131.95, 139.11, 141.75; FTIR (film) 3600–3000 (OH), 2108 (NCS), 1621, 1502 cm^{-1}; HRFABMS (M + 1)$^+$ calcd for $C_{23}H_{29}N_2O_4S$ 429.1848, obsd 429.1836. Anal. ($C_{23}H_{28}N_2O_4S{\cdot}HCl$) C, H, N.

Opioid Receptor Binding. Radioreceptor binding assays were carried out as described previously using guinea pig brain homogenate.[12] The radioligand used was [^{3}H]bremazocine (New England Nuclear) (37.0 Ci/mmol) at a concentration of 0.5 nM for determination of total opioid binding sites. For μ-binding sites 1.0 nM [^{3}H]DAGO [D-Ala^2-NMePhe^4-Gly-ol^5-enkephalin] (Amersham) (60.0 Ci/mmol) was used. For κ-binding sites, 0.5 nM [^{3}H]bremazocine was used in the presence of 100 nM unlabeled DAGO and 100 nM unlabeled DPDPE to block μ and δ sites, respectively. For δ-binding sites 1.0 nM [^{3}H]DPDPE [D-Pen^2-D-Pen^5-enkephalin] (New England Nuclear) (34.3 Ci/mmol) was used. Nonspecific binding was determined using naloxone (10 μM). Stock solutions of each test compound were prepared immediately prior to the assay by dissolving the free amine in 50% acetic acid (200 μL) and then were serially diluting with water. Nine concentrations of each ligand to be tested were examined in competition experiments with each radioligand. The samples were incubated in 50 mM Tris-HCl buffer (pH 7.4) at 25 °C for 1 h and then rapidly filtered through Whatman GF/B filters, which were rinsed twice with cold buffer (2 mL each) and after standing overnight in Aquasol II scintillation fluid (10 mL) were counted in a scintillation counter. IC_{50} values were determined using log-probit analysis.

Irreversibility and Protection Studies. The studies were carried out as described previously.[12] Membrane preparations were incubated with drug to be tested for 1 h at 25 °C. For protection studies, naloxone was added at a concentration of 1 μM (recovery was checked with naloxone alone). After incubation, the samples were diluted 4-fold with buffer and centrifuged for 15 min at 20000g. The supernatant was removed, and the pellet was resuspended in 3 times the original volume of buffer and incubated at 37 °C for 15 min, centrifuged again, and resuspended in the original volume of buffer. A binding assay using [^{3}H]-bremazocine, [^{3}H]DAGO, or [^{3}H]DPDPE was carried out as described above.

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Ring Substituted and Other Conformationally Constrained Tyrosine Analogues of [D-Pen^2,D-Pen^5]enkephalin with δ Opioid Receptor Selectivity[1]

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The conformationally restricted, cyclic disulfide-containing δ opioid receptor selective enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin (DPDPE) was modified by 2′ (CH_3) and 3′ (I, OCH_3, NO_2, NH_2) ring substitutions and by β-methyl conformationally constrained β-methyltyrosine derivatives in the 1 position. The potency and selectivity of these analogues were evaluated by bioassay in the mouse vas deference (MVD, δ receptor assay) and guinea pig ileum (GPI, μ receptor assay) assays and by radioreceptor binding assays in the rat brain using [^{3}H]CTOP (μ ligand) and [^{3}H][p-ClPhe^4]DPDPE (δ ligand). The analogues showed highly variable potencies in the binding assays and in the bioassays. Aromatic ring substituents with positive Hammett constants had decreased potency, while substituents with negative Hammett constants has increased potency for the opioid receptor. The most potent and most selective compound based on the binding was [2′-MeTyr^1]DPDPE (IC_{50} = 0.89 nM and selectivity ratio 1310 in the binding assays). The 6-hydroxy-2-aminotetralin-2-carboxylic acid-containing analogue, [Hat^1]DPDPE, also was highly potent and selective in both assays, demonstrating that significant modifications of tyrosine in enkephalins are possible with maintenance of high potency and δ opioid receptor selectivity. Of the β-methyl-substituted Tyr^1 analogues, [(2S,3R)-β-MeTyr^1]DPDPE was the most potent and δ receptor selective. The results with substitution of β-MeTyr or Hat instead of Tyr also demonstrate that topographical modification in a conformationally restricted ligand can significantly modulate both potency and receptor selectivity of peptide ligands that have multiple sites of biological activity.

Since the discovery of enkephalins in 1975[2] numerous studies have been made to elucidate the structure–activity relationship of enkephalins and other opioid peptides. Although several thousand peptide analogues have been synthesized (for reviews[3–6]), highly receptor selective *and* potent peptide analogues only have been obtained during the last 10 years which confirm the postulate of a multiplicity of opioid receptors.[7,8]

One approach for the design of highly selective ligands involves the incorporation of conformational constraints.[9] In our laboratory, this approach has led to the development of highly selective ligands for both μ and δ opioid receptors.[10,11] One of the most selective analogues for the δ opioid receptors was the cyclic analogue [D-Pen^2,D-

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977–983). All optically active amino acids are of L variety unless otherwise noted. Other abbreviations used are β-MeTyr, β-methyltyrosine; Hat, 6-hydroxy-2-aminotetralin-2-carboxylic acid; Pen, penicillamine; CTP, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH$_2$; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$; [p-ClPhe^4]DPDPE, Tyr-D-Pen-Gly-p-ClPhe-D-Pen; GPI, guinea pig ileum; MVD, mouse vas deferens; FAB-MS, fast atom bombardment mass spectrometry; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; D-Tic, tetrahydroisoquinoline-2-carboxylic acid; Ad, adamantyl; SPPS, solid-phase peptide synthesis.

