Newly Discovered Stereochemical Requirements in the Side-Chain Conformation of δ Opioid Agonists for Recognizing Opioid δ Receptors

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Topographic design of peptide ligands using specialized topographically constrained amino acids can provide new insights into the stereochemical requirements for δ opioid receptors. A highly constrained tyrosine derivative, (2*S*,3*S*)-β-methyl-2′,6′-dimethyltyrosine [(2*S*,3*S*)-TMT], was prepared by asymmetric synthesis and incorporated in [D-Pen^2,D-Pen^5]enkephalin (δ_1) and Deltorphin I (δ_2). The results of binding assays and bioassays showed that the two analogues (**3** and **4**) acted very differently at δ opioid receptors. Further pharmacological evaluations suggested that they actually interact primarily with the δ_1 and δ_2 receptor subtypes, respectively. These results, and conformational studies using NMR and computer-assisted modeling, provided insights into the different stereochemical requirements for these two δ opioid ligands to recognize the δ opioid receptor and its subtypes.

Introduction

Numerous studies have been made to elucidate the structure–activity relationship of enkephalin and other opioid peptides since the discovery of enkephalin in 1975.[1] One approach for the design of highly selective ligands involves the incorporation of conformational constraints.[2] In our laboratory, this approach has led to the development of one of the most selective analogues for the δ opioid receptors the cyclic analogues [D-Pen^2,D-Pen^5]enkephalin (DPDPE), a peptide with a highly constrained 14-membered ring.[3] Structure–activity studies led to the suggestion that the Tyr^1 and Phe^4 residues were two critical pharmacophores of DPDPE, and this has led to a second generation of analogues of DPDPE with modification of the parent peptide in the Tyr^1 and Phe^4 residues.[4-6] The rationale for these modifications has been based on our examination of the conformation and dynamics of DPDPE which indicate that the Tyr^1 and Phe^4 aromatic side chain groups were on the same surface of the structure in relatively close proximity with some differences between suggested solution and "bioactive" conformations.[7] The need to develop analogues with appropriate topographical constraints in χ (χ_1, χ_2, etc.) space thus became evident. An example of this approach for DPDPE includes [6-hydroxy-2-aminotetralin-2-carboxylic acid^1]DPDPE ([Hat^1]-DPDPE), which had about the same binding affinity as its parent compound at δ opioid receptors, but was 5 times less potent in the MVD assay.[8] Incorporation of Hat^1 into Leu-enkephalin also led to a potent analogue.[9] The β-methyltyrosine^1 (β-MeTyr) and β-methylphenylalanine^4 (β-MePhe) analogues also served as useful templates to examine the effects of the topographical arrangements of the aromatic side chain groups in DPDPE on δ opioid receptor selectivity and affinity.[8,10] In the case of the incorporation of β-MeTyr^1 isomers into DPDPE a moderately potent and selective analogue, [(2*S*,3*R*)-β-MeTyr^1]-DPDPE, was obtained.[8] Since both the χ_1 and χ_2 torsinal angles were constrained in Hat, but only the χ_1 angle was constrained in β-MeTyr, we decided it was necessary to design a new class of Tyr derivatives that can better examine χ_1 and χ_2 constraints in structures more closely related to tyrosine. This seemed important since almost all modifications of the Tyr^1 residue in δ opioid ligands have led to decreases in opioid δ receptor affinity, including *N*-alkylation of the α-amino group by methyl or pentyl,[11] iodination,[12] and substitution of hydrogens with methoxy groups in the aromatic moiety of Tyr^1 residue.[13] One exception was the introduction of lipophilic alkyl groups into the Tyr^1 aromatic ring, especially at the 2′- and 6′-positions which offered a more potent but less selective DPDPE analogue.[14]

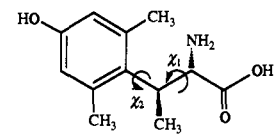
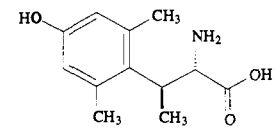


Figure 1. (2*S*,3*S*)-2′,6′-Dimethyl-β-methyltyrosine [(2*S*,3*S*)-TMT].

In this study, we demonstrate for the first time the differential stereochemical requirements of the aromatic side chains of δ opioid peptide agonists for δ opioid receptor recognition. We report that by topographic modification of the Tyr^1 residue in both cyclic D-Pen^2,D-Pen^5]enkephalin (DPDPE, **1**) and in Deltorphin I (DELT I, **2**), significant differences in binding affinity as well as δ opioid receptor selectivity are obtained. A conformationally constrained tyrosine derivative, (2*S*,3*S*)-β-methyl-2′,6′-dimethyltyrosine [(2*S*,3*S*)-TMT] (Figure 1), was designed and was prepared by asymmetric synthesis.[15] Because of the



3: (2*S*,3*S*)-TMT-D-Pen-Gly-Phe-D-Pen-OH
4: (2*S*,3*S*)-TMT-D-Ala-Phe-Asp-Val-VAl-Gly-NH_2

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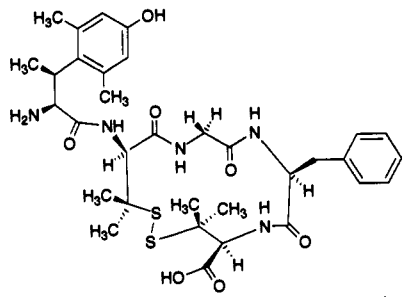

Figure 2. Schematic structure of [(2*S*,3*S*)-TMT[1]]DPDPE.

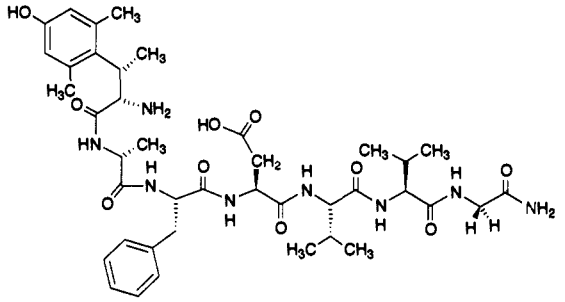

Figure 3. Schematic structure of [(2*S*,3*S*)-TMT[1]]Deltorphin I.

methyl substitutions in this tyrosine derivative, rotation about both the χ_1 and χ_2 torsion angles is restricted (Figure 1).[16] Methodologies used for the asymmetric synthesis of (2*S*,3*S*)-TMT were modified from those reported previously for other aromatic amino acids[17-20] to obtain (2*S*,3*S*)-TMT in high chiral purity. The peptides were prepared by solid-phase synthesis. Structures of [(2*S*,3*S*)-TMT[1]]-DPDPE (**3**) and [(2*S*,3*S*)-TMT[1]]Deltorphin I (**4**) are shown in Figure 2 and 3, respectively.

Results

Binding Assays against Radiolabeled δ_1 Ligand. Incorporation of (2*S*,3*S*)-TMT into DPDPE led to a 130-fold decrease in binding affinity and a 100-fold decrease in selectivity for δ opioid receptors (Table 1), but resulted in hardly any changes in binding affinity to the μ receptor, thus providing a nonselective ligand. On the other hand, in [(2*S*,3*S*)-TMT[1]]Deltorphin I, though the binding affinity for the δ receptor decreased 5-fold, it remained in the nanomolar range for the δ receptor, and its binding affinity for the μ receptor decreased 8-fold, thus providing a highly potent and a more δ-receptor-selective ligand than the native compound DELT I (**2**).

Bioassays. The results obtained for compounds **1**–**4** in the *in vitro* guinea pig ileum (GPI, μ) and mouse vas deferens (MVD, δ) bioassays are shown in Table 1. Incorporation of (2*S*,3*S*)-TMT into DPDPE led to an analogue which showed a 40-fold decrease in potency in the MVD (δ) assay and a 25-fold increase in potency in the GPI (μ) assay which provided a 1000-fold decrease in selectivity for δ opioid receptors (Table 1), thus giving a nonselective ligand. On the other hand, for [(2*S*,3*S*)-TMT[1]]Deltorphin I, the potencies in both the GPI and MVD assays were very similar to those for DELT I (Table 1), thus providing a highly potent and δ-receptor-selective ligand.

