The Design, Synthesis and Evaluation of Peptide Ligands to Study Opioid Receptors

BY

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Dedicated to my greatest supporter

Brian R. Judson
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Abbreviations


Amino Acids are in the L-configuration except where indicated otherwise. Additional abbreviations used in this dissertation are as follows:

5-RhodB: 5-carboxytetraethylrhodamine, 5-carboxyrhodamine B;
Alloc: allyloxycarbonyl;
BAL: backbone amide linker;
β-CNA: beta-chlornaltrexamine;
β-FNA: beta-funaltrexamine;
Boc: *tert*-butoxycarbonyl;
BNTX: 7-benzylidenenaltrexone;
cAMP: cyclic adenosine monophosphate;
cDNA: complementary DNA;
Cha: cyclohexylalanine;
CHCA: α-cyano-4-hydroxycinnamic acid;
CHO: Chinese hamster ovary;
CPM: cyclopropyl methyl;
CNS: central nervous system;
CSU: confocal-scanning unit;
CTOP: D-Phe-*cyclo*[Cys-Tyr-D-Trp-Orn-Thr-Pen]-Thr-NH₂;
Del I: deltorphin I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂;
Del II: deltorphin II, Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂;
DADLE: [D-Ala^2,D-Leu^5]enkephalin;
DALCE: [D-Ala^2,Leu^5,Cys^6]enkephalin;
DALDA: Tyr-D-Arg-Phe-Lys-NH_2;
DALECK: [D-Ala^2,Leu^5]enkephalin;
DAMGO: [D-Ala^2,MePhe^4,glyol]enkephalin;
DCM: dichloromethane;
DHB: 2,5-dihydroxybenzoic acid;
DIC: 1,3-diisopropylcarbodiimide;
DIEA: N,N-diisopropylethylamine;
DMF: N,N-dimethylformamide;
Dmt: 2,6-dimethyltyrosine;
DOR: δ opioid receptor;
DPDPE: cyclo[D-Pen^2,D-Pen^5]enkephalin;
DSB: d-desthiobiotin;
DSLET: [D-Ser^2,Leu^5,Thr^6]enkephalin;
Dyn A: dynorphin A;
EL: extracellular loop;
Enk: Met/Leu-enkephalin, Tyr-Gly-Gly-Phe-Leu/Met;
ESI-MS: electrospray ionization mass spectrometry;
FBS: fetal bovine serum;
FCS: fetal calf serum;
Fmoc: 9-fluorenylmethoxycarbonyl;
FMPB AM Resin: 4-(4-Formyl-3-methoxyphenoxy)butyryl Amino Methyl Resin;
FIT: fentanyl isothiocyanate;
GIRK: gated inward rectifying potassium channel;
GNTI: Guanidinonattrindole;
GPCR: G-protein coupled receptor;
GPI: guinea pig ileum;
GRK: G-protein coupled kinase;
HATU: N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylmethanaminium hexafluorophosphate Noxide;
HOAt: 7-aza-1-hydroxybenzotriazole;
HOBt: 1-hydroxybenzotriazole;
HEK 293 cells: Human embryonic kidney 293 cells;
HPLC: high-performance liquid chromatography;
Hmb; 2-hydroxy-4-methoxybenzyl;
i.c.v.: intracerebroventricular;
i.t.: intrathecal;
IL: intracellular loop;
ivDde: 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl;
KDAN: Kappa Delta Agonist Antagonist;
KDN: Kappa DOR Antagonist;
KMN: Kappa Mu Antagonist;
KOR: κ opioid receptor;
LC-MS: liquid chromatography mass spectrometry;
MALDI: matrix assisted laser desorption ionization;
MDAN: Mu Delta Agonist Antagonist;
MeIm: methylimidazole;
MFP: multifunctional peptide;
MOR: µ opioid receptor;
MSNT: 1-(mesitylene-2-sulphonyl)-3-nitro-1H-1,2,4-triazole;
Mtt: 4-methyltrityl;
MVD: mouse vas deferens;
NDA: 2,3-Naphthalenedicarboxyaldehyde;
NLX: naloxone;
NMR: nuclear magnetic resonance;
NTB: naltriben;
NTI: naltrindole;
NTII: naltrindole isothiocynate;
NTX: naltrexone;
oNB: ortho-nitrobenzyl;
OPA: ortho-phthaldehyde;
ORL-1: opioid-receptor like-1;
PAL: peptide amide linker, 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeryl;
PBS: phosphate buffered saline;
PC peptide: photocleavable multifunctional peptide;
PEG: polyethylene glycol;
Pen: penicillamine;
PS: polystyrene;
PyBOP: benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate;
PyClock: 6-chloro-benzotriazole-1-yloxy-tris-pyrrolidino-phosphonium hexafluorophosphate;
RP-HPLC: reversed-phase high performance liquid chromatography;
SA: sinapinic acid;
s.c.: subcutaneous;
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis;
SEM: standard error of mean;
SPPS: solid-phase peptide synthesis;
SUPERFIT: (+)-cis-3-methylfentanyl isothiocyanate;
TEAA: triethylammonium acetate;
TEAP: triethylammonium phosphate;
TFA: trifluoroacetic acid;
THF: tetrahydrofuran;
Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid;
TICP: Tyr-Tic-Cha-Phe;
TIPP: Tyr-Tic-Phe-Phe;
TIPP[Ψ]: Tyr-TicΨ[CH₂NH]Phe-Phe;
TIS: triisopropylsilane;
TM: transmembrane;
TMOF: trimethyl orthoformate;
WRIB: wash-resistant inhibition of binding
Abstract

The δ opioid receptor (DOR) is involved in the modulation of µ opioid receptor (MOR) agonist mediated side effects such as the development of tolerance, and MOR and DOR may interact to form heterodimers capable of unique pharmacological signaling. Understanding the molecular interactions involved in ligand recognition by opioid receptors will aid in the design of novel therapeutics that target the opioid receptors with fewer side effects.

The aim of this research was to develop and use opioid peptide ligands to examine ligand-receptor interactions at the molecular level. We designed a multifunctional peptide-based affinity label derivative of the high affinity DOR selective peptide antagonist TIPP (Tyr-Tic-Phe-Phe, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) to irreversibly interact with DOR. The multifunctional peptide also contains a fluorescent group and multiple tags to aid in the detection and isolation of labeled DOR. The initial solid phase peptide synthesis of the multifunctional peptide generated multiple side products and required extensive evaluation and optimization to increase the efficiency of peptide synthesis. A model peptide that contained only the affinity label and a fluorescent tag (at 65 nM) demonstrated 95% wash resistant inhibition of binding to DOR suggesting that this peptide is an affinity label for DOR.

We also report the synthesis and initial binding analysis of a heterobivalent peptide-based affinity label targeting the proposed MOR-DOR heterodimer. This peptide demonstrated nanomolar affinity to both DOR and MOR in radioligand competition assays, and at 96 nM exhibited 94% wash resistant inhibition of binding to DOR, suggesting that the heterobivalent peptide is an affinity label for DOR.
Thus, we successfully prepared two series of novel opioid peptide ligands which appear to be affinity labels for DOR. One series of peptides contains multiple functional groups to aid in DOR isolation, detection and analysis. The second peptide consists of two pharmacophores incorporated into a single entity to study proposed MOR-DOR heterodimers. These peptides will be useful pharmacological tools to study opioid receptors.
Chapter 1. Literature Review

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.
1. Opioid Receptors

1.1. Introduction

Opioid receptors are involved in a variety of physiological processes but are most recognized for their role in the pain response, especially as targets of analgesic agents. The prototypical opioid ligand morphine was isolated from the opium poppy in the 1800s, but it was not until the 1970s that the opioid receptors were established. To this day understanding the exact molecular interactions that occur between the opioid receptors and their ligands remains a major goal in opioid research. Knowledge of the molecular interactions involved in ligand recognition by opioid receptors is important for the design of novel therapeutics targeting the opioid receptors.

1.2. Multiple Receptor Types

Beckett and Casy first proposed that the opioids interacted with a surface receptor in the 1950s. The existence of the opioid receptors in nervous tissue was later demonstrated by the stereospecific binding of radiolabeled ligands. Early pharmacological investigations by Martin and co-workers defined the mu opioid receptor (MOR) and the kappa opioid receptor (KOR) based on binding to morphine and ketocyclazocine, respectively. The delta opioid receptor (DOR) was later identified in the mouse vas deferens from which it derives its name (δ for deferens). Opioid like receptor-1 (ORL-1), identified by Mollereau et al., has 60% homology to the three opioid receptors. The classic opioid ligands do not have high affinity for ORL-1, and the roles this receptor plays in the analgesic pathway have yet to be completely defined. All three opioid receptors, MOR, DOR and KOR, have been cloned from multiple species, and extensive pharmacological analysis has been performed including studying
the effects of changing the amino acid residues in the receptors on ligand binding and activity.\textsuperscript{13, 14} In general, the binding affinities and activities of ligands for the opioid receptor clones are in agreement with those reported for the same receptors in brain preparations.\textsuperscript{12, 15}

Two groups independently cloned DOR utilizing radiolabeled DOR-selective ligands to screen cDNA libraries generated from NG 108-15 cells, which express DOR, but not MOR or KOR.\textsuperscript{16, 17} The identity of the isolated DOR receptor was further demonstrated in binding assays, which gave results that were in agreement with the previously observed pharmacology of this receptor. MOR and KOR were subsequently cloned using cDNA screening based on sequence homology to DOR.\textsuperscript{12, 18}

1.3. Significance of DOR

All three opioid receptors are involved in the analgesic response, and each receptor has been the target of therapeutic agents.\textsuperscript{19} Morphine (1, Figure 1.1) and the majority of narcotic analgesics on the market produce analgesia through activation of MOR. The severity of side effects associated with morphine and its derivatives, such as respiratory depression, addiction liability and constipation, has driven the pursuit of effective analgesics with reduced side effect profiles.\textsuperscript{19}

It has become increasingly evident that the opioid system is a complex organization of signaling pathways that involves actions of all three receptor types. DOR seems to play a particularly important role in the modulation of MOR

\textbf{Figure 1.1.} Morphine.
mediated side effects.\textsuperscript{20-24} Furthermore, DOR may itself prove to be a target of analgesics and other drug classes in its own right.\textsuperscript{25}

DOR has become an attractive target for the treatment of a variety of conditions, especially as a target of analgesics for the treatment of inflammatory pain,\textsuperscript{26} since activation of this receptor results in fewer side effects, such as respiratory depression, inhibition of GI motility and physical dependence, compared to MOR. Even more, DOR agonists have the potential to treat neuropathic pain, a condition that has long eluded effective therapeutics.\textsuperscript{25} Therefore, understanding how ligands interact with DOR is an important step in the design of new therapeutic agents targeting this receptor.

1.4. Structural Elements of the Opioid Receptors

Sequence analysis of the cloned opioid receptors indicated that these receptors belong to the rhodopsin family of G-protein coupled receptors (GPCRs), and there is extensive evidence that the opioid receptors are coupled to G-proteins.\textsuperscript{19, 25, 27} As members of the rhodopsin (class A) family of GPCRs the opioid receptors are characterized by seven transmembrane helices (TM 1-7) connected by three intracellular loops (IL1-3) and three extracellular loops (EL1-3) as well as an extracellular N-terminus and intracellular C-terminus (Figure 1.2).\textsuperscript{13} The amino acid sequence identity between the receptors varies between the domains. For instance, while the overall identity between the opioid receptor types is 60%, it is greater than 70% in the transmembrane domains and less than 40% in the extracellular loops.\textsuperscript{25}

Only a limited number of class A GPCR’s have been successfully crystallized and analyzed by X-ray crystallography. Structures have been obtained for rhodopsin and more recently opsin, the A\textsubscript{2A} adenonsine receptor and modified structures of the beta-1
and beta-2-adrenergic receptors.\textsuperscript{28-38} The difficulty in obtaining crystal structures of GPCRs lies in their extremely low expression levels, hydrophobicity and a basal level of activity that leads to structural instability.\textsuperscript{31, 39}

\textbf{Figure 1.2.} Serpentine diagram of DOR from ref.\textsuperscript{13} Yellow star indicates location of the DRY motif.

In the absence of crystallographic data for the opioid receptors studies of the interactions that are important between ligands and the receptors have relied heavily on pharmacological data and mutational analysis. In addition to amino acid mutation, the generation of chimeric receptors has provided important information regarding the regions of the opioid receptors that are important for ligand binding and selectivity.\textsuperscript{15, 40} Chimeric receptors are generated by swapping out various domains of the opioid receptors with each other. For instance, TM1 of MOR may be grafted into DOR so that
the resulting chimeric receptor is made up of the ILs ELs, termini and TM2-TM7 of DOR but has the amino acid residues that correspond to MOR in TM1.

There are several conserved residues among the opioid receptors including a cysteine residue in EL1 and one in EL2 that are thought to form a disulfide linkage. In addition, there are residues and motifs that are conserved among all class A receptors including the DRY motif at the cytoplasmic end of TM3 (yellow star, Figure 2.2). The most divergent sequences between the receptors are found in the extracellular loops, which are thought to be the site of ligand discrimination between the different receptor types.

Extensive mutational analysis and chimeric receptor analysis of all three opioid receptors indicates that different regions in the receptors are important for establishing ligand selectivity. In general, DOR selectivity is attributed to EL3 whereas MOR appears to rely on EL1 and EL3. The specific amino acids in EL3 of DOR implicated in imparting selectivity for the DOR ligands SNC80, naltrexone (NTI), [D-Pen², D-Pen⁵]enkephalin (DPDPE) and deltorphin II are Trp284, Val296, and Val297. Studies of MOR/DOR chimeric receptors implicated TM5-7 in DOR ligand selectivity. An aromatic binding pocket comprised of amino acids in TM3-7 was proposed based on the effects of an alanine scan on peptide and non-peptide ligand binding. Specific amino acids that impart ligand selectivity were determined by mutational analysis. Lys108 of DOR prevents the binding of the MOR selective peptide DAMGO ([D-Ala², N-Me-Phe⁴, Gly-ol]enkephalin) and an alanine scan of 20 amino acid residues demonstrated the importance of three amino acids in EL3, namely Trp284, Val296 and Val297, for the binding of DOR selective ligands.
There is evidence that peptide versus non-peptide, agonist versus antagonist and equilibrium versus non-equilibrium ligands have different binding/activation requirements, further demonstrating the need to study ligand-receptor interactions encompassing a diverse array of ligand types for the opioid receptors.\textsuperscript{14} Chimeric DOR receptors containing EL3 of MOR showed decreased binding to both the small molecule SNC80 and peptide based DOR selective ligands DPDPE and deltorphin II (del II)\textsuperscript{41} while the binding of the DOR selective non-peptide affinity label SUPERFIT was dependent on EL1 and TM3.\textsuperscript{14, 43} Mutation of Ser177 to Leu in DOR resulted in the inhibition of adenylyl cyclase by naloxone and naltrexone (i.e. imparted agonist activity) without affecting affinity. However, this mutation did not influence the efficacy of Tyr-TicΨ[CH\textsubscript{2}NH]-Phe-Phe-OH (TIPP[Ψ]).\textsuperscript{44} Thus, chimeric studies and mutational analysis of the opioid receptors have provided valuable insight into the interactions between ligands and these receptors. However, caution must be taken when interpreting these results as changes to ligand binding and efficacy may be a result of modification of structural elements rather than the direct disruption of ligand-receptor interactions.\textsuperscript{14}

1.5. Molecular Modeling and Ligand Docking

The lack of a crystal structure for any of the opioid receptors has placed a great importance on the ability to establish computational models of receptor-ligand interactions. The majority of models for the opioid receptors are based on the structure of rhodopsin.\textsuperscript{45} Recent reports have compared these models to those based on the β\textsubscript{2}-adrenergic structures and in general there was not a significant difference in model parameters.\textsuperscript{46, 47} This is not a surprise in light of the similarity between these structures and the observed superimposition of the TM helices.\textsuperscript{48, 49} Pairing molecular modeling
with experimental evidence generated from mutational/chimeric receptor studies and affinity labeling is proposed to increase the likelihood of generating valid parameters of ligand binding and interactions with these receptors.\textsuperscript{50, 51}

Receptor modeling by Mosberg and co-workers suggested that there was one common binding site in the opioid receptors for agonists and antagonists.\textsuperscript{50} A binding pocket in all three opioid receptors was proposed to consist of the following residues: Asp (3.32), Tyr (3.33), Lys (5.39), Phe (5.47), Trp (6.48), Ile (6.51), His (6.52), Ile (6.53), Ile (7.39) and Tyr (7.43) (Weinstein nomenclature).\textsuperscript{45} Common interactions were observed for peptide and non-peptide agonists and antagonists such as the interaction of the alkaloid tyramine moiety or Tyr\textsuperscript{1} of peptides with the conserved Asp residue in TM III and His in TM VI. A cyclic peptide (Tyr-c(S-CH\textsubscript{2}-S)[D-Cys-Phe-2-Nal-Cys]NH\textsubscript{2}) with mixed MOR agonist/DOR partial agonist/antagonist activity was docked into models of MOR and DOR.\textsuperscript{47} Steric overlap was observed between the Nal residue of the peptide and Trp284 (6.48) in the active conformation of DOR, but not in the inactive conformation. This is in agreement with the efficacy profile of this peptide as a DOR partial agonist/antagonist. Docking of DOR antagonists NTI, naltriben (NTB) and 7-benzylidenenaltrexone (BNTX) into three different DOR models also demonstrated the importance of Val296, Val297 and Leu300 in forming a hydrophobic binding pocket.\textsuperscript{51} The involvement of these residues in ligand binding is in agreement with mutational analysis of DOR.\textsuperscript{14}

Since changes in the properties of ligand binding in chimeric and mutational studies may be a result of changing structural features of the receptor and not necessarily
direct receptor-ligand interactions, affinity label studies are important counterparts for providing direct evidence of receptor-ligand interactions at the molecular level.\textsuperscript{52}

1.6. Signaling Cascade

Prior to the cloning of the opioid receptors, Blume \textit{et al.} demonstrated that GTP was required for opioid-mediated inhibition of adenylyl cyclase and that the binding affinity of selective agonists was decreased in the presence of GTP.\textsuperscript{53} Agonist binding to the opioid receptors stimulates coupling to a G-protein, followed by dissociation of the G-protein subunits and subsequent cellular signaling events (Figure 1.3).\textsuperscript{40, 54, 55} G-proteins are comprised of subunits denoted α, β and γ. The G\textsubscript{α} subunit is a GTPase that is occupied by GDP in the inactive state. Upon agonist binding to a GPCR, exchange of GDP for GTP in the G\textsubscript{α} subunit results in the subsequent dissociation of the heterotrimeric subunits of the G protein into G\textsubscript{α} and G\textsubscript{βγ}. G\textsubscript{α} (and in some cases G\textsubscript{βγ}) then couples to down stream effectors leading to a physiological response. Signal transduction is regulated by the hydrolysis of GTP to GDP, which is either a result of spontaneous hydrolysis or interaction with GTPase activating protein (GAP).\textsuperscript{56-59} The mechanism by which GPCR activation is relayed to the G-

\textbf{Figure 1.3.} G protein activation pathway from ref.\textsuperscript{60} RGS = regulators of G-protein signaling.
protein is not fully understood and proposed mechanisms range from large shifts in helices to more recent proposals involving conserved water molecules which may act as prosthetic groups.\textsuperscript{59-61} DOR is coupled to pertussis toxin-sensitive G proteins (G\textsubscript{i} or G\textsubscript{o}), and receptor activation is associated with a decrease in adenylyl cyclase activity, inhibition of voltage-gated Ca\textsuperscript{2+} channels and stimulation of gated inward rectifying K\textsuperscript{+} channels (GIRKs), phospholipase C\textbeta, and protein kinases A and C.\textsuperscript{40} All three opioid receptor types display a basal level of constitutive activity which is independent of G protein activation.

Analgesia associated with activation of the opioid receptors is the result of modulation of neuronal activity affecting the transmission in the central nervous system and the peripheral nervous system.\textsuperscript{54, 55} The inhibition of Ca\textsuperscript{2+} channels decreases Ca\textsuperscript{2+} influx and therefore neurotransmitter release, particularly substance P.\textsuperscript{62} The increase in the activity of GIRK increases K\textsuperscript{+} efflux, which results in the hyperpolarization of postsynaptic neurons, thus preventing further stimulation from presynaptic release of nociceptive signals. Inhibition of adenylyl cyclase modulates ion channels in dorsal root ganglia (DRG) neurons by decreasing their excitability and therefore neurotransmission of primary afferent neurons.\textsuperscript{63}

The coupling of DOR to downstream effectors is dependent on the system under study. For example, activation of DOR in neuroblastoma cells lead to modulation of adenylyl cyclase, Ca\textsuperscript{2+} channels and phospholipase C, but in rat DRG and pituitary preparations these receptors are not efficiently coupled to Ca\textsuperscript{2+} channels.\textsuperscript{64}
1.7. Pharmacology

Our current understanding of the function of the opioid receptors is a result of extensive pharmacological analysis. Many of the early pharmacological assays relied on isolated tissue preparations such as mouse vas deferens (MVD) and guinea pig ileum (GPI), which express more than one opioid receptor type. The identification of selective antagonists aided in studies that employed these preparations. The successful cloning of the opioid receptors permitted extensive pharmacological and mutational analysis with the advantage of examining homogenous populations of receptors. Knockout (K/O) mice that lack each of the opioid receptors have been instrumental in relating in vitro and in vivo data and in supplying information on the origin of proposed receptor subtypes (see chapter 3).

Activation of the opioid receptors leads to a variety of physiological responses depending on which receptor is targeted and include analgesia, respiratory depression, euphoria, feeding, hormone release, decreased GI transit and effects on anxiety, depression and addiction. The opioid receptors are also relevant in a variety of other physiological functions such as inflammation. Opioid receptors have been found on cells of the immune system such as T lymphocytes, human granulocytes and monocytes suggesting that opioids are also involved in modulation of the immune response.

DOR has been implicated in the regulation of the development of tolerance due to the action of MOR agonists. MOR and DOR receptors may be co-localized on neurons allowing for the possibility that agonist effects of MOR ligands may be modulated by direct interaction between MOR and DOR. It is also possible that DOR regulates MOR signaling via downstream signaling events. The development of
morphine tolerance and dependence is attenuated or even abolished by selective antagonism of DOR and in DOR K/O mice.\textsuperscript{22}

DOR selective antagonists NTI and TIPP[Ψ] and the DOR selective agonists del II, DPDPE and (+)BW373U86 all reversed respiratory depression caused by the MOR agonist alfentanil without decreasing antinociception.\textsuperscript{23} Interestingly, administration of these DOR ligands alone had no observable effect on respiration.

DOR selective analgesics display synergistic effects when co-administered with MOR agonists. [Leu\textsuperscript{5}]enkephalin was shown to potentiate morphine-mediated analgesia even when the peptide was given at sub-antinociceptive doses.\textsuperscript{71} This effect may allow administration of even lower doses of MOR agonists thereby reducing side effects associated with activation of MOR. DOR selective ligands may also be useful in the treatment of mental diseases such as schizophrenia, bipolar disorder and manic depression.\textsuperscript{40}

Since DOR antagonists also modulate MOR mediated side effects such as the development of tolerance and physical dependence, compounds with mixed MOR agonist and DOR antagonist activity may be useful analgesics.\textsuperscript{71} The first compound reported with mixed MOR agonist/DOR antagonist activity was TIPP-NH\textsubscript{2}.\textsuperscript{72} The pseudopeptide TIPP[Ψ], which is a potent DOR antagonist, attenuated the development of tolerance and dependence when administered intracerebroventricularly (i.c.v.) with concomitant subcutaneous (s.c.) injection of morphine.\textsuperscript{73} The suppression of MOR mediated side effects was comparable to experiments where DOR was targeted by antisense oligonucleotides.\textsuperscript{71}
While DOR agonists lack many of the side effects possessed by MOR agonists, the side effect profile of many DOR selective agents has precluded their use in clinical settings. These side effects range from seizure to the rapid development of tolerance.\(^{19,74}\)

Opioid receptor subtypes have been proposed based on pharmacological and clinical observations.\(^{75,76}\) Clinical and experimental observations such as a lack of cross-tolerance between related opioids have led to the proposal of opioid receptor subtypes. The source of the pharmacological subtypes has yet to be defined. Different populations of opioid receptors may result from alternative splicing, post-translational modification, differential distribution or oligomerization.\(^{40}\) There is mounting evidence that homo- and hetero-oligomerization may be responsible for many of the observed pharmacological profiles.\(^{77}\)

1.8. DOR Trafficking

Altering the surface expression of a GPCR is proposed as a mechanism by which cells can regulate their response to various signals. Trafficking of receptors to and from the cell surface can either diminish or enhance a GPCRs interaction with external stimuli.\(^{78}\) The development of opioid tolerance is thought to be associated with the internalization of DOR in response to agonist stimulation.\(^{74}\) This process is initiated by agonist stimulation, which results in phosphorylation of the receptor and subsequent association with beta-arrestin. This association prevents interaction of DOR with its G-protein and ultimately leads to receptor internalization and degradation. The kinases involved in GPCR internalization, G-protein-coupled kinases\(^{79}\) (GRKs), are recruited by the \(G_{\beta\gamma}\) subunit. However, Traynor and co-workers demonstrated that phosphorylation and subsequent internalization of DOR occurred in the absence of \(G_{\beta\gamma}\) recruitment,
depending on the agonist identity.\textsuperscript{74} Internalization of DOR, was observed in HEK293 cells in both the presence and absence of pertussis toxin following treatment with DPDPE but not TAN67. [Met\textsuperscript{5}]enkephalin, DPDPE, deltorphin II and (+)BW373U86 caused internalization of DOR that was sensitive to pertussis toxin. The latter three ligands were shown to stimulate phosphorylation of the receptor. Naltrindole, oxymorphindole (OMI), TAN67 and morphine did not result in internalization of DOR, and receptor phosphorylation was not observed following treatment with morphine or OMI.\textsuperscript{74}

In addition to receptor internalization, trafficking of receptors to the cell surface is also observed under various conditions. For example, DOR surface expression may be increased during cellular response to external signals such as those involved in the inflammation response and under conditions of chronic pain.\textsuperscript{78} This may explain the observation that analgesia associated with DOR is specific to certain types of painful stimuli. An increase in DOR cell surface expression is also observed in response to agonist stimulation of MOR. Receptor trafficking of DOR and MOR is further discussed in Chapter 3.

1.9. Endogenous Ligands

The first endogenous ligands identified for the opioid receptors were [Met\textsuperscript{5}]- and [Leu\textsuperscript{5}]enkephalin which preferentially interact with DOR.\textsuperscript{80} The enkephalins were isolated from porcine brain and sequenced via Edman degradation in conjunction with mass spectrometry followed by comparison to synthetic peptides. The dynorphins, which show slight selectivity for KOR, the \(\beta\)-endorphins, which bind with high affinity to both DOR and MOR, the endomorphins which selectively interact with MOR, and the enkephalins comprise the mammalian peptides known to be endogenous ligands for the
opioid receptors (Table 1.1).\textsuperscript{19} A comparison of the mammalian peptides, with the exception of the endomorphins, reveals a common N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe) which is the minimal sequence required for opioid activity that is specifically antagonized by naloxone and which Goldstein proposed as the “message” portion of the endogenous opioid peptides.\textsuperscript{81} The highly divergent and variable length C-termini of the endogenous opioid peptides comprises the “address” and are responsible for directing the endogenous ligands to the appropriate receptor. Highly selective and potent peptides were later isolated from frog skin leading to the discovery of the MOR selective peptide dermorphin and the DOR selective deltorphins (Table 1.1).\textsuperscript{82} The amphibian peptides differ from mammalian peptides by the presence of a D-amino acid in position 2, which arises from post-translational modification, and the consensus tripeptide sequence Tyr-D-Xaa-Phe which comprises the “message” portion of these peptides.\textsuperscript{83}

The endogenous mammalian opioid peptides are derived from precursor proteins encoded by three distinct genes.\textsuperscript{84} Proenkephalin A encodes four copies of [Met\textsuperscript{5}]enkephalin, one copy of [Leu\textsuperscript{5}]enkephalin, one copy of [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{6}-Phe\textsuperscript{7} and one copy of [Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{6}-Gly\textsuperscript{7}-Leu\textsuperscript{8}. Dynorphin A, dynorphin B, α- and β-neoendorphin are derived from prodynorphin, and the β-endorphins are encoded in pro-opiomelanocortin.\textsuperscript{19, 27} The origin of the endomorphins has yet to be resolved.\textsuperscript{85}

1.10. Exogenous Ligands

The opium poppy \textit{Papaver somniferum} was the source of the opiate analgesics for thousands of years and was used by ancient civilizations for a variety of ailments including the relief of pain.\textsuperscript{19, 62} Morphine was isolated from opium in the early 1800’s by
Table 1.1. The endogenous opioid peptides. The precursor proteins for the mammalian peptides are shown in bold.\textsuperscript{19, 27}

<table>
<thead>
<tr>
<th>Peptide Type</th>
<th>Sequence</th>
<th>Ki (nM)</th>
<th>IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MOR</td>
<td>DOR</td>
</tr>
<tr>
<td><strong>Proenkephalin</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>[Met\textsuperscript{5}]enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Met</td>
<td>9.5</td>
<td>0.91</td>
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<tr>
<td>[Leu\textsuperscript{5}]enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
<td>19</td>
<td>1.2</td>
</tr>
<tr>
<td>[Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}-Phe\textsuperscript{6}</td>
<td>Tyr-Gly-Gly-Phe-Met-Arg-Phe</td>
<td>3.7</td>
<td>9.4</td>
</tr>
<tr>
<td>[Met\textsuperscript{5}]enkephalin-Arg\textsuperscript{4}-Gly\textsuperscript{3}-Leu\textsuperscript{8}</td>
<td>Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu</td>
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<td>4.8</td>
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<td><strong>Prodynorphin</strong></td>
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<tr>
<td>Dynorphin A</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Trp-Asp-Asn-Gln</td>
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<td>2.4</td>
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<tr>
<td>Dynorphin B</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr</td>
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<tr>
<td>α-neoendorphin</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Arg-Pro-Lys</td>
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<tr>
<td>β-neoendorphin</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Arg-Pro</td>
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<tr>
<td><strong>Proopioi melanocortin</strong></td>
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<tr>
<td>β\textsubscript{7}-endorphin</td>
<td>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</td>
<td>2.1</td>
<td>2.4</td>
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<td><strong>Endorphins</strong></td>
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<tr>
<td>Endomorphin-1</td>
<td>Tyr-Pro-Trp-Phe-NH\textsubscript{2}</td>
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<td>Endomorphin-2</td>
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<td><strong>Amphibian Peptides</strong></td>
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<td>Dermorphin</td>
<td>Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH\textsubscript{2}</td>
<td>0.70</td>
<td>62</td>
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<tr>
<td>A (deltorphin)</td>
<td>Tyr-D-Met-Phe-His-Leu-Met-Asp-NH\textsubscript{2}</td>
<td>1,630</td>
<td>2.4</td>
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<tr>
<td>B (deltorphin II)</td>
<td>Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH\textsubscript{2}</td>
<td>2.450</td>
<td>0.71</td>
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<tr>
<td>C (deltorphin I)</td>
<td>Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH\textsubscript{2}</td>
<td>3,150</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Freidrich Sertürner who named the compound after Morpheus the Greek god of sleep. Despite a heroic effort by medicinal chemists to develop analgesics with reduced side effects, morphine remains the gold standard for the treatment of severe pain two centuries after its discovery.

