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Modification of the Phe^3 aromatic moiety in delta receptor-selective dermorphin/deltorphin-related tetrapeptides

Effects on opioid receptor binding

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The previously described cyclic delta opioid receptor-selective tetrapeptide H-Tyr-D-Cys-Phe-D-Pen-OH (JOM-13) was modified at residue 3 by incorporation of both natural and unnatural amino acids with varying steric, electronic, and lipophilic properties. Effects on mu and delta opioid receptor binding affinities were evaluated by testing the compounds for displacement of radiolabeled receptor-selective ligands in a guinea pig brain receptor binding assay. Results obtained with the bulky aromatic 1-Nal^3 and 2-Nal^3 substitutions suggest that the shape of the receptor subsite with which the side chain of the internal aromatic residue interacts differs for delta and mu receptors. This subsite of either receptor can accommodate the transverse steric bulk of the 1-Nal^3 side chain but only the delta receptor can readily accept the more elongated 2-Nal^3 side chain. Several analogs with pi-excessive heteroaromatic side chains in residue 3 were examined. In general, these analogs display diminished binding to mu and delta receptors, consistent with previous findings for analogs with residue 3 substitutions of modified electronic character. Several analogs with alkyl side chains in residue 3 were also examined. While delta receptor binding affinity is severely diminished with Val^3, Ile^3, and Leu^3 substitutions, Cha^3 substitution is very well tolerated, indicating that, contrary to the widely held belief, an aromatic side chain in this portion of the ligand is not required for delta receptor binding. Where possible, comparison of results in this delta-selective tetrapeptide series with those reported for analogous modification in the cyclic delta-selective pentapeptide [D-Pen^2, D-Pen^5]enkephalin (DPDPE) and linear pentapeptide enkephalins reveals similar trends.

Key words: aromatic side chain; conformational restriction; delta opioid receptor; deltorphin analog; dermorphin analog; disulfide bond; enkephalin analog; heteratom; mu opioid receptor; opioids

The heterogeneity of opioid receptors is well-established and the existence of a least three opioid receptor types, designated mu, delta and kappa, is widely accepted. In recent years, a body of evidence has accumulated which indicates that the mu and delta receptor types differ in their structural and conformational requirements for peptide ligand binding (1, 2) and may mediate different pharmacological and physiological events (3). Conformational restriction of enkephalin analogs has proven a valuable means for assessing disparities between the

Abbreviations recommended by IUPAC-IUB Commission on Biochemical Nomenclature have been used. Other abbreviations: ACN, acetonitrile; Boc, t-butyloxycarbonyl; COSY, correlation spectroscopy; DAMGO, [D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DPDPE, [D-Pen^2, D-Pen^5]enkephalin; DPM, disintegrations per minute; FAB-MS, fast atom bombardment-mass spectrometry; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reverse phase-high performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TSP, (trimethylsilyl)propionic acid; Tris, tris[hydroxylmethyl]aminomethane; U69,593, 5α,7α,8β-(−)-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide. Unusual amino acids: Cha, 3-(cyclohexyl)alanine; 1-Nal, 3-(1-naphthyl)alanine; 2-Nal, 3-(2-naphthyl)alanine; Pal, 3-(3-pyridyl)alanine; Pen, penicillamine; Thi, 3-(2-thienyl)alanine.

peptide binding characteristics at each of the opioid receptor types, since peptide analogs designed with appropriate conformational constraints may possess the ability to adopt the conformation required for interaction at one receptor type but not another. We have previously described (4) a series of disulfide-containing tetrapeptides (des-Gly^3 enkephalin analogs) which can be viewed as truncated, cyclic analogs of the frog skin derived heptapeptides, dermorphin (5) and deltorphin (6, 7). The lead compound in this series, Tyr-D-Cys-Phe-D-PenOH (JOM-13), exhibits considerable delta receptor selectivity and a higher delta binding affinity than the standard delta selective ligand, [D-Pen^2, D-Pen^5]enkephalin (DPDPE (8)) (4). In addition, JOM-13 and its analogs possess a greater degree of structural rigidity than DPDPE due to the absence of the central glycine residue found in the pentapeptide and might therefore prove to be even more amenable to conformational analysis than DPDPE. In an effort to further investigate this tetrapeptide series, to allow comparison with some of the cyclic disulfide-containing pentapeptide enkephalin analogs, and to explore the possibility that the Phe^3 residue of dermorphin/deltorphin-like tetrapeptides and the Phe^4 residue of enkephalin-like pentapeptides interact differently at mu and/or delta opioid receptors (9), we have prepared several analogs of JOM-13 with modifications designed to assess the effect of substitution of amino acids with varying steric, electronic, and lipophilic properties at each residue. These alterations of the cyclic parent tetrapeptide, particularly at the aromatic Phe residue, provide interesting insights into binding requirements of this series to the mu and delta opioid receptors. Both the Tyr^1 and Phe^4 (or Phe^3) aromatic side chains are believed to play a critical role in the enkephalin binding conformation, each residue interacting at the receptor (2). Analogs described here were designed to probe the steric, electronic, and lipophilic requirements of the residue 3 side chain aromatic function by replacement of the Phe^3 residue of JOM-13 with larger aromatic groups, with heteroatomic aromatic side chains, or with nonaromatic aliphatic moieties.

