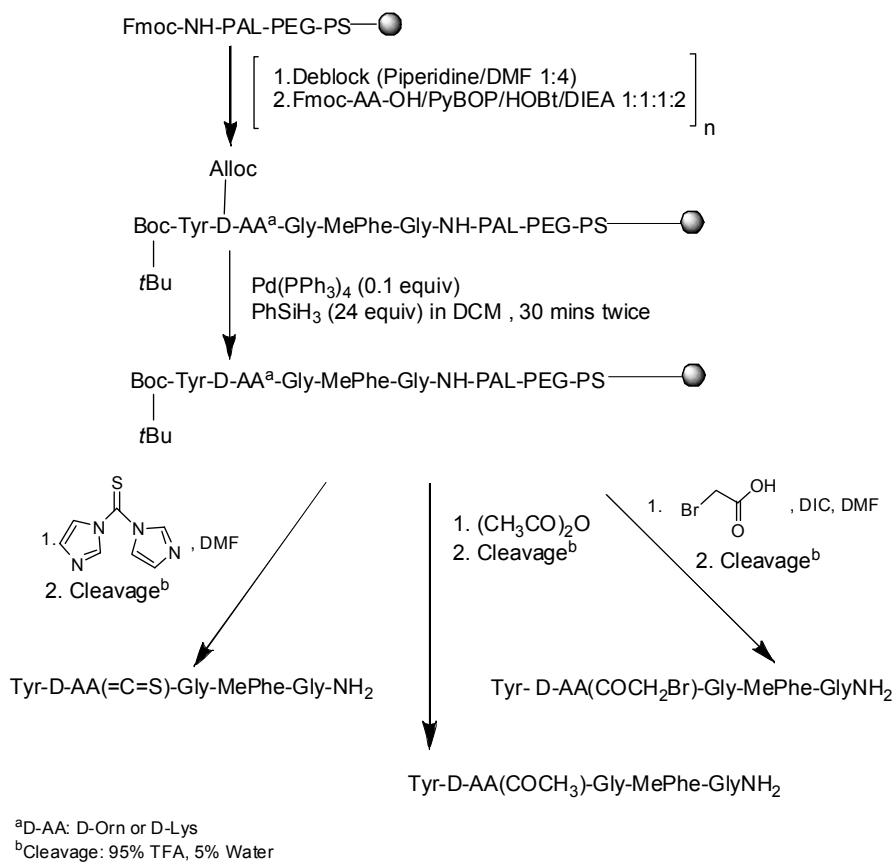


Figure 4.10. Modifications incorporated in the DAMGA analogs

derivatives of DAMGO was successfully overcome by replacing the glycol with a glycylamide. The isothiocyanate analogs **7** and **10** (Table 4.6) were successfully synthesized and purified without any formation of side product.



Scheme 4.2. Synthesis of DAMGA analogs

Table 4.6 Analytical data for DAMGA analogs **7-12**

Compound no.	DAMGA Analogs	System 1 ^a	System 2 ^b	Calculated M+H ⁺	Observed M+H ⁺
		t _R /Purity(%)	t _{R (min)} / Purity (%)		
7	[D-Orn(=C=S) ²]-	19.48/ 100	17.32/ 100	612.3	612.3
8	[D-Orn(COCH ₂ Br) ²]-	12.76 /100	10.22/ 94.9	690.2	690.2, 692.2
9	[D-Orn(COCH ₃) ²]-	9.49/ 100	7.29/ 100	612.3	612.3
10	[D-Lys(=C=S) ²]-	20.61/ 100	19.02/ 100	626.3	626.3
11	[D-Lys(COCH ₂ Br) ²]-	14.10/ 100	11.77/ 100	704.2	704.2,706.2
12	[D-Lys(COCH ₃) ²]-	11.29/100	8.82/ 97.1	626.3	626.3

A 5-50% gradient of MeCN over 45 min at a flowrate of 1 mL / min was used.

^aSystem 1 = 0.1% TFA in aq. MeCN

^bSystem 2 = 0.09 M TEAP and MeCN

The binding affinities of the DAMGA derivatives **7-12** to MOR and DOR were then evaluated in radioligand binding assays as described previously. All of the modified analogs **7-12** exhibited subnanomolar affinity for MOR (Table 4.7), comparable to that of parent peptide DAMGO, indicating that the C-terminal

Table 4.7 Binding affinities of DAMGA derivatives for MOR and DOR

DAMGA Analogs	IC ₅₀ (nM ± SEM)		IC ₅₀ ratio DOR / MOR	Relative Affinity ^a
	MOR	DOR		
7 [D-Orn(=C=S) ²]	0.35 ± 0.05	9.69 ± 0.75	28	1.45
8 [D-Orn(COCH ₂ Br)]	0.85 ± 0.32	23.4 ± 2.2	27	0.48
9 [D-Orn(COCH ₃) ²]	0.38 ± 0.13	26.5 ± 2.9	70	1.30
10 [D-Lys(=C=S) ²]	0.57 ± 0.05	74.5 ± 7.6	129	0.89
11 [D-Lys(COCH ₂ Br) ²]	0.45 ± 0.02	30.6 ± 0.7	67	1.10
12 [D-Lys(COCH ₃) ²]	0.71 ± 0.04	105 ± 11	148	0.72
DAMGO	0.51 ± 0.01	281 ± 20	550	1.0

^a Relative to DAMGO

replacement by the alcohol to amide was well tolerated by MOR. However, most of the DAMGA analogs (**7-9** and **11**) showed considerable affinity for DOR as well, in the range of 10-30 nM (Table 4.7). All of the analogs show significantly higher affinity for DOR when compared to DAMGO ($IC_{50} = 281$ nM). Therefore, the C-terminal glycol functionality plays an important role in conferring selectivity for MOR by decreasing affinity for DOR. The [D-Lys²]DAMGA series of analogs, however, showed greater selectivity for MOR compared to the [D-Orn²]DAMGA series of analogs. The loss in selectivity for MOR resulting from the replacement of glycol with the glycylamide functionality appears to be counterbalanced to some extent by the extra methylene unit in the side chain of D-Lys in the case of **10** (IC_{50} for DOR = 74 nM) and **12** (IC_{50} for DOR = 105 nM) but not **11**.

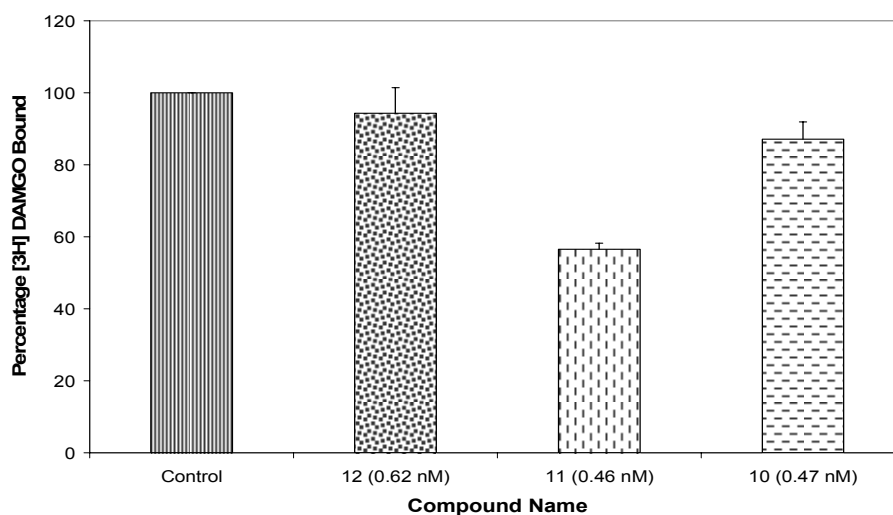


Figure 4.11. Wash-resistant inhibition of binding by [D-Lys²]DAMGA analogs **10** and **11**

The DAMGA analogs were also evaluated for wash-resistant inhibition of binding to MOR according to the established protocol described previously. Among the [D-

Lys²]DAMGA analogs, as seen in Figure 4.11, the bromoacetamide analog **11** ([D-Lys(COCH₂Br)²]DAMGA) exhibited 44% wash-resistant inhibition of [³H]DAMGO binding compared to untreated control membranes. This suggests that this compound could be binding irreversibly to MOR and thus be an affinity label. The reversible compound, **12**, did not exhibit any wash-resistant inhibition of binding, confirming the efficiency of the washing procedures in these experiments. The isothiocyanate analog, **10**, however, was removed from the membrane by the washing procedure (>90% [³H]DAMGO binding in presence of **12** at its IC₅₀ concentration, Figure 4.11). Although **11** appears to exhibit irreversible binding to MOR at its IC₅₀ value (0.46 nM), its lower selectivity for MOR over DOR (DOR / MOR = 67, Table 4.7) could be an impediment in utilizing **11** as an affinity label for MOR at higher concentrations. Wash-resistant inhibition of [³H]DPDPE binding of **11** to DOR needs to be carried out to determine whether **11** may interact irreversibly with DOR. For the [D-Orn²]DAMGA analogs, in initial wash-resistant inhibition of binding experiments the reversible control compound **9** was not removed by the washing procedure, and therefore the washing procedure may need to be modified and these experiments will need to be repeated.

4.4 Conclusions

A new series of affinity label analogs were successfully prepared using Fmoc-based solid phase synthesis procedure by replacing the D-Ala² of DAMGO with either D-Orn² or D-Lys² and attaching either a bromoacetamide (–COCH₂Br) or an isothiocyanate (=C=S) as an electrophilic functionality to the side chain amine of

either D-Orn² or D-Lys². During the purification of both [D-Orn(=C=S)]²DAMGO, **1**, and [D-Lys(=C=S)]²DAMGO, **4**, the formation of an intramolecular cyclic O-alkyl thiocarbamate side product occurred. The side reaction was verified by characterization of the products obtained from the attempted synthesis of **4** by HPLC, MS, FT-IR and NMR. The data obtained from the spectroscopic analysis of the side product supports an intramolecular attack of the C-terminal glycol on the isothiocyanate in [D-Orn(=C=S)]²DAMGO or [D-Lys(=C=S)]²DAMGO with the formation of the cyclic O-alkyl thiocarbamates. The bromoacetamide derivatives (**2** and **5**) as well as the reversible controls (**3** and **6**) were successfully synthesized, purified and evaluated for their binding affinities for MOR. The bromoacetamide analogs **2** and **5** exhibited subnanomolar binding affinities (IC₅₀ = 0.45 nM) and selectivity for MOR over DOR (IC₅₀ ratio 75-230). Interestingly only **2** showed wash-resistant inhibition of binding (>40% at its IC₅₀ concentration) of [³H]DAMGO to MOR while **5** failed to inhibit binding to MOR in a wash-resistant manner, suggesting that only **2** binds irreversibly to MOR.