Ligands that interact with all three opioid receptor types have been identified through modifications of both small molecule ligands and the endogenous peptides including the generation of DOR selective agonists and antagonists. Agonists and antagonists are also valuable pharmacological tools that have been extensively used to characterize the opioid receptors.

1.10.1. DOR Selective Non-peptide Agonists

The first DOR selective non-peptide agonist was BW373U86 (2, Figure 1.4), but this compound, which is a racemic mixture, also has affinity for MOR and demonstrates at least partial agonist activity at both MOR and DOR in vivo (Table 1.2). R- (+)-BW373U86 demonstrated enhanced potency for DOR compared to (-)-BW373U86. SNC80 (3) is the methyl ether derivative of (+)-BW373U86 and displays DOR mediated antinociceptive activity. SNC80 and (+)-BW373U86 also demonstrate antidepressant activity in rodent models.

Small molecule DOR agonists have been shown to produce non-lethal seizures in several species including rodents and rhesus monkeys. Administering either DOR antagonists or therapies for absence epilepsy blocked SNC80 induced seizures. Antidepressant effects of SNC80 were examined in a forced swim test in mice, and antidepressant activity was shown to be separable from the convulsant activity. In a recent investigation of the convulsant activity of SNC80, seizures were observed in only
Figure 1.4. Non-peptide DOR selective agonists.

Table 1.2. Affinities and potencies of selected agonists at MOR and DOR.\textsuperscript{19}

<table>
<thead>
<tr>
<th>Agonist</th>
<th>IC\textsubscript{50} or Ki (nM)</th>
<th>IC\textsubscript{50} (nM)</th>
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</thead>
<tbody>
<tr>
<td>BW373U86</td>
<td>0.92* 46* 0.2</td>
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</tr>
<tr>
<td>SNC 80</td>
<td>1.0* 2500* 2.7</td>
<td></td>
</tr>
<tr>
<td>TAN 67</td>
<td>1.1 2300 6.6</td>
<td></td>
</tr>
<tr>
<td>(+) TAN 67</td>
<td>11 815 74</td>
<td></td>
</tr>
<tr>
<td>(-) TAN 67</td>
<td>0.47 70 150</td>
<td></td>
</tr>
<tr>
<td>SIOM\textsuperscript{a}</td>
<td>4.1 88 22</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} From ref.\textsuperscript{93, 94}
one of four monkeys tested at 10 mg/kg SNC80.\textsuperscript{90} When the same monkey that demonstrated seizure activity with SNC80 at 10 mg/kg was re-tested with the same dose one year after the initial study, this monkey did not display any observable seizure activity. The dose of SNC80 required to observe seizures is generally 3- to 10-fold higher than the ED\textsubscript{50} for antinociception.\textsuperscript{90}

TAN67 (4) produces antinociception in the acetic acid writhing assay and binds to DOR with high affinity and selectivity.\textsuperscript{91} Enantiomerically pure (-) TAN67 is a full agonist in the MVD and is antinociceptive in the tail flick assay after i.c.v. administration to diabetic mice\textsuperscript{92} and i.t. administration to normal mice.\textsuperscript{93} (+) TAN67 produced nociception following i.t. administration. The nociceptive behavior associated with (+) TAN67 was blocked by both (-) TAN67 and naltrindole (8, Figure 1.5).

SIOM (5) demonstrates selectivity for one of the proposed DOR subtypes (DOR-1) and is an agonist at high doses but at lower doses displays antagonist activity at DOR-1 and also acts as a MOR antagonist.\textsuperscript{27, 94-96} For a detailed discussion of opioid receptor subtypes see Chapter 3.

1.10.2. DOR Selective Non-peptide Antagonists

Opioid antagonists have played a critical role in deciphering the pharmacology of the opioid receptors. For example, selective blockade by the antagonist naloxone (6, Figure 1.5) is a criterion for the classification of opioid receptor mediated activity in pharmacological assays.\textsuperscript{65} Naloxone binds to all three opioid receptor types and was the first example of a “pure opioid antagonist”. Naltrexone (7), which is the N-cyclopropyl methyl (CPM) derivative of naloxone, is a more potent antagonist than the parent compound but also binds to all three receptor types. Naltrindole (8) (NTI) was the first
non-peptide DOR selective antagonist reported in the literature and has served as a lead compound for the synthesis of a variety of compounds targeting DOR (Table 1.3). NTI was designed by Portoghese and co-workers using the “message-address” concept whereby the oxymorphone core comprises the “message” and the indole acts as both the “message” and “address” akin to Phe in the enkephalins.

![Chemical structures of Naloxone and Naltrexone](image)

**Figure 1.5.** Non-peptide antagonists.

**Table 1.3.** Affinities and potencies of selected antagonists.

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>$K_i$ (nM)</th>
<th>$K_e$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOR</td>
<td>MOR</td>
</tr>
<tr>
<td>Naloxone</td>
<td>23</td>
<td>1.8</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>6.6</td>
<td>1.1</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>0.12</td>
<td>11</td>
</tr>
</tbody>
</table>
1.10.3. DOR Selective Peptides

Synthetic peptide ligands have played a vital role in the pharmacological analysis of the opioid receptors. Advances in the formulation and modification of peptides are also improving their potential as useful therapeutic agents.\textsuperscript{98,99}

1.10.3.1. Agonists

The enkephalins have been extensively modified resulting in ligands that display increased affinity, potency and metabolic stability. Common modifications include the incorporation of a D-amino acid into position 2, as found in the endogenous amphibian peptides, and/or cyclization of the peptide. DADLE, DSLET and DTLET (Table 1.4) are linear analogs of enkephalin that demonstrate enhanced selectivity for DOR.\textsuperscript{19} DSLET and DTLET produce antinociception and do not demonstrate cross tolerance in mice that are acutely tolerant to morphine.\textsuperscript{25} DPDPE (9, Figure 1.6 and Table 1.4) is a highly DOR selective cyclic enkephalin analog which contains the modified amino acid D-penicillamine at both positions 2 and 5 linked together through a disulfide bond.\textsuperscript{19} DPDPE is also antinociceptive when administered to mice, but does not cause the GI effects or Straub tail associated with opioid analgesics such as morphine.\textsuperscript{25}

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_i$ (nM)</th>
<th>$IC_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DOR</td>
<td>MOR</td>
<td>KOR</td>
</tr>
<tr>
<td>DADLE</td>
<td>Tyr-D-Ala-Gly-Phe-D-Leu</td>
<td>2.1</td>
<td>14</td>
</tr>
<tr>
<td>DSLET</td>
<td>Tyr-D-Ser-Gly-Phe-Leu-Thr</td>
<td>1.8</td>
<td>39</td>
</tr>
<tr>
<td>DTLET</td>
<td>Tyr-D-Thr-Gly-Phe-Leu-Thr</td>
<td>2.7</td>
<td>34</td>
</tr>
<tr>
<td>DPDPE</td>
<td>Tyr-D-Pen-Gly-Phe-D-Pen</td>
<td>2.7</td>
<td>710</td>
</tr>
</tbody>
</table>

\textit{Table 1.4. Affinities and potencies of DOR selective peptide agonists.}\textsuperscript{19}
Figure 1.6. DOR selective peptide ligands.
1.10.3.2. Antagonists

The first DOR selective peptide-based antagonists were N,N-dialkylated enkephalin derivatives. One of the early pharmacologically useful derivatives of enkephalin was ICI-174,864 (10, Figure 1.6) which is a potent antagonist in the MVD assay (Table 1.5). This peptide showed selective antagonism of DPDPE following i.c.v. administration in rodents. However, at high concentrations ICI-174,864 demonstrated agonist activity.

The DOR selective antagonist Tyr-Tic-Phe-Phe (TIPP) where Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (11, Figure 1.6 and Table 1.5), has been extensively modified and is a promising scaffold for the development of multilabeled derivatives to study DOR. Schiller and co-workers synthesized TIPP while investigating the effect of conformational constraint on opioid receptor selectivity. A shift in selectivity from MOR to DOR was observed when the constrained phenylalanine derivative Tic was substituted for D-Phe\(^2\) in the MOR selective peptide Tyr-D-Phe-Phe-Phe-NH\(_2\). The C-terminal amide derivative TIPP-NH\(_2\) (12, Figure 1.6) is a full agonist in the guinea pig ileum (GPI) and was the first ligand reported to have mixed MOR agonist and DOR antagonist activity.

<p>| Table 1.5. Affinities and potencies of DOR selective peptide antagonists.(^{19}) |
|----------------|--------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>K(_i) (nM)</th>
<th>K(_e) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICI 174,864</td>
<td>N,N-diallyl-Tyr-Aib-Aib-Phe-Leu</td>
<td>29,600</td>
<td>190</td>
</tr>
<tr>
<td>TIPP</td>
<td>Tyr-Tic-Phe-Phe</td>
<td>1,720</td>
<td>1.2</td>
</tr>
<tr>
<td>TIPP[[^1]]</td>
<td>Tyr-Tic[^1][[^1]N[^1]]-Phe-Phe</td>
<td>3,230</td>
<td>0.31</td>
</tr>
</tbody>
</table>

\(^{19}\) This section refers to the earlier text on the first DOR selective peptide-based antagonists and their properties.
The peptide acids TIPP and TIP are the prototypical DOR selective peptide antagonists. The TIP(P) peptides are stable in aqueous buffer but undergo degradation in DMSO and MeOH via Tyr-Tic diketopiperizine formation and cleavage of the Phe$^3$-Tic$^2$ amide bond. The pseudopeptide TIPP[Ψ] (Figure 1.6) has a reduced amide bond between Tic$^2$ and Phe$^3$ to prevent diketopiperizine formation and is a more potent DOR antagonist than the parent peptide. TIP(P) derivatives extended at the C-terminus were investigated as potential ligands for the incorporation of multiple labels to aid in studies of DOR. Incorporation of amino acid residues with acidic or basic side chains, such as aspartic or glutamic acid and lysine, into peptide ligands is useful for the addition of labels as the side chain can be selectively deprotected and functionalized during solid phase peptide synthesis (SPPS). C-Terminal extension of TIPP to yield the peptides TIPP-D/L-Asx/Glx (Asx = Asn or Asp, Gsx = Gln or Glu) maintained the selectivity of these peptides for DOR. The stereochemistry of the acidic amino acid in the 5$^{th}$ position had little effect on binding affinity to DOR in the Asx derivatives, although a slight preference was demonstrated for D-Glx over L-Glx. The addition of a lysine residue, which was acetylated to mask the positive charge and therefore mimic the functionalization of the side chain amine, was also well tolerated by DOR. Both TIPP-D-Lys(Ac) and TIPP-L-Lys(Ac) retained nanomolar affinity for DOR. These studies

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>$K_i$(nM)</th>
<th>$K_c$(nM)</th>
<th>IC$_{50}$(nM)</th>
<th>MOR</th>
<th>DOR</th>
<th>MVD</th>
<th>GPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIPP-NH$_2$</td>
<td>Tyr-Tic-Phe-Phe-NH$_2$</td>
<td>79</td>
<td>3.0</td>
<td>18.0</td>
<td>1700</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIPP-NH$_2$</td>
<td>Dmt-Tic-Phe-Phe-NH$_2$</td>
<td>1.2</td>
<td>0.12</td>
<td>0.20</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.6. Affinities and potencies of TIPP derivatives.
indicated that C-terminal extension of TIPP with hydrophilic residues was well tolerated by DOR.\textsuperscript{105}

\section*{1.11. Ligands to Study the Delta Opioid Receptors}

Affinity labels are ligands that bind to their receptors with non-equilibrium kinetics and display an extremely slow off rate and a $K_d$ less than $1 \times 10^{-12}$ M. The binding of an affinity label to its target receptor is proposed to occur in a two-step recognition process (Figure 1.7).\textsuperscript{92} The first step is characterized by reversible binding of the ligand to the receptor followed by an irreversible, non-equilibrium binding interaction which typically results from the formation of a covalent bond. These ligands are useful probes for receptor studies because the labeling of the receptor is irreversible and therefore the interaction is preserved even after biochemical manipulation such as receptor isolation. For example, affinity labels have been utilized to purify and image their receptor targets.\textsuperscript{106, 107} In general, affinity labels can be divided into two categories: those that require photolysis for receptor labeling (photoaffinity) and those that require interaction with a nucleophilic residue in the receptor target for irreversible binding (electrophilic). Both types offer distinct advantages and disadvantages over one another.\textsuperscript{107}

Photoaffinity labels rely on the application of radiation typically from a UV source to transform a functional group such as an azide or a diazo compound into a highly reactive nitrone or carbene intermediate.\textsuperscript{107} An advantage of these labels is their ease of design as the species generated by photolysis can react with multiple functional groups.
The first peptide based affinity labels for the opioid receptors typically utilized phenylalanine derivatives with a para-azido substitution. However, the UV irradiation required for photoaffinity label activation can also damage the opioid receptors. Therefore, more recent publications have focused on the synthesis and utilization of electrophilic affinity labels.

\[
D + R \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} DR \rightarrow DR^* \\
k_{-1}/k_1 = K_d
\]

**Figure 1.7.** Proposed interaction of an electrophilic affinity label with its target receptor. A. Electrophilic group of ligand is in appropriate proximity to a nucleophilic residue in the receptor for a second irreversible binding step to occur. B. Ligand does not bind in the vicinity of a nucleophilic receptor. C. Electrophilic group of ligand is not in appropriate proximity of a nucleophilic residue and the irreversible binding step does not take place. Adapted from Takemori and Portoghese.

The successful generation of an electrophilic affinity label requires proper placement of an electrophilic group such that the reactive functionality of the ligand is in
close proximity to a nucleophilic residue on the receptor (Figure 1.7). Though the design of electrophilic affinity labels may require a more extensive examination of the proper placement of the reactive group in each respective ligand, the selectivity with which these ligands label their targets is enhanced over the photoaffinity labels. This ‘recognition amplification’ is exemplified by the MOR affinity label β-funaltrexamine (β–FNA 14, Figure 1.8) which demonstrates reversible binding to all three opioid receptors but covalently label only MOR at low ligand concentrations.\textsuperscript{107}

Opioid antagonists have thus far been more amenable than agonists to affinity label derivatization. β-Chlornaltrexamine (β-CNA, 15, Figure 1.8), which is a nitrogen mustard derivative of naltrexone, was the first antagonist-based affinity label reported for opioid receptors and was reported to bind irreversibly to all three receptors, with the highest affinity for MOR followed by KOR and lastly DOR.\textsuperscript{107} Receptor labeling by the highly reactive aziridine intermediate of β-CNA is similar to that observed with a photoaffinity label. β-FNA, a fumarate derivative of naltrexamine, is an irreversible MOR antagonist and a reversible KOR agonist.\textsuperscript{109} While reversible MOR antagonists were able to block covalent labeling of MOR by β-FNA, reversible agonists were ineffective at preventing alkylation. These data suggest that agonists and antagonists bind differently to MOR.

Kinetic analysis of β-FNA binding to MOR revealed that the majority of initial binding interactions were reversible with only a small subset leading to a covalent interaction.\textsuperscript{110} This demonstrates the importance of designing ligands that have high affinity in the first reversible recognition step (Figure 1.7). The residues involved in the covalent attachment of β-FNA to MOR were determined using CNBr cleavage in
Figure 1.8. Non-peptide affinity labels.
conjunction with SDS-PAGE to characterize the receptor fragment bound to tritiated β-FNA.\textsuperscript{52} Site directed mutagenesis was then employed to determine the specific residue involved in irreversible labeling. When Lys233 was replaced with Ala, β-FNA did not bind irreversibly to MOR suggesting that this residue is the site of covalent attachment. Initial binding studies of β-FNA with chimeric receptors revealed that the region from the middle of IL3 to the C-terminus was required for covalent labeling of MOR by β-FNA.\textsuperscript{111} Since this region does not contain Lys233 but has been shown to be important for morphine binding to MOR, the authors concluded that Lys233 was likely not part of the MOR ligand-binding pocket. The studies of β-FNA binding demonstrate the complimentary nature of the various techniques available to study receptor ligand interactions, but also the importance of determining the site of affinity label attachment. Chimeric receptor studies delineate the region of a receptor important in ligand binding while site directed mutagenesis hones in on specific amino acid residues that confer ligand binding and selectivity. Affinity labels allow the direct detection of interactions between receptor residues and ligands.\textsuperscript{107}

Fentanyl isothiocyanate (FIT) (\textsuperscript{17}, Figure 1.8), which is an isothiocyanate derivative of the MOR agonist fentanyl, selectively alkylates DOR. A more selective derivative (+)-\textit{cis}-3-methyl FIT (SUPERFIT, \textsuperscript{18}) was synthesized by Burke et al.\textsuperscript{112} SUPERFIT has been used to purify DOR from NG108-15 cells and chimeric receptor studies indicate that the region of the receptor required for irreversible labeling ranges from IL1 to the middle of TM3.\textsuperscript{43, 107} SUPERFIT blocked diprenorphine binding to all three cloned receptor types, but displays 200-fold selectivity for DOR over MOR and KOR. The isothiocyanate is proposed to interact with a cysteine or lysine residue of
DOR, and since IL1, TM2 and TM3 have high homology between MOR and DOR the authors proposed that the selectivity of SUPERFIT binding is due to EL1. SUPERFIT provides an excellent example of the selectivity that may be conferred to a ligand as a result of selectivity amplification, as the parent compound fentanyl is a reversible MOR agonist.43

Portoghese and co-workers first described the use of a reporter affinity label for MOR based on the cross-linking of Cys and Lys residues by naphthalene dialdehyde (Figure 9A).113 The isoindole that results from Cys and Lys cross-linking is fluorescent, hence the name “reporter” affinity label. PNTI (19, Figure 1.9B) is an affinity label derivative of NTI which contains ortho-phthaldehyde (OPA).114 The binding of PNTI to DOR was examined by flow cytometry of Chinese hamster ovary (CHO) cells stably expressing the receptor. The specific fluorescence of the isoindole moiety indicated that this compound bound covalently to DOR. Cross-linking of the Cys216 and Lys214 residues located in TM5 was proposed as the mechanism of irreversible binding. Remarkably, PNTI, which is a derivative of the prototypical small molecule DOR antagonist NTI, acts as a full agonist in the MVD assay with an IC50 of 0.12 nM. The agonist activity was found to be irreversible following extensive washing, but was attenuated by pre-treating the MVD preparation with NTI, indicating specific labeling of DOR. A drawback to using the OPA group is the overlap between the autofluorescence of cells and the specific fluorescence of OPA.115 To overcome this disadvantage Portoghese and co-workers designed OPA derivatives of NTI appended to a naphthalene dialdehyde (NDA-NTI, 20).116 The attachment of the napthyl moiety to the dialdehyde115 lowers the energy required to excite the isoindole that results from covalent binding to the
Figure 1.9. A. Mechanism of isoindole formation. B. Reporter affinity labels.
side chains of Cys and Lys residues. NDA-NTI retained the agonist activity observed with PNTI, however binding to DOR did not afford the fluorescent isoindole but did result in an irreversible complex with the receptor. The authors propose Schiff base formation with the side chain amine of Lys214 in TM5, which results in pseudo-irreversible binding of this derivative.

Peptides offer the synthetic advantage of modular assembly, which makes these ligands easily amenable to incorporation of numerous labels including affinity labels. The first peptide-based electrophilic affinity label reported in the literature was a derivative of enkephalin, [D-Ala²,Leu⁵]enkephalin chloromethyl ketone (DALECK), which demonstrated agonist activity. DALECK displayed irreversible binding in the GPI and MVD, and the agonist activity was blocked by the opioid antagonist naloxone.

DALCE ([D-Ala²,Leu⁵,Cys⁶]enkephalin) is an affinity label derivative of enkephalin that relies on the formation of a disulfide bond for irreversible binding to DOR. Addition of the reducing agent DTT to the binding buffer reduces the blockade of DOR binding sites providing evidence for the formation of a disulfide between DALCE and DOR.

Many of the early electrophilic affinity labels were comprised of chloromethyl ketone (DALECK) or cysteine residues (DALCE). More recently, the isothiocyanate and bromoacetamide functional groups have been utilized in the preparation of peptide based affinity labels for both MOR and DOR. The identification of affinity labels for DOR has been particularly promising with derivatives of enkephalin and the DOR selective antagonist TIPP.
Early efforts aimed at the identification of affinity label derivatives of DOR agonists relied on para-substitution of Phe\(^3\) in deltorphin I derivatives. Both the bromoacetamide ([D-Ala\(^2\), Phe(p-NHCOCH\(_2\)Br)]del I) and the isothiocyanate ([D-Ala\(^2\), Phe(p-NCS)]del I) derivatives displayed a loss in affinity to DOR compared with the parent peptide (TABLE 1.7).\(^{119}\) In fact, para substitution of Phe\(^3\) with the bromoacetamide and isothiocyanate groups caused a general decrease in affinity of peptide ligands for DOR.\(^{120}\)

### Table 1.7. Peptide based affinity labels.\(^{118, 119}\)

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>IC(_{50}) DOR</th>
<th>IC(_{50}) MOR</th>
<th>WRIB(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DALCE</td>
<td>[D-Ala(^2), Leu(^5), Cys(^6)]enkephalin</td>
<td>4.1 nM</td>
<td>55 nM</td>
<td>50% @ 3 μM</td>
</tr>
<tr>
<td>Deltorphin derivatives:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Ala(^2), Phe(p-NHCOCH(_2)Br)(^3)]del I</td>
<td>3800 nM</td>
<td>&gt; 10000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Ala(^2), Phe(p-NCS)(^3)]del I</td>
<td>83 nM</td>
<td>&gt; 10000</td>
<td>45% @ 80 nM</td>
<td></td>
</tr>
<tr>
<td>[D-Ala(^2), Phe(p-NHCOCH(_2)Br)(^5)]del I</td>
<td>140 nM</td>
<td>4000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[D-Ala(^2), Phe(p-NCS)(^5)]del I</td>
<td>300 nM</td>
<td>&gt; 10000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Multiple derivatives of TIP(P) have been prepared in the Aldrich research group.\(^{105, 108, 120-123}\) Derivatives of TIPP in which the para position of Phe\(^3\) was substituted with either isothiocyanate or bromoacetamide displayed a decrease in affinity for DOR (Table 1.8).\(^{108}\) However, despite the small decrease in binding affinity to DOR, TIP(p-NCS)P displayed an IC\(_{50}\) of 12 nM and 55\% wash resistant inhibition of binding (WRIB). When the Phe(p-NCS) amino acid was moved to Phe\(^4\) of TIPP ligands with high affinity and selectivity were identified and these peptides exhibited 50\% WRIB and an IC\(_{50}\) of 5 nM compared to 6 nM for the parent peptide TIPP (Table 1.7).\(^{123}\) The corresponding bromoacetamide derivatives TIP(p-NHCOCH\(_2\)Br)P and TIPP(p-NHCOCH\(_2\)Br) demonstrated IC\(_{50}\) values of 65 nM and 5.4 nM, respectively. While the Phe(p-
NHCOCH₂Br)³ derivative did not demonstrate WRIB, the Phe(ρ-NHCOCH₂Br)⁴ TIPP derivative displayed 85% inhibition of [³H]DPDPE binding to DOR.¹²²

Table 1.8. TIPP based affinity labels.¹¹⁸,¹¹⁹

<table>
<thead>
<tr>
<th>R¹</th>
<th>R²</th>
<th>DOR IC₅₀ (nM)</th>
<th>WRIBᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂</td>
<td>H</td>
<td>87.7</td>
<td>20%</td>
</tr>
<tr>
<td>NCS</td>
<td>H</td>
<td>12.4</td>
<td>55%</td>
</tr>
<tr>
<td>H</td>
<td>NH₂</td>
<td>11.8</td>
<td>20%</td>
</tr>
<tr>
<td>H</td>
<td>NCS</td>
<td>5.4</td>
<td>50%</td>
</tr>
<tr>
<td>NH₂</td>
<td>H</td>
<td>36.2</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td>NHCOCH₂Br</td>
<td>H</td>
<td>65.0</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td>H</td>
<td>NH₂</td>
<td>19.9</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td>H</td>
<td>NHCOCH₂Br</td>
<td>14.1</td>
<td>85%</td>
</tr>
</tbody>
</table>

a. Inhibition of [³H]DPDPE binding.

To identify a general solid phase synthetic strategy for the preparation of TIPP-based probes for studies of DOR the commonly used tag biotin was incorporated at the C-terminus of TIPP via a hydrophilic diamine linker. The resulting peptide TIPP-Asp-NH(CH₂CH₂O)₂CH₂CH₂NH-biotin had a Kᵢ = 12 nM which was comparable to that of TIPP (Kᵢ = 6.1 nM) and TIPP-Asp-NH₂ (Kᵢ = 9.5 nM).¹²¹ This biotinylated peptide served as a scaffold for the introduction of an affinity label in the synthesis of dual labeled TIPP derivatives. The affinity labels TIPP(ρ-NCS)-Asp-NH(CH₂CH₂O)₂CH₂CH₂NH-biotin and TIPP(ρ-NHCOCH₂Br)-Asp-
NH(CH₂CH₂O)₂CH₂CH₂NH-biotin demonstrated high affinity for DOR with IC₅₀ values of 41 and 10 nM, respectively.¹²⁰ Both affinity labels exhibited WRIB with 56% inhibition of [³H]DPDPE binding for the isothiocyanate and 69% for the bromoacetamide containing derivatives.

The combination of an affinity label and a fluorophore offers the advantage of utilizing covalent attachment in conjunction with the capability to visualize receptors in a variety of preparations (as described above) without the use of radioisotopes.¹²⁴

A variety of techniques have been utilized to gain an understanding of ligand-receptor interactions of the opioid receptors. These techniques are complimentary in their nature, and each technique has its own merits and pitfalls. The focus of the research presented in this thesis is on the design, synthesis and evaluation of peptide-based affinity labels targeting DOR. Our goal is to utilize these ligands to obtain a more detailed picture of the nature of the molecular interactions that occur between these ligands and DOR.

1.12. References


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Chapter 2. Design and Synthesis of Multi-labeled TIPP (Tyr-Tic-Phe-Phe, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) Derivatives Targeting DOR

*Note that the compound numbers used in this chapter are only applicable to this chapter.
2.1. Literature Review

2.1.1. Introduction

There are three opioid receptor types, mu, kappa and delta, all of which produce analgesia.\(^1\) The opioid receptors are G-protein coupled receptors (GPCRs), which mediate their action through inhibitory G proteins. Since there is no crystal structure for any of the opioid receptors, computational models are primarily based on the crystal structure of bovine rhodopsin, which is a related GPCR.\(^2\) Mutagenesis of the opioid receptors has been employed to identify receptor domains and specific amino acids that are involved in ligand binding.\(^3\) These studies are limited due to the possibility of changing structural elements within the receptor and must be cautiously evaluated, especially when loss of ligand affinity results from changing amino acid residues.\(^4\) Furthermore, mutagenesis generally does not give information on the portion of the ligand that is interacting with the receptor.

We are interested in studying ligand-receptor interactions of opioid peptides with the opioid receptors. The endogenous ligands of the opioid receptors are peptides, and there is evidence that peptides may have different binding requirements compared to small molecule ligands.\(^3,\ 5,\ 6\) Developing an understanding of the molecular interactions between the opioid receptors and both peptide and non-peptide ligands will provide complimentary information to aid in the design of therapeutics targeting the opioid receptors.

The delta opioid receptor (DOR) is particularly interesting due to its modulation of the mu opioid receptor (MOR).\(^7\-\(^{11}\) Both agonists and antagonists at DOR result in
attenuation of side effects, for example tolerance and respiratory depression mediated by MOR agonists such as morphine.

2.1.2. Techniques to Study Ligand-Receptor Interactions: Affinity Labels

There are significant limitations to the techniques available to study receptor-ligand interactions of GPCRs. The standard approach used in the study of soluble proteins is to determine an X-ray structure that, if possible, is co-crystallized with a ligand of interest, to determine molecular interactions via electron density maps. GPCRs are not amenable to the current crystallization process due to their high hydrophobic content, instability due to basal level activity and low expression levels. As a result only a relatively small number of GPCRs have been successfully crystallized and examined by X-ray crystallography. In lieu of a crystal structure, alternative techniques such as mutagenesis and affinity labeling have been employed to examine ligand-receptor interactions of the opioid receptors.

Affinity labels are ligands that interact with their targets in a non-equilibrium manner and have emerged as useful tools to study receptors, particularly in cases where a crystal structure is unavailable. Electrophilic affinity labels are proposed to bind to their target receptors in a two-step process involving the reversible recognition of the ligand by the receptor, followed by a second irreversible interaction (Figure 2.1). The second step leads to selectivity amplification since the receptor labeling will only occur when the electrophilic group is in appropriate proximity to a nucleophilic residue of the receptor. The covalent attachment of an affinity label to its target receptor is useful because the attached ligand can be used in the detection and isolation of receptors that are bound to the affinity label. Identifying specific amino acid residues in the opioid receptors that
bind covalently to affinity label peptides will yield information about the binding domain of the receptor for that ligand. Information about ligand orientation can also be deduced by deciphering the binding interactions between an affinity label and its target receptor. This information will aid in improving computational models of ligand receptor interactions, which will be useful for the design of new drugs targeting these receptors.