METHODS

Peptides

Peptides were synthesized by standard solid phase methods similar to those previously described for the synthesis of JOM-13 (4), using chloromethylated polystyrene (Merrifield) resin crosslinked with 1% divinylbenzene. Trifluoroacetic acid (TFA) was used for deprotection, and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were employed to facilitate coupling. Alpha-amino functions were t-butyloxycarbonyl (Boc) protected, and *p*-methylbenzyl protection was employed for the labile side chain sulfhydryl groups of Cys and Pen. Simultaneous deprotection and cleavage from the resin were accomplished by treatment with anhydrous hydrogen fluoride in the presence of 5% *p*-cresol and 5% *p*-thiocresol (10) for 45 min at 0°. Prior to cyclization, linear peptides were purified by reverse phase-high performance liquid chromatography (RP-HPLC) on a Vydac 218TP C-18 column (2.5 × 22 cm) using the solvent system 0.1% TFA in water/0.1% TFA in acetonitrile, by a gradient of 10–50% organic component in 40 min. Cyclization to disulfide analogs was accomplished by treatment of an aqueous solution (pH 8.5) of the linear free sulfhydryl-containing species with $K_3Fe(CN)_6$ for ca. 1 h. Cyclized peptides were then purified by RP-HPLC as described above, and pure fractions were lyophilized.

*Tyr-D-Cys-(1-Nal)-D-PenOH (**1**).* The title peptide was prepared as described for JOM-13 (4) using Boc-3-(1-naphthyl)-L-alanine (1-Nal) in place of Boc-Phe in the solid phase synthesis protocol. A 1.3 g sample of the protected, resin-bound peptide was treated with 12 mL HF, 0.6 g cresol, and 0.6 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 01% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 113 mg of pure linear peptide. A sample of 43.0 mg of this peptide was oxidized as described above, yielding after RP-HPLC, 20.1 mg of the title disulfide-containing peptide.

*Tyr-D-Cys-(2-Nal)-D-PenOH (**2**).* The title peptide was prepared as described for JOM-13 (4) by substituting Boc-3-(2-naphthyl)-L-alanine (2-Nal) for Boc-Phe. A 1.3 g sample of the protected, resin-bound peptide was treated with 11 mL HF, 0.6 g *p*-cresol, and 0.6 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 79.5 mg of pure linear peptide. A sample of 43.0 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 14.5 mg of the disulfide-containing peptide.

*Tyr-D-Cys-Thi-D-PenOH (**3a** and **3b**).* The title peptide was prepared as described for JOM-13 (4) using Boc-3-(2-thienyl)-D,L-alanine (Thi) in place of Boc-Phe. A 1.1 g sample of the protected, resin-bound precursor peptide was treated with 10 mL HF, 0.6 g *p*-cresol, and 0.6 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 2 pools of impure diastereomeric peptide, each greatly enriched in one component, with masses of 26.7 mg and 45.9 mg, respectively. In each case, the entire sample was subjected to oxidation as described above. The earlier-eluting disulfhydryl peptide yielded, after oxidation and

RP-HPLC, 11.4 mg of cyclized peptide **3a**. The later-eluting linear peptide yielded, after oxidation and purification 7.2 mg of disulfide-containing peptide **3b**.

Tyr-D-Cys-Trp-D-PenOH (4). The peptide was prepared as described for JOM-13 (4) using Boc-L-tryptophan (Trp) in place of Boc-Phe. It should be noted that no indole-N protection was employed for this residue. Instead, anisole was used as a cation scavenger in the TFA deprotection solution, to protect N^{in}-alkylation. A 1.2 g sample of the protected, resin-bound precursor peptide was treated with 11 mL HF, 0.6 g *p*-cresol, and 0.6 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 115 mg of pure linear peptide. A sample of 49.5 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 25.0 mg of the disulfide-containing peptide.