The formation of the cyclic O-alkyl thiocarbamate side product from the isothiocyanate derivatives was successfully overcome by replacing the C-terminal glycol functionality of DAMGO with a glycyl amide. The corresponding DAMGA series of affinity labels were successfully synthesized and examined for receptor affinity as well as wash-resistant inhibition of binding. The bromoacetamide derivative, **11** bound to MOR in a wash resistant manner. However the isothiocyanate

analog, **12**, failed to demonstrate wash-resistant inhibition of binding at its IC₅₀ concentration.

Although all of the DAMGA analogs exhibited subnanomolar binding affinity (IC₅₀ = 0.3-0.8 nM) to MOR in initial radioligand binding experiments, most of the DAMGA analogs (**7-9** and **11**) also had nanomolar affinity for DOR (IC₅₀ = 10-30 nM). Therefore, the C-terminal glycol functionality plays an important role in imparting selectivity for MOR over DOR, and replacing it with a glycylamide resulted in significant decreases in MOR selectivity.

4.5 Experimental

The DAMGO-based affinity label analogs were prepared using solid phase peptide synthesis methodology described in the previous chapter (Chapter 3, section 3.7.1). The differences between the two synthetic schemes were in the choice of resin and loading of Fmoc-glyol onto the DHP-HM resin.

Materials

The DHP-HM resin (3,4-dihydro-2H-pyran-2ylmethoxymethyl polystyrene, 100-200 mesh) was obtained from Novabiochem (San Diego, CA). Fmoc-Gly-ol was obtained from AnaSpec Inc (Fermont, CA). Pyridinium *p*-toluenesulfonate (PPTS) was obtained from Sigma-Aldrich (Saint Louis, MO). The Fmoc-protected amino acids, the remaining chemicals and the HPLC grade solvents were obtained from the sources listed in Chapter 3 (section 3.7.1).

Synthesis of DAMGO-based analogs

4.5.1 Loading of Fmoc-Glyol on to DHP-HM Resin and SSPS of DAMGO Derivatives (Figure 4.4)³⁸

The first step in the synthesis of the DAMGO-based analogs (Figure 4.3) was loading of Fmoc-Gly-ol onto the 3,4-dihydro-2H-pyran-2-ylmethoxymethyl polystyrene (DHP-HM) resin (1.3 mmol/ g). The required amount of resin (670 mg) was swollen in dichloroethane (DCE, 6 mL) for 1 h. Fmoc-Glyol (740 mg, 3 equiv) and PPTS (327 mg, 1.5 equiv) were added to the swollen resin and the mixture stirred at 80°C for 48 h. After 48 h the resin was drained, washed with DCM (4 x 1 min), DMF (4 x 1 min) and DCM (3 x1 min) and dried under vacuum overnight.

The loading of the resin was determined by quantitative analysis of the Fmoc group deprotection.⁴⁰ The deprotection reaction rapidly generates dibenzovulvene, which is scavenged by piperidine to afford an adduct that absorbs at 302 nm. The resin substitution was calculated according to equation 1

$$\text{Substitution in mmol/ g} = (A * V * 10^3) / 7800 * W \quad (1)$$

where A= Absorbance

V= Volume (10 mL)

W = Weight of the sample resin.

For the Fmoc deprotection and quantitation reaction, three different samples were analyzed. These were Fmoc-PAL-PEG-PS resin (3-4 mg) of known substitution (0.38 mmol/g), a blank and three samples of DHP-HM resin (3-4 mg each) following

loading of Fmoc-Glyol as described above. In a 10 mL volumetric flask DCM (0.4 mL) and piperidine (0.4 mL) were added to the resin and the mixture was kept for 30 min at room temperature. Then, methanol (1.6 mL) was added to each sample and the solutions diluted to 10 mL with DCM. The resulting samples were then filtered and the absorbance of each sample recorded at 302 nm.

The substitution of DHP-HM resin was determined to be 0.87 mmol/ g following this protocol; based on the theoretical substitution (0.95 mmol/ g), and the percent loading was 91%. Following determination of the resin loading, ~650 mg of this resin was used to synthesize the analogs **1-6** (100 mg for each analog) according to the solid phase peptide synthesis procedure described previously (Chapter 3, section 3.7.1).

4.5.2 Solid Phase Peptide Synthesis of the DAMGA Derivatives

The synthesis of the DAMGA derivatives (**7-12**) started with 500 mg of Fmoc-PAL-PEG-PS (0.38 mmol/g) resin. The Fmoc solid phase peptide synthesis procedure described earlier (Chapter 3, section 3.7.1) was used to synthesize all of the analogs.

4.5.3 Cleavage from Resin

The affinity label peptides (**1, 2, 4, 5, 7, 8, 10, and 11**) were cleaved from resin using 95% TFA and 5% water, as described previously in Chapter 3.7.2, and the peptides lyophilized to give the crude products. Owing to their lower hydrophobicity, the reversible controls (**3, 6, 9, and 12**) were diluted with 3-4 mL of 10% aqueous acetic acid and then back extracted with ether (2 x 2 mL). The aqueous layers were collected and lyophilized to give the crude peptides.

4.5.4 Purification and Analysis

The crude peptides were purified by reversed phase preparative HPLC as described in Chapter 3 (section 3.7.3) using a linear gradient of 5-50% aqueous MeCN containing 0.1% TFA over 45 min at a flow rate of 20 mL/min. After purification of the affinity label analogs (**1**, **2**, **4**, **5**, **7**, **8**, **10** and **11**) the fractions were collected and immediately lyophilized to avoid potential degradation of the affinity label analogues by water. The fractions were then analyzed by analytical HPLC and ESI-mass spectrometry according to the protocol described in Chapter 3 (section 3.7.3). For **3**, **6**, **9** and **12** the pure fractions were then combined and lyophilized to get the pure peptide. For **2**, **5**, **7**, **8**, **10** and **11** the pure lyophilized fractions were combined and re-lyophilized to get the pure peptides. The purity of the final peptides were verified in analytical HPLC using two different systems: 0.1% TFA in aq. MeCN and 0.09 M TEAP and MeCN

4.5.5 Instrumentation

The FTIR spectra of KBr pellets were collected over a range of 500 to 4000 cm^{-1} on a Shimadzu FTIR-8400S spectrophotometer. Proton NMR spectra were recorded on a Bruker 500 MHz spectrophotometer in d_6 -DMSO.

4.5.6 Pharmacological Assays:

The radioligand binding and wash-resistant inhibition of binding of all of the analogs (**2**, **3**, **5**, **6** and **7-12**) were carried out with Chinese hamster ovary (CHO) cells expressing MOR and DOR according to procedures described earlier (Chapter 3,

section 3.7.4). [³H]DAMGO and [³H]DPDPE were used as the radioligands for MOR and DOR, respectively.

4.6 Bibliography

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Chapter 5.

Design, Synthesis and Evaluation of a Dermorphin-Based Multifunctional Affinity Label Probe for Mu Opioid Receptors

* Note that each chapter has compound numbers.

5.1 Introduction

The cloning of the opioid receptors in the early 1990s¹⁻⁵ led to significant advancements in understanding opioid receptor structures and receptor-ligand interactions at the molecular level.

Affinity labels, ligands that bind to their target receptors in an irreversible manner,⁶ have been useful biochemical tools to study opioid receptors. There have been reports in the literature of a large number of affinity labels, mostly nonpeptide ligands, for characterizing different opioid receptor types.^{6, 7} Nonpeptide affinity labels for opioid receptors have been predominantly electrophilic affinity labels. β -Chlornaltrexamine (β -CNA),⁸ labels all three opioid receptors, and β -funaltrexamine (β -FNA) alkylates μ opioid receptors (MOR).⁹

β -FNA, a MOR selective antagonist, was the first affinity label for opioid receptor whose point of attachment to its receptor was successfully determined by Liu-Chen and coworkers using molecular biology and protein isolation techniques. Initially, based on the binding of [³H] β -FNA to MOR / κ opioid receptor (KOR) receptor chimeras, a region of MOR spanning from the third intracellular loop to the C-terminus was determined to be essential for irreversible binding.¹⁰ However, upon isolation and partial purification of the labeled receptor, the point of attachment was found to be in the extracellular loop 2 (EL)-transmembrane 5 (TM5) region. Subsequently, site directed mutagenesis of amino acid residues in this region identified Lys233, a conserved residues in all three opioid receptors, as the attachment point.¹¹ This illustrates the challenges and limitations of studying

receptor-ligand interactions and further underscores the importance of obtaining direct experimental evidence pertaining to receptor-ligand interactions. The only other affinity label whose point of attachment has been determined is the ‘reporter affinity label’ naltrexamine naphthalene dialdehyde derivative NNA where the attachment points to MOR were evaluated using site-directed mutagenesis by Portoghese and coworkers.¹² Lys233 and the adjacent Cys235 at the top of TM5 were determined to be the residues involved in formation of a fluorescent isoindole with NNA indicating covalent binding of the reporter affinity label. Mutation of either of these residues resulted in the loss of fluorescence. Although this method allows the determination of attachment points without isolating the labeled receptor, a critical limitation to this approach is the requirement for two specific nucleophilic receptor residues, Lys and Cys, in close proximity to one another to form the fluorescent product.

Peptide-based affinity labels, on the other hand, have been predominantly photoaffinity labels, e.g. the azide derivatives of several enkephalin analogs.^{6, 7} The main disadvantage of using the azido group as a photoaffinity label for opioid receptors is that the shorter UV wavelength (254 nm) generally used to generate the reactive species can inactivate opioid receptors.¹³ Reports of peptide-based electrophilic affinity labels in literature have been limited. Prior to isothiocyanate derivatives of opioid peptides as electrophilic affinity labels designed in our group,¹⁴⁻¹⁶ opioid peptide derivatives containing electrophilic affinity labels were limited to chloromethylketone derivatives of the enkephalins,^{17, 18} the cysteine-containing

enkephalin analog of DALCE ([D-Ala²,Leu⁵Cys⁶]enkephalin Tyr-D-Ala-Gly-Phe-Leu-CysOH)¹⁹ and enkephalin analogs containing melphanan (Mel),²⁰ the nitrogen mustard derivative of *p*-aminophenylalanine.

