Extended TIP(P) Analogues as Precursors for Labeled δ-Opioid Receptor Ligands

Vivek Kumar, 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 17, 2000

Tyr-Tic-Phe-Phe-OH (TIPP) and the shorter Tyr-Tic-Phe-OH (TIP) peptides are potent and highly selective antagonists at the δ-opioid receptor and, therefore, are ideal candidates for the attachment of labels to assist in the study of δ-opioid receptors. Peptides extended at the C-terminus with residues which can be used as handles for further modification and/or labeling (i.e. Asx, Glx, and Lys) were synthesized. The TIPP-D/L-Asx/Glx derivatives exhibited similar δ-receptor affinity to TIPP (K_i = 5−10 nM vs K_i = 6 nM), and neither the location of the carboxylic acid moiety nor the stereochemistry of the C-terminal residue significantly affected the δ-receptor affinity of these derivatives. Extension of TIPP with an additional residue did not increase μ-receptor affinity, even though the position of the acidic group, which imparts δ-receptor selectivity to TIPP, was shifted relative to the carboxylic acid moiety of TIPP. The δ-receptor affinities of the TIP-D/L-Asx/Glx derivatives were found to be influenced mainly by the position of the carboxylic acid function rather than the stereochemistry of the C-terminal residue. TIP(P)-D/L-Lys(Ac)-OH derivatives exhibited moderate δ-receptor affinity (K_i^δ = 16−28 nM). The most potent compounds found in the extended TIP(P) series were TIPP-D-Gln-OH and TIP-D-Gln-OH (K_i^δ = 5 nM) which had similar affinities to TIPP.

Introduction

Opioid receptors play a critical role in the mediation and modulation of analgesia. Morphine and its derivatives are widely used clinically to alleviate pain, but utilization of these μ-selective opiates is limited due to their inherent side effects such as physical dependence and respiratory depression. δ-Receptor agonists are being increasingly investigated because they produce analgesia without the dependence or respiratory depression associated with μ-selective opiates.[1] δ-Receptor antagonists have been examined for possible therapeutic use as immunosuppressants[2] and in the treatment of cocaine and alcohol addiction.[3,4]

The availability of highly selective ligands for individual receptor types aids in the development of potential therapeutic agents. Moreover such ligands, either agonist or antagonist, are valuable pharmacological tools to understand the pharmacophoric requirements for binding with different receptors and the various biological effects produced by individual receptor types.[5] Strategically labeled ligands (e.g. with a fluorescent label) have been used as pharmacological tools to study receptor function and to aid in the identification of individual receptor types. Peptide ligands for opioid receptors, particularly enkephalins, have been labeled with fluorescent functionalities such as rhodamine,[6] pyrene,[7] dansyl,[8,9] and fluorescein.[10,11] Similarly, nonpeptide opiates have been labeled with nitrobenzoxadiazole (NBD)[12] and BODIPY.[13] Fluorescent probes for other receptors have been utilized to study the kinetics of receptor−ligand association and dissociation rates,[14] as well as the interactions between ligand, receptor, and G-proteins (GTP-binding proteins).[15,16] Other receptor properties, such as the localization of the receptor-binding domain,[17] have also been examined using fluorescent-labeled ligands. In addition, opioid peptides labeled with biotin have been used in the study of ligand−receptor complexes.[11,18−21] These labels can be readily attached to either a free carboxylic acid or an amino group on the peptides in one of two ways: either to a side chain functional group of a noncritical residue or by extending the peptide backbone in a manner which has minimal influence on binding at the receptor binding site.

We are interested in developing potent and selectively labeled opioid peptides as pharmacological tools to study δ-opioid receptor structure and function. The first δ-receptor antagonists were *N*,*N*-dialkylated enkephalin derivatives: e.g. *N*,*N*-diallylleucine enkephalin (ICI 154,129), *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864)[22,23] (later shown to be an inverse δ-agonist),[24] and *N*,*N*-dibenzylleucine enkephalin.[25] Subsequently, TIPP-OH (Tyr-Tic-Phe-Phe-OH), which contains a 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) residue at the 2-position, was identified.[26] This peptide represents the prototype of a new class of potent and highly selective δ-opioid antagonists.[26] 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 (MVD) assay (K_e = 3−5 nM).[26] Also, TIPP does not display any μ- or κ-antagonist properties in the guinea pig ileum (GPI) assay at concentrations as high as 10 μM. Removal of the Phe residues from TIPP led to the identification of the tri- and dipeptide antagonists Tyr-Tic-Phe-OH (TIP) and

* To whom correspondence should be addressed. Phone: (410) 706-6863. Fax: (410) 706-0346. E-mail: jaldrich@umaryland.edu.
† University of Georgia.