Tyr-D-Cys-His-D-PenOH (5). The title peptide was prepared as described for JOM-13 (4) using Boc-N^{im}-tosyl-L-histidine (His). Since it is sufficiently acidic to cleave the tosyl protecting group, HOBt was eliminated from every coupling. A 1.5 g sample of the protected, resin-bound precursor peptide was treated with 13 mL HF, 0.7 g *p*-cresol, and 0.7 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, as described above; however, due to the high polarity of the His^3 substituted peptide and the large sample volume, only partial purification was effected. Following lyophilization, the crude material was subjected to further isocratic RP-HPLC purification at 8% organic component, and 38.0 mg of pure linear peptide was obtained. This entire sample was subjected to oxidation as described above, yielding after extensive RP-HPLC (standard conditions, followed by isocratic conditions at 14% organic component on a 1.0 cm × 25 cm column), 5.5 mg of the desired disulfide-containing peptide.

Tyr-D-Cys-Pal-D-PenOH (6). The peptide was prepared as described for JOM-13 (4) using Boc-3-(3-pyridyl)-L-alanine (Pal) in place of Boc-Phe. A 1.1 g sample of the protected, resin-bound precursor peptide was treated with 10 mL HF, 0.5 g *p*-cresol, and 0.5 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 99.6 mg of pure linear peptide. A sample of 42.0 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 14.5 mg of the disulfide-containing peptide.

Tyr-D-Cys-Cha-D-PenOH (7). The title peptide was prepared as described for JOM-13 (4) employing Boc-3-(cyclohexyl)-L-alanine (Cha). A 1.3 g sample of the protected, resin-bound precursor peptide was treated with 12 mL HF, 0.6 g *p*-cresol, and 0.6 g *p*-thiocresol, and after extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC. This yielded, after lyophilization, 67.5 mg of pure linear peptide. A sample of 41.8 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 23.0 mg of the target disulfide-containing peptide.

Tyr-D-Cys-Val-D-PenOH (8). The title peptide was prepared as described for JOM-13 (4) by substituting Boc-L-valine (Val) for Boc-Phe. A 1.3 g sample of the protected, resin-bound peptide was treated with 11 mL HF, 0.6 g *p*-cresol, and 0.6 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 89.0 mg of linear peptide. A sample of 39.0 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 6.5 mg of the target disulfide-containing peptide.

Tyr-D-Cys-Leu-D-PenOH (9). The title peptide was prepared as described for JOM-13 (4) by substituting Boc-L-leucine (Leu) for Boc-Phe. A 1.1 g sample of the protected, resin-bound peptide was treated with 10 mL HF, 0.5 g *p*-cresol, and 0.5 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 125 mg of linear peptide. A sample of 32.0 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 16.8 mg of the target disulfide-containing peptide.

Tyr-D-Cys-Ile-D-PenOH (10). The title peptide was prepared as described for JOM-13 (4) by substituting Boc-L-isoleucine (Ile) for Boc-Phe. A 1.1 g sample of the protected, resin-bound peptide was treated with 10 mL HF, 0.5 g *p*-cresol, and 0.5 g *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 42.5 mg of pure linear peptide. A sample of 20.5 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 5.0 mg of the target disulfide-containing peptide.

Peptide analysis

Final product purity was assessed by analytical RP-HPLC on a Vydac C-18 column (4.6 mm × 250 mm) by a gradient of 10–70% organic component over 30 min,

with a flow rate of 1 mL/min. Peaks were monitored at 230 nm and 280 nm and analyzed with Waters Maxima 820 software. Peptide purity was >95% in each case. All analytical RP-HPLC gradients were run using the solvent system 0.1% TFA in water/0.1% TFA in acetonitrile. Peaks which also appeared in chromatograms in which no peptide was injected were considered to be artifacts and were ignored. Peptide purity was then assessed by peak integration. In addition, all newly reported peptides were subjected to thin-layer chromatography (TLC) on precoated silica gel plates in three solvent systems: (solvent ratios are volume:volume) (A) *n*-butanol:acetic acid:water (4:1:5, organic component only); (B) *n*-butanol:water (containing 3.5% acetic acid and 1.5% pyridine) (1:1, organic component only); (C) *n*-amyl alcohol:pyridine:water (7:7:6). Three methods of visualization (ninhydrin, ultraviolet absorption, iodine vapor) detected a single spot after TLC in each solvent system.

To assure that the final peptides had been successfully cyclized, the presence of free sulfhydryl groups in final product peptides was assessed by testing with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). When combined with free sulfhydryl-containing species, this reagent forms an adduct giving a characteristic yellow color and absorbance at 412 nm (11). In all cases, absorbances of the final peptides at this wavelength were indistinguishable from sulfhydryl-free controls confirming the absence of free sulfhydryl groups.