Since pain relief is mediated mainly through MOR, it is important to understand the interactions between MOR ligands and their receptor. The endogenous ligands of the opioid receptors are peptides, and studies of chimeric opioid receptors and site-directed mutagenesis suggest that peptide ligands may interact differently with opioid receptors than nonpeptide ligands.²¹ Therefore, structural information obtained from interactions of peptide ligands with opioid receptors can be complimentary to that obtained for nonpeptide ligands.

There have been very few reports of electrophilic peptide-based affinity labels selective for MOR. The chloromethyl ketone of Tyr-D-Ala-Gly-(NMe)Phen (DAMK, IC₅₀ = 1-5 μM for dose-dependent irreversible inhibition of [³H]naloxone binding)²² and Tyr-D-Ala-Gly-Phe-Leu(CH₂SNpys) (Npys = 3-nitro-2-pyridinesulphenyl, IC₅₀ = 19 nM for dose-dependent irreversible inhibition of [³H]DAMGO binding)²³ are the only examples of peptide-based electrophilic affinity labels for MOR reported in the literature. Previous attempts in our group to prepare affinity labels for MOR by incorporating an electrophilic functionality such as a bromoacetamide or isothiocyanate at the para position of either Phe³ or Phe⁴ in endomorphin-2 (Tyr-Pro-Phe-PheNH₂) were unsuccessful because the modified analogs exhibited large (40- to 80-fold) decreases in MOR binding affinity compared to the parent peptide endomorphin-2.²⁴ Earlier attempts were also made to identify electrophilic affinity

labels based on dermorphin, an endogenous heptapeptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-SerNH₂) derived from South American frog skin that is very potent and highly selective for MOR.²⁵ Previously, the para position of Phe³ or a Phe in position 5 of dermorphin and [Lys⁷]dermorphin were modified to introduce an electrophilic functionality, i.e. a bromoacetamide or isothiocyanate group.²⁶ Modification in the ‘message’ domain (Phe³) resulted in a >1000-fold decrease in MOR affinity. While modification of a Phe in position 5 in the ‘address’ domain of dermorphin and [Lys⁷]dermorphin was well tolerated and the peptides retained nanomolar affinity for MOR, none of these modified analogs exhibited wash-resistant inhibition of binding to MOR, and therefore are not affinity labels for these receptors.²⁶

In a continued effort to develop MOR selective affinity labels, we have recently discovered dermorphin-based affinity label analogs that show exceptionally high affinity (IC₅₀ = 0.1-5 nM) for MOR. These analogs were designed by modifying position 2 of dermorphin by incorporating D-Orn or D-Lys and attaching a bromoacetamide or an isothiocyanate as the electrophilic functionality (see Chapter 3 for details), which is a new strategy for designing peptide-based affinity label derivatives of opioid peptides that has not been previously reported. This resulted in a substantial improvement in binding affinity (between 10- to 100-fold) compared to the previous dermorphin-based analogs synthesized in our laboratory.²⁶ Importantly, three of these four affinity labels, [D-Orn(=C=S)²]-, [D-Orn(-COCH₂Br)²]- and [D-Lys(=C=S)²]dermorphin appear to have higher affinity (approximately 3-20 fold) than the well-studied nonpeptide MOR affinity label β-FNA (IC₅₀ = 2.2 nM in standard

binding assays)^{10, 27} and exhibit wash-resistant inhibition of binding at very low (≤ 1 nM) concentrations equal to their IC_{50} values.²⁸ To our knowledge, these peptides are the highest affinity peptide-based affinity labels for MOR reported to date.

The long range goal of this project is to determine the attachment point of a peptide-based affinity label to MOR. In this project, [D-Lys(=C=S)²]dermorphin, one of the recently discovered dermorphin-based MOR affinity label (see Chapter 3), was selected as the lead peptide for designing a multifunctional probe with the goal of identifying the attachment point of this peptide to MOR. This ligand was selected due to its high affinity ($IC_{50} = 0.38$ nM), selectivity (IC_{50} ratio DOR/MOR = 250) and its ability to bind to MOR in a wash-resistant manner (see Chapter 3).

Peptides, due to their polymeric nature, provide definite advantages over nonpeptides in isolation studies of affinity labeled receptors. For example, appropriate residues in the peptide can be used to attach a purification tag such as biotin or d-desthiobiotin (DSB). This may then be used to assist in receptor enrichment via affinity purification (e.g., with streptavidin for biotinylated peptides). A biotinylated derivative of β -endorphin has been used to purify MOR.²⁹ Opioid receptors are transmembrane in nature are expressed at very low concentration in different cell lines.²¹ Although MOR has been expressed in *Escherichia coli*³⁰ and insect cell lines,^{31, 32} the quantities obtained from such expression were not enough to carry out spectroscopic studies.³³ Affinity purification via d-desthiobiotin-streptavidin interaction would enrich the available receptors. The lower affinity of the biotin precursor DSB for the biotin binding proteins,³⁴ e.g., streptavidin, compared to biotin

offers a distinct advantage over biotin. It is very difficult to dissociate biotinylated compounds from streptavidin due to the extremely high affinity of biotin for streptavidin ($K_d = 4 \times 10^{-14}$ M),³⁵ which further complicates the analysis of labeled receptors. This problem can be overcome by using DSB. Although there is no agreement in the literature on how much lower the affinity of DSB is compared to biotin, it is believed to be at least several orders of magnitude.³⁶⁻⁴⁰

To aid in the detection of the labeled receptor in microscopy experiments, a fluorescent group incorporated into our lead affinity label peptide [D-Lys(=C=S)²]dermorphin would be very useful. Previously, fluorescent derivatives (Alexa 488 or BIODIPY) attached to either the Lys side chain in [Lys⁷]dermorphin⁴¹ or the C-terminus of dermorphin⁴² have been reported to be well tolerated by MOR. Hence, we expect attachment of a fluorescent label to our dermorphin-based affinity label peptide will also be well tolerated. To minimize nonspecific interactions with the labeled receptor a hydrophilic fluorescent group, i.e. 5-carboxyrhodamine B or Oregon Green, was selected. These fluorophores are also insensitive to pH in the physiological range⁴³ which minimizes possible alterations of the spectrofluorometric properties of the fluorophores with any change in pH (e.g. inside the cell) during microscopy experiments.

5.2 Design of a Dermorphin-Based Multifunctional Affinity Label:

Design Strategy

To develop a multifunctional probe for MOR, the lead peptide [D-Lys(=C=S)²]dermorphin was further modified to incorporate DSB as a purification tag, Oregon Green or 5-carboxyrhodamine B as a fluorescent tag, and poly(ethylene glycol) (PEG)-like linkers to decrease the hydrophobicity of the peptide (Figure 5.1). Figure 5.2 shows the design of the dermorphin-based multifunctional affinity label peptide for MOR.

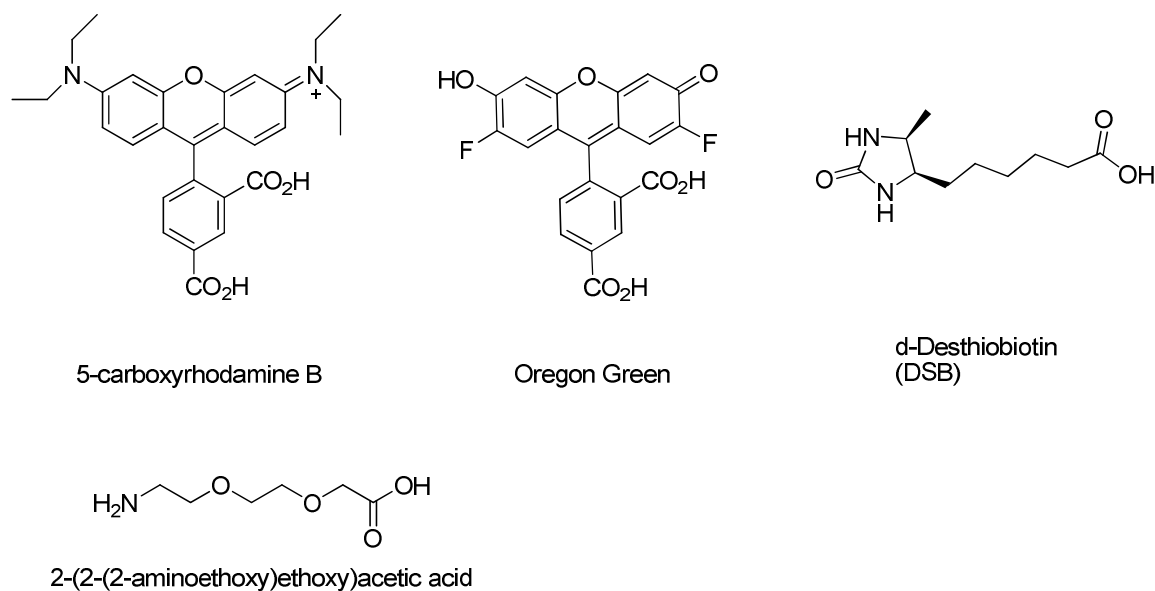


Figure 5.1. The fluorescent and purification tags and the PEG-like linker for incorporation into multifunctional dermorphin derivatives