10.1021/jm000362h CCC: $19.00
Published on Web 11/29/2000

Tyr-Tic-OH, which exhibit weaker affinity ($K_i^\delta = 9$ and 128 nM, respectively) for the δ-receptor compared to TIPP.[26−28] Both TIPP and TIP are more potent than ICI-174,864 and are more δ-selective than both ICI-174,864 and the nonpeptide δ-receptor antagonist naltrindole.[26]

Structure−activity relationship (SAR) studies have led to numerous modifications of residues in TIP(P).[26] A variety of aromatic and nonaromatic amino acids have been substituted for Phe^3.[27,29−31] These substitutions showed that an aromatic residue in position 3 is not critical for δ-antagonist activity.[27] Replacement of Phe^3 in TIPP by aliphatic residues such as Leu, Ile, or Nva (norvaline) had little effect on δ-receptor antagonist potency and selectivity; however, substitution with cyclohexylalanine improved both affinity and selectivity.[29] Several substitutions on the phenyl ring of Phe^4 (e.g. *p*-NO_2 and *p*-Cl, -Br, -I) are also well-tolerated.[31] *N*-Alkyl and *N*-aralkyl modifications of the Tyr residue of TIPP resulted in peptides with increased potency and selectivity for the δ-receptor.[31] Substitution of 2′,6′-dimethyltyrosine (Dmt) for Tyr in TIPP increases affinity for both μ- and δ-receptors, with the larger increases in μ-receptor affinity reducing δ-receptor selectivity.[31] An important TIPP analogue TIPP[Ψ],[32] in which the peptide bond between Tic^2 and Phe^3 was reduced, was synthesized to overcome the chemical instability of TIP(P) in organic solvents such as DMSO and MeOH.[33] Although TIP(P) is stable in aqueous buffer (pH 7.7), it undergoes slow conversion to the Tyr-Tic diketopiperazine in organic solvents. TIPP[Ψ] was found to be a potent δ-selective antagonist and resistant to both chemical and enzymatic degradation.[32]

To date there are only scant reports in the literature dealing with C-terminal modified or extended TIP(P) peptides.[34] Amidation of TIPP-OH to give TIPP-NH_2 results in minimal reduction in δ-receptor affinity but increased affinity for μ-receptors.[26] In smooth muscle preparations, TIPP-NH_2 is a mixed μ-agonist/δ-antagonist.[26] The TIP(P) sequence has been introduced into the *N*-terminus of deltorphin I,[35,36] dermorphin,[36] and dynorphin A-(1−11)NH_2,[36] resulting in peptides which exhibit enhanced affinity and selectivity for δ-receptors compared to the parent peptides.

The aim of the current study was to identify lead peptides for incorporation of labels such as fluorescein for subsequent studies of the δ-opioid receptor. 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. To label the peptides without reducing their δ-receptor affinity and/or selectivity, we synthesized peptides extended at the C-terminus. The additional residues examined contained either an acidic or amine functionality, which could be further modified to incorporate the labeling group; a C-terminal acidic functionality was maintained in the peptides in order to retain δ-receptor selectivity. TIP also exhibits high δ-receptor affinity and selectivity, and since extended TIP peptides would contain an acidic functionality in the same position as TIPP, extended TIP peptides were also prepared. We wanted to evaluate optimal stereochemistry for the extending residue and at the same time determine whether the side chain or backbone extension was best suited for incorporating the label. Thus D/L-Asn/Gln-extended TIP(P) derivatives are simplified analogues of labeled peptides where the label is on the side chain, and peptides extended with D/L-Asp/GluNH_2 correspond to labeled derivatives in which the label is attached to the backbone. Attaching groups to Lys is a popular method to incorporate labels into peptides, and therefore TIP(P)-D/L-Lys(Ac)-OH were prepared as simplified analogues of these labeled peptides. Here we present results for these extended TIP(P) analogues Tyr-Tic-Phe-(Phe)-X, where X = D/L-Asx, -Glx, or -Lys(Ac). To determine the optimal residues, these initial peptides were evaluated for affinity at δ- and μ-receptors. Those molecules with the best affinity and selectivity for the δ-receptors will be further modified in future studies by attaching a labeling functionality such as a fluorescent group.

