Solid Phase Synthesis and Evaluation of Tyr-Tic-Phe-Phe(*p*-NHCOCH$_2$Br) ([Phe(*p*-bromoacetamide)^4]TIPP), a Potent Affinity Label for δ Opioid Receptors

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Abstract: Derivatives of the δ opioid receptor selective peptide Tyr-Tic-Phe-Phe-OH (TIPP) containing a *p*-bromoacetamide moiety on the phenyl ring of Phe^3 or Phe^4 were prepared by solid phase synthesis. [Phe(*p*-NHCOCH$_2$Br)^4]TIPP exhibited high affinity for cloned δ receptors (IC_{50} = 5.4 nM), and incubation with only 2.5 nM resulted in 85% wash resistant inhibition of radioligand binding to δ receptors. Therefore, this peptide is a potent affinity label for further study of δ opioid receptors.

Affinity labels are ligands that bind to their target in a nonequilibrium manner, generally by covalent attachment. These irreversible ligands are important pharmacological tools in studying biological effects produced by different receptors. They bind to their targets in a two step process and must first recognize the binding site (reversible binding) before irreversible interaction can occur.[1,2] The reactive group must be suitably located on a potent ligand such that it can react covalently with an appropriate functionality on the receptor. Affinity label ligands fall into two categories, those containing an electrophilic group (e.g., isothiocyanates, haloacetamides, Michael acceptors, and nitrogen mustards) and ones containing a photoaffinity label (i.e., a precursor of a nitrene or carbene).

A variety of affinity labels, mostly nonpeptide ligands, have been prepared for the three opioid receptors (μ, δ, and κ).[3] The isothiocyanate group has been used extensively in nonpeptide affinity labels for opioid receptors, including naltrindole isothiocyanate (NTII),[4] fentanyl isothiocyanate (FIT),[5] and *cis*-(+)-3-methylfentanyl isothiocyanate (SUPERFIT),[6] as affinity labels for δ opioid receptors. Recently, a novel derivative of naltrindole was described as a "reporter affinity label" for δ receptors.[7]

Peptide-based affinity labels for opioid receptors have been mainly photoaffinity labels, such as the azide derivative of DTLET.[2,3,8] However, the use of photoaffinity labels for opioid receptors is limited because these receptors are susceptible to inactivation by UV irradiation.[9] The development of opioid peptides containing electrophilic affinity labels has been limited to a few compounds, namely, for the δ receptor the chloromethyl ketone DALECK (Tyr-D-Ala-Gly-Phe-LeuCH$_2$-Cl),[10,11] the maleimide derivative of DSLET,[12] and enkephalin derivatives containing the nitrogen mustard melphalan.[13,14] DALCE (Tyr-D-Ala-Gly-Phe-Leu-Cys),[15] containing a C-terminal cysteine residue, has been used extensively as an affinity label for δ opioid receptors, while the derivative with cysteine protected as the 3-nitro-2-pyridinesulfenyl (Npys) derivative was shown to irreversibly bind to the μ receptor;[16] these peptides are thought to result in disulfide bond formation with a Cys residue on the receptor and can be reversed by treatment with a thiol reagent such as dithiothreitol. Micromolar concentrations of these peptide ligands were used to demonstrate wash resistant inhibition of binding to δ receptors.

Results from site-directed mutagenesis studies of opioid receptors provide evidence that different ligands may interact differently with δ opioid receptors (see, for example, refs 17−19). Therefore, affinity labels based on different types of compounds (peptide and nonpeptide, agonist and antagonist) are complimentary to one another, and studying the differences and similarities in their binding to opioid receptors could provide valuable insights into receptor−ligand interactions, which could greatly facilitate the design of new opioid ligands.