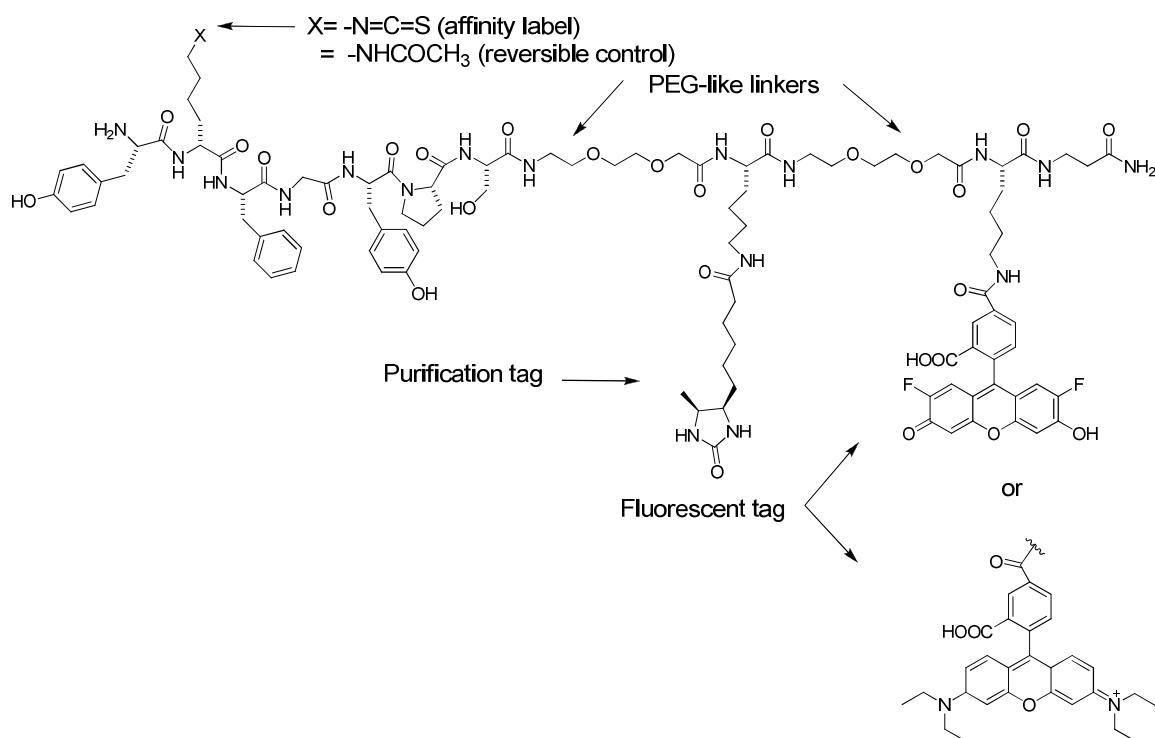


Figure 5.2. Design of dermorphin-based multifunctional affinity labels for MOR

The lead peptide [D-Lys(=C=S)²]dermorphin was extended at the C-terminus by two additional Lys residues. The Lys residues were separated from each other and the peptide by hydrophilic (PEG)-like linkers. The Lys residues were used as handles to incorporate the purification and fluorescent tags attached to the side chain amines of these residues (Figure 5.2). The fluorescent group was attached to the Lys closest to the C-terminus to prevent potential interference with receptor binding. A shorter β -alanine linker was incorporated at the C-terminus for attachment to the resin.

5.3.1 Synthesis of the Multifunctional [D-Lys(=C=S)²]dermorphin Derivative

5.3.

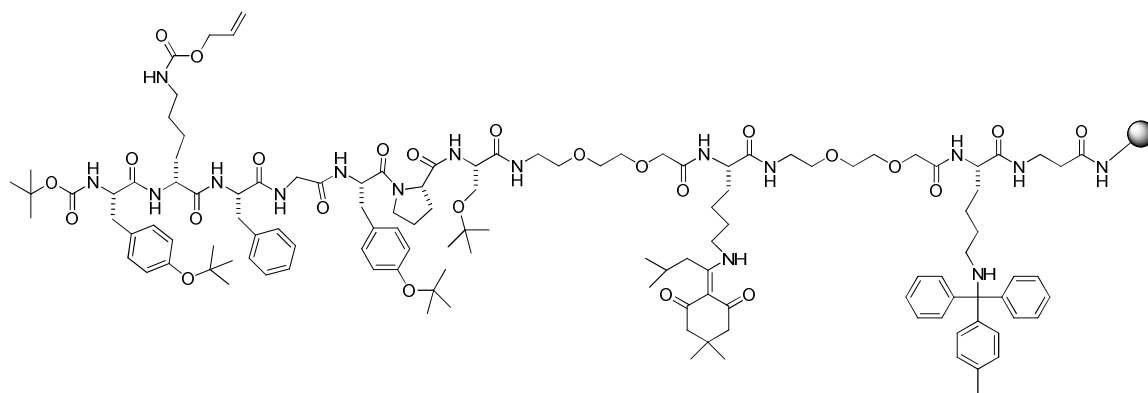


Figure 5.3 Structure of the fully protected dermorphin intermediate.

incorporate the purification tag. Next the resin was treated with 3% TFA in the presence of TIPS (triisopropylsilane) to remove the Mtt group from the Lys residue closest to the C-terminus. The free amine thus obtained was then treated with the fluorophore Oregon Green 488 5-carboxylic acid to incorporate the fluorescent tag. Residual amine groups were acetylated afterwards using acetic anhydride.

Since the cost of commercially available single isomer fluorescent dyes is very high (\$130/ 5 mg for Oregon Green), only one equivalent of this reagent was used for incorporation of Oregon Green. Alternatively, 5-carboxyrhodamine B was also used as the fluorescent tag. Although the single isomers of carboxyrhodamine are also very expensive (\$160/10 mg), the sodium salt of a mixture of 5- and 6-carboxyrhodamine B is quite inexpensive. It is commercially available as a 20% aqueous solution (\$12.60/kg, Abbey Color, Philadelphia, PA) and is marketed as Rhodamine WT (for water tracer).⁴⁶ Acidic workup of Rhodamine WT with HCl precipitates a mixture of the 5- and 6-carboxy isomers which are easily separated by preparative HPLC;⁴⁶ the resulting individual isomers were characterized by analytical HPLC, ESI-MS and NMR. However, sample preparation for purification initially met with some difficulty, as a mixture of the carboxyrhodamine B isomers was not readily soluble in a mixture of MeCN/H₂O. After trying different solvents, a 50:50 mixture of isopropanol and H₂O turned out to be the most suitable solvent; 1 mL of this mixture was used to dissolve 40 mg of the sample. Thus, 80 mg of the mixture of carboxyrhodamine B isomers could be purified at a time, resulting in ~20 mg of pure

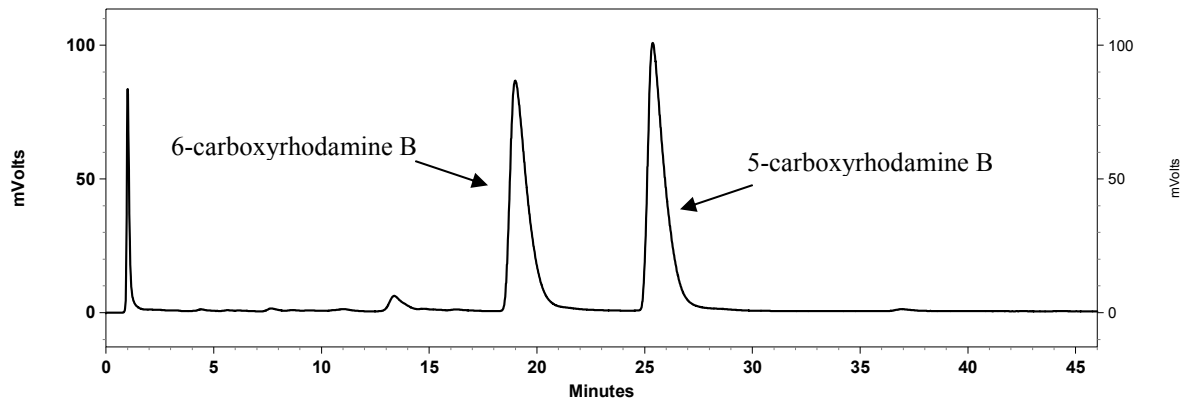


Figure 5.4. Separation of 5- and 6-carboxy isomers of rhodamine B in 20-50% MeCN containing 0.1% TFA over 60 mins at a flow rate of 1 mL/min.

5-carboxyrhodamine B. 5- and 6-carboxy isomers. The 6-carboxy isomer elutes first from the HPLC column, followed by the 5-carboxy isomer as verified by ^1H NMR spectroscopy. Similar to the coupling of Oregon Green to the multilabeled peptide, 1 equiv of 5-carboxyrhodamine B was coupled to the free amine side chain of Lys obtained after deprotection of the Mtt group with 3% TFA. The reaction was run overnight in the dark to prevent possible degradation of the fluorophore in the presence of light. Residual amine groups were acetylated afterwards using acetic anhydride. The rest of the synthesis was also carried out in the dark for the reason described above. After the coupling of 5-carboxyrhodamine B the rest of the synthesis (Aloc deprotection and incorporation of the affinity label) was carried out following the established procedures⁴⁷ (Scheme 5.1). The final cleavage of the crude peptides was carried out in 95% TFA and 5% H_2O ; extraction with ether was avoided due to their high hydrophobicities.

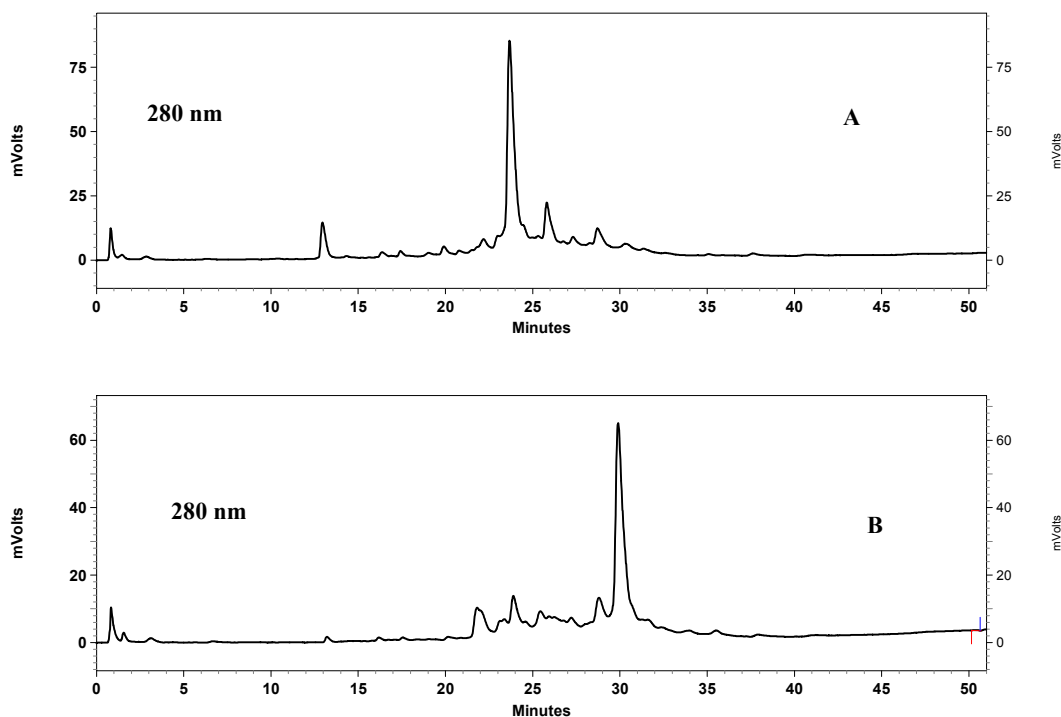


Figure 5.5. HPLC spectra of the crude multilabeled peptide. **A.** HPLC after coupling of 5-carboxyrhodamine and Aloc deprotection. **B.** Multilabeled peptide **3** after sequential coupling of 5-carboxyrhodamine, Aloc deprotection and incorporation of the isothiocyanate functionality. A 10-60% gradient of aqueous MeCN containing 0.1% TFA over 50 min at a flow rate of 1 mL/min was used for HPLC analysis.