Liu-Chen and co-workers reported the first successful identification of the specific amino acid involved in the covalent attachment of an affinity label to an opioid receptor. Beta-funaltrexamine (β-FNA, 1, Figure 2.2A) is a selective alkylating agent of MOR, but binds reversibly to the kappa opioid receptor (KOR). The third intracellular loop (IL3) through the C-terminus of MOR was defined as the sequence

**Figure 2.1.** Proposed interaction of an electrophilic affinity label with its target receptor. A. Electrophilic group of the ligand is in appropriate proximity to a nucleophilic residue in the receptor for a second irreversible binding step to occur. B. The electrophile does not have the appropriate reactivity to label the receptor. C. The electrophilic group of the ligand is not in the proximity of a nucleophilic residue and the irreversible binding step does not take place. Adapted from Takemori and Portoghes ref. 23
required for irreversible binding of β-FNA in chimeric receptor studies. However, site directed mutagenesis revealed that Lys233 (Figure 2.2B), which is located in transmembrane 5 (TM5) and outside of the region defined in the chimeric receptor studies, was the site of covalent attachment of β-FNA to MOR.

Zhu et al. examined the irreversible binding of SUPERFIT (2, Figure 2.2A) to DOR using chimeric receptors comprised of MOR and DOR sequences. The region involved in covalent labeling was reported to be between the first intracellular loop (IL1) through the middle of the third transmembrane helix (TM3). Since TM2 of MOR and DOR have identical sequences, the selective labeling of DOR by SUPERFIT was likely a result of interactions with extracellular loop 1 (EL1) through TM3. There is no report of the specific amino acid residue involved in the covalent attachment of SUPERFIT to DOR.

Recently Roth and co-workers used molecular modeling in conjunction with mass spectrometry and mutagenesis to determine the site of attachment of a thiocyanate derivative of the KOR selective agonist salvinorin A (3, Figure 2.2A) to KOR. The point of attachment was proposed to be Cys315, which is located in TM7.

The addition of a fluorescent tag to an affinity label ligand could potentially aid in the detection of ligand-bound receptors, and fluorescence is commonly used in protein research as an alternative to radiolabeling. There are limited examples of ligands that contain an affinity label and a fluorescent reporter. The closest examples for the opioid receptors are reporter affinity labels for DOR based on the interactions between the sulfhydryl and amine groups of Cys and Lys residues, respectively, with the o-phthaldehyde group. In this case, the covalent interaction between the ligand and the
Figure 2.2. A. Affinity labels for MOR, DOR and KOR. B. Position of covalent attachment of β-FNA to Lys233 of MOR. From ref. 4.
receptor affords the fluorescent isoindole derivative. In addition to incorporating fluorescent tags, purification and/or isolation tags can also be included in ligands to aid in receptor isolation.\textsuperscript{35}

\subsection*{2.1.3. Receptor Isolation and Analysis}

The isolation, purification and analysis of GPCRs is complicated by their low expression levels, even in engineered cell lines, and their extreme hydrophobicity which can lead to aggregation upon isolation from the cellular membrane.\textsuperscript{36} Howells and co-workers have utilized dual-tagged receptors in conjunction with several forms of chromatography to isolate the opioid receptors.\textsuperscript{37-39} DOR, MOR and KOR were engineered with an N-terminal FLAG-tag and a C-terminal hexahistidine (His)-tag and expressed in human embryonic kidney (HEK) 293 cells. After solubilizing the HEK 293 cell membranes using n-dodecyl-β-D-maltoside, the authors used extensive chromatography involving sequential isolation by different combinations of wheat germ agglutin agarose chromatography, gel filtration chromatography, immunoaffinity chromatography, immobilized metal ion chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to isolate the opioid receptors. Following isolation and in-gel digestion by trypsin or chymotrypsin, the receptors were characterized by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). A drawback to using such an extensive purification protocol is the need to start with very large amounts of membrane preparation (for example, 50 cell culture plates were used to isolate DOR\textsuperscript{38}).
2.1.4. Bioanalytical Tags and Techniques

Biotin\(^{40}\) (4, Figure 2.3) and its derivative d-desthiobiotin\(^{41}\) (DSB, 5) are commonly used to isolate receptors through affinity chromatography. Biotin has also been coupled with many of the tags described in the following section, including photocleavable groups, as a means of introducing a purification and/or detection tag to biomolecular targets and their ligands.\(^{42,43}\)

![Biotin and d-Desthiobiotin](image)

**Figure 2.3.** Purification tags biotin and its derivative desthiobiotin.

“Click chemistry” has been increasingly utilized to tag biomolecules with various probes to assist in the analysis of complex mixtures such as those encountered in biological samples.\(^{44-46}\) The copper catalyzed modification of the Huisgen cycloaddition of an azide to an alkyne\(^{47-49}\) (Scheme 2.1) is the most commonly encountered “click” reaction in

![Click chemistry](image)

**Scheme 2.1.** Proposed steps in the formation of a triazole in the copper mediated addition of an azide to an alkyne. Adapted from ref.\(^{49}\)
the current scientific literature, especially with regard to biological applications. This reaction has been employed in protein isolation,\textsuperscript{50, 51} and both azide and alkyne functionalized probes are commercially available for click-based detection of tagged biomolecules.

Combinations of the various tags described above into a single molecular entity have been used in numerous biological applications, ranging from enzyme profiling and proteomics to DNA sequencing. The addition of a photocleavable group allows for the release of specific groups prior to detection (or as a means of detecting) molecules of interest. One of the most commonly encountered photoreactive groups is the \textit{o}-nitrobenzyl group (\textit{o}NB).\textsuperscript{52} Derivatives of \textit{o}NB have been employed in a diverse array of applications including solid phase organic synthesis, biomolecular detection and oligonucleotide sequencing. Common substitutions to the \textit{o}NB core (Figure 2.4) are the introduction of an \textit{α}-methyl at the benzylic carbon, alkoxy substitution of the benzene ring (meta to the NO\textsubscript{2} and benzylic alcohol) and substitution of the benzyl alcohol with benzylamine.\textsuperscript{53} Many of these substitutions lead to a bathochromic shift in the absorption (>350 nm) and in some cases enhanced rates of photolysis. An hydroxyacid photolinker was synthesized by Whitehouse et al.\textsuperscript{54} to introduce the photocleavable linker onto the Wang resin (hydroxy based solid support) for solid phase organic synthesis.

While the \textit{o}NB group has been extensively applied to organic synthesis, it has also enjoyed ample application in biologically oriented experiments. \textit{o}NB esters and amides have been used to for the spatial and temporal protection (caging) of substrates for mechanistic studies in biochemistry.\textsuperscript{52} Barltrop and Schofield originally described the light mediated release of glycine from benzyloxy carbonyl glycine in the early 1960s.\textsuperscript{55}
Photochemical caging has since been applied to peptides, nucleotides, neurotransmitters, enzyme substrates and a host of other biologically relevant substrates.\textsuperscript{52}

The oNB group has been useful for on-ChIP DNA sequencing with photocleavable fluorescent nucleotides used in conjunction with clickable DNA templates.\textsuperscript{56, 57} The DNA templates were immobilized on a glass chip via the azide/alkyne click reaction. The fluorescent photocleavable nucleotides were then incorporated into the growing DNA chain by DNA polymerase and detected by fluorescence. The fluorophore was cleaved from the nucleotide by UV irradiation, followed by incorporation of the next photocleavable fluorescent nucleotide, and the cycle was repeated to determine the sequence of the DNA template.

\textbf{Figure 2.4.} Common derivatives of the oNB group.

The combination of purification tags, fluorophores and photocleavable groups has also been used extensively in protein analysis. Proteomics involves the identification and quantitation of proteins from complex biological matrices.\textsuperscript{58} Isotope coded affinity tags (ICAT) have been used to label cysteine-containing proteins with isotopic labels for MS quantitation using tandem mass spectrometry (MS/MS).\textsuperscript{59} More recently, labels such as biotin and fluorescein have been incorporated to allow for visualization of the tagged
proteins in the visible ICAT (VICAT) method.\textsuperscript{60-62} Since some of these tags confound the mass spectral data, photocleavable groups have been incorporated to release the tag prior to MS/MS analysis. In addition to acting as a detection tag, fluorescein conjugates were also used as a substitute for radioisotopes in VICAT because the fluorescein tag acted as a reporter ion in MS/MS experiments.\textsuperscript{62}

Matrix assisted desorption ionization (MALDI)-MS is a standard technique to study complex biological samples. MALDI-MS uses a pulsed laser to excite matrix ions which then transfer energy to analytes embedded within the matrix, resulting in the formation and ejection of analyte ions.\textsuperscript{63, 64} Samples are spotted onto the surface of a MALDI target plate and co-crystallized with a MALDI matrix. MALDI matrices are typically derivatives of benzoic or cinnamic acids (Figure 2.5), which form crystalline

![Chemical structures](image)

**Figure 2.5.** Common organic acids used as MALDI matrices.
solids and absorb in the region corresponding to the MALDI laser (typically 337-355 nm). The tag-mass technique is a recent development, which utilizes MADLI-MS for tissue imaging of biomolecules such as mRNA or low abundance membrane proteins. The tags used in the tag-mass MALDI experiments were comprised of a biomolecular recognition unit (probe), such as an antibody, conjugated to a reporter mass tag (usually a peptide) via a photocleavable linker. An aminomethyl derivative of the oNB group was utilized in the tag-mass application because the absorption band of this group (~340 nm) was very close to that of the MALDI N$_2$ laser (337 nm). Following incubation with the tissue of interest, the mass tag was cleaved from the structure using the MALDI laser to activate the photocleavable group. The reporter mass was detected only when the probe tag was capable of interacting with its biomolecular target. The benefits of this technique were decreased background over traditional fluorescent-based techniques and increased ease of handling compared to traditional radioisotope techniques. Furthermore, the potential combination of probes and mass tags is nearly unlimited so that a diverse array of targets can potentially be imaged using this approach.

Ehring et al. demonstrated that sample ionization could occur by both thermal and photochemical MALDI processes. To decipher between these two mechanisms, Ehring and co-workers modified a MALDI-time of flight (TOF) mass spectrometer (Figure 2.6) so that the sample could be irradiated directly from the front (photo- and thermal ionization) or from the back (thermal ionization). Different populations of analyte and matrix ions were observed depending on the laser geometry. In particular, molecular ions [M+H]$^+$ were detected when sample was irradiated from the front but when samples were irradiated from the back only preformed cationized species [M+Na]$^+$, [M+K]$^+$ were
detected. These experiments demonstrated that laser induced photochemistry involving the photochemical excitation of matrix and subsequent reaction with analyte played a major role in the desorption process.

The photolytic processes of the MALDI laser have been exploited to screen a combinatorial library. Peptides immobilized on a solid support were sequenced in on-bead MALDI experiments reported by Semmler et al.\textsuperscript{67} as a means of increasing the throughput of peptide identification. The peptides were synthesized on a solid support that contained a substituted oNB group. Single beads were immobilized on a MALDI sample plate, and the MALDI laser was used to cleave the peptides from the resin. The peptides were then sequenced by MALDI Fourier transform ion cyclotron resonance tandem mass spectrometry (FTICR-MS).

**Figure 2.6.** Ehring’s setup for MALDI-MS to distinguish photochemical versus thermal mechanisms of analyte fragmentation. The Nd-YAG laser (355 nm) was used to irradiate the “backside” of the sample plate, resulting in ionization as a result of thermal MALDI processes only. The N\textsubscript{2} laser (337 nm) was used in the standard MALDI setup for “front-side” irradiation resulting in both photolytic and thermolytic MALDI processes. From ref.\textsuperscript{66}
The combination of analytical techniques and chemistries for the analysis of biomolecules has been especially beneficial to the field of proteomics. The analysis of membrane proteins such as the opioid receptors can potentially be aided by such combinations of tags, which allow for increased sensitivity of detection, improved isolation of labeled receptors and the ability to pursue these objectives from a variety of approaches.

2.2. Rationale

We are interested in utilizing peptides that possess a fluorescent tag along with various isolation tags and an electrophilic affinity label to aid in the elucidation of the specific DOR amino acids involved in binding opioid selective peptides. Our proposed strategy involves biological techniques coupled with mass spectrometry to identify DOR receptor fragments covalently labeled with our peptides. As such, the peptides described in this chapter were designed to aid in receptor isolation studies.

The multifunctional peptides were derived from a series of dual labeled peptides previously synthesized in our laboratory. Dual labeled TIPP derivatives with bromoacetamide at the para position of Phe⁴ [Tyr-Tic-Phe-Phe(p-NHCOCH₂Br), Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid] extended at the C terminus with the purification tag biotin³⁵ or d-desthiobiotin (DSB) (Aldrich and Wang, unpublished results) display high affinity and selectivity for DOR. Biotin exhibits femtomolar affinity for streptavidin, forming a nearly irreversible complex. DSB is a derivative of biotin that exhibits decreased affinity to streptavidin and therefore improved elution efficiency from streptavidin-based affinity supports compared to biotin.⁴¹ The dual labeled TIPP
derivatives with the DSB tag (Figure 2.7) are the parent peptides from which our multifunction peptides are derived.

A photocleavable multifunctional peptide (PC peptide, Figure 2.8) was designed to label and isolate DOR using a combination of the tags described in Section 2.1.4. We incorporated lysine residues at the C-terminus of the TIPP-based affinity label derivative to provide handles for further modification through functionalization of the side chain amines. In addition, polyethylene glycol (PEG)-like linkers were included to enhance water solubility and to separate the DOR selective peptide TIPP from the various functional groups incorporated at the C-terminus. 5-Carboxyrhodamine B (5-RhodB) was coupled to the side chain amine of one lysine to aid in detection of peptide-bound receptors through fluorescence imaging. Our choice of fluorophore was based on the desired characteristics such as the ease of incorporation into the peptide, quantum yield, Stokes shift, and cost of obtaining the fluorophore in sufficient quantities for SPPS. Commercially available fluorophores commonly used for protein detection and peptide labeling are often prohibitively expensive with regard to their use in solid phase peptide synthesis (SPPS). Rhodamine B (RhodB) has a quantum yield of 0.5 (in EtOH)\textsuperscript{68,69}}

Figure 2.7. Dual labeled TIPP derivative 10 and the corresponding amine control 9.
Figure 2.8. The photocleavable multifunctional peptides. Each functionality is highlighted with a bracket and a description. The lysine residues where the purification tag (a) and the fluorophore (b) are incorporated are also labeled in the structure.
is a commonly used fluorophore. The carboxyl derivatives of RhodB are available as a bulk solution of the sodium salts of 5- and 6-RhodB, which substantially reduces the cost of obtaining either isomer in sufficient quantities for peptide synthesis. The two isomers are readily separated using high performance liquid chromatographic (HPLC) in aqueous acetonitrile containing 0.1 % TFA. The low cost of obtaining large amounts of 5-RhodB for use in SPPS was a substantial driving force in our choice of this fluorophore, which has a Stokes shift of 65 nm (λ_{ex} of 520 nm and λ_{em} of 585). We are also interested in exploring the use of a click-based isolation technique for the enrichment of peptide bound receptors prior to enzymatic digestion. Therefore, we incorporated an alkyl azide into the peptide via a PEG-like linker. The azide will be used to covalently attach the peptide-based affinity label to an alkyne functionalized chromatographic support. In order to recover the ligand-receptor complex following click-based purification, a photocleavable linker was incorporated between the azide and the remainder of the peptide. As discussed in the introduction to this chapter, the oNB group has been used extensively in biological systems and was incorporated into our initial series of multifunctional peptide ligands targeting DOR. Upon activation at 365 nm the photocleavable linker undergoes photolysis to give the peptide acid 13 (Scheme 2.2) and the nitroso ketone product of the linker 14.

The PC peptide is a composite of the TIPP-Asp peptide-based affinity label, DSB, 5-RhodB, oNB and an alkyl azide. Each functional group is activated using parameters such as exposure to light (to cleave oNB or produce fluorescence from 5-RhodB), reaction with specific chemical entities (Cu and alkyne for the azide click reaction), or
simply incubation with the appropriate proteins (e.g. DOR for TIPP affinity label, streptavidin for DSB).

Scheme 2.2. The photocleavage of the oNB group leads to the formation of the peptide acid 13 and the corresponding nitroso ketone 14.
2.3. Results and Discussion

Preface to Results

As described in the rationale section of this chapter (section 2.2), the goal of this project was to design and synthesize peptides for the isolation of DOR for mass spectrometric analysis. During the initial synthesis of our photocleavable multifunctional peptide (PC peptide), several side products were observed in HPLC and electrospray ionization mass spectrometric (ESI-MS) analyses. Many of these side products were not identified despite our efforts to decipher their structures based on mass spectrometric analysis. In order to address the synthetic challenges and to increase the yield of the PC peptide, we designed and synthesized several model peptides including a series of dual labeled derivatives. An examination of the synthesis and where applicable, the biological evaluation, of these peptides is presented in the following sections.

This discussion will begin with the modified synthesis of the linker and its application to the synthesis of the parent dual-labeled peptides followed by an extensive discussion of the PC and related multifunctional peptides.

2.3.1. A Revised Synthesis of the Diamino-PEG-Like Linker

A revised synthetic strategy for obtaining the diamino-PEG-like linker, which was previously used in the preparation of the biotinylated TIPP derivatives\textsuperscript{35, 71} and in the synthesis of the multifunctional peptides (MFPs) presented here, was investigated to improve product yields. The original synthesis\textsuperscript{71} (Scheme 2.3) involved the mono-Alloc protection of a symmetric diamine (15), which resulted in a mixture of starting material, the desired mono-protected amine (16) and diprotected amine (17). The diprotected product is readily removed by extraction, however separating the starting material from
the mono-protected product via column chromatography results in further loss of the target product and low yields (in my hands often less than 20%). We developed a synthetic strategy that avoids the use of a symmetrical starting material and generated linker intermediates on a 1-10 g scale, which allowed for significant scale up of peptide synthesis. The revised synthesis also aided in the design of a novel series of multifunctional peptides targeting DOR (see sections 2.3.3 - 2.3.9 below).

![Scheme 2.3. Synthesis of mono-Alloc protected diamine.](image)

The PEG-like aminoazide linker (Scheme 2.4) was prepared following a modification of a procedure reported by Bertozzi et al. Nucleophilic displacement of chloride on 2-[2-(2-chloroethoxy)ethoxy]ethanol by phthalimide was followed by methanesulfonate esterification of the alcohol and subsequent nucleophilic attack by azide. The phthalimide protected linker 20 was then purified by column chromatography (69% yield over three steps). An Ing-Manske deprotection of the phthalimide afforded the PEG-like amino azide linker 21, which was taken on in the synthesis of the MFPs (section 2.3.4) and a previously described dual labeled TIPP derivative (TIPPAsp-Linker-DSB, section 2.3.2).
2.3.2. Modified Synthesis of a Dual Labeled TIPP Derivative (TIPP(X)-Asp-Linker-DSB)

Our revised strategy for preparation of the linker permitted the synthesis of the dual labeled TIPP derivatives 9 and 10 (Figure 2.7, originally synthesized by X. Wang) starting with over a gram of resin. Accessing the amine for further amino acid couplings required transformation of the azide in the diamine linker (in 23, Scheme 2.5) via a solid phase Staudinger reduction (Scheme 2.5). Azides have been utilized as a protecting group for amines in SPPS employing Nα-azido acids. After coupling of the first Nα-azido acid, PMe3 was used to reduce the azide to the α-amine, and this reaction was employed in the synthesis of the TIPP derivatives reported here. The azido-amino-PEG-like linker 21 (Scheme 2.4) was first loaded onto an aldehyde-based resin (FMPB AM).
Scheme 2.5A. Synthesis of dual labeled TIPP derivative 10 and amine control 9.
Scheme 2.5B. Synthesis of dual labeled TIPP derivatives 10 and amine control 9.
by reductive amination using NaBH(OAc)₃ (to give 22, Scheme 2.4) following a previously described procedure.⁷¹ D-Desthiobiotin (DSB) was then coupled to the secondary amine using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBT) and N,N-diisopropylethylamine (DIEA) in DCM/DMF (1:1). Following the solid phase Staudinger reduction (Scheme 2.5), the linear sequence of the dual labeled TIPP derivative 27 was assembled by standard Fmoc SPPS using Nα-fluorenylmethoxycarbonyl (Fmoc) protected amino acids, with the exception of the N-terminal residue which was protected with t-butoxycarbonyl (Boc). The allyloxycarbonyl (Alloc) group was removed from Phe(p-NHAlloc)⁴ using Pd(PPh₃) and phenylsilane in DCM.⁷⁵, ⁷⁶ The resin was then split into two portions, and one portion was cleaved in trifluoroacetic acid (TFA) to yield the reversible control peptide 9. Bromoacetic acid was then preactivated with N,N-diisopropylcarbodiimide (DIC) and added to the second portion of resin to afford the bromoacetamide affinity label peptide 10. The synthesis of the dual labeled TIPP derivative proceeded without significant generation of side products as detected by HPLC or ESI-MS analyses (Figures 2.9 and 2.10). The final peptide 10 was cleaved from the resin and purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) using a gradient of aqueous acetonitrile containing 0.1% TFA.
Figure 2.9. A. ESI-MS spectra and B. HPLC chromatogram (monitored at 214 nm) of peptide 9. The desired peptide has a $m/z = 1091.5$ [M+H]$^+$ and 1113.5 [M+Na]$^+$ and $t_R = 21.7$ min (HPLC: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-50% B over 45 min). The peak in the HPLC chromatogram with $t_R = 27.5$ and $m/z = 1147.6$ [M+H]$^+$ in the ESI-MS, is the tBu protected peptide which is a result of premature termination of the cleavage reaction and is not an indication of issues with the synthesis.
Figure 2.10. A. ESI-MS spectra and B. HPLC chromatogram (monitored at 214 nm) of an aliquot of peptide 10. The desired peptide has a $m/z = 1211.5$ [M+H]$^+$ and a $t_R = 25.1$ min (HPLC: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-50% B over 45 min).
2.3.3. Synthesis and Evaluation of a Photocleavable Multifunctional Peptide Ligand Targeting DOR

The PC peptide (12, Figure 2.8) was designed to aid in our isolation of labeled DOR fragments. Therefore, multiple functional groups were incorporated into this peptide in addition to the affinity label and DSB isolation tag. Fmoc-protected amino acids comprise the majority of the amino acids used to assemble the linear sequence of the PC peptide affinity label 12 and the amine control peptide 11 with the exception of the N-terminal amino acid, which was protected with Boc at the α-amine. The use of Boc at the N-terminus was essential to avoid degradation of the bromoacetamide affinity label during removal of the Fmoc group with piperidine. The ε-amine of the lysine residues (Figure 2.8) at positions 7 (Lys\textsuperscript{a}) and 9 (Lys\textsuperscript{b}) were protected with the ivDde and Mtt groups, respectively (see Scheme 2.6). The aniline of Phe(p-NH\textsubscript{2}) at position 4 was protected with the Alloc group. These groups were chosen based on the ability to selectivity remove them in the presence of the protecting groups required for peptide assembly. The sequence of selective deprotection and side chain functionalization was chosen to maximize efficient use of the fluorophore. Due to the reactivity of the electrophilic affinity label, the bromoacetamide was introduced in the last step of peptide synthesis.

The synthesis of the PC peptide (Scheme 2.6) was initiated with reductive amination of an aldehyde-based resin with the amino-azide-PEG-like linker as previously described using NaBH(OAc)\textsubscript{3} in DCM/TMOF (2:1).\textsuperscript{71} The resulting secondary amine 22 was subsequently coupled to the photocleavable linker using PyBOP, HOBt and DIEA in DCM/DMF (1:1), followed by capping with benzoic acid. Ester bond formation between
Scheme 2.6A. Coupling the photocleavable linker and β-Ala to the amino-azido-PEG-like linker.
Scheme 2.6B. Linear assembly of the PC peptide precursor.
Scheme 2.6C. Side chain functionalization of the precursor to the PC peptide.
Scheme 2.6D. Side chain functionalization of the precursor to the PC peptide continued.
Scheme 2.6E. Side chain functionalization (continued) and cleavage of the PC peptide amine control 11 and bromoacetamide affinity label 12 from the resin.
the secondary alcohol of the photocleavable linker and Fmoc-β-alanine (Fmoc-β-Ala) was accomplished using 1-(mesitylene-2-sulfonyl)-3-nitro1-H-1,2,4-triazole (MSNT) and 1-methylimidazole (Melm). Unreacted alcohol was capped following the standard procedure using benzoic anhydride and pyridine. Complete linear assembly of the multifunctional peptide was performed using standard Fmoc solid phase peptide synthesis using a mixed solvent system comprised of DCM/DMF (1:1). Subsequent functionalization of the side chains commenced with deprotection of the ε-amine of Lys(ivDde) by 2% hydrazine in DMF followed by coupling to d-desertiobiotin (DSB) using PyBOP, HOBt and DIEA in DCM/DMF (1:1). The Mtt protecting group was then removed from Lys using 3% TFA and 5% triisopropylsilane (TIS) in DCM. The free epsilon amine was subsequently coupled to 5-RhodB using PyBOP, HOBt and DIEA in DCM/DMF (1:1). The Alloc group at Phe(p-NH2) was then removed using Pd(PPh3)4 and phenylsilane in DCM. At this stage, the resin was aliquoted into two parts; one aliquot was cleaved in TFA to afford the amine control. Bromoacetic acid was preactivated with DIC and reacted with the second aliquot to yield the PC peptide bromoacetamide affinity label. The multifunctional peptide with the amine, and the bromoacetamide at the para position of Phe, were characterized by HPLC and ESI-MS (Figures 2.11-2.16).

In the initial synthesis of the PC peptide, multiple side products were observed in HPLC chromatograms and mass spectra of aliquots of peptide cleaved from the resin (Figure 2.11). The abundance of side products lowered the yield of the PC peptide and resulted in complications during purification of the peptide by preparative RP-HPLC. The stability of 29, comprised of the azide-based linker and the photocleavable group,
Figure 2.11. A-B. ESI-MS spectra and C. HPLC chromatogram (monitored at 214 nm) of an aliquot of peptide cleaved from the resin following assembly of affinity label 12. The desired peptide has a $m/z = 868.7$ [M+2H]$^{3+}$ and $t_R$ of 46.8 min (HPLC: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 20-50% B over 60 min).
was assessed by HPLC analysis of an aliquot of the compound cleaved from the resin. This product was stable in MeCN/H$_2$O containing 0.1% TFA at room temperature for 2 days (Figure 2.12). This indicates that decomposition of the linker at this stage does not contribute to the side products observed during synthesis of the PC peptide. ESI-MS (LC and direct injection) was used to determine the identity of some of the side products in different synthetic preparations of the peptides and at various stages throughout the syntheses (for example see Figure 2.13). The major contributing factors to the low yields of the PC peptide were incomplete Tyr coupling to Tic and incomplete deprotection of the Alloc and ivDde groups. Incomplete Mtt deprotection and/or incomplete 5-RhodB coupling were also identified as possible contributions to the generation of multiple peaks.
observed in HPLC analysis of earlier syntheses. The use of the azabenzotriazoles, \(N\)-(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylnitrobenzotriazoles, N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylnitrobenzotriazoles, N-oxido (HATU) and 7-aza-1-hydroxybenzotriazole (HOAt) for Boc-Tyr coupling drove this reaction to near completion. These reagents are more reactive compared to their benzotriazole counterparts and produce more efficient coupling reactions as a result of anchimeric assistance.\(^{81}\) We also performed double couplings of amino acids at several of the positions of the PC peptide in subsequent syntheses to limit the potential contribution of deletion products to decreased peptide yields.

![LC-MS total ion chromatogram (TIC) of the Alloc protected intermediate 34 from an early synthesis of peptide 12. Observed m/z values: \(t_R = 8.1\) min, \(m/z = 456.3 [M+H]^+\) corresponds to the mass of the PC-linker product 29; \(t_R = 9.5\) min, \(m/z = 1051.6 [M+2H]^{2+}\) corresponds to the mass of the des-5-RhodB product 33; \(t_R = 13.6\), \(m/z = 1056.1 [M+2H]^{2+}\) corresponds to the mass for the protected peptide 32; \(t_R = 18.6\) min, \(m/z = 1285 [M+H]^{2+}\) corresponds to the desired intermediate 34. There are also several other peaks that have yet to be identified.](image-url)
In ensuing syntheses of the PC peptide, the extent of Mtt removal was assessed by removing an aliquot of the Mtt deprotected peptide on resin and acetylation the resulting free amine with acetic anhydride. If the deprotection was complete, the peak corresponding to the free amine of Lys\(^b\) was absent in HPLC and ESI-MS analyses of the peptide cleaved from the resin. Incomplete removal of the Mtt group did not appear to be an issue in subsequent syntheses and the des-5-RhodB peak observed in an earlier synthesis was likely a result of incomplete 5-RhodB coupling. To address the incomplete deprotection of ivDde, we used a fresh bottle of hydrazine and examined the order of reagent addition in this reaction. Since our peptide contained the Alloc protecting group, allyl alcohol was added in the reaction to avoid possible diimide reduction of the Alloc double bond.\(^{82}\) Since alcoholic reagents shrink polystyrene based resins, the use of allyl alcohol may have decreased the extent of the ivDde deprotection by hydrazine. Furthermore, this reaction is performed in DMF, which does not efficiently swell polystyrene-based resins.\(^{83}\) The use of a new bottle of hydrazine and changing the order of reagent addition such that the hydrazine was added prior to allyl alcohol also improved the deprotection of the ivDde group.

Despite the improvement in obtaining the amine control, several side products were generated in the final step of peptide synthesis during bromoacetamide formation at the para amine of Phe\((p\text{-NH}_2)\)\(^4\), which substantially decreased the yield of the final peptide (Figure 2.14). When fresh bromoacetic acid and DIC were used to form the affinity label 12, the number of side products observed in the HPLC chromatogram was not substantially different between the amine control 11 and the affinity label peptides (Figure 2.15 compared to 2.16).
To further optimize generation of the PC peptide, an alternate solvent system for the purification was investigated. While acetonitrile/water (0.1% TFA) was capable of removing the majority of impurities, peaks with overlapping retention times were identified when “purified” peptide was analyzed by HPLC in both the triethylammonium phosphate (TEAP) and ammonium acetate (5 mM, pH 3 acetic acid) solvent systems. HPLC of the peptide in triethylammonium acetate (TEAA, pH 5.5)/acetonitrile was performed to determine if the resolution of the impurities could be retained while still utilizing a solvent system with favorable properties such as high volatility and non-nucleophilic characteristic. Following the attempted purification of ~15 mg of the bromoacetamide functionalized multifunctional peptide in the TEAA/acetonitrile solvent

Figure 2.14. HPLC chromatogram (monitored at 214 nm) with overlaid traces corresponding to 11 (blue, $t_R = 38.9$ min, Area % = 35.3) and 12 (red, $t_R = 41.5$ min, Area % = 17.5). HPLC conditions: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min, gradient started at 5 min.
Figure 2.15. A-B. ESI-MS spectra and C. HPLC chromatogram (monitored at 214 nm) of an aliquot of peptide cleaved from the resin following assembly of amine control 11 ($m/z = 828.4 \ [M+2H]^3^+$, $t_R = 34.6 \text{ min, Area } \% = 52.3$). A. ESI-MS spectrum ($m/z = 100-2000$) and B. enlargement of the peaks corresponding to the desired peptide 11 with a $+3$ charge state. HPLC conditions: Solvent A = $\text{H}_2\text{O}$ containing (0.1\% TFA), Solvent B = acetonitrile containing (0.1\% TFA), gradient: 20-50\% B over 60 min.
Figure 2.16. A-B. ESI-MS spectra and C. HPLC chromatogram (monitored at 214 nm) of an aliquot of peptide cleaved from the resin following assembly of affinity label 12 (m/z = 1302.6 [M+H]^{2+}, and m/z = 1313.6 [M+H+Na]^{2+}, t_R = 39.7 min, Area % = 39.8). A. ESI-MS spectrum (m/z = 100-2000) showing low mass fragments and B. enlargement of the peaks corresponding to the desired peptide 12 with a +2 charge state. HPLC conditions: Solvent A = H_2O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 20-50% B over 60 min.
system (pH 5.5), MALDI-MS and LC-MS analysis confirmed degradation of the peptide due to acetate ion attack at the bromoacetamide.