Results and Discussion

Chemistry. All peptides were prepared by solid-phase synthesis using the standard Fmoc/*tert*-butyl protection strategy. The D/L-Asp-NH_2 derivatives of TIP(P) were synthesized by attaching Fmoc-D/L-Asp-(O*t*Bu) to the PAL-PEG-PS (peptide amide linker-poly(ethylene glycol)polystyrene) resin. The D/L-Asn derivatives of TIP(P) were synthesized by attaching the side chain acidic functionality of Fmoc-D/L-Asp-O*t*Bu to the same resin. Similar strategies were utilized to obtain the D/L-Glu and D/L-Gln derivatives. The D/L-Lys(Ac) derivatives were prepared on the Wang resin to yield the peptide acids. *N,N*-Diisopropylcarbodiimide (DIC) and hydroxybenzotriazole (HOBt) were used for all couplings involving standard amino acids. This reagent can give poor results in couplings with hindered amino acids; therefore (benzotriazol-1-yloxy)tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP),[37] which is reported to be a highly efficient coupling reagent, was used for coupling Boc-Tyr(O*t*Bu)-OH to the hindered secondary amine of Tic^2 in the peptides. Following synthesis and purification, the identity and purity of the final compounds were verified using mass spectrometry and analytical HPLC; the analytical data for the peptides is shown in Table 1.

Opioid Receptor Affinities. The peptides were evaluated for their binding affinity to δ- and μ-receptors in radioligand binding assays using cloned receptors stably expressed in CHO (Chinese hamster ovary) cells. The affinities for the δ- and μ-receptors, determined by competitive inhibition of the radioligands [^{3}H]DPDPE and [^{3}H]DAMGO, respectively, are summarized in Table 2, compared to the parent peptides TIPP and TIP. The δ-opioid receptor affinity of TIPP and TIP determined in our laboratory using cloned receptors in CHO cells were somewhat lower than those reported by Schiller and co-workers ($K_i^\delta = 1.22$ and 9.07 nM, respectively) for binding of TIPP and TIP to rat brain membranes.[26]

Chain extensions of TIPP with D/L-Asx and D/L-Glx caused less than a 2-fold decrease in δ-receptor affinity compared to TIPP. For the TIPP-Asx derivatives, changing the stereochemistry of Asx did not influence δ-receptor affinity or selectivity. Similarly, changing the position of the carboxylic acid moiety, from the α-carbon in the case of TIPP-D/L-Asn-OH to the β-carbon in the case TIPP-D/L-Asp-NH_2, did not significantly affect affinity. Among the Glx derivatives, the affinity for the δ-recep-

Table 1. Analytical Data for Extended TIP(P) Derivatives

peptide	FAB-MS [M + H$^+$] found	FAB-MS [M + H$^+$] calcd	HPLC t_R (min)[a] A	B	C
TIPP	635.3	635.3	22.9	15.5	7.6
TIPP-Asp-NH_2	749.3	749.3	20.3	13.0	10.2
TIPP-Asn-OH	749.4	749.3	20.5	13.7	10.5
TIPP-D-Asp-NH_2	749.3	749.3	20.3	13.2	10.4
TIPP-D-Asn-OH	749.6	749.3	20.2	13.0	10.3
TIPP-Glu-NH_2	763.4	763.3	31.0	12.8	10.3
TIPP-Gln-OH	763.4	763.3	27.7	13.5	12.9
TIPP-D-Glu-NH_2	763.4	763.3	23.0	12.9	12.8
TIPP-D-Gln-OH	763.4	763.3	25.7	12.7	11.1
TIPP-Lys(Ac)OH	805.3	805.4	24.5	13.7	10.4
TIPP-D-Lys(Ac)OH	805.4	805.4	21.2	15.0	12.7
TIP	488.2	488.2	28.8	11.3	7.1
TIP-Asp-NH_2	602.3	602.3	21.5	9.1	8.2
TIP-Asn-OH	602.3	602.3	21.7	9.5	7.2
TIP-D-Asp-NH_2	602.3	602.3	19.9	9.3	7.2
TIP-D-Asn-OH	602.2	602.3	18.8	8.6	6.8
TIP-Glu-NH_2	616.2	616.3	16.5	8.7	6.9
TIP-Gln-OH	616.3	616.3	20.5	8.4	6.9
TIP-D-Glu-NH_2	616.3	616.3	17.9	8.6	10.3
TIP-D-Gln-OH	616.2	616.3	27.9	8.6	7.0
TIP-Lys(Ac)OH	658.3	658.3	19.9	15.2	7.0
TIP-D-Lys(Ac)-OH	658.4	658.3	21.0	13.8	9.1

[a] See Experimental Section for HPLC solvent systems.