^{1}H NMR spectra, recorded on General Electric GN-500 and IBM WP 270 SY spectrometers, operating at 500 MHz and 270 MHz, respectively, were used analytically for structure confirmation of the final peptides. Samples contained 1–2 mg of the compound in D_2O, acidified D_2O or DMSO solvent, with 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionic acid, sodium salt (TSP-d_4) added as the internal standard. Diagnostic resonances arising from the methyl groups and the alpha proton of penicillamine and the aromatic resonances of tyrosine confirmed the presence of these residues. No resonances were observed that could not be accounted for, and all resonances pertaining to specifically-modified residue 3 amino acids were present (peak patterns consistent with appropriate aromatic and nonaromatic residues). Some analogs were subjected to 2D COSY analysis to establish intraresidue connectivities and/or to 2D NOESY experiments to obtain primary sequences from intraresidue NOE interactions. In each case, the anticipated sequence was confirmed. All peptides displayed NH-αCH-βCH$_2$ connectivities consistent with the presence of cysteine (half cystine).

Final products were subjected to fast atom bombardment-mass spectrometry (FAB-MS) to determine product molecular weights. In all cases the appropriate, predicted molecular weights were confirmed.

Receptor binding assays

Binding assays were based on the displacement by the test compounds of radiolabeled ([^{3}H]) [D-Ala^2, NMe-Phe^3, Gly^5-ol]enkephalin (DAMGO) (mu ligand), DPDPE (delta ligand), and U69,593 (kappa ligand) (all from Amersham) from opioid receptors in guinea pig brain membrane receptors. For the membrane preparations, 10 frozen guinea pig brains (Pel-Freez Biologicals), weighing approximately 36 g, were thawed at 5° for 3 h. The brains were then suspended in cold 50 mM Tris buffer, pH 7.4, at a concentration of 0.1 g/mL (approximately 360 mL total) and homogenized for 20 s on a Brinkman Polytron Homogenizer (model # PT 10–35, probe model # PT 20 ST) at one-half maximum speed. The homogenate was centrifuged for 15 min at 14000 × *g* at 4°, and the supernatant was discarded. The pellets were resuspended in 360 mL cold 50 mM Tris buffer by homogenization for 20 s at one-quarter maximum speed. Centrifugation at 4° was repeated at 14000 × *g* for 15 min, and the supernatant was again discarded. The pellets were again resuspended by homogenization (20 s at one-quarter speed) in 360 mL 50 mM Tris buffer at 25°, pH 7.4. After incubation at 37° for 30 min to release endogenous opioids, the homogenate was centrifuged at 14000 × *g* for 15 min at 4°. Pellets of brain membrane finally were resuspended in 400 mL 50 mM Tris buffer on ice, pH 7.4, by homogenization for 20 s at one-quarter speed. The membrane suspension was divided into aliquots of the appropriate size for a set of binding assays (approximately 10 mL) and stored in capped plastic test tubes at −80°. Before use in a binding assay, each aliquot was thawed, homogenized (8 strokes in a glass homogenizer with a Teflon pestle), and stored on ice.

Receptor binding assays, done in triplicate, were performed as follows: In polypropylene tubes in an ice bath, assay components were carefully pipetted in the following order: 25 μL water (total binding), displacing ligand in water (nonspecific binding), or test compound (varying concentrations prepared by serial dilutions) in water; then, 200 μL membrane preparation (determined in control experiments to be in the linear range of the specific binding vs. protein concentration dependence). These were gently vortexed and preincubated in a temperature regulated water bath for 15 min at 25°, then placed on ice for the addition of 25 μL of the radioligand solution in 50 mM Tris buffer (pH 7.4). The assay suspension in each tube was again gently vortexed and incubated in a water bath to reach equilibrium (75 min for assays using 0.6 nM [^{3}H]DAMGO and 1.8 nM [^{3}H]DPDPE, 90 min for assays using 0.9 nM [^{3}H]U69,593, each determined from time dependence studies of specific binding) at 25°. Before harvesting the samples, the filter paper sheet was pretreated for 1 min with amyl alcohol-saturated water, followed by one Tris buffer wash. Subsequently, the samples were diluted, rapidly filtered, and washed twice with 4 mL cold Tris buffer (pH 7.4), using a 24-tube Brandel Cell

Harvester (model M-24R). Filter discs were removed with forceps and placed in scintillation vials, to which 1 mL ethanol and 10 mL Scintiverse liquid scintillant were added. Capped vials were shaken by hand for 10 s and allowed to sit several hours. Radioactivity on the filters was then determined by liquid scintillation counting for 10 min per sample. Radioligand stock solution aliquots also were counted similarly after transfer into vials and addition of 10 mL Scintiverse. Inhibition of radiolabeled ligand binding by the test compounds was computed from maximal specific binding, determined with an appropriate excess of unlabeled ligand (10 μM DPDPE or U69,593; 5 μM DAMGO). At the concentrations employed for the assays, total binding of each radioligand to the membrane preparation typically measured ~2000 DPM, while nonspecific binding was approximately 200 DPM for [^{3}H]DAMGO and [^{3}H]U69,593 and 400 DPM for [^{3}H]DPDPE. IC_{50} values were obtained by linear regression from plots relating inhibition of specific binding to the log of 12 different ligand concentrations, using the computer programs LIGAND (12) (Biosoft Software). K_i values were similarly calculated using values for K_D of each ligand, determined by analysis of saturation binding experiments. Values of K_D so determined were: [^{3}H]DPDPE, K_D = 2.97 nM; [^{3}H]DAMGO, K_D = 0.85 nM. For binding to kappa receptors, expected to be weak for all analogs, the protocol was altered to include only 5 ligand concentrations (in duplicate). Analysis by LIGAND, using K_D = 0.9 nM for [^{3}H]U69,593, yielded estimates or lower limits for K_i.

