Int. J. Peptide Protein Res. 44, 1994, 420–426
Printed in Belgium – all rights reserved

INTERNATIONAL JOURNAL OF
PEPTIDE & PROTEIN RESEARCH
ISSN 0367-8377

Substitution of aromatic and nonaromatic amino acids for the Phe^3 residue in the δ-selective opioid peptide deltorphin I: Effects on binding affinity and selectivity

DEBORAH L. HEYL[1], SHARON J. SCHMITTER[1], HASSIBA BOUZIT[1], THOMAS W. JOHNSON[1], ANGELA M. HEPP[1], KATHLEEN R. KURTZ[1] and CAROL MOUSIGIAN[2]

[1] *Department of Chemistry, Eastern Michigan University, Ypsilanti, Michigan, USA and* [2] *College of Pharmacy, The University of Michigan, Ann Arbor, Michigan, USA*

Received 3 November 1993, accepted for publication 8 April 1994

Deltorphins I and II (Tyr-D-Ala-Phe-Asp-Val-Val-Gly NH_2 and Tyr-D-Ala-Phe-Glu-Val-Val-Gly NH_2) display a high degree of δ-opioid receptor selectivity. Since they lack the intervening Gly^3 residue found between the Tyr and Phe aromatic moieties in pentapeptide enkephalins, deltorphins I and II resemble a previously described series of cyclic tetrapeptides based on Tyr-c[D-Cys-Phe-D-Pen] (JOM-13). With the goal of development of structure–activity relationships for deltorphins and comparison with that of the cyclic tetrapeptides, ten analogs of deltorphin I were synthesized in which Phe^3 was replaced with specific aromatic and nonaromatic amino acids with varying physicochemical properties. Results indicated that analogs containing the bicyclic aromatic amino acids 3-(1-naphthyl)-L-alanine [1-Nal; $K_i(\mu) = 767$ nM, $K_i(\delta) = 7.70$ nM], 3-(2-naphthyl)-L-alanine [2-Nal; $K_i(\mu) = 1910$ nM, $K_i(\delta) = 49.2$ nM], tryptophan [$K_i(\mu) = 1250$ nM, $K_i(\delta) = 23.9$ nM], and 3-(3-benzothienyl)-L-alanine [Bth; $K_i(\mu) = 112$ nM, $K_i(\delta) = 3.36$ nM] were fairly well tolerated at μ- and δ-receptors, though affinity was compromised to varying degrees relative to deltorphin I. Shortening the Phe side chain by incorporation of phenylglycine (Pgl) was detrimental to both μ ($K_i = 4710$ nM) and δ ($K_i = 15.6$ nM) binding, while extension of the side chain with homophenylalanine (Hfe) enhanced μ binding ($K_i = 67.8$ nM), leaving δ affinity unaffected ($K_i = 2.64$ nM). Substitution with nonaromatic amino acids valine and isoleucine led expectedly to poor opioid binding [$K_i(\mu) = \geq 10\,000$ nM for each, $K_i(\delta) = 160$ and 94.7 nM, respectively], while peptides containing cyclohexylalanine (Cha) and leucine surprisingly retained affinity at both μ ($K_i = 322$ and 1240 nM, respectively) and δ ($K_i = 10.5$ and 12.4 nM, respectively) sites. In general, these trends mirror those observed for similar modification in Tyr-c[D-Cys-Phe-D-Pen].

Key words: aromatic side chain; bicyclic side chain; δ-opioid receptor; deltorphin analog; enkephalin analog; μ-opioid receptor; opioids; phenylalanine

Abbreviations recommended by IUPAC–IUB Commission on Biochemical Nomenclature have been used. Other abbreviations: ACN, acetonitrile; Boc, *tert*-butyloxycarbonyl; DAMGO, [D-Ala^2,NMePhe^3,Gly^5-ol]enkephalin; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; FAB-MS, fast atom bombardment mass spectroscopy; HOBt, 1-hydroxybenzotriazole; MBHA, *p*-methylbenzhydrylamine; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; U69,593, 5a,7a,8b-(–)-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro(4,5)dec-8-yl]benzeneacetamide. Unusual amino acids: Bth, 3-(3-benzothienyl)alanine; Cha, 3-(cyclohexyl)alanine; Hfe, homophenylalanine; 1-Nal, 3-(1-naphthyl)alanine; 2-Nal, 3-(2-naphthyl)alanine; Orn, ornithine; Pgl, 2-phenylglycine.