Table 2. Opioid Receptor Affinities for Extended TIP(P) Analogues[a]

peptide	K_i^δ (nM)	K_i^μ (nM)
TIPP	6.1 ± 0.5	>4500
TIPP-Asp-NH_2	9.5 ± 1.7	>4000
TIPP-Asn-OH	7.1 ± 1.2	>4500
TIPP-D-Asp-NH_2	10.3 ± 1.9	3280 ± 720
TIPP-D-Asn-OH	8.1 ± 1.3	3200 ± 540
TIPP-Glu-NH_2	9.9 ± 2.5	>4500
TIPP-Gln-OH	9.7 ± 3.4	>4500
TIPP-D-Glu-NH_2	7.8 ± 0.6	1950 ± 200
TIPP-D-Gln-OH	5.1 ± 0.4	>4500
TIPP-Lys(Ac)OH	21.5 ± 5.4	>4500
TIPP-D-Lys(Ac)OH	16.3 ± 1.7	>4500
TIP	148.0 ± 25.8	>4500
TIP-Asp-NH_2	21.4 ± 4.9	>4500
TIP-Asn-OH	61.1 ± 23.3	>4500
TIP-D-Asp-NH_2	69.0 ± 12.7	>4500
TIP-D-Asn-OH	13.7 ± 1.5	>4500
TIP-Glu-NH_2	19.9 ± 1.6	4500 ± 280
TIP-Gln-OH	37.7 ± 4.8	>4500
TIP-D-Glu-NH_2	34.9 ± 11.7	>4500
TIP-D-Gln-OH	5.0 ± 1.6	>4500
TIP-Lys(Ac)OH	27.8 ± 4.6	>4500
TIP-D-Lys(Ac)-OH	21.6 ± 1.1	>4500

[a] K_i values are the average ± SEM of 3−7 independent determinations.

tors was affected to a small extent by stereochemistry. Thus, TIPP-D-Gln-OH/D-Glu-NH_2 (K_i = 5 and 8 nM) had slightly higher affinity than TIPP-L-Gln-OH/L-Glu-NH_2 (K_i = 10 nM). Also TIPP-D-Gln-OH/D-Glu-NH_2 exhibited slightly higher affinity than the corresponding TIPP-D-Asn-OH/D-Asp-NH_2 derivatives (K_i = 8−10 nM). TIPP-L-Glu-NH_2/L-Gln-OH and TIPP-L-Asp-NH_2/L-Asn-OH had similar affinities (K_i = 7−9 nM). Schiller and coworkers have reported that modification of the carboxylic acid moiety, i.e. amidation, reduced δ-receptor selectivity by improving μ-affinity. Therefore, it was anticipated that extending the peptide chain by an additional residue or changing the position of the carboxylic acid functionality in the extended TIPP derivatives (from the α-carbon to the β- or γ-carbon) would influence μ-receptor affinity. However, of these

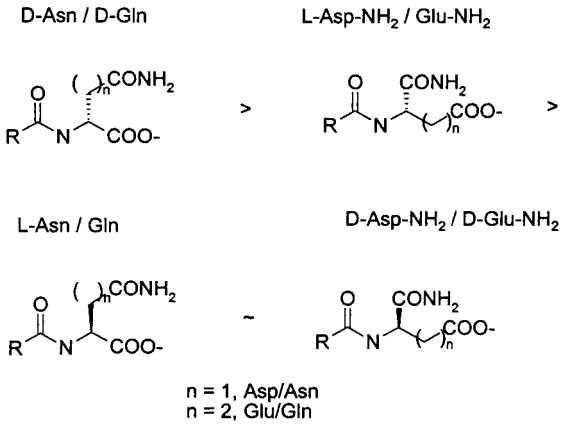

Figure 1. Comparison of the structures of D/L-Asx/Glx residues and the relative potencies of TIP-D/L-Asx/Glx analogues.

analogues, only TIPP-D-Glu-NH_2 showed increased μ-receptor affinity (K_i^μ = 1950 nM) and therefore decreased δ-receptor selectivity (K_i^μ/K_i^δ = 249).

It has been demonstrated in the past that labels can be easily attached to Lys residues within peptide chains using solid-phase techniques. Thus the extended peptides TIPP-D/L-Lys(Ac)-OH were also synthesized and evaluated. The acetyl group was used to eliminate the basicity of the ϵ-amine and mimic the attachment of a labeling group. TIPP-D/L-Lys(Ac)-OH (K_i^δ = 16−21 nM) derivatives exhibited a larger (3−4-fold) decrease in δ-receptor affinity compared to the TIPP-D/L-Asx/Glx; a change in stereochemistry of the Lys residue had only a minimal effect on δ-receptor affinity.