The purification of the PC peptide by tandem purification was then investigated using TEAP (pH 2.5)/acetonitrile as the first system and acetonitrile (0.1% TFA)/H₂O (0.1% TFA) as the second purification system. Using this method of purification, the amine control PC peptide was isolated in a sufficient amount to analyze its affinity for DOR in a binding assay. This was not the case for peptide 12 for which < 1 mg was isolated > 95% pure.

The final synthesis of the PC peptide produced the highest HPLC purity of both the amine control and the bromoacetamide affinity label. The improved purity may negate the need for the tandem HPLC purification of the PC control and bromoacetamide affinity label peptides.

In order to investigate the possible contribution of the various functional groups of the PC peptide to the generation of side products observed during peptide synthesis, we designed and synthesized various analogs, which will be discussed in section 2.3.4.

The interaction of 12 (200 nM) with Chinese hamster ovary (CHO) cells expressing DOR (CHO-DOR) was assessed using fluorescence microscopy (Figure 2.17). Cells labeled with the PC peptide exhibited fluorescence associated with the cell membrane, even after extensive washing to remove noncovalently bound peptide (Figure 2.17A). TIPP was not able to displace the multifunctional peptide when CHO-DOR cells preincubated with the PC peptide were subsequently incubated with TIPP (Figure 2.17C), which strongly suggested that the multilabeled peptide bound covalently to DOR. When CHO-DOR were pretreated with TIPP followed by incubation with the multifunctional
peptide the cells were protected from fluorescent labeling (Figure 2.17B), indicating that TIPP was able to block PC peptide binding to DOR. CHO cells that do not express DOR did not exhibit fluorescence after incubation with 12 (400 nM, Figure 2.17D), also indicating that DOR is required for the fluorescent labeling.

The binding affinity of the amine control 11 was determined in radioligand competition assays using CHO-DOR cells. This peptide exhibited only modest binding affinity to DOR with an IC₅₀ value of 459 ± 149 nM.

Figure 2.17. Fluorescence microscopy results from incubation of CHO cells with the PC peptide bromoacetamide affinity label 12. A. CHO cells treated with 200 nM peptide alone 12. B. Cells pretreated with TIPP (2 µM), followed by treatment with peptide 12 (200 nM). C. Cells treated with peptide 12 (200 nM), followed by incubation with TIPP (2 µM). D. CHO cells without DOR treated with peptide 12 (400 nM).
2.3.4. Design and Synthesis and Evaluation of Multifunctional Peptides to Address Synthetic Challenges and Probe Side Reactions of the PC Peptide

2.3.4.1. Synthesis of a Multifunctional Peptide Without the Photocleavable Linker

A multifunctional peptide (MFP) containing the affinity label, fluorophore, DSB tag and azide, but without the photocleavable linker (36, Figure 2.18), was synthesized to probe the influence of the photocleavable linker on the purity of the final peptide. This peptide may also be used in our studies as an alternative to the PC peptide; in this case the photocleavable linker would be incorporated into the solid support. The synthetic route to the MFP (Scheme 2.7) was similar to that of the PC peptide, with the exception that the photocleavable group was not incorporated into the peptide. In addition, the "clickable" linker (N$_3$(CH$_2$CH$_2$O)$_3$CH$_2$CH$_2$NH$_2$) used in the synthesis of the MFP was commercially available. The synthesis of the MFP proceeded smoothly up to the final

![Figure 2.18. Structure of the MFP 36 with the Lys residues where the DSB and 5-RhodB tags are incorporated are designated as a and b, respectively.](image-url)
Scheme 2.7A. Linear assembly of the MFP precursor.
Scheme 2.7B. Side chain functionalization and cleavage of 42 to yield the MFP 36.
amino acid coupling of Boc-Tyr(tBu)-OH to the Tic residue of the peptide (Scheme 2.7, 38 to 39). This coupling was performed twice using the standard coupling reagents PyBOP and HOBT with DIEA as the base in DCM/DMF (1:1). Despite the double coupling and prolonged reaction time (overnight), starting material was present in an aliquot of the peptide cleaved from the resin (Figure 2.19B). Since the Fmoc protected Tic (Figure 2.19A) was not observed in HPLC analysis of the aliquot of peptide, the incomplete reaction was due specifically to incomplete coupling of Tyr to Tic and not incomplete Fmoc deprotection. Difficult couplings, such as in this case where a secondary amine is the reactive group, can often be overcome with the use of stronger activating reagents such as HATU in combination with HOAt and DIEA. Again, despite multiple couplings and prolonged reaction times, starting material was still present in an HPLC chromatogram of an aliquot of the peptide cleaved from the resin (Figure 2.19C). Aggregation of the peptide on resin is often to blame when couplings prove to be challenging to drive to completion. Aggregation is most often a result of H-bonding and/or beta sheet formation. Polar solvents such as DMF are often capable of disrupting these interactions but since a mixed solvent system (DCM/DMF, 1:1) was used to ensure proper resin swelling in all amino acid couplings described for the multifunctional peptides, a second approach was employed. Chaotropic agents such as LiCl can disrupt aggregation and are used in either washing steps and/or included with coupling reagents during peptide synthesis of difficult sequences. The resin was split into three portions (portions A, B and C) and one portion (portion A) was washed with a 0.8 M solution of LiCl in DMF, followed by coupling to Boc-Tyr(tBu)-OH using HATU, HOAt and DIEA (2 X). The result of this combination did not offer much improvement.
in the ratio of starting material to product (Figure 2.19D). In a final attempt to drive the reaction to completion, a series of solvent washes similar to that used in our Alloc deprotection was used (DCM 3 X 1 min, THF 3 X 1 min, DCM 3 X 1 min, DMF 1 X 15 min, DCM 3 X 1 min, MeOH 3 X 1 min, DCM 3 X 1 min) followed by amino acid coupling using HATU/HOAt/DIEA in DCM/DMF (1:1). An aliquot of the peptide cleaved from the resin after the final coupling demonstrated the desired product as the predominant peak (Figure 2.19E). Since the MFP was subjected to 11 coupling reactions, the exact influence of each step is difficult to determine.

A portion of the peptide on resin (portion B) was reacted with acetic anhydride (10 equiv) and DIEA in DMF to determine if capping the unreacted secondary amine of Tic (38) would lead to overlapping peaks of the desired and capped peptides (this was performed to ensure that these peaks would be separable upon HPLC purification of the final peptide). This capping step did not influence the retention time of the peak corresponding to starting material in HPLC analysis and there were no peaks corresponding to the Tic acetylated peptide in ESI-MS analysis. Since the acetic anhydride group is very small, the inability to cap the Tic residue suggests that this

**Figure 2.19.** A. HPLC chromatogram (monitored at 214 nm) of the Fmoc protected peptide Fmoc-38 (t_R = 50.3 min, area % = 78.4).
Figure 2.19. B-E. HPLC chromatograms of coupling reactions with Boc-Tyr(tBu)-OH using various combinations of coupling reagents. B. Coupling reaction: Boc-Tyr-OH/PyBOP/HOBt/DIEA (4:4:4:8) X 2. C. Coupling reaction: Boc-Tyr-OH/HOAt/HATU/DIEA (1:1:0.9:2) 3X, Boc-Tyr-OH/HOAt/HATU/DIEA (4:4:3.6:8). D. Following LiCl (0.8 M in DMF) and coupling reaction: Boc-Tyr-OH/PyBOP/HOBt/DIEA (4:4:4:8) in DCM/DMF/0.8 M LiCl in DMF (1:1:1) X 3. E. Following an extensive series of washing steps, the reaction was driven to product formation with Boc-Tyr-OH/PyBOP/HOBt/DIEA (4:4:4:8) in DCM/DMF (1:1). The t<sub>R</sub> of the desired peptide is 36.7 min and the starting material is 31.5 min. The area percents for the peaks are given for the two major peaks only. HPLC conditions: Solvent A = H<sub>2</sub>O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min, gradient started at 5 min.
Figure 2.20. A. HPLC chromatograms (monitored at 214 nm) of the MFP intermediate 39 following removal of the ivDde group (t_R = 27.0 min, area % = 78.6), and B. DSB coupling (t_R = 30.9 min, area % = 55.6). HPLC conditions: Solvent A = H_2O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min gradient started at 5 min.
peptide is in a conformation that prevents even small groups from reacting with the secondary amine of Tic and this is likely the reason that completing the coupling of Boc-Tyr(tBu)-OH to Tic proved to be extremely difficult.

It was hoped that the extensive washing of the resin with various solvents would be enough to disrupt any conformation of the peptide leading to decreased coupling efficiency. The third portion of the resin (portion C) was subjected to the series of washes described for portion A (i.e.: DCM (3 X 1 min), THF (3 X 1 min), DCM (3 X 1 min), DCM/DMF (1:1, 3 X 1 min), DMF (1 X 15 min), DCM (3 X 1 min), MeOH (3 X 1 min), and finally DCM (3 X 1 min), then reacted with Boc-Tyr(tBu)-OH, HATU, HOAt and DIEA in DCM/DMF. There was not a significant decrease in the ratio of the peaks for starting material vs. product in this reaction suggesting that this series of washes alone was not sufficient to drive the reaction to completion as discussed above for portion A.

Portion A of the resin was employed in the continuation of the synthesis of MFP 36. The deprotection of the ivDde group using 2% hydrazine and subsequent DSB coupling proceeded without difficulty (Figure 2.20). Mtt deprotection and 5-RhodB coupling resulted in the generation of three predominant peaks at 214 nm in HPLC analysis (Figure 2.21). The peptide was analyzed by LC-MS (Figure 2.22) and two peaks were identified as the desired peptide (t<sub>R</sub> = 46.4 min, m/z = 1167 ([M+H]<sup>2+</sup>)) and des-5-RhodB product (t<sub>R</sub> = 36.8 min, m/z = 933 ([M+H]<sup>2+</sup>)). The third peak corresponds to an as yet unidentified product with a base peak of m/z = 935 in the mass spectrum (Figure 2.22). When the peptide was subjected to the conditions for Alloc deprotection, further generation of side product peaks was observed in the HPLC analysis of an aliquot of the peptide cleaved from the resin (Figure 2.23). LC-MS analysis of the peptide aliquot
Figure 2.21. A-B. ESI-MS and C. HPLC chromatogram (214 nm) of an aliquot of the MFP following 5-RhodB coupling (peptide 41 cleaved from the resin). A. Full mass spectrum and B. enlargement of mass spectrum showing desired peptide (m/z = 1166.1 [M+H]²⁺), side product peaks. C. The desired peptide has a tᵣ = 39.57 min, area % = 26.1 and the two side products have tᵣ (and area percents) = 30.8 min (11.3%) and 47.4 min (8.8%). HPLC conditions: Solvent A = H₂O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min, gradient started at 5 min.
Figure 2.22. LC-MS of an aliquot of peptide 41 cleaved from the resin. A. HPLC chromatogram (monitored at 254 nm) and B. total ion chromatogram (TIC). C-E. Mass spectra corresponding to the three major peaks in A and B. C. 36.7 min peak, base peak $m/z = 933$ [M+H]$^{2+}$, which corresponds to the des-5-RhodB peptide, D. 46.4 min peak, base peak $m/z = 779$ [M+H]$^+$, and 1167 [M+H]$^{2+}$, which is the desired peptide; and E. 55.2 min peak, base peak $m/z = 935$, which has not yet been identified. HPLC conditions: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-65% B over 60 min, gradient started after 5 min.
Figure 2.23. A-B. ESI-MS spectra and C. HPLC chromatogram (214 nm) of peptide 36 following the removal of the Alloc group from peptide 41. HPLC conditions: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min, gradient started at 5 min. A. ESI mass spectrum of m/z peaks from 100-2000 and B. in the range 1100-1200. The desired peptide has an m/z = 1124.1 [M+H]$^{2+}$, the acetylated product has an m/z = 1145.1 [M+H]$^{2+}$. Multiple sodium adducts for the desired peptide (1135.1 [M+Na]$^{2+}$; 1141.1 [M+2Na]$^{2+}$) and for the acetylated side product (1156.1 [M+Na]$^{2+}$; 1167.6 [M+2Na]$^{2+}$) were also observed in ESI-MS.
(Figures 2.24-2.25) produced m/z values corresponding to the desired peptide (t_R = 41.7 min, m/z = 1125 ([M+H]^2+)), an acetylated product (t_R = 43.2 min, m/z = 1147 ([M+H]^2+), +43 Da of the molecular weight of the desired peptide) and several as yet unidentified products. These results indicate that the simple exclusion of the oNB group from the PC peptide scaffold did not enhance peptide synthesis under these conditions. Our results suggest that the current synthetic strategy for obtaining the MFP 36 needs significant revision and the highly substituted polystyrene based resin (0.79-1 mmol/g, FMPB AM resin) may not be the optimal support for the synthesis of this peptide.

Figure 2.24. LC-MS of the MFP (36) following the removal of the Alloc group. A. HPLC chromatogram (monitored at 254 nm) and B. TIC. HPLC conditions: Solvent A = H_2O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-65% B over 60 min, gradient started after 5 min.
Figure 2.25. Mass spectra corresponding to peaks in TIC and HPLC chromatogram of the MFP 36. The retention times of the peaks are in parentheses. The desired peptide has a $t_R = 41.7$ min and a base peak with $m/z = 751 \ [M+2H]^3+$. The acetylated product has a $t_R = 43.2$ min and a base peak of $m/z = 765 \ [M+2H]^3+$. There are also several peaks for which the structures have yet to be assigned.
2.3.4.2. Fluorescent Dual Labeled TIPP Derivative

A dual labeled TIPP derivative containing only the affinity label and the fluorophore 5-RhodB (Figure 2.26) was designed to determine whether the fluorophore was undergoing a side reaction during formation of the bromoacetamide affinity label. In addition, this dual labeled peptide was submitted for binding analysis to determine if placement of the fluorophore one position away from the TIPP peptide influenced DOR binding. The dual labeled peptide was synthesized by standard Fmoc SPPS on the Peptide Amide Linker-polyethylene glycol-polystyrene resin (PAL-PEG-PS resin) as outlined in Scheme 2.8. Fmoc-Lys(Mtt) was coupled directly to the linker using HOBr, PyBOP and DIEA in DCM/DMF (1:1) followed by complete linear assembly of the peptide. Side chain functionalization was initiated with the removal of the Mtt group using 3% TFA followed by coupling to 5-RhodB using standard coupling reagents for

![Figure 2.26. Structures of the fluorescent dual label (FDL) peptide 44 and the corresponding amine control 43.](image-url)
peptide synthesis (HOBt, PyBOP, DIEA). The Alloc group was then removed from Phe(p-NHAlloc)\(^4\) using Pd(PPh\(_3\))\(_4\) and phenylsilane in DCM. The resin was then split into two portions. One portion was cleaved in TFA to yield the amine control 43 (Figure 2.26). Bromoacetic acid was preactivated with DIC in DCM and added to second portion of resin to introduce the bromoacetamide group to the peptide at Phe\(^4\). The synthesis of the dual labeled peptide amine control 43 and bromoacetamide affinity label 44 proceeded smoothly without significant generation of side products (Figures 2.27 and 2.28). These results indicate that 5-RhodB did not significantly contribute to the side products observed in the synthesis of the PC peptide, at least not by reacting with the reagents used to functionalize the remaining side chains. However, the influence of the fluorophore on secondary structure formation and/or steric hindrance during synthesis of the PC peptide cannot be ruled out as contributing to the difficulties encountered in the synthesis of the peptide.

The fluorescent bromoacetamide peptide based affinity label 44 was subjected to HPLC analysis in methanol (0.1% TFA) to assess the stability of the electrophilic group. These conditions did not lead to significant degradation of the peptide as monitored by HPLC. The DSB derivative 9 also did not undergo degradation during HPLC analysis in acidic methanol. The results of this analysis indicate that acidic methanol is not able to displace bromine in these peptides. These results also suggest that MeOH (0.1% TFA) can be used as a second solvent system for the analysis of bromoacetamide containing affinity labels as an alternative to the TEAP/acetonitrile solvent system. Nevertheless, the TEAP/acetonitrile solvent system is preferred in syntheses with questionable purity,
Scheme 2.8. SPPS of the fluorescent TIPP derivatives 43 and 44.
Figure 2.27. A. ESI-MS ($m/z = 753.4$, [M+H]$^{2+}$, 502.6, [M+2H]$^{3+}$) and B. HPLC chromatogram of the amine control 43 (crude peptide, $t_R = 30.8$ min, area % = 60.8). HPLC conditions: Solvent A = H$_2$O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-50% B over 45 min gradient started at 1 min.
Figure 2.28. A. ESI-MS (m/z = 813.3 [M+H]^2+) and B. HPLC chromatogram of the affinity label 44 (crude peptide, t_R = 34.6 min, area % = 40.5). HPLC conditions: Solvent A = H_2O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-50% B over 45 min, gradient started at 1 min.
as TEAP provides higher resolution of the multifunctional peptides described in this thesis when compared to the methanol solvent system.

The fluorescent dual labeled TIPP derivative 44 and corresponding amine control 43 were examined for binding affinity to CHO-DOR cells. A decrease in binding affinity to DOR (Table 2.1) was observed compared to the parent dual label peptides (9 and 10), indicating that the fluorophore should be farther separated from the TIPP portion of the peptide to minimize interference with receptor binding. The amine control peptide 43 and bromoacetamide affinity label 44 were also evaluated for wash resistant inhibition of binding\(^{89}\) (WRIB) of \(^{3}\text{H}\)DPDPE binding to DOR. CHO cell membranes were incubated with the peptides followed by a series of washes involving centrifugation and resuspension of the membranes to remove non-bound peptide. Following this washing procedure, the binding of \(^{3}\text{H}\)DPDPE to DOR was evaluated using a standard binding assay.\(^{89}\) At 65 nM, 44 exhibited 95% WRIB compared to 17% for the reversible amine

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC(_{50}) DOR (nM) ± SEM(^{a})</th>
<th>IC(_{50}) MOR (nM) ± SEM(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>459 ± 149</td>
<td>ND</td>
</tr>
<tr>
<td>43</td>
<td>93.4 ± 14.4</td>
<td>1060 ± 97</td>
</tr>
<tr>
<td>44</td>
<td>80.5 ± 8.8</td>
<td>1170 ± 60</td>
</tr>
<tr>
<td>TIPP(p-NH(_2))-Asp-NH(CH(_2)CH(_2)O)(_2)CH(_2)CH(_2)NH-DSB(^{c})</td>
<td>38.4 ± 4.9</td>
<td>&gt; 10000</td>
</tr>
<tr>
<td>TIPP(p-NHCOCH(_2)Br)-Asp-NH(CH(_2)CH(_2)O)(_2)CH(_2)CH(_2)NH-DSB(^{c})</td>
<td>5.2 ± 2.7</td>
<td>6280 ± 840</td>
</tr>
</tbody>
</table>

a. The IC\(_{50}\) values are the mean ± SEM of three independent experiments
b. The IC\(_{50}\) values are the mean ± SEM of two independent experiments
c. From X. Wang’s Dissertation.
control peptide 43 at 76 nM (Figure 2.29). Therefore, this peptide will potentially be useful to label and visualize DOR in cellular and tissue preparations.

**Figure 2.29.** Percent WRIB of [3H]DPDPE binding to DOR of the fluorescent amine control 43 and affinity label 44. The affinity label 44 (65 nM) exhibited 95 ± 7 % WRIB following a 90 min preincubation (at room temperature) with membranes from CHO cells that express DOR. The control compound 43 (76 nM) was efficiently washed from the membranes and demonstrated 17 ± 5 % WRIB under the same conditions as peptide 44. The results are an average of three independent experiments ± %SEM.
2.3.4.3. Synthesis of an Extended Fluorescent TIPP Derivative

Due to the decreased binding affinity exhibited by the fluorescent dual label 44 and amine control 43 compared to TIPP-Asp-DSB 7 and 9, we designed and synthesized an extended fluorescent TIPP peptide (56, Scheme 2.9). In this series, the fluorophore is separated from the TIPP portion of the peptide by two linkers and a lysine residue to determine if the placement of the fluorophore farther away from the C-terminus of TIPP-Asp would enhance the binding affinity of these fluorescently labeled peptides. This set of peptides will also be useful in the synthesis of peptides with different fluorophores in order to examine the possible influence of fluorophore identity with regard to binding affinity. Peptide 56 was manually synthesized by Fmoc SPPS on the PAL-PEG-PS resin. As with the MFP 36, the final coupling of Boc-Tyr-OH to Tic also proved to be difficult with this peptide. A double coupling of Tyr to Tic using HATU, HOAt, DIEA and 0.8 M LiCl in DMF resulted in the formation of product, but starting material was still present (Figure 2.30A). The same coupling step in the synthesis of the PC peptide proceeds smoothly using HATU, HOAt and DIEA in DMF with a reaction time of 1.5 h, suggesting that the photocleavable linker in the PC peptide may assist in preventing aggregation. Following the extensive series of washes described for 38 to 39 in section 2.3.4.1, coupling of Boc-Tyr(tBu)-OH to Tic using HATU, HOAt and DIEA afforded the desired peptide (Figure 2.30B). The linear assembly of peptide 56 (Scheme 2.9) was similar to the synthesis of the amine control 43 except Lys(ivDde) and a second PEG-like linker were incorporated into the peptide chain. Side chain functionalization of 52 was achieved following the procedure described for the multifunctional peptides except that Lys\(^a\) was acetylated using acetic anhydride and DIEA in DMF. Unlike the MFP 36, the
Scheme 2.9. SPPS of the extended fluorescent TIPP derivative 56. The Lys residues where acetyl and 5-Rhod B were coupled are labeled a and b, respectively.
Figure 2.30. A. HPLC chromatogram of peptide 52 following Boc-
Tyr(tBu)-OH coupling using HATU and B. HPLC chromatogram of 52
following Boc-Tyr(tBu)-OH coupling following extensive washing. The t<sub>R</sub>
of the desired peptide is 34.4 min and the starting material is 29.0 min. The
area percents for the peaks are given for the two major peaks only. HPLC
conditions: Solvent A = H<sub>2</sub>O containing (0.1% TFA), Solvent B =
acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min,
gradient started at 5 min.
final Alloc deprotection yielded the peptide 56 without significant generation of side products (Figure 2.31). Since the MFP was synthesized on a polystyrene resin, these results suggest that the use of the PAL-PEG-PS resin was beneficial to the synthesis of the extended TIPP peptide 56.

**Figure 2.31.** A. ESI-MS \( (m/z = 911 \ [\text{M+H}]^{2+}) \) and B. HPLC chromatogram of 56 (crude peptide, \( t_R = 33.2 \text{ min, area %} = 64.3 \)). HPLC conditions: Solvent A = H\(_2\)O containing (0.1% TFA), Solvent B = acetonitrile containing (0.1% TFA), gradient: 5-60% B over 55 min, gradient started at 5 min.
2.3.5. Synthesis of a Model Peptide and Initial Mass Spectrometry Experiments

Evidence for cleavage of the PC peptide at the photocleavable linker by MALDI-MS (see for example Figure 2.32), suggested that the MALDI laser could potentially act as the source of photolysis for the selective cleavage of the peptides from a resin in our proposed receptor isolation experiments. The PC peptide showed ions corresponding to both the parent peptide (for example, see Figure 2.32, \( m/z = 2484 \)) and the peptide fragment corresponding to cleavage of the PC linker (\( m/z = 2047 \)) in MALDI-MS. The corresponding cleavage products were also observed in ESI-MS (Figure 2.15, parent peptide, \( m/z = 829 \) \([\text{M+H}]^3+\) and cleavage products, \( m/z = 690 \) \([\text{M+H+Na}]^3+\) (peptide

![MALDI-MS spectrum](image)

**Figure 2.32.** MALDI-MS of the PC peptide amine control 11 demonstrating cleavage from the oNB linker. The \( m/z \) of the desired peptide is 2484 (MW = 2486) and the fragment resulting from cleavage of oNB is 2047 (MW = 2048).
acid, 13, Scheme 2) and the linker 478 [M+H+Na]$^+$ (for structure see 29, Scheme 2.6). Since ESI-MS has a lower mass range of detection compared to MALDI (due to matrix interference at $m/z < 600$), we observed a peak with an $m/z = 478$, which likely corresponds to the PC linker cleaved by in-source decay in the ESI chamber. As discussed in the literature review (section 2.1.4) MALDI induced cleavage of the oNB group, which can potentially occur by thermal and photolytic MALDI processes, has been reported and used in various applications including tag based mass mapping. Therefore, a model peptide was used in our initial investigation of cleavage of the peptide from the FMPB AM resin using the MALDI N$_2$ laser.

A multilabeled peptide (57 and 59, Figures 2.33-2.34) containing the fluorophore, azide and the photocleavable linker was synthesized following the procedure described for the PC peptide (section 2.3.3) except that Lys(ivDde) and DBS were not incorporated, the Alloc protecting group was not removed and the N-terminal residue was Fmoc-Tyr(tBu) rather than Boc-Tyr(tBu). The N-terminal coupling of Fmoc-Tyr(tBu)-OH also proved to be difficult with this peptide and the coupling had to be repeated multiple times under various conditions. Fmoc-Tyr(tBu)-OH was coupled to the secondary amine of Tic using PyBOP, HOBt and DIEA in DCM/DMF (1:1) followed by a reaction using a stronger activating agent, 6-chloro-benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyClock), in place of PyBOP and HOBt. Finally, HATU in combination with HOAt and DIEA in DCM/DMF (1:1) afforded the final linear sequence of the peptide. Following the removal of the Mtt group, the side chain amine of lysine was coupled to 5-RhodB. The peptide was either cleaved from the resin with TFA and lyophilized or reserved for on bead MALDI analysis. Since this peptide was synthesized
for use in on-bead MALDI model studies, it was not evaluated for binding affinity or efficacy at DOR.

![Structure](image-url)

**Figure 2.33.** Structure of the TFA cleaved model peptide 57 (A) and the corresponding fragment resulting from cleavage at the ester bond 58 (B).

Release of peptide 59 from the resin was explored in on-bead MALDI experiments following the protocol reported by Semmler et al., in which the photocleavable linker was exploited for cleavage via the MALDI N₂ laser (337 nm). The resin was either spotted onto the MALDI plate and crushed with a spatula or directly covered in matrix (following crushing, the resin was covered in matrix). Peptide that had been cleaved in TFA 57 was also spotted onto the plate and covered with matrix. The matrix used in these experiments was DHB as this matrix was reported to give the highest ion count by Semmler et al. These experiments were also attempted using sinapinic
acid (7, Figure 2.5) and CHCA (8, Figure 2.5) but as reported by Semmler, DHB (6, Figure 2.5) gave superior ion intensities in our experiments as well. We also attempted three methods of bead preparation for spotting which included spotting whole beads, spotting beads and crushing them on the MALDI plate with a spatula, and finally crushing the beads in a vial then spotting. Crushing the beads on the MALDI plate, followed by covering them in matrix gave the highest peak intensities for the peptide fragment. Figure 2.35 shows the MALDI-MS of the FMPB AM resin without peptide. There are peaks generated from the resin alone in the m/z range of 1200 through 1650. The MALDI MS of the peptide on resin from plate-crushed beads is given in Figure 2.36.
The peak at $m/z = 1998$ corresponds to the side chain protected peptide acid (Figure 2.34) that has been cleaved from the resin as a result of cleavage from the $o$NB group. Therefore, we successfully released our protected peptide from the FMPB AM resin using the MALDI laser to cleave the peptide from the $o$NB group. MALDI-MS of the peptide that was first cleaved from the resin by TFA (57), and then spotted onto the MALDI plate, produced peaks for the parent peptide and peptide fragment 58. Increasing laser intensity increased the ion intensity of 58 (Figure 2.37). Whether this cleavage was a result of photolytic vs. thermolytic processes is unclear. The results of these experiments had significant implications on our ability to decrease the number of steps in sample preparation of labeled DOR because we have the potential to directly detect labeled receptor fragments on a solid support using MALDI MS.

**Figure 2.35.** MALDI-MS of FMPB AM resin without peptide (ion intensity = 5105).
Figure 2.36. MALDI-MS of 59 on resin. A. Resin was crushed on the MALDI plate (ion intensity = 1.1 x 10^4). B. Resin was crushed in a vial prior to spotting the MALDI plate (ion intensity = 2.8 x 10^4). The samples were irradiated with the MALDI laser at an intensity of 2700 giving rise to the protected peptide cleaved from the resin (m/z = 1998, MW = 1997) and peaks corresponding to sequential loss of the tBu protecting groups.
Figure 2.37. MALDI-MS of 57 cleaved from resin by TFA using different laser intensities. A. The sample was irradiated with a laser intensity of 2600 giving rise to both the full length peptide ($m/z = 2323.0$, MW = 2323) and the fragment resulting from cleavage at the $o$NB group ($m/z = 1886$, MW = 1885, base peak ion intensity = $1.5 \times 10^3$). B. The sample was irradiated with a laser intensity of 2700 which led to an increase in the fragment ion intensity ($m/z = 1886$, base peak ion intensity = $2.6 \times 10^4$).
2.4. Conclusions

The key to synthesis of multifunctional peptides was the use of various protecting groups that allowed for selective functionalization of side chain amines during solid phase peptide synthesis. It is also well established that high purity reagents are required in SPPS, since intermediates are not purified during peptide assembly on the solid support and any side products will be carried through the entire synthesis. The resin type and structure of the peptides also impacted the purity of the peptide syntheses. Despite significant challenges encountered in the synthesis of the photocleavable multifunctional peptide, a final synthesis using fresh reagents and HPLC analysis at every step in the synthesis afforded both the amine control 11 and the bromoacetamide affinity label 12 with purities of 52% and 40%, respectively, by HPLC of the crude peptide following cleavage from the resin.