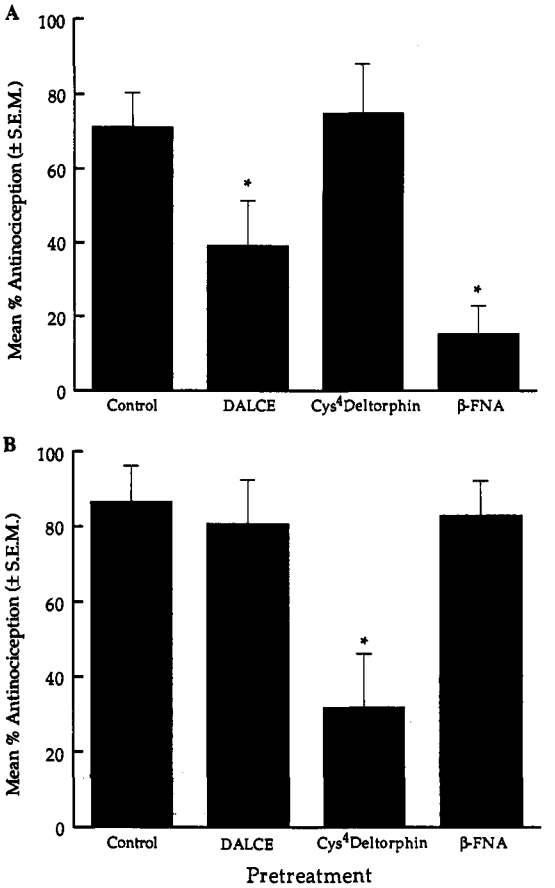

Figure 4. (A) Antinociceptive effect of icv [(2*S*,3*S*)-TMT[1]]-DPDPE (**3**) (30 nmol at + 10 min) in control animals and animals pretreated with DALCE (4.4 nmol at −24 h), [Cys[4]]Deltorphin (3 nmol at −24 h), and β-FNA (18 nmol at −24 h) in the mouse tail-flick test. (B) Antinociceptive effect of icv [(2*S*,3*S*)-TMT[1]]-DELT I (**4**) (30 nmol at + 10 min) in control animals and animals pretreated with DALCE (4.4 nmol at −24 h), [Cys[4]]Deltorphin (3 nmol at −24 h) and β-FNA (19 nmol at −24 h) in the mouse tail-flick test (an asterisk, *, indicates a significant difference versus control, $p < 0.05$).

Antinociception Assay. The DPDPE analogue **3** and the DELT I analogue **4** were tested for their antinociceptive effects using [D-Ala[2],Leu[5],Lys[6]]enkephalin (DALCE), [Cys[4]]Deltorphin and β-funaltrexamine (β-FNA) as selective antagonists for δ_1, δ_2, and μ receptors, respectively.[21-26] Both analogues **3** and **4** produced an analgesic effect in control assays. The results (Figure 4) demonstrate that the analgesic effect of [(2*S*,3*S*)-TMT[1]]DPDPE (**3**) was significantly antagonized by DALCE (from 71.3% ± 9.1% to 39.2% ± 12.0%), a δ_1 antagonist, as well as by β-FNA, a μ antagonist (from 71.3% ± 9.1% to 15.2% ± 7.6%). Thus, the DPDPE analogue **3** has agonist effects at both δ_1 and μ opioid receptors. On the other hand, the analgesia effect of the DELT I analogue **4** was only antagonized by

Table 1. Binding Affinities and Biological Potencies

	binding data IC_{50} (nM) ± SEM			bioassay data EC_{50} (nM) ± SEM		
peptide[a]	[^{3}H]CTOP	[^{3}H][*p*-ClPhe[4]]DPDPE	selectivity (μ/δ)	GPI (μ)	MVD (δ)	selectivity (μ/δ)
DPDPE (1)	609 ± 70	1.6 ± 0.2	380	7300 ± 1700	4.1 ± 0.46	1780
Deltorphin I (2)	2100 ± 690	0.60 ± 0.30	3500	2900 ± 250	0.36 ± 0.04	8060
3	722 ± 126	211 ± 33	3.4	293 ± 0.70	168 ± 37.2	1.74
4	17100 ± 3900	2.98 ± 0.14	5740	3840 ± 850	0.66 ± 0.063	5820

[a] **3** = [(2*S*,3*S*)-TMT[1]]DPDPE; 4 = [(2*S*,3*S*)-TMT[1]]DELT I.

Table 2. ^{1}H Chemical Shifts (δ, ppm) and Coupling Constants (J, Hz) for [(2S,3S)-TMT^1]DPDPE (3) and [(2S,3S)-TMT^1]Deltorphin I (4) (T = 305 K, DMSO-d_6)[a]

	NH		H_α		H_β		H_γ	
residue	3	4	3	4	3	4	3	4
(2S,3S)-TMT^1			4.17 $J_{\alpha\beta}$ = 10.1	4.19 $J_{\alpha\beta}$ = 11.3	3.28	3.31	1.15 $J_{\beta\gamma}$ = 7.1	1.09 $J_{\beta\gamma}$ = 7.3
D-Pen^2, D-Ala^2	8.67 $J_{NH\alpha}$ = 6.3 (8.4)	8.95 $J_{NH\alpha}$ = 7.9 (6.2)	4.57	4.53 $J_{\alpha\beta}$ = 7.0		0.92	1.50 1.26	
Gly^3, Phe^3	8.51 $J_{NH\alpha}$ = 9.3 $J_{NH\alpha'}$ = 2.6 (3.8)	8.44 $J_{NH\alpha}$ = 8.9 (8.2)	4.33 (α) 3.27 (α') $J_{\alpha\alpha'}$ = 14.6	4.64 $J_{\alpha\beta}$ = 2.8 $J_{\alpha\beta'}$ = 11.4		3.06 (β) 2.68 (β') $J_{\beta\beta'}$ = 13.7		
Phe^4, Asp^4	8.77 $J_{NH\alpha}$ = 7.6 (7.5)	8.59 $J_{NH\alpha}$ = 7.6 (6.1)	4.30 $J_{\alpha\beta}$ = 3.8 $J_{\alpha\beta'}$ = 11.3	4.61 $J_{\alpha\beta}$ = 5.4 $J_{\alpha\beta'}$ = 8.6	3.09 (β) 2.84 (β') $J_{\beta\beta'}$ = 14.3	2.69 (β) 2.51 (β') $J_{\beta\beta'}$ = 16.9		
D-Pen^5, Val^5	7.28 $J_{NH\alpha}$ = 8.6 (0.0)	7.66 $J_{NH\alpha}$ = 8.6 (4.2)	4.32	4.24 $J_{\alpha\beta}$ = 6.4		1.99	1.37 1.31	0.81 $J_{\beta\gamma}$ = 6.8
Val^6	N/A	7.86 $J_{NH\alpha}$ = 8.5 (5.4)	N/A	4.10 $J_{\alpha\beta}$ = 6.8		1.97		0.85 $J_{\beta\gamma}$ = 6.7
Gly^7	N/A	8.04 $J_{NH\alpha}$ = 5.7 $J_{NH\alpha'}$ = 5.7 (6.9)	N/A	3.66 (α) 3.60 (α') $J_{\alpha\alpha'}$ = 16.5	N/A	N/A		

[a] Chemical shifts of (2S,3S)-TMT^1 aromatic protons and 2',6'-dimethyl protons are 6.40 (3)/6.41, 6.38 (4) ppm, 2.32, 2.22 (3)/2.29, 2.23 (4) ppm, respectively; chemical shifts of Phe aromatic protons are 7.2–7.3 (3) and 7.1–7.3 (4) ppm, respectively. Temperature coefficients of NH protons (–ppb/K) are given in parentheses.

