Synthesis and Evaluation of Isothiocyanate-Containing Derivatives of the δ-Opioid Receptor Antagonist Tyr-Tic-Phe-Phe (TIPP) as Potential Affinity Labels for δ-Opioid Receptors

Dean Y. Maeda,[†] Fred Berman,[‡] Thomas F. Murray,[‡] and Jane V. Aldrich*[,†]

Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201, and Department of Physiology and Pharmacology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602

Received August 10, 2000

Derivatives of the δ-opioid receptor-selective peptide antagonist H-Tyr-Tic-Phe-Phe-OH (TIPP) containing an isothiocyanate moiety at the para position of either Phe[3] or Phe[4] were prepared as potential affinity labels for δ-opioid receptors. The synthesis was accomplished using a general solution-phase synthetic procedure which allows for introduction of affinity labeling groups late in the synthesis of a variety of small peptide substrates. The target peptides and their corresponding amines were then evaluated in radioligand binding experiments using Chinese hamster ovary (CHO) cells expressing δ- and μ-opioid receptors. The peptides [Phe(*p*-NCS)[3]]TIPP (**2**) and [Phe(*p*-NCS)[4]]TIPP (**4**) showed affinity for δ-receptors comparable to the parent compound TIPP (IC_{50} = 12 and 5 nM, respectively, vs 6 nM for TIPP). Both peptides **2** and **4** were able to inhibit radioligand binding to δ-receptors in a wash-resistant manner at a concentration of 10 nM. Therefore, the peptides [Phe(*p*-NCS)[3]]TIPP (**2**) and [Phe(*p*-NCS)[4]]-TIPP (**4**) represent two affinity labels that may prove useful in the study of δ-opioid receptors.

Introduction

Affinity labels are ligands that commonly interact with receptors via a covalent linkage. Due to their irreversible nature, affinity labels exhibit a number of advantages over traditional reversible ligands in various receptor studies. By forming a covalent linkage with the receptor, an affinity label can irreversibly block a receptor for in vivo studies or, if radiolabeled, facilitate receptor purification and mapping of the binding site. Affinity labels have greatly aided the study and characterization of the μ-, κ-, and δ-opioid receptors.[1] Affinity labels that have been used in the study of δ-opioid receptors include the nonpeptide opiates fentanyl isothiocyanate (FIT),[2] *cis*-(+)-3-methylfentanyl isothiocyanate (SUPERFIT),[3] and naltrindole isothiocyanate (NTII).[4] Peptide-based affinity labels for δ-opioid receptors include [D-Ala[2],Cys[6]]enkephalin (DALCE)[5] and its *S*-3-nitropyridinesulfenyl (Npys) protected derivative [D-Ala[2],-Cys(Npys)[6]]leucine enkephalin,[6] [D-Ala[2],D-Leu[5]]enkephalin chloromethyl ketone (DALECK),[7] and, most recently, Tyr-D-Ser-Gly-Phe-Leu-Thr-NH-Gly-maleoyl.[8]

In the study of δ-opioid receptors, irreversible ligands have been used to demonstrate δ-receptor-mediated analgesia,[9] and the δ-receptors from NG108-15 cells labeled with SUPERFIT were purified to apparent homogeneity.[10] Most recently, SUPERFIT has been used in binding assays with chimeric receptors to identify domains of the δ-opioid receptor which are important for the high-affinity binding of this ligand.[11] The binding pockets of opioid receptors are usually made up of noncontiguous amino acid residues, and a variety of affinity labels that interact with different receptor-based nucleophiles could help depict a clearer picture of the δ-opioid receptor binding site.

For the development of an ideal affinity label, the parent compound should exhibit high affinity for the receptor to be studied. We previously prepared an isothiocyanate-containing analogue of *N*,*N*-dibenzylleucine enkephalin which exhibits dose-dependent wash-resistant inhibition of radioligand binding at concentrations of 0.1−1 μM.[12] In an ongoing effort to develop effective affinity labels for δ-opioid receptors, the potent and selective tetrapeptide antagonist Tyr-Tic-Phe-Phe (TIPP, Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid)[13,14] was chosen as the parent peptide for this study due to its higher affinity and selectivity for δ-opioid receptors compared to *N*,*N*-dibenzylleucine enkephalin. TIPP is comprised entirely of aromatic residues, and its development helped illustrate the differences and similarities in μ- and δ-receptor binding requirements.[13,14] The higher δ-opioid receptor affinity and selectivity of TIPP could lead to more potent affinity labels upon isothiocyanate derivatization.

The goal of this project was to prepare TIPP derivatives **2** and **4** containing an isothiocyanate moiety at the para position of either Phe[3] or Phe[4], respectively (Figure 1), utilizing a general synthetic methodology which we developed[12] for the preparation of peptide-based affinity labels. The amine-containing peptides **1** and **3** were also synthesized as reversible controls for the evaluation of wash-resistant inhibition of binding. These peptides were then tested for inhibition of radioligand binding under both equilibrium and nonequilibrium conditions using Chinese hamster ovary (CHO) cells stably transfected with δ- and μ-opioid receptors. These assays were used to determine whether an isothiocyanate is well-tolerated at the para positions of the phenyl ring in the 3- and 4-positions and whether the isothiocyanate

* To whom correspondence should be addressed. Tel: 410-706-6863. Fax: 410-706-0346. E-mail: jaldrich@umaryland.edu.
[†] University of Maryland.
[‡] University of Georgia.