Chain extension of TIP led to peptides with significantly improved binding affinity for the δ-receptor compared to the tripeptide. The binding affinity of TIP-D-Gln-OH (K_i = 5 nM) was identical to that of TIPP, indicating that the δ-receptor could tolerate a hydrophilic residue in position 4. The effect of amino acid substitution was significantly greater in the TIP-X series than observed in the TIPP-X series. The δ-receptor affinities varied from equipotent with TIPP for the most potent analogue TIP-D-Gln-OH to 12−15-fold lower affinity than TIPP (for TIP-Asn-OH and TIP-D-Asp-NH_2). The identity of the amino acid significantly affected δ-receptor affinity, with the order of potency being D-Gln-OH > D-Asn-OH > L-Glu-NH_2 ~ L-Asp-NH_2 > L-Gln-OH > D-Glu-NH_2 > L-Asn-OH ~ D-Asp-NH_2. These results suggested that the position of the carboxylic acid (Figure 1) was more important than the absolute stereochemistry of the amino acid. None of the TIP-X analogues displayed increased μ-receptor affinity compared to TIP or TIPP. The Lys-containing TIP derivatives, TIP-D/L-Lys(Ac)-OH (K_i^δ = 21−28 nM), possessed affinities similar to those of TIPP-D/L-Lys(Ac)-OH; they were intermediate in potency compared to other TIP-X derivatives, where X = D/L-Asx/Gln.

Conclusions

Extension of TIPP with hydrophilic residues yielded a number of peptides with high δ-receptor affinity and selectivity. Extension of TIPP with an additional residue did not increase μ-receptor affinity, even though the position of the acidic group, which imparts δ-receptor selectivity to TIPP, was shifted relative to the carboxylic

acid moiety of TIPP. The influence of the residue in position 4 of TIP-X was considerably more variable. For the TIP-Asx/Glx series, δ-receptor affinity appeared to correlate with the relative position of the acidic functionality rather than the stereochemistry of the amino acid. TIP(P)-D/L-Lys(Ac)-OH derivatives exhibited moderate δ-receptor affinity (16−28 nM) but were less potent than the most potent TIP(P)-Asx/Glx derivatives (K_i^{δ} = 5 nM). TIPP-D-Gln-OH and TIP-D-Gln-OH exhibited the highest δ-receptor affinity of the analogues examined and were equipotent with TIPP, suggesting these as lead peptides for attachment of a labeling functionality. These studies are ongoing in our laboratory.

Experimental Section

General Methods. All Fmoc-protected amino acids were purchased from Bachem (King of Prussia, PA) or Perseptive Biosystems, Inc. (Framingham, MA). The phenol of Tyr was protected as the *tert*-butyl ether. The Wang resin, HOBt, and PyBOP were obtained from Novabiochem USA (San Diego, CA). *N*,*N*-Diisopropylethylamine (DIPEA) and PAL-PEG-PS (peptide amide linker-poly(ethylene glycol) polystyrene) resin were purchased from Perseptive Biosystems, Inc. Trifluoroacetic acid (TFA) was obtained from Pierce Chemical Co. (Rockford, IL), and DIC and piperidine were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade solvents (dichloromethane (DCM), acetonitrile, and *N*,*N*-dimethylacetamide (DMA)) for synthesis and HPLC analysis were from Burdick & Jackson Inc., Muskegon, MI, or EM Sciences.

Stepwise solid-phase peptide synthesis was carried out on a Biosearch 9500 automated peptide synthesizer by the standard Fmoc/*tert*-butyl strategy.[38] The D/L-Asp, -Asn, -Glu, and -Gln derivatives of TIP(P) were synthesized using the Fmoc-PAL-PEG-PS resin (0.5 g, 0.4 mmol/g), whereas the D/L-Lys(Ac) derivatives were synthesized on a Wang resin (0.5 g, 0.56 mmol/g). Fmoc-D-Lys(Ac)-OH or Fmoc-L-Lys(Ac)-OH (1.15 g, 10 equiv) was attached to the Wang resin as the symmetric anhydride prepared using DIC (0.22 mL, 5 equiv) along with *N*,*N*-(dimethylamino)pyridine (34 mg, 0.1 equiv) for 16 h. The resin was swelled by washing with DCM (3 × 2 min), followed by DMA/DCM (1:1) (3 × 2 min). The amino acids (4-fold excess) were coupled to the resin sequentially using DIC/HOBt as the coupling reagent for all amino acids except the *N*-terminal Boc-Tyr(O*t*Bu)-OH residue. The completeness of the coupling reactions was monitored by the qualitative ninhydrin test. Boc-Tyr(O*t*Bu)-OH (4 equiv) was coupled using PyBOP/HOBt/DIPEA (4:4:8 equiv) until a negative chloranil test[39] was obtained (6 h). After coupling the last amino acid, the resin was washed with DMA, DCM and MeOH, dried in vacuo, and cleaved with 90% aqueous TFA for 2 h. The resin was then filtered and washed with a small amount of TFA (1−2 mL), and the TFA was removed in vacuo. The resulting yellow oil was dissolved in 10% acetic acid (a small amount of MeCN was also used to aid in the dissolution) and the peptide lyophilized. Due to the high hydrophobicity of the peptides, extraction with ether was avoided.

