Synthesis and Evaluation of *N,N*-Dialkyl Enkephalin-Based Affinity Labels for δ Opioid Receptors

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To develop affinity labels for δ opioid receptors based on peptide antagonists, the Phe[4] residues of *N,N*-dibenzylleucine enkephalin and *N,N*-diallyl[Aib[2],Aib[3]]leucine enkephalin (ICI-174,864) were substituted with either Phe(*p*-NCS) or Phe(*p*-NHCOCH$_2$Br). A general synthetic method was developed for the conversion of small peptide substrates into potential affinity labels. The target peptides were synthesized using Phe(*p*-NH$_2$) and a Boc/Fmoc orthogonal protection strategy which allowed for late functional group conversion of a *p*-amine group in the peptides to the desired affinity labeling moieties. A key step in the synthesis was the selective deprotection of a Boc group in the presence of a *tert*-butyl ester using trimethylsilyl trifluoromethanesulfonate (TMS-OTf). The target peptides were evaluated in radioligand binding experiments in Chinese hamster ovary (CHO) cells expressing δ or μ opioid receptors. The δ receptor affinities of the *N,N*-dibenzylleucine enkephalin analogues were 2.5−10-fold higher than those for the corresponding ICI-174,864 analogues. In general, substitution at the para position of Phe[4] decreased binding affinity at both δ and μ receptors in standard radioligand binding assays; the one exception was *N,N*-dibenzyl[Phe(*p*-NCS)[4]]leucine enkephalin (**2**) which exhibited a 2-fold increase in affinity for δ receptors (IC_{50} = 34.9 nM) compared to *N,N*-dibenzylleucine enkephalin (IC_{50} = 78.2 nM). The decreases in μ receptor affinities were greater than in δ receptor affinities so that all of the analogues tested exhibited significantly greater δ receptor selectivity than the unsubstituted parent peptides. Of the target peptides tested, only *N,N*-dibenzyl[Phe(*p*-NCS)[4]]leucine enkephalin (**2**) exhibited wash-resistant inhibition of radioligand binding to δ receptors. To our knowledge, **2** represents the first peptide-based affinity label to utilize an isothiocyanate group as the electrophilic affinity labeling moiety. As a result of this study, enkephalin analogue **2** emerges as a potential affinity label useful for the further study of δ opioid receptors.

Introduction

For centuries, humans have used the extract of the opium poppy, *Papaver somniferum*, for the alleviation of pain. Although morphine (the major constituent of opium) and many other synthetic analogues are used clinically, these analgesics have also been associated with serious side effects including physical dependence, respiratory depression, and tolerance.[1,2] Opioids interact with three major opioid receptor types (mu (μ), kappa (κ), delta (δ)), and many of the undesired side effects associated with opioid usage have been associated with activation of the μ receptor.[3] Activation of the κ receptor also exhibits side effects including dysphoria, diuresis, and sedation. Because of these factors, there is considerable interest in studying δ receptor ligands for the alleviation of pain and other therapeutic implications.[4]

Interest in the δ receptor is not limited to its potential for mediating analgesia. Subanalgesic doses of δ agonists have been shown to potentiate morphine antinociception,[5,6] and δ antagonists are effective at decreasing the development of tolerance and dependence to morphine.[7−9] The δ opioid system interacts with many reward pathways,[10] and antagonists may help attenuate alcoholism[11,12] and cocaine addiction.[13] Antagonists at the δ receptor also exhibit immunosuppressive effects[14] and may represent a novel way to treat autoimmune disorders and organ rejection in transplant patients.[15] Although considerable research has been performed on δ opioid receptors, there is still much to be learned about δ receptors and their physiological roles.

Affinity labels are ligands which bind to receptors in a nonequilibrium manner and can be useful as pharmacological tools.[16] Nonpeptide affinity labels that have been used in the study of δ opioid receptors include fentanyl isothiocyanate (FIT),[17] *cis*-(+)-3-methylfentanyl isothiocyanate (SUPERFIT),[18] and naltrindole isothiocyanate (NTII).[19] Peptide-based affinity labels for δ opioid receptors include [D-Ala[2],Cys[6]]-enkephalin (DALCE)[20] and its *S*-3-nitropyridinesulfenyl (Npys) protected derivative [D-Ala[2],Cys(Npys)[6]]-leucine enkephalin,[21] [D-Ala[2],D-Leu[5]]enkephalin chloromethyl ketone (DALECK),[22] and, most recently, Tyr-D-Ser-Gly-Phe-Leu-Thr-NH-Gly-maleoyl.[23]

The irreversible nature of an affinity label offers several advantages over a reversible ligand for certain receptor studies. Opioid affinity labels have been used to visualize the distribution of δ receptors in the rat neostriatum[24] and for the long-term blockade of opioid receptor activation.[25] Recently, affinity labels have been

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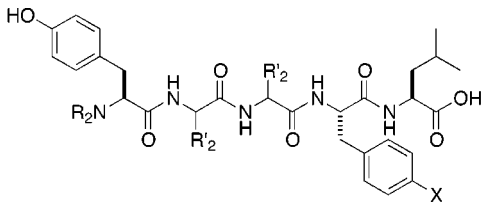
used to investigate the binding requirements of the δ opioid receptor. Liu-Chen and co-workers have identified the region of the δ opioid receptor responsible for the irreversible binding of SUPERFIT,[26] thereby gaining new information about possible residues involved in receptor/ligand interactions. The affinity labels currently in use for δ opioid receptors are based on both peptide agonists and nonpeptidic ligands (both agonists and antagonists). Due to potential differences in binding modes between peptides and nonpeptides and agonists versus antagonists, affinity labels based on δ-selective peptide antagonists may yield additional structural information about the binding site of δ opioid receptors.