RESULTS AND DISCUSSION

The following modified and naturally occurring amino acids were incorporated as residue 3 substitutions in the lead tetrapeptide, Tyr-D-Cys-Phe-D-PenOH (JOM-13): 3-(1-naphthyl)-L-alanine (1-Nal) (**1**), 3-(2-naphthyl)-L-alanine (2-Nal) (**2**), 3-(2-thienyl)-D,L-alanine (Thi) (**3a** and **3b**), L-tryptophan (Trp) (**4**), L-histidine (His) (**5**), 3-(3-pyridyl)-L-alanine (Pal) (**6**), 3-(cyclohexyl)-L-alanine (Cha) (**7**), L-valine (Val) (**8**), L-leucine (Leu) (**9**), and L-isoleucine (Ile) (**10**). Each of these modifications was designed to test the influences of varying steric and electronic character and lipophilicity in this region of the peptide molecule; we have reported previously (13) that each of these determinants appears to play an important role in interaction with the opioid binding sites. Receptor binding affinities for mu, delta, and kappa opioid receptors, as measured by K_i values (± standard errors of the mean) against [^{3}H]DAMGO, [^{3}H]DPDPE, and [^{3}H]U69,593, respectively, for these peptides are provided in Table 1 as are comparable data for DPDPE and the parent tetrapeptide, JOM-13. As can be seen from Table 1, K_i values for kappa binding for all peptides reported here indicate low affinity for the kappa receptor site. Values

TABLE 1

Opioid receptor binding profiles of cyclic tetrapeptides

Peptide	Analog No.	Binding K_i (nM)			$K_i(\mu)/K_i(\delta)$
		DAMGO	DPDPE	U69,593	
Tyr-D-Pen-Gly-Phe-D-PenOH	(DPDPE)	810 ± 66	3.98 ± 0.46	> 10000	204
Tyr-D-Cys-Phe-PenOH	(JOM-13)	107 ± 1.0	1.79 ± 0.11	> 10000	60
Tyr-D-Cys-(1-Nal)-D-PenOH	**1**	91.5 ± 6.5	2.85 ± 0.33	~1500	32.1
Tyr-D-Cys-(2-Nal)-D-PenOH	**2**	590 ± 90	2.23 ± 0.20	~3000	265
Tyr-D-Cys-Thi-D-PenOH	**3a**	1880 ± 608	65.3 ± 7.2	> 10000	28.8
Tyr-D-Cys-Thi-D-PenOH	**3b**	180 ± 17	9.96 ± 1.8	> 10000	18.1
Tyr-D-Cys-Trp-D-PenOH	**4**	183 ± 19	2.49 ± 0.11	~600	73.5
Tyr-D-Cys-His-D-PenOH	**5**	10700 ± 860	56.3 ± 4.5	> 10000	200
Tyr-D-Cys-Pal-D-PenOH	**6**	1610 ± 350	132 ± 17	> 10000	12.2
Tyr-D-Cys-Cha-D-PenOH	**7**	20.2 ± 1.7	4.89 ± 0.54	> 10000	4.13
Tyr-D-Cys-Val-D-PenOH	**8**	7400 ± 1180	827 ± 85	> 10000	8.95
Tyr-D-Cys-Leu-D-PenOH	**9**	413 ± 44	188 ± 44	> 10000	2.20
Tyr-D-Cys-Ile-D-PenOH	**10**	543 ± 70	350 ± 41	> 10000	1.55

DAMGO = [^{3}H][D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin.
DPDPE = [^{3}H][D-Pen^2, D-Pen^5]enkephalin.

were typically >10000 nM and in all cases reflected substantially weaker binding than at either mu or delta sites. Also presented in Table 1 is the ratio of the mu and delta K_i values for each compound, which provides a measure of the delta (vs. mu) selectivity of the compound. Physicochemical data for newly reported peptides are presented in Table 2.