Figure 5.5 shows the HPLC spectra of the crude multilabeled peptide and an intermediate. The crude peptides were successfully purified by preparative HPLC; the purity of all four peptides **1-4** (>97%) was determined by analytical HPLC in two different solvent systems and molecular weights were verified by ESI-MS (Table 5.1).

Table 5.1 Analytical data for multilabeled dermorphin analogs **1-4**

Multilabeled Dermorphin Analogs	System 1 ^a	System 2 ^b	ESI-MS (<i>m/z</i>)	
	<i>t_R</i> /Purity(%)	<i>t_R</i> /Purity(%)	Calculated	Observed
1 [D-Lys(=C=S) ²]Oregon Green	21.16/ 100	17.78/ 100	[M+3H] ³⁺ = 703.9 [M+2H] ²⁺ = 1055.5	[M+3H] ³⁺ = 703.9 [M+2H] ²⁺ = 1055.5
2 [D-Lys(COCH ₃) ²]Oregon Green	15.61/ 97.1	12.92/ 100	[M+3H] ³⁺ = 703.9 [M+2H] ²⁺ = 1055.5	[M+3H] ³⁺ = 703.9 [M+2H] ²⁺ = 1055.5
3 [D-Lys(=C=S) ²]5-carboxyrhodamine	24.87/ 100.0	20.91/ 99.8	[M+3H] ³⁺ = 729.0 [M+2H] ²⁺ = 1093.1	[M+3H] ³⁺ = 729.0 [M+2H] ²⁺ = 1093.1
4 [D-Lys(COCH ₃) ²]5-carboxyrhodamine	19.95/ 97.4	16.17/ 99.0	[M+3H] ³⁺ = 729.0 [M+2H] ²⁺ = 1093.0	[M+3H] ³⁺ = 728.7 [M+2H] ²⁺ = 1093.1

A 15-50% gradient of MeCN over 35 min at a flowrate of 1 mL/min was used.

^bSystem 2 = 0.09 M TEAP (triethylamine phosphate) and MeCN

^aSystem 1 = 0.1% TFA in aqueous. MeCN

5.3.2 Results from Preliminary Microscopy Experiments

To demonstrate possible binding of the multilabeled fluorescent peptide **1** containing Oregon Green as the fluorophore to MOR, initial microscopic experiments were carried out utilizing SH-SY5Y cells, a human neuroblastoma cell line, stably expressing MOR and DOR.⁴⁸ Panel A in Figure 5.6 shows the fluorescence obtained after incubating the SH-SY5Y cells with 400 nM of compound **1** for 90 min at 4° C followed by extensive washing of the cells. In panel C, the cells were first incubated with 400 nM of **1** at 4° C for 90 min followed by incubation with 10 μM of naloxone, which is an antagonist with high affinity for MOR,⁷ for 30 min. The fluorescence due to the peptide did not appear to decrease as shown in panel C (Figure 5.6, indicated

by arrows), indicating that naloxone could not displace the fluorescent peptide from

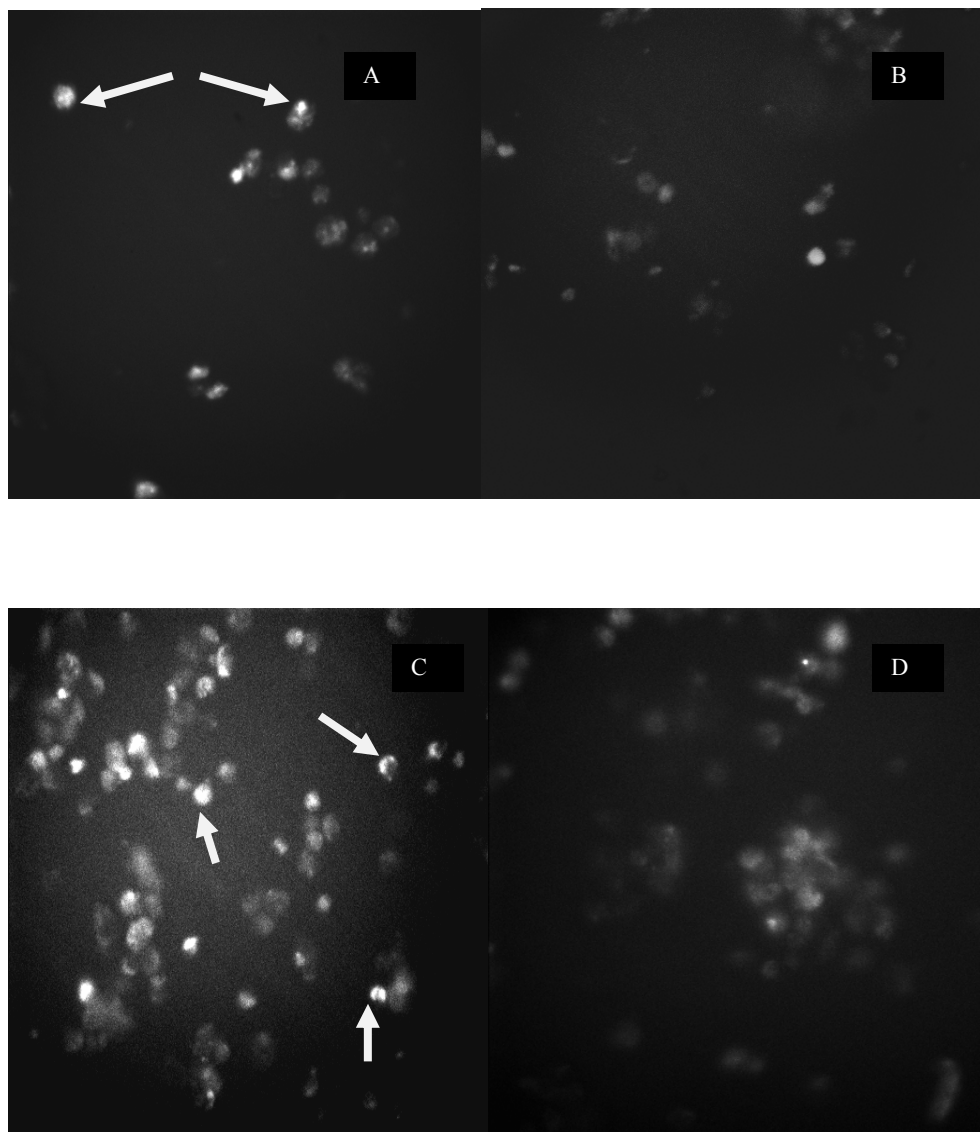


Figure 5.6. Labeling of SH-SY5Y cells by the [D-Lys(N=C=S)²dermorphin derivative **1** containing Oregon Green. A: Labeling of cells with the 400 nM of the fluorescent peptide **1** alone at 4° C for 90 min (indicated by arrows). B: Protection experiment in which cells were first incubated with naloxone (10 μM) for 30 min followed by labeling with **1** at 4° C for 90 min. C: Labeling of cells with the peptide (indicated by arrows) for 90 min, followed by incubation with naloxone. D: Cells not incubated with peptide or naloxone for 90 min. Cells were extensively washed following incubation. The fluorescence of Oregon Green was measured following excitation at 494 nm and emission at 531 nm.

MOR and suggesting that the peptide may bind irreversibly to MOR. To verify whether the fluorescent peptide **1** and naloxone are interacting with the same receptor, a protection experiment was carried out, where cells were first treated with 10 μ M of naloxone for 30 min followed by a treatment with 400 nM of **1** for 90 min. As can be seen in panel B of Figure 5.6 minimal fluorescence above background was observed consistent with peptide **1** and naloxone interacting with the same receptor. To detect any auto-fluorescence of the SH-SY5Y cells in the labeling experiments, cells were treated with buffer only (panel D of Figure 5.6). Therefore the results obtained from this preliminary microscopy experiment suggest labeling of MOR by the dermorphin-based multifunctional peptide **1**. The inability of naloxone to displace the fluorescent peptide from MOR further suggests that the peptide binds irreversibly to MOR, and thus demonstrates the utility of this approach.

5.4. Conclusions

A multifunctional affinity label peptide was designed by modifying [D-Lys(=C=S)²]dermorphin which exhibits high affinity ($IC_{50} = 0.38$ nM), selectivity and wash resistant inhibition of binding to MOR (Chapter 3). The design involved incorporating additional C-terminal Lys residues as handles to attach a purification tag and fluorescent tags to aid in receptor isolation and detection of the labeled receptor, respectively. A solid-phase synthetic methodology utilizing selective deprotection of different protecting groups on the side chain amines of Lys residues permitted the incorporation of multiple labels (Oregon Green or 5-carboxyrhodamine B and DSB) to yield the multilabeled dermorphin derivatives. Preliminary

microscopy experiments examining the interaction of the fluorescent peptide **1** with MOR on SH-SY5Y cells suggests irreversible binding of the multifunctional affinity label dermorphin derivative, and thus demonstrates the utility of this approach. Radioligand binding assays of **1-4** will be carried out in the near future to determine the affinity and confirm the wash-resistant inhibition of binding of the multilabeled [D-Lys(=C=S)²]dermorphin derivatives. Additional microscopic experiments will also be performed to verify the results of the initial studies, to evaluate the effects of different ligands on fluorescent labeling and also to study receptor trafficking.