We previously attempted to develop affinity labels based on δ-selective peptide antagonists by introducing a nitrogen mustard into N-terminal dialkylated enkephalins. In an initial study of the structure−activity relationships (SAR) for N-terminal substituents on leucine enkephalin, the *N,N*-dibenzyl derivative exhibited the highest potency and selectivity for the δ opioid receptor.[27] Therefore in a subsequent attempt to prepare peptide-based affinity labels, the Phe[4] residues of this pentapeptide and the related δ-selective peptide antagonist *N,N*-diallyl[Aib[2],Aib[3]]leucine enkephalin (ICI-174,864, Aib = 2-aminoisobutyric acid)[28] were substituted with melphalan (Mel), the para-substituted nitrogen mustard analogue of phenylalanine.[29] Mel was sufficiently stable to be directly incorporated into the growing peptide chain. Unfortunately, neither of the Mel-containing peptides synthesized exhibited significant wash-resistant antagonism in the mouse vas deferens (MVD) smooth muscle assay. The labeling process is thought to occur in two discrete steps: first, reversible binding of the affinity label to the receptor occurs, followed by covalent linkage with the receptor.[16] The lack of irreversible binding of the Mel-containing peptides might be due to potential problems in both steps. The large, sterically demanding nitrogen mustard moiety of the Mel residue may have had a deleterious effect on the reversible binding of the synthesized peptides. In addition, the relatively low reactivity of the aromatic nitrogen mustard may have hindered the process of covalent bond formation with the receptor.

To further investigate N,N-dialkylated enkephalin derivatives as potential affinity labels, we have incorporated more reactive and less sterically demanding functional groups at the para position of Phe[4]. A second goal in the syntheses of these peptide derivatives was the development of a general methodology flexible enough for the incorporation of various labeling moieties into a target peptide. The functional groups incorporated in these analogues were the isothiocyanate and the bromoacetamide groups. While these groups have shown their effectiveness in various nonpeptidic affinity labels for opioid receptors,[17−19,30] prior to this study they have not been incorporated into opioid peptide derivatives. The target peptides for this study included the *p*-isothiocyanate and *p*-bromoacetamide derivatives of *N,N*-dibenzylleucine enkephalin and ICI-174,864 (see Figure 1). The amine-containing derivatives were also prepared as reversible control compounds for the washing procedures used in the assay for wash-resistant inhibition of binding. The assays for inhibition of radioligand binding under both reversible and nonequilibrium conditions used Chinese hamster ovary (CHO) cells stably transfected with the mouse δ and rat μ opioid receptors.[31]



N,N-dibenzyl leucine enkephalin: R = Bn; R' = H; X = H
1: R = Bn; R' = H; X = NH_2
2: R = Bn; R' = H; X = NCS
3: R = Bn; R' = H; X = $NHCOCH_2Br$

ICI-174,864: R = Allyl; R' = CH_3; X = H
4: R = Allyl; R' = CH_3; X = NH_2
5: R = Allyl; R' = CH_3; X = NCS
6: R = Allyl; R' = CH_3; X = $NHCOCH_2Br$

Figure 1. Target enkephalin-based affinity labels and related peptides.

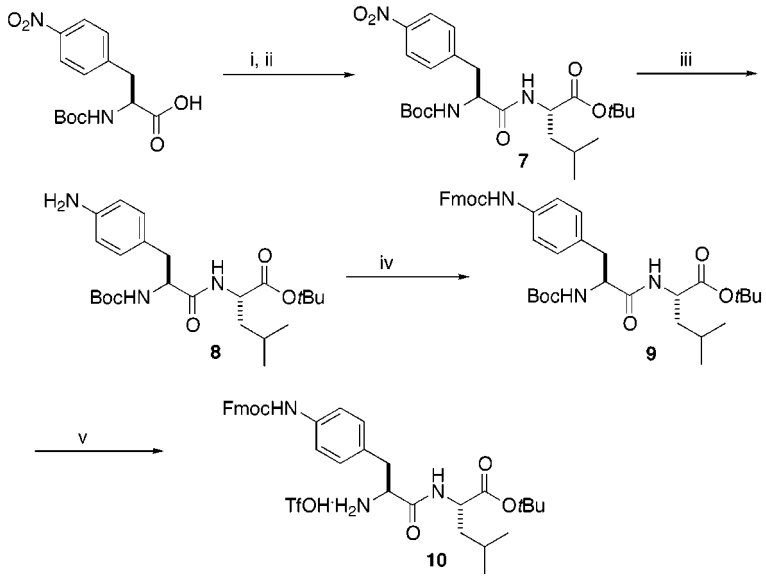
Synthetic Strategy

In the synthetic scheme for these potential affinity labels, a major consideration was the strategy for introducing the electrophilic groups. In an earlier study, the nitrogen mustard of Mel lent itself very well to standard conditions of solution-phase peptide synthesis and could be directly incorporated into the growing peptide chain with no special protection or reaction conditions.[29] Unfortunately, the more reactive isothiocyanate and bromoacetamide moieties were not expected to be stable to multiple peptide couplings and certain functional group deprotections. Therefore a synthetic scheme was devised that would allow functional group conversion late in the peptide synthesis. *p*-Nitrophenylalanine was chosen as the precursor of the peptide-based affinity labels because the nitro group can be reduced to an amine which can then be transformed into a number of labeling moieties, including the aforementioned isothiocyanate and bromoacetamide. Late reduction of the nitro group to the amine was not an option in the synthesis of these target peptides due to the susceptibility of the N-terminal substituents to hydrogenolysis.

An important aspect for these syntheses was the selective deprotection of an amine group in the presence of other protecting groups. Because the *tert*-butyl protecting group is easily and effectively removed using trifluoroacetic acid (TFA), this protecting group was chosen for semipermanent protection of the peptides due to the mild conditions required for final deprotection. This then required a transient protecting group for the *p*-amino functionality which could be selectively deprotected to allow electrophilic functional group introduction. Our laboratory initially explored the use of some hyperacid labile protecting groups, such as Bpoc (1-methyl-1-(4-biphenyl)ethoxycarbonyl), Ppoc (2-triphenylphosphonoisopropoxycarbonyl), and Ddz (2-(3,5-dimethoxyphenyl)-2-propyloxycarbonyl), for these syntheses. These protecting groups are generally introduced as the azides or active esters, but unfortunately these reagents were not reactive enough to protect the aniline functionality of Phe(*p*-NH_2) (unpublished results).

We then explored possible orthogonal protection strategies using base-labile protecting groups for the *p*-amino group in conjunction with the semipermanent

Scheme 1[a]



[a] Conditions: (i) *i*BuOCOCl, NMM; (ii) HCl·H-Leu-O*t*Bu, NMM (81%); (iii) NH_4HCO_2, 10% Pd/C (94%); (iv) Fmoc-Cl, 10% $NaHCO_3$, dioxane (80%); (v) TMS-OTf, toluene (97%).

tert-butyl-type groups. One such group is the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group,[32] which is removed through β-elimination using an organic base, most commonly piperidine. This protection strategy allowed for the deprotection of the *p*-amine on phenylalanine near the end of the synthesis, and therefore the electrophilic groups would not be subjected to a lengthy synthetic process.