10.1021/jm000345s CCC: $19.00
Published on Web 11/28/2000

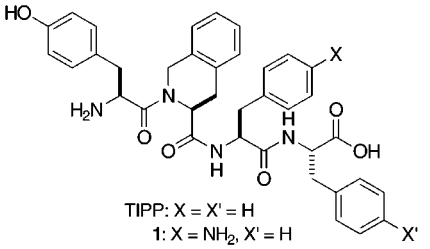
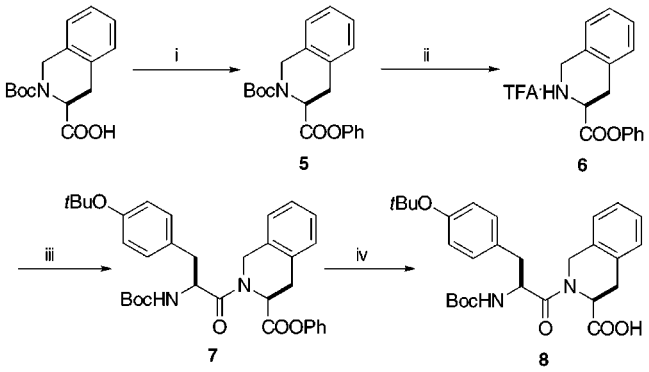


Figure 1. TIPP and related target peptides.

Scheme 1[a]



[a] (i) DCC, phenol, CH_2Cl_2 (86%); (ii) 40% TFA/CH_2Cl_2 (69%); (iii) Boc-Tyr(O*t*Bu)-OH, PyBOP, DIPEA, CH_2Cl_2 (90%); (iv) H_2O_2, 1 M NaOH, dioxane (72%).

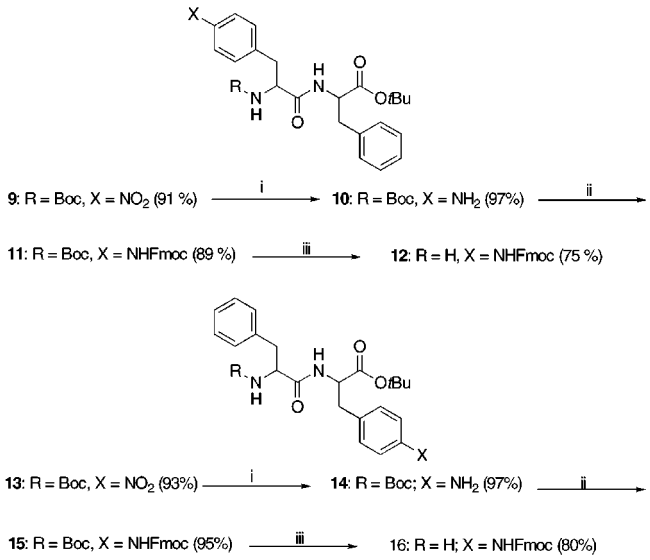
derivatives bind to δ-opioid receptors in a nonequilibrium manner.

Synthesis

A [2+2] convergent synthesis was utilized in which the C-terminal dipeptides containing an Fmoc-protected anilide amine at either Phe[3] or Phe[4] were coupled to the common Boc-protected N-terminal sequence of Tyr-Tic. The protected N-terminal dipeptide was initially synthesized as the methyl ester, but saponification of the methyl ester required long reaction times and resulted in low yields of the desired peptide. The possibility for racemization of the C-terminal residue under such prolonged basic conditions prompted us to utilize the phenyl ester as the carboxyl-protecting group (Scheme 1) since it requires mild deprotection conditions.[15,16] The synthesis of the N-protected Tyr-Tic fragment began with the esterification of Boc-Tic with dicyclohexylcarbodiimide (DCC) and phenol to provide the fully protected amino acid **5**. The Boc group was then removed using 30% trifluoroacetic acid (TFA) in CH_2Cl_2 to provide the carboxyl-protected Tic derivative **6** in 69% yield.

Since the α-amine of Tic is a secondary amine, difficulties in coupling the Tyr residue to Tic due to increased steric hindrance were expected, and therefore the coupling reagent (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)[17] was used for this reaction. PyBOP has been shown to be very effective in the coupling of *N*-methyl and other sterically hindered amino acids.[17] The protected phenyl ester **6** was coupled to Boc-Tyr(O*t*Bu)-OH using PyBOP to give dipeptide **7** in 90% yield, and the phenyl ester was saponified with excess hydrogen peroxide and 1 equiv of NaOH in dioxane/H_2O[15,16] to give the desired N-terminal dipeptide **8** in 72% yield.

Scheme 2[a]



[a] (i) NH_4HCO_2, 10% Pd/C, MeOH; (ii) Fmoc-Cl, 10% Na_2CO_3, dioxane; (iii) TMS-OTf, toluene, CH_2Cl_2.