Preliminary fluorescence microscopy experiments with the PC peptide bromoacetamide affinity label 12 demonstrated that the bromoacetamide affinity label was a useful tool for visualizing DOR expressed on CHO cells. The binding affinity of the amine control 11 was modest compared to the parent compound 9 suggesting that the current scaffold for incorporating the various tags may not be optimal. A series of multifunctional peptides derived from the PC peptide were prepared to address synthetic challenges posed by the PC peptide. A dual labeled derivative, which contained only the affinity label and the fluorophore was synthesized without significant generation of side products and both the amine control 43 and bromoacetamide affinity label 44 peptides were obtained in mg quantities with relative ease. While the IC$_{50}$ values for the amine control and affinity label were modest at 93 and 81 nM respectively, the affinity label
peptide exhibited nearly 95% WRIB of $[^3]$HDPDPE binding to DOR at 65 nM peptide. The amine control was efficiently washed from the membranes (< 20% WRIB), suggesting that the WRIB exhibited by the affinity label was specifically due to interactions between DOR and the bromoacetamide group. This dual labeled TIPP derivative will likely be useful as a probe for the detection of labeled DOR in microscopy and other bioanalytical studies of DOR.

2.5. Future Work

The PC peptide 12 produced from the most recent synthesis will require further purification to yield sufficient quantities of the peptide for biological evaluation. Since the HPLC purity of this synthesis is higher than previous syntheses, the first approach will be to purify the peptide in aqueous acetonitrile containing 0.1% TFA, followed by HPLC analysis in the TEAP system. If side products are determined to co-elute with the desired peptide, a tandem preparative HPLC purification will be carried out as described in section 2.3.3. The initial fluorescence microscopy experiments with the PC peptide need to be repeated with the amine control 11 and PC peptide 12 to verify the efficiency of the washing procedure to remove noncovalently bound peptide. Additional fluorescence-based experiments, such as determining a time course for peptide binding, may also be useful. Full binding analysis of the PC peptide amine control and bromoacetamide affinity label also needs to be performed in CHO-DOR cells. The wash resistant inhibition of $[^3]$HDPDPE binding by 12 compared to 11 in CHO-DOR cells will also be determined.

Model studies for the attachment of a peptide onto an alkyne functionalized solid support also need to be completed. Peptide 57 was purified for use in these studies and
two solid supports (PEGA and ChemMatrix rink amide resin) have been functionalized with propargyl glycine. The peptide will be “clicked” onto each of these supports followed by Fmoc quantitation to determine the efficiency of the reaction under biologically relevant conditions (binding buffer see section 2.6.7.1). Following the immobilization of the peptide onto the solid support, the resin will be examined in on-bead MALDI experiments similar to those reported in section 2.7.

Since the PC peptide amine control demonstrated only modest affinity for DOR, peptides 56 and 36 will be evaluated for binding affinity at DOR. If the MFP 36 has significantly improved affinity for DOR over the PC peptide, an alternate route for the synthesis of this peptide should be explored. Since the synthesis of peptide 56, which was performed on the PAL-PEG-PS resin, proceeded smoothly following extensive washing with multiple solvents (section 2.3.4.1), the MFP synthesis may benefit using a similar support. The backbone amide linker (BAL) can be coupled to the PAL-PEG-PS Resin or to the amino-Chem Matrix resin to enable reductive amination of the resulting aldehyde with the amino-azido-PEG-like linker. The lower substitution and enhanced swelling properties of these resins compared to the FMPB AM resin may result in the formation of fewer side products in the synthesis of this peptide.

Since the dual labeled peptide 44 with the bromoacetamide affinity label and 5-RhodB fluorophore demonstrated promising results in WRIB analysis, this peptide should also be explored in fluorescence microscopy experiments with CHO cells expressing DOR.
2.6. Experimental

2.6.1. Materials

4-(4-Formyl-3-methoxyphenoxy)butyryl AM resin (FMPB AM, 0.79 - 1.0 mmol/g), benzotriazole-1-yl-oxo-tris-pyrrolidine-phosphonium hexafluorophosphate (PyBOP), \( N\)-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-\(N\)-methylmethanaminium hexafluorophosphate \(N\)-oxide (HATU) and MSNT were purchased from Novabiochem (San Diego, CA). All 9-fluorenylmethoxycarbonyl (Fmoc) and \( tert\)-butoxycarbonyl (Boc) protected amino acids were purchased from Novabiochem, Peptides International (Louisville, KY) or Bachem (King of Prussia, PA). 1-Hydroxylbenzotriazole (HOBt) and Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-NH(CH\(_2\)CH\(_2\)O)\(_2\)CH\(_2\)CO\(_2\)H) were purchased from Peptides International. Acetonitrile, dichloromethane (DCM), \( N,N\)-dimethylformamide (DMF), methanol, \( N,N\)-diisopropylethylamine (DIEA), glacial acetic acid and diethyl ether were purchased from Fisher Scientific (Pittsburg, PA). Methylimidazole (MelM) and 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy]butyric acid were purchased from Fluka. Rhodamine WT was purchased from Abbey Color (Philadelphia, PA). Potassium phthalimide, 2-[2-(2 chloroethoxy)ethoxy]ethanol, trimethyl orthoformate (TMOF), 11-azido-3,6,9-trioxaundecan-1-amine, methanesulfonyl chloride, anhydrous DMF, d-desthiobiotin (DSB), piperidine, bromoacetic acid, \( N,N\)-diisopropylcarbodiimide (DIC), triphenyl phosphine, trimethyl phosphine (1M in toluene), sodium azide and hydrazine were purchased from Sigma Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockville, IL). 7-Aza-1-hydroxybenzotriazole (HOAt) was purchased from AAPPTec (Louisville, KY). The low load (0.19 mmol/g) Fmoc- Peptide
Amide Linker-poly(ethylene glycol)-polystrene (PAL-PEG-PS) resin was purchased from Applied Biosystems (Foster City, CA). Chinese hamster ovary (CHO) cells stably expressing DOR were a kind gift from Dr. Lee-Yuan Liu-Chen (Temple University, Philadelphia, PA).

2.6.2. Linker Synthesis

Potassium phthalimide (8.25 g, 44.6 mmol, 1 equiv) was dissolved in anhydrous DMF (15 mL) and 2-[2-(2 chloroethoxy)ethoxy]ethanol (7.5 g, 44.6 mmol, 1 equiv) was added. The reaction was heated to 80 °C and stirred over 2 days. DMF was removed in vacuo, and the resulting yellow oil was dissolved in ethyl acetate/ether (3:1) and washed with sat. NaCl (3X). The aqueous layer was back extracted with ethyl acetate/ether (3:1). The organic layer was dried and concentrated via rotary evaporation and placed under high vacuum to remove residual solvent.

The phthalimide protected amine ($m/z = 279.29$, $R_f = 0.33$ (9.0:0.5, DCM/MeOH)) was dissolved in DCM (15 mL) and cooled to 0 °C over ice. DIEA (7.8 mL, 44.6 mmol, 1 equiv) was added to the stirring solution and methanesulfonyl chloride (MsCl) (2.3 mL, 29.7 mmol 0.7 equiv) in DCM (5 mL) was added drop wise over 20 min. The reaction was brought to room temperature and stirred for 1 h. Additional MsCl (1 mL, 13 mmol, 0.3 equiv) was added in two batches (0.5 mL each) and stirred for 1 h to bring the reaction to completion ($R_f = 0.7$ (9.0:0.5, DCM/MeOH)). The solution was washed with 5% NaHCO₃ (2X) and sat. NaCl (2X). The organic layer was dried, concentrated via rotary evaporation and placed under high vacuum to remove residual solvent.
The mesylate product was dissolved in dry DMF (20 mL) and solid NaN$_3$ (1.9 g, 29.7 mmol, 0.7 equiv) was added. The reaction was heated to 80 °C for 2 h and 60 °C overnight. Additional NaN$_3$ (1 g, 15 mmol, 0.3 equiv) and DMF (20 ml) were added to the reaction and the mixture stirred at 60 °C for 3 h. The DCM and DMF were removed in vacuo and the product was dissolved in ethylacetate/diethyl ether (3:1) and washed with water (3X) and dilute NaCl (3X). The organic layer was dried with magnesium sulfate, concentrated in vacuo and purified by silica gel column chromatography using a gradient of DCM/MeOH (9:0.25 to 9:1). The aza-phthalimide product 20 was obtained in 69% yield (9.4 g, 31 mmol) over three steps ($m/z = 326.9$ [M+Na]$^+$, $R_f = 0.8$, DCM/MeOH, 9.5:0.5). (*The azido-intermediates generated during synthesis of the diamine linker should be handled with care. Never heat or store concentrated solutions; these are capable of exploding if mishandled.)

Compound 20 (4 g, 13.2 mmol, 1 equiv), was dissolved in 100% ethanol (30 mL) and hydrazine (1 mL, 31.6 mmol, 2.4 equiv) was added dropwise. The reaction was refluxed for 1.5 h and monitored by TLC and ESI-MS ($m/z = 175.1$ [M+Na]$^+$; $R_f = 0.1$ (DCM/MeOH, 9.5:0.5)). KOH (100 mL) was added and the aqueous layer was extracted with chloroform (4 X 20 mL). The organic layer was washed with sat. NaCl and dried with magnesium sulfate. The chloroform was removed by rotary evaporation and finally under high vacuum. The aza-amine product, 21, (0.7 g, 4 mmol, 5 equiv) was coupled to the FMPB AM resin (0.79 mmol of a 0.79 mmol/g substituted resin) by reductive amination with NaBH(OAc)$_3$ (0.8 g, 4 mmol, 5 equiv) in DCM/TMOF (2:1) following a previously described procedure.$^{71}$ 21 was reacted with the resin for 1 h, NaBH(OAc)$_3$
was then added and the reaction was mixed for an additional 2 h. After 3 h the reaction was terminated by filtration and the resin was washed with DCM/TMOF (2:1) and DCM.

2.6.3. Synthesis of Fmoc-Phe(p-NHAlloc)-OH

Fmoc-Phe(p-NHAlloc)-OH was synthesized following a modified protocol by Leelasvatanaki and Aldrich\(^90\) as reported by Angela Peck.\(^91\) The para amine of H-Phe(p-NH\(_2\))-OH was selectively protected with Alloc by dissolving the amino acid (2 g, 9 mmol, 1 equiv) in citrate buffer (16 mL, pH 4.6) followed by drop wise addition of allyl chloroformate (1 mL, 9 mmol, 1 equiv). The solution was stirred at room temperature overnight to afford H-Phe(p-NHAlloc)-OH which precipitated upon formation. The precipitate was then filtered, washed with cold citrate buffer (40 mL, pH 4.6) and cold water (40 ml) then dried under high vacuum to afford the Alloc-protected amino acid (2.7 g, 54% yield).

Fmoc-OSu (2.9 g, 8.5 mmol, 1.4 equiv) was dissolved in dioxane and added drop wise to H-Phe(p-NHAlloc)-OH (2.7 g, 6 mmol, 1 equiv) in 10% Na\(_2\)CO\(_3\)/dioxane (1:1, 60 mL) at 0°C for 1h. The reaction was then warmed to room temperature and stirred overnight. The solution was poured into ice water and the mixture extracted with ether. The aqueous layer was cooled over ice and acidified with HCl to afford a white precipitate which was filtered and washed with cold 0.1 N HCl and cold water then dried under high vacuum to give Fmoc-Phe(p-NHAlloc)-OH (77% yield, \(m/z = 509.2\) [M+Na]\(^+\), \(t_R = 24.3\) min, gradient 5-60% B over 55 min, gradient started after 5 min, solvent A = H\(_2\)O containing 0.1% TFA, solvent B = acetonitrile containing 0.1% TFA).
2.6.4. **RP-HPLC Purification of Rhodamine Isomers**

Rhodamine WT was purified as described by Kruger et al. A solution of Rhodamine WT (10 mL) was made acidic with 2 equivalents of HCl and water (10 mL) was added to the resulting precipitate followed by freezing (-80 °C) and lyophilization. The lyophilisate, which was a mixture of the 5- and 6-carboxy isomers, was purified by RP-HPLC in acetonitrile containing 0.1% TFA (solvent B) and H$_2$O (solvent A) using a gradient of 25-55% B over 60 min (acetonitrile (0.1% TFA) was held at 5% for 7 min while the sample was loaded onto the column, followed a gradient of 5-25% B over 5 min then 25-55% B over 60 min). The isomers demonstrated baseline resolution with retention times of 19.0 and 25.4 min for isomer 6 and 5, respectively in analytical HPLC analysis using an analytical gradient of 20-50% organic in aqueous acetonitrile containing 0.1% TFA. Following purification, the isomers (Figure 2.38) were identified by $^1$H NMR (400 MHz, CD$_3$OD) 5-carboxyrhodamine B: δ 8.63 (H$_1$), δ 8.32 (H$_2$), δ 7.54 (H$_3$); 6- carboxyrhodamine B: δ 7.18 (H$_1$), δ 7.15 (H$_2$), δ 6.73 (H$_3$), which were in

![Figure 2.38.](image-url) Structures of 5- and 6-carboxyrhodamine B isomers with the proton positions labeled corresponding to NMR shifts.
agreement with the published chemical shifts.\textsuperscript{92} Typical yields for the purification of 80 mg crude precipitate was 20 mg of pure 5-RhodB. The purity of the isomer was assessed in two solvent systems: System 1. aqueous acetonirile containing 0.1 % TFA (20-50% organic over 30 min, $t_R = 19.1$ min, purity varied from 97.5 - 100%) and system 2. aqueous MeOH containing 0.1% TFA (25-70% organic over 35 min, $t_R = 23.3$ min, purity varied from 98-100%). The mass was verified by ESI-MS ($m/z = 487.22$ [M+H]$^+$, 510.21 [M+Na]$^+$) using a Waters LCT-premiere ESI-TOF-MS.

2.6.5. Peptide Synthesis

Peptides were synthesized manually on the CHOIR\textsuperscript{93} assembly by solid phase peptide synthesis using Fmoc-protected amino acids except the N-terminal residue, which was the Boc-protected derivative. Unless otherwise indicated, amino acid couplings were performed using a 4-fold excess of amino acid, PyBOP and HOBt and an 8-fold excess of DIEA in a mixed solvent system (DCM/DMF, 1:1) for 3 to 16 h. Following amino acid couplings the resin was washed with DCM/DMF, followed by DMF. When amino acid couplings were performed for 16 h, extra base (1-2 equiv) was added 3 and 8 h following the start of the reaction to replace base that had evaporated. Unless otherwise indicated, equivalents are reported relative to resin substitution. The Fmoc group was removed using 20% piperidine in DMF (3 X 15 min) followed by washing with DMF, DCM/DMF and finally DCM. Reactions were monitored by ninhydrin\textsuperscript{94} (primary amines) or choranil\textsuperscript{95} (secondary amines) tests in addition to HPLC and ESI-MS of aliquots of the peptide cleaved from the resin.
2.6.5.1. TIPP(X)-Asp-linker-DSB 9 and 10

Following reductive amination of 1.5 g of FMPB AM, (0.79 mmol/g) with 21, DSB (3 equiv) was coupled to the secondary amine using HOBt (3 equiv), PyBOP (3 equiv) and DIEA (6 equiv) in DCM/DMF (1:1). The resin was transferred to a round bottom flask, which was subsequently flushed with N₂ and anhydrous DCM/DMF (1:4) added. The flask was then cooled over ice and PMe₃ (11 equiv, 1 M in toluene) was added dropwise to the resin and the reaction mixed for 30 min. The reaction was then warmed to room temperature and mixed for an additional 3 h. H₂O (16 mL/g resin) was added to the flask and the reaction mixed for 1 h. The resin was then transferred back to the (clean) solid phase reaction vessel, drained and washed with DCM (3 X 5 min), MeOH (2 X 2 min), DCM (2 X 5 min) and MeOH (3 X 2 min).

The resin was swollen in DCM/DMF (1:1) and washed with DCM/DMF (1:1), then Fmoc-Asp(OtBu)-OH (1.5 equiv), PyBOP (1.5 equiv), HOBt (1.5 equiv) and DIEA (6 equiv) were added and the reaction was mixed for 6 h. The resin was drained, washed and the full linear sequence of the peptide was assembled following standard Fmoc SPPS using a 4-fold excess of Fmoc-amino acid, PyBOP and HOBt and an 8-fold excess of DIEA in DCM/DMF (1:1). Double couplings were performed at positions 2 (Fmoc-Tic-OH), 3 (Fmoc-Phe-OH) and 4 (Fmoc-Phe(p-NHAlloc)) and three coupling reactions were performed for position 1 (Boc-Tyr(tBu)-OH).

The Alloc group of Phe(p-NHAlloc)⁴ was removed by treating the peptide on resin with phenylsilane (0.12 equiv/mg resin) for 5 min with N₂ on the CHOIR followed by the addition of Pd(PPh₃)₄ (0.1 equiv) and mixing for 30 min. The resin was filtered and fresh reagents were added and the reaction was mixed for 1 h. The resin was again
filtered, fresh reagents were added and the reaction was mixed for an additional 1 h. The resin was split into 2 portions and one portion was cleaved in 90% TFA/10% H₂O to yield the amine control peptide 9.

Bromoacetic acid was preactivated with DIC (10 equiv) in DCM for 20 min and added to the second portion of the resin. The reaction proceeded with shaking for 16 h after which the resin was filtered and washed with DMF (3 X 30 sec), DCM/DMF (1:1, 5 X 2 min) and finally DCM (5 X 2 min). The bromoacetamide functionalized peptide was then cleaved in 90% TFA/10% H₂O and purified by RP-HPLC (section 2.6.7) using a gradient of 15-50%B over 35 min (Solvent A = H₂O (0.1% TFA), Solvent B = acetonitrile (0.1% TFA). The pure peptide was analyzed in two HPLC solvent systems (Table 2.2) and verified by ESI-MS.

2.6.5.2. PC Peptides 11 and 12

Following reductive amination of the FMPB AM resin, the photocleavable (PC) linker 4-[4-(1-hydroxyethyl)-2-methoxy-5-nitophenoxy]butyric acid was coupled to the secondary amine of the linker using a 1.5-fold excess of the PC linker, HOBt and PyBOP and 3-fold excess of DIEA in DCM/DMF (1:1). The reaction proceeded for 16 h with addition of base (2 equiv) 3 and 8 h following the start of the reaction. The resin was then filtered and washed with DCM/DMF (1:1) and DCM. Benzoic acid (4 equiv), HOBt (4 equiv) and PyBOP (4 equiv) were dissolved in DCM/DMF (1:1) and DIEA (8 equiv) then the mixture was added to the resin and reacted for 45 min to cap any unreacted secondary amine of the linker.

Following the capping step and washing with DCM/DMF (1:1) and DCM, the ester between β-alanine and the secondary alcohol of the photocleavable linker was
formed following a standard procedure. The peptide on resin was transferred to a round bottom flask, under N₂ atmosphere. β-Alanine (5 equiv) was dissolved in a minimum amount of anhydrous DCM/DMF (1:1) and MeIm (3 equiv) was added followed by MSNT (5 equiv) dissolved in a minimum amount of DCM. The mixture was added to the resin and reacted for 16 h on a shaking arm. The resin was then transferred to a solid phase reaction vessel, filtered and washed with DCM/DMF (1:1) and DCM. Any remaining secondary alcohol from the PC linker was capped with benzoic anhydride (5 equiv) and pyridine (1 equiv) in DMF for 30 min following the standard protocol.

Fmoc quantitation was performed in triplicate to determine the substitution level following the usual procedure. The measured substitution was 0.33 mmol/g (33% theoretical) and this substitution was used in subsequent calculations of the amounts of amino acids and coupling reagents used to assemble the peptide. The Fmoc group was removed from Fmoc-β-Ala following the standard deprotection protocol (section 2.6.5) and the linear sequence of the peptide was assembled using Fmoc SPPS.

Lys⁸ (in compounds 11 and 12) was coupled to the resin as Fmoc-Lys(ivDde)-OH using standard coupling conditions (see above). Lys⁹ (in compounds 11 and 12) was coupled as the Fmoc-Lys(Mtt)-OH amino acid. Phe⁴ was coupled as Fmoc-Phe(p-NHAlloc)-OH. Double couplings were performed for residues at positions 2 (Fmoc-Tic-OH), 3 (Fmoc-Phe-OH), 4 (Fmoc-Phe(p-NHAlloc-OH), 6 (Fmoc-NH(CH₂CH₂O)₂CH₂-CO₂H) and 9 (Fmoc-Lys(Mtt)-OH). Boc-Tyr(tBu)-OH was coupled to the peptide using HATU (3.6 equiv), HOAt (4 equiv) and DIEA (8 equiv) in DCM/DMF (1:1) for 2.5 h. Following the assembly of the full linear sequence of the peptide (32, Scheme 6) the side chains were functionalized following the sequence in Scheme 6.
Lys(ivDde) was deprotected using a modification of the standard procedure for deprotection of ivDde in the presence of the Alloc protecting group. Hydrazine (2% in DMF) was added to the resin followed immediately by allyl alcohol (200 equiv) and mixed for 5 min, the reaction was drained and fresh reagents were added (8 X 5 min). Following the deprotection and washing with DMF, DCM/DMF (1:1) and DCM, DSB (3 equiv) was coupled to the ε-amine of Lys using HOBt, PyBOP and DIEA (3:3:6) in DCM/DMF (1:1). Benzoic anhydride (5 equiv) and pyridine (5 equiv) in DMF were added to the resin and reacted for 30 min to cap any unreacted amine.

The Mtt group was then removed from Lys using 3% TFA and 5% TIPS in DCM (1 x 10 min, 3 x 2 min, 1 x 10 min). The resin was washed extensively with DCM and DCM/DMF and finally with DCM. 5-RhodB (0.7 equiv relative to theoretical substitution first, then a second time with 0.2 equiv) was coupled to the free amine of Lys using HOBT, PyBOP and DIEA at a ratio of 1:1:4 to 5-RhodB in DCM/DMF (1:1). Unreacted amine was capped with benzoic anhydride (5 equiv) and pyridine (5 equiv) in DMF for 30 min.

The Alloc group of Phe(p-NHAlloc) was removed by treating the peptide on resin with phenylsilane (0.12 equiv/mg resin) for 5 min with N₂ mixing, followed by the addition of Pd(PPh₃)₄ (0.1 equiv) and mixing by shaking overnight. The resin was split into two portions and one portion was cleaved in 90% TFA/10% H₂O for 2 h then filtered and washed with TFA. The filtrate was evaporated, diluted with H₂O and lyophilized.

Bromoacetic acid (10 equiv) was preactivated with DIC (10 equiv) for 20 min in DCM then added to the second portion of the peptide on resin. Coupling of the activated bromoacetic acid was performed for 16 h by shaking. The peptide was subsequently
cleaved from the resin in 90% TFA/10% H₂O for 2 h. The resin was then filtered, washed with TFA and the TFA was evaporated, followed by addition of water and lyophilization of the filtrate to afford the crude peptide.

The amine control 11 and bromoacetamide 12 were purified by tandem RP-HPLC. System 1 was TEAP (solvent A = 0.09 M triethylammonium phosphate, pH 2.5, solvent B = acetonitrile) with a gradient of 10-20% B over 5 min then 20-60% B over 80 min. The fractions with the highest purity (typically > 70 area %) were combined and lyophilized. The combined fractions were then purified in system 2 (aqueous acetonitrile containing 0.1% TFA) using a gradient of 5-20% B over 5 min then 20-60% B over 80 min (solvent A = H₂O (0.1% TFA), solvent B = acetonitrile (0.1% TFA)). Pure fractions were then analyzed in two solvent systems (see Table 2.2).

2.6.5.3. Multifunctional Peptide 36

Peptide 36 was prepared similarly to the PC peptide except that the PC linker was not incorporated and the “clickable” linker was N₃(CH₂CH₂O)₃CH₂CH₂NH₂. Also, the coupling of Boc-Tyr(tBu)-OH was performed multiple times (12 times) under various conditions. Boc-Tyr(tBu)-OH (4 equiv), PyBOP (4 equiv) and HOBt (4 equiv) were dissolved in DCM/DMF (1:1) and DIEA (8 equiv) was added. The mixture was added to the resin and reacted for 3 h, followed by analysis of an aliquot of the peptide cleaved from the resin. The reaction was repeated using a 4-fold excess of Boc-Tyr(tBu)-OH, PyBOP and HOBt and an 8-fold excess of DIEA for 16 h in DCM/DMF (1:1) followed by HPLC and ESI-MS analysis of an aliquot of peptide cleaved from the resin. The coupling reaction was then performed using HATU (0.9 equiv), HOAt (1 equiv), Boc-Tyr(tBu)-OH (1 equiv) and DIEA (2 equiv) in DCM/DMF (1:1) for 3 h then the reaction
was drained fresh reagents were added and the reaction was repeated 2 X for 16 h then again using a 4-fold excess of HATU, HOAt and Boc-Tyr(tBu)-OH and 8-fold excess of DIEA in DCM/DMF (1:1). In between the reactions, an aliquot of peptide was cleaved from the resin and analyzed by HPLC and ESI-MS. Following the sixth attempt to couple Boc-Tyr(tBu)-OH to Tic, the peptide on resin was then divided into three portions (aliquots A, B and C). The first portion of the resin (aliquot A) was washed with LiCl (0.8 M in DMF) X 2 then the coupling reaction was repeated with Boc-Tyr(tBu)-OH (4 equiv), HATU (3.6 equiv), HOAt (4 equiv) and DIEA (8 equiv) in DMC:DMF:0.8 M LiCl in DMF (1:1:1) for 3 h then 16 h X 2 in DMC:DMF:0.8 M LiCl in DMF (1:1:1) and 2 X in 1:1 DCM/DMF (without LiCl). The peptide on resin was washed extensively with DCM (3 X 1 min), THF (3 X 1 min), DCM (3 X 1 min), DCM/DMF (1:1, 3 X 1 min), DMF (1 X 15 min), DCM (3 X 1 min), MeOH (3 X 1 min), and finally DCM (3 X 1 min). The washing cycle was repeated and the coupling was performed again using Boc-Tyr(tBu)-OH (4 equiv), HATU (3.6 equiv), HOAt (4 equiv) and DIEA (8 equiv) in DCM/DMF (1:1) for 16 h. Following the final coupling reaction, the peptide was functionalized with DSB and 5-RhodB at Lys residues 7 and 9, respectively, and bromoacetamide at Phe(p-NH$_2$)$_4$ as described for the PC peptide (section 2.6.5.2).

The second portion of the resin (aliquot B) was reacted with acetic anhydride (10 equiv) and DIEA (5 equiv) for 30 min, followed by HPLC and ESI-MS analysis of an aliquot of peptide cleaved from the resin.

The third portion of the resin (aliquot C) was subjected to the series of washes described for aliquot A (DCM (3 X 1 min), THF (3 X 1 min), DCM (3 X 1 min), DCM/DMF (1:1, 3 X 1 min), DMF (1 X 15 min), DCM (3 X 1 min), MeOH (3 X 1 min),
and finally DCM (3 X 1 min)). The wash cycle was repeated and Boc-Tyr(tBu)-OH (4 equiv), HATU (3.6 equiv) and HOAt (4 equiv) were dissolved in DMF and DIEA (8 equiv) was added. The resin was covered in DCM and the coupling reagents were added (final solvent was 1:1 DCM/DMF); the reaction was stopped after 3 h and analyzed by HPLC and ESI-MS of an aliquot of peptide cleaved from the resin.

2.6.5.4. Fluorescent TIPP Derivatives 43, 44 and 56

The fluorescent dual label peptide 44 and corresponding amine control 43 were prepared manually using standard Fmoc SPPS on a PAL-PEG-PS resin (0.19 mmol/g). The Mtt deprotection and 5-RhodB coupling were performed as described for the PC peptide (see Section 2.6.5.2) except that 1 equiv of 5-RhodB (relative to theoretical resin substitution) with coupling reagents at a ratio of 1:1:4 (HOBt, PyBOP, DIEA) was used and the reaction was performed once in DCM/DMF (1:1). Deprotection of Phe(p-NHAllo)c4 and subsequent introduction of the bromoacetamide was performed following the protocol described for the PC peptide. The amine control 43 and bromoacetamide 44 peptides were cleaved from the resin in 90% TFA/10% H2O for 2 h, then filtered, washed with TFA, the filtrate diluted with H2O and lyophilized. The peptides were purified by RP-HPLC (section 2.6.6) in aqueous acetonitrile containing 0.1% TFA using a gradient of 10-60% organic over 50 min. The retention times and observed m/z values for the peptides are given in Table 2.2.

Peptide 56 was synthesized manually on the PAL-PEG-PS (0.19 mmol/g) resin using the standard protocol for Fmoc SPPS except that the coupling of Boc-Tyr(tBu)-OH required multiple couplings (5 total) using HOBt, PyBOP, DIEA (1X) and HATU, HOAt, and DIEA (4 X). The peptide was treated with Boc-Tyr(tBu)-OH (4 equiv), HOBt (4
equiv), PyBOP (4 equiv) and DIEA (8 equiv) in DCM/DMF for 16 h. Then the peptide on resin was reacted with Boc-Tyr(tBu)-OH (4 equiv), HATU (3.6 equiv), HOAt (4 equiv), DIEA (8 equiv) in DCM/DMF for 5 h. The resin was washed with 0.8 M LiCl and the coupling with Boc-Tyr(tBu)-OH (4 equiv), HATU (3.6 equiv), HOAt (4 equiv), DIEA (8 equiv) in DCM/DMF was performed for 16 h X 2. The resin was then washed extensively with DCM, DMF, MeOH and THF as described for the MFP 36 (see section 2.6.5.3) and the coupling reaction was repeated using a 4-fold excess of Boc-Tyr(tBu)-OH, HATU and HOAt and an 8-fold excess of DIEA for 16 h in DCM/DMF (1:1).