Heterogeneity in the opioid receptor system has long been established (1); μ, δ and κ receptor types demonstrate varying specificities for both endogenous and synthetic ligands, therefore differing in their structural and conformational requirements for peptide binding at the molecular level (2, 3).

The enkephalins (Tyr-Gly-Gly-Phe-LeuOH and Tyr-Gly-Gly-Phe-MetOH), natural ligands for opioid receptors in the central nervous system (4), are perhaps the best known class of endogenous neuropeptides. It is generally accepted that the aromatic side chains of Tyr and Phe in these pentapeptides are responsible for interacting at receptors, and their topographical relationship to one another is important (5). Structure–activity relationship (SAR) studies on series of cyclic

pentapeptides based on Tyr-c[D-Pen-Gly-Phe-D-Pen] (DPDPE) (6–9) as well as the des-Gly^3 tetrapeptide Tyr-c[D-Cys-Phe-D-Pen] (JOM-13) (9–12) have been reported.

Three heptapeptides which were isolated from amphibian skin possess some of the highest opioid potencies and δ-selectivities demonstrated thus far; these include dermenkephalin (Tyr-D-Met-Phe-His-Leu-Met-AspNH$_2$), deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-GlyNH$_2$), and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-GlyNH$_2$) (13). Like the JOM-13 tetrapeptide series noted above, the aromatic residues of these peptides are separated by only one amino acid residue. Evidence indicates that the high δ selectivity of the deltorphins is due in part to the anionic side chain at position 4, which reduces μ binding without affecting δ affinity (14). However, it should be noted that dermenkephalin, while also δ-selective, contains a histidine in place of this anionic residue. The hydrophobic *C*-terminal tripeptide appears necessary for high δ binding affinity (15), and it has been proposed that this *C*-terminal portion plays a conformational role (16). It is likely that the conformational restriction imposed by cyclization in JOM-13 achieves a similar conformational effect (17). SAR results from our laboratories (9–12) and others (18, 19) suggest possible differences in the manner in which the Phe^3 residue of dermorphin/deltorphin-like opioid peptides and the Phe^4 residue of enkephalin-like peptides interact at both μ and δ opioid receptors. In light of more recent evidence for subtypes of the δ receptor (20, 21) and in an attempt to elucidate molecular requirements for opioid receptor binding and specificity, we began to investigate the δ-selective peptide deltorphin I, primarily by replacement of the Phe^3 amino acid with other commercially available amino acids which vary in steric, electronic and lipophilic properties. This work focuses on the essential aromatic Phe residue by modification of side chain length and replacement with nonaromatic and bicyclic aromatic amino acids. In addition to expanding the SAR knowledge base for the deltorphins, results allow comparison with SAR trends observed for the conformationally restricted opioid tetrapeptide JOM-13.

EXPERIMENTAL PROCEDURES

Peptides

The peptides were prepared on a manual shaker using standard solid-phase techniques for *N*-α-Boc protected amino acids on MBHA resin. Resins and protected amino acids were purchased from Bachem California and Bachem Bioscience. The side chains of Tyr and Asp were protected as the 2,6-dichlorobenzyl ether and benzyl ester, respectively. The deprotection solution was 30% TFA in DCM; the resin was shaken for 30 min to remove Boc protection, then neutralized with 10% DIEA in DCM. DCC and HOBt were used as coupling agents. Simultaneous deprotection and cleavage from the resin were accomplished by treatment with 90% anhydrous hydrogen fluoride (Immunodynamics apparatus) and 10% anisole scavenger at 0 °C for 1 h. HF removal and resin drying were achieved by nitrogen stream. The peptide resin was then washed with diethyl ether and the peptide was extracted with 70% ACN/30% water (with 0.1% TFA), concentrated under reduced pressure, diluted with water, and lyophilized. Crude peptides were purified to homogeneity on a Waters preparative RP-HPLC instrument on a Vydac 218TP C-18 column (2.2 × 25.0 cm, 10 mL/min) with a linear gradient of 0.1% TFA in water to 0.1% TFA in acetonitrile (10–50% organic component in 2 h). Quantities of pure peptide are reported after lyophilization of pure fractions as analyzed by RP-HPLC.

Tyr-D-Ala-(1-Nal)-Asp-Val-Val-GlyNH$_2$ (1). The title peptide was prepared starting with 0.79 g resin (1.1 mmol/g) using Boc-3-(1-naphthyl)-L-alanine (1-Nal) for the appropriate coupling. A 1.0 g sample of the protected, resin-bound peptide was treated with 10 mL HF and 1 mL anisole. The crude peptide (200 mg) yielded 32.9 mg of pure peptide.