Since the target peptides share a common C-terminal dipeptide fragment, the synthetic scheme involved a convergent [3+2] fragment condensation of the common C-terminal dipeptide fragment to the two different N-terminal fragments to yield the desired precursor pentapeptides. The electrophilic groups were then introduced into the protected peptides, followed by final TFA deprotection to yield the desired peptides.

Synthesis

The synthesis of the potential affinity labels began with preparing the common C-terminal dipeptide fragment **10**. Initial attempts to prepare **10** through the selective *p*-amine protection of Phe(*p*-NH_2) with the Fmoc group using pH control[33] resulted in a compound with poor solubility, and attempts to protect Boc-Phe-(*p*-NH_2)-OH with the Fmoc group resulted in low yields. Therefore derivatization at the dipeptide stage was pursued. The synthesis (Scheme 1) began with the isobutyl chloroformate-mediated coupling of Boc-Phe-(*p*-NO_2)-OH to HCl·H-Leu-O*t*Bu to give dipeptide **7** in 81% yield. Reduction of the *p*-nitro group by catalytic transfer hydrogenation[34] provided the amine derivative **8** in near quantitative yield, and protection of the resulting amine using Fmoc-Cl under standard conditions gave the fully protected dipeptide derivative **9** in 80% yield.

In a key step of the synthesis, the Boc group was removed in the presence of a *tert*-butyl ester. This selective deprotection was achieved using 1 equiv of trimethylsilyl trifluoromethansulfonate (TMS-OTf) in toluene.[35] After addition of the reagent, the product **10** (as the triflate salt) precipitated out of solution as a gel.

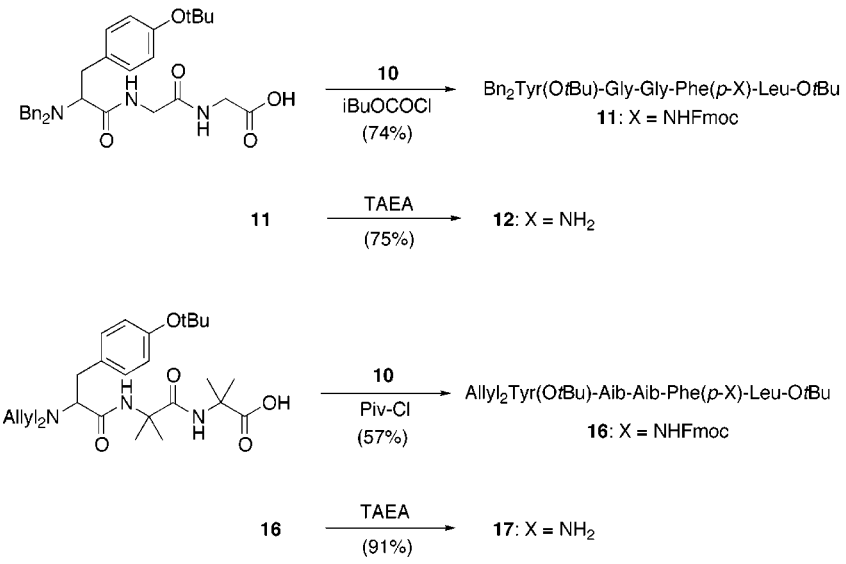
The N-terminal tripeptides were prepared using previously described methods[29] and coupled to the C-terminal dipeptide **10** using either *i*BuOCOCl (for *N,N*-dibenzyl-Tyr-Gly-Gly-OH) or pivaloyl chloride[36] (for *N,N*-diallyl-Tyr-Aib-Aib-OH) to provide pentapeptides **11** and **16**, respectively (Scheme 2). Next the Fmoc group was removed to expose the *p*-amine for electrophile introduction. Tris(2-aminoethylamine) (TAEA), which was developed by Carpino and co-workers[37] to simplify the removal of the dibenzofulvene byproduct during solution-phase synthesis, was used to remove the Fmoc group from **11** and **16** to yield the protected pentapeptide amines **12** and **17**, respectively.

The penultimate step in the synthetic strategy was the incorporation of the reactive functional groups into the protected pentapeptides (Scheme 3). During the synthesis of the dibenzylleucine enkephalin derivatives, functional group conversion of the amine to the bromoacetamide initially used bromoacetyl chloride. Extended reaction times resulted in halide exchange and led to the isolation of chloroacetamide **14**, which was identified by high-resolution mass spectrometry. This problem was circumvented in subsequent syntheses through the use of bromoacetyl bromide. The isothiocyanates **13** and **18** were formed by reaction of **12** and **17** with thiophosgene, respectively. Final deprotection of the peptide derivatives using TFA and anisole as a *tert*-butyl cation scavenger yielded the target peptides **1**−**6** for pharmacological evaluation.

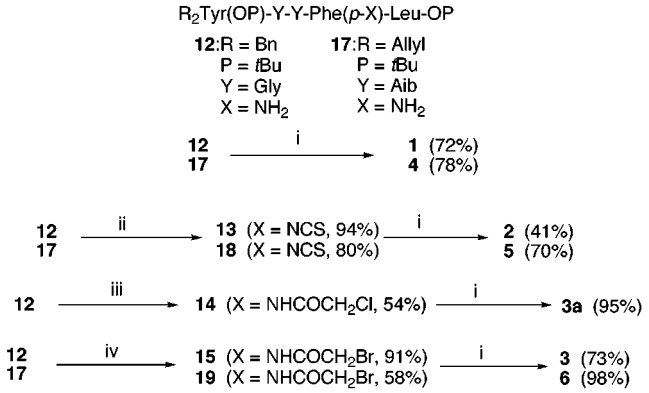
Pharmacological Evaluation

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 (Table 1).[31] In all cases, the dibenzyl derivatives exhibited higher (2.5−10-fold) affinity for δ opioid receptors than the corresponding diallyl derivatives, while the diallyl derivatives displayed higher δ selectivity by virtue of greater decreases in binding to μ opioid receptors. It was

Scheme 2



Scheme 3[a]



[a] Conditions: (i) 10% anisole/TFA; (ii) thiophosgene, DIPEA; (iii) bromoacetyl chloride, DIPEA, overnight at rt; (iv) bromoacetyl bromide, DIPEA, 2 h at 0 °C.