5.5 Experimental

5.5.1 Synthesis of Dermorphin-based Multifunctional Affinity Label

The dermorphin-based multifunctional affinity label derivatives were prepared by solid phase peptide synthesis methodology described in Chapter 3. The methodology for selective incorporation of different functional tags (purification and fluorescent tags) is described below.

Materials

The Fmoc-mini-PEG linker (2-(2-(2-aminoethoxy)ethoxy)acetic acid) was purchased from Peptides International (Louisville, KY). Fmoc-βAla-OH, Fmoc-Lys(Mtt)-OH and Fmoc-Lys(ivDde)-OH were purchased from Novabiochem (San Diego, CA). Oregon Green 488, Rhodamine WT and d-desthiobiotin were obtained from Molecular Probes (Eugene, OR), Abbey Color (Philadelphia, PA) and Sigma-Aldrich

(St. Louis, MO), respectively. Hydrazine was obtained from Sigma-Aldrich (St. Louis, MO). The remaining chemicals, Fmoc protected amino acids, Fmoc-PAL-PEG-PS resin, and HPLC grade solvents were obtained from sources listed in Chapter 3.

Synthesis of dermorphin-based multifunctional affinity labels

In addition to the Fmoc-protected amino acids mentioned in Chapter 3, Fmoc- β Ala-OH, Fmoc-Lys(Mtt)-OH, Fmoc-mini-PEG-linker and Fmoc-Lys(ivDde)-OH were used in the synthesis. The synthesis started with Fmoc-PAL-PEG-PS resin (300 mg, 0.19–0.21 mmol/g). Following Fmoc deprotection of the resin using piperidine and DMF (1:4), Fmoc- β Ala-OH, Fmoc-Lys(Mtt)-OH, Fmoc-mini-PEG-linker and Fmoc-Lys(ivDde)-OH were sequentially coupled following the Fmoc solid phase peptide synthesis procedures described in Chapter 3 (Scheme 5.1). The peptide chain was then further elongated with Fmoc-mini-PEG-linker and the fully protected dermorphin peptide sequence assembled on the resin as per the protocol in Chapter 3. The linkers, Ser⁷, Pro⁶ and the N-terminal Tyr¹ were coupled twice and required overnight reaction for the second coupling. The completion of the couplings of amino acids and linker were monitored by the ninhydrin test for coupling to a primary amine and by the chloranil test if coupled to a secondary amine.

After assembly of the entire peptide chain on the resin, the ivDde protecting group from the C-terminal Lys was selectively removed by treatment with 2% hydrazine in DMF twice for 10 min. The resin was then washed with DCM/DMF (1:1, 10 x 1 min). DSB was then coupled to the resulting primary amine with PyBOP, and HOBT

as the coupling agents and DIEA as base. DSB (3 equiv) was first dissolved in a minimum amount of DMSO: DMF (1:1). PyBOP (3 equiv) and HOBt (3 equiv) were dissolved in a minimum amount of DMSO: DMF (1:1) separately and the solution then added to the DSB followed by DIEA (6 equiv). The mixture was then added to the resin and the reaction run overnight. The completion of the coupling was verified by a negative ninhydrin test. Next the Mtt group from the second Lys from the C-terminus was selectively deprotected by treatment with 3% TFA and 1% TIPS in DCM for 30 mins. The resin was then washed with DCM/DMF (1:1, 10 x 1 min). The resultant free amine was reacted with Oregon Green 488, PyBOP, HOBt and DIEA (1:1:1:2) overnight in the dark. Residual amine groups were acetylated afterwards using acetic anhydride. Cleavage of an aliquot (~15 mg) and analysis by HPLC and ESI-MS were carried out to verify the product. At this stage, the Alloc group of the side chain of D-Lys² was deprotected with a catalytic amount of Pd(PPh₃)₄ plus phenyl silane according to the procedure described previously.^{47, 49} The resin containing the free amine on the side chain of D-Lys² was then divided into two parts (100 mg each). One part was treated with a large excess of acetic anhydride (20 equiv) in DMF to obtain the acetylated control compound. The second part was reacted with thiocarbonyl diimidazole (4 equiv) in DMF to yield the desired isothiocyanate affinity label as per the protocol described earlier in Chapter 3.

To synthesize the dermorphin-based multifunctional affinity label containing 5-carboxyrhodamine B, the 5-isomer was isolated from the mixture of the two isomers (5- and 6-carboxyrhodamine B) in Rhodamine WT (see section 5.5.4 below). The

peptide was synthesized following the same procedure described above. Aliquot analysis by HPLC (Figure 5.5) and ESI-MS was carried out after reaction of 5-carboxyrhodamine B as described above to verify the product. Alloc deprotection and subsequent synthesis of the reversible control and affinity label were carried out following the protocol described above.

It is important to note that all synthetic steps following incorporation of the fluorescent functionalities Oregon Green or 5-carboxyrhodamine B were carried out in the dark to prevent possible degradation of the fluorophore.

5.5.2 Cleavage from Resin

The peptides were cleaved from the resin with 95% TFA and 5% H₂O for 2 h in the dark. Due to the considerable hydrophobicities of these peptides ether extraction was not carried out. Instead, following filtration of the resin excess TFA was removed by evaporation. The resulting peptide was diluted 10 fold with 10% aqueous acetic acid and lyophilized to get the crude products.

5.5.3 Purification and Analysis

The crude peptides (**1-4**, 30-40 mg each) were purified by reversed phase preparative HPLC as described in chapter 3 using a linear gradient of 15-70% aqueous MeCN containing 0.1% TFA over 55 min at a flow rate of 20 mL/min. The samples were prepared by dissolving the crude peptides in 1:9 MeCN: H₂O. After purification of the affinity label analogs (**1** and **3**) the fractions were collected and immediately lyophilized to avoid potential degradation of the affinity label. The fractions were then analyzed by analytical HPLC and ESI-mass spectrometry

according to the protocol described in chapter 3 (section 3.5.3). For the affinity labels **1** and **3** the pure lyophilized fractions were dissolved, combined and re-lyophilized to give the pure peptides. For the reversible control peptides **2** and **4**, the pure fractions were combined and lyophilized to give the pure peptides. The purity of the final peptides (**1-4**) was verified by analytical HPLC using two different solvent systems: 0.1% TFA in aqueous MeCN and 0.09 M TEAP (triethylamine phosphate) and MeCN (see Table 5.1).

5.5.4 Separation of the Isomers from Rhodamine WT.

Rhodamine WT (Abby Color) was obtained as the sodium salt as a 20% aqueous solution. Acidification of Rhodamine WT (25 mL) by dropwise addition of 2 equiv of 3 M HCl precipitated a mixture of the 5- and 6-carboxy isomers. The precipitate was lyophilized after adding a few mL of water to give the crude product. The retention times of the two isomers were 19.01 and 25.37 min by analytical HPLC (20-50% gradient of aq MeCN containing 0.1% TFA over 60 min). For purification of 5-carboxyrhodamine B from the mixture, 40 mg of the crude sample was dissolved per mL of isopropanol/water in the ratio of 1:1. The solution was then sonicated and centrifuged for 10 min. The purification was carried out by reversed phase preparative HPLC using 25-55% aqueous MeCN (containing 0.1% TFA) over 60 min. The fractions for the two isomers were collected separately, lyophilized and analyzed by HPLC and ESI-MS. The identities of the isomers were determined using proton NMR analysis in deuterated methanol of each pure isomer. The chemical shifts (ppm) of the protons shown in Figure 5.7 for the two isomers of

carboxyrhodamine B are as follows: for 5-carboxyrhodamine B, the chemical shifts were δ 8.63 (H_1), δ 8.32 (H_2), δ 7.54 (H_3) and for 6-carboxyrhodamine B, the chemical shifts were δ 7.18 (H_1), δ 7.15 (H_2) and δ 6.73 (H_3).⁵⁰ Based on the NMR, the peaks from HPLC were assigned as 6-carboxyrhodamine (t_R = 19.01 min) and 5-carboxyrhodamine B (t_R = 25.37 min). From 80 mgs of crude Rhodamine B, 20 mg of pure

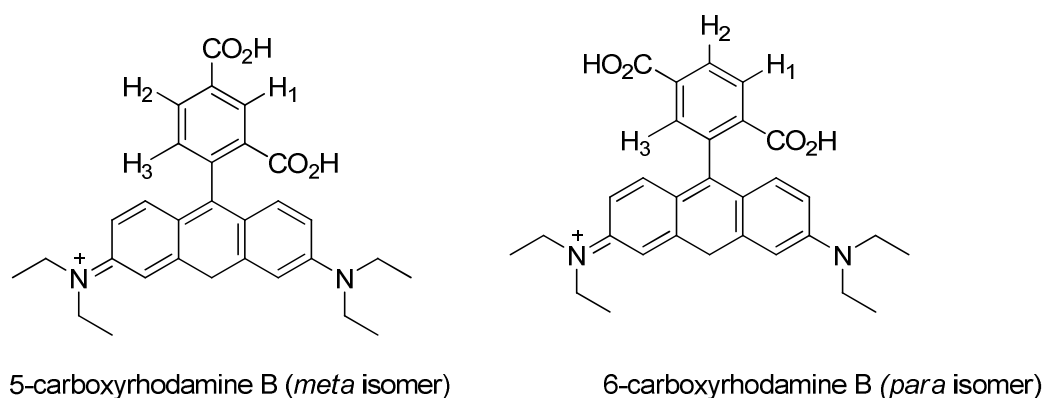


Figure 5.7. Isomers of carboxyrhodamine B

obtained. The purity of 5-carboxyrhodamine B was verified by analytical HPLC using two different solvent systems: 0.1% TFA in aqueous MeCN (20-50% over 30 min, t_R = 11.92 min, purity = 97.8%) and 0.1% TFA in aqueous MeOH (t_R = 23.25 min, purity = 98.6%). The molecular weight of 5-carboxyrhodamine B was verified by ESI-MS mass spectroscopy (m/z = 487.88 for $[M+H]^+$) using a Waters LCT Premier time of flight mass spectrometer.