Compounds **1–6** were designed to assess the effects of altered size, lipophilicity, and electronic character of the residue 3 side chain, while maintaining aromaticity. The naphthylalanine-containing analogs **1** and **2** differ in the point of side chain attachment of the aromatic naphthyl system to the β-carbon and, therefore, differ in the orientation of the aromatic function. Replacement of the Phe phenyl ring with a naphthyl moiety increases the steric bulk and lipophilicity at this crucial residue, as reflected by relatively late RP-HPLC elution times. Further, the pharmacological consequences of the differing orientation of the aromatic naphthyl moiety relative to the backbone allow the nature of the receptor subsite with which the residue 3 side chain interacts to be probed. When compared to JOM-13, **1**, containing 1-Nal, is essentially equiactive at the mu receptor while suffering only a minor loss of affinity at the delta type. The 2-Nal-containing analog, **2**, on the other hand, retains similar delta activity but displays a five fold reduction in affinity at the mu receptor; consequently, the delta selectivity of this analog is enhanced ca. fourfold relative to JOM-13 and, in fact, **2** displays increased delta receptor selectivity and affinity relative to DPDPE. The similar delta binding affinities of both naphthylalanines, each comparable to that observed for JOM-13, suggest that the delta receptor binding pocket for this element of the pharmacophore is both "broad" and "deep" and can thus tolerate rather bulky, flat aromatic side chains at this position. By contrast, the reduced mu binding affinity of **2** compared with **1** suggests that this receptor binding subsite is broad but not deep. The mu receptor results observed here are consistent with previous observations in cyclic, mu receptor selective tetrapeptides as well as in pentapeptide enkephalin analogs. In the former case, Schiller has observed in his mu selective series of structure: Tyr-D-Orn-X-GluNH_2, that The 1-Nal[3] analog displays comparable mu receptor affinity relative to Tyr-D-Orn-Phe-GluNH_2, while the 2-Nal[3] compound exhibits a reduction in mu binding affinity (14). For pentapeptide enkephalins, it has been reported that in linear enkephalinamide analogs the 2-Nal[4] substitution, com-

TABLE 2

Physiochemical data for cyclic tetrapeptides

Peptide	Analog No.	HPLC		Purity (%)[c]	[O]Yield (%)[d]	$TLCR_f$[e]			FAB-MS Mol wt
		I[a]	II[b]			A	B	C	
Tyr-D-Cys-(1-Nal)-D-PenOH	**1**	17	35	100	47	0.70	0.62	0.66	610
Tyr-D-Cys-(2-Nal)-D-PenOH	**2**	17	36	100	34	0.71	0.71	0.73	610
Tyr-D-Cys-Thi-D-PenOH	**3a**	16	31	96	43	0.54	0.39	0.59	566
Tyr-D-Cys-Thi-D-PenOH	**3b**	14	29	98	16	0.56	0.49	0.62	566
Tyr-D-Cys-Trp-D-P-enOH	**4**	16	31	99	50	0.61	0.59	0.67	599
Tyr-D-Cys-His-D-PenOH	**5**	12	25	98	15	0.02	0.02	0.18	550
Tyr-D-Cys-Pal-D-PenOH	**6**	12	26	99	35	0.23	0.18	0.67	561
Tyr-D-Cys-Cha-D-PenOH	**7**	16	32	100	55	0.78	0.76	0.79	566
Tyr-D-Cys-Val-D-PenOH	**8**	13	26	96	17	0.41	0.35	0.64	512
Tyr-D-Cys-Leu-D-PenOH	**9**	14	28	99	53	0.68	0.51	0.69	526
Tyr-D-Cys-Ile-D-PenOH	**10**	13	27	99	25	0.69	0.52	0.70	526

[a] HPLC elution on a Vydac 218TP C-18 column (0.46 cm × 25 cm) using a linear gradient of 10–70% organic component in 30 min at a flow rate of 1 mL/min. Solvent system was 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. The solvent front ("breakthrough peak") eluted at 3.0 min.

[b] HPLC elution time on a Vydac 218TP C-18 column (0.46 cm × 25 cm) using a linear gradient of 0–70% organic component in 70 min at a flow rate of 1 mL/min. Solvent system was 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. The solvent front ("breakthrough peak") eluted at 3.0 min.

[c] Purity of final cyclized peptide as assessed by HPLC peak integration from chromatograms at 230 nm.

[d] Yield in peptide cyclization/oxidation reaction, after purification.

[e] R_f values for thin-layer chromatograms in solvent systems: (A) *n*-butanol:acetic acid:water (4:15, organic component only); (B) *n*-butanol:water (containing 3.5% acetic acid and 1.5% pyridine) (1:1, organic component only); (C) *n*-amyl alcohol:pyridine:water (7:7:6).

pared with 1-Nal[4] substitution, results in a 10-fold potency drop in the guinea pig ileum (GPI) bioassay (15), a prototypical mu receptor assay (16).