We are interested in developing potent and selective labeled opioid peptides as pharmacological tools to study opioid receptor−ligand interactions and receptor function, with an emphasis on δ receptors. Because affinity labels must first bind reversibly prior to covalent attachment, the development of potent affinity label peptide derivatives requires high affinity parent peptides. TIPP (Tyr-Tic-Phe-Phe-OH, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) is the prototype of a new class of potent and highly selective δ opioid ligands.[20] TIPP exhibits high δ receptor affinity (K_i = 1.22 nM) and selectivity (K_i^{μ}/K_i^{δ} = 1410) in radioligand binding assays, as well as high antagonist potency against various δ agonists in the mouse vas deferens assay (K_e = 3−5 nM).[20] Also, TIPP does not display any μ or κ antagonist properties in the guinea pig ileum assay at concentrations as high as 10 μM. Recently, however, TIPP was reported to exhibit agonist activity in adenylyl cyclase assays using cells containing both endogenous and transfected δ opioid receptors.[21] The high δ receptor affinity and selectivity exhibited by TIPP makes it an ideal candidate for labeling to assist in the study of δ opioid receptors. We recently reported the synthesis of the isothiocyanate derivative of the δ opioid peptide antagonist *N*,*N*-dibenzyl enkephalin, which exhibited wash resistant inhibition of binding to cloned δ opioid receptors at 100 nM.[22] Similarly, derivatives of TIPP containing the *para*-isothiocyanate on Phe at positions 3 and 4 were also synthesized using solution phase chemistry.[23] Both [Phe(*p*-NCS)^3]- and [Phe(*p*-NCS)^4]TIPP exhibited high affinity for δ receptors (IC_{50}

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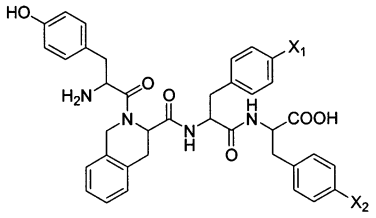
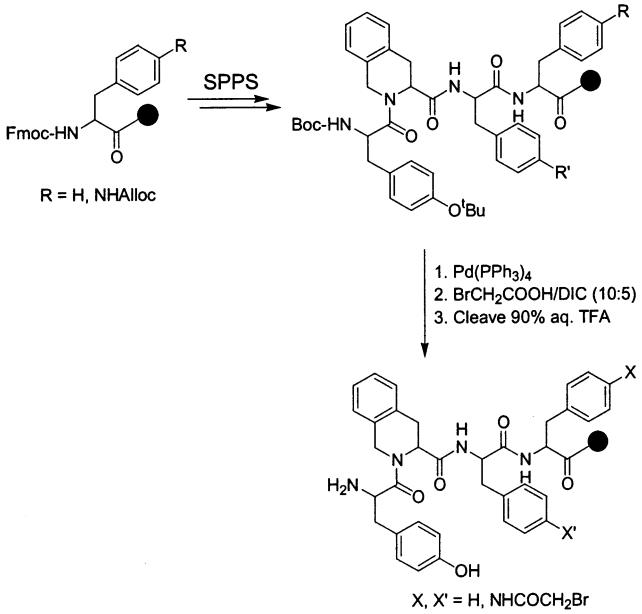


1. X_1 = -NH_2, X_2 = -H
2. X_1 = -$NHCOCH_2Br$, X_2 = -H
3. X1 = H, X_2 = -NH_2
4. X1 = H, X_2 = -$NHCOCH_2Br$
TIPP X_1 = X_2 = -H

Figure 1. TIPP derivatives.

Scheme 1



= 12 and 5 nM, respectively), comparable to the parent peptide TIPP, and inhibited binding in a wash resistant manner by 50−55% at a concentration of 10 nM.[23]