Following complete linear assembly, the ivDde group was removed from the ε-amine of Lys by the addition of 2% hydrazine in DMF and allyl alcohol (200 equiv) (6 x 5 min). The resulting free amine was acetylated with acetic anhydride (10 equiv) and DIEA (5 equiv) in DMF for 30 min. The Mtt group was removed as described for the PC peptide and 5-RhodB (1 equiv relative to initial resin substitution) was coupled to the ε-amine using HOBt, PyBOP and DIEA at a ratio of (1:1:4) in DCM/DMF (1:1). The Alloc group was removed following the procedure described for the PC peptide (section 2.6.5.2).

2.6.5.5. Model Peptide 57

Peptide 57 was also prepared similar to the PC peptide except that Lys(ivDde)-OH and a second PEG-like linker were not incorporated. Also, the Alloc group was not deprotected and the N-terminal residue was Fmoc-Tyr(tBu)-OH. The coupling of Fmoc-Tyr(tBu)-OH to Tic was performed using multiple reactions. The coupling was first performed using Fmoc-Tyr(tBu)-OH (4 equiv), PyBOP (4 equiv), HOBt (4 equiv) and DIEA (8 equiv) in DCM/DMF (1:1) for 3 h. The resin was filtered, fresh reagents were
added and the coupling reaction was performed for 16 h. Following HPLC analysis of an aliquot of peptide cleaved from the resin, Fmoc-Tyr(tBu)-OH (3 equiv) and PyClock (3 equiv) were dissolved in DCM/DMF (1:1) and DIEA (6 equiv) was added. The mixture was added to the resin and mixed for 3 h, the resin was then filtered and fresh reagents were added and the reaction was mixed for 16 h. Finally, Fmoc-Tyr(tBu)-OH, HATU (0.9 equiv) and HOAt (1 equiv) were dissolved in DCM/DMF (1:1), DIEA (2 equiv) was added and the mixture was added to the resin and reacted for 3 h. The resin was drained, fresh reagents were added and the reaction was repeated for 16 h. The Mtt group was then removed from the side chain amine of lysine, and 5-RhodB was coupled as described for the PC peptide.

2.6.6. Peptide Purification and Analysis

Crude peptides were purified by preparative reversed-phase HPLC (Shimadzu equipped with two LC-6AD pumps and a SDP-10AVP UV detector) on a Vydac C18 column (10 µ, 300 Å, 22 x 250 mm) using the gradients and solvents indicated in subsections of section 2.6.5. HPLC fractions were collected manually and immediately frozen and lyophilized. Analysis of the peptides was performed by analytical HPLC (Shimadzu equipped with 2 LC-10ATVP pumps and a SPD-10-AVP UV detector or an Agilent 1200 series HPLC equipped with a quaternary pump and a variable wavelength detector) with a Vydac C18 column (5 µ, 300 Å, 4.6 x 50 mm) in two solvent systems for pure peptides.

The molecular weights of the peptides were verified by ESI-MS using a Waters LCT Premier time of flight (TOF) mass spectrometer. LC-MS analysis was performed on two LC MS instruments: 1) Micromass Quattro microTM triple quadrupole mass
spectrometer (Micromass Ltd, Manchester UK) equipped with a Z-spray interface Waters 2690 LC module with either a Vydac C\textsubscript{18} column (5 µ, 300 Å, 1 x 50 mm); mobile phase gradient of acetonitrile in 0.1% formic acid; flow rate, 0.2 ml/min or with an Agilent C\textsubscript{18} column (5 µ, 300 Å, 4.6 x 150 mm) mobile phase gradient of acetonitrile in 0.1% TFA; flow rate, 1 ml/min and 2) Q-Tof-2 (Micromass Ltd, Manchester UK) equipped with a Waters Acquity LC module with an Inertsil ODS-3 C\textsubscript{18} column (2.1 x 50 mm); mobile phase gradient 20-50% acetonitrile in 0.06% formic acid in 30 min; flow rate, 0.4 ml/min. MALDI-MS was performed using either a 4700 Proteomics Analyzer with TOF-TOF optics (Applied Biosystems, Foster City, CA) or with a Voyager DE STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA).

**Table 2.2.** Peptide HPLC retention times (t\textsubscript{R}) and observed/ calculated m/z values with the corresponding charge state.

<table>
<thead>
<tr>
<th>Peptide #</th>
<th>System 1 t\textsubscript{R} (min)/% purity</th>
<th>System 2 t\textsubscript{R} (min)/% purity</th>
<th>ESI-MS m/z [charge state]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Calc.</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25.3\textsuperscript{a} / 99.1</td>
<td>24.2\textsuperscript{b} / 96.4</td>
<td>1211.5 [M+H]\textsuperscript{+}</td>
</tr>
<tr>
<td>11</td>
<td>34.2\textsuperscript{c} / &gt;99</td>
<td>32.6\textsuperscript{a} / 97</td>
<td>828.7 [M+2H]\textsuperscript{2+}</td>
</tr>
<tr>
<td>12</td>
<td>39.5\textsuperscript{c} / &gt;99</td>
<td>-----------------------------</td>
<td>868.7 [M+2H]\textsuperscript{3+}</td>
</tr>
<tr>
<td>43</td>
<td>33.1\textsuperscript{a} / &gt;99</td>
<td>42.6\textsuperscript{c} / &gt;99</td>
<td>753.4 [M+H]\textsuperscript{2+}</td>
</tr>
<tr>
<td>44</td>
<td>36.6\textsuperscript{a} / &gt;99</td>
<td>38.3\textsuperscript{f} / 97.8</td>
<td>813.3 [M+H]\textsuperscript{2+}</td>
</tr>
</tbody>
</table>

a. Gradient 5-50% B over 45 min (Solvent A = H\textsubscript{2}O (0.1% TFA), Solvent B = acetonitrile (0.1% TFA))
b. TEAP gradient = 5-50% over 45 min (Solvent A = 0.09 M triethylammonium phosphate, pH 2.5, Solvent B = acetonitrile)
c. Gradient 20-50% B over 60 min (Solvent A = H\textsubscript{2}O (0.1% TFA), Solvent B = acetonitrile (0.1% TFA))
d. Gradient 25-70% B over 35 min, gradient started after 5 min (Solvent A = H\textsubscript{2}O (0.1% TFA), Solvent B = methanol (0.1% TFA))
e. Gradient 10-60% B over 50 min (Solvent A = H\textsubscript{2}O (0.1% TFA), Solvent B = methanol (0.1% TFA))
2.6.7. Biological Analysis

2.6.7.1. Fluorescence Microscopy

CHO-DOR cells were seeded into 8-well microscope slides containing DMEM:F12 media with 10% fetal calf serum (FCS), 1000 U/mL penicillin/streptomycin and 100 U/mL geneticin. The cells were incubated at 37 °C in 5% CO₂ for 36-48 h, then washed with PBS (X 2) and incubated with binding buffer (phenol-free RPMI 1640 media containing 10% FCS and protease inhibitors). Peptide 12 (200 nM) was added to cells followed by 30 min incubation at room temperature, then binding buffer or TIPP (2 µM) was added and the cells were incubated for an additional 90 min at room temperature. A separate set of cells was incubated with TIPP (2 µM) for 30 min followed by 90 min incubation with the peptide at room temperature. CHO cells that did not express the receptor were incubated with the peptide for 30 min followed by addition of binding buffer and an additional 90 min incubation at room temperature. Following the incubation all cells were washed with ice cold PBS four times and imaged by fluorescence microscopy. Imaging was performed in epifluorescence mode (λₑₓ = 520 nm, λₑᵐ = 580 nm) using a custom built spinning disk confocal microscope (Intelligent Imaging Innovations, Denver CO, CSU-10-Based) equipped with an Olympus IX-81 inverted fluorescence microscope frame laser.

2.6.7.2. Binding Analysis

Radioligand competition assays were performed following a previously described procedure with CHO cell membranes from CHO cells stably expressing either DOR or MOR using [³H]DPDPE or [³H]DAMGO as the radioligands for DOR or MOR, respectively. Cells were harvested at confluency in 50 mM Tris buffer (pH 7.4, 4 °C),
homogenized and centrifuged (45000 X g, 10 min); the pellet was then washed by repeated suspension and centrifugation (4X). The final pellet was resuspended in Tris buffer (pH 7.4, 4 °C) to a total protein concentration of 30-60 µg/mL. The peptide ligands were then incubated at various concentrations (ranging from 0.1 nM to 10 µM) with 100 µg membrane protein in the presence of the radioligands (at their approximate $K_d$ values, 0.5 nM of DPDPE for DOR and 0.64 nM of DAMGO for MOR), 3 mM Mg$^{2+}$ and a peptidase inhibitor cocktail (10 µM bestatin, 30 µM captopril, and 50 µM L-leucyl-L-leucine) for 90 min at 22 °C. Nonspecific binding was determined using 10 µM of non-radioactive DPDPE or DAMGO for DOR or MOR, respectively. The reactions were terminated by filtration over Whatman GF/B fiber filters using a Brandel cell harvestor. The filters were incubated with scintillation cocktail for 6 h, and radioactivity was determined by scintillation counting.

2.6.7.3. Wash Resistant Inhibition of Binding Assay

WRIB analysis of the peptide was performed as previously described. Membranes from CHO cells stably expressing DOR were incubated in the presence or absence of the TIPP derivatives (peptides 43 at 94 nM and 44 at 96 nM). The membranes were incubated with the peptides for 90 min (at room temperature), then centrifuged (40,000 g X 15 min, 4 °C) and resuspended in 50 mM Tris buffer (pH 7.4, 4 °C). The membranes were washed 5 times by alternating recentrifugation and resuspension steps. Following the final centrifugation step, the pellets were resuspended in 50 mM Tris buffer (pH 7.4, 4°C) and examined for [$^3$H]DPDPE binding as described in section 2.6.2. The results were presented as the percent of [$^3$H]DPDPE binding to untreated control membranes.
2.7. On-Bead MALDI Studies

Peptide 59 on the FMPB AM resin (10 mg) was mixed with acetonitrile/H$_2$O (100 µL containing 0.1% TFA, 2:1). The beads were spotted onto a MALDI plate (1 µL, 10-40 beads) and either covered with 1 µL of MALDI matrix consisting of DHB (10 mg in 100 µL acetonitrile/H$_2$O (0.1% TFA, 2:1) or smashed with a spatula then covered with 1 µL of the DHB matrix. In addition, a subset of the resin with the peptide was smashed in a vial containing the ACN/H$_2$O mixture then spotted as described above. Blank resin (FMPB AM resin) was also spotted in a similar manner except the beads were not crushed in the vial. An aliquot of the peptide on resin was cleaved in 90% TFA/10% H$_2$O. The lyophilized peptide was then dissolved in the ACN/H$_2$O (0.1% TFA) solution and 1 µL was spotted, dried and covered with 1 µL of Matrix.

MALDI-MS analysis was performed using a Voyager DE STR Biospectrometry Workstation (Applied Biosystems, Framingham, MA) with a pulsed N$_2$ laser (337 nm) in reflector mode using delayed extraction (200 nsec). The samples were irradiated with 30 laser shots per spectrum at laser intensities varying from 2600 - 2700 at a grid voltage of 71% and a guide wire voltage of 0.05%.

2.8. References


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Chapter 3. The Design, Synthesis and Evaluation of Bivalent Ligands Targeting Opioid Receptor Dimers

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.
3.1. Literature Review

3.1.1. Introduction

Multiple subtypes of the opioid receptors have been described based on pharmacological and clinical data. For instance, early studies in mice demonstrated that certain opioid ligands lack cross tolerance and the proposed subtypes demonstrated different sensitivities to various antagonists. Furthermore, opioid analgesics do not always show cross tolerance in patients undergoing opioid therapy. Since only one gene product has been identified for each of the opioid receptors, these subtypes could be due to splice variants, post-translational modifications or receptor oligomerization. There is mounting evidence that oligomerization of the opioid receptors may be the origin of the proposed subtypes. Ligands designed to study the pharmacological behavior of opioid receptor oligomers will increase our knowledge of opioid oligomerization and ultimately aid the development of novel therapeutics for these receptor complexes.

3.1.2. Oligomerization of G-Protein Coupled Receptors

Oligomerization of G-protein coupled receptors (GPCRs) has been extensively reviewed. Dimer formation and higher order oligomerization may be a way for the cell to increase the number of functional receptor units without the need for an increased number of gene products. GPCR oligomers can have altered ligand binding properties, both in affinity and activation, and may have assorted G-protein partners.

Oligomerization is essential for the proper function and cell surface expression of a number of receptors such as the class C GPCRs GABA<sub>B</sub>, calcium sensing receptor and the receptors for sweet and umami tastes. Of the class A GPCR family, oligomerization has been reported for rhodopsin, melatonin receptors, glycoprotein hormone receptors,
adrenergic receptors, dopamine receptors, opioid receptors and others. The opioid receptors are reported to homo- and heterodimerize resulting in pharmacologically distinct profiles, some of which correlate with the proposed receptor subtypes.23-27

3.1.3. Proposed Delta Opioid Receptor Subtypes

Multiple subtypes have been proposed for each of the opioid receptors, but this discussion will focus on those pertaining to the delta opioid receptor (DOR). The initial evidence that the three opioid receptor types, DOR, the mu opioid receptor (MOR) and the kappa opioid receptor (KOR), could be further divided into receptor subtypes was described 20 years ago based on pharmacological characterization.3, 23, 28-31 Further evidence that the proposed opioid receptor subtypes could result from receptor oligomerization has been provided by co-immunoprecipitation experiments, receptor imaging and knockout (K/O) animal studies.15, 24

Different notations for the postulated DOR subtypes are used throughout the literature (DOR-1, DOR-2, DOR\textsubscript{cx}, DOR\textsubscript{ncx}, MOR-DOR complex and the KOR-DOR complex) and it is not clear which of these subtypes, if any, correspond to one another.3, 5, 23, 24, 31-34 For example, the DOR-2 subtype has been proposed to be the same as the DOR-MOR complex and also DOR\textsubscript{cx}, but ligand selectivity for DOR-2 does not always overlap with the profile for MOR-DOR/DOR\textsubscript{cx} ligand selectivity.3, 23, 31 Moreover, the DOR-1 subtype has also been proposed to be the equivalent of the MOR-DOR complex\textsuperscript{35} as well as a DOR-KOR heterodimer.32

Whether the proposed subtypes of the opioid receptors are a result of oligomerization and to what extent the opioid receptors oligomerize \textit{in vivo} is also debated.2, 36-38 Further, heterodimerization is not observed in all tissues or cell lines that
express both MOR and DOR, even in situations where DOR appears to modulate MOR surface expression and downstream coupling to effectors.\(^{39}\) In fact, there is debate as to whether the majority of reports on GPCR oligomerization are pharmacologically relevant and not simply a result of experimental conditions, especially with regard to resonance energy transfer and co-immunoprecipitation experiments.\(^ {17,21,40-42}\)

### 3.1.4. Evidence for Delta Opioid Receptor Subtypes and Receptor Oligomerization

Early studies of the proposed opioid receptor subtypes relied mainly on the pharmacological characterization of opioid ligands \textit{in vivo} or in tissue preparations such as the mouse vas deferens (MVD), guinea pig ileum (GPI) and brain preparations. Rothman and co-workers originally described the DOR\(_{\text{cx}}\) binding site as a high affinity site for the DOR selective agonist \([D-\text{Pen}^2,\text{D-Pen}^5]\)enkephalin (DPDPE, 1, Figure 3.1) and demonstrated that MOR ligands were non-competitive inhibitors of this site.\(^ {43,44}\) The characterization of DOR binding sites in the MOR-DOR complex, denoted DOR\(_{\text{cx}}\), was differentiated from the non-complexed DOR binding site DOR\(_{\text{ncx}}\) via depletion of DOR\(_{\text{ncx}}\) by the DOR selective affinity label (+)-\textit{trans}-SUPERFIT (3, Figure 3.1) in rat brains. The DOR\(_{\text{cx}}\) site was labeled with tritiated \([D-\text{Ala}^2,\text{D-Leu}^5]\)enkephalin ([\(^{3}\text{H}\)]DADLE), and this labeling was non-competitively inhibited by MOR ligands, but competitively inhibited by DOR ligands.

The DOR selective ligands DPDPE and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH\(_2\), del II) did not demonstrate cross-tolerance in mice, providing evidence that these ligands were activating subtypes of DOR.\(^ {29}\) Antagonism of the DOR ligands \([D-\text{Ser}^2,\text{Leu}^5]\)enkephalin-Thr\(^6\) (DSLET) and DPDPE also differed depending on the DOR selective antagonist administered. Naltriben (NTB, 4, Figure 3.1) effectively antagonized
In the mouse tail flick assay, DSLET antagonized DPDPE, but did not antagonize DPDPE.\textsuperscript{30} In contrast, DPDPE was antagonized by 7-benzylidenenaltrexone (BNTX, 5, Figure 3.1) in the mouse tail flick assay but DSLET was not.\textsuperscript{45} In guinea pig membranes, BNTX also

\textbf{Figure 3.1.} DOR ligands used to study the proposed receptor subtypes.
demonstrated high affinity binding to DOR-1 (DPDPE) binding sites which was 100-fold higher than that for DOR-2 (DSLET) binding sites.\textsuperscript{45}

In the MVD preparation, the proposed DOR-2 subtype was characterized by high affinity binding to del II and antagonism by NTB. The proposed DOR-1 subtype exhibited high affinity for DPDPE, which was antagonized by BNTX.\textsuperscript{32} DPDPE was also proposed to bind to and act as an agonist at DOR-1 in mouse spinal cord preparations. The DOR antagonist BNTX and the KOR antagonist norBNI (7, Figure 3.2) were both antagonists of DPDPE.\textsuperscript{32} These results led to the proposal that the DOR-1 subtype was a heterodimer comprised of DOR and KOR.

Experiments employing engineered cell lines and endogenous cell lines that express the opioid receptors have also been used to classify the proposed opioid receptor subtypes. Rothman and co-workers recently conducted binding experiments in Chinese hamster ovary (CHO) cells that co-expressed MOR and DOR and in CHO that expressed only one receptor type.\textsuperscript{11} DPDPE was a competitive inhibitor of DADLE in SUPERFIT (2, Figure 3.1) treated MOR-DOR CHO cells, whereas morphine was a non-competitive inhibitor. These results support the earlier conclusion by Rothman et al. that

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3.2.png}
\caption{KOR ligands.}
\end{figure}
DOR\textsubscript{cx} is a heterodimer of DOR and MOR\textsuperscript{43,44}.

Law and co-workers examined the binding affinity of MOR ligands in ECR 293 cells that co-expressed MOR and DOR\textsuperscript{46}. The authors reported a decrease in the binding affinities of [D-Ala\textsuperscript{2},NMePhe\textsuperscript{4},Gly-ol\textsuperscript{5}]enkephalin (DAMGO), morphine and endomorphin-1 (Tyr-Pro-Trp-Phe-NH\textsubscript{2}) and an increase in binding affinity of endomorphin-2 (Tyr-Pro-Phe-Phe-NH\textsubscript{2}) for MOR when DOR expression levels matched MOR levels in this cell line. Antagonists such as naloxone (9, Figure 3.3) and naltrexone (10, Figure 3.3) did not exhibit a similar change in binding affinity. The changes in ligand affinity were not a result of the ligands binding to DOR, and were attributed to ligand interactions with the MOR-DOR dimer. Ligand potencies were generally not affected except for an increase in the potency of endomorphin-2 inhibition of forskolin-stimulated cyclic AMP production\textsuperscript{46}.

![Figure 3.3. General opioid antagonists.](image)

9 Naloxone \( R = \text{Allyl} \)
10 Naltrexone \( R = \text{Cyclopropylmethyl} \)

The effects of DOR ligands on MOR ligand activity have also been examined \textit{in vivo} in conjunction with cellular analysis to correlate the \textit{in vivo} observations with receptor behavior in cells. Co-administration of morphine and the DOR antagonist TIPP[Ψ] (11, Figure 3.4) resulted in an increase in the potency of morphine in the tail flick assay\textsuperscript{47}. The authors proposed that the increase in morphine potency was likely a result of an increase in the number of binding sites as a consequence of receptor
dimerization. A bioluminescence resonance energy transfer (BRET) assay (see section 3.1.5.2) was used to demonstrate co-localization of MOR and DOR receptors in human embryonic kidney (HEK) 293 cells. Co-immunoprecipitation (see section 3.1.5.1) was used to isolate MOR-DOR dimers from mouse spinal cord membranes, further supporting the conclusion that the receptor system under study was a dimer of DOR and MOR. The DOR selective ligands TIPP[Ψ] and del II increased the number of MOR binding sites (B_{max}) in radioligand binding assays with [^{3}H]DAMGO. The increase in B_{max} was only observed in HEK 293 cells that co-expressed MOR and DOR and not in cells that expressed a single receptor type. The DOR agonists DPDPE and SNC80 (13, Figure 3.5) did not have a similar effect on DAMGO binding. Interestingly, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-The-NH$_2$ (CTOP), which is a MOR antagonist, caused a similar increase in B_{max} observed with [^{3}H]del II only in cells that expressed both MOR and DOR. Since del II is proposed to be a DOR-2 selective ligand and DPDPE is proposed to be a DOR-1 selective ligand, these results suggest that the DOR-2 subtype corresponds to the MOR-DOR complex (in contrast to reports by van Rijn et al. that the MOR-DOR corresponds to the DOR-1 subtype; see section 3.1.9 for details). Given that an increase in binding of
morphine and DAMGO to MOR was observed when cells were co-treated with specific DOR selective ligands regardless of whether the ligand was an antagonist (TIPPP and NTB), agonist (del II) or inverse agonist (ICI 174 864, 6, Figure 3.1), ligand occupancy of DOR appeared to be the main criteria for the observed increase in MOR binding sites.5

3.1.5. Co-immunoprecipitation and Imaging of Opioid Receptor Dimers

3.1.5.1. Co-immunoprecipitation Experiments

Co-immunoprecipitation experiments involve the isolation of a receptor complex using antibody recognition of an epitope from one receptor to form the immunoprecipitate followed by Western blot analysis with an antibody specific for the epitope from the second receptor.48 These experiments are commonly performed with epitope-tagged receptors, usually the FLAG, His6, myc and c-myc epitopes. However, co-immunoprecipitation can also be performed using endogenous sources such as tissue preparations, but these experiments require antibodies that are specific to their receptor targets and must lack cross reactivity with other proteins.48

Figure 3.5. DOR small molecule agonists.
Homodimerization of DOR was examined by cross-linking the receptors in a cell line that expressed both FLAG and myc-tagged DOR followed by immunoprecipitation and immunoblotting.\textsuperscript{49} Since cross-linking can couple transient receptor complexes, whether these studies reflect true dimers is a subject of debate.\textsuperscript{19}

Unlike DOR homodimers, MOR-DOR heterodimers do not require cross-linking to be stable to detergent.\textsuperscript{5} Co-immunoprecipitation of myc-tagged DOR and FLAG-tagged MOR was performed using the lysate of HEK 293 cells that co-expressed both tagged receptors.\textsuperscript{5} A 150 kDa heterodimer was observed in Western blot analysis and pretreatment with a reducing agent decreased the level of dimerization between the receptors. Lysates from HEK 293 cells that individually expressed either tagged MOR or tagged DOR were mixed followed by immunoprecipitation. Western analysis of the mixture did not produce the dimer bands, indicating that co-expression of the receptors was required for dimerization. The authors also demonstrated that the heterodimer was a functional complex with altered ligand binding properties in cells that co-expressed wild type receptors (SKNSH) and in CHO cells that stably the receptors (co-expression vs. individual expression). Fan et al. reported similar results using co-immunoprecipitation of FLAG-tagged DOR and myc-tagged DOR in COS-7 monkey kidney cells.\textsuperscript{10}

Co-immunoprecipitation and Western blot analysis have been used extensively in engineered cells lines, but the examination of receptor oligomers in native tissues requires the use of antibodies specific for each receptor.\textsuperscript{48} Antibodies for DOR have been shown to be cross reactive with other proteins,\textsuperscript{36} therefore limiting the utility of co-immunoprecipitation for the analysis of DOR oligomerization in native cellular and tissue preparations. Critics of the results obtained by co-immunoprecipitation studies have also
questioned the contribution of the solubilization process to aggregation of GPCRs, which tend to aggregate upon isolation from the cell membrane.\textsuperscript{17,50}

3.1.5.2. Opioid Receptor Imaging: BRET

Fluorescence (Förster) resonance energy transfer (FRET) and bioluminescence energy transfer (BRET) have been utilized to demonstrate the dimerization of GPCRs, including the opioid receptors, in live cells.\textsuperscript{18,48,50} The energy transfer process requires that the fluorophores be within 100 Å of each other. Both FRET and BRET rely on the use of fluorophores with overlapping emission and excitation wavelengths such that the emission of one fluorophore (donor) overlaps with the excitation wavelength of the second fluorophore (acceptor) leading to observable emission from the second fluorophore. Yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) are commonly used variants of the green fluorescent protein (GFP) in FRET experiments. In BRET, a luciferase (Luc) substrate is typically the donor and GFP or YFP are typical acceptor fluorophores.\textsuperscript{48} BRET\textsuperscript{2} is a more recent development in BRET technology that utilizes a modified GFP (GFP\textsuperscript{2}) in combination with a modified luciferase substrate (DeepBlueC).\textsuperscript{48,50} BRET\textsuperscript{2} has the advantage of decreased background compared to traditional BRET as a result of more complete overlap between donor emission and acceptor excitation wavelengths and larger separation between donor and acceptor emission wavelengths.

Devi and co-workers demonstrated heterodimerization of MOR and DOR using BRET of YFP-tagged MOR or DOR in combination with luciferase-tagged DOR or MOR, respectively, in HEK 293 cells.\textsuperscript{47} An increase in BRET signal was observed in cells that co-expressed MOR and DOR but not in cells that co-expressed luciferase-
tagged CC5 chemokine receptors with YFP-tagged MOR or DOR, suggesting that the increase in BRET signal was a result of dimerization of MOR and DOR and not a result of non-specific receptor interactions.

Sadèe and co-workers reported homo- and heterooligomerization for all three opioid receptor types in HEK 293 cells expressing tagged receptors. The authors used BRET in combination with radioligand binding assays and co-immunoprecipitation to demonstrate that the oligomeric receptors were functional dimers and not higher order oligomers.

Despite advances in BRET technology, the quantum yield of BRET is decreased compared to traditional BRET and requires higher levels of receptor expression. Since BRET requires recombinant receptors fused with the donor-acceptor pair, this method is not applicable to endogenous opioid systems. The site of incorporation of the energy transfer reporter in the GPCR for BRET may also influence signaling, especially with regard to conformational changes that occur after ligand binding. This may lead to ambiguity in deciphering whether changes in BRET signal are a result of dimerization vs. conformational changes within a single receptor induced by ligand binding. Furthermore, BRET signals can arise from random events and care must be taken to decipher between signal resulting from interacting receptors and those from random interactions of the fluorophores.

3.1.6. G-Protein Fusions

The ability of one receptor in a receptor dimer to modulate signaling though a G protein fused to a second receptor was investigated with pertussis toxin insensitive G-protein fusions of MOR and DOR. The heterodimers were comprised of either DOR-G-
protein fusion and FLAG-MOR or MOR-G-protein fusion and FLAG-DOR. Co-immunoprecipitations in combination with Western analysis were used to confirm the identity of the heterodimers. Cells were treated with pertussis toxin to block endogenous $G_\alpha$ and prevent interaction of the non-fused receptor with any non-fused G-proteins. Cells were then treated with ligands selective for each receptor type to examine the ability of the non-fused receptor in the dimer to cause activation of the G-protein that was fused to the second receptor. Agonists selective for the non-fused receptor were able to activate G-protein signaling. Since there was no measurable change in $K_i$ or $EC_{50}$ for any of the ligands tested, the authors propose that contact dimers (see Section 3.1.8) are the likely form of MOR-DOR heterodimers in this system.

3.1.7. Receptor Trafficking

Receptor trafficking is a means by which a cell can regulate responses to external stimuli. In both recombinant cell lines and endogenous cellular preparations, DOR exhibits limited surface expression.$^{53}$ This surface expression was increased in the presence of nociceptive stimulation$^{54}$ or exposure to certain lipophilic opiates such as naltrexone (NTX, 10, Figure 3.3).$^{55}$ DOR trafficking has been implicated in the development of morphine tolerance$^{56}$ and DOR knockout mice demonstrate a decrease in the development of morphine tolerance compared to wild type mice.$^{34}$ Furthermore, DOR selective antagonists have demonstrated the ability to modulate the pharmacological profile of MOR selective ligands.$^{53, 57}$ There is growing evidence that trafficking of MOR and DOR are interconnected.$^{54}$ Researchers are also uncovering a diverse array of proteins that interact with and modulate the degradation, surface expression and interaction of MOR and DOR.$^{58}$
Receptor trafficking of MOR was evaluated in cells that co-expressed wild type MOR and a chimeric MOR receptor (D MOR), which has a C-terminal sequence corresponding to the C-terminal cytoplasmic residues of DOR. Heterodimers of MOR and D MOR were identified by co-immunoprecipitation in cells that co-expressed each receptor type, and dimerization was found to be ligand dependent. Morphine did not induce receptor internalization in cells that expressed only MOR, but in the engineered cell line expressing both MOR and D MOR internalization was observed when cells were treated with morphine. These results provide evidence that the carboxyl terminus of DOR is involved in heterodimerization and trafficking of MOR following exposure to morphine.

The surface expression of both DOR and MOR was decreased due to retention in Golgi compartment in Neuro2A cells transfected with FLAG-tagged MOR and myc-tagged DOR. Surface expression of both receptors was rescued with co-expression of the receptor transport protein RTP4. RTP4 also decreased ubiquitination of MOR and DOR and appears to regulate the formation of MOR-DOR heterodimers as observed by a decrease in G\textsubscript{i} signaling by the receptors. NTB was also capable of decreasing receptor ubiquitination and increasing cell surface expression similar to NTX, suggesting that these ligands may act as an exogenous chaperones of MOR-DOR dimers.
3.1.8. Models of Opioid Receptor Dimerization

GPCR oligomerization can occur by two different mechanisms, either domain swapping or through formation of contact dimers (Figure 6).\textsuperscript{61, 62} Domain swapping would result in one orthosteric binding site, whereas contact dimers would result in two binding sites. The majority of GPCR oligomers are assumed to form via contact dimerization based on the available data.