The synthesis of both C-terminal dipeptides followed the same procedure (Scheme 2). For the synthesis of the Phe[3]-substituted derivative H-Phe(*p*-NHFmoc)-Phe-O*t*Bu (**12**), Boc-Phe(*p*-NO_2)-OH was first coupled to H-Phe-O*t*Bu using isobutyl chloroformate, which provided dipeptide **9** in 91% yield. The coupling was followed by reduction of the nitro group by catalytic transfer hydrogenation[18] and the resulting amine **10** then protected using 9-fluorenylmethyl chloroformate (Fmoc-Cl) to give the fully protected dipeptide **11**. Trimethylsilyl trifluoromethanesulfonate (TMS-OTf; 1 equiv)[19] was then used to selectively remove the Boc group in the presence of a *tert*-butyl ester. This method was used in the previous syntheses of enkephalin analogues containing affinity labeling groups[12] and provided the desired C-terminal dipeptide fragment **12** in 75% yield. The synthesis of the Phe[4]-substituted dipeptide **16** followed the same procedures (Scheme 2).

The common N-terminal dipeptide fragment **8** was then coupled to the Phe[3]- and Phe[4]-substituted C-terminal dipeptides **12** and **16** using PyBOP (Scheme 3). The Fmoc group was removed by treatment of the peptides with piperidine to yield the amine precursors **18** and **21**. Thiophosgene treatment provided the corresponding isothiocyanate derivatives **19** and **22**. Peptides **18**, **19**, **21**, and **22** were then deprotected using TFA containing anisole as a scavenger to give the target peptides **1**−**4**, respectively.

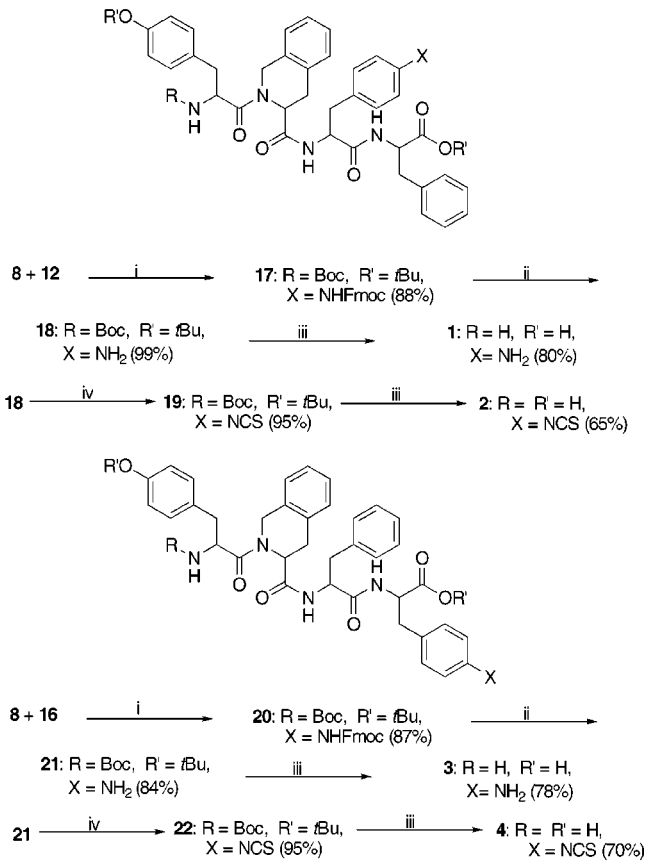
Pharmacological Evaluation

These final peptides were subjected to radioligand binding assays under standard conditions using cloned δ- and μ-receptors expressed in CHO cells and [^{3}H]Tyr-*cyclo*[D-Pen-Gly-Phe-D-Pen] (DPDPE) and [^{3}H]Tyr-D-Ala-Gly-MePhe-Leu-Gly-ol (DAMGO), respectively, as the radioligands. All of the derivatives exhibited nanomolar affinity and excellent selectivity for δ-receptors (Table 1), which was expected from the pharmacological profile of the parent peptide TIPP. Peptides **2**−**4** exhib-

Table 1. Binding Affinities of Peptides **1**−**4** under Standard Assay Conditions

compd	$IC_{50} \pm SEM$ (nM)	
	δ	μ
TIPP	5.8 ± 1.2	22700 ± 1500
[Phe(*p*-NH_2)^3]TIPP, **1**	87.7 ± 15.3	>10000
[Phe(*p*-NCS)^3]TIPP, **2**	12.4 ± 2.4	>10000
[Phe(*p*-NH_2)^4]TIPP, **3**	11.8 ± 2.5	>10000
[Phe(*p*-NCS)^4]TIPP, **4**	5.4 ± 1.3	>10000

Scheme 3[a]



[a] (i) PyBOP, DMF; (ii) piperidine, DMF; (iii) TFA/anisole; (iv) thiophosgene, CH_2Cl_2.

ited comparable δ-opioid receptor affinity (IC_{50} = 5−12 nM) to TIPP (IC_{50} = 6 nM), and the μ-receptor affinity was low (IC_{50} > 10 μM) in all cases. In general, substitutions at the para position of Phe^3 and Phe^4 of TIPP were well-tolerated by δ-opioid receptors. An exception to this was [Phe(*p*-NH_2)^3]TIPP (**1**), where amine substitution at Phe^3 resulted in a 10-fold decrease in δ-opioid receptor affinity.