In analogs **3a**, **3b**, **4**, **5**, and **6** the electronic and lipophilic character of the residue 3 side chain was modified considerably by the incorporation of various heteroaromatic moieties. For analog **5**, the possible partial positive charge (at physiological pH and under assay conditions at pH 7.4) on the 5-membered imidazole ring of the His[3] side chain makes it quite polar, and this hydrophilic analog elutes earliest on RP-HPLC of all members of the series (although, it should be kept in mind that under the acidic conditions of RP-HPLC, the charged nature of this residue is more evident than in the binding assay). Most likely as a consequence of this hydrophilicity, the mu and delta receptor binding affinities of analog **5** are reduced 100-fold and 30-fold, respectively. Similarly, compound **6**, which contains Pal, is very hydrophilic. The pK_a for the Pal side chain, however, is approximately 5.3 (16), so the positively charged form should be virtually unpopulated under assay conditions. Nonetheless, its delta binding is compromised 74-fold relative to JOM-13, and a 15-fold reduction in mu affinity is observed. As a result, this modification proves to be even more detrimental than His[3] substitution for delta receptor interaction, though mu binding is better for the Pal[3] analog. Compounds **3a**, **3b**, and **4** display intermediate lipophilic character when compared to other tetrapeptides in the series. Each of these aromatic modifications in fairly well-accommodated in this tetrapeptide series. Compound **3a** probably contains the D-Thi amino acid, which would account for its lower binding affinity than the diastereomeric peptide, **3b**, which displays only a slight to moderate reduction in opioid binding (1.5-fold reduction at the mu type and fivefold reduction at the delta receptor). Trp proves an even better substitution at residue 3 in this series, as analog **4** retains similar delta affinity to the lead compound and suffers only a slight decline in mu binding. A similar benign effect of Trp[4] for Phe[4] substitution in linear pentapeptide enkephalins has been reported. One further point pertaining to this particular group of analogs should be noted. The electronic character of the 5-membered heterocyclic aromatic systems for compounds **3a**, **3b**, and **5** should be expected to be pi-excessive. The resultant decline in delta binding affinity would be consistent with results of affinity reduction observed upon substitution on the Phe aromatic ring with electron releasing groups and the corresponding affinity enhancement associated with electron withdrawing substituents (13). Data for analog **6**, however, tend to contradict this analogy, since the 6-membered pi system of the Pal heterocycle should be electron deficient, and this analog displays less delta binding affinity than the aforementioned electron-rich analogs. This poor binding in general may be attributed to the hydrophilicity of the residue 3 side chain, consistent with the simultaneous requirement for lipophilicity in the Phe[3] substituted aromatic analogs (13).

Previous studies in the linear enkephalins have led to the proposal that the Phe aromatic ring is required for delta, but not mu, interaction (18); in fact, saturation of the phenyl ring resulting in Cha, a bulky and lipophilic side chain substituent, is well tolerated at the mu receptor (19). Compound **7** and the less bulky alkyl side chain-containing analogs **8**, **9**, and **10** were designed to examine this tendency in the cyclic tetrapeptide series. The Val[3] analog, **8**, exhibits a drastic reduction in binding affinity at both receptor types, 69-fold at the mu receptor and 462-fold at the delta type, relative to JOM-13. For the Leu[3] substitution in this series, both lipophilicity and side chain length of the non-aromatic residue are increased beyond those of Val. As a result, compound **9** apparently can achieve a greater binding interaction at both mu and delta opioid sites. However, binding affinities at both receptor types are still reduced relative to JOM-13, by a rather modest factor of 4 at mu and a more significant factor of 105 at delta sites. Similarly, the Ile[3] analog, **10**, displays poor delta receptor affinity (reduced 196-fold), while mu binding is only slightly reduced. While the results obtained with analogs **8–10** are in accord with previous reports suggesting a requirement for a residue 3/4 aromatic side chain (Phe or substitute) for delta but not mu binding, the binding data for **7**, the Cha[3] analog, are quite different. For this peptide, a fivefold enhancement in affinity at the mu receptor and an unanticipated retention of delta activity (affinity reduced only 2.7-fold relative to JOM-13) are exhibited. This implies that aromaticity of the third residue *per se* is not a requirement for binding of this series to the delta receptor, provided that the side chain is sufficiently lipophilic and reaches into the binding pocket. Apparently, the Val, Leu, and Ile side chains do not fit one or both of these criteria (or provide some other unfavorable interaction, e.g. due perhaps to steric effects resulting from β-substitution in the cases of Ile and Val), resulting in fairly inactive compounds. The side chain cyclohexyl functionality of **7** most likely takes on a chair conformation, and thus in shape approximates a flat aromatic moiety, able to extend into the binding pocket. The potent delta binding affinity of **7** suggests that either the long accepted hypothesis that an aromatic side chain (Phe or appropriate substitute) is required for delta binding is incorrect or that the tetrapeptide residue 3 and pentapeptide residue 4 side chains interact differently with the delta receptor, perhaps binding to different subsites. An explanation similar to this latter possibility has been proposed for mu ligand-receptor interactions to explain discrepancies in mu binding affinities of tetrapeptides and pentapeptide opioids (9); however, as we have previously reported (20), no correspondingly convincing evidence yet exists for delta receptor interactions. Recent evidence, in fact, suggests that the hypothesis that an internal aromatic residue is required for delta recep-