There are a variety of methods for modeling GPCR dimers.\textsuperscript{61} Filizola and Weinstein used subtractive correlated mutation analysis to generate models of opioid receptor dimers that were in agreement with experimental data.\textsuperscript{63, 64} This method utilizes multiple sequence alignments to determine residues that show compensatory changes during evolution with the underlying assumption that if residues at one protein interface mutate, residues at the second site of the interface must also mutate in order to maintain an interaction.\textsuperscript{61} Modeling of the DOR-DOR homodimer predicted that contacts would be predominantly between the transmembrane (TM) domains, specifically TM4-TM4, TM4-TM5 or TM5-TM5.\textsuperscript{63} The sites of contact in a MOR-DOR heterodimer were predicted to be between MOR (TM1) and DOR.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3_6.png}
\caption{Possible arrangement of GPCRs in the cell membrane. A. Receptor monomers. B. Domain swapped dimer. C. 5,6-Contact dimer. Adapted from ref.\textsuperscript{62}}
\end{figure}
In protein-protein docking experiments, Liu et al. predicted two models for the MOR-DOR dimer. In one model, MOR and DOR form contacts through an interface comprised of MOR (TM1, 7) and DOR (TM4, 5), with the majority of interactions occurring between MOR (TM1) and DOR (TM4). They also proposed a second model in which the interface of the dimer was comprised of MOR (TM6, 7) and DOR (TM4, 5), with more frequent interactions occurring between MOR (TM6) and DOR (TM4). Both of these models also show close contacts between the C-termini.

While the models of MOR-DOR dimerization presented here are also in agreement with experimental data, Micovic et al. have suggested that the DOR-1 and DOR-2 subtypes represent different binding sites on a single receptor. Using ligand docking the authors proposed that subtype selective ligands ‘fit’ into different conformations of DOR and suggest that these alternative conformations may be the origin of the observed subtypes. Mosberg and co-workers have demonstrated that monomeric MOR is fully functional and therefore does not require dimerization to activate its G-protein. However, these results do not negate the possibility that opioid receptor dimerization may lead to novel, functional signaling units.

3.1.9. Significance of Opioid Receptor Subtypes

Opioid receptor subtypes are proposed to provide novel targets for analgesics and other drugs aimed at modulating the opioid system. van Rijn and Whistler provided evidence that the DOR-1 and DOR-2 subtypes have divergent effects on alcohol consumption in mice. While the DOR-2 antagonist NTB decreased alcohol consumption, antagonism of both DOR-2 and DOR-1 by NTI or NTX had either no
effect or a limited effect on alcohol consumption, respectively. Further, agonism by TAN67 (14, Figure 3.5), which is a proposed DOR-1 ligand, also resulted in decreased alcohol consumption. The ability of TAN67 to decrease alcohol consumption in mice that lacked either MOR or DOR was then compared to wild type mice. The effects of TAN67 required the expression of both MOR and DOR but the effects of NTB only required expression of the DOR gene. The authors also provided evidence that the DOR-1 subtype was a heterodimer comprised of MOR and DOR using co-immunoprecipitation and ligand binding studies in human embryonic kidney (HEK) 293 cells that co-expressed both receptors.  

Portoghese and co-workers proposed that the tissue selective analgesia produced by 6’-GNTI (8, Figure 3.2) occurs via binding to the proposed DOR-KOR heterodimer in the spinal cord of mice. 68 6’-GNTI was originally described as a KOR agonist. 69 However, signaling by 6’-GNTI was antagonized by both norbinaltorphimine (norBNI), a KOR antagonist, and naltrindole (NTI), a DOR antagonist, in cells that co-express KOR and DOR and in vivo in mice. 68 Furthermore, while 6’-GNTI is an agonist in the GPI where it binds KOR, it is not in the MVD even though all three opioid receptor types are present in this tissue, further suggesting that the DOR-KOR heterodimer is tissue specific. The KOR-DOR heterodimer was proposed to be limited to the mouse spinal cord and absent from the brain. KDN-21 (15, Figure 3.7), a bivalent ligand (see section 3.1.10 for a discussion of bivalent ligands), which has a KOR antagonist (5’-GNTI) appended to a DOR antagonist (NTI), is an antagonist of 6’-GNTI-mediated analgesia in vivo. 68 The antagonist activity of KDN-21 is proposed to occur via interaction with a DOR-KOR heterodimer originating from the DOR-1 and KOR-2 subtypes. These results
are in agreement with the report by Gomes et al.\textsuperscript{47} that the DOR-2 subtype corresponds to the MOR-DOR heterodimer and in opposition to those of van Rijn and Whistler\textsuperscript{35} (described above) that the DOR-1 subtype is equivalent to the MOR-DOR heterodimer.

3.1.10. Bivalent Ligands Targeting the Opioid Receptors

3.1.10.1. Definitions and General Introduction to Bivalent Ligands

The term bivalent ligand refers to a class of compounds that house two pharmacophoric entities within a single structure. Bivalent ligands can be homobivalent, whereby the two pharmacophores are identical, or heterobivalent and therefore possess two distinct pharmacophores. Bifunctional ligands possess a single pharmacophore but have multiple sites of action. For example, Tyr-Tic-Phe-Phe-NH\textsubscript{2} (TIPP-NH\textsubscript{2} \textsuperscript{16}, Figure 3.8) is a bifunctional peptide and was the first compound reported in the literature with mixed MOR agonist and DOR antagonist properties.\textsuperscript{70}

Bivalent ligands targeting the opioid receptors have been reviewed.\textsuperscript{57, 71-73} There are four types of ligand combinations to produce bivalent compounds targeting the opioid receptor system: 1) Peptide-peptide opioids, 2) Peptide-non-peptide opioids, 3) Non-peptide-nonpeptide opioids and 4) opioid-non-opioid combinations. In addition to

\textbf{Figure 3.7.} Structure of KDN-21.
enhanced affinity and activity at their target receptors, many peptide bivalent ligands also display increased metabolic stability.\textsuperscript{71}

![TIPP-NH\textsubscript{2} 16](image1.png) ![TENA 17](image2.png)

**Figure 3.8.** Example of a bifunctional ligand (16) and a bivalent ligand (17).

In designing a bivalent ligand one should consider 1) individual pharmacophore selectivity and affinity for the target receptor(s), 2) the ease with which a linker can be incorporated into the structure and 3) linker length and composition.\textsuperscript{74} The purpose of the bivalent ligand and the desired outcome of the interaction with its target receptor should also be considered. Bivalent ligands can be designed to interact with individual receptors or simultaneously with receptor oligomers; both of these scenarios are discussed below. The utility of bivalent ligand design in improving pharmacological profiles is also included in this discussion.

### 3.1.10.2. Bivalents Ligands and the Effect of Linker Length and Composition

The early literature on bivalent ligands predates the proposal of opioid receptor dimers. As such, the majority of these ligands were not designed to bridge receptors and all of these ligands bind reversibly to the receptors. The purpose of this discussion is to survey the design of bivalent ligands and examine the effects of various linkers on the
affinity and activity at the opioid receptors. Furthermore, with the exception of Portoghese’s MDAN series of bivalent ligands (section 3.1.10.3), many of the bivalent ligands in the literature that are reported to bridge opioid receptors were designed for binding to the DOR-KOR heterodimer. Nevertheless, these ligands were studied for the design of our bivalent peptides that target the MOR-DOR heterodimer. Portoghese’s early work with 6β,6β’-[ethylenebis(oxyethyleneimino)] bis [17-(cyclopropylmethyl)-4,5α-epoxymorphinan-3,14-diol] (TENA, 17, Figure 3.8) and the corresponding monomeric-linker conjugates demonstrated that the selectivity, affinity and potency of a pharmacophore could be enhanced by incorporation into a bivalent ligand.75, 76 Also in these early publications, Portoghese and co-workers suggested that incorporating a linker of appropriate length would allow bridging of neighboring receptors by bivalent ligands.

One of the first bivalent peptide ligands reported for opioid receptors was biphalin ((Tyr-D-Ala-Gly-Phe-NH)_2), a homodimeric peptide comprised of two modified enkephalin tetrapeptides linked at the C-termini by hydrazine, and was described in the literature over 20 years ago.77, 78 Biphalin was designed to have increased metabolic stability and increased affinity for opioid receptors. This ligand is 9000-fold more potent than [Met^5]enkephalin and is a more potent analgesic than morphine in the hotplate assay.74, 75 Furthermore, biphalin has high metabolic stability, demonstrating the effect of ligand combination on pharmacokinetics.71, 79, 80

Numerous linkers have been investigated for the incorporation of two pharmacophores into a single compound.71, 72, 81 In the mid 1980s, Costa et al. prepared bivalent peptides comprised of homodimeric enkephalin by linking the peptides at the C-termini to give X-(CH_2)_n-X, where X was either [D-Ala^2]enkephalin-NH (1-4) (DTE_n) or
In this series both DTE\textsubscript{2} and DPE\textsubscript{2} displayed increased affinity for DOR. Increasing the linker length between the two peptides resulted in a decrease in potency and affinity for MOR but did not have a similar effect on the potency and affinity for DOR up to DTE\textsubscript{12} and DPE\textsubscript{10}.

Similar dermorphin (Tyr-D-Ala-Phe-Gly-Pro-Ser-NH\textsubscript{2}) -based ligands were prepared by Lazarus and co-workers by linking the C-termini of dermorphin 1-4 or 1-5 by either a hydrazine or a (poly)ethylenediamine linker.\textsuperscript{83} Although monomeric dermorphin is MOR selective, when the spacer was hydrazine both ligands showed increased binding affinity for MOR and DOR. As with the enkephalin bivalent ligands described above, binding affinity at MOR decreased with increasing linker length.\textsuperscript{83}

More recently, homobivalent analogs of endomorphin-2 (Tyr-Pro-Phe-Phe-NH\textsubscript{2}) with a diamino-polyglycine linker were prepared by Gao et al.\textsuperscript{84} The length of the spacer varied from 0 (i.e. hydrazine) to 18 atoms. The dimeric compounds showed a decrease in MOR affinity compared to endomorphin-2 but displayed increased affinity for DOR. The highest affinity for DOR was observed with a linker length of 12 atoms. Since endomorphin-2 is a MOR selective ligand, this study provides evidence that a switch in ligand selectivity is possible by combining a MOR selective ligand into a homobivalent compound.

Dmt (2’,6’-dimethyl-L-tyrosine) was linked at the C-terminus by an aminoalkyl chain to yield a homobivalent ligand with high affinity for MOR (18, Figure 3.9).\textsuperscript{85} The highest binding affinity for MOR and DOR was associated with linkers varying from 4 to 8 methylene units. Replacing the diaminomethylene linker with a 2(1H)-pyrazinone linker (19, Figure 3.9) resulted in compounds with enhanced affinity, activity and
selectivity for MOR. Since this series of Dmt-based ligands are likely to be too short to bridge neighboring receptors, they are proposed to bind a single receptor site in MOR. Extension of Dmt with Tic in homobivalent peptides linked at the C-termini by either diaminopolymethylene or diaminoalkyl 2(1H)-pyrazinone resulted in extremely potent antagonists at both MOR and DOR. The capability of bridging receptors by these ligands is proposed to be limited to between 8 and 18Å and they are likely too short to bridge opioid receptor dimers.

[Dmt\textsuperscript{1}]DALDA (Dmt-D-Arg-Phe-Lys-NH\textsubscript{2}) a MOR agonist, and TICP[Ψ](12, Figure 3.4) a DOR antagonist, were joined through an ethylenediamine linker giving rise to an analgesic with decreased tolerance and dependence. This compound, however, demonstrated decreased binding affinity and activity at MOR and DOR compared to the parent peptides. The authors attribute the decrease observed in binding affinity to MOR to steric bulk arising from the linker and the decrease in affinity at DOR was attributed to the lack of an acidic functionality at the C-terminus in the bivalent peptide.

Hruby and co-workers have prepared bivalent ligands\textsuperscript{89, 90} designed to increase bioavailability of enkephalin and take advantage of agonism of both DOR and MOR for the treatment of pain with reduced side effects.\textsuperscript{57} [D-Ala\textsuperscript{2}]enkephalin (1-4) was conjugated to an amino-substituted fentanyl scaffold through either β-Ala\textsuperscript{89} (20, Figure 3.9) or 3-hydrazinocarbonyl propionyl (21, Figure 3.9).\textsuperscript{90} The bivalent ligand with the β-Ala linker was a potent agonist at MOR and DOR \textit{in vitro}.\textsuperscript{89} Selectivity for MOR was increased when β-Ala was replaced with 3-hydrazinocarbonyl propionyl.\textsuperscript{90} The selectivity was shifted to DOR over MOR when Tyr-D-Ala-Gly-Phe was linked to fentanyl by coupling Phe\textsuperscript{4} directly to the piperidine ring of the fentanyl scaffold (22,
Figure 3.9. Examples of bivalent ligands.
Figure 3.9). Substitution of Tyr$^1$ with Dmt enhanced the selectivity of the compound for MOR, which provided a compound with “balanced” activity at both MOR and DOR.$^{90}$

Portoghese and co-workers described bivalent ligands comprised of the small molecule opioid agonist oxymorphone joined to the dipeptide address sequence of Leu enkephalin (Phe-Leu) or dynorphin 4-8 (Phe-Leu-Arg-Arg-Ile) by hydrazone.$^{91, 92}$ These compounds were designed to enhance affinity and selectivity of oxymorphone for DOR and KOR, respectively. The enkephalin-based ligands displayed increased affinity for DOR whereas the dynorphin-based ligands displayed enhanced affinity at KOR; neither series demonstrated a change in affinity for MOR compared to oxymorphone.

The characteristics of the linker used to bridge pharmacophores in a bivalent ligand such as hydrophobicity/hydrophilicity, conformational flexibility and of course length, can significantly affect the ability of these compounds to bind to their target receptor(s). In general, the diaminomethylene-based linkers have provided a general scaffold for the generation of potent and selective bivalent ligands. However, when considering possible linkers, the bivalent ligand must be considered as a whole since the optimal linker in one series may not be appropriate for other ligand combinations.

3.1.10.3. Bivalent Ligands Designed to Bridge Opioid Receptor Dimers

Bivalent ligands that specifically target receptor heterodimers are expected to have greater affinity in systems that express both receptor types.$^{57, 71, 93-95}$ This is due in part to an increase in the effective concentration of the ligand at one receptor when one pharmacophore in the bivalent ligand binds to the second receptor in the dimer (B, Figure 3.10).$^{95}$ Since the individual pharmacophores of bivalent ligands that contain a linker capable of spanning two receptors in a dimer should be able to interact independently
with their receptor, there are two possible pathways of monovalent binding to the receptor complex that can lead to a bivalent receptor state (D, Figure 3.10). Therefore, the thermodynamics (ΔG°) should also be more favorable for bivalent ligand binding compared to their monomeric counterparts. If binding occurs in a stepwise process (A to B, Figure 3.10) and if the linker length is the optimal spacer for bridging receptors in the dimer, the entropic barrier for association of the second ligand (B to D, Figure 3.10) will be substantially reduced compared to monomeric ligands.

Figure 3.10. Illustration of the possible binding mechanism for binding of a bivalent ligand to a receptor dimer. The bivalent ligand can interact with the receptor dimer in a stepwise fashion A to B which increases the effective concentration of the second pharmacophore at its target receptor leading to D. Two bivalent ligands can also occupy the receptor dimer (C). From ref.95

In the late 1970’s Coy et al. reported the synthesis of Nα,Nε-bis ([D-Ala², Met⁵]enkephalin)-Lys amide, which is a homobivalent ligand that was designed to simultaneously bind to multiple receptors.96 This peptide was 4-fold more potent than the parent peptide ([D-Ala², Met⁵]enkephalin) in producing analgesia in the rat tail flick assay. This study predates the literature examining oligomerization of the opioid receptors. As noted above, the majority of bivalent ligands reported since Coy are
ligands that bind to individual receptors. The recent increase in the number of publications regarding ligands specifically designed to bridge opioid receptor dimers is largely due to Portoghese’s work on the MDAN, KDAN, KDN and KMN series of bivalent ligands.

Portoghese and co-workers have employed a common strategy for the design of bivalent ligands targeting opioid receptor heterodimers.9, 92, 95, 97-99 Two pharmacophores, one selective for each receptor, are joined together by variable length spacers (ranging from 16-21 atoms) usually consisting of a central diamine that bridges diglycolic acid (O(CH₂COOH)₂) or polyglycine moieties. In order to determine possible effects of the linker, monomeric controls in which only one pharmacophore is conjugated to the linker were prepared and examined in the binding and activity assays.

The MDAN (MOR-DOR agonist antagonist) ligands98 were developed based on the ability of DOR antagonists to decrease the tolerance and dependence associated with MOR agonists without significantly decreasing analgesia.34, 53, 57, 100, 101 Oxymorphone and NTI were coupled via a diglycolic amine linker with lengths varying from 16 to 21 atoms (Figure 3.11). An increase in linker length was associated with increased antinociceptive activity when compounds were administered intracerebroventricularrayly (i.c.v.) in mice. Each bivalent ligand was synthesized in conjunction with a monomeric control (denoted MA and DN) in which only one pharmacophore was attached to the linker. The MA monomeric controls displayed similar ED₅₀ to oxymorphone and MA-19 (27, Figure 3.11) co-administered with DN-20 (28, Figure 3.11) resulted in antinociception comparable to administration of MA-19 alone. Chronic i.c.v. administration of MDAN-16, -17, -18 and MA-19 (Figure 3.11) resulted in the
Figure 3.11. The MDAN (23-28) and KDAN (29-30) series of ligands designed by Portoghese and co-workers.
development of tolerance. However, no significant development of tolerance was observed following chronic i.c.v. administration of MDAN-19, -20 or -21 (Figure 3.11). Co-administration of NTI increased the potency of MDAN-19 but not of MDAN-21. The monovalent ligands were more potent than their bivalent counterparts, which the authors attribute to possible negative allosteric cooperativity between MOR and DOR.

The KDAN (KOR-DOR agonist antagonist) series of bivalent ligands was designed by Portoghese and co-workers to probe the proposed DOR-KOR heterodimer. NTI (DOR antagonist) and ICI-199441 (KOR agonist) were coupled via an oligoglycyl linker that varied from 12 to 20 atoms. Each bivalent ligand was synthesized in conjunction with a monomeric control. The monomeric controls were evaluated to determine any effect of linker on binding affinity and/or activity of the compounds. The KDAN ligands and the KOR monomeric controls were full agonists and KDAN-12 (29, Figure 3.11) and -18 (30) were antagonized by norBNI. NTB antagonized the effect of KDAN-18, which was attributed to disruption of the KOR-DOR dimers resulting in a univalent binding state, thereby lowering the potency of KDAN-18.

The KMN (KOR-MOR antagonist) series of bivalent ligands, which target the proposed MOR-KOR dimer, was comprised of the KOR antagonist 5’-guanidinonaltrindole (5’-GNTI) and the MOR antagonist β-naltrexamine. The bivalent ligands were analyzed for antagonist activity in HEK 293 cells that co-expressed MOR and KOR, and the optimal linker length in the KMN series was found to be 21-atoms.

3.1.10.4. Concluding Remarks

The current bivalent ligands for the opioid receptors reported in the literature are all reversible ligands, and the majority of these compounds were not designed with the
intent of bridging receptor dimers. In this thesis, the term chimeric is used to describe our series of heterobivalent ligands comprised of two different peptides connected by various linkers. Our chimeric peptide ligands represent the first example of heterobivalent peptide-based affinity labels targeting opioid receptor dimers. Here, we present a novel solid phase technique for the generation of bivalent ligands targeting the MOR-DOR heterodimer and biological analysis in CHO cells expressing each receptor type.

### 3.2. Rationale

Our chimeric ligands were designed to bind MOR and DOR receptors simultaneously during receptor-receptor interactions. These ligands bear an affinity label on one of the peptides in the bivalent ligand to irreversibly anchor it to one receptor during analysis of ligand binding. The current bivalent ligands reported in the literature for MOR and DOR all bind reversibly to these receptors. Therefore, these ligands will dissociate and re-associate to both receptor targets in the MOR-DOR dimer (Figure 3.12A). Since our chimeric peptides possess an affinity label in one of the ligands, covalent binding of the peptide-based affinity label to one receptor will anchor the peptide and reversible binding will only be observed with the second ligand and its receptor (Figure 3.12B). This offers an advantage in our proposed pharmacological experiments (section 3.5.2) since we will only be concerned with competitive inhibition of a single receptor-binding site in the dimer.
3.3. Results and Discussion

3.3.1. Peptide Design

Our initial series of bivalent ligands contain the DOR selective peptide antagonist TIPP (Tyr-Tic-Phe-Phe, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) linked to the MOR selective agonist dermorphin via a poly-amino acid-ethylenediamine linker.
These peptides were chosen based on previous work in the Aldrich research group in which TIPP and dermorphin affinity label derivatives were identified that exhibited high affinity and wash resistant inhibition of binding (WRIB) to DOR and MOR, respectively.\textsuperscript{102, 103} The chimeric peptides reported in this thesis have the affinity label on the TIPP portion of the peptide. An Asp residue was incorporated at the C-terminus of TIPP to maintain selectivity of this portion of the peptide for DOR.\textsuperscript{104} Ethylenediamine acts as a bridge to which Gly and/or β-Ala can be coupled in various combinations to yield linkers from 10 to 22 atoms corresponding to approximately 13 Å and 27 Å, respectively (when linkers are in an extended conformation). Portoghese and co-workers demonstrated that 25 Å was the optimal linker length for the MDAN series of bivalent ligands.\textsuperscript{98} However, these distances are not directly comparable between non-peptide ligands and peptide ligands of different lengths. Molecular modeling of the distance between binding sites of MOR homodimers suggested lengths between 27 Å and 32 Å depending on which transmembrane helices comprise the dimer interface.\textsuperscript{95} We chose to first synthesize the bivalent peptides with the 16-atom polyglycine-ethylenediamine linker (approximately 20 Å) to establish the synthetic methodology.

We are also exploring the effect of incorporating a flexible polyethylene glycol (PEG)-like linker on both the synthesis and binding affinity of the peptides to DOR. TIPP-Asp derivatives previously synthesized in our laboratory were extended with a linker at the C-terminus of Asp\textsuperscript{105} and did not have an amide bond in close proximity to the C-terminus of this residue. Therefore, we designed a series of monomeric peptides to assess the influence of the proximity of the amide bond from the linker adjacent to the Asp residue in the TIPP-Asp portion of the peptides on DOR affinity. In this series of
peptides, the PEG-like linker is either coupled directly to the Asp$^5$ residue of the TIPP(X)-Asp peptide or is coupled to Gly, β-Ala or γ-amino isobutyric acid (GABA). The PEG-like linkers for this series contain either 2- or 3-ethylene oxide (-CH$_2$CH$_2$O-) units. The chimeric and monomeric peptides can be visually divided into two portions, a northern half and a southern half (Figure 3.13). Each region (north or south) has an associated portion of the linker. The central portion of each linker is a monoprotected

**Figure 3.13.** A. Illustration of the chimeric ligands targeting the MOR-DOR dimer and B. monomeric control peptides. C. A representative peptide labeled with the northern, southern and central regions of the peptide.
diamine to which the remaining portion of the linker and the peptides are coupled. The synthesis of these peptides starts with assembly of the non-affinity label peptide (or just the acetylated linker in the case of monomeric controls) followed by deprotection of the central diamine linker and subsequent assembly of the peptide-based affinity label (TIPP(X)-Asp, X= NH₂ or NHCOCH₂Br). Throughout this discussion the TIPP(X)-Asp portion of the peptide and the linker associated with the central amine where TIPP(X)-Asp is assembled will be referred to as the northern portion of the peptide. The portion of the linker associated with the amine of the central diamine linker where dermorphin (or just the acetylated linker in monomeric controls) is assembled will be referred to as the southern portion of the peptide.

3.3.2. Synthesis of Chimeric and Monomeric Peptides with an Ethylenediamine-Linker

Chimeric peptides with an ethylenediamine-based linker were successfully prepared by solid phase peptide synthesis (SPPS). The strategy for our synthesis involved the use of orthogonal protecting groups, which allowed for the selective deprotection and subsequent selective assembly of each portion of the chimeric and monomeric peptides (Scheme 3.1). Peptide assembly was initiated with the reductive amination of an aldehyde-based resin 4-(4-formyl-3-methoxyphenoxy)butyryl AM resin (FMPB AM) with a mono-Alloc protected ethylenediamine linker (NH₂CH₂CH₂NH-Alloc) using NaBH(OAc)₃ in dichloromethane (DCM):trimethyl ortho-formate (TMOF) (2:1) following a previously described method. The resulting secondary amine was then coupled to Nα-9-fluorenylmethoxycarbonyl- (Fmoc) Gly-OH using 1-hydroxybenzotriazole (HOBt), benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium
Scheme 3.1A. Synthesis of chimeric peptides.

1. Fmoc-AA, PyBOP, HOBt, DIEA (4:4:4:8) in DCM/DMF (1:1)
2. Piperidine/DMF (1:4)
Scheme 3.1B. Synthesis of chimeric peptides continued.

35
1. Boc-Tyr(fBu)-OH, PyBOP, HOBt, DIEA (4:4:4:8) in DCM:DMF (1:1)
2. Piperidine:DMF (1:4)*

36
Pd(PPh₃)₄ (0.1 equiv), phenylsilane (0.12 equiv/mg resin) in DCM

37
DIC (10 equiv), BrCH₃CO₂H (10 equiv) in DCM

38
90% TFA

39
90% TFA

40
* Step was not included in initial synthesis of chimeric peptides but was included in subsequent syntheses.
hexafluorophosphate (PyBOP) and N,N-diisopropylethylamine (DIEA) in DCM: N,N-dimethylformamide (DMF) (1:1). The southern portion of the linker and the dermorphin sequence were then assembled using standard SPPS with Fmoc-protected amino acids, except for the N-terminal Tyr residue, which was protected with t-butyloxycarbonyl (Boc) at the α-amine. Following successful assembly of the dermorphin portion of the chimeric peptide, the Alloc group was removed using Pd(PPh₃)₄ and phenylsilane in DCM to free the monoprotected amine of the ethylenedimine linker. The northern portion of the linker and the TIPP-Asp peptide were then assembled using standard Fmoc SPPS in DCM/DMF (1:1).

The chimeric and monomeric peptides with an Asp-Gly in the peptide sequence were prone to aspartimide formation.¹⁰⁷-¹⁰⁹ Therefore, the Gly residue C-terminal to Asp was coupled as N¹-Fmoc-N¹-(2-Fmoc-oxy-4-methoxybenzyl)-glycine (Fmoc-(Fmoc-Hmb)Gly-OH) to avoid this side reaction (Figure 3.14A). The 2-hydroxy-4-methoxybenzyl (Hmb) group is an acid labile protecting group that is used to prevent aspartimide formation and is also incorporated into peptides to prevent aggregation during peptide synthesis.¹¹⁰-¹¹² During amino acid couplings, the o-hydroxyl of the Hmb group can be acylated.¹¹²,¹¹³ In fact, the mechanism by which the first amino acid couples to the Hmb protected Gly residue is proposed to occur through an O to N acyl transfer reaction.¹¹² The acylated Hmb is stable to 90% TFA, but the ester is base labile (Figure 3.14B).¹¹³ During Fmoc-based peptide synthesis this does not pose an issue since O-deacylation of the Hmb occurs during base-mediated Fmoc deprotection, reforming the acid labile Hmb group. However, in our syntheses the N-terminal amino acid was Boc protected to afford orthogonal protection during side chain functionalization, and the final
Figure 3.14. A. Structure of Fmoc-(Fmoc-Hmb)Gly-OH. B. O-Acylation with R group (amino acid or anhydride) and O-deacylation lead to different products when cleaved in 90% TFA.
Figure 3.15. A. HPLC chromatogram of the Hmb O-acylated chimeric amine control (42) and B. affinity label (43).
peptide was typically not subjected to base treatment prior to acid-mediated cleavage from the resin. In the initial synthesis of the chimeric series the bis-Tyr acylated peptides were the major products for both the amine control (42, Figure 3.15) and the affinity label peptide (43, Figure 3.15). Therefore, in subsequent syntheses, following full linear assembly and prior to introduction of the affinity label, the peptides were treated with 20% piperidine in DMF to O-deaclylate the Hmb protecting group.\textsuperscript{113}

Following linear assembly and O-deaclylation of Hmb, the Alloc protecting group on Phe(\(\rho\)-NHAlloc)\textsuperscript{4} was removed using \(\text{Pd(PPh}_3)\text{)}_4\) and phenylsilane in DCM. The peptide on resin was then split into two portions and one portion was cleaved from the resin to yield the control peptide (38, Figure 3.16). The control peptide was purified to > 95% purity by RP-HPLC in aqueous acetonitrile containing 0.1% TFA and analyzed by ESI-MS. The pure fractions were combined, lyophilized, analyzed in two solvent systems (section 3.6.6) and submitted for pharmacological analysis (section 3.3.4).

Bromoacetic acid was preactivated with \(N,N\)-diisopropylcarbodiimide (DIC) and the mixture was added to the second portion of the peptide on resin to form the bromoacetamide at the para position of Phe\textsuperscript{4}. The bromoacetamide functionalized peptide (40, Figure 3.16, Table 3.1A) was then cleaved from the resin and purified by RP-HPLC as described above for the amine control. This peptide degraded over a period of hours when left at room temperature in aqueous acetonitrile containing 0.1% TFA resulting in two new peaks (three total) in the HPLC chromatogram (Figure 3.16C, HPLC).

A monomeric control peptide with the 16-atom spacer was prepared similarly except that the N-terminal Gly of the southern portion of the linker was acetylated with
Figure 3.16. A. HPLC chromatogram of the chimeric amine control (38) and B. affinity label (40). C. Degradation of 40 upon sitting at room temperature in aqueous acetonitrile/H₂O (containing 0.1% TFA).
acetic anhydride. The TIPP portion of the peptide and the northern portion of the linker were prepared as described above. The synthesis of the monomeric peptide proceeded smoothly (50, Scheme 3.2). The peptide was cleaved from the resin using TFA and purified by RP-HPLC in aqueous acetonitrile containing 0.1% TFA.

**Scheme 3.2.** Synthesis of the monomeric control peptide.
### Table 3.1A. HPLC retention times, observed m/z of the purified chimeric (38 and 40) and monomeric peptide (50).