5.5.5 Microscopy Experiments

SH-SY5Y cells stably expressing MOR and DOR were purchased from ATCC (American Type Culture Collection). SH-SY5Y cells were seeded onto a 384 well plate in phenol-free RPMI media containing 10% FBS (fetal bovine serum) and high glucose (4500 mg/L), and the cells incubated for 36 hours. Following incubation, the cells were washed with PBS (phosphate buffered saline) and the plate was centrifuged for 5 min at 1000 rpm using a Sorvall centrifuge to make certain that the SH-SY5Y cells were attached to the plate surface.

After centrifugation, a group of cells was treated with the affinity label (**1**) (400 nM) for 90 min at 4° C. For protection experiments, another group of cells was first treated with naloxone (10 µM) for 30 min at 4° C followed by treatment with **1** (400 nM) for 90 minutes at 4° C. A third group of cells was treated with **1** for 90 min at 4° C followed by naloxone incubation (10 µM) for 30 min at 4° C, and finally a fourth group of cells was treated with only PBS for 90 min at 4° C. Following all of the treatments, all groups of cells were washed with ice-cold PBS 5 times and the plate was kept on ice until microscopy was performed.

Images of each group of cells were then taken by epifluorescence microscopy using 494 nm as the excitation wavelength and 531 nm as the emission wavelength appropriate for Oregon Green. Imaging was performed on a custom-built spinning disk confocal microscope (Intelligent Imaging Innovations, Denver CO, CSU-10-Based) using an Olympus IX-81 inverted fluorescence microscope frame laser

appropriate for excitation of Oregon Green. Cells were imaged using a Hamamatsu Back-Thinned EMCCD (512 x 512) for epifluorescence using an appropriately matched emission filter and a high speed (<8 ms) emission filter wheel.

5.6 Bibliography

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Chapter 6.
Conclusions and Future Work

6.1 Introduction

The objective of this dissertation work was to design, and synthesize peptide-based affinity labels for μ opioid receptors (MOR). Affinity labels, compounds that bind to their target receptor in an irreversible manner, can be very useful tools to study receptor-ligand interactions.¹ Since the endogenous ligands for opioid receptors are peptides, information obtained from such peptide-based affinity labels could provide valuable insights that can facilitate the development of novel therapeutic agents.

Towards this goal, dermorphin and DAMGO were selected as parent peptides in this research for developing affinity labels, including multifunctional affinity labels, for MOR. The methodology, synthesis and results obtained from these studies have been described in detail in Chapters 3, 4 and 5. Here the important conclusions and significance of the projects will be summarized and future work described.

6.2 Conclusions from Research Projects

6.2.1 Project 1

Discovery of Dermorphin-Based Affinity Labels with Subnanomolar Affinity for Mu Opioid Receptors (Chapter 3)

A series of affinity label analogs with high affinity and selectivity for MOR were discovered by substituting D-Ala² of dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-SerNH₂) with either D-Orn² or D-Lys² and attaching either an isothiocyanate or a bromoacetamide onto the side chain amine of these two modified analogs.^{2, 3} All four

potential affinity label derivatives exhibited very high affinity (0.1-5 nM, Table 6.1) for MOR in standard radioligand binding assays. This was a substantial improvement in binding affinity (between 10- to 100-fold) compared to the previous dermorphin-based analogs synthesized in our laboratory in which the para position of Phe³ or a Phe in position 5 of dermorphin or [Lys⁷]dermorphin were modified.⁴ Furthermore, three of these four potential affinity labels showed subnanomolar binding affinity ($IC_{50} < 1$ nM) indicating that these modifications are well tolerated in the binding pocket of MOR. From the differences in the binding affinities observed in the case of the bromoacetamide derivatives in the D-Orn vs. D-Lys series of derivatives (Table 6.1), it appears that the different lengths of the side chains in D-Lys and D-Orn as well as the identity of the attached functionality play important roles in determining the affinities of these dermorphin analogs for MOR.

Table 6.1: Binding affinities of dermorphin derivatives for MOR and DOR

Dermorphin Analogs	IC ₅₀ (nM ± SEM)		^a Rel. MOR affinity	IC ₅₀ ratio DOR/MOR
	MOR	DOR		
1 [D-Orn(=C=S) ²]	0.81 ± 0.29	23.8 ± 2.1	0.89	29
2 [D-Orn(COCH ₂ Br) ²]	0.11 ± 0.02	342 ± 20	6.54	3110
3 [D-Orn(COCH ₃) ²]	4.25 ± 0.35	272 ± 23	0.17	64
4 [D-Lys(=C=S) ²]	0.38 ± 0.08	97.1 ± 4.9	1.89	255
5 [D-Lys(COCH ₂ Br) ²]	5.23 ± 2.31	382 ± 22	0.14	73
6 [D-Lys(COCH ₃) ²]	29.8 ± 7.6	436 ± 34	0.02	15
Dermorphin ^b	0.72 ± 0.07	197 ± 28	1.0	274

^a Relative to dermorphin. ^b From ref. 4.

In the wash-resistant inhibition of binding experiments, all four potential affinity labels exhibit 30-40 % inhibition of [³H]DAMGO in a wash-resistant manner, three of them (**1**, **4** and **5**) at concentrations equal to their IC₅₀ values. One analog, **2**,

required higher concentration (1 nM) than its IC₅₀ value (0.11 nM) to exhibit wash-resistant inhibition of binding to MOR. These results indicate these four modified analogs of dermorphin are electrophilic affinity labels that likely bind irreversibly to MOR. In addition, analogs **2** and **4** also exhibited concentration-dependent wash resistant inhibition of binding when evaluated at concentrations higher than their IC₅₀ values.

To the best of our knowledge, the peptide-based affinity labels discovered in the present study have the highest binding affinity for MOR among the peptide-based affinity labels reported in the literature. These peptides exhibit 4- to 190-fold higher affinity compared to the Tyr-D-Ala-Gly-Phe-Leu(CH₂SNpys), reported to date to have the highest affinity for MOR (IC₅₀ = 19 nM in standard radioligand binding assays).⁵

6.2.2 Project 2

Synthesis and Evaluation of DAMGO-Based Affinity Labels for MOR and

Discovery of an Unexpected Side Reaction (Chapter 4)

The objective of this project was to design, synthesize and evaluate DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol)-based affinity labels for MOR. With the successful discovery of dermorphin-based affinity labels described above (Project 1), the same design strategy was applied to prepare DAMGO-based affinity labels. This would serve two purposes: 1) To establish whether this design strategy, i.e. attachment of an affinity label group to the side chain amine of a D-amino acid (e.g. D-Orn or D-Lys) at the 2nd position of these peptide ligands, could be a general approach for

generating affinity label derivatives of opioid peptides, and 2) from the binding data, to examine whether this residue may bind similarly to MOR when incorporated in peptides with different message sequences, i.e Tyr-X-Gly-Phe (X = D-amino acid) present in frog skin peptides e.g. dermorphin,⁶ and DAMGO, a synthetic analog of enkephalin,⁷ with the Tyr-Gly-Gly-Phe sequence present in endogenous mammalian opioid peptides.⁸

Of the four affinity labels (**7**, **8**, **10**, and **11**), two (**8** and **11**) showed exceptionally high binding affinity and moderate selectivity for MOR (Table 6.2). Of these two, only **8** exhibited significant (>40%) wash-resistant inhibition of binding of [³H]DAMGO to MOR at a concentration equal to its IC₅₀ value, suggesting possible irreversible binding of **8** to MOR. Analog **11**, on the other hand, did not remain in the receptor membrane after the washing procedure. Interestingly purification and

Table 6.2: Binding affinities of DAMGO and DAMGA derivatives for MOR and DOR

DAMGO Analogs	IC ₅₀ (nM ± SEM)		IC ₅₀ ratio	Relative Affinity ^a
	MOR	DOR	DOR/MOR	
7 [D-Orn(=C=S) ²]	^b			
8 [D-Orn(COCH ₂ Br) ²]	0.45 ± 0.06	33.1 ± 0.9	73	1.1
9 [D-Orn(COCH ₃) ²]	0.58 ± 0.11	102 ± 6	176	0.88
10 [D-Lys(=C=S) ²]	^b			
11 [D-Lys(COCH ₂ Br) ²]	0.45 ± 0.25	103 ± 1	229	1.1
12 [D-Lys(COCH ₃) ²]	1.13 ± 0.22	268 ± 28	237	0.45
DAMGA Analogs				
13 [D-Orn(=C=S) ²]	0.35 ± 0.05	9.69 ± 0.75	28	1.46
14 [D-Orn(COCH ₂ Br)]	0.85 ± 0.37	23.4 ± 2.2	26	0.60
15 [D-Orn(COCH ₃) ²]	0.38 ± 0.13	26.5 ± 2.9	70	1.34
16 [D-Lys(=C=S) ²]	0.57 ± 0.05	74.5 ± 7.6	129	0.89
17 [D-Lys(COCH ₂ Br) ²]	0.45 ± 0.02	30.6 ± 0.7	67	1.13
18 [D-Lys(COCH ₃) ²]	0.71 ± 0.04	105 ± 11	148	0.72
DAMGO	0.51 ± 0.01	281 ± 20	550	1.0

^a Relative to DAMGO, ^b Side reaction

analysis of the isothiocyanate-containing analogs (**7** and **10**) revealed an unexpected side reaction with the formation of cyclic O-alkyl thiocarbamates. This side reaction involves the C-terminal gly-ol functionality of DAMGO acting as a nucleophile and attacking the electrophilic isothiocyanate functionality attached to the side chain of either D-Orn or D-Lys. This leads to the formation of the cyclic O-alkyl thiocarbamate derivative (Figure 6.1).⁹

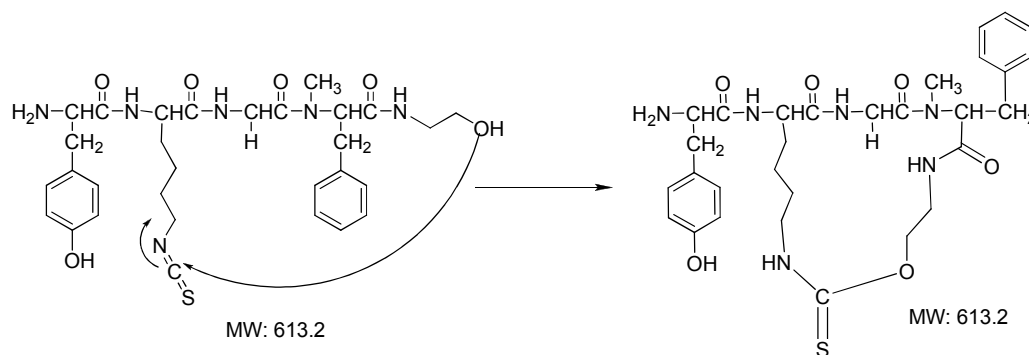


Figure 6.1. Cyclization reaction leading to the cyclic O-alkyl thiocarbamate side product. Reaction of [D-Lys(=C=S)²]DAMGO is shown as the example.