Table 1. Binding Affinities of Peptides **1**−**6** under Standard Assay Conditions

	$IC_{50} \pm$ SEM (nM)		IC_{50} ratio
compd	δ	μ	μ/δ
N,*N*-dibenzyl-Leu enkephalin	78.2 ± 1.1	1600 ± 1100	20
1	486 ± 1	23000 ± 1300	47
2	34.9 ± 3.3	1900 ± 1300	54
3	149 ± 3	9100 ± 1300	61
3a (*p*-$NHCH_2Cl$)	145 ± 4	19000 ± 1200	131
N,*N*-diallyl[Aib^2,Aib^3]Leu enkephalin (ICI-174,864)	703 ± 1	18900 ± 1100	27
4	1394 ± 1	87700 ± 1900	63
5	361 ± 3	33400 ± 1300	92
6	392 ± 3	>100000	>255

interesting to note that *N*,*N*-dibenzylleucine enkephalin exhibited a 10-fold greater affinity for δ receptors than the widely used ICI-174,864. This is in contrast to earlier studies[29] in which *N*,*N*-diallyl[Aib^2,Aib^3]leucine enkephalin was reported to be more potent than *N*,*N*-dibenzylleucine enkephalin in antagonizing the effects of [D-Ala^2,D-Leu^5]enkephalin (DADLE) in the MVD smooth muscle assay. This reversal in rank order potency suggests differences between the δ receptors present in the MVD and cloned receptor binding assays, and may be due to δ receptor subtypes.[38,39]

Substitution at the para position of Phe^4 on the two enkephalin derivatives had varying effects on affinity. For the dibenzyl derivatives, substitution at the para position of Phe^4 generally caused a decrease in δ receptor binding affinity; the notable exception was the isothiocyanate derivative **2**, which exhibited the highest δ receptor affinity of all of the peptides tested. Substitution at the para position was better tolerated by the diallyl derivatives, as the isothiocyanate- and bromoacetamide-containing peptides demonstrated 2-fold higher affinity for the δ receptor than the unsubstituted parent compound, *N*,*N*-diallyl[Aib^2,Aib^3]leucine enkephalin. Although the amine is the smallest substituent, peptides **1** and **4** displayed the lowest affinity in their respective series, indicating that the decreases in affinity at the δ receptor for the other analogues were not due to steric bulk alone. Within a series, the large acetamide groups in peptides **3**, **3a**, and **6** caused a decrease in μ binding affinity, thereby increasing the δ selectivity of these compounds with respect to either the isothiocyanate (**2** and **5**) or the amine (**1** and **4**) derivatives. The different substituent effects, particularly the different effects of the same substituent in the two series, may suggest subtle differences in the interactions between these two series of peptides and the δ receptor.

The peptides were then examined for wash-resistant inhibition of [^{3}H]DPDPE binding to δ receptors (Figure 2). The experiments involved incubation of the test compounds with CHO cell membranes for 90 min at room temperature, followed by washing the membranes via centrifugation, decanting, and resuspension in fresh buffer. The efficiency of the washing procedure was examined following incubation of the membranes with a high concentration (3 μM) of the amine-containing peptide **1**, which cannot bind covalently to the receptors. These initial studies indicated that a series of five washes was sufficient to remove the majority of this peptide without an appreciable loss of protein from the CHO cell membranes. The other peptide derivatives were initially assessed for wash-resistant inhibition of binding at a concentration of 1 μM to identify compounds which deserved further evaluation. The results after the washing procedure indicated that binding

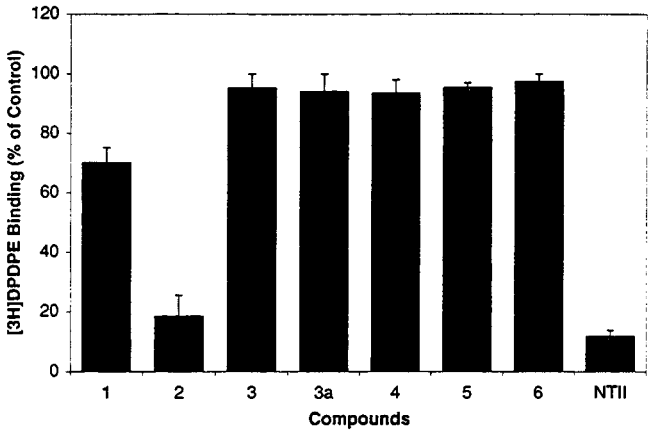


Figure 2. Wash-resistant inhibition of binding. Results are percent [^{3}H]DPDPE binding (±SEM) following incubation with peptides **1**−**6** or NTI and subsequent washing. All compounds were tested at 1.0 μM, except for compound **1**, which was tested at 3 μM.

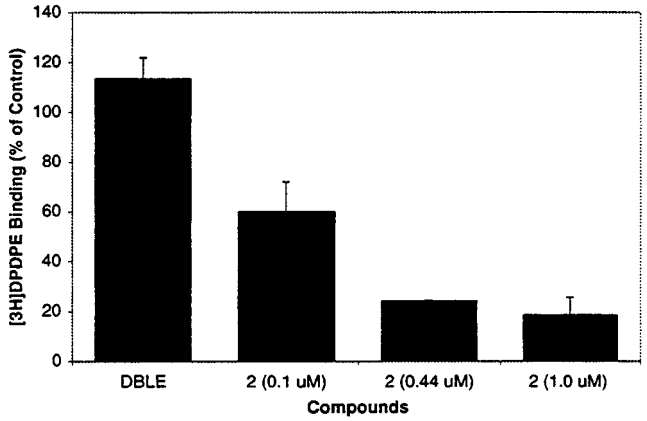


Figure 3. Dose-dependent wash-resistant inhibition of [^{3}H]-DPDPE binding by compound **2**. Results are percent [^{3}H]-DPDPE binding (±SEM) following incubation with compound **2** (tested at the concentrations in parentheses) or 1.0 μM *N,N*-dibenzylleucine enkephalin (DBLE).