HPLC Analysis. Analytical HPLC analysis of the peptides was carried out on a Beckman system GOLD consisting of a programmable solvent module 126 and a diode array detector model 168. The HPLC analysis and purification were performed with a binary solvent system, where solvent A was aqueous 0.1% TFA and solvent B was MeCN containing 0.1% TFA. Analytical HPLC employed a Vydac 218-TP column (4.6 × 250 mm) equipped with a Vydac guard cartridge using a standard linear gradient of 5−80% solvent B over 50 min, at a flow rate of 1 mL/min, for the crude peptides (system A, Table 1); the elution was monitored at 214 and 280 nm. Purification of the peptides was performed on a Rainin HPXL HPLC system equipped with a Shimadzu SPD-10A detector and utilized a Vydac column 218-TP column (22 × 250 mm) with a Vydac guard cartridge using a linear gradient of 20−50% solvent B over 30 min; purification was monitored at 214 nm. The purity of the final peptides was also verified in two HPLC solvent systems using a flow rate of 1 mL/min: system B, 20−50% linear gradient of aqueous MeCN containing 0.1% TFA over 20 min, and system C, 40−70% linear gradient of aqueous MeOH containing 0.1% TFA over 20 min. The final purity of all peptides by both analytical systems was >98%. Molecular weights of the compounds were determined by fast atom bombardment mass spectrometry (FAB-MS) on a Kratos MS50RF mass spectrometer (Environmental Health Sciences Center, Oregon State University, Corvallis, OR).

Radioligand Binding Assays.[40] Opioid receptor binding studies were performed on membranes derived from CHO cells expressing cloned δ- and μ-receptors stably. Binding assays were carried out in the presence of peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+}. Incubations were performed with varying concentrations of the peptides up to 10 μM for 90 min at 22 °C using [^{3}H]DPDPE and [^{3}H]DAMGO as the radioligands for δ- and μ-receptors, respectively. Nonspecific binding was determined in the presence of 10 μM unlabeled ligand. IC_{50} values were derived from nonlinear regression analyses of competitive curves using GraphPad software (Prism) and then converted to dissociation constants (K_i) using the Cheng−Prusoff equation;[41] K_D values for [^{3}H]DPDPE and [^{3}H]DAMGO were 0.5 and 0.644 nM, respectively.

Acknowledgment. This research was supported by a grant from the National Institute on Drug Abuse (DA 10035). The authors also thank Jennif Chandler for performing the radioligand binding assays and Brian Arbogast for FAB-MS analysis of the peptides.

References

(1) Rapaka, R. S.; Porreca, F. Development of Delta Opioid Peptides as Nonaddicting Analgesics. *Pharm. Res.* **1991**, *8*, 1−8.
(2) Arakawa, K.; Akami, T.; Okamoto, M.; Akioka, K.; Nakai, I.; Oka, T.; Nagase, H. Immunosuppression by Delta Opioid Receptor Antagonist. *Transplant Proc.* **1993**, *25*, 738−740.
(3) Reid, L. D.; Glick, S. D.; Menkens, K. A.; French, E. D.; Bilsky, E. J.; Porreca, F. Cocaine Self-Administration and Naltrindole, a Delta-Selective Opioid Antagonist. *Neuroreport* **1995**, *6*, 1409−1412.
(4) Krishnan-Sarin, S.; Jing, S. L.; Kurtz, D. L.; Zweifel, M.; Portoghese, P. S.; Li, T. K.; Froehlich, J. C. The Delta Opioid Receptor Antagonist Naltrindole Attenuates Both Alcohol and Saccharin Intake in Rats Selectively Bred for Alcohol Preference. *Psychopharmacology (Berlin)* **1995**, *120*, 177−185.
(5) Aldrich, J. V. Analgesics. In *Burger's Medicinal Chemistry and Drug Discovery*, 5th ed.; Wolff, M. E., Ed.; John Wiley & Sons: New York, 1996; pp 321−441.
(6) Hazum, E.; Chang, K. J.; Shecter, Y.; Wilkinson, S.; Cautrecasas, P. Fluorescent and Photoaffinity Enkephalin Derivatives: Preparation and Interaction with Opiate Receptors. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 841−846.
(7) Mihara, H.; Lee, S.; Shimohigashi, Y.; Aoyagi, H.; Kato, T.; Izumiya, N.; Costa, T. δ and μ Opioid Receptor Probes: Fluorescent Enkephalins with High Receptor Affinity and Specificity. *FEBS Lett.* **1985**, *193*, 35−38.
(8) Fournie-Zaluski, M. C.; Gacel, G.; Roques, B. P.; Senault, B.; Lecomte, J. M.; Malfroy, B.; Swerts, J. P.; Schwartz, J. C. Fluorescent Enkephalin Derivatives with Biological Activity. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 300−305.
(9) Correa, F. M.; Innis, R. B.; Rouot, B.; Pasternak, G. W.; Snyder, S. H. Fluorescent Probes of alpha- and beta-Adrenergic and Opiate Receptors: Biochemical and Histochemical Evaluation. *Neurosci. Lett.* **1980**, *16*, 47−53.
(10) Kolb, V. M.; Koman, A.; Terenius, L. Fluorescent Probes for Opioid Receptors. *Life Sci.* **1983**, *33*, 423−426.
(11) Goldstein, A.; Nestor, J. J.; Naidu, A.; Newman, S. R. "DAKLI": A Multipurpose Ligand with High Affinity and Selectivity for Dynorphin (κ Opioid) Binding Sites. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7375−7379.
(12) Archer, S.; Medzihradsky, F.; Seyed-Mozaffari, A.; Emmerson, P. J. Synthesis and Characterization of 7-Nitrobenzo-2-oxa-1,3-diazole (NBD)-Labeled Fluorescent Opioid. *Biochem. Pharmacol.* **1992**, *43*, 301−306.
(13) Emmerson, P. J.; Archer, S.; El-Hamouly, W.; Mansour, A.; Akil, H.; Medzihradsky, F. Synthesis and Characterization of 4,4-Difluoro-4-bora-3a,4a-diaza-*s*-Indacene (BODIPY)-Labeled Fluorescent Ligands for the Mu Opioid Receptor. *Biochem. Pharmacol.* **1997**, *54*, 1315−1322.