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Table I. Inhibitory Potency and Selectivity of DPDPE Analogues in GPI and MVD Bioassays

Peptide	IC_{50} (nM)		% E_{max}[a]	selectivity ratio[b]
	GPI	MVD		
1, DPDPE	7300 ± 1700	4.1 ± 0.46	90	3270
2, [3'-ITyr[1]]DPDPE	24600 ± 9100	262 ± 10.6	59	208
3, [3'-OCH_3Tyr[1]]DPDPE	~600000	137 ± 19.4	NA	~4300
4, [3'-NO_2Tyr[1]]DPDPE	~90000	1010 ± 176	NA	~70
5, [3'-NH_2Tyr[1]]DPDPE	>100000	111 ± 15	NA	>90
6, [2'-CH_3-L-Tyr[1]]DPDPE	431 ± 83	4.52 ± 0.60	90	111
7, [2'-CH_3-D-Tyr[1]]DPDPE	6610 ± 537	763 ± 199	92	9.8
8, [Hat[1]]DPDPE (peak 1)	6600 ± 653	137 ± 23	31	176
9, [Hat[1]]DPDPE (peak 2)	3500 ± 200	22 ± 3.7	79	220
10, [(2S,3S)-β-MeTyr[1]]DPDPE	7180 ± 210	243 ± 33	10	67
11, [(2R,3R)-β-MeTyr[1]]DPDPE	>100000	20703 ± 11000	NA	>5
12, [(2S,3R)-β-MeTyr[1]]DPDPE	3400 ± 2280	14.6 ± 2.1	58	540
13, [(2R,3S)-β-MeTyr[1]]DPDPE	49100 ± 8630	698 ± 68	46	256

[a] The drug effects at the guinea pig ileum are expressed as absolute % inhibition of contraction according to the expression % inhibition = 100 × control − test/control, where control is the contraction measured in the absence of analogue and test is the contraction strength after addition of analogue. The mean data were subjected to nonlinear regression analysis to obtain the maximum inhibition (E_{max}) and potency represented by the IC_{50} (concentration of drug producing 50% of E_{max}). In all cases, the data was fitted adequately by an equation describing a rectangular hyperbola, i.e. a Hill equation or logistic function with a slope of 1. [b] The selectivity ratio represents the ratio of concentration required to produce *equal* degrees of inhibition in the GPI and the MVD. It should be noted that the simple ratio of IC_{50} in the GPI and MVD would give a different (misleading) result in many cases as the IC_{50} value is dependent on the E_{max} in the GPI, which varied considerably for the analogues measured in the GPI. [c] NA = not applicable.

Pen[5]]enkephalin (DPDPE), a peptide with a highly constrained 14-membered ring.

We have prepared a second generation of analogues of DPDPE with modification of the parent peptide in the Phe[4] residue.[12-14] The rationale for these modifications was based on our examination of the conformation and dynamics of DPDPE[15] which indicated that the Tyr[1] and Phe[4] aromatic side chain groups were on the same surface of the structure in relatively close proximity. This suggested that the topographical relationships between these two aromatic rings might be important for receptor recognition and transduction. Examination of the conformational model indicated that the substitution of the Phe[4] residue in the para position would be possible without disruption of the conformation, and indeed the para halogenated Phe[4] analogues were found to possess higher δ opioid receptor potency and selectivity than DPDPE, indicating the importance of the side chain conformation of Phe[4] and the stereoelectronic property of substituents on Phe[4].[12] In this study, the p-ClPhe[4] analogue has proven to be a useful tool for examining the properties of δ opioid receptor being available in tritiated form.[16]

The relationship of the Tyr[1] and Phe[4] side chain groups in DPDPE depends on both the Phe[4] residue and the Tyr[1] residue. However, previous examinations suggested that any modification of the Tyr[1] residue would decrease the opioid receptor affinity. The only exception was N-alkylation of the α-amino group by methyl or pentyl[17,18] (in

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Table II. Binding Affinities and Selectivities of DPDPE Analogues in Competition with [^{3}H]CTOP and [^{3}H][*p*-ClPhe^4]DPDPE

peptide	IC_{50} (nM) [^{3}H]CTOP	IC_{50} (nM): binding vs [^{3}H][p-ClPhe^4]DPDPE		ratio IC_{50}^{a} μ/δ
1, DPDPE	609 ± 70	1.6 ± 0.2	5.83 ± 0.7^b	380
2, [3'-ITyr^1]DPDPE	5530 ± 600	ND	54.6 ± 6^b	101^a
3, [3'-OMeTyr^1]DPDPE	4110 ± 225	50 ± 2.0	20.3 ± 3^b	76
4, [3'-NO_2Tyr^1]DPDPE	3300 ± 200	720 ± 231	521.0 ± 60^b	4.6
5, [3'-NH_2Tyr^1]DPDPE	8000	ND	93.0 ± 10^b	86^a
6, [2'-Me-L-Tyr^1]DPDPE	1170 ± 419	0.89 ± 0.18		1310
7, [2'-Me-D-Tyr^1]DPDPE	16600 ± 3000	1130 ± 77		14.7
8, [Hat^1]DPDPE (peak 1)	2820 ± 328	24.5 ± 3.5		115
9, [Hat^1]DPDPE (peak 2)	1440 ± 353	2.36 ± 0.46		610
10, [(2*S*,3*S*)-β-MeTyr^1]DPDPE	18600 ± 4050	440 ± 211		42.3
11, [(2*R*,3*R*)-β-MeTyr^1]DPDPE	22800 ± 2090	426 ± 137		53.4
12, [(2*S*,3*R*)-β-MeTyr^1]DPDPE	>20000	84.8 ± 21.6		>235
13, [(2*R*,3*S*)-β-MeTyr^1]DPDPE	>40000	~20000		

^{a} In compounds 2 and 5, the ratios reported are for data obtained using [^{3}H]DPDPE as the radiolabeled ligand for the δ receptor. In all other cases [^{3}H][*p*-ClPhe^4]DPDPE is used as the radiolabeled ligand for binding studies (see Experimental Section) at the δ receptor and for calculation of the selectivity ratios. ND = not determined. ^{b} IC_{50} vs [^{3}H]DPDPE.

the cases studied, the ligands were more selective for μ-opioid receptors).

Only a few enkephalin analogues modified on the aromatic ring of Tyr^1 have been examined.[5] Radioiodinated Tyr analogues could be important research tools, but the [3'-ITyr^1]enkephalins were $^1/_{10}$ the potency of the unsubstituted ligands on opioid receptors and the diiodo derivatives were inactive.[19,20] Therefore, these ligands have not been useful tools for radioreceptor binding assays because of their low affinity for opioid receptors and their high nonspecific binding. In 1983, Deekes et al.[21] reported that replacing the N-terminal tyrosine of Leu-enkephalin with the conformationally constrained Tyr analogue 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) gave an analogue that was 7 times more potent than Leu-enkephalin in the GPI assay but 30 times less active than Leu-enkephalin in the MVD assay.