Tyr-D-Ala-(2-Nal)-Asp-Val-Val-GlyNH$_2$ (2). The peptide was prepared from 0.79 g resin (0.80 mmol/g) by using Boc-3-(2-naphthyl)-L-alanine (2-Nal) for the appropriate coupling. A 1.0 g sample of the protected, resin-bound peptide was treated with 10 mL HF and 1 mL of anisole. 200 mg of crude peptide yielded 9.1 mg of pure peptide **2**.

Tyr-D-Ala-Trp-Asp-Val-Val-GlyNH$_2$ (3). The title peptide was prepared from 0.60 g resin (1.1 mmol/g) using Boc-L-tryptophan (Trp) without side-chain protection, since indole was used as a scavenger in the deprotection solution after the Trp coupling. A 0.8 g sample of the protected, resin-bound precursor peptide was treated with 8 mL HF, 0.8 mL anisole and 100 mg indole. The crude sample (179 mg) yielded 71.7 mg of pure product.

Tyr-D-Ala-Bth-Asp-Val-Val-GlyNH$_2$ (4). The title peptide was prepared from 0.60 g resin (1.1 mmol/g) employing Boc-3-(3-benzothienyl)-L-alanine (Bth) for the appropriate coupling. A 0.8 g sample of the protected, resin-bound precursor peptide was treated with 8 mL HF and 0.8 mL anisole, and 200 mg of the crude peptide yielded 41.0 mg of pure peptide **4**.

Tyr-D-Ala-Cha-Asp-Val-Val-GlyNH$_2$ (5). The peptide was prepared from 0.50 g resin (1.1 mmol/g) using Boc-3-(cyclohexyl)-L-alanine (Cha) for the pertinent coupling. A 0.85 g sample of the protected, resin-bound precursor peptide was treated with 9 mL HF and 0.85 mL anisole scavenger. The crude peptide (177 mg) yielded 60.0 mg of pure peptide **5**.

Tyr-D-Ala-Val-Asp-Val-Val-GlyNH$_2$ (6). The title peptide was prepared using 0.50 g resin (1.1 mmol/g) by employing Boc-L-valine (Val) at the relevant coupling. A 0.35 g sample of the protected, resin-bound peptide was treated with 4 mL HF and 0.35 mL anisole. The crude peptide (200 mg) yielded 85.6 mg of pure peptide **6**.

Tyr-D-Ala-Leu-Asp-Val-Val-GlyNH$_2$ (7). The peptide was synthesized from 0.50 g resin (1.1 mmol/g) using Boc-L-leucine (Leu) for the appropriate coupling. A 0.7 g sample of the protected, resin-bound precursor peptide was treated with 7 mL HF and 0.7 mL anisole. The crude peptide (169 mg) yielded 54.6 mg of pure product **7**.

Tyr-D-Ala-Ile-Asp-Val-Val-GlyNH$_2$ (8). The title peptide was prepared from 0.53 g resin (1.1 mmol/g) by employing Boc-L-isoleucine (Ile) for the pertinent coupling. A 0.7 g sample of the protected, resin-bound peptide was treated with 7 mL HF and 0.7 mL anisole. The crude peptide (180 mg) yielded 61.4 mg of pure peptide.

Tyr-D-Ala-Pgl-Asp-Val-Val-GlyNH$_2$ (9). The title peptide was prepared from 0.50 g resin (1.1 mmol/g) by using Boc-2-phenyl-L-glycine (Pgl) at the appropriate time. A 0.70 g sample of the protected, resin-bound peptide was treated with 7 mL HF and 0.7 mL anisole. The lyophilized crude peptide (200 mg) yielded 94.0 mg of pure peptide.

Tyr-D-Ala-Hfe-Asp-Val-Val-GlyNH$_2$ (10). The peptide was prepared from 0.50 g resin (1.1 mmol/g) using Boc-L-homophenylalanine (Hfe) at the pertinent place. A 0.71 g sample of the protected, resin-bound precursor peptide was treated with 7 mL HF and 0.7 mL anisole. 200 mg of crude material yielded 31.0 mg of pure peptide.