levels of [^{3}H]DPDPE for the treated membranes were close to those for untreated membranes, except for the membranes treated with the isothiocyanate-containing dibenzyl compound **2**. In these membranes, [^{3}H]DPDPE binding was only approximately 19% of untreated control membrane. This level of wash-resistant inhibition of binding was close to that observed following treatment with naltrindole 5′-isothiocyanate (NTII) at a similar concentration (Figure 2). Since the affinity of *N,N*-dibenzylleucine enkephalin for δ receptors is similar to that observed for **2** under standard binding assay conditions, *N,N*-dibenzylleucine enkephalin was used as the control compound in subsequent examination of **2** for wash-resistant inhibition of binding. *N,N*-Dibenzylleucine enkephalin and **2** were examined for wash-resistant inhibition of binding at concentrations which should give comparable levels of receptor saturation (1.0 and 0.44 μM, respectively). The washing procedure effectively removed 1.0 μM *N,N*-dibenzylleucine enkephalin, while preincubation with 0.44 μM peptide **2** still resulted in 76% wash resistant inhibition of [^{3}H]-DPDPE binding (Figure 3). Even following preincubation with 100 nM **2**, [^{3}H]DPDPE binding was only approximately 60% of untreated control membranes (Figure 3). These data suggest that the isothiocyanate-containing dibenzyl compound **2** may be useful as an affinity label in the study of δ opioid receptors.

Conclusions

Utilizing a convergent [3+2] synthetic strategy, potential affinity labels based on the δ-selective peptide antagonists *N,N*-dibenzylleucine enkephalin[27] and ICI-174,864[28] were prepared. An Fmoc group was used to protect a *p*-amine functionality on Phe^4 prior to derivatization to the isothiocyanate or bromoacetamide groups. A key step in the synthesis was the selective removal of a Boc group in the presence of a *tert*-butyl ester, which was accomplished with an equimolar amount of TMS-OTf in toluene. The orthogonal Boc/Fmoc protection strategy utilized in these syntheses should prove to be a versatile method for the preparation of a wide variety of affinity labels based on small peptides.

The derivatized peptides **1**−**6** displayed a wide range of affinities for δ opioid receptors, with IC_{50} values varying from 34 nM to 1.4 μM under standard radioligand binding assay conditions. Despite the wide range of affinities, all of the peptides were selective for δ over μ opioid receptors. Substitution at the para position of Phe of the *N,N*-dibenzyl enkephalin analogues generally decreased binding affinity at the δ opioid receptor, but isothiocyanate and bromoacetamide functionalization increased δ opioid receptor binding for the ICI-174,864 derivatives. This would suggest that the differences in N-terminal and backbone substitution between the *N,N*-dibenzyl and ICI-174,864 derivatives resulted in subtle differences in their interactions with δ opioid receptors.

Of the compounds tested, only the isothiocyanate-containing derivative of *N,N*-dibenzylleucine enkephalin (**2**) exhibited wash-resistant inhibition of binding to CHO cells expressing δ receptors. The corresponding ICI-174,864 derivative **5** did not exhibit wash-resistant inhibition of binding, despite its structural similarity to **2**. The affinity labeling process begins with reversible binding of the ligand to the receptor, followed by covalent bond formation if a receptor-based nucleophile is in proper alignment with the electrophilic moiety. Different spatial orientations during binding, which may be due to differences in the N-terminal and backbone substitutions in **5**, may either decrease reversible binding to the receptor and/or prohibit proper alignment of the isothiocyanate with a receptor-based nucleophile.

From these observations, enkephalin analogue **2** emerges as an affinity label selective for the δ opioid receptor. Peptide **2** represents the first peptide-based affinity label to utilize an isothiocyanate group as the electrophilic labeling moiety for opioid or any receptors. Further pharmacological studies with **2** in conjunction with other δ-selective affinity labels may provide further insight on the binding requirements and physiological roles of δ opioid receptors. These studies are ongoing in our laboratory.

Experimental Section

All amino acids except for Boc-Phe(*p*-NO_2) and Aib were purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic reagents and the amino acids Boc-Phe(*p*-NO_2) and Aib were purchased from Aldrich Chemical Co. (Milwaukee, WI). General laboratory solvents were obtained from Mallinckrodt

Baker Inc. (Phillipsburg, NJ), and HPLC-grade solvents were obtained from Burdick and Jackson (Muskegon, MI).

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

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

The purity of intermediates and the final peptides were determined by high-performance liquid chromatography (HPLC) analysis. The system consisted of a Beckman model 431A HPLC using either a Vydac (C_4, 300 Å, 5 μm, 4.6 × 250 mm, column A) or a Zorbax Protein Plus (C_3, 300 Å, 10 μm, 4.6 × 250 mm, column B) analytical column. The compounds were eluted using a linear gradient of 0−75% B over 50 min at a flow rate of 1.5 mL/min and detected at 214 nm; solvent A was aqueous 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA.

Isobutyl Chloroformate-Mediated Peptide Coupling (Method I).[40] Under an N_2 atmosphere, a 0.25 M solution of the carboxyl component (1.0 equiv) in dry tetrahydrofuran (THF) was cooled to −15 °C (dry ice/MeOH) and neutralized with one equivalent of *N*-methylmorpholine (NMM). A stoichometric amount of isobutyl chloroformate was then added, followed 90 s later by a 0.5 M solution of the amine component and NMM (each 1.0 equiv) in *N*,*N*-dimethylformamide (DMF). The reaction mixture was kept at −15 °C for 0.5 h and then allowed to warm to room temperature. When the reaction was complete, the THF was evaporated and the residue suspended in EtOAc. The organic layer was washed with H_2O, 5% $KHSO_4$, H_2O, 5% $NaHCO_3$, H_2O, and then finally with saturated NaCl. The organic layer was then dried over $MgSO_4$ and the EtOAc evaporated to give the crude peptide.

Final Deprotection with TFA (Method II). The peptide was treated with a 10% anisole/TFA for 1 h at room temperature. The TFA was then evaporated and the product precipitated with cold ether. The precipitate was washed five times with cold ether, and then dried in vacuo.