Hansen et al.[22] introduced alkyl groups into the Tyr^1 ring of enkephalin analogues, producing significant increases in both in vitro and in vivo potencies in Tyr-D-Met-Gly-Phe-NH-Ad and Tyr-D-Met-Gly-Phe-NH-$(CH_2)_5CO_2Me$. The 2',6'-dimethyl substitution was particularly important in its effect on opioid binding. Unfortunately, there was no data given on the selectivity of these analogues for μ and δ receptors. Stammer et al.[23] prepared 2,3-methanotyrosine analogues of DALE, but the best isomer was 10 times less potent than Leu-enkephalin in the MVD assay.

To examine the relationship of Tyr^1 and Phe^4 in DPDPE for the receptor affinity and selectivity, we have synthesized several ring-substituted and otherwise conformationally constrained Tyr^1 analogues of DPDPE, and evaluated their opioid receptor affinities in the rat brain as well as their potencies and selectivities in MVD and GPI bioassays.

Results

All of the new analogues (see Table I for structures) of DPDPE were prepared by the solid-phase method of peptide synthesis using methods very similar to those used previously in the synthesis of DPDPE and its Phe^4- and β-MePhe^4-substituted analogues.[10,12,13] In the case of the 2'-MeTyr and 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) residues, they were added as racemic mixtures. The crude diastereomeric products generally were purified by gel filtration on a Sephadex G-10 column followed by reversed-phase high-pressure liquid chromatography (RP-HPLC) (see the Experimental Section for complete details for each compound). The configuration of the Tyr analogues in the peptides was identified by an enzymatic method using L-amino acid oxidase or chiral TLC methods very similar to those used previously for the [β-MePhe^4]DPDPE isomers.[12,24] The configuration of the Hat in its analogues could not be determined unambiguously because the above enzymatic method did not work (there is no α-proton in this amino acid), and optically pure 6-hydroxy-2-aminotetraline-2-carboxylic acids are not available for chiral TLC evaluation. The structure and purity of the peptides were assessed by thin-layer chromatography, analytical RP-HPLC, and fast-atom-bombardment mass spectrometry. Amino acid analysis and ^{1}H NMR also were used for identification of the structures.

The potencies of the DPDPE analogues to inhibit electrically evoked contraction of the myenteric plexus longitudinal muscle of the guinea pig ileum (GPI) and of the mouse vas deferens (MVD) are summarized in Table I. The results are compared with those obtained for [D-Pen^2,D-Pen^5]enkephalin (DPDPE). All of the new analogues were more potent in the mouse vas deference assay system (where the δ opioid receptors are predominant) than in the GPI assay which contains μ and κ opioid receptors (Table I). The effects of all of the compounds in the MVD assay were antagonized by ICI 174864, a highly δ selective antagonist (data not shown) and hence all of the analogues were found to be selective.

As can be seen from Table I, the peptides containing

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Table III. Macroscopic pK_a for Phenolic Hydroxyl Ionization in Tyrosine and Tyrosine Derivatives

compound	pK_a	refs	compound	pK_a	refs
Tyr	10.1, 10.14, 10.16	46, 43, 47	3'-ITyr	8.2	43
erythro-β-MeTyr	10.20	46	3'-NO_2Tyr	7.2	43
threo-β-MeTyr	10.17	46	3'-NH_2Tyr	10.0	43, 48
Hat	10.48	46	3'-OMeTyr	9.7	49
2'-MeTyr	10.28	46			

D-amino acid derivatives in the 1 position (**7**, **11**, **13**) were less potent than the peptides with L-tyrosine derivatives (**6**, **10**, **12**). These findings support the idea that an L configuration of Tyr[1] in the enkephalins is important for high potency at opioid receptors.

The analogue which contains the conformationally constrained tyrosine analogue Hat (**9**, which is the diastereoisomeric peptide from the other HPLC peak (**8**) and probably contains the L amino acid) is more potent than analogue **8**. The lower potency of **8** in both assays is consistent with the idea that the peptide from the second peak contains the tyrosine analogue with an L configuration.

The substitution on the tyrosine modified the potency of analogues in both assays. Substituents with a positive Hammett constant (I, NO_2, OCH_3) decreased the potency in both assays, while the analogue with the methyl group in the 2' position on the aromatic ring of Tyr[1] (**6**) is slightly more potent as a δ ligand than the parent DPDPE in the binding assay and almost equipotent in the MVD assays. Interestingly, however, whereas **6** is considerably less selective than DPDPE in the peripheral bioassay systems (Table I), in the brain binding assays (Table II) it is much more selective for δ receptors than DPDPE.

We have systematically modified the conformation of the Tyr side chain moiety in DPDPE by preparing all four isomers of β-MeTyr[1]-substituted DPDPE. The substitution of a β-MeTyr for Tyr[1] in DPDPE has a large effect on its bioactivity. Although all four analogues are less potent than the parent compound, there are large differences in the IC_{50} values, particularly in the MVD and in the binding assays. The L-threo compound [(2*S*,3*R*)-β-MeTyr]DPDPE (**12**) was the most active and selective for the δ opioid receptor. The D-threo-containing analogue [(2*R*,3*S*)-β-MeTyr]DPDPE (**13**) was very inactive in both assays. Interestingly, the L-erythro-containing analogue [(2*S*,3*S*)-β-MeTyr]DPDPE (**10**) is essentially equipotent to the D-erythro analogue **11** in the δ binding assay (Table II), but 5–15 times less potent than **12**, the L-threo analogue, in both binding and bioassays. It is clear that the topographical features at position 1 can have dramatic effects on the interaction of DPDPE with the δ receptor. Finally, the interaction of **10**–**13** with the μ receptor based on both binding and bioassays is greatly decreased relative to DPDPE.

Some trends observed in the rat brain membrane binding assays (Table II) deserve further comment. All of the analogues inhibited the binding of [³H][*p*-ClPhe⁴]DPDPE[16] or [³H]DPDPE (δ selective ligands) and [³H]CTOP (highly μ opioid selective ligand),[25] but the IC_{50} values against [³H]-δ ligands were always lower than the IC_{50} values against [³H]CTOP, showing the δ opioid receptor selectivity of all of these analogues (Table II). [L-2'-MeTyr[1]]DPDPE (**6**) was the most potent and most selective in the radioligand assays. This compound was 3 times more selective and 2 times more potent than DPDPE. The DPDPE analogues that had substituents on the aromatic ring of tyrosine with positive Hammett constants (I, NO_2, OCH_3) had significantly decreased binding affinity in both assays. The IC_{50} values of [3'-NH_2Tyr[1]]DPDPE were relatively high in the binding assay against [³H][*p*-ClPhe⁴]DPDPE.