Table 3. ^{13}C NMR Chemical Shifts (δ ppm) for [(2S,3S)-TMT^1]DPDPE (3) and [(2S,3S)-TMT^1]Deltorphin I (4) (T = 305 K, DMSO-d_6)[a]

	C_α		C_β		C_γ	
residue	3	4	3	4	3	4
(2S,3S)-TMT^1	59.6	55.2 (1.6 ± 0.3)	37.0	36.5	16.8	16.1
D-Pen^2, D-Ala^2	59.1	48.3	N/A	19.1	26.4 27.9	N/A
Gly^3, Phe^3	41.9	53.5	N/A	38.2	N/A	N/A
Phe^4, Asp^4	56.2	50.0	36.5	36.3	N/A	N/A
D-Pen^5, Val^5	62.1	57.7	N/A	30.6	26.0 27.7	18.3
Val^6	N/A	58.4	N/A	30.4	N/A	18.3
Gly^7	N/A	41.9	N/A	N/A	N/A	N/A

[a] Chemical shifts of (2S,3S)-TMT^1 aromatic carbons and 2',6'-dimethyl carbons are: 115.4, 117.4 (3)/115.9, 117.6 (4) ppm, 21.3, 21.5 (3)/21.5 (4) ppm, respectively; chemical shifts of Phe aromatic carbons are 126.4, 128.9 (3)/128.4, 129.7 (4) ppm, respectively. The long-range proton–carbon coupling constants ($J_{H_\alpha C_\gamma}$) (Hz) of (2S,3S)-TMT^1 in 4 are given in parentheses.

[Cys^4]Deltorphin (from 86.6% ± 9.6% to 31.9% ± 14.2%), a δ_2 antagonist, and was not affected by DALCE (δ_1) or β-FNA (μ). Thus the DELT I analogue 4 is a pure δ_2 selective agonist.

NMR Studies. 1D proton NMR spectroscopy, z-filtered total correlation spectroscopy (zf-TOCSY),[27] rotating frame exchange spectroscopy (ROESY),[28] heteronuclear single quantum relay spectroscopy (HSQC)[29] and temperature dependence of NH proton chemical shift experiments were performed on DPDPE, DELT I and analogues 3 and 4. Details of the methods are described in the Experimental Section. The proton chemical resonances were assigned by using 2D zf-TOCSY and 2D ROESY spectra. 1D proton NMR spectroscopy and 2D zf-TOCSY were used to measure homonuclear vicinal coupling constants. The results are listed in Table 2. z-Filtered carbon-coupled HSQC-TOCSY experiments were used for the assignment of carbon resonances, and for evaluation of long-range heteronuclear coupling constants. The low concentration of peptide 3 allowed only the assignment of protonated carbons (Table 3) by HMQC. Observed NOEs from 2D ROESY spectra are shown in Tables 4 and 5, and results of temperature-dependence studies of NH proton chemical shifts are listed in Table 2. The rotamer populations of side-chain conformations were obtained by calculations using both homonuclear ($^3J_{H_\alpha H_\beta}$) and heteronuclear ($^3J_{H_\alpha C_\gamma}$) coupling constants (details in the Experimental Section). In the case of peptide 3, a special method using the γ-effect (details in the Experimental Section) was applied to determine the side-chain rotamer populations of TMT.[1] Details of the calculations of the rotamer populations of side chains are described in the Experimental Section, and the results are listed in Table 6.

Discussion

It has been suggested based on pharmacological studies that DPDPE and Deltorphin II binded preferentially to two different subtypes of δ opioid receptors.[21–26] We have demonstrated in binding assays and bioassays that compounds 3 and 4, both derived from highly selective δ opioid ligands and with the same modifications of the Tyr^1 residue, behaved very differently on interacting with the opioid δ receptors. These results strongly suggest that these two analogues interact differently at the two subtypes of δ opioid receptors. Because there is no reliable binding

Table 4. Observed NOE Correlations of [(2S,3S)-TMT[1]]DPDPE (T = 305 K, DMSO-d_6)[a]

residue	NH	H_α	H_β
(2S,3S)-TMT[1]			(2S,3S)-TMT[1]-2′,6′-Me (m), 2.32, 2.22 ppm, (2S,3S)-TMT[1]-β-Me (m)
D-Pen[2]		D-Pen[2]-Me (m), *pro-R*, 1.5 ppm, D-Pen[2]-Me (m), *pro-S*, 1.26 ppm	
Gly[3]	D-Pen[2]-H_α (s), Gly[3]-$H_{\alpha'}$ (w), *pro-R*, 3.27 ppm, D-Pen[2]-Me (w), *pro-R*, 1.5 ppm		
Phe[4]	Gly[3]-H_α (m), *pro-S*, 4.33 ppm, Phe[4]-H_α (w), Phe[4]-$H_{\beta'}$ (w), *pro-R*	Phe[4]-H_β (s), *pro-S*, 3.09 ppm, Phe[4]-$H_{\beta'}$ (w), *pro-R*, 2.84 ppm, Phe[4]-aromatic-Hs (m-w)	
D-Pen[5]	Phe[4]-NH (m), D-Pen[5]-H_α (m-w), Phe[4]-H_α (w), D-Pen[5]-Me (w), *pro-S*, 1.37 ppm	D-Pen[5]-Me (m), *pro-S*,1.37 ppm, D-Pen[5]-Me (m), *pro-R*,1.31 ppm	

[a] The strength of the NOE correlations are shown in parentheses; w = weak, m = medium, s = strong.

Table 5. Observed NOE Correlations of [(2S,3S)-TMT[1]]Deltorphin I (T = 305 K, DMSO-d_6)[a]

residue	NH	H_α	H_β
(2S,3S)-TMT[1]		(2S,3S)-TMT[1]-aromatic-Me (m-w), (2S,3S)-TMT[1]-H_β (w)	(2S,3S)-TMT[1]-aromatic-Me (m-w), (2S,3S)-TMT[1]-β-Me (m-w)
D-Ala[2]	D-Ala[2]-H_α (w), (2S,3S)-TMT[1]-H_α (s), D-Ala[2]-Me (w)		
Phe[3]	D-Ala[2]-H_α (s), Phe[3]-H_α (m-w), Phe[3]-aromatic-Hs (w), Phe[3]-$H_{\beta'}$, *pro-R*, (s-m), D-Ala[2]-Me (w)	Phe[3]-H_β, *pro-S* (m), Phe[3]-$H_{\beta'}$, *pro-R* (w)	
Asp[4]	Phe[3]-H_α (s), Asp[4]-H_α (w), Phe[3]-H_β, *pro-S* (w), Asp[4]-$H_{\beta'}$, *pro-S*, (m-w), Val[5]-NH (w), Phe[3]-NH (w)	Asp[4]-$H_{\beta'}$, *pro-S* (w), Asp[4]-H_β, *pro-R* (m)	
Val[5]	Asp[4]-H_α (s-m), Val[5]-H_α (m-w), Val[5]-H_β (w), Val[5]-H_γ (w), Phe[3]-H_β, *pro-S* (vw), Asp[4]-H_β, *pro-R*, (vw)	Val[5]-H_β (m), Val[5]-H_γ (m-w)	
Val[6]	Val[5]-H_α (s-m), Val[6]-H_α (w), Val[5]-NH (m-w), Val[6]-H_β (m-w)	Val[6]-H_β (m), Val[6]-H_γ (w)	
Gly[7]	Val[6]-H_α (m), Gly[7]-$H_{\alpha'}$ (m), *pro-S*, Val[6]-NH (m-w), Val[6]-H_β (vw)	$H_{\alpha'}$ (pro-*S*) with D-Ala[2]-Me (w), H_α (*pro-R*) with Val[5]-Me (m-w), H_α (*pro-R*) with Val[6]-Me (m-w)	

[a] The strength of the NOE correlations are shown in parentheses; vw = very weak, w = weak, m = medium, s = strong.