(14) Carraway, K. L.; Cerione, R. A. Fluorescent-labeled Growth Factor Molecules Serve as Probes for Receptor Binding and Endocytosis. *Biochemistry* **1993**, *32*, 12039−12045.
(15) Fay, S. P.; Posner, R. G.; Swann, W. N.; Sklar, L. A. Real-Time Analysis of the Assembly of Ligand, Receptor, and G-Protein by Quantitative Fluorescence Flow Cytometry. *Biochemistry* **1991**, *30*, 5066−5075.
(16) Tota, M. R.; Daniel, S.; Sirotina, A.; Mazina, K. E.; Fong, T. M.; Longmore, J.; Strader, C. D. Characterization of a Fluorescent Substance P Analogue. *Biochemistry* **1994**, *33*, 13079−13086.
(17) Carraway, K. L.; Koland, J. G.; Cerione, R. A. Location of the Epidermal Growth Factor Binding Site on the EGF Receptor. A Resonance Energy Transfer Study. *Biochemistry* **1990**, *29*, 8741−8747.
(18) Koman, A.; Terenius, L. Bifunctional Enkephalin Analogues for Affinity Separation Purposes. *FEBS Lett.* **1980**, *118*, 293−295.
(19) Hochhaus, G.; Gibson, B. W.; Sadee, W. Biotinylated Human β-Endorphins as Probes for the Opioid Receptor. *J. Biol. Chem.* **1988**, *263*, 92−97.
(20) Hochhaus, G.; Patthy, A.; Schwietert, R.; Santi, D. V.; Sadee, W. [Biocytin^{13}]Dynorphin A 1−13 Amide: A Potential Probe for the κ-Opioid Receptor. *Pharm. Res.* **1988**, *5*, 790−794.
(21) Eppler, C. M.; Hulmes, J. D.; Wang, J. B.; Johnson, B.; Corbett, M.; Luthin, D. R.; Uhl, G. R.; Linden, J. Purification and Partial Amino Acid Sequence of a mu-opioid Receptor from Rat Brain. *J. Biol. Chem.* **1993**, *268*, 26447−26451.
(22) Cotton, R.; Giles, M. G.; Miller, L.; Shaw, J. S.; Timms, D. ICI 174864: A Highly Selective Antagonist for the Opioid Delta-Receptor. *Eur. J. Pharmacol.* **1984**, *97*, 331−332.
(23) Corbett, A. D.; Gillan, M. G.; Kosterlitz, H. W.; McKnight, A. T.; Paterson, S. J.; Robson, L. E. Selectivities of Opioid Peptide Analogues as Agonists and Antagonists at the Delta-Receptor. *Br. J. Pharmacol.* **1984**, *83*, 271−279.
(24) Costa, T.; Herz, A. Antagonists with Negative Intrinsic Activity at Delta Opioid Receptors Coupled to GTP-binding Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7321−7325.
(25) Aldrich Lovett, J.; Portoghese, P. S. *N*,*N*-Dialkylated Leucine Enkephalins as Potential Delta Opioid Receptor Antagonists. *J. Med. Chem.* **1987**, *30*, 1144−1149.
(26) Schiller, P. W.; Nguyen, T. M. D.; Weltrowska, G.; Wilkes, B. C.; Marsden, B. J.; Lemieux, C.; Chung, N. N. Differential Stereochemical Requirements of μ vs δ Opioid Receptors for Ligand Binding and Signal Transduction: Development of a Class of Potent and Highly δ-Selective Antagonists. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11871−11875.
(27) Temussi, P. A.; Salvadori, P. A.; Bianchi, C.; Guerrini, R.; Tomatis, R.; Lazarus, L. H.; Picone, D.; Tancredi, T. Selective Opioid Dipeptides. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 933−939.
(28) Mosberg, H. I. Pharmacophore Elements of the TIPP Class of Delta Opioid Receptor Antagonists. *Lett. Pept. Sci.* **1994**, *1*, 69−72.