The aim of this project was to synthesize and analyze TIPP derivatives containing a bromoacetamide moiety at the para position of either Phe^3 or Phe^4 (Figure 1). The peptides were prepared using the solid phase synthetic strategy developed earlier in our laboratory[24] (Scheme 1). Even for these small tetrapeptides, this solid phase methodology is much more efficient, both in terms of overall product yield and time, than the labor intensive solution phase methodology used initially for the synthesis of the isothiocyanate derivatives of *N*,*N*-dibenzyl enkephalin[22] and TIPP.[23] To introduce the affinity labels, Fmoc-Phe(*p*-NHAlloc) (Alloc = allyloxycarbonyl) was incorporated into either position 3 or 4 of the peptide. For the Phe-substituted^4 peptide, Fmoc-Phe(*p*-NHAlloc) was synthesized as described earlier[24] and attached to the Wang resin.[25] *N*,*N*-Diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were used for coupling Fmoc-Phe and Fmoc-Tic, and benzotriazole-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP),[26] which is a highly efficient coupling reagent, was used for the final coupling of Boc-Tyr(O*t*Bu) to the hindered secondary amine of Tic^2. Following assembly of the tetrapeptide sequence, the Alloc group was removed using Pd(PPh$_3$)$_4$ in 92.5% CH_2Cl_2/5% AcOH/2.5% *N*-methylmorpholine[24] in the penultimate step, and the bromoacetyl function was introduced by reaction with bromoacetic acid (10 equiv) and DIC (5 equiv) in *N*,*N*-dimethylacetamide (DMA) for 6 h. The peptides were cleaved from the solid support using 90% trifluoroacetic acid (TFA) containing 10% water for 2 h and purified and analyzed by reversed-phase high-performance liquid chromatography (HPLC) as reported earlier.[27,28] The final purity of all peptides by two analytical systems (HPLC and capillary electrophoresis CE) was >98%. Molecular weights of the compounds were verified by fast atom bombardment mass spectrometry (FAB-MS).

Table 1. δ Opioid Receptor Binding Affinities of the TIPP Analogues[a]

	peptide	IC_{50} ± SEM (nM)[b]
1	[Phe(*p*NH_2)^3]TIPP	36.2 ± 1.6
2	[Phe(*p*-$NHCOCH_2Br$)^3]TIPP	65.0 ± 5.7
3	[Phe(*p*-NH_2)^4]TIPP[c]	19.9 ± 2.6
4	[Phe(*p*-$NHCOCH_2Br$)^4]TIPP[c]	5.4 ± 0.6
	TIPP	14.1 ± 0.4

[a] [^{3}H]DPDPE was used as the radioligand. IC_{50} values are the average ± SEM of 3−7 independent determinations. [b] IC_{50} > 10 000 nM at μ receptors (determined using [^{3}H]DAMGO as the radioligand) for all compounds except [Phe(*p*-$NHCOCH_2Br$)^4]TIPP (IC_{50} = 5390 ± 1220 nM). [c] IC_{50} > 10 000 nM at κ receptors (determined using [^{3}H]diprenorphine as the radioligand).

These peptides were tested for inhibition of radioligand binding under both standard and nonequilibrium conditions[22] using Chinese hamster ovary (CHO) cells stably transfected with δ opioid receptors. These assays were used to determine whether a *para*-bromoacetamide was tolerated at the 3- or 4-positions and whether the bromoacetyl derivatives could bind to δ opioid receptors in a nonequilibrium manner; the amine-containing peptides[23] were used as reversible controls for wash resistant inhibition of binding.

The peptides were initially examined under standard binding assay conditions[29] for their affinities for δ, μ, and κ receptors (Table 1). All of the derivatives exhibited nanomolar affinity for δ receptors. Substitution of a bromoacetamide or amine at the para position on the phenyl ring of Phe^4 provided peptides with higher affinity than peptides with these substituents on Phe^3. Both a *para*-bromoacetyl or an isothiocyanate[23] group on Phe^4 are well-tolerated by the δ opioid receptor (IC_{50} = 5−5.4 nM), and a *para* amino group (IC_{50} = 20 nM) also had little effect on δ receptor affinity. Thus, steric factors did not appear to influence the affinities of these analogues. In contrast, substitution of Phe^3 with a *para* amino group decreased δ receptor affinity 2-fold (IC_{50} = 36 nM), with an additional 2-fold loss in affinity when the steric bulk was increased by introducing the bromoacetyl group (IC_{50} = 65 nM); only the *para*-isothiocyanate derivative[23] (IC_{50} = 12 nM) retained affinity comparable to TIPP. These results suggest that steric bulk may play a role in affinity when substitutions are made at the para position of Phe^3.