Table 6. Population of Side-Chain Rotamers between Gauche (−), Trans, and Gauche (+) along the χ_1 Torsional Angle

peptide	amino acid residue	population (%) gauche (−)	trans	gauche (+)
DPDPE	Tyr[1]	42 (39)[b]	30 (60)[b]	28 (1)[b]
	Phe[4]	72 (69)[b]	7 (17)[b]	21 (14)[b]
(TMT[1])[a]-DPDPE	(TMT[1])[a]	63	5	32
	Phe[4]	75	3	22
DELT I	Tyr[1]	31	20	49
	Phe[3]	86	4	10
	Asp[4]	53	10	37
(TMT[1])[a]-DELT I	(TMT[1])[a]	75	3	22
	Phe[3]	80	2	18
	Asp[4]	55	25	20

[a] (2S,3S)-TMT. [b] Data in parentheses were reported previously by Mosberg *et al.* (see ref 35).

assays that can be used to demonstrate the heterogeneity of opioid δ receptor subtypes, we used a well-accepted *in vivo* antinociceptive assay to examine the possible δ opioid receptor subtype selectivities of analogues **3** and **4**. Both analogues **3** and **4** produced analgesia in these assays (Figure 4). Pretreatment of mice with DALCE (an δ_1 antagonist) significantly antagonized the analgesic effect of analogue **3**. On the other hand, pretreatment of mice with [Cys[4]]Deltorphin (a δ_2 antagonists) did not antagonize the analgesic effect of peptide **3**. These results demonstrated that the DPDPE analogue **3** is a δ_1 selective agonist. It is noted that β-FNA (a μ antagonist) did antagonize the analgesia effect of compound **3** in antinociceptive assay suggesting that compound **3** has a mixed analgesia effect mediated through both δ and μ opioid receptors. This observation also is supported by binding and bioassay data which showed considerable potency for analogue **3** at μ opioid receptors. Since only pretreatment of mice with [Cys[4]]Deltorphin but not DALCE or β-FNA could significantly antagonize the analgesic effect of DELT I analogue **4**, [(2S,3S)-TMT[1]]DELT I appears to be a pure, highly potent and selective agonist for the opioid δ_2 receptor subtype. Thus, this study enables us to demonstrate for the first time that a DPDPE analogue **3** and a DELT I analogue **4** may actually interact differently (quantitatively) with the two subtypes of δ opioid receptors. More importantly, because we have used a highly constrained Tyr[1] residue that is located in the message domain of these δ opioid ligands, conformational analysis can provide insights into the differential stereochemical requirements of the message domain pharmacophores for recognizing the two opioid δ receptor subtypes, respectively.

2D NMR techniques[30] and computer-assisted modeling have been used to determine the favored solution (DMSO-d_6) conformations of analogues **3** and **4**. Although the low-energy conformations we obtained are not necessarily the bioactive conformations, the approach taken here can provide possible insights into the bioactive conformation. For [(2S,3S)-TMT[1]]DPDPE, the NMR data are consistent with the backbone conformation derived from the X-ray crystal structure of DPDPE.[31] Therefore, the initial backbone conformation used for molecular modeling of

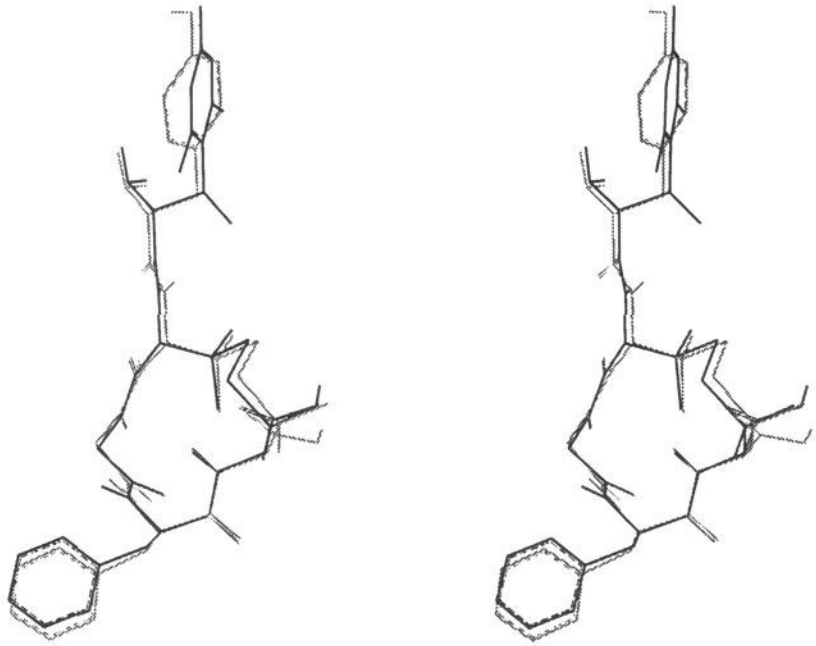

Figure 5. Stereoview of the superposition (two molecules were matched with five α-carbons) of preferred conformers of [(2*S*,3*S*)-TMT[1]]DPDPE (in thick darker lines) and the DPDPE crystal structure (in fainted lines) after energy minimization (TRIPOS force field, Delre charges, dielectric constant = 45, gradient = 0.05 kcal/mol, RMS = 0.2).

[(2*S*,3*S*)-TMT[1]]DPDPE was taken directly from the crystal structure of DPDPE. The side-chain conformations of the (2*S*,3*S*)-TMT[1] and Phe[4] residues were derived from the measured homonuclear $J_{H_\alpha H_\beta}$ and heteronuclear $J_{H_\alpha C_\gamma}$ vicinal coupling constants and the conformational dependence of the γ-effect.[32-34] In our study, the observed side-chain rotamers of Tyr[1] residue in DPDPE (Table 6) were almost equally distributed between gauche (−), trans, and gauche (+). It is noted that in a previous NMR study of DPDPE (in DMSO) a slightly preference for trans (60%) rotamer was suggested;[35] this difference may be caused by the slightly different experimental conditions used. Our results show (Table 6) that for both the (2*S*,3*S*)-TMT[1] and the Phe[4] residues, by far the most populated conformation for the χ_1 torsion angle is gauche (−), and that gauche (+) also is populated. Computer-assisted modeling has given further insights into the topographic structure of this peptide. The program, SYBYL 6.01, was used, and energy minimization was carried out after applying NMR constraints. The side-chain conformations were modeled utilizing the NMR data. One preferred conformer was found, which has a gauche (−) conformation about the χ_1 torsion angle both for the (2*S*,3*S*)-TMT[1] and Phe[4] residues. In Figure 5, the superposition of this conformer of [(2*S*,3*S*)-TMT[1]]DPDPE and the crystal structure of DPDPE is shown. It correlates exceptionally well with the DPDPE crystal structure (RMS = 0.2 Å) except for a minor alteration of the χ_2 angle for the (2*S*,3*S*)-TMT[1] side chain (χ_2 = 100°) when compared to that of Tyr[1] in DPDPE (χ_2 = 125°). The energy barrier between the rotomers about the χ_2 angle in (2*S*,3*S*)-TMT[1] was previously determined from temperature-dependent NMR studies[16] and is approximately 15–20 kcal/mol, which indicated there was a slow rotation along the χ_2 angle of TMT.[16] The similarity of backbone conformation of [(2*S*,3*S*)-TMT[1]]DPDPE and of DPDPE suggests that these two analogues should bind to opioid δ receptors similarly. Thus their differences in binding affinity may be due to the significantly different preferred side-chain conformations of their *N*-terminal residue (Table 6) (though the steric effect cannot be completely discounted). The bioassay and binding affinity results (Table 1) strongly support this suggestion. An examination of the spacial relationships between the TMT (Tyr[1] in the DPDPE crystal structure) and Phe[4] has been made by defining two planes using the planar aromatic moieites as templates in the model developed. We found that in the DPDPE crystal structure, the plane angle between the Tyr[1] aromatic moiety and Phe[4] aromatic moiety was 45°, and the centroid distance between these two aromatic ring was 15.2 Å; in [(2*S*,3*S*)-TMT[1]]DPDPE the plane angle between TMT[1] aromatic moiety and Phe[4] aromatic moiety was 64°, and centroid distance between these two aromatic ring was 15.3 Å. The difference in spacial relationship between the two aromatic pharmacophores, especially in the plane angle differences, also may be important for the decrease in potency of the DPDPE analogue **3**. It was previously noted that the increase of the lipophilicity of the aromatic moiety in Tyr[1] where 2,6-dimethyltyrosine (DMT) was incorporated can increase the potency of the DPDPE analogue.[8] Since we did not see an increase of potency of the DPDPE analogue **3** with increased lipophilicity of aromatic moiety in Tyr[1] by methyl substitution, we believe the χ space orientation of the Tyr[1] side chain and its stereochemistry can play a major role for DPDPE and its analogues recognizing δ opioid receptors. In addition, the steric effect of the β-methyl substitution previously noted in β-MeTyr[1] analogues of DPDPE[8] also may be important.