One of the 6-hydroxy-2-aminotetralin-2-carboxylic acid (Hat) containing analogues, **9**, also was very potent in the binding assay against [³H][*p*-ClPhe⁴]DPDPE, demonstrating its strong binding to the δ receptor. Interestingly, the related diastereoisomer **8** also was quite potent in this binding assay, supporting the idea that the conformation of the side chain of both Hat enantiomeric in DPDPE analogues is quite similar, in contrast to the results observed for other 1 position D- and L-amino acid-containing peptide analogues.

Discussion

From earlier work,[14,15] it is quite likely that the relative proximity of aromatic rings in the Phe and Tyr residues of DPDPE is critical for δ versus μ receptor selectivity, and thus it is critical that each of these side chains is involved in favorable binding interactions at the receptor.[15,26] The effect of modification of the Phe⁴ residue of DPDPE has been investigated in detail in our laboratory and others,[12-14,27,28] and in this context, it was found that modifications that alter the backbone conformation and/or the orientation of Phe aromatic ring relative to the peptide backbone have deleterious effects that decrease the binding affinity and MVD potency. However, many modifications that do not disturb the orientation of the residue 4 aromatic side chain from the favored gauche (−) side chain conformation can lead to enhanced binding affinity and MVD potency. In this paper, we investigated the effects of ring substitutions and side chain conformational constraints on the Tyr[1] residue in DPDPE on receptor binding affinity and selectivity.

In contrast to the results observed in Phe⁴-modifications on the aromatic ring in the para position, in which it was found that substituents with positive Hammett constants increased the binding affinity in the rat brain and the MVD potency, in L-Tyr[1] analogues, these substituents (I, NO_2) in the 3' position decreased the binding affinity and

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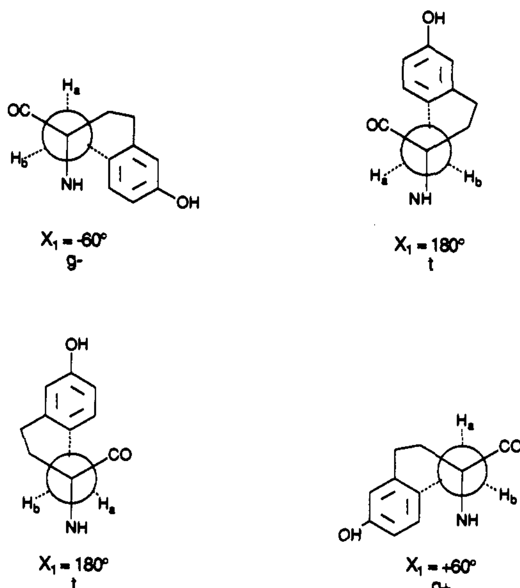

Figure 1. Newman projection of the side chain conformations of (2*S*)-Hat (top) and (2*R*)-Hat (bottom).

MVD potency. However, a methyl substituent in the 2′ position increased potency in the binding assay. In Table III, we summarized the macroscopic pK_a values for the phenolic hydroxyl group of the substituted tyrosines. As can be seen from Table III, the electron-withdrawing substituents increase the ionization of the phenolic hydroxyl group, and the electron-donating substituent (CH_3) decrease it. The most active analogue was [2′-MeTyr]-DPDPE (**6**). This suggests that the phenolic hydroxyl group must be un-ionized for δ opioid receptor recognition. However, it should be pointed out that the low potency for analogues substituted in the 3′ position may be due to steric effects of that position relative to the 2′ position when the analogues are interacting with the δ receptor.

It is interesting to note that the peptides containing the conformationally constrained tyrosine derivative L- or D-6-hydroxy-2-aminotetralin-2-carboxylic acid in the 1 position had relatively high binding affinities vs [^{3}H][*p*-ClPhe^4]DPDPE, being 2.36 and 24.5 nM, respectively. This observation is consistent with previous reports.[20,28] However, in these earlier papers, the substitution of Hat for Tyr^1 increased the μ selectivity of Leu-enkephalin,[21] and retained the high potency and μ selectivity of dermorphin analogues.[28] In our case, the selectivity for the δ receptor was not significantly different from that of DPDPE. In the bioassays (MVD and GPI), these analogues were less selective for the δ receptor than DPDPE, but in binding assays, **9** was 2 times more δ selective than DPDPE. These tyrosine analogues have two rotatable side chain bonds (see Figure 1). Therefore, only two discrete conformations for the side chain of Hat can exist, trans and gauche (−), or trans and gauche (+), respectively, depending on the configuration at the C_α-carbon. In this regard, it is interesting to note that the *k*′ values of compounds **8** and **9** were identical in acetonitrile–0.1% TFA buffer eluent (See Table IV), and the separation also was very difficult in methanol–water. The *k*′ values, binding affinity, and MVD assay data all suggest that the three-dimensional structure of the L- and D-constrained tyrosine analogues are similar. These findings support the idea that the Hat side chain conformation in DPDPE is predominantly trans.

As with the Hat^1-containing analogues, the β-MeTyr^1 analogues of DPDPE are good templates to examine the effects of the topographical arrangements of side chain groups in DPDPE on receptor selectivity and affinity. We have utilized substitution at the diastereotopic β hydrogens of tyrosine as a way of biasing the side chain group to particular side chain torsional angles. Similar examinations were made in our laboratory using all four β-MePhe isomers in [β-MePhe^4]DPDPE[13] and in using the D-Tic (tetrahydroisoquinoline-2-carboxylic acid, a conformationally constrained analogue of Phe) in μ opioid receptor selective analogues of CTP (D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2).[11]

The affinity and selectivity of the [β-MeTyr^1]DPDPE analogues depends greatly on the side chain conformation of β-MeTyr. Placing β-MeTyr in the 1 position of DPDPE significantly lowered the binding affinity vs tritiated δ ligands and the potency in the MVD bioassay. In the L-amino acid-containing [(2*S*,3*S*)-β-MeTyr^1]DPDPE a gauche (−) conformation would be predicted to be preferred for the side chain conformation of β-MeTyr based on steric considerations. Similarly, in [(2*S*,3*R*)-β-MeTyr^1]DPDPE, the preferred side chain conformation of the β-MeTyr would be trans. On the basis of the bioactivities of these analogues, the preferred side chain conformation for Tyr^1 in the DPDPE appears to be trans, as [(2*S*,3*R*)-β-MeTyr^1]DPDPE is greater than 10 times more active in the MVD, and about 5 times more active in the binding assay than [(2*S*,3*S*)-β-MeTyr^1]DPDPE.