Peptide analysis

Peptide purity (Table 2) was assessed by analytical RP-HPLC using Waters instrumentation on a Vydac C-18 column (4.6 mm × 250 mm) by gradients of 20–86% organic component over 22 min and 0–50% organic component over 50 min, with a flow rate of 1 mL/min. Peaks were monitored at 214, 230 and 280 nm. All compounds were at least 95% pure as analyzed by peak integration at each wavelength. The solvents used in all cases were 0.1% TFA in water and 0.1% TFA in ACN. Peaks which appeared in chromatograms where no peptide was injected were considered artifacts and were ignored. In addition, all peptides were subjected to thin-layer chromatography (TLC) on precoated silica sheets in the following 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). The plates were visualized by ninhydrin spray, iodine vapor and ultraviolet absorption, and a single spot was detected in each solvent system.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were performed on a Bruker spectrometer at 250 MHz. Samples (*ca.* 5 mg) were dissolved in DMSO. Diagnostic resonances and peak patterns confirmed the presence of all indicated residues, and no extra resonances were observed. Final product confirmation was obtained from fast-atom bombardment-mass spectroscopy (FAB-MS), which in all cases yielded the appropriate molecular weight.

Receptor binding assays

Binding assays were based on the displacement by the test compounds of radiolabelled ([^{3}H]) DAMGO (μ ligand, purchased from Multiple Peptide Systems), DPDPE (δ ligand, purchased from New England Nuclear), and U69,593 (κ ligand, purchased from Amersham) from opioid receptors in guinea pig brain. Frozen guinea pig brains were purchased from Keystone Biologicals. This procedure has been described previously (11). Receptor binding assays were performed in triplicate, and results reported are the average of two experiments. Radioligand concentrations used were 1.2 nM [^{3}H]DAMGO, 2.5 nM [^{3}H]DPDPE and 1.0 nM [^{3}H]U69,593. Saturation binding experiments determined the K_D of each ligand as follows: [^{3}H]DAMGO = 1.31–1.35 nM; [^{3}H]DPDPE = 1.60–1.72 nM; [^{3}H]U69,593 = 1.13–1.25 nM.

RESULTS AND DISCUSSION

The μ, δ and κ receptor binding affinities of all analogs, as measured by K_i values ($\pm$ standard errors of the mean) against [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69,593, respectively, are provided in Table 1, along with the corresponding results for deltorphin I. As can be noted, all analogs demonstrated low affinity for the κ opioid receptor, with values greater than 10000 nM. A measure of the δ selectivity is provided as well, as a ratio of $K_i(\mu)$ and $K_i(\delta)$ values for each analog. Table 2 provides physicochemical data for each newly reported peptide.

Our binding assays have demonstrated that the heptapeptide deltorphin I binds with high affinity (K_i = 1.73 nM) to δ opioid receptors and minimally to μ receptors (K_i = 677 nM), thus displaying considerable δ receptor selectivity (nearly 400-fold). We prepared analogs of this peptide with modifications of the phenylalanine (Phe) residue to study the structural role of this residue in deltorphin I for receptor recognition and activation. In addition, comparison of SAR data tends to confirm that Tyr-c[D-Cys-Phe-D-Pen] (JOM-13), a synthetic peptide developed prior to the isolation of the naturally occurring deltorphins, is a δ ligand analogous to the des-Gly^3 deltorphins rather than the enkephalins.

TABLE 1

Opioid receptor binding affinities of deltorphin analogs

Peptide	Cmpd. No.	Binding K_i (nM)[a]			$K_i(\mu)/K_i(\delta)$
		DAMGO[b]	DPDPE[c]	U69.593	
Y-a-F-D-V-V-G-NH_2	Deltorphin I	677 ± 66.0	1.73 ± 0.210	> 10000	391
Y-a-(1-Nal)-D-V-V-G-NH_2	**1**	767 ± 46.3	7.70 ± 0.402	> 10000	99.6
Y-a-(2-Nal)-D-V-V-G-NH_2	**2**	1910 ± 184	49.2 ± 3.40	> 10000	38.8
Y-a-(Trp)-D-V-V-G-NH_2	**3**	1250 ± 71.6	23.9 ± 1.23	> 10000	52.3
Y-a-(Bth)-D-V-V-G-NH_2	**4**	112 ± 7.90	3.36 ± 0.728	> 10000	33.3
Y-a-(Cha)-D-V-V-G-NH_2	**5**	322 ± 21.2	10.5 ± 5.54	> 10000	30.7
Y-a-(Val)-D-V-V-G-NH_2	**6**	> 10000	160 ± 5.57	> 10000	> 62.5
Y-a-(Leu)-D-V-V-G-NH_2	**7**	1240 ± 213	12.4 ± 0.892	> 10000	100
Y-a-(Ile)-D-V-V-G-NH_2	**8**	> 10000	94.7 ± 2.78	> 10000	> 106
Y-a-(Pgl)-D-V-V-G-NH_2	**9**	4710 ± 510	15.6 ± 2.50	> 10000	302
Y-a-(Hfe)-D-V-V-G-NH_2	**10**	67.8 ± 4.71	2.64 ± 0.532	> 10000	25.7