In [(2*S*,3*S*)-TMT[1]]DELT I, the most populated conformation for the χ_1 torsion angle is gauche (−) for both the (2*S*,3*S*)-TMT[1] and for the Phe[3] residues, as was found for [(2*S*,3*S*)-TMT[1]]DPDPE. [(2*S*,3*S*)-TMT[1]]DELT I was modeled based on the NMR data. Vicinal coupling constants ($^3J_{NH_\alpha}$) and temperature coefficients $\Delta\delta_{NH}/\Delta T$ obtained for [(2*S*,3*S*)TMT[1]]DELT I in DMSO (Tables 2 and 3) are generally consistent with the same parameters obtained for DELT I under somewhat different experi-

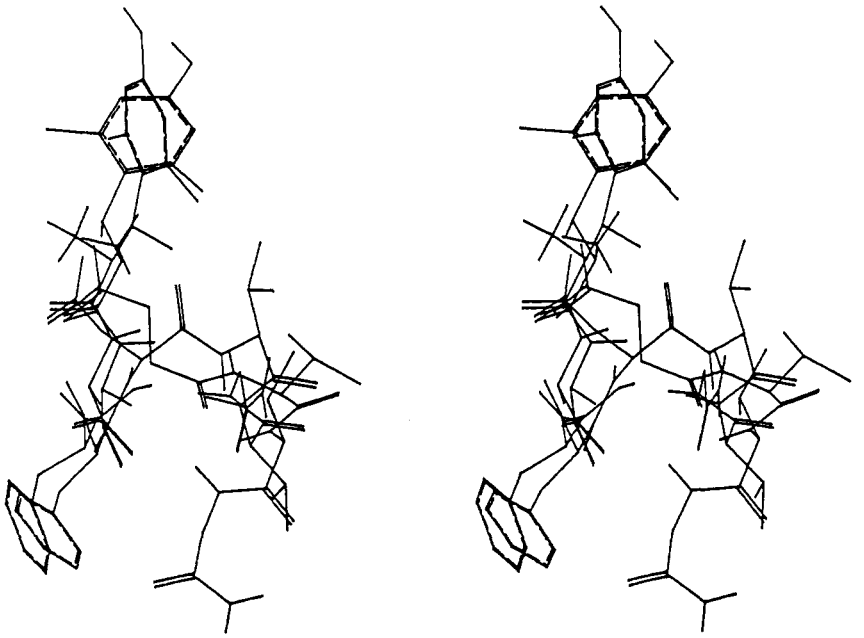

Figure 6. Superimposed stereoview of two conformations of [(2*S*,3*S*)-TMT^1]Deltorphin I derived from NMR constraints (DMSO-d_6). Energy was minimized by using the TRIPOS force field, Delre charges, and dielectric constant = 45.

mental conditions.[36-38] The temperature coefficients range from −4.2 to −8.8 ppb/K, which indicate that none of the amide protons are involved in a stable intramolecular hydrogen bond. The observed NOEs between backbone protons of neighboring residues are indicative of a time-averaged solution conformation. In particular, the relatively intense $C_\alpha H_i$ – NH_{i+1} and NH_i – NH_{i+1} NOEs simultaneously observed for residues 4–7 in [(2*S*,3*S*)-TMT^1]DELT I cannot be expected to have their origin from the same conformer.[39] On the other hand, three consecutive NH_i – NH_{i+1} NOEs in the C-terminal part of [(2*S*,3*S*)-TMT^1]DELT I and several medium- and long-range NOEs, especially the NOEs between the $C_\beta H_3$ of D-Ala^2 and $C_\alpha H$ of Gly^7, strongly suggest that highly ordered, folded conformers of [(2*S*,3*S*)-TMT^1]DELT I exist in equilibrium with extended disordered conformers. Similar conclusions have been put forward in previous NMR and conformational studies of DELT I.[36-38] The multiple NH_i – NH_{i+1} connectivities in C-terminal part are common for [(2*S*,3*S*)-TMT^1]DELT I and DELT I studied under different conditions. However, medium- and long-range NOEs observed for [(2*S*,3*S*)-TMT^1]DELT I differ from those observed previously for DELT I under somewhat different conditions.[36-38] Moreover, different relative intensities of NOE cross-peaks were observed for DELT I at 295 and 310 K.[38]

Molecular modeling carried out in this study was aimed at determining low-energy conformers of [(2*S*,3*S*)-TMT^1]-DELT I, which might help explain the observed nonsequential NOEs, including the three consecutive NOEs between the amide protons of Asp^4, Val^5, Val^6, and Gly^7, a long-range NOE between the $C_\beta H_3$ of D-Ala^2 and the $C_\alpha H$ or Gly^7, and two medium-range NOEs between the other α-proton of Gly^7 and the γ-methyl protons of Val^5 and Val^6. The modeling was based on 20 low-energy conformers of a DELT I analogue obtained previously[40] from the ECEPP/2 force field.[41,42] In order to model first the long-range contact between D-Ala^2 and Gly^7, we performed energy minimization of these conformers of DELT I analogue, applying the one-side penalty potential to constrain the distance between the C_β of D-Ala^2 and the C_α of Gly^7. A conformer was found which satisfied this constraint, as well as most of the local NOE constrains. Visual inspection of this conformer revealed that a small modification can bring into close proximity of the C_α of Gly^7 and a C_γ atom of either Val^5 or Val^6. Therefore, energy minimization based on this structure was repeated several times with an additional constraining potential applied between the C_α of Gly^7 and one of the C_γ atoms of Val^5 or Val^6. As a result, two conformers of DELT I analogue 4 were found that satisfied the long-range NOE between D-Ala^2 and Gly^7, as well as one of the two medium-range NOEs between C_α of Gly^7 and the γ-protons of Val^{5,6}. Finally, the two conformers of [(2*S*,3*S*)-TMT^1]DELT I were transferred into the SYBYL 6.01 program with modification of Tyr^1 to (2*S*,3*S*)-TMT^1 and refined by energy minimization with the TRIPOS force field,[43] which included the distance constraints applied to the atom pairs corresponding to the medium- and long-range NOEs observed in DMSO.