Finally, it is clear from these investigations that some modifications of Tyr^1 in DPDPE can be made without disturbing the "bioactive conformation" of DPDPE. The replacement of Tyr^1 by 2′-MeTyr or Hat in DPDPE actually results in more potent analogues. [2′-MeTyr^1]-DPDPE (**6**) has comparable potency and selectivity for δ opioid receptors in radioreceptor assays with our parahalogenated Phe^4-DPDPE analogues[12] and the recently discovered δ opioid peptides, the deltorphins.[29,30] However, in contrast to these latter compounds, it is interesting to note that whereas **6** is more selective than DPDPE in the binding assay, it is less selective than DPDPE in the bioassays. One possible explanation is that this reflects subtypes of δ receptors in these tissues. Alternatively, it could be that **6** does bind with great selectivity for δ receptors, but is more efficatious at μ receptors than DPDPE. Recent pharmacological studies (see for example refs 31, 32) strongly suggest the presence of multiple δ opioid receptors, suggesting the former explanation is more likely.

Experimental Section

General Method for Synthesis of DPDPE Analogues. All of the analogues were synthesized by solid phase peptide methods using procedures similar to those previously used for DPDPE and

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Table IV. Analytical Characterization of Tyr^1-Substituted DPDPE Analogues

	TLC[a] R_f values				HPLC (k')		FAB/MS (M + 1)$^+$	
peptide	I	II	III	IV	V	VI	calcd	found
1	0.43	0.66	0.86	0.81	0.91			
2	0.47	0.67	0.89	0.84	2.74		772	772
3	0.36	0.68	0.86	0.81	1.05		676	676
4	0.40	0.67	0.83	0.82	2.81		691	691
5	0.30	0.39	0.80	0.62	0.63		661	661
6	0.48	0.63	0.81	0.80		5.23	660	660
7	0.51	0.63	0.81	0.80		3.21	660	660
8	0.50	0.59	0.81	0.61	8.59[c]	3.75	672	672
9	0.50	0.59	0.81	0.61	7.97[c]	3.75	672	672
10	0.47	0.63	0.83	0.80	4.57		660	660
11	0.47	0.62	0.80	0.80			660	660
12	0.47	0.63	0.78	0.80		5.60	660	660
13	0.49	0.63	0.78	0.81		2.75	660	660

[a] Merck DC-Fertigplatten Kieselgel 60 F_{254} plates. Solvent systems are as follows: I, butanol–acetic acid–water 4:1:1; II, butanol–acetic acid–pyridine–water 13:3:12:10; III, 2-propanol–ammonia–water 3:1:1; IV, butanol–acetic acid–ethyl acetate–water 1:1:1:1. [b] Capacity factor for the following systems: Vydac 218TP104 C18 column (25 × 0.4 cm) with V (0.1% TFA–CH_3CN 75:25) and VI (0.1% TFA–CH_3CN 80:20) at a flow rate of 1.5 mL/min at 280 nm. [c] Eluent was methanol–water (25:75).

its analogues.[10,12,13] Chloromethylated (1.3 mmol/g resin) polystyrene resin 1% cross-linked with divinylbenzene (Lab. Systems, San Mateo, CA) was used as a solid support. N^α-*tert*-Butyloxycarbonyl (Boc) protected amino acids were used throughout. The unprotected amino acids (Tyr, Gly, 3′-ITyr) were obtained from Aldrich (Milwaukee, WI), the 3′-methoxytyrosine was obtained from Chemical Dynamics Corp. (South Plainfield, NJ). The β-methyltyrosine isomers[33] D,L-2′-methyl-Tyr,[34] 3′-NO_2-Tyr,[35] D,L-6-hydroxy-2-aminotetraline-2-carboxylic acid[21,36] were synthesized in our laboratory and were converted to their N^α-*tert*-butyloxycarbonyl derivatives with di-*tert*-butyl dicarbonate (Fluka) following literature procedures.[37] The N^α-Boc-D-Pen-(*S*-*p*-MeBzl) was obtained as the DCHA salt (Peptides International, Louisville, KY) and liberated just prior to use. The N^α-Boc-D-Pen(*S*-*p*-MeBzl) was attached to the resin by Gysin's method.[38] Diisopropylcarbodiimide and 1-hydroxybenzotriazole (Aldrich, Milwaukee, WI) were used in the coupling reactions which were monitored by the ninhydrin test.[39] Following completion of the synthesis, the peptides were cleaved from the resin with anhydrous HF (10 mL/g resin) with anisole added as a scavenger (2 mL/g resin) for 60 min at 0 °C. The peptide was extracted from the resin by first washing (3 × 10 mL) with anhydrous ethyl ether, then stirring the resin under N_2 suspended in 30 mL of glacial acetic acid. The resin was then washed with 30% acetic acid and water. The combined solutions were lyophilized. The linear peptide was then cyclized using a 0.1 N solution of $K_3[Fe(CN)_6]$ according to procedures published previously.[10] The analogues were purified by gel filtration on Sephadex G-10 using 15% acetic acid and by RP-HPLC (Spectra Physics) using a Vydac 218TP1010 C18 reversed-phase column (25 cm × 1 cm) and a linear gradient of 20–40% CH_3CN in 0.1% aqueous trifluoroacetic acid, 1%/min at a flow rate of 4 mL/min with UV detection at 280 nm. The purity was detected by TLC in four solvent systems on silica gel and by analytical HPLC (see details in Table IV). Amino acid analysis were performed on a 420A amino acid analyzer (ABI). The unusual amino acid D-Pen was not determined. The (M + 1)$^+$ molecular ions and fragmentation patterns were obtained by FAB-MS and were in agreement with the calculated molecular weights for each peptide. ^{1}H-NMR spectra were obtained for each analogue and were consistent with the amino acid sequence and structure of the peptides.