[a] Average values determined from two assays performed in triplicate, ± standard errors of the mean.
[b] DAMGO = [^{3}H][D-Ala^2, NMePhe^4, Gly^5-ol]enkephalin.
[c] DPDPE = [^{3}H][D-Pen^5]enkephalin.

TABLE 2

Physicochemical data for deltorphin analogs

Peptide	Cmpd. No.	HPLC		Purity (%)[c]	TLC R_f[d]			FAB-MS Mol.wt.
		I[a]	II[b]		A	B	C	
Y-a-(1-Nal)-D-V-V-G-NH_2	**1**	4.03	11.3	95	0.85	0.44	0.66	818.4
Y-a-(2-Nal)-D-V-V-G-NH_2	**2**	4.06	12.9	95	0.59	0.41	0.66	818.4
Y-a-(Trp)-D-V-V-G-NH_2	**3**	3.42	9.85	98	0.66	0.38	0.64	807.9
Y-a-(Bth)-D-V-V-G-NH_2	**4**	3.55	9.88	99	0.54	0.32	0.62	824.0
Y-a-(Cha)-D-V-V-G-NH_2	**5**	3.81	11.0	95	0.49	0.42	0.68	773.9
Y-a-(Val)-D-V-V-G-NH_2	**6**	1.13	8.24	99	0.56	0.30	0.59	720.8
Y-a-(Leu)-D-V-V-G-NH_2	**7**	2.71	9.06	98	0.33	0.39	0.63	734.9
Y-a-(Ile)-D-V-V-G-NH_2	**8**	2.52	8.91	95	0.60	0.34	0.60	734.9
Y-a-(Pgl)-D-V-V-G-NH_2	**9**	2.65	9.00	99	0.59	0.33	0.56	754.7
Y-a-(Hfe)-D-V-V-G-NH_2	**10**	3.71	9.82	95	0.46	0.42	0.60	782.9

[a] HPLC K' on a Vydac 218TP C-18 column (0.46 cm × 25 cm); gradient of 20–86% organic component in 22 min; flow rate of 1 mL/min. Solvent system was 0.1% TFA in water, 0.1% TFA in acetonitrile. Solvent front breakthrough at 3.1 min.
[b] HPLC K' on a Vydac 218TP C-18 column (0.46 cm × 25 cm); gradient of 0–50% organic component in 50 min; flow rate of 1 mL/min. Solvent system was 0.1% TFA in water, 0.1% TFA in acetonitrile. Solvent front breakthrough at 3.4 min.
[c] Purity of final product peptide as assessed by RP-HPLC peak integration at 230 nm.
[d] R_f values for thin layer chromatograms in the following solvent systems: (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).

In analogs **1–4**, phenylalanine was replaced with bicyclic aromatic amino acids. Substitution of 3-(1-naphthyl)-L-alanine (1-Nal, **1**) or 3-(2-naphthyl)-L-alanine (2-Nal, **2**) resulted in compounds with δ receptor K_i values of 7.70 and 49.2 nM, respectively. Binding to the μ receptor was relatively unaffected, although a three-fold reduction in affinity was observed for the 2-Nal modification. Analog **1** retained considerable δ selectivity. These analogs contain a flat aromatic side chain at residue 3 which increases steric bulk and lipophilicity (as reflected by relatively late RP-HPLC elution times), both of which appeared to be well tolerated. Since these analogs differ in the point of attachment of the naphthyl moiety and therefore have a different orientation of the side chain naphthyl group with respect to the peptide backbone, the data give an indication as to the size and shape of the δ binding pocket, which may be broad rather than deep. A similar observation can be made for the μ binding pocket. These results are in general agreement with previous studies that have found linear enkephalins with naphthylalanine substitutions to retain activity (22). Also, in pen-

tapeptide enkephalinamides, analogs containing 2-Nal show a 10-fold drop in potency in the μ receptor guinea pig ileum (GPI) bioassay relative to 1-Nal analogs (23). A similar reduction in μ affinity upon substitution of Phe with 2-Nal was observed for the μ-selective cyclic tetrapeptide Tyr-c[D-Orn-X-Glu]NH_2, while the comparable 1-Nal analog retained μ binding (24). Most significantly, effects of this modification on the μ receptor binding of deltorphin I are consistent with SAR for the δ-selective cyclic tetrapeptide Tyr-c[D-Cys-Phe-D-Pen] (JOM-13); however, little difference was noted between the δ binding of the 1-Nal and the 2-Nal tetrapeptide analogs, both displaying only slightly reduced δ binding affinity relative to the lead compound (11).