Superposition of the two resulting conformers is shown in Figure 6. Both models have similar conformations of the N-terminal tetrapeptide and differ in the C-terminal moiety. Both conformers violate one of the medium-range NOEs between Val^5 or Val^6 and Gly^7, as well as some local constraints imposed by $C_\alpha H_i$ – NH_{i+1} NOEs. The results of molecular modeling shows that the NMR data obtained for [(2*S*,3*S*)-TMT^1]DELT I cannot be explained either by a single conformer or even by a family of related conformers. In addition, both conformers differ from those suggested for DELT I on the basis of NMR measurements in a mixture DMSO/H_2O at low temperature.[36,37] These discrepancies probably reflect a complex conformational equilibrium which exists for these linear peptides in solution. Differences in NMR data obtained for DELT I and [(2*S*,3*S*)-TMT^1]DELT I indicate that both structural modifications and different experimental conditions can change the statistical weights of backbone conformers. In these circumstances no clear conclusion can be drawn on the relationship between solution and biologically active conformations of DELT I analogues: a receptor environment can select any conformer contributing into an equilibrium in solution. However, the two *N*-terminal conformers of [(2*S*,3*S*)-TMT^1]DELT I selected here as

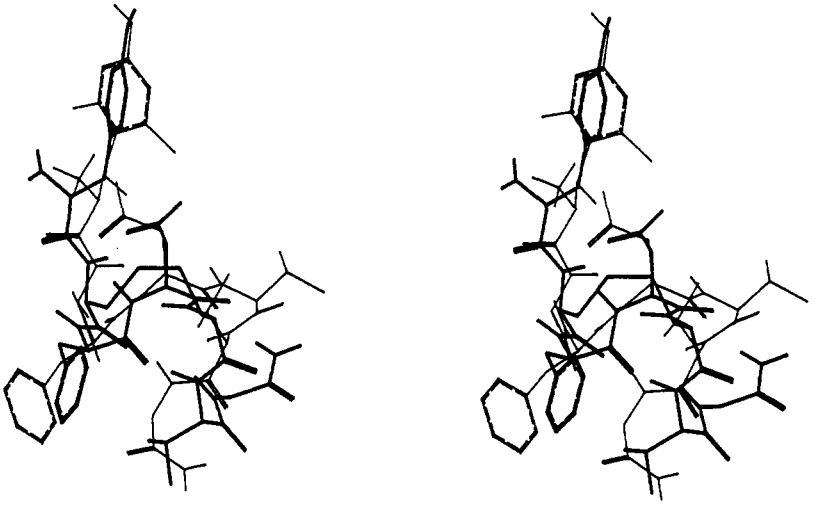

Figure 7. Superimposed (residues 1–4 in both molecules) stereoview of one conformation of [(2*S*,3*S*)-TMT[1]]Deltorphin I (thinner lines) derived from NMR constraints (DMSO-d_6) and model of cyclic [D-Cys[2],Cys[5]]Deltorphin I (thicker lines) derived from calculations. Energy was minimized by using the Tripos force field, Delre charges, and dielectric constant = 45.

possible contributors to the solution equilibrium, show some resemblance to the biologically active conformation suggested earlier for the potent cyclic analog [D-Cys[2],Cys[5]]-Deltorphin I.[40] The RMS deviation of the C_α and the C_β atoms of residues 1–4 between the biologically active conformation and one of the conformers found in this work was 0.65 Å; the corresponding superposition is shown in Figure 7. This comparison suggests that the conformational motif found in this preliminary modeling may be involved in δ opioid receptor binding of [(2*S*,3*S*)-TMT[1]]-DELT I.

On the basis of the NMR results and biological testing data, we postulate that the gauche (−) conformation of Tyr[1] in DPDPE as found to be the most populated in analogue **3** does not provide a favorable topography for potent and selective interaction with opioid δ receptors. A populated trans side-chain conformation for Tyr[1] in DPDPE tends to afford a potent and selective DPDPE analogue as suggested by previous NMR studies[35,7a] and in a proposed bioactive conformation based on theoretical calculations.[7b] On the other hand, in the DELT I analogue **4**, preference for a gauche (−) side-chain conformation led to a selective and potent δ opioid agonist. A receptor-bound conformation of Dermenkephalin, a Deltorphin like δ opioid agonist, was previously suggested based on theoretical calculations, and a gauche (−) conformation was found to be the preferred conformation of the Tyr[1] side chain in the bioactive conformations of Dermenkephalin and its analogues.[44] These results suggest in general that a gauche (−) side-chain conformation of Tyr[1] is preferred for Deltorphin I analogues to bind to δ opioid receptors, while a gauche (−) side-chain conformation of Tyr[1] may not a preferred conformation for DPDPE analogues (Tables 1 and 6). Hence, the stereochemical requirements of the Tyr[1] residues are different for DPDPE (and its analogue) and DELT I (and its analogue) to recognize their respective opioid δ receptor subtypes. Thus we propose that the stereochemical requirements for recognizing the δ_1 opioid receptor subtype are different from those for the δ_2 opioid receptor subtype.

Attempts to superimpose the crystal structure of the DPDPE and the suggested solution conformation of the [(2*S*,3*S*)-TMT[1]]DELT I were not successful. The centroid distance between two aromatic rings (TMT[1] and Phe[3]) in the two models of DELT I analogue **4** was 11.5 Å and 10.9 Å, respectively, they were 3 to 4 Å closer than that in DPDPE and its analogue **3**. These may also reflect the different stereochemical requirements for DPDPE and DELT I to recognize opioid δ receptors. It should be noted that although there is strong pharmacological support for classification of opioid δ receptor subtypes,[22–26] unequivocal demonstration of δ receptor subtypes using radioligand techniques has not been totally achieved yet. Radioligand binding studies have been reported that suggest the existence of subtypes of opioid δ receptor,[21,45] although other interpretation of these data appear possible. Additionally, to date the molecular structure of only one δ receptor has been reported,[46] although additional δ receptors may yet be identified. In consequence, a definitive identification of the different affinities of the analogues as due to the subtypes of opioid δ receptors is not possible. Studies attempting to evaluate the binding of these compounds to the cloned δ receptor, and to mutants of this receptor using radiolabeled derivatives, are currently underway and should yield insights as to how these compounds recognize different opioid receptor types and subtypes differently and to possible reasons for the observed dramatic changes in selectivity seen in the two analogues reported here.

Conclusions

Incorporation of (2*S*,3*S*)-TMT into δ opioid peptide agonists with subtype selectivity provided restrictions to the rotation about the tyrosine side chain both at the χ_1 and χ_2 torsion angles and elimated one of the rotamers about the χ_1 torsion angle. We have shown that topographic modification of the Tyr[1] residue with TMT can provide new insights into the different topographical requirements for DPDPE and Deltorphin I to recognize the δ opioid receptor and its subtypes. A reasonable interpretation of the binding and bioassay results suggests that [(2*S*,3*S*)-TMT[1]]DPDPE and [(2*S*,3*S*)-TMT[1]]DELT I primarily bind to the δ_1 and δ_2 receptors, respectively.

Table 7. Analytical Characterization of DPDPE, Deltorphin I, and Their Tyr¹-Modified Analogues

peptide	TLC[a] R_f values				HPLC k' (T_R, min)[b]		FAB-MS (M + 1)⁺	
	I	II	III	IV	V	VI	calcd	found
1	0.27	0.64	0.33	0.67	6.69 (19.1)	5.08 (15.1)	646	646
2	0.22	0.65	0.30	0.71	6.56 (18.8)	4.87 (14.6)	769	769
3	0.43	0.67	0.44	0.69	8.05 (22.4)	6.71 (19.1)	688	688
4	0.32	0.71	0.44	0.74	7.38 (20.8)	5.93 (17.2)	811	811

[a] Merck DC-Fertigplatten Kieselgal 60 F_{254} plates (ninhydrin monitored). Solvent systems are as follows: I, butanol–acetic acid–water, 4:1:5; II, butanol–pyridine–acetic acid–water, 15:10:3:12; III, butanol–water (3.5% acetic acid and 1.5% pyridine), 1:1; IV, 1-amyl alcohol–pyridine–water, 7:7:6. [b] Capacity factors (k') and retention times (T_R, min) were recorded from the following systems: Vydac 218TP104 C18 column (25 × 0.4 cm) with V (linear gradient 0–55% of CH_3CN in 30 min with 0.1% TFA) and VI (linear gradient 10–55% of CH_3CN in 30 min with 0.1% TFA) at a flow rate of 1.5 mL/min at 280 nm.