(29) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N. N.; Zelent, B.; Wilkes, B. C.; Carpenter, K. A. Structure-Agonist/Antagonist Activity Relationships of TIPP Analogues. In *Peptides: Chemistry, Structure and Biology*; Kaumaya, T. P., Hodges, R. S., Eds.; Mayflower Scientific Inc.: Kingswinford, England, 1996; pp 609−611.
(30) Tourwe, D.; Mannekens, E.; Diem, T. N. T.; Verheyden, P.; Jaspers, H.; Toth, G.; Peter, A.; Kertesz, I.; Torok, G.; Chung, N. N.; Schiller, P. W. Side Chain Methyl Substitution in the δ-Opioid Receptor Antagonist TIPP has an Important Effect on the Activity Profile. *J. Med. Chem.* **1998**, *41*, 5167−5176.
(31) Schiller, P. W.; Weltrowska, G.; Berezowska, I.; Nguyen, T. M.-D.; Wilkes, B. C.; Lemieux, C.; Chung, N. N. The TIPP Opioid Peptide Family: Development of δ Antagonists, δ Agonists, and Mixed μ Agonist/δ Antagonists. *Biopolymers* **1999**, *51*, 411−425.
(32) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Wilkes, B. C.; Chung, N. N.; Lemieux, C. TIPP[psi]: A Highly Potent and Stable Pseudopeptide Delta Opioid Receptor Antagonist with Extraordinary Delta Selectivity. *J. Med. Chem.* **1993**, *36*, 3182−3187.
(33) Marsden, B. J.; Nguyen, T. M.-D.; Schiller, P. W. Spontaneous Degradation via Diketopiperazine Formation of Peptides Containing a Tetrahydroisoquinoline-3-carboxylic acid Residue in the 2-Position of the Peptide Sequence. *Int. J. Pept. Protein Res.* **1993**, *41*, 313−316.
(34) Schmidt, R.; Kalman, A.; Chung, N. N.; Lemieux, C.; Horvath, C.; Schiller, P. W. Structure−activity Relationships of Dermorphin Analogues Containing N-Substituted Amino Acids in the 2-Position of the Peptide Sequence. *Int. J. Pept. Protein Res.* **1995**, *46*, 47−55.
(35) Schiller, P. W.; Weltrowska, G.; Nguyen, T. M.-D.; Wilkes, B. C.; Chung, N. N.; Lemieux, C. Conformationally Restricted Deltorphin Analogues. *J. Med. Chem.* **1992**, *35*, 3956−3961.
(36) Schmidt, R.; Chung, N. N.; Lemieux, C.; Schiller, P. W. Tic(2)-Substitution in Dermorphin, Deltorphin-I and Dynorphin-A Analogues − Effect on Opioid Receptor-Binding and Opioid Activity In-vitro. *Regul. Pept.* **1994**, *54*, 259−260.
(37) Coste, J.; Le-Nguyen, D.; Castro, B. PyBOP: A New Peptide Coupling Reagent Devoid of Toxic Byproduct. *Tetrahedron Lett.* **1990**, *31*, 205−208.
(38) Snyder, K. R.; Story, S. C.; Heidt, M. E.; Murray, T. F.; DeLander, G. E.; Aldrich, J. V. Effect of Modification of the Basic Residues of Dynorphin A-(1−13) Amide on Kappa Opioid Receptor Selectivity and Opioid Activity. *J. Med. Chem.* **1992**, *35*, 4330−4333.
(39) Vojkovsky, T. Detection of Secondary Amines on Solid Phase. *Pept. Res.* **1995**, *8*, 236−237.
(40) Arttamangkul, S.; Ishmael, J. E.; Murray, T. F.; Grandy, D. K.; DeLander, G. E.; Kieffer, B. L.; Aldrich, J. V. Synthesis and Opioid Activity of Conformationally Constrained Dynorphin A Analogues. 2. Conformational Constraint in the "Address" Sequence. *J. Med. Chem.* **1997**, *40*, 1211−1218.
(41) Cheng, Y.; Prusoff, W. H. Relationship Between the Inhibition Constant (K_i) and the Concentration of Inhibitor which Causes 50 Percent Inhibition (I_{50}) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099−3108.

JM000362H