The compounds all exhibited negligible affinity for μ receptors, and the Phe-substituted^4 peptides also exhibited negligible affinity for κ receptors (Table 1). Thus, these TIPP derivatives retained excellent selectivity for δ opioid receptors.

[Phe(*p*-$NHCOCH_2Br$)^4]TIPP was also evaluated in the adenylyl cyclase assay[30] using cloned δ receptors and compared to TIPP. As previously reported,[21] TIPP was

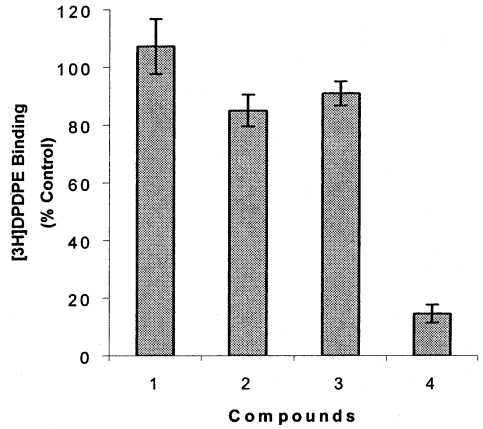


Figure 2. Wash resistant inhibition of binding to cloned δ opioid receptors. Results are percent [^{3}H]DPDPE binding ($\pm$ SEM of at least three independent determinations) following incubation with the peptides and subsequent washing (five times) of the membranes. Peptides **1**−**4** were tested at concentrations close to their IC_{50} values (30, 100, 10, and 2.5 nM, respectively).

a partial agonist in this assay, exhibiting 75 $\pm$ 2% maximum inhibition of cyclic adenosine 3′,5′-monophosphate (cAMP) formation relative to the full agonist DPDPE. [Phe(*p*-$NHCOCH_2Br$)^4]TIPP exhibited somewhat lower efficacy than TIPP (65 $\pm$ 13% of the maximum inhibition of TIPP) but was still a partial agonist in this assay.

The derivatized peptides were then subjected to wash resistant inhibition of [^{3}H]DPDPE binding to δ receptors (Figure 2). The peptides were preincubated with CHO cell membranes for 90 min at room temperature, followed by washing the membranes (five times) by dilution followed by centrifugation.[22] The membranes were then evaluated for [^{3}H]DPDPE binding as compared to untreated control membranes. Because of the high lipophilicity of the peptides being examined, low concentrations (approximately equal to the IC_{50} values of the TIPP derivatives) were used in these experiments. At these concentrations, the amine control compounds **1** and **3**, which cannot bind covalently, were efficiently removed by the washing procedure, resulting in recovery of >80% radioligand binding as compared to the control membranes. The Phe(*p*-bromoacetamide)^3 derivative **2** was also removed by the washing procedure, indicating that this compound does not bind covalently to δ opioid receptors. In contrast, incubation of membranes with the Phe^4 affinity-labeled derivative **4** at the low concentration of 2.5 nM resulted in recovery of only 15% of control specific binding.

In conclusion, substituting Phe^4 with the bromoacetamide group at the para position resulted in a peptide with high affinity (IC_{50} = 5.4 nM) for δ receptors, and wash resistant inhibition binding experiments suggested that this compound interacts irreversibly with these receptors. The demonstration of 85% wash resistant inhibition of binding to δ receptors at a low nanomolar concentration is in sharp contrast to earlier peptide-based affinity labels,[10−13,15] where micromolar concentrations were required to inhibit binding to δ receptors in a wash resistant manner to a similar extent. This extensive wash resistant inhibition of binding by **4** at low nanomolar concentrations is comparable to that observed for the most potent of the nonpeptide affinity labels for the δ opioid receptor.[6] Thus, [Phe(*p*-$NHCOCH_2Br$)^4]TIPP represents a very promising affinity label for δ opioid receptors, which is complementary to our earlier reported isothiocyanate derivatives of TIPP[23] and *N*,*N*-dibenzyl enkephalin.[22] Further studies with these affinity labels are ongoing in our laboratory.

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