[D-Pen^2,D-Pen^5]enkephalin (DPDPE, 1). The title compound was prepared by the methods described above and was found to be identical to the compound previously synthesized.[10]

[3′-ITyr^1]DPDPE (2). (a) Synthesis of [3′-ITyr^1]DPDPE by SPPS. N^α-Boc-*S*-*p*-MeBzl-D-Pen-resin (1.61 g, 1.5 mmol) was used as the starting material, and the following protected amino acids were added in a stepwise fashion to the growing peptide chain: N^α-Boc-Phe, N^α-Boc-Gly, N^α-Boc-D-Pen(*S*-*p*-MeBzl), and N^α-Boc-3′-ITyr. After coupling of the last amino acid, the N^α-Boc-protecting group was removed by TFA solution, the peptide-resin was neutralized with diisopropylethylamine, and the resulting 3′-ITyr-D-Pen(*S*-*p*-MeBzl)-Gly-Phe-D-Pen(*S*-*p*-MeBzl)-resin was dried in vacuo. Cleavage of all side-chain protection groups and the peptide from the resin was achieved with liquid HF (approximately 20 mL) and 2 mL of anisole, followed by stirring for 60 min at 0 °C. The HF was rapidly removed by vacuum aspiration at 0 °C, the product was washed with ethyl ether (3 × 20 mL), and the peptide was extracted with 40 mL of glacial acetic acid and washed with 10% acetic acid (3 × 20 mL), and both fractions were lyophilized separately. The crude linear peptide product was dissolved in 2 L of deaerated water (using 3 mL of DMF to dissolve completely) and the pH was adjusted to 8.5 with aqueous ammonia. The solution was stirred and 0.10 N $K_3Fe(CN)_6$ added until the yellow color persisted for 20 min. The pH was decreased to 5 with a few drops of acetic acid, and the ferro and excess ferricyanide were removed by stirring the solution with a 30-mL settled volume of Amberlite IR-45 (Cl^- form). Then the mixture was stirred for 1 h and filtered off and washed with 10% acetic acid. The solution was evaporated down to 200 mL and lyophilized. The residue was dissolved in 10 mL of 15% acetic acid and centrifuged at 4000 rpm to precipitate a small amount of ferricyanide that had not been removed previously. The supernatant was applied to a Sephadex G-10 gel filtration column (50 × 3.2 cm). The major peak was isolated and lyophilized. A portion of the powder (100 mg) was dissolved in 2 mL of 20% acetonitrile in 0.1% trifluoroacetic acid and purified on a Vydac 218TP1010 C18 RP-HPLC column (25 cm × 1 cm) with linear gradient elution of 20–40% CH_3CN in 0.1% trifluoroacetic acid 1%/min at a flow rate of 3 mL/min. The more lipophilic impurities were washed from the column with 80%

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CH_3CN in 0.1% TFA for 5 min, and after equilibrium (5 min, 20% CH_3CN) the column was ready to use. The major peak was isolated as a white lyophilized powder; yield 12%. Amino acid analysis: 3′-ITyr 0.90 (1.00), Gly 1.00 (1.00), Phe, 1.00 (1.00). The analytical data are presented in Table IV.

(b) Iodination of DPDPE. The iodination of DPDPE was carried out by the method of Hunter and Greenwood[40] as modified by Miller et al.[20] DPDPE TFA salt (0.83 mg, 1.1 μmol) was dissolved in phosphate buffer (pH 7.2, 0.4 M, 1.5 mL). Then 20 μL of NaI in water (0.224 mg, 1.5 μmol) was added at 0 °C. The reaction was started with addition of 20 μL of aqueous solution of chloramine T (0.82 mg, 2.9 μmol) in water. The mixture was separated by HPLC using a Vydac 218TP1010 C18 column with linear gradient elution; see above. Unreacted DPDPE, [3′-ITyr[1]]DPDPE, a trace of [3′,5′-diiodo-Tyr[1]]DPDPE, and an unknown product were detected (k' values in these conditions: 1.77, 2.6, 3.12, and 1.18, respectively). The third peak was identical with [3′-ITyr[1]]DPDPE obtained by SPPS.

[3′-OCH_3Tyr[1]]DPDPE (3). The title compound was prepared as for [3′-ITyr[1]]DPDPE by SPPS in a 1-mmol scale except that N^{α}-Boc-3′-OMeTyr-OH (mp 127 °C) was coupled to the N-terminal position. The workup and purification were the same procedures as for [3′-ITyr[1]]DPDPE. Yield: 13%. Amino acid analysis: 3′-OCH_3Tyr 0.95 (1.00), Gly 1.00 (1.00), Phe 0.98 (1.00). TLC, HPLC, and FAB-MS data are presented in Table IV.

[3′-NO_2Tyr[1]]DPDPE (4). (a) N^{α}-Boc-S-p-MeBzl-D-Pen-resin (1.17 g, 1 mmol) was used for synthesis which was carried out in a manner similar to that used for [3′-ITyr[1]]DPDPE (3) except that N^{α}-Boc-3′-NO_2Tyr-OH [mp: 99 °C. ^1H NMR ([$^2H_6$]DMSO): δ 7.78 (s, 1 H, aromatic), 7.43 (d, 1 H, aromatic), 7.04 (d, 1 H, aromatic), 4.06 (m, 1 H, αH), 3.01 (dd, 1 H, βH), 2.80 (dd, 1 H, β′H), 1.31 (s, 9 H, t-Bu)] was added to the peptide chain instead of N^{α}-Boc-3′-ITyr-OH. Workup and purification were as in 2 to give the title peptide 4. The yield was 15%. Amino acid analysis: 3′-NO_2Tyr 0.98 (1.00), Gly 1.00 (1.00), Phe 0.96 (1.00). TLC, HPLC, and FAB-MS data are presented in Table IV.

(b) Nitration of DPDPE. Nitration of DPDPE was carried out by the method of Riordan et al.,[41] which was slightly modified according to Guillette et al.[42] DPDPE TFA salt (10.4 mg, 13.6 μmol) was dissolved in 5 mL of a mixture of ethanol–0.01 M ammonium acetate, pH 7 (1:1), and stirred at room temperature. Tetranitromethane (Sigma Chemical Corp., 120 mg, 614 μmol, 45 times excess) in 5 mL of ethanol was added slowly over 30 min and stirred for 5 h. The reaction was checked by RP-HPLC every 30 min, using a Vydac 218TP1010 C18 column [Conditions: linear gradient elution, 20–35% CH_3CN in 0.1% trifluoroacetic acid, 1%/min, at 280 nm at a flow rate of 4 mL/min.]. After 5 h, the pH was adjusted to 4 with acetic acid to stop the reaction. The mixture of products was separated by HPLC (see above). [3′,5′-$(NO_2)_2$Tyr[1]]DPDPE was not detected. Amino acid analysis: 3′-NO_2Tyr 0.95 (1.00), Gly 1.00 (1.00), Phe 1.00 (1.00). TLC, HPLC, FAB-MS and binding data as well as in vitro bioassay in MVD and GPI data were the same as for [3′-NO_2Tyr[1]]DPDPE obtained by SPPS.