***N*$^{\alpha}$-*tert*-Butyloxycarbonyl-L-*p*-nitrophenylalanyl-L-leucine *tert*-Butyl Ester (7).** *N*-*tert*-Butyloxycarbonyl-L-*p*-nitrophenylalanine (1.18 g, 3.80 mmol) was coupled to L-leucine *tert*-butyl ester hydrochloride (0.85 g, 3.80 mmol) according to method I to give 1.48 g (81%) of **7** as a white solid: R_f (EtOAc) = 0.68; R_f [butanol/acetic acid/water, (BAW) 3:1:1] = 0.87; HPLC (column A) t_R = 37.5 min; mp 161−163 °C; $[\alpha]_D$ = −15.9° (c = 0.94, MeOH); ^{1}H NMR ($CDCl_3$) δ = 0.88 (d, 3H, J = 1.7 Hz), 0.91 (d, 3H, J = 1.4 Hz), 1.39 (s, 9H), 1.41 (s, 9H), 1.55 (m, 3H), 3.09 (dd, 2H, J = 6.7 Hz, 14.3 Hz), 4.10 (m, 2H), 5.08 (d, 1H), 6.21 (d, 1H), 7.35 (d, 2H, J = 8.7 Hz), 8.12 (d, 2H, J = 8.7 Hz); FAB-MS [M + H]$^+$ 480.1 (calcd 480.3).

***N*$^{\alpha}$-*tert*-Butyloxycarbonyl-L-*p*-aminophenylalanyl-L-leucine *tert*-Butyl Ester (8).** Ammonium formate (1.5 g, 24.5 mmol) was added to a solution of **7** (1.30 g, 2.70 mmol) in MeOH (10 mL). Once the ammonium formate had dissolved, a suspension of 10% Pd/C (0.26 g) in MeOH/H_2O (1:1, 1 mL) was then added dropwise. After 0.5 h, the reaction mixture was filtered through Celite, and the MeOH evaporated. The residue was then dissolved in EtOAc (15 mL), and the organic layer was washed with H_2O (10 mL) and saturated NaCl (10 mL) and then dried over $MgSO_4$. The EtOAc was evaporated to yield dipeptide **8** as a white solid (1.15 g, 94%): R_f (EtOAc) = 0.57; R_f (EtOAc/hexane, 9:1) = 0.52; HPLC (column A) t_R = 27.2 min; mp 158−160 °C; $[\alpha]_D$ = −19.0° (c = 1.04, MeOH); ^{1}H NMR ($CDCl_3$) δ = 0.88 (d, 3H, J = 2.1 Hz), 0.90 (d, 3H, J = 1.8 Hz), 1.39 (s, 9H), 1.42 (s, 9H), 1.54 (m, 3H), 2.94 (d, 2H, J = 6.5 Hz), 4.24 (broad s, 1H), 4.41 (m, 1H), 4.95 (broad s, 1H), 6.28 (d, 1H, J = 8.4 Hz), 6.65 (d, 2H, J = 8.4 Hz), 6.98 (d, 2H, J = 8.3 Hz); FAB-MS [M + H]$^+$ 450.2 (calcd 450.3).

***N*$^{\alpha}$-*tert*-Butyloxycarbonyl-L-*p*-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine *tert*-Butyl Ester (9).** Ten percent Na_2CO_3 was added to a solution of **8** (1.10 g, 2.40 mmol) in dioxane (10 mL) to adjust the pH to 8. A solution of 9-fluorenylmethyl chloroformate (0.56 g, 2.2 mmol) was added, and the pH was maintained using additional 10% Na_2CO_3; a light, white precipitate formed immediately. The reaction was allowed to proceed overnight. The resultant suspension was then diluted with H_2O (10 mL) and the precipitate filtered and washed with 0.1 N HCl and H_2O. The crude solid (1.53 g, 105%) was dried and recrystallized from MeOH/H_2O to yield 1.29 g (80%) of **9** as a white solid: R_f (EtOAc) = 0.69; HPLC (column A) t_R = 43.7 min; mp 207−210 °C; $[\alpha]_D$ = −6.2° (c = 0.45, MeOH); ^{1}H NMR ($CDCl_3$) δ = 0.88 (d, 3H, J = 3.3 Hz), 0.89 (d, 3H, J = 8.9 Hz), 1.40 (s, 9H), 1.41 (s, 9H), 1.51 (m, 3H), 3.01 (d, 2H, J = 6.5 Hz), 4.25 (t, 2H, J = 6.5 Hz), 4.31(m, 1H), 4.41 (m, 1H), 4.51 (d, 2H, J = 6.6 Hz), 6.22 (d, 1H, J = 7.9 Hz), 6.56 (s, 1H), 7.12 (d, 2H, J = 8.2 Hz), 7.31 (t, 4H, J = 7.5 Hz), 7.40 (t, 2H, J = 7.3 Hz), 7.59 (d, 2H, J = 7.2 Hz), 7.76 (d, 2H, J = 7.4 Hz); FAB-MS [M + H]$^+$ 672.3 (calcd 672.4).

L-*p*-Amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine *tert*-Butyl Ester (10). A solution of TMS-OTf in toluene (0.358 M, 4.77 mL, 1.66 mmol) was added dropwise to a solution of **9** (1.10 g, 1.70 mmol) in CH_2Cl_2 (15 mL). The product (as the triflate salt) began to precipitate out as a gelatinous mass following addition. After 4 h, the gel was filtered, washed with additional CH_2Cl_2, and then dried to give 0.94 g (97%) of intermediate **10** as a white solid: R_f (EtOAc) = 0.68; HPLC (column A) t_R = 24.3 min; $[\alpha]_D$ = −14.5° (c = 1.00, MeOH); ^{1}H NMR ($CDCl_3$) δ = 0.90 (d, 6H, J = 3.3 Hz), 1.45 (s, 9H), 1.57 (m, 3H), 1.78 (broad s, 1H), 4.25 (t, 1H, J = Hz), 4.45 (m, 1H), 4.56 (d, 2H, J = Hz), 6.65 (s, 1H), 7.12 (d, 2H, J = 8.2 Hz), 7.31 (t, 4H, J = 7.5 Hz), 7.40 (t, 2H, J = 7.3 Hz), 7.59 (d, 2H, J = 7.2 Hz), 7.76 (d, 2H, J = 7.4 Hz); FAB-MS [M + H]$^+$ 572.3 (calcd 572.3).

***N*,*N*-Dibenzyl-*O*-*tert*-butyl-L-tyrosylglycylglycyl-L-*p*-amino(9-fluorenylmethoxycarbonyl)phenylalanyl-L-leucine *tert*-Butyl Ester (11).** The tripeptide *N*,*N*-dibenzyl-*O*-*tert*-butyl-L-tyrosylglycylglycine[27] (310 mg, 0.7 mmol) was coupled to **10** as described in method I, except that DMF was used instead of THF/DMF as the solvent. The crude material was purified by flash silica gel chromatography using EtOAc as the eluent to give 0.57 g (74%) of **11** as a foam: R_f (EtOAc) = 0.43; HPLC (column A) t_R = 42.3 min; $[\alpha]_D$ = −8.63° (c = 1.2, $CHCl_3$); FAB-MS [M + H]$^+$ 1085.7 (calcd 1085.6).