The derivatized peptides were then examined for wash-resistant inhibition of [^{3}H]DPDPE binding to δ-receptors (Figure 2). The experiment involved incubation of the test compounds with CHO cell membranes for 90 min at room temperature, followed by washing the membranes five times via centrifugation, decanting, and resuspension in fresh buffer. Due to the highly lipophilic nature of these peptides, which can make it difficult to wash out noncovalently bound compound, low concentrations approximating respective IC_{50} values of the TIPP derivatives were examined (60 nM for peptide **1** and 10 nM for peptides **2**−**4**). At these concentrations, the reversible amine control compounds **1** and **3** (which

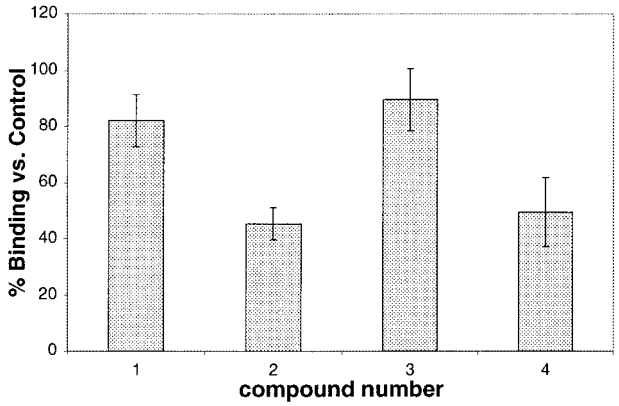


Figure 2. Wash-resistant inhibition of binding. Results are percent [^{3}H]DPDPE binding (±SEM) following incubation with peptides **1**−**4** and subsequent washing. Peptide **1** was tested at a concentration of 60 nM; peptides **2**−**4** were tested at a concentration of 10 nM.

cannot bind covalently to the receptors) were efficiently removed by the washing procedure, resulting in recovery of >80% radioligand binding compared to control membranes. In membranes treated with peptides **2** and **4**, radioligand binding was only 45% and 50% (respectively) of untreated membrane control binding values. These data indicate that peptides **2** and **4** may be interacting with δ-opioid receptors in a nonequilibrium fashion.

Discussion

In our continuing efforts to synthesize affinity labels for the δ-opioid receptor based on peptide antagonists, we again used the Boc/Fmoc orthogonal protection strategy described previously,[12] highlighting its overall versatility for the introduction of affinity labeling moieties into a variety of peptide substrates. A key step in the synthesis was the selective removal of a Boc protecting group in the presence of a *tert*-butyl ester, which was accomplished using 1 equiv of TMS-OTf in toluene. Difficulty in saponifying a methyl ester during the synthesis of the common N-terminal dipeptide fragment prompted us to use the phenyl ester, which requires mild deprotection conditions, for carboxylic acid protection.

In radioligand binding assays at the δ-receptor, isothiocyanate substitution at the para position on either Phe^3 or Phe^4 was well-tolerated, and both peptide derivatives **2**−**4** exhibited wash-resistant inhibiton of radioligand binding. Peptides **2** and **4** were tested at concentrations close to that of their IC_{50} values (10 nM), and the observed wash-resistant inhibition of radioligand binding of close to 50% suggests a very efficient labeling process occurring with the two ligands. The previously synthesized *N*,*N*-dibenzyl[Phe(*p*-NCS)^4]leucine enkephalin affinity label inhibited radioligand binding in a wash-resistant fashion to approximately 60% of control values at a concentration of 100 nM.[12] The labeling process is thought to occur in two discrete steps: first, reversible binding to the receptor occurs, followed by covalent linkage with the receptor. The higher δ-opioid receptor affinity of these TIPP derivatives (IC_{50} = 12 and 5 nM vs 35 nM for *N*,*N*-dibenzyl-[Phe(*p*-NCS)^4]leucine enkephalin) may enhance the first

step in the labeling process and thereby facilitate covalent reaction in the second step, resulting in potent affinity labels for δ-opioid receptors.

In conclusion, the isothiocyanate group was shown to be effective in conferring wash-resistant inhibition of radioligand binding to δ-selective peptide antagonists, and [Phe(*p*-NCS)^3]TIPP (**2**) and [Phe(*p*-NCS)^4]TIPP (**4**) are affinity labels which may be useful pharmacological tools in the study of δ-opioid receptors. The complementary nature of the TIPP derivatives [Phe(*p*-NCS)^3]TIPP (**2**) and [Phe(*p*-NCS)^4]TIPP (**4**) and the previously described *N*,*N*-dibenzyl[Phe(*p*-NCS)^4]leucine enkephalin[12] may provide additional information as to the important residues necessary for ligand binding to δ-opioid receptors. This information, coupled with current computational models of δ-opioid receptors,[20−22] could help in the development of therapeutically important δ-opioid receptor ligands. Further studies with these promising affinity labels for δ-receptors are ongoing in our laboratory.

Experimental Section

PyBOP was purchased from Advanced Chemtech (Louisville, KY). All other materials for synthesis and purification and instrumentation used for NMR and fast atom bombardment mass spectrometry (FAB-MS) analyses are the same as previously described.[12]