[3′-NH_2Tyr[1]]DPDPE (5). (a) Reduction of [3′-NO_2Tyr[1]]-DPDPE by $Na_2S_2O_4$ by the Method of Sokolovsky et al.[43] [3′-NO_2Tyr[1]]DPDPE·TFA salt (5 mg in 2 mL of Tris buffer, pH 8.0, 0.5 M) was added to sodium dithionite (5.2 mg, 30 μmol, 4 times excess) in 0.5 mL of Tris buffer. The yellow color of the solution of [3′-NO_2Tyr[1]]DPDPE disappeared immediately. The reaction mixture was purified by HPLC on a Vydac 218TP1010 C18 column using the same conditions used for [3′-NO_2Tyr[1]]-DPDPE. Yield: 80%. Amino acid analysis: 3′-NH_2Tyr[1] 0.95 (1.00), Gly 1.00 (1.00), Phe 0.98 (1.00). TLC, HPLC, and FAB-MS data are given in Table IV.

(b) Hydrogenation of [3′-NO_2Tyr[1]]DPDPE. [3′-NO_2Tyr[1]]DPDPE·TFA salt (2.9 mg, 3.6 μmol) was hydrogenated in the presence of 7 mg of Pd on charcoal (5%) for 2 h at about 40 psi of H_2 gas. Before filtration of catalyst, 20 μL of dithioethane was added to the reaction mixture to protect it from oxidation and to displace the product absorbed on the catalyst. The catalyst was filtered off and the solution was lyophilized. The trace of dithioethane was removed by HPLC using similar conditions as above. Yield: 50%. TLC, HPLC, and FAB-MS data were the same as for [3′-NH_2Tyr[1]]DPDPE obtained by reduction with $Na_2S_2O_4$.

[L-2′-MeTyr[1]]DPDPE (6) and [D-2′-MeTyr[1]]DPDPE (7). A 1.2-g (1 mmol) samples of N^{α}-Boc-S-p-MeBzl-D-Pen-O-resin was used for the synthesis as for 2 except that N^{α}-Boc-D,L-2′-MeTyr[1]-OH [(mp: 139–140 °C. ^1H NMR: δ 7.09 (d, 1 H, 6′ aromatic, 6.78 (d, 1 H, 3′ aromatic), 6.71 (dd, 1 H, 5′ aromatic), 3.96 (dd, 1 H, αH), 3.28 (dd, 1 H, βH), 2.94 (dd, 1 H, β′H), 2.29 (s, 3 H, CH_3), 1.31 (s, 9 H, t-Bu)][34] was added to the growing peptide chain instead of N^{α}-Boc-3′-ITyr-OH. The obtained peptide-resin was worked up as for 2. The crude cyclic peptide was dissolved in 2 mL of 20% acetonitrile in 0.1% trifluoroacetic acid and purified on a Vydac 218TP1010 C18 RP-HPLC column starting with an isocratic elution of 21% CH_3CN in 0.1% TFA for 20 min at a flow rate of 4 mL/min at 280 nm. The more lipophilic impurities were washed with 90% CH_3CN in 0.1% TFA for 5 min; finally after equilibration with 21% CH_3CN in 0.1% TFA the column can be used for a new purification. There were two major peaks (t_R = 10.38 and 14.66 min, respectively) corresponding to the two diastereomeric peptides each isolated as a white powder. The yield of the first eluted was 26 mg and the yield of the second peak was 39.8 mg.

The determination of the identity of the optically pure enantiomers was carried out by enzymatic[13] and chiral TLC[24] methods using amino acid mixtures obtained from the hyrolyzed peptides of the first or second peak. After enzymatic digestion with L-amino acid oxidase (Sigma, St. Louis, MO), 2′-MeTyr could be detected only from the peptide obtained from the first peak. Therefore, the first peptide eluted corresponds to the D-isomer of [2′-MeTyr[1]]DPDPE and the second peptide eluted is the L-isomer of [2′-MeTyr[1]]DPDPE. Confirmatory results were obtained by chiral TLC.[24] Amino acid analysis: 6, 2′-MeTyr 0.98 (1.00), Gly 1.00 (1.00), Phe 1.02 (1.00); 7, 2′-MeTyr 0.95 (1.00), Gly 1.00 (1.00), Phe 1.00 (1.00). See Table IV for analytical data of the purified products.

[L-and D-Hat[1]]DPDPE (8, 9). The title compounds were prepared from 0.8 mmol of N^{α}-Boc-D-Pen(S-p-MeBzl)-O-resin as for 2 except that racemic N^{α}-Boc-DL-Hat-OH[36] [mp: 150–151 °C. ^1H NMR ([$^2H_6$]DMSO): δ 6.90 (s, 1 H, NH), 6.77 (m, 1 H, aromatic), 6.49 (m, 2 H, aromatic), 3.37 (bs, 2 H, βCH_2), 2.61 (m, 2 H, β′CH_2), 1.86 and 2.23 (m, 2 H, γCH_2), 1.34 (s, 9 H, t-Bu)] was used in place of N^{α}-Boc-3′-ITyr-OH in the synthesis of the peptide chain. A peptide resin was treated with HF as before and the peptide was isolated and cyclized to disulfide. Then 0.2 g of crude peptide was purified by RP-HPLC using a linear gradient elution with 20–40% acetonitrile in 0.1% TFA for 20 min. The more lipophilic impurities were washed with 90% CH_3CN in 0.1% TFA at a flow rate of 4 mL/min. One main peak (t_R = 16.07 min) was collected to give the diastereomeric peptides in one peak. Separation of these peptides in aqueous solvents containing acetonitrile were unsuccessful. Therefore, methanol was used instead of acetonitrile. It was found that 25% methanol in water was the optimal eluent in isocratic mode at a flow rate of 3 mL/min. The retention times of the two peaks were 41.7 and 44.8 min, respectively. We could not determine unambiguously the configuration of Hat in the two peptide peaks because the L-amino acid oxidase assay did not work here, apparently because there is no α-hydrogen in this amino acid. The yield for the peptides obtained in the first and second peak was 8% each. Amino acid analysis: 8, Hat 0.98 (1.00), Gly 1.00 (1.00), Phe 1.03 (1.00); 9, Hat 0.95 (1.00), Gly 1.00 (1.00), Phe 1.02 (1.00). Analytical data for the products are given in Table IV.