***N*,*N*-Dibenzyl-*O*-*tert*-butyl-L-tyrosylglycylglycyl-L-*p*-aminophenylalanyl-L-leucine *tert*-Butyl Ester (12).** Compound **11** (269 mg, 0.25 mmol) was dissolved in CH_2Cl_2 (2.0 mL) and TAEA (1.8 mL, 12.5 mmol) was then added. A white precipitate formed immediately. After 3 h, the solvent was removed in vacuo, and the residue was dissolved in EtOAc (30 mL). The EtOAc layer was washed with saturated NaCl, 1 M phosphate buffer at pH 5.5 (3 × 10 mL), and saturated NaCl and then dried over $MgSO_4$. The solvent was removed in vacuo, and the crude residue was applied to a flash silica gel column with EtOAc as the eluent to give 160 mg (75%) of **12** as a clear glass: R_f (EtOAc) = 0.14; R_f [$CHCl_3$/MeOH/acetic acid, (CMA) 95:5:1] = 0.18; R_f (BAW, 3:1:1) = 0.67; HPLC (column A) t_R = 34.0 min; FAB-MS [M + H]$^+$ 863.5 (calcd 863.5).

***N*,*N*-Dibenzyl-*O*-*tert*-butyl-L-tyrosylglycylglycyl-L-*p*-isothiocyanatophenylalanyl-L-leucine *tert*-Butyl Ester (13).** Compound **12** (37 mg, 0.04 mmol) was dissolved in CH_2Cl_2 (0.5 mL). *N*,*N*-Diisopropylethylamine (DIPEA; 6.5 μL, 0.08 mmol) was added to the solution, followed by thiophosgene (6.5 μL, 0.08 mmol). After 4 h, the solvent was removed under a stream of N_2, and the crude residue was applied to silica gel flash chromatography with EtOAc as the eluent to give 38 mg (94%) of **13** as a clear glass: R_f (EtOAc) = 0.48, R_f (CMA 95:5:1) = 0.28; HPLC (column A) t_R = 41.2 min; FAB-MS [M + H]$^+$ 905.8 (calcd 905.5).

***N*,*N*-Dibenzyl-*O*-*tert*-butyl-L-tyrosylglycylglycyl-L-*p*-chloroacetamidophenylalanyl-L-leucine *tert*-Butyl Ester (14).** Compound **12** (77 mg, 0.09 mmol) was dissolved in CH_2Cl_2 (1.0 mL) and chilled to 0 °C in an ice water bath. DIPEA

(31 μL, 0.18 mmol) was added to the solution, followed by bromoacetyl chloride (11 μL, 0.14 mmol) A thick white vapor formed upon addition of the bromoacetyl chloride, and the solution quickly turned a dark color. After 1 h at 0 °C, the solution was allowed to warm to room temperature and the reaction continued overnight. The solvent was then removed under an N_2 stream, and the residue was applied to silica gel flash chromatography with EtOAc as the eluent to give 49 mg (54%) of **14** as a clear glass: HPLC (column A) $t_R = 37.0$ min; FAB-MS $[M + H]^+$ 939.5 (calcd 939.5); high-resolution FAB-MS (calcd for $C_{52}H_{69}ClN_6O_8$) 939.4787, found 939.4778.

***N,N*-Dibenzyl-*O*-*tert*-butyl-L-tyrosylglycylglycyl-L-*p*-bromoacetamidophenylalanyl-L-leucine *tert*-Butyl Ester (15).** Compound **12** (21 mg, 0.02 mmol) was dissolved in CH_2Cl_2 (0.5 mL) and chilled to 0 °C in an ice water bath. DIPEA (8.5 μL, 0.05 mmol) was added to the solution, followed by bromoacetyl bromide (3.2 μL, 0.04 mmol). After 2 h at 0 °C, the solution was allowed to warm to room temperature for 1 h. The solvent was then removed in vacuo, and the residue was purified by silica gel flash chromatography with EtOAc as the eluent to give 16 mg (91%) of **15** as a glass: R_f (EtOAc) = 0.62; HPLC (column A) $t_R = 37.8$ min; FAB-MS $[M + H]^+$ 985.4 (calcd 985.0);

***N,N*-Dibenzyl-L-tyrosylglycylglycyl-L-*p*-aminophenylalanyl-L-leucine (1).** Compound **12** (20.0 mg, 0.02 mmol) was deprotected using method II to yield 16.0 mg (72%) of **1** as a white solid: HPLC (column A) $t_R = 46.6$ min (94% purity); FAB-MS $[M + H]^+$ 751.4 (calcd 751.4).

***N,N*-Dibenzyl-L-tyrosylglycylglycyl-L-*p*-isothiocyanatophenylalanyl-L-leucine (2).** Compound **13** (38.0 mg, 0.04 mmol) was deprotected using method II to yield 15.4 mg (41%) of **2** as a white solid: HPLC (column A) $t_R = 32.0$ min (95% purity); FAB-MS $[M + H]^+$ 793.3 (calcd 793.3).

***N,N*-Dibenzyl-L-tyrosylglycylglycyl-L-*p*-chloroacetamidophenylalanyl-L-leucine (3a).** Compound **14** (20.0 mg, 0.02 mmol) was deprotected using method II to yield 19.1 mg (95%) of **3a** as a white solid: HPLC (column A) $t_R = 27.4$ min (87% purity); FAB-MS $[M + H]^+$ 827.4 (calcd 827.4).

***N,N*-Dibenzyl-L-tyrosylglycylglycyl-L-*p*-bromoacetamidophenylalanyl-L-leucine (3).** Compound **15** (20.0 mg, 0.02 mmol) was deprotected using method II to yield 14.6 mg (73%) of **3** as a white solid: HPLC (column A) $t_R = 27.8$ min (98% purity); FAB-MS $[M + H]^+$ 873.3 (calcd 873.3).