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

Phenyl *tert*-Butyloxycarbonyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (5). *tert*-Butyloxycarbonyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (2.6 g, 9.4 mmol) and phenol (0.88 g, 9.4 mmol) were dissolved in EtOAc (20 mL) and cooled to −15 °C in a dry ice/MeOH bath. DCC (2.13 g, 10.3 mmol) suspended in EtOAc (5 mL) was added to the chilled solution, and the reaction mixture was allowed to warm to room temperature and stirred overnight. Following the addition of a few drops of glacial acetic acid, the dicyclohexylurea reaction byproduct was removed by filtration, and the filtrate was washed with 5% $NaHCO_3$, H_2O, 0.1 N HCl, H_2O, and brine. The organic layer was then concentrated by rotary evaporation and filtered through a plug of silica gel with 30% hexane/EtOAc as the eluent. The filtrate was then collected and evaporated to give 2.86 g (86%) of **5** which crystallized spontaneously during the evaporation process: mp 75−78 °C; R_f (EtOAc) = 0.65; HPLC t_R = 25.7 min (96% purity); ^{1}H NMR ($CDCl_3$) δ 1.51 (s, 9H), 1.55 (s, 9H), 3.31 (d, 2H, J = 5.3 Hz), 3.33 (dd, 2H, J = 5, 10 Hz), 4.63 (overlapping dd, 4H), 5.02 (t, 1H, J = 5.2 Hz), 5.35 (q, 1H, J = 5 Hz), 6.82 (m, 5H), 7.25 (m, 4H); FAB-MS $[M + H]^+$ 354 (calcd 354).

Phenyl L-1,2,3,4-Tetrahydroisoquinoline-3-carboxylate (6). Amino acid derivative **5** (1.00 g, 2.8 mmol) was dissolved in CH_2Cl_2 (5 mL), and TFA (3 mL) was added dropwise. Vigorous bubbling occurred, which ceased after approximately 10 min. After a total reaction time of 0.5 h, the CH_2Cl_2 and TFA were removed by rotary evaporation followed by repeated dilution (5 × 5 mL aliquots) with ethyl ether. During the final dilution, white crystals spontaneously began to form. These crystals were filtered and washed with additional ethyl ether to yield 0.72 g (69%) of **6** as the TFA salt: mp 106 °C dec; R_f (EtOAc) = 0.44; HPLC t_R = 20.8 min (93% purity); $[\alpha]_D$ = −56.4° (c = 1.32, DMSO); ^{1}H NMR ($CDCl_3$) δ 2.68 (broad s, 1H), 3.27 (dd, 2H, J = 5, 10 Hz), 4.08 (dd, 1 H, J = 4.9, 10 Hz), 4.24 (d, 2H, J = 4.6 Hz), 7.17 (m, 5H), 7.30 (m, 2H), 7.46 (m, 2H); FAB-MS $[M + H]^+$ 254 (calcd 254).

Phenyl *tert*-Butyloxycarbonyl-L-*O*-*tert*-butyltyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylate (7). *tert*-Butyloxycarbonyl-L-*O*-*tert*-butyltyrosine (0.33 g, 1.0 mmol), amino acid **6** (0.30 g, 1.0 mmol) and PyBOP (0.42 g, 1.0 mmol) were dissolved in CH_2Cl_2 (5 mL) at room temperature. After a few minutes, *N*,*N*-diisopropylethylamine (DIPEA; 420 μL, 3 mmol) was added dropwise. After reacting overnight, the mixture was diluted with EtOAc (15 mL) and washed with H_2O, 5% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and brine. The organic layer was dried over $MgSO_4$, concentrated by rotary evaporation and passed through a silica gel plug with EtOAc as the eluent. The filtrate was evaporated to give 0.42 g (90%) of **7** as a whitish foam: mp 110−113 °C; R_f (EtOAc) = 0.69; HPLC t_R = 47.5 min (98% purity); $[\alpha]_D$ = −5.2° (c = 1.24, toluene); FAB-MS $[M + H]^+$ 573 (calcd 573). Anal. ($C_{34}H_{40}N_2O_6$) C, H, N.

***tert*-Butyloxycarbonyl-L-*O*-*tert*-butyltyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carboxylic Acid (8).** Protected dipeptide fragment **7** (0.40 g, 0.69 mmol) was dissolved in dioxane (5 mL) and 30% H_2O_2 (785 μL) was added, followed by 1 N NaOH (700 μL). The reaction was allowed to proceed overnight, the mixture then chilled in an ice bath, and neutralized using 1 N HCl. The solution was concentrated by rotary evaporation, and the resulting residue was dissolved in EtOAc. The organic solution was washed with saturated Na_2SO_3, brine, and dried over $MgSO_4$. Evaporation of EtOAc yielded 0.25 g (72%) of **8** as a clear glass: R_f (EtOAc) = 0.21−0.40; HPLC t_R = 38.0 min (73% purity); $[\alpha]_D$ = −53.8° (c = 1.19, MeCN); FAB-MS (negative mode) $[M - H]^-$ 495 (calcd 495).