Experimental Section

General Methods for Peptide Synthesis and Purification. All of the analogues were synthesized by solid-phase peptide methods using procedures similar to those previously used for DPDPE, Deltorphin I, and their analogues.[3,4,8,10] Chloromethylated (0.7 mmol/g) polystyrene resin 1% cross-link with divinylbenzene (Peptides International, Louisville, KY) was used as a solid support for the syntheses of the DPDPE analogue. 4-Methylbenzhydrylamine polystyrene resin (0.51 mmol/g) 1% cross-link with divinylbenzene (Bachem California, Torrance, CA) was used as a solid support for the syntheses of Deltorphin I and its analogue.[47] All syntheses were carried out on a Vega 1000 semiautomatic peptide synthesizer. N^{α}-*tert*-Butyloxycarbonyl (Boc)-protected amino acids were used throughout. The unprotected amino acids (Gly, Val, Phe) were obtained from Aldrich (Milwaukee, WI), the D-Pen-*S*-*p*-MeBzl was obtained from Peptides International (Louisville, KY), and all of them were converted to their N^{α}-*tert*-butyloxycarbonyl derivatives with *di*-*tert*-butyl dicarbonate (Bachem California, Torrance, CA) following literature procedures. The N^{α}-Boc-D-Ala and the N^{α}-Boc-L-Asp-β-benzyl ester were obtained from Bachem California (Torrance, CA). The (2*S*,3*S*)-2′,6′-dimethyl-β-methyltyrosine was prepared in our laboratory[15] and was converted to its N^{α}-*tert*-butyloxycarbonyl derivative with *di*-*tert*-butyl dicarbonate (Bachem California, Torrance, CA) following literature procedures.[48] The N^{α}-Boc-D-Pen-*S*-*p*-MeBzl was attached to the chloromethylated polystyrene resin by Gysin's method.[49] Diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBT, Aldrich, Milwaukee, WI) was prepared as 1 M solution in DMF prior to use in the coupling reactions which were monitored by the ninhydrin test.[50] (Benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent, Bachem California, Torrance, CA) and diisopropylethylamine (DIEA, Aldrich, Milwaukee, WI) in 1-methyl-2-pyrolidinone (NMP, Aldrich, Milwaukee, WI) was used to couple N^{α}-Boc-(2*S*,3*S*)-TMT. Following completion of the synthesis, the peptides were cleaved from the resin with anhydrous HF (10 mL/g resin) with *p*-cresol and thiocresol as scavengers (0.5 g each/g resin) for 60 min at 0 °C. The cleaved resin was washed with anhydrous ether (3 × 60 mL) and was then extracted with glacial acetic acid (5 × 60 mL). The acetic acid solution was combined, frozen, and lyophilized to afford the crude peptide. The linear DPDPE analogue was then cyclized using a 0.1 M solution of $K_3[Fe(CN)_6]$. The details are as following: the linear DPDPE was dissolved in CH_3CN and transferred to a 50-mL syringe, the peptide solution was added (with a rate of 1.0 mL/h) to 50 mL of a degassed 0.1 M solution of $K_3[Fe(CN)_6]$ at pH = 8.3–8.7 (adjusted by addition of 6% NH_4OH solution) under argon. After all the linear peptide solution had been transferred to the $K_3[Fe(CN)_6]$ solution, the iron anions were removed by anion-exchange resin (Cl^- form). The peptide analogues were purified by RP-HPLC (Perkin Elmer) using a Vydac 218TP1010 C_{18} reverse-phase column (25 cm × 1 cm) and a linear gradient of 15–75% CH_3CN in 0.1% aqueous TFA, at a flow rate of 3 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 Table 7). Amino acid analyses were performed on a 420A amino acid analyzer (ABI). The (M + 1)⁺ molecular ions and fragmentation patterns were obtained by FABMS and were consistent with the amino acid sequence and structure of the peptides.

[D-Pen²,D-Pen⁵]Enkephalin (DPDPE, 1). The title compound was prepared by the methods described above and was found to be identical to the compound previously synthesized.[3,4,10]

Deltorphin I (2). The title compound was prepared by the methods described above (without cyclization procedure) and was found to be identical to the compound previously synthesized.[47,51]

[(2*S*,3*S*)-TMT¹]DPDPE (3). N^{α}-Boc-S-*p*-MeBzl-D-Pen-resin (0.74 g, 0.68 mmol/g, 0.5 mmol) was used as 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 optically pure N^{α}-Boc-(2*S*,3*S*)-TMT. Analytical data of N^{α}-Boc-(2*S*,3*S*)-TMT: mp 156.5–157 °C. ^{1}H-NMR ($CDCl_3$, TMS): δ 6.49 (s, broad, 2H, 3′, 5′ aromatic-Hs), 4.64–4.48 (m, 1H, C_{α}-H), 3.53 (m, 1H, C_{β}-H), 2.36 (s, Ar-CH_3), 2.30 (s, Ar-CH_3′), 1.51 (d, J = 23.74 Hz, 3H, C_{β}-CH_3), 1.33 (s, 9H, *t*-Bu). HR-EIMS: calcd for $C_{17}H_{25}NO_5$, 323.1733; found (M⁺ + 1), 323.1728. $[\alpha]^{25}_D$ = −18.5° (c 0.14, $CHCl_3$). All the N^{α}-*tert*-butyloxycarbonyl (Boc) protected amino acids (2 equiv) except for N^{α}-Boc-(2*S*,3*S*)-TMT were coupled to the growing peptide chain by using DIC (2.5 equiv) and HOBT (2.5 equiv) as coupling reagents. N^{α}-Boc-(2*S*,3*S*)-TMT (1.2 equiv) was added to the growing peptide chain using BOP reagent (1.44 equiv) and DIEA (1.7 equiv) in 1-methyl-2-pyrolidinone (NMP) as solvent. After coupling of the last amino acid, the resin was washed with dichloromethane (6 × 30 mL) and methanol (4 × 35 mL) and was dried by nitrogen gas flow (9 psi) for 10 min. The resin was then stored *in vacuo* for 24 h. Cleavage of all side chain protecting groups and the peptide from the resin was achieved with liquid HF (approximately 10 mL) and 0.5 g of *p*-cresol and 0.5 g of thiocresol, followed by stirring for 60 min at 0 °C. The HF was rapidly evaporated by vacuum aspiration at 0 °C to room temperature over 15 min. The product was washed with anhydrous ether (6 × 30 mL), and the peptide was extracted with glacial acetic acid (8 × 25 mL). The acetic acid fractions were combined and lyophilized. The crude linear peptide was dissolved in a 30-mL acetonitrile and water mixture (2:1, v/v) and was transferred to a syringe. The peptide solution was added to a degassed 0.1 M solution of $K_3[Fe(CN)_6]$ (2 equiv of crude linear peptide) at pH = 8.3–8.7 at a rate of 0.1 mL/h via a syringe pump. The pH value was maintained at 8.3–8.7 at a rate of 0.1 mL/h via a syringe pump. The pH value was maintained at 8.3–8.7, and the entire process of cyclization was done under the protection of bubbling argon. After all the peptide solution had been transferred to the $K_3[Fe(CN)_6]$ solution, the pH was adjusted to 4 by addition of acetic acid solution, and the ferro- and excess ferricyanide were removed by stirring the solution with a 9-mL settled volume of Amberlite IR-45 anion-exchange resin (Cl^- form). The mixture was stirred for 1.75 h, and the anion-exchange resin was filtered and washed by 50% acetonitrile/water mixture (v/v) (8 × 30 mL). The solution was evaporated down to *ca.* 100 mL and lyophilized. The residue was dissolved in acetonitrile and 0.1% TFA water solution mixture (15:85, v/v) and purified on a Vydac 218TP1010 C_{18} RP-HPLC column (25 cm × 1 cm) with a linear gradient elution of 15–75% CH_3CN in 0.1% trifluoroacetic acid (aqueous solution) for 1 min at a flow rate of 3 mL/min. The more lipophilic impurities were washed from the column with 95–100% CH_3CN in 0.1% TFA for 10 min, and after equilibrium (11 min, 15% CH_3CN) the column was ready for use again. The UV detector was set at 280 nm during the

entire purification process. The major peak was isolated and lyphilized to afford a white powder. Yield 13%. Amino acid analysis: (2S,3S)-TMT 0.95 (1.00), Gly 1.04 (1.00), Phe 1.00 (1.00). The analytical data are presented in Table 7.