The design of the affinity label derivatives was then modified to overcome the formation of this side product by replacing the C-terminal glyol group by a glycylamide functionality to give [D-Ala²,NMePhe⁴,Gly⁵]enkephalinamide (DAMGA). The new series of analogs (**13-18**, Table 6.2) containing the isothiocyanate and bromoacetamide functionalities were then successfully prepared and evaluated for their binding affinities to MOR. As shown in Table 6.2, although the new series of analogs maintain extremely high affinity (IC₅₀ = 0.3-0.8 nM) for MOR, the selectivity of most of these ligands for MOR dropped significantly (3- to 4-

fold) compared to the DAMGO derivatives. This suggests that the C-terminal glycol functionality plays an important role in conferring selectivity for MOR, as replacing it with the glycylamide functionality resulted in increased affinity for DOR.

The present study successfully established a general approach for designing affinity labels based on MOR-selective peptide ligands containing a D-amino acid at position 2. The modifications were well tolerated and the resulting peptides showed very high affinity for MOR. One of the DAMGO-based analogs, **8**, showed >40% wash-resistant inhibition of binding of [³H]DAMGO to MOR at concentration equal to its IC₅₀ value (0.45 nM), suggesting irreversible binding of this analog to MOR. Of the [D-Lys²]DAMGA series of analogs (**16** – **18**), **17** exhibited 44% wash-resistant inhibition of binding at concentration equal to its IC₅₀ value suggesting this peptide may interact irreversibly with MOR. The wash-resistant inhibition of binding of [D-Orn²]DAMGA derivatives (**13** – **15**) will be carried out soon.

6.2.3 Project 3

Design, Synthesis and Evaluation of a Dermorphin-Based Multifunctional Affinity Label Probe for Mu Opioid Receptors (Chapter 5)

With the long range goal of identifying the point of attachment of a peptide-based affinity label to MOR, the objective of this project was to design and synthesize a MOR selective affinity label peptide as a multifunctional probe which would facilitate the process of receptor isolation and identification of the attachment point of the affinity label to MOR.

Towards this goal, a MOR-selective peptide-based affinity label [D-Lys(=C=S)²]dermorphin (Project 1, Chapter 3, compound **4**) was chosen as the lead peptide for incorporating additional functionalities, namely d-desthiobiotin (DSB) as a purification tag, and Oregon Green or 5-carboxyrhodamine B as a fluorescent tag (Figure 6.2) to facilitate receptor isolation and aid in visualizing the labeled receptor, respectively. This particular analog was chosen due to its high binding affinity (IC_{50} = 0.38 nM), selectivity (DOR/MOR = 250) and wash-resistant inhibition of binding to

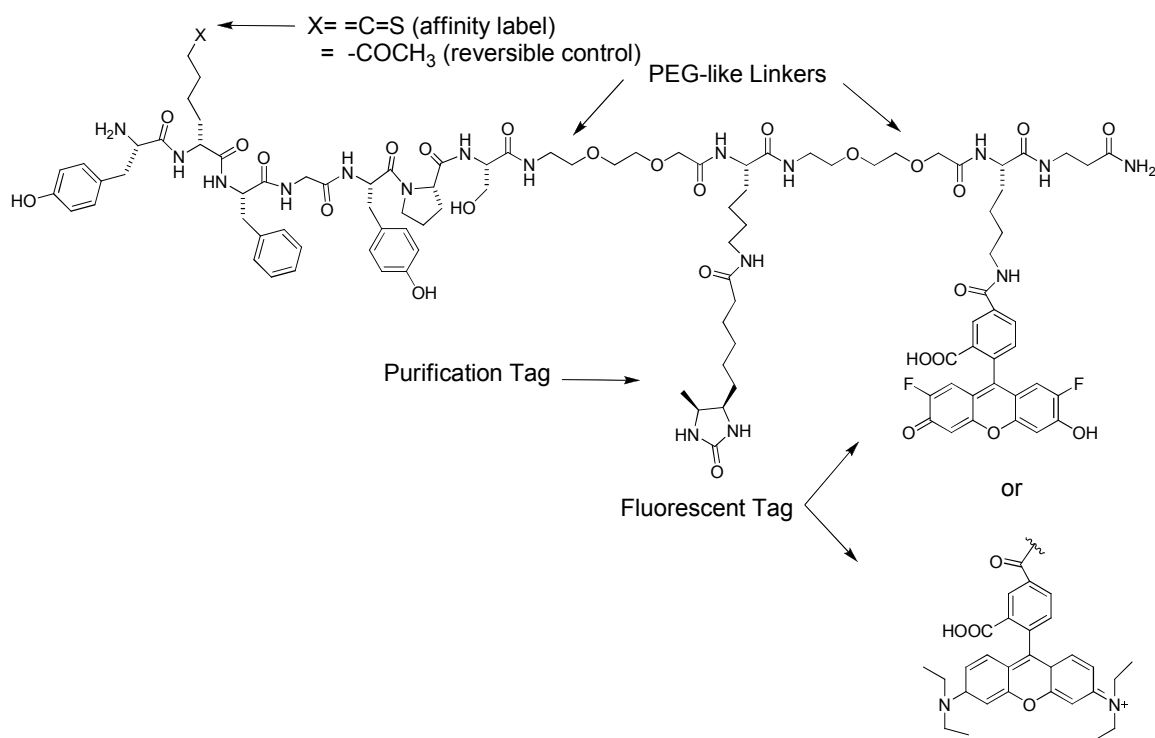


Figure 6.2. The design of the dermorphin-based multifunctional affinity label for MOR

MOR in a concentration-dependent manner (see Chapter 3). The design of this multifunctional affinity label probe included additional Lys residues that served as handles to incorporate the additional tags. The purification tag and the fluorescent tag

were separated from each other and from the peptide by a hydrophilic poly(ethylene glycol) (PEG)-like linker to increase the water solubility of the peptide and decrease nonspecific binding.

The synthetic methodology to prepare this multifunctional affinity label peptide on a solid support involved choosing protecting groups for the three different Lys side chain amines present in the multilabel peptide so that each protecting group could be selectively removed and the appropriate label incorporated without affecting the other protecting groups present in this peptide. Employing this synthetic strategy the multilabeled analogs were successfully synthesized and obtained in >97% purity based on analytical HPLC following purification.

With the successful synthesis of the multifunctional affinity label peptides, preliminary microscopic experiments were performed by labeling SH-SY5Y cells that stably express MOR with the multilabeled [D-Lys(=C=S)²]dermorphin derivative containing Oregon Green (Compound **1**, Chapter 5). Preliminary microscopic results obtained by labeling SH-SY5Y cells with **1**, including protection and displacement experiments with the MOR antagonist naloxone, suggest irreversible binding of **1** to MOR. Thus, the multifunctional probe was successfully used to label and visualize MOR, demonstrating the utility of this approach.

6.3 Future Work

The multilabeled affinity label peptide containing Oregon Green or 5-carboxyrhodamine B will first be evaluated for MOR affinity and selectivity in standard radioligand binding assays using CHO cells stably expressing MOR and

DOR to determine their affinities relative to parent affinity label derivative of dermorphin (compound **4**, Chapter 3). The wash-resistant inhibition of binding by the multifunctional peptides (compound **1** and **3**, Chapter 5) will be examined to optimize concentration, and incubation time.¹⁰ To ensure effective removal of noncovalently bound compound and to assess efficiency of the washing procedure, reversible control multilabeled peptides (**2** and **4**, Chapter 5) will also be evaluated for wash-resistant inhibition of binding. Optimizing the washing procedure is particularly important since addition of DSB and fluorescent group could make difficult to remove noncovalently bound peptide from MOR.

The microscopy experiments with the multifunctional peptide described in Chapter 5 will be repeated using CHO cells expressing MOR. Wash-resistant fluorescent labeling of cells with the peptides **1** and **3** (Chapter 5) will be evaluated using confocal microscopy. Like the wash-resistant inhibition of binding assay, parallel experiments will also be performed with the reversible control compounds (**2** and **4**, Chapter 5) to evaluate the effectiveness of the washing procedure.

The long range goal of this project is to identify the attachment point of the peptide-based affinity label to MOR. After the binding affinities are determined in radioligand binding assays and wash-resistant inhibition of binding and wash-resistant fluorescent labeling evaluated as described above, receptor isolation studies will be undertaken to determine the attachment point of the multifunctional peptide to MOR. The CHO cells stably expressing MOR will be labeled with the fluorescent peptide at an appropriate concentration. Epitope tagged MOR will be used for such

isolation experiments and immunoprecipitation of the labeled receptor using an appropriate antibody against the epitope will also be included in the isolation procedure. Since the peptide contains a fluorescent label, SDS-PAGE of the labeled receptor followed by fluorescent imaging would help identify the desired band. The labeled receptor band thus isolated could then be enzymatically cleaved and the labeled receptor fragment could be further isolated from other unlabeled fragments through binding of the DSB group to streptavidin¹¹ and the unlabeled fragments removed by washing. The isolated labeled fragments will then be analyzed by MALDI-TOF-MS (matrix-assisted laser desorption/ionization-time of flight-mass spectrometry) to attempt to determine the attachment point.¹¹⁻¹³ The optimum conditions (compatibility with detergents, concentration, incubation time, elution efficiency from the streptavidin bound peptides, etc) will be first established based on detailed model studies with the multilabeled peptides based on literature procedures used in the purification of MOR.¹⁴ For these studies deuterated multilabeled peptides would be prepared to assist detection labeled fragments by MALDI-TOF-MS.¹⁵

Another important application of the multilabeled fluorescent peptide will be to study receptor internalization. After labeling MOR expressed on CHO cells with dermorphin-based fluorescent peptides at a low temperature (4° C), internalization can be monitored by confocal microscopy following raising the temperature to 35° C.¹⁶ Since dermorphin is an agonist, the multilabeled derivative would be expected to result in receptor internalization.

6.4 Bibliography

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