***tert*-Butyl *tert*-Butyloxycarbonyl-L-4-nitrophenylalanyl-L-phenylalaninate (9).** Under an N_2 atmosphere, a solution of *tert*-butyloxycarbonyl-L-4-nitrophenylalanine (1.40 g, 4.50 mmol) in dry THF (20 mL) was cooled to −15 °C (dry ice/MeOH) and neutralized with *N*-methylmorpholine (NMM; 0.99 mL, 4.5 mmol). Isobutyl chloroformate (0.44 mL, 4.5 mmol) was then added, followed 1.5 min later by a solution of L-phenylalanine *tert*-butyl ester hydrochloride (1.08 g, 4.20 mmol) and NMM (0.99 mL, 4.5 mmol) in DMF (5 mL). The reaction mixture was kept at −15 °C for 30 min, and then allowed to warm to room temperature. After completion of the reaction, the THF was evaporated, and the residue was suspended in EtOAc. The organic layer was washed with H_2O, 5% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and finally with saturated NaCl. The organic layer was then dried over $MgSO_4$ and the EtOAc evaporated to yield the crude peptide as a white solid (1.95 g, 91%). This was recrystallized from hot MeOH to give 1.64 g (76%) of **9** as a white solid: mp 125−127 °C; R_f (EtOAc) = 0.59; R_f (EtOAc/hexane, 4:1) = 0.51; R_f [chloroform/methanol/acetic acid (CMA), 85:10:5] = 0.80; HPLC t_R = 26.2 min (91% purity); $[\alpha]_D$ = −1.6° (c = 1.23, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.36 (s, 9H), 1.39 (s, 9H), 3.02 (d, 2H, J = 6.0 Hz), 3.08 (signal obscured by doublet at 3.02, 1H), 3.16 (dd, 1H, J = 6.8, Hz), 4.35 (m, 1H), 4.62 (q, 1H, J = 6.1 Hz), 4.95 (broad s, 1H), 6.18 (broad d, 1H), 7.07 (m, 2H), 7.21 (m, 3H), 7.32 (d, 2H, J = 8.6 Hz), 8.09 (d, 2H, J = 8.7 Hz); FAB-MS $[M + H]^+$ 514.3 (calcd 514.3).

***tert*-Butyl *tert*-Butyloxycarbonyl-L-4-aminophenylalanyl-L-phenylalaninate (10).** Dipeptide fragment **9** (1.02 g, 2.00 mmol) and ammonium formate (1.13 g, 18.0 mmol) were dissolved in MeOH (15 mL), and 10% Pd/C (0.10 g) was then added as a suspension in MeOH/H_2O (1:1, 1 mL). The reaction mixture was stirred for 0.5 h, filtered through Celite and the MeOH was evaporated. The residue was dissolved in EtOAc (10 mL) and the organic solution was washed with H_2O and brine, dried over $MgSO_4$ and evaporated to give **10** as a white solid (0.93 g, 97%): R_f (EtOAc) = 0.42; HPLC t_R = 14.5 min (94% purity); $[\alpha]_D$ = −4.1° (c = 1.28, MeOH); FAB-MS $[M + H]^+$ 484.1 (calcd 484.3).

***tert*-Butyl *tert*-Butyloxycarbonyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalaninate**

(11). Fmoc-Cl (0.25 g, 1.0 mmol) in dioxane (1 mL) was added to a solution of dipeptide **10** (0.48 g, 1.0 mmol) in dioxane (7 mL), followed by dropwise addition of a 10% Na_2CO_3 solution to maintain a pH of 8. A white precipitate formed upon addition of Na_2CO_3. Once the pH of the suspension stabilized, the reaction mixture was allowed to proceed overnight. The white precipitate was then filtered, washed with cold 10% citric acid, H_2O, and dried to give **11** as a white solid (0.56 g, 89%): mp 173−174 °C; R_f (EtOAc) = 0.70; R_f (EtOAc/hexane, 2:3) = 0.29; $[\alpha]_D$ = 9.9° (*c* = 0.45, DMSO); ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 9H), 1.39 (s, 9H), 3.00 (m, 4H), 4.25 (t, 1H, *J* = 6.6 Hz), 4.29 (signal obscured by triplet at 4.25, 1H), 4.51 (d, 2H, *J* = 6.7 Hz), 4.62 (q, 1H), 5.11 (broad s, 1H), 6.32 (broad d, 1H), 6.67 (s, 1H), 7.04 (m, 4H), 7.21 (m, 2H), 7.29 (t, 4H, *J* = 8.0 Hz), 7.39 (t, 2H, *J* = 7.3 Hz,), 7.59 (d, 2H, *J* = 7.4 Hz), 7.74 (d, 1H, *J* = 8.1 Hz), 7.75 (d, 2H, *J* = 7.4 Hz); FAB-MS $[M + H]^+$ 706 (calcd 706).

***tert*-Butyl L-4-Amino(9-fluorenylmethoxycarbonyl)-phenylalanyl-L-phenylalaninate (12).** Dipeptide fragment **11** (66 mg, 0.09 mmol) was dissolved in CH_2Cl_2 (0.5 mL) and TMS-OTf (0.967 M solution in toluene, 100 μL, 0.09 mmol) was added dropwise via syringe. After 3 h, the reaction mixture was diluted with EtOAc (10 mL) and the organic layer was washed with 5% $NaHCO_3$, H_2O, and saturated NaCl. The organic layer was dried over $MgSO_4$ and concentrated under vacuum. The residue was then applied to a silica gel flash column and eluted with EtOAc to give 45 mg (75%) of **12** as a whitish foam: ^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9H), 2.54 (dd, 1H, *J* = 4.5, 13.8 Hz), 3.04 (d, 2H, *J* = 6.3 Hz), 3.09 (partially obscured dd, 1H, *J* = 3.7 Hz), 3.53 (dd, 1H, *J* = 3.9, 9.3 Hz), 4.25 (t, 1H, *J* = 6.7 Hz), 4.51 (d, 2H, *J* = 6.6 Hz), 4.74 (q, 1H, *J* = 6.3 Hz), 6.84 (broad s, 1H), 7.08 (m, 4H), 7.21 (m, 2H), 7.29 (t, 4H, *J* = 8.0 Hz), 7.39 (t, 2H, *J* = 7.3 Hz), 7.59 (d, 2H, *J* = 7.4 Hz), 7.74 (d, 1H, *J* = 8.1 Hz), 7.75 (d, 2H, *J* = 7.4 Hz); FAB-MS $[M - t\text{Bu} + H]^+$ 550.6 (calcd 550.2).