***N,N*-Diallyl-*O*-*tert*-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-amino(9-fluorenylmethoxycarbonyl)-phenylalanyl-L-leucine *tert*-Butyl Ester (16).** *N,N*-Diallyl-*O*-*tert*-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyric acid (250 mg, 0.48 mmol) was dissolved in toluene (2 mL) and cooled in an ice bath. Triethylamine (166 μL, 1.19 mmol) was added to the solution, followed by trimethylacetyl chloride (60 μL, 0.48 mmol). The reaction was kept at 0 °C for 1 h and then allowed to warm to room temperature overnight. The reaction mixture was then filtered and concentrated by rotary evaporation. The dipeptide fragment **10** (175 mg, 0.24 mmol) was dissolved in EtOAc and the solution washed with 5% $NaHCO_3$, H_2O, and saturated NaCl, dried over Na_2SO_4 and evaporated to yield 132 mg (0.23 mmol) of L-*p*-amino(9-fluorenylmethoxycarbonyl)phenylalanine-L-leucine *tert* butyl ester as the free amine. A solution of the free amine in toluene (1 mL) was then added to the anhydride intermediate, and the reaction was left at room temperature overnight. Following the standard workup, the crude product was applied to a silica gel flash column with EtOAc/hexane (4:1) as the eluent to yield 140 mg (57%) of **16** as a foam: R_f (EtOAc) = 0.61; HPLC (column B) $t_R = 37.2$ min; FAB-MS $[M + H]^+$ 1041.6 (calcd 1041.6).

***N,N*-Diallyl-*O*-*tert*-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-aminophenylalanyl-L-leucine *tert*-Butyl Ester (17).** Compound **16** (221 mg, 0.21 mmol) was deprotected using TAEA as described in the preparation of **12**. The crude peptide was applied to a flash silica gel column with EtOAc as the eluent to yield 158 mg (91%) of **17** as a clear glass: R_f (EtOAc) = 0.25; HPLC (column B) $t_R = 35.0$ min; FAB-MS $[M + H]^+$ 819.6 (calcd 819.5).

***N,N*-Diallyl-*O*-*tert*-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-isothiocyanatophenylalanyl-L-leucine *tert*-Butyl Ester (18).** Compound **17** (15.0 mg, 0.02 mmol) was treated as described in the preparation of **13** to yield 13.3 mg (80%) of **18** as a clear glass: R_f (EtOAc) = 0.54; HPLC (column B) $t_R = 25.0$ min; FAB-MS $[M + H]^+$ 861 (calcd 861.5).

***N,N*-Diallyl-*O*-*tert*-butyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-bromoacetamidophenylalanyl-L-leucine *tert*-Butyl Ester (19).** Compound **17** (14.0 mg, 0.02 mmol) was treated as described in the preparation of **15** to yield 9.1 mg (58%) of **19** as a clear glass: HPLC (column B) $t_R = 40.9$ min; FAB-MS $[M + H]^+$ 941 (calcd 941.5).

***N,N*-Diallyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-aminophenylalanyl-L-leucine (4).** Compound **17** (9.9 mg, 0.01 mmol) was deprotected as described in method II to yield 8.9 mg (78%) of **4** as a white solid: HPLC (column B) $t_R = 22.5$ min (96% purity); FAB-MS $[M + H]^+$ 707.4 (calcd 707.4).

***N,N*-Diallyl-L-tyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-isothiocyanatophenylalanyl-L-leucine (5).** Compound **18** (13.0 mg, 0.01 mmol) was deprotected as described in method II to yield 9.1 mg (70%) of **5** as a white solid: HPLC (column B) $t_R = 33.3$ min (98% purity); FAB-MS $[M + H]^+$ 749.3 (calcd 749.4).

***N,N*-Diallyltyrosyl-α-aminoisobutyryl-α-aminoisobutyryl-L-*p*-bromoacetamidophenylalanyl-L-leucine (6).** Compound **19** (9.0 mg, 0.01 mmol) was deprotected as described in method II to yield 8.5 mg (98%) of **6** as a white solid: HPLC (column B) $t_R = 40.9$ min (98% purity); FAB-MS $[M + H]^+$ 829.3 (calcd 829.3).

Binding Assays. Radioligand binding assays of the potential affinity label derivatives were performed with Chinese hamster ovary (CHO) cells stably transfected with either mouse δ or rat μ opioid receptors.[31] Cells were harvested 48−72 h following plating in 50 mM Tris buffer, pH 7.4, at 4 °C and homogenized using a Dounce homogenizer. The homogenate was then centrifuged at 45000*g* for 10 min at 4 °C. The pellet was washed twice by resuspension and recentrifugation as in the previous step. The pellet was resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C to yield a protein concentration of 30−60 μg/mL. Incubations were performed for 90 min at 22 °C with [^{3}H]DPDPE and [^{3}H]DAMGO for δ and μ receptors, respectively. 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). Binding assays were carried out in mixtures containing 100 μg of membrane protein, 3 mM Mg^{2+}, and peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) in a final volume of 2 mL 50 mM Tris buffer, pH 7.4, at 22 °C. Nonspecific binding was determined in the presence of 10 μM unlabeled DPDPE and DAMGO for the δ and μ binding assays, respectively. The reactions were terminated by rapid filtration over Whatman GF/B glass fiber filters using a Brandel M48-R cell harvester, then washed with 5 × 2 mL of ice-cold Tris buffer. The filters were presoaked for at least 2 h in 0.5% polyethylenimine to decrease nonspecific binding. The filter disks were then placed in minivials with 4 mL of Cytoscint (ICN Radiochemicals) and allowed to elute for at least 6 h before counting in a Beckman LS 6800 scintillation counter. IC_{50} values were then derived from nonlinear regression analysis of competition curves 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 enkephalin derivatives for 90 min at room temperature. The homogenates were then centrifuged at 40000*g* for 15 min at 4 °C and the pellet was resuspended in 7 mL of 50 mM Tris buffer, pH 7.4, at 4 °C. The centrifugation and resuspension steps were repeated four times as the

washing protocol. After the fifth resuspension, the homogenate was recentrifuged and the final pellet resuspended in 50 mM Tris buffer, pH 7.4, at 4 °C. These final CHO cell membrane homogenates were then subjected to radioligand binding assays as described above. The radioligand binding to membranes treated with the enkephalin analogues were then expressed as percent binding of untreated control membranes (±SEM). The data for the initial screening of the peptides and *N*,*N*-dibenzylleucine enkepahlin are the average of at least two experiments, and the data for the subsequent evaluation of peptide **2** are the average of three experiments.

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