Variation of lipophilic and electronic character was accomplished by substitution of Phe with L-tryptophan (Trp, **3**) and 3-(3-benzothienyl)-L-alanine (Bth, **4**). In linear enkephalins, Trp substitution lowers δ binding without much effect on μ affinity (25). While **3** retained reasonable δ binding (K_i = 23.9 nM), its affinity was reduced 14-fold relative to deltorphin I, and its δ selectivity suffered, since μ affinity was reduced by only a factor of two. This is a more drastic reduction in δ binding than is observed for the cyclic tetrapeptide JOM-13 (11). Surprisingly, the Bth-containing analog, **4**, retained excellent δ binding affinity (K_i = 3.36 nM), at the expense of selectivity, since μ affinity was enhanced. This may be attributable to electronic differences between the heteroatoms. Bth substitutions in JOM-13 have not been reported for comparison, but replacement of Phe with the monocyclic aromatic sulfur-containing amino acid, 3-(2-thienyl)alanine, is well tolerated; δ binding is compromised only five-fold (11).

In the second group of analogs (**5–8**), Phe was substituted with nonaromatic amino acids. Only 3-cyclohexyl-L-alanine (Cha, **5**) and L-leucine (Leu, **7**) proved to be adequate substitutions for Phe, with δ receptor K_i values of 10.5 and 12.4, respectively. However, compound **5** displayed nearly four-fold higher affinity for the μ receptor than **7**, compromising its δ-selectivity, while that of the Leu-containing analog was retained. Since the chair form cyclohexyl side chain of **5** should occupy similar van der Waals space as that of the flat Phe aromatic ring of deltorphin I, the fact that it is accommodated is not remarkable. However, the observation that the Leu modification is just as well accepted as the δ site was rather unexpected. Analogs containing L-valine (Val, **6**) and L-isoleucine (Ile, **8**) did not bind measurably to μ receptors, and δ opioid affinities were substantially reduced as well (δ K_i = 160 and 94.7 nM, respectively). The relative trends reported here for deltorphin I are similar to those obtained with JOM-13 analogs, though the magnitude of affinity reduction at the δ receptor type varies considerably (11). In JOM-13, replacement of Phe by Val or Ile is three to five times more detrimental to δ binding with respect to the parent compound (11). Even more surprising is the Leu^3 substitution, which reduces δ binding 105-fold in JOM-13 (11) and only seven-fold deltorphin I. Effects from substitutions with the bulkier and lipophilic Cha, however, are quite similar in the two series (11). SAR data from linear enkephalin analogs indicates that an aromatic ring at the Phe^4 position is required for binding at the δ receptor, while μ affinity is relatively unaffected with bulky, nonaromatic amino acids like Cha at this position (26); in cyclic pentapeptides, however, Cha^4 is well tolerated (27). Data suggest that aromaticity is not a prerequisite for δ receptor binding of deltorphin I or JOM-13; however, a minimal amount of steric bulk and lipophilicity seem to be required at the binding site for optimum interaction, possibly by van der Waals or hydrophobic forces. It is likely that the shorter Val side chain of **6** fails to reach the binding pocket. Differing van der Waals distributions of the residue 3 side chains of **7** and **8** may account for their contrasting binding properties. It also is possible that β-substitution in the cases of Val and Ile provides an adverse steric effect (11).

In analogs **9** and **10**, the effect of varying the Phe side-chain length was explored. Both of these modifications might be expected to alter the relative spatial relationship of the critical Tyr and Phe aromatic residues due to changes in the orientation of the phenyl side chain with respect to the peptide backbone. The length of the Phe side chain was shortened by one methylene unit by substitution with 2-phenyl-L-glycine (Pgl, **9**). Direct attachment of the aromatic ring to the α carbon may decrease conformational mobility of the side chain as well as cause deviations in backbone conformation. This modification resulted in a compound which unexpectedly retained δ binding affinity (K_i = 15.6 nM), approximately nine-fold less than that of deltorphin I. Since μ binding also was compromised to the same extent, this analog retained the best δ-selectivity (over 300-fold) of all those reported here, excluding deltorphin I itself. This modification has been shown to reduce δ binding by more than 100-fold in cyclic tetrapeptides based on JOM-13 (10) and even more drastically in linear (26) and cyclic (10) pentapeptides. However, little effect on δ affinity was reported for this substitution in the dermorphin-related, μ-selective cyclic tetrapeptide Tyr-c[D-Orn-X-Asp]NH_2 (18).