***tert*-Butyl *tert*-Butyloxycarbonyl-L-phenylalanyl-L-4-nitrophenylalaninate (13).** *tert*-Butyloxycarbonyl-L-phenylalanine (1.19 g, 4.50 mmol) was coupled to L-4-nitrophenylalanine *tert*-butyl ester (1.16 g, 4.50 mmol) as described for the preparation of **9** to yield 2.15 g (93%) as a white solid. The crude product was then recrystallized from hot MeOH to give 1.69 (73%) of **13** as a white solid: mp 147−149 °C; R_f (EtOAc) = 0.64; HPLC t_R = 25.7 min (97% purity); $[\alpha]_D$ = −15.3° (*c* = 1.16, MeOH); ^{1}H NMR ($CDCl_3$) δ 1.34 (s, 9H), 1.39 (s, 9H), 3.01 (dd, 2H, *J* = 3.3, 6.8 Hz), 3.12 (t, 2H, *J* = 6.2 Hz), 4.29 (q, 1H, *J* = 6.3 Hz), 4.62 (q, 1H, *J* = 6.5 Hz), 4.88 (broad s, 1H), 6.37 (broad d, 1H), 7.23 (m, 7H), 8.07 (d, 2H, *J* = 8.7 Hz); FAB-MS $[M + H]^+$ = 514.3 (calcd 514.3).

***tert*-Butyl *tert*-Butyloxycarbonyl-L-phenylalanyl-L-4-aminophenylalaninate (14).** Dipeptide **13** (1.02 g, 2.00 mmol) was reduced by catalytic transfer hydrogenation as described for the preparation of **10** to yield 0.94 g (97%) of **14** as a white solid: mp 166−167 °C; R_f (EtOAc) = 0.48; HPLC t_R = 13.5 min (98% purity); $[\alpha]_D$ = −4.8° (*c* = 1.31, MeOH); FAB-MS $[M + H]^+$ 484.1 (calcd 484.3).

***tert*-Butyl *tert*-Butyloxycarbonyl-L-phenylalanyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalaninate (15).** Dipeptide **14** (0.48 g, 1.0 mmol) was protected as the Fmoc carbamate and purified as described for the preparation of **11** to yield 0.74 g (95%) of **15** as a white solid: mp 113−115 °C; R_f (EtOAc) = 0.28; HPLC t_R = 26.2 min (87% purity); $[\alpha]_D$ = −5.3° (*c* = 1.19, MeOH); FAB-MS $[M + H]^+$ 706.3 (calcd 706.3).

***tert*-Butyl L-Phenylalanyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalaninate (16).** Fully protected dipeptide fragment **15** (96 mg, 0.14 mmol) was selectively deprotected using TMS-OTf as described for the preparation of **12** to yield 70 mg (80%) of **16** as a foam: R_f (EtOAc/hexane, 4:1) = 0.10−0.21; FAB-MS $[M + H]^+$ 606 (calcd 606).

***tert*-Butyl *tert*-Butyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-phenylalaninate (17).** The N-terminal dipeptide fragment **8** (61 mg, 0.12 mmol), C-terminal dipeptide fragment **12** (93 mg, 0.12 mmol), and PyBOP (64 mg, 0.12 mmol) were dissolved in DMF (1.0 mL) at room temperature. After a few minutes, DIPEA (64 μL, 0.36 mmol) was added dropwise to the reaction mixture, and the reaction was allowed to proceed overnight. The DMF was removed in vacuo, and the residue dissolved in EtOAc (10 mL). The organic layer was washed with 5% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and brine, and dried over $MgSO_4$. The organic layer was concentrated, and the resulting oil was applied to a silica gel flash column with CH_2Cl_2/hexane (4:1) as the eluent to give 117 mg (88%) of **17** as a whitish foam: R_f (EtOAc) = 0.67; HPLC t_R = 39.7 min (94% purity); FAB-MS $[M + Na]^+$ 1106 (calcd 1106). Anal. ($C_{65}H_{73}N_5O_{10}\cdot 0.5H_2O$) C, H, N.

***tert*-Butyl *tert*-Butyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-4-aminophenylalanyl-L-phenylalaninate (18).** Tetrapeptide **17** (70 mg, 0.063 mmol) was dissolved in DMF (1.0 mL) and piperidine (60 μL) was then added. The reaction was allowed to proceed at room temperature for 1.5 h, and then the reaction mixture was concentrated under vacuum. The residue was purified by silica gel flash chromatography with EtOAc/hexane (3:1) as the eluent to give **18** (54 mg, 99%) as a foam: R_f (EtOAc/hexane, 3:1) = 0.47; HPLC t_R = 25.3 min (91% purity); FAB-MS $[M + H]^+$ 862 (calcd 862).

***tert*-Butyl *tert*-Butyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-4-isothiocyanatophenylalanyl-L-phenylalaninate (19).** Tetrapeptide **18** (14 mg, 0.016 mmol) was dissolved in CH_2Cl_2 (1.0 mL) at room temperature. Thiophosgene (1.9 μL, 0.024 mmol) was added to this solution, followed by the slow addition of DIPEA (7.0 μL, 0.04 mmol). The reaction was allowed to proceed for 1.5 h, and then the reaction mixture was concentrated under vacuum. The product was purified by silica gel flash chromatography with EtOAc/hexane (4:1) as the eluent to give **19** (13.9 mg, 95%) as a clear glass: HPLC t_R = 39.0 min (89% purity); FAB-MS $[M + H]^+$ 904.2 (calcd 904.2).