tor binding is no longer tenable. We have reported that Cha[4] substitution in a series of pentapeptide enkephalines related to DPDPE results in only a fourfold decrease in delta binding affinity (21), in excellent agreement with the observations reported here for analog **7**.

In summary, the results presented here suggest differences in the topology of the mu and delta receptor subsites responsible for binding the residue 3 (in dermorphin/deltorphin-like opioids) or residue 4 (in enkephalin analogs) side chain, with the delta receptor subsite consisting of a deeper hydrophobic pocket. The high delta receptor affinity of the Cha[3] analog, **7**, demonstrates that an aromatic residue, *per se*, is not required for interaction with this subsite of the delta receptor as long as a relatively flat, lipophilic side chain is available for this interaction. Finally, the data presented here are consistent with our working model that posits interaction with the same delta receptor binding loci for both tetrapeptide and pentapeptide opioids.

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REFERENCES

1. Hruby, V.J. & Gehrig, C.A. (1989) *Med. Res. Rev.* **9**, 343–401
2. Schiller, P.W. (1984) in *The Peptides: Analysis, Synthesis, Biology* (Udenfriend, S. & Meienhofer, J. eds.), pp. 220–268, Academic Press, New York
3. Olson, G.A., Olson, R.D. & Kastin, A.J. (1990) *Peptides* **11**, 1277–1304
4. Mosberg, H.I., Omnaas, J.R., Medzihradsky, F. & Smith, C.B. (1988) *Life Sci.* **43**, 1013–1020
5. Montecucchi, P.C., De Castiglione, R., Piani, S., Gozzini, L. & Erspamer, V. (1981) *Int. J. Peptide Protein Res.* **17**, 275–283
6. Mor, A., Delfour, A., Sagan, S., Amiche, M., Pradelles, P., Rossier, J. & Nicolas, P. (1989) *FEBS Lett.* **255**, 269–274
7. Erspamer, V., Melchiorri, P., Falconieri-Erspamer, G. Negri, L., Corsi, R., Severini, C., Barra, D., Simmaco, M. & Kreil, G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5188–5192
8. Mosberg, H.I., Hurst, R., Hruby, V.J., Gee, K., Yamamura, H.I., Galligan, J.J. & Burks, T.F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5871–5874
9. Schiller, P.W., Nguyen, T.M.D. & Lemieux C. (1989) in *Peptides 1988* (Jung, G. & Bayer, E., eds.), pp. 613–615, W. deGruyter, Berlin
10. Heath, W.F., Tam, J.P. & Merrifield, R.B. (1986) *Int. J. Peptide Protein Res.* **28**, 498–507
11. Ellman, G.L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
12. Munson, P.J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
13. Heyl, D.L. & Mosberg, H.I. (1991) *Proc. 12th American Peptide Symposium*, in press
14. Schiller, P.W. (1991) presented at 12th American Peptide Symposium, Cambridge, MA
15. Mierke, D.F., Said-Nejad, O.E., Schiller, P.W. & Goodman, M. (1990) *Biopolymers* **29**, 179–196
16. Hoes, C., Hoogerhout, P., Bloemhoff, W. & Kerling, K.E.T. (1979) *Recl. Trav. Chim. Pays-Bas* **98**, 137–139
17. Schiller, P.W., Yam, C.F. & Prosmanne, J. (1978) *J. Med. Chem.* **21**, 1110–1116
18. Fournie-Zaluski, M.-C., Gacel, G., Maigret, B., Premilat, S. & Roques, B.P. (1981) *Mol. Pharmacol.* **20**, 484–491
19. Audigier, Y., Mazarguil, H., Gout, R. & Cros, J. (1980) *European J. Pharmacol.* **63**, 35–46
20. Mosberg, H.I., Heyl, D.L., Haaseth, R.C., Omnaas, J.R., Medzhiradsky, F. & Smith, C.B. (1990) *Mol. Pharmacol.* **38**, 924–928
21. Hansen, D.W., Peterson, K.B., Chandrakumar, N.S., Tsymbalov, S., Hallinan, E.A., Stapelfeld, A., Williamson, N.L., Reichman, M., Heyl, D.L., Omnaas, J.R. & Mosberg, H.I. (1991) presented at 12th American Peptide Symposium, Cambridge, MA

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