[(2S,3S)-β-MeTyr[1]]DPDPE (10) and [(2R,3R)-β-MeTyr[1]]DPDPE (11). The title compounds were prepared from

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0.9 mmol of N^α-Boc-D-Pen(*S*-*p*-MeBzl)-*O*-resin the same way as 2 except that N^α-Boc-*erythro*-D,L-β-MeTyr-OH [mp: 138–139 °C. ^{1}H NMR ([$^2H_6$]DMSO): δ 6.99 (d, 2 H, aromatic), 6.64 (d, 1 H, aromatic), 6.56 (d, 1 H, aromatic), 3.97 (m, 1 H, αH), 2.94 (m, 1 H, βH), 1.27 (s, 9 H, *t*-Bu), 1.11 (d, 3 H, βCH_3). MS (EI): calcd, 295; found, 295][34] was used instead of N^α-Boc-3′-ITyr-OH in the coupling scheme. Workup and purification as for **6** and **7** gave the title compounds **10** and **11** from two separate peaks. Enzymatic and chiral TLC methods[12,24] reveal that the first peptide eluted by HPLC corresponded to the (2*R*,3*R*)-β-MeTyr-containing diastereoisomer **11**, and the second peptide eluted was [(2*S*,3*S*)-β-MeTyr^1]DPDPE (**10**). Yields were 7% for **10** and 5% for **11**. Amino acid analysis: **10**, β-MeTyr 0.95 (1.00), Gly 1.00 (1.00), Phe 1.02 (1.00); **11**, β-MeTyr 0.93 (1.00), Gly 1.00 (1.00), Phe 1.01 (1.00). Analytical data for the purified compounds are given in Table IV.

[(2*S*,3*R*)-β-MeTyr^1]DPDPE (12) and [(2*R*,3*S*)-β-MeTyr^1]DPDPE (13). The title compounds were synthesized from 1.25 mmol of N^α-Boc-D-Pen(*S*-*p*-MeBzl)-*O*-resin as for **10** and **11** except that racemic N^α-Boc-*threo*-β-MeTyr-OH [mp: 100–102 °C. ^{1}H NMR ([$^2H_6$]DMSO): δ 7.03 (d, 2 H, aromatic), 6.72 (d, 1 H, aromatic), 6.62 (d, 1 H, aromatic), 4.28 (m, 1 H, αH), 3.16 (m, 1 H, βH), 1.39 (s, 9 H, *t*-Bu), 1.27 (d, 3 H, βCH_3). MS (EI): Calcd, 295; found, 295][34] was added to the growing peptide chain instead of racemic N^α-Boc-*erythro*-β-MeTyr-OH. The workup, purification, and identification of the configuration of the *threo*-β-MeTyr in the peptides eluted first or second were similar to those for **10** and **11**. According to the enzymatic and chiral TLC method,[12,24] the peptide eluted first on HPLC was [(2*R*,3*S*)-β-MeTyr^1]DPDPE (**13**) and the peptide eluted second corresponded to [(2*S*,3*R*)-β-MeTyr^1]DPDPE (**12**). Yields were 8% for **12** and 6% for **13**. Amino acid analysis: **12**, β-MeTyr 0.98 (1.00), Gly 1.00 (1.00), Phe 1.00 (1.00); **13**, β-MeTyr 0.94 (1.00), Gly 1.00 (1.00), Phe 0.98 (1.00). Analytical data for the purified compounds are given in Table IV.

GPI and MVD Bioassays. Electrically induced smooth muscle contractions of mouse vas deferens and guinea pig ileum longitudinal muscle-myenteric plexus were used as bioassays.[44] Tissues came from male ICR mice weighing 25–30 g and from male Hartley guinea pigs weighing 150–400 g. The tissues were tied to gold chains with suture silk, suspended in 20-mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium-free for the MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1-g tension (0.5 g for MVD) and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz for 0.4-ms pulses (2.0-ms pulses for MVD) and supramaximal voltage. Drugs were added to the baths in 20–60-μL volumes. The agonists remained in contact with the tissue for 3 min and the baths were then rinsed several times with fresh Krebs solution. Tissues were given 8 min to re-equilibrate and regain predrug contraction height. Antagonists were added to the bath 2 min prior to the addition of the agonist. Percent inhibition was calculated by dividing height for 1 min preceding the addition of the agonist by the contraction height 3 min after exposure to the agonist. IC_{50} values represent the mean of not less than four tissues. IC_{50} estimates and relative potency estimates were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method.[45] In some cases, the weak μ agonist actions of these analogues did not permit completion of dose–response curves in the GPI.

Radioreceptor Assay. Adult male Sprague–Dawley rats (200–250 g) were sacrificed and the brains immediately removed and placed on ice. Whole brains including cerebellum was homogenized with a motor-driven Potter-Evjen tissue grinder seven strokes). The homogenate was preincubated at 25 °C for 30 min to remove endogenous opioids and centrifuged two times at 48000*g* for 15 min before use in the radioreceptor assay.

Binding affinities versus [^{3}H]DPDPE or [^{3}H][*p*-ClPhe^4]DPDPE,[16] and [^{3}H]CTOP (New England Nuclear, Boston, MA) were measured by a rapid-filtration technique. A 100-μL aliquot of rat brain homogenate (0.5% final) was incubated with either 1.0 nM [^{3}H]DPDPE, 0.8 nM [^{3}H][*p*-ClPhe^4]DPDPE, or 0.5 nM [^{3}H]CTOP in a total volume of 1 mL of 50 mM Tris-HCl, pH 7.4, containing bovine serum albumin (1 mg/mL), bacitracin (50 μg/mL), bestatin (30 μM), captopril (10 μM), and phenylmethanesulfonyl fluoride (100 μL). Steady state binding experiments were carried out at 25 °C for 180 min. All binding measurements were done in duplicate, and the radioligand displaced by 1 μM naltrexone hydrochloride was defined as specific tissue binding. The binding reaction was terminated by rapid filtration of samples with a Brandel cell harvester through GF/B Whatman glass-fiber filter strips pretreated with 0.1% polyethylenimine solution. This was followed immediately by three rapid washes with 4-mL aliquots of ice-cold saline solution. Filters were removed and allowed to dry before assaying filter-bound radioactivity by liquid scintillation spectrophotometry (45% efficiency). IC_{50} values were calculated by nonlinear regression using a computer program developed by Susan Yamamura.

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