L-Tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-4-aminophenylalanyl-L-phenylalanine (1). Tetrapeptide **18** (14 mg, 0.016 mmol) was dissolved in 10% anisole/TFA (5 mL) for 1 h at room temperature. The TFA was then evaporated and the product precipitated with cold ether (10 mL). The precipitate was washed five times with cold ether and then dried in vacuo to yield **1** (11 mg, 80%) as a white solid: HPLC (0−75% B over 50 min) t_R = 25.5 min (96% purity); FAB-MS $[M + H]^+$ 650 (calcd 650).

L-Tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-4-isothiocyanatophenylalanyl-L-phenylalanine (2). Tetrapeptide **19** (11 mg, 0.010 mmol) was deprotected as described for the preparation of **1** to yield peptide **2** (7 mg, 65%) as a white solid: HPLC (0−75% B over 50 min) t_R = 32.5 min (96% purity); FAB-MS $[M + H]^+$ 692 (calcd 692).

***tert*-Butyl *tert*-Butyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-phenylalanyl-L-4-amino(9-fluorenylmethoxycarbonyl)phenylalaninate (20).** The N-terminal dipeptide fragment **8** (130 mg, 0.26 mmol) was coupled to C-terminal dipeptide fragment **16** (140 mg, 0.19 mmol) using PyBOP (136 mg, 0.26 mmol) as described for the preparation of **17** to give **20** (174 mg, 87%) as a whitish foam: HPLC t_R = 39.9 min (95% purity); FAB-MS $[M + H]^+$ 1085 (calcd 1085). Anal. ($C_{65}H_{73}N_5O_{10}\cdot H_2O$) C, H, N.

***tert*-Butyl *tert*-Butyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-phenylalanyl-L-4-aminophenylalaninate (21).** Tetrapeptide **20** (100 mg, 0.09 mmol) was treated with piperidine (200 μL) and purified as described for the preparation of **18** to yield **21** (67 mg, 84%) as a foam: HPLC t_R = 24.3 min (85% purity); FAB-MS $[M + H]^+$ 862 (calcd 862).

***tert*-Butyl *tert*-Butyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-phenylalanyl-L-4-isothiocyanatophenylalaninate (22).** Tetrapeptide **21** (14 mg, 0.016 mmol) was treated with thiophosgene (1.9 μL, 0.024 mmol) and purified as described in the preparation

of **19** to yield **22** (13.9 mg, 95%) as a clear glass: HPLC t_R = 38.8 min (97% purity); FAB-MS $[M + H]^+$ 905 (calcd 905).

L-Tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-phenylalanyl-L-4-aminophenylalanine (3). Tetrapeptide **21** (17 mg, 0.02 mmol) was deprotected as described in the preparation of **1** to yield peptide **3** (13 mg, 78%) as a white solid: HPLC (0−75% B over 50 min) t_R = 29.3 min (94% purity); FAB-MS $[M + H]^+$ 650 (calcd 650).

L-Tyrosyl-L-1,2,3,4-tetrahydroisoquinoline-3-carbonyl-L-phenylalanyl-L-4-isothiocyanatophenylalanine (4). Tetrapeptide **22** (10 mg, 0.011 mmol) was deprotected as described for preparation of **1** to yield peptide **4** (6 mg, 70%) as a white solid: HPLC (0−75% B over 50 min) t_R = 32.3 min (97% purity); FAB-MS $[M + H]^+$ 692 (calcd 692).

Binding Assays. Radioligand binding assays of the potential affinity label derivatives were performed with [^{3}H]DPDPE (for δ) and [^{3}H]DAMGO (for μ) using CHO cells stably transfected with either mouse δ- or rat μ-opioid receptors as previously described.[12,23] The concentrations of [^{3}H]DPDPE and [^{3}H]DAMGO ranged from 0.5 to 0.8 nM in all binding assays. These radioligand concentrations approximate the K_D values of DPDPE and DAMGO for these receptors (0.5 and 0.64 nM, respectively). IC_{50} values were then derived from nonlinear regression analysis of competition curves using nine concentrations of the peptides; results are reported as ±SEM of 3−4 experiments.

Wash-Resistant Binding Assays. Potential affinity label derivatives for the δ-opioid receptor were examined for wash-resistant inhibition of binding to opioid receptors. CHO cell membranes expressing δ-receptors were incubated in the absence or presence of the TIPP derivatives for 90 min at room temperature. The membranes were washed by centrifugation and resuspension[12] and then subjected to radioligand binding assays as described above. The radioligand binding to membranes treated with the TIPP analogues were then expressed as percent binding to untreated control membranes (±SEM). All compounds were evaluated for wash-resistant inhibition of radioligand binding using 4 experiments, except for compound **1**, which was evaluated using 3 experiments.

Acknowledgment. The authors thank Elizabeth Olenchek and Jennif Chandler for their assistance in the pharmacological evaluation of these peptides and Brian Arbogast for obtaining the mass spectral data. This research was supported by the National Institute of Drug Abuse (Grant DA10035). Additional financial assistance from the American Foundation for Pharmaceutical Education (AFPE) in the form of a predoctoral fellowship (D.Y.M.) was also greatly appreciated.

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JM000345S