In compound **10**, substitution with L-homophenylalanine (Hfe) extended the side chain by one methylene unit. This analog bound to the δ receptor with essentially equal affinity (K_i = 2.64 nM) as deltorphin I; μ binding was enhanced, thereby reducing δ-selectivity. This result corroborates earlier findings that side chain lengthening is not detrimental to the μ or δ binding of des-Gly^3 opioid peptides like JOM-13 (10, 18), although it destroys δ receptor binding 100-fold in the cyclic pentapeptide DPDPE (10). The longer side chain may interfere with receptor interaction in the peptides where the Tyr and Phe aromatic moieties are spaced farther apart, yet allow the appropriate arrangement

of aromatic rings in deltorphin-related peptides. Like the results derived from SAR studies on JOM-13, data reported here support the observation that varying the aromatic 3/4 residue side-chain length and/or orientation is better tolerated in deltorphin-like opioids (where Tyr and Phe are separated by only one amino acid residue) than in enkephalin-like opioids (where the aromatic rings are separated by two residues) (10). These findings lend support to the theory of possible differing binding conformations, and perhaps different receptor subsites or even subtypes, for enkephalin-like pentapeptides and dermorphin/deltorphin-like peptides (10). In fact, reports of a lack of cross-tolerance between DPDPE and deltorphin II (21), as well as differential antagonism of the two peptides in mice (20), make it tempting to ascribe these results to the presence of two subtypes of δ receptors, one which interacts with enkephalin-like peptides and one with opioids related to the deltorphins. Still, other explanations are conceivable. Indeed, binding studies indicate that DPDPE, JOM-13, and deltorphins I and II must bind to the same site, as evidenced by the displacement of [^{3}H]DPDPE by each peptide.

In summary, all modifications reported here proved at least slightly detrimental to δ receptor affinity relative to deltorphin I, with the notable exceptions of Bth (**4**) and Hfe (**10**). However, most modifications, whether steric, lipophilic, or electronic, were reasonably accommodated, with the only extreme reductions in δ affinity resulting from substitution of Phe with less bulky, nonaromatic amino acids (Val, **6** and Ile, **8**). Binding to the μ receptor varied, and analogs **4** and **10** again demonstrated the best affinity. In most cases, δ opioid receptor selectivity was reduced, but the Pgl-containing analog (**9**) retained excellent selectivity. Most consequences arising from modifying deltorphin I were consistent with those observed upon identical modification in the cyclic, δ-selective opioid tetrapeptide, JOM-13, although the magnitudes of these effects were sometimes variable. In general, data support the hypothesis that JOM-13 and the deltorphins bind in a similar fashion to the same δ opioid receptor subtype, which may or may not differ from the binding mode or site of pentapeptide enkephalins.

ACKNOWLEDGMENTS

Opioid receptor binding assays were performed under the direction of Dr. Henry I. Mosberg at The University of Michigan. ^{1}H-NMR and FAB-MS analyses were provided by Parke-Davis/Warner-Lambert in Ann Arbor, MI. D.L.H. is the recipient of a Bristol-Myers Squibb Company Award of Research Corporation. Additional funding for this research has been provided by the Eastern Michigan University (EMU) chemistry department, a Provost's Research Support Award for New Faculty at EMU, and an EMU Spring-Summer Faculty Research Award. Student support for A.M.H. and K.K. was provided by the Research Experience for Undergraduates program through the National Science Foundation.