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC t&lt;sub&gt;r&lt;/sub&gt; (min) / % Purity</th>
<th>m/z (ESI-MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>System 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>System 2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>38</td>
<td>25.8 / 97.8</td>
<td>44.2&lt;sup&gt;d&lt;/sup&gt; / &gt; 99</td>
</tr>
<tr>
<td>40</td>
<td>29.6 / &gt; 99</td>
<td>28.9&lt;sup&gt;c,d&lt;/sup&gt; / &gt; 99</td>
</tr>
<tr>
<td>50</td>
<td>19.5&lt;sup&gt;e&lt;/sup&gt; / 99.1</td>
<td>33.4&lt;sup&gt;e&lt;/sup&gt; / &gt; 99</td>
</tr>
</tbody>
</table>

Unless otherwise noted, the HPLC gradient was 5 - 50% B over 45 min, gradient started at 5 min.

a. System 1: Solvent A = Water (0.1% TFA), Solvent B = Acetonitrile (0.1% TFA)
b. System 2: Solvent A = Water (0.1% TFA), Solvent B = Methanol (0.1% TFA)
c. System 3: Solvent A = 0.09 M Triethylammonium phosphate (TEAP), pH 2.5, Solvent B = acetonitrile
d. HPLC gradient was 5 - 50% B over 45 min., gradient started after 1 min.
e. HPLC gradient was 5 - 60% B over 55 min, gradient started after 1 min.

### 3.3.3. Monomeric Peptides with a PEG-Like Linker

A series of monomeric control peptides with a PEG-like linker was synthesized to determine the effect of introducing flexibility into the linker on DOR affinity. These peptides were also designed to examine the effect of the proximity of the amide bond in the linker adjacent to Asp<sup>5</sup> of the TIPP-Asp peptide on DOR affinity. The synthesis of these peptides was carried out similar to the ethylenediamine-based peptides except that the linkers were comprised of either 8-amino-3,6-dioxo-octan-1-amine (NH<sub>2</sub>(CH<sub>2</sub>CH<sub>2</sub>O)<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) or 11-amino-3,6,9-trioxa-decan-1-amine (NH<sub>2</sub>(CH<sub>2</sub>CH<sub>2</sub>O)<sub>3</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>) and various combinations of Gly, β-Ala and GABA.

The synthesis of the monomeric peptides with a PEG-like linker is illustrated in Scheme 3 and the HPLC chromatograms and ESI-MS spectra of the final peptides are shown in Figures 17-21. Following reductive amination of the aldehyde-based resin
(FMPB AM) with the amino-azide PEG-like linker N$_3$(CH$_2$CH$_2$O)$_2$CH$_2$CH$_2$NH$_2$ (compounds $60$-$62$, Scheme 3.3) or N$_3$(CH$_2$CH$_2$O)$_3$CH$_2$CH$_2$NH$_2$ (compounds $63$ and $64$), Fmoc-Gly-OH was coupled to the secondary amine using PyBOP, HOBt and DIEA in DCM/DMF (1:1). Unreacted amine was capped with benzoic anhydride and pyridine. This capping step led to the unintended capping of the α-amine of Gly to give a benzamide, which resulted in the formation of a side product in addition to the desired peptides. Since Fmoc is reported to be stable to pyridine, this unintended capping may be a result of premature Fmoc group removal as a result of reagent contamination or a side reaction involving the secondary amine of the linker. This discussion is further elaborated in the future work section of this chapter (section 3.5). The Fmoc group was removed from the remaining uncapped Gly with 20% piperidine in DMF and the resulting free amine was acetylated with acetic anhydride and DIEA in DMF. The azide was then reduced to the amine by a solid phase Staudinger reduction using PMe$_3$. The northern portion of the linker and the TIPP-Asp peptide were then assembled using Fmoc SPPS. The monomeric peptide with the 16 atom linker was prepared by first coupling Fmoc-(Fmoc-Hmb)Gly-OH to the linker (Scheme 3.3). The remaining TIPP(p-NHAlloc)-Asp peptide was then assembled using Fmoc SPPS to give the final linear sequence of the monomeric control peptide $60$ with two predominant peaks in the HPLC chromatogram and ESI-MS gave masses for the desired product ($m/z = 1136.08$ [M+H]$^+$) plus a mass 62 Da higher than expected ($m/z = 1198.36$ [M+H]$^+$) corresponding to the benzamide side product. Monomeric peptide $60$ was then treated with 20% piperidine in DMF to O-deacylate the Hmb group. The monomeric peptide with the 17 atom linker $61$ was prepared similar to $60$ except that Fmoc-β-Ala-OH was coupled to the linker instead
Scheme 3.3A. Synthesis of the monomeric controls peptides.
* Fmoc-(Fmoc-Hmb)Gly-OH used in synthesis of 60.
54 \[\text{Boc-Tyr(\text{Bu})-OH, PyBOP, HOBt, DIEA (4:4:4:8) in DCM:DMF (1:1)}\]

1. Boc-Tyr(\text{Bu})-OH, PyBOP, HOBt, DIEA (4:4:4:8) in DCM:DMF (1:1)
2. Piperidine:DMF (1:4)\(^*\)

\[\begin{align*}
\text{54} & \quad 55 \\
\text{56} & \quad \text{Pd}(\text{PPh}_3)_4 (0.1 \text{ equiv}), \text{phenylsilane (0.12 equiv/mg resin)} \text{ in DCM} \\
\text{58} & \quad \text{90\% TFA} \\
\end{align*}\]

\[\begin{align*}
\text{60} & \quad n = 1, m = 2 \\
\text{61} & \quad n = 2, m = 2 \\
\text{62} & \quad n = 3, m = 2 \\
\text{63} & \quad n = 2, m = 3 \\
\end{align*}\]

* Step included in synthesis of 60 only.
of Fmoc-(Fmoc-Hmb)Gly-OH (Scheme 3.3). The remaining TIPP(\(p\)-NHA\(\text{Alloc}\))-Asp peptide was then synthesized using standard Fmoc SPPS in DCM/DMF (1:1) to give the final linear sequence of 61 (Scheme 3). The monomeric peptide with the 18-atom linker 62 was also prepared similar to 60 except that Fmoc-GABA-OH was coupled to the linker (Figure 17). TIPP(\(p\)-NHA\(\text{Alloc}\))-Asp peptide was then assembled using Fmoc SPPS to give the final linear sequence of 62 (Scheme 3.3). The Alloc group was removed from the peptides using Pd(PPh\(_3\))\(_4\) and phenylsilane in DCM to afford the free amine at the para position of Phe\(^4\). HPLC and ESI-MS of the final products confirmed the presence of starting material as well as the benzamide side products (Figures 3.17-3.21). The monomeric peptide with the 20-atom linker (63, Scheme 3.3) was prepared by first coupling Fmoc-\(\beta\)-Ala-OH to the linker (N\(_3\)(CH\(_2\)CH\(_2\)O)\(_3\)CH\(_2\)CH\(_2\)NH\(_2\)) followed by Fmoc SPPS to assemble the remaining TIPP(\(p\)-NHA\(\text{Alloc}\))-Asp peptide. The monomeric peptide with the 16 atom ((-NH\(_2\)(CH\(_2\)CH\(_2\)O)\(_3\)CH\(_2\)CH\(_2\)NH\(_2\)Gly-) linker was prepared by coupling Fmoc-Asp(O\(t\)Bu)-OH directly to the linker (Scheme 3.3), followed by Fmoc SPPS to assemble the remaining TIPP(\(p\)-NHA\(\text{Alloc}\))-Asp peptide to give the final linear sequence of 64 (Scheme 3.3). The peptides were then cleaved from the resin using 90% TFA and purified by RP-HPLC in aqueous acetonitrile containing 0.1% TFA.

The synthesis of the monomeric peptides with PEG-like linkers proceeded smoothly except for the generation of a side product with a mass 62 Da higher than that of the desired peptides, which was present in the syntheses of all the monomeric peptides in this series and resulted from a capping step using benzoic anhydride and pyridine, apparently due to unintended Fmoc removal during this reaction. The HPLC chromatograms of these peptides (Figures 3.17-3.21) show one predominant peak for
compounds 63 and 64 and two major peaks for compounds 60, 61 and 62. ESI-MS analysis confirmed the presence of the desired peptides and also the corresponding benzamide side products (Figures 3.17-3.21). Nevertheless, these peptides were purified to >95% purity (Table 3.1B) with relative ease in a single RP-HPLC purification and submitted for pharmacological analysis.

Table 3.1B. HPLC retention times and observed m/z of the purified monomeric peptides 60-64.

<table>
<thead>
<tr>
<th>Compound</th>
<th>HPLC t_R (min) / % Purity</th>
<th>m/z (ESI-MS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>System 1 ¹</td>
<td>System 2 ²</td>
</tr>
<tr>
<td>60</td>
<td>20.6 / &gt; 99</td>
<td>34.8 / &gt; 99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61</td>
<td>20.7 / &gt; 99</td>
<td>34.9 / &gt; 99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>20.7 / &gt; 99</td>
<td>35.0 / &gt; 99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>21.1 / &gt; 99</td>
<td>36.0 / &gt; 99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>21.1 / &gt; 99</td>
<td>35.4 / &gt; 99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The HPLC gradient was 5 - 50%B over 45 min, gradient started at 5 min.

a. System 1: Solvent A = Water (0.1% TFA), Solvent B = Acetonitrile (0.1% TFA)
b. System 2: Solvent A = Water (0.1% TFA), Solvent B = Methanol (0.1% TFA)
Figure 3.17. A. Structures of the desired monomeric control (60) and the benzamide side product (65). B. ESI-MS and C. HPLC of the crude peptide cleaved from the resin.
Figure 3.18. A. Structures of the desired monomeric control (61) and the benzamide side product (66). B. ESI-MS and C. HPLC of the crude peptide cleaved from the resin.
Figure 3.19. A. Structures of the desired monomeric control (62) and the benzamide side product (67). B. ESI-MS and C. HPLC of the crude peptide cleaved from the resin.
Figure 3.20. A. Structures of the desired monomeric control (63) and the benzamide side product (68). B. ESI-MS and C. HPLC of the crude peptide cleaved from the resin.
Figure 3.21. A. Structures of the desired monomeric control (64) and the benzamide side product (69). B. ESI-MS and C. HPLC of the crude peptide cleaved from the
3.3.4. Pharmacological Analysis of the Chimeric and Monomeric Peptides

The binding affinities of the peptides were determined in radioligand competition assays using \[^3\text{H}\text{DPDPE}\] as the radioligand for DOR or \[^3\text{H}\text{DAMGO}\] for MOR and CHO cells that expressed either DOR or MOR. Compounds 38 and 40 were then evaluated for wash resistant inhibition of binding (WRIB) of \[^3\text{H}\text{DPDPE}\] to DOR.

Chimeric peptides 38 and 40 as well as the monomeric control 50, exhibited nanomolar affinity for DOR. Peptides 38 and 40 had a decreased affinity for DOR compared to TIPP\((p-\text{NH}_2)\)\(^{102}\) and to the monomeric control 50 (Table 3.2). The chimeric peptide-based affinity label 40 exhibited 94% WRIB at a concentration of 96 nM. Since the control peptide 38 resulted in 23% WRIB (at 94 nM), the washing procedure used in these experiments removed nearly 80% of non-covalently bound peptide. Thus, while the conjugation of TIPP\((p-\text{NH}_2)\)-Asp to the ethylenediamine-based linker comprised of a 16-atom spacer and to dermorphin decreased affinity at DOR compared to TIPP\((p-\text{NH}_2)\), this extension did not prevent the interaction of the affinity label 40 with DOR. These results also indicate that peptide 40 is an affinity label for DOR and therefore, may be a useful probe in studies of MOR-DOR heterodimers. The monomeric control 50 also exhibited a decrease in DOR binding affinity compared to TIPP\((p-\text{NH}_2)\) (Table 3.2). This indicates that conjugating TIPP\((p-\text{NH}_2)\)-Asp to the linker decreased binding affinity to DOR. Since the binding affinity of the monomeric control is approximately 2-fold greater than that of either chimeric peptide, the incorporation of dermorphin into the chimeric peptides also decreased binding affinity to DOR.

The monomeric peptides with the PEG-like linker (60-64) also demonstrated decreased binding affinity for DOR compared to TIPP\((p-\text{NH}_2)\) (Table 3.2). These
Table 3.2. Affinities of chimeric peptides, monomeric peptides and parent peptides for DOR and MOR.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Peptide Sequence</th>
<th>IC$_{50}$ DOR (nM) ± SEM$^a$</th>
<th>IC$_{50}$ MOR (nM) ± SEM$^a$</th>
</tr>
</thead>
</table>
| 38       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-Gly-Gly-NHCH$_2$  
           | Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Gly-NHCH$_2$ | 136 ± 12 | 37.2 ± 3.0 |
| 40       | Tyr-Tic-Phe-Phe(p-NHCOCH$_2$Br)-Asp-Gly-Gly-NHCH$_2$  
           | Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-Gly-Gly-NHCH$_2$ | 146 ± 12 | 67.1 ± 11.4 |
| 48       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-Gly-Gly-NHCH$_2$  
           | Ac-Gly-Gly-NHCH$_2$ | 60.7 ± 4.9 | 6330 ± 680$^d$ |
| 58       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-Gly-NH(CH$_2$CH$_2$O)$_2$CH$_2$  
           | Ac-Gly-NHCH$_2$ | 99.2 ± 17.4 | ND |
| 59       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-β-Ala-NH(CH$_2$CH$_2$O)$_2$CH$_2$  
           | Ac-Gly-NHCH$_2$ | 71.9 ± 5.6 | ND |
| 60       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-GABA-NH(CH$_2$CH$_2$O)$_2$CH$_2$  
           | Ac-Gly-NHCH$_2$ | 88.0 ± 18.2 | ND |
| 61       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-β-Ala-NH(CH$_2$CH$_2$O)$_2$CH$_2$  
           | Ac-Gly-NHCH$_2$ | 67.8 ± 7.9 | ND |
| 62       | Tyr-Tic-Phe-Phe(p-NH$_2$)-Asp-NH(CH$_2$CH$_2$O)$_2$CH$_2$  
           | Ac-Gly-NHCH$_2$ | 64.1 ± 6.4 | ND |
| TIPP(p-NH$_2$)$^b$ | Tyr-Tic-Phe-Phe(p-NH$_2$) | 19.9 | >10000 |
| Dermorphin$^c$ | Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$ | 197 | 0.72 |

a. The IC$_{50}$ values are reported as the mean ± SEM of three independent experiments.
b. From ref.$^{102}$
c. From ref.$^{103}$
d. n = 2, awaiting data.
peptides had very similar binding affinities (Table 3.2) to each other and had affinities that were generally comparable to monomeric control 50. These results suggest that the proximity of the amide bond C-terminal to Asp had little effect on affinity and that introducing PEG units into the linker did not prevent the binding of these compounds to DOR.

Peptides 38 and 40 were also evaluated in radioligand competition assays with MOR. As expected, the monomeric peptide 50 retained selectivity for DOR and exhibited micromolar affinity to MOR (Table 3.2). While the affinity of the chimeric amine control 38 for MOR was 50-fold lower than of dermorphin, this peptide had approximately 4-fold higher affinity for MOR over DOR. Since the binding affinity of

![Figure 3.22. Percent WRIB of [³H]DPDPE binding to DOR of the chimeric amine control 38 and affinity label 40. The affinity label 40 (96 nM) exhibited 94 ± 1 % WRIB following a 90 min preincubation (at room temperature) with membranes from CHO cells that express DOR. The control compound 38 (94 nM) was washed from the membranes and demonstrated 23 ± 5 %](image)
for MOR was still in the nanomolar range, conjugating dermorphin to the linker and TIPP(p-NH₂)-Asp did not inhibit binding to MOR.

3.4. Conclusions

We successfully prepared the first series of heterobivalent peptides that were designed to bind covalently to MOR-DOR heterodimers. Our chimeric affinity label represents the first example of a heterobivalent peptide based affinity label targeting the opioid receptors. The chimeric peptide comprised of TIPP(p-NH₂)-Asp and dermorphin had nanomolar affinity for both DOR and MOR. As expected, the monomeric control peptide in this series retained selectivity for DOR over MOR and had an IC₅₀ value of 60.7 nM for DOR. The chimeric peptide with a bromoacetamide affinity label at the para position of Phe⁴ in the TIPP-Asp portion of the peptide displayed 94% WRIB of [³H]DPDPE binding to DOR at 96 nM peptide.

Our monomeric peptides with a PEG-like linker also exhibited nanomolar affinity for DOR. Radioligand binding analyses with these peptides demonstrated that introduction of an amide bond C-terminal to Asp⁵ of TIPP-Asp does not considerably decrease binding affinity for DOR and that various linker compositions are tolerated by DOR.

3.5. Future Work

The chimeric peptides, and their monomeric counterparts, reported in this thesis are the first in this series of chimeric peptide-based affinity labels for the MOR-DOR dimer. As such, the linker length employed in our initial syntheses may not be the appropriate length to bridge the receptors and the synthesis and evaluation of peptides
bearing different length linkers will need to be addressed as outlined in this section of the chapter.

3.5.1. Synthesis

Chimeric peptides with the PEG-like linker need to be prepared with a 16-atom spacer so that comparisons can be made with the peptides that possess the ethylenediamine-based linker. The synthesis of a chimeric peptide with a PEG-like linker containing a 20-atom spacer (Scheme 3.4) is nearly complete. Following purification, this peptide will be analyzed in our pharmacological assays. In order to compare the effect of the PEG-like linker to the ethylenediamine-based linker on receptor binding, the corresponding chimeric peptides with the ethylenediamine-based linker also need to be prepared with a 20-atom linker. We also need to synthesize the peptides with

![Scheme 3.4. Chimeric peptides with the 20-atom spacer.](image_url)
linkers that are likely too short to bridge receptors (e.g. 10 atom linker) as controls for the pharmacological assays.

The benzamide capped peptides that were encountered following the capping step with benzoic anhydride and pyridine in the synthesis of the monomeric controls with the PEG-like linker needs to be addressed. The most likely scenario is unintended Fmoc deprotection of the Gly residue followed by acylation. It is possible that the pyridine that was utilized in this step was contaminated with a secondary amine. Because the initial resin substitution was almost 1 mmol/g, it is also possible that unreacted secondary amine from the linker was involved in a rearrangement of the Fmoc protecting group. In order to differentiate between these two possibilities, fresh pyridine should be used for capping following Fmoc-Gly coupling. If the benzamide side product is not observed then the contamination scenario is the likely culprit, but if the side product is formed it is likely a result of the rearrangement of the Fmoc protecting group. There is no evidence of capped linker in the ESI-MS spectra of these peptides, indicating that the unreacted amine is a minor contributor to side products in final cleavages of the peptides from the resin. Therefore, this capping step can be avoided in future syntheses.

3.5.2. Pharmacological Evaluation

Our initial series of chimeric peptides and monomeric peptides will be evaluated in cells that co-express MOR and DOR. As noted in the literature review, bivalent ligands such as our chimeric peptides may display lower affinity to their target receptors when analyzed in systems that express only a single receptor type. Therefore, we may observe improved affinity of the chimeric peptides with the 16-atom spacer in cells that co-express MOR and DOR if this spacer length allows bridging of the receptors in a
dimer. These peptides will also be explored in radioligand competition assays with the proposed subtype-selective ligands. Since the population of the affinity labeled receptors can be enriched by either long incubations or the inclusion of washing steps to remove non-covalently bound ligand, the competitive inhibition experiments will only be concerned with a single monovalent state (Figure 3.12B, from $k_{2B}$) and the bivalent state (Figure 3.12B, from $k_{7B}$) of the receptor dimer. The affinity labeling should be blocked when receptor preparations are incubated first with DOR ligands that are selective for the dimer but should exhibit WRIB when the cells are first incubated with DOR ligands that are selective for the non-complexed receptor. The binding of the chimeric affinity label to DOR will also be examined in the presence and absence of MOR selective ligands. When the linker length between the pharmacophores is optimal for bridging receptor dimers, the MOR ligands are expected to exhibit increased IC$_{50}$ values. Since the affinity label in this series of peptides is selective for DOR, an increase in the IC$_{50}$ of MOR ligands would suggest a physical interaction between the receptors. These experiments will further delineate the ligand selectivity of the MOR-DOR dimer and give further information on receptor-ligand interactions involving the opioid receptors.

3.6. Experimental

3.6.1. Materials

4-(4-Formyl-3-methoxyphenoxy)butyryl AM resin (FMPB AM), 0.79 - 1.0 mmol/g and benzo triazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) were purchased from Novabiochem (San Diego, CA). All N$^\alpha$-9-fluorenymethoxycarbonyl (Fmoc) and t-butyloxycarbonyl (Boc) protected amino acids were purchased from Novabiochem, Peptides International (Louisville, KY) or Bachem
1-Hydroxybenzotriazole (HOBr) was purchased from Peptides International. Acetonitrile, dichloromethane (DCM), \(N,N\)-dimethylformamide (DMF), methanol, \(N,N\)-diisopropylethylamine (DIEA), glacial acetic acid, diethyl ether, \(N\)-Alloc-ethylenediamine, reaction vessels and frits were purchased from Fisher Scientific (Pittsburg, PA). Piperidine, bromoacetic acid, diisopropylcarbodiimide (DIC), triisopropylsilane (TIS), phenylsilane, trimethyl \textit{ortho}-formate (TMOF), 11-azido-3,6,9-trioxaundecan-1-amine, sodium diethylthiocarbamate (NaDDTC) and hydrazine were purchased from Sigma Aldrich (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockville, IL).

8-Azido-3,6-dioxaoctan-1-amine and Fmoc-Phe(pNHAloc)-OH were prepared as described in sections 2.6.2 and 2.6.3, respectively.

### 3.6.2. Synthesis of Chimeric Peptides with Polyglycine-ethylenediamine Linkers 38 and 40

Unless otherwise noted, all reagent equivalents are relative to resin substitution, which varied from 0.33 to 1 mmol/g. \(N\)-Alloc-ethylenediamine was reacted with the FMPB AM resin via reductive amination with NaBH(OAc)\(_3\) in DCM/TMOF (2:1) following a previously described procedure.\(^{105}\) The linker (5 equiv) was first reacted with the resin for 1 h then NaBH(OAc)\(_3\) (5 equiv) was added and the reaction was mixed for an additional 2 h followed by washing with DCM/TMOF (2:1) and DCM. The amino acid portion of the linker and dermorphin were then assembled off the resulting secondary amine using a 4-fold excess of amino acids, PyBOP and HOBr and 8-fold excess of DIEA. Amino acid couplings were performed in a mixed solvent system (DCM/DMF, 1:1) for 3 to 16 h by \(\text{N}_2\) bubbling using the CHOIR assembly\(^{115}\) in a polypropylene reaction vessel fitted with a polyethylene frit. Reaction completion was
qualitatively monitored by the ninhydrin test\textsuperscript{116} (for primary amines), the choranil test\textsuperscript{117} (for secondary amines) or by cleaving aliquots of the peptide from resin (see section 3.6.5) for HPLC and mass spectrometric analysis. All amino acids were Fmoc protected except for the N-terminal amine, which was Boc protected. Following assembly of dermorphin, the Alloc protecting group was removed using Pd(PPh\textsubscript{3})\textsubscript{4} (0.1 equiv) and phenylsilane (0.12 equiv/mg resin).\textsuperscript{118,119} The resin was covered in the minimum amount of DCM required for efficient mixing and phenylsilane was then added. The solution was mixed for 5 min, Pd(PPh\textsubscript{3})\textsubscript{4} was dissolved in a minimal amount of DCM and added to the reaction. The solution was mixed on the CHOIR for 1 h, the reaction was drained and fresh reagents were added. The vessel was capped with a septum, fitted with a needle and placed on a shaking arm overnight. The resin was then washed with DCM (5 x 2 min), THF (4 x 2 min), DCM (3 x 2 min), DMF (3 x 2 min), 0.5% DIEA in DMF (3 x 2 min), 0.02 M NaDDTC in DMF (3 x 15 min), DCM/DMF (1:1, 5 x 2 min), DCM (3 x 2 min) and finally MeOH (3 x 1 min). The progress of the deprotection reaction was assessed by cleaving an aliquot of the peptide from the resin (see sections 3.6.5) and monitoring for starting material. In general, the removal of the Alloc group required 2 full cycles for complete deprotection of the amine. The remaining portion of the linker and the TIPP-Asp portion of the peptide were then assembled using the same conditions as described above, except Fmoc-(Fmoc-Hmb)-Gly-OH was used for the coupling at the position C-terminal to Asp. Following amino acid couplings, aliquots of the peptide were cleaved from the resin (see section 3.6.5) and examined by HPLC to determine if a second coupling was required. Double couplings were typically required for Fmoc-(FmocHmb)Gly-OH and Boc-Tyr(OtBu)-OH to Tic. Following complete linear synthesis
of the peptide, the resin was treated with 1:4 piperidine/DMF (2 x 10 min) to O-deacylate any Hmb that reacted with Boc-Tyr(tBu)-OH in the last amino acid coupling step. After washing to remove piperidine and DMF the Alloc group was removed from Phe(p-NHAlloc)$^4$ with Pd(PPh$_3$)$_4$ (0.1 equiv) and phenylsilane (0.12 equiv/mg resin) and the resin was washed as described above. The resin was then divided into two portions and one portion was cleaved in TFA (see section 3.6.5) to give the amine control peptide 38. The remaining portion was coupled to bromoacetic acid to give the affinity label at the para position of Phe$^4$. Bromoacetic acid (10 equiv) was preactivated with DIC (10 equiv) for 20 min in DCM, the mixture was added to the remaining portion of the resin and the reaction was mixed overnight on a shaking arm. The resin was filtered and washed extensively with DCM, DCM/DMF, and again with DCM.

3.6.3. Synthesis of a Monomeric Peptide with a Polyglycine-ethylenediamine Linker 50

The monomeric control peptide was synthesized similarly, except the N-terminal Gly of the southern portion of the linker was acetylated with acetic anhydride (10 equiv) and DIEA (5 equiv) in DCM/DMF for 30 min. Following acetylation, the TIPP portion of the peptide was assembled as described above (section 3.6.2).

3.6.4. Synthesis of Monomeric Peptides with PEG-Like Linkers 60-64

11-Azido-3,6,9-trioxaundecan-1-amine (for compounds 63 and 64) or 8-azido-3,6-dioxaoctan-1-amine (compounds 60-62) was reacted with the FMPB AM resin via reductive amination as described above (section 3.6.2). Fmoc-Gly-OH was then coupled to the resulting secondary amine using the general procedure for amino acid coupling described above (section 3.6.2). Benzoic anhydride (5 equiv) was dissolved in
DCM/DMF (1:1) and pyridine (1 equiv) was added to the mixture. The mixture was added to the resin, reacted for 30 min then the resin was washed with DCM/DMF (1:1) and DCM. The Fmoc group was then removed with 1:4 piperidine in DMF (3 x 15 min) followed by washing with DMF, DCM/DMF (1:1) and DCM. The resulting free amine was subsequently acetylated with acetic anhydride (15 equiv) and DIEA (2 equiv) in DMF for 30 min. The resin was then washed with DCM/DMF (1:1) and DCM then covered in 1:4 anhydrous DCM/DMF (approximately 1 mL/100 mg resin) and trimethylphosphine (1 M in toluene, 1 equiv) was added. The reaction was mixed by N₂ bubbling for 2 h. Water (1 equiv) was then added to the reaction followed by mixing for 30 min by N₂ bubbling. The resin was filtered and the reaction was repeated with 2 equiv of PMe₃ and H₂O for compounds 60-62 and 3 equiv for compounds 63-64, followed by extensive washing with DCM and DMF. The formation of the amine was monitored by HPLC and ESI-MS of aliquots of peptide cleaved from the resin (see section 3.6.5). The remaining portion of the linker and the TIPP portion of the peptide were then assembled using the same conditions as described above for the general SPPS of the chimeric peptides (section 3.6.2) except that double couplings were generally not required.

3.6.5. Cleavage Conditions

The chimeric amine control 38 and monomeric peptide 60 were cleaved in 90% TFA/8%TIS/2% H₂O (2 mL/100 mg) for 2 h. The resin was then filtered and washed with TFA (2 mL). The eluent was collected and the TFA was evaporated. Aqueous acetic acid (10% v/v, 2 mL) was added and the aqueous solution was extracted with diethyl ether (2 mL). The aqueous solutions were then frozen to -80°C and lyophilized to dryness. Since the only protecting group present on the final sequence of the monomeric
peptides 61-64 was t-Bu and Boc, the TIS scavenger was not required in the cleavage cocktail. These peptides (61-64), monomeric control 50 and the bromoacetamide affinity label 40 were cleaved in 90% TFA/10% H₂O (2 mL/ 100 mg) for 2h, after which the TFA was evaporated followed by addition of water and lyophilization. Although the chimeric peptides 38 and 40 and the monomeric controls 50 and 60 have the Hmb group, TIS is not required in the cleavage cocktail and these peptides can be cleaved in 90% TFA/10% H₂O in future preparations of crude peptide for purification by HPLC. Aliquots of the peptides for HPLC and ESI-MS analysis were cleaved from the resin similarly using approximately 10 mg of resin.

### 3.6.6. Peptide Purification and Analysis

Crude peptides were purified by preparative reversed-phase HPLC (Shimadzu equipped with two LC-6AD pumps and a SDP-10AVP UV detector) on a Vydac C₁₈ column (10 µ, 300 Å, 22x250 mm) using the gradients and solvents indicated in Table 3.3. HPLC fractions were collected manually and immediately frozen and lyophilized. Analysis of the peptides was performed by analytical HPLC (Shimadzu equipped with 2 LC-10ATVP pumps and a SPD-10-AVP UV detector) with a Vydac C₁₈ column (5 µ, 300 Å, 4.6 x 50 mm) in two solvent

### Table 3.3. Preparative RP-HPLC gradients.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Preparative HPLC Gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>10-50 % B over 80 min</td>
</tr>
<tr>
<td>40</td>
<td>10-50 % B over 80 min</td>
</tr>
<tr>
<td>50</td>
<td>5-50 % B over 55 min¹</td>
</tr>
<tr>
<td>60</td>
<td>5-40% B over 70 min</td>
</tr>
<tr>
<td>61</td>
<td>5-50% B over 90 min</td>
</tr>
<tr>
<td>62</td>
<td>5-40% B over 70 min</td>
</tr>
<tr>
<td>63</td>
<td>5-40% B over 70 min</td>
</tr>
<tr>
<td>64</td>
<td>5-40% B over 70 min</td>
</tr>
</tbody>
</table>

Solvent A = Water (0.1% TFA)  
Solvent B = Acetonitrile (0.1% TFA)  
¹. Gradient start = 1 min
systems (see Table 3.1 for details). The molecular weights of the peptides were verified by ESI-MS using a Waters LCT Premier time of flight (TOF) mass spectrometer.

3.6.7. Pharmacological Analysis

The radioligand binding$^{120}$ and WRIB$^{121}$ assays were performed as described in Chapter 2 using CHO cells stably expressing either DOR or MOR and [$^3$H]DPDPE or [$^3$H]DAMGO as the radioligands for DOR and MOR, respectively.

3.7. References