REFERENCES

1. Lord, J.A.H., Waterfield, A.A., Hughes, J. & Kosterlitz, H.W. (1977) *Nature (London)* **267**, 495–499
2. Olson, G.A., Olson, R.D. & Kastin, A.J. (1990) *Peptides* **11**, 1277–1304
3. Schiller, P.W. & DiMaio, J. (1982) *Nature (London)* **297**, 74–76
4. Hughes, J. & Kosterlitz, H.W. (1975) *Nature (London)* **258**, 577–579;
5. Schiller, P.W (1984) in *The Peptides: Analysis, Synthesis, and Biology* (Udenfriend, S. & Meienhofer, J., eds.), pp. 220–268, Academic Press, NY
6. Mosberg, H.I., Hurst, R., Hruby, V.J., Gee, K., Yamamura, H.I., Galligan, J.J. & Burks, T.F. (1983) *Proc. Natl. Acad. Sci.* **80**, 5871–5874
7. Mosberg, H.I., Hruby, V.J., Hurst, R., Galligan, J.J., Burks, T.F. & Yamamura, H.I. (1983) *Life Sci.* **32**, 2656–2659
8. Mosberg, H.I., Hurst, R., Hruby, V.J., Galligan, J.J., Burks, T.F., Gee, K. & Yamamura, H.I. (1983) in *Peptides: Structure and Function* (Hruby, V.J. & Rich, D.H., eds.), pp. 279–282, Pierce Chemical Co., Rockford, IL
9. Mosberg, H.I., Heyl, D.L., Omnaas, J.R., Haaseth, R.C., Medzihradsky, F. & Smith, C.B. (1990) *Mol. Pharmacol.* **38**, 924–928
10. Heyl, D.L., Omnaas, J.R., Sobczyk-Kojiro, K., Medzihradsky, F., Smith, C.B. & Mosberg, H.I. (1991) *Int. J. Peptide Protein Res.* **37**, 224–229
11. Heyl, D.L. & Mosberg, H.I. (1992) *Int. J. Peptide Protein Res.* **39**, 450–457
12. Heyl, D.L. & Mosberg, H.I. (1992) *J. Med. Chem.* **35**, 1535–1541
13. Erspamer, V., Melchiorri, P., Falconieiri-Erspamer, G., Negri, L., Corsi, R., Severni, C., Barra, D., Simmaco, M. & Kreil, D. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5188–5192
14. Lazarus, L.H., Salvadori, S., Santagada, V., Tomatis, R. & Wilson, W.E. (1991) *J. Med. Chem.* **34**, 1350–1355
15. Melchiorri, P., Negri, L., Falconieri-Erspamer, G., Severini, C., Corsi, R., Soaje, M., Erspamer, V. & Barra, D. (1991) *Eur. J. Pharmacol.* **195**, 201–207
16. Balboni, G., Marastoni, M., Picone, D., Salvadori, S., Tancredi, T., Temussi, P.A. & Tomatis, R. (1990) *Biochem. Biophys. Res. Commun.* **169**, 617–622
17. Mosberg, H.I., Kroona, H.B., Omnaas, J.R., Sobczyk-Kojiro, K., Bush, P. & Mousigian, C. (1994) *Proc. 13th American Peptide Symposium*, 514–516
18. Schiller, P.W., Nguyen, T.M.-D., Maziak, L.A., Wilkes, B.C. & Lemieux, C. (1987) *J. Med. Chem.* **30**, 2094–2099
19. Schiller, P.W., Nguyen, T.M.-D., DiMaio, J. & Lemieux, C. (1983) *Life Sci.* **33**, 319–322
20. Jiang, Q., Takemori, A.E., Sultana, M., Portoghese, P.S., Bowen, W.D., Mosberg, H.I. & Porreca, F. (1990) *J. Pharmacol. Exp. Ther.* **257**, 1069–1075
21. Mattia, A., Vanderah, T., Mosberg, H.I. & Porreca, F. (1990) *J. Pharmacol. Exp. Ther.* **258**, 583–587
22. Gesellchen, P.D., Frederickson, R.C.A., Tafur, S. & Smiley, D. (1981) in *Peptides: Structure and Function* (Rich, D.H. & Gross, E., eds.), pp. 621–624, Pierce Chemical Co., Rockford, IL
23. Mierke, D.F., Said-Nejad, O.E., Schiller, P.W. & Goodman, M. (1990) *Biopolymers* **29**, 179–196
24. Schiller, P.W. (1991) presented at 12th American Peptide Symposium, Cambridge, MA
25. Schiller, P.W., Yam, C.F. & Prosmanne, J. (1978) *J. Med. Chem.* **21**(11), 1110–1116
26. Fournie-Zaluski, M.C., Gacel, G., Maigret, B., Premilat, S. & Roques, B.P. (1981) *Mol. Pharmacol.* **20**, 484–491

27. 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

Address:

Deborah L. Heyl
Department of Chemistry
225 Jefferson Science Building
Eastern Michigan University
Ypsilanti, MI 48197
USA