[(2S,3S)-TMT^1]Deltorphin I (4). 4-Methylbenzhydrylamine polystyrene resin (0.51 mmol/g) 1% cross-link with divinylbenzene (Bachem California, Torrance, CA) was used as a solid support for the syntheses of the title compound 4, and the following protected amino acids were added in a stepwise fashion to the growing peptide chain: N^{α}-Boc-Gly, N^{α}-Boc-Val, N^{α}-Boc-Val, N^{α}-Boc-Asp(O-Bzl), N^{α}-Boc-Phe, N^{α}-Boc-D-Ala, and optically pure N^{α}-Boc-(2S,3S)-TMT. An excess (2 equiv) of protected amino acids [except for N^{α}-Boc-(2S,3S)-TMT], HOBT, and DIC was used for the coupling reactions, which were monitored by ninhydrin tests. N^{α}-Boc-(2S,3S)-TMT (1.2 equiv) was added to the growing peptide chain using BOP reagent (1.44 equiv) and DIEA (1.7 equiv) in NMP for 15 h. The resin was washed and dried, the protecting groups were removed, and the peptide was cleaved from the resin in a similar fashion as that for preparation of the DPDPE analogue 3. The crude product was dissolved in acetonitrile and 0.1% TFA water solution mixture (15:85, v/v) and purified on a Vydac 218TP1010 C_{18} RP-HPLC column (25 cm × 1 cm) with linear gradient elution of 15-70% CH_3CN in 0.1% trifluoroacetic acid (aqueous solution) for 1 min at a flow rate of 3 mL/min. The more lipophilic impurities were washed from the column with 95–100% CH_3CN in 0.1% TFA for 10 min, and after equilibrium (11 min, 15% CH_3CN) the column was ready for use again. The UV detector was set at 280 nm during the entire purification process. The major peak was isolated and lyphilized to afford a white powder; yield 45%. Amino acid analysis: (2S,3S)-TMT 0.98 (1.00), D-Ala 1.01 (1.00), Phe 1.00 (1.00), Asp 1.08 (1.00), Val 1.95 (2.00), Gly 1.04 (1.00). The analytical data are presented in Table 7.

Radioligand Binding Assay. Membranes were prepared from whole brains taken from adult male Sprague–Dawley rats (250–300 g) obtained from Harlan Sprague–Dawley, Inc. (Indianapolis, IN). Following decapitation, the brain was removed, dissected, and homogenized at 0 °C in 20 volumes of 50 mM Tris-HCl (Sigma, St. Louis, MO) buffer adjusted to pH 7.4 using a Teflon-glass homogenizer. The membrane fraction obtained by centrifugation at 48000g for 15 min at 4 °C was resuspended in 20 volumes of fresh Tris buffer and incubated at 25 °C for 30 min to dissociate any receptor-bound endogeneous opioid peptides. The incubated homogenate was centrifuged again as described and the final pellet resuspended in 20 volumes of fresh Tris-HCl buffer.

Radioligand binding inhibition assay samples were prepared in an assay buffer consisting of 50 mM Tris-HCl, 1.0 mg/mL bovine serum albumin, 30 μM bestatin, 50 μg/mL bacitracin, 10 μM captopril, and 0.1 mM toluenesulfonyl fluoride, pH 7.4 (all from Sigma, St. Louis, MO) except bestatin which was obtained from Peptides International (Louisville, Kentucky). The radioligands used were cyclic [^{3}H][D-Pen^2,*p-Cl*-Phe^4,D-Pen^5]-enkephalin[52] at a concentration of 0.75 nM and [^{3}H]CTOP (New England Nuclear, Boston, MA) at a concentration of 0.5 nM.[53] Peptide analogues were dissolved in assay buffer (or prior to that in DMSO or methanol according to the chemist's recommendations and did not exceed 5% of the total volume) and added to duplicate assay tubes at 10 concentrations over an 800-fold range. Control (total) binding was measured in the absence of any inhibitor while nonspecific binding was measured in the presence of 10 μM naltrexone (Sigma, St. Louis, MO). The final volume of the assay samples was 1.0 mL, of which 10% consisted of the memberane preparation in 0.1 mL of Tris-HCl buffer. Incubations were performed at 25 °C for 3 h, after which the samples were filtered through polyethylamine (0.5% w/v, Sigma, St. Louis, MO) treated GF/B glass fiber filter strips (Brandel, Gaithersburg, MD). The filters were washed three times with 0.4 mL of ice-cold normal saline before transfer to scintillation vials. The filtrate radioactivity was measured after adding 7–10 mL of cocktail [Ecolite (+), ICN Biomedicals, Inc.] to each vial and allowing the samples to equilibrate over 8 h at 4 °C.

Binding data were analyzed by nonlinear least-square regression analysis program named Inplot 4.03 (GraphPad, San Diego, CA). Statistical comparisons between one and two site fits were made using *F*-ration test using a *p* value of 0.05 as the cut-off for significance.[54] Data best fitted by a one-site model was reanalyzed using the logistic equation.[55] Data obtained from independent measurements are presented as the arithmetic mean ± SEM.

***In Vitro* Bioassays.** Electrically induced smooth muscle contractions from mouse vas deferens (MVD) and guinea pig ileum (GPI) longitudinal muscle-myenteric plexus were used for bioassays.[56] 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 equilibrated 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 equilibrated 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 tissue baths for 3 min and were removed by rinsing 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 agonists. 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. Estimates and relative potency estimates were determined by fitting the mean data to the Hill equation by using a computerized nonlinear least-squares method.[57] In some cases, the weak μ agonist action of these analogues did no permit completion of dose-response curves in the GPL.

Method of Antinociceptive Assay. Male ICR mice weighing 20–30 g were used throughout these studies. All testing was performed in accordance with the recommendation and policies of the International Association for the Study of Pain (IASP) and National Institute of Health (NIH) and the University of Arizona Guidelines for the care and use of laboratory animals. Antinociception was assessed in mice using the warm water tail flick assay. In the tail flick assay, tails were dipped in 55 °C water, and the latency to a rapid flick was recorded with the baseline cut-off and maximal possible latencies set at 5 and 15 s, respectively. Percent antinociception was calculated according to the following formula: 100 × (test–control latency)/(15 s–control). The compounds 3, 4, DALCE, [Cys^4]Deltorphin, and β-FNA were dissolved in 10% dimethyl sulfoxide. Intracerebroventricular (icv) administration was performed using the methods of Haley and McCormick[58] as modified by Porreca *et al.*[59] Briefly, mice were lightly anesthetized with ether, and a small incision was made in the scalp. A Hamilton microsyringe was inserted to a depth of 3 mm at a point 2 mm caudal and 2 mm lateral from bregma. Compounds were injected in a 5 μL volume for all icv administrations. In studies assessing the effects of the selective antagonists DALCE (4.57 nmol), [Cys^4]Deltorphin (3 nmol), and β-FNA (19 nmol), compounds were injected 24 h prior to agonist administration. These times and doses have previously been shown to produce selective blockade of δ_1, δ_2, and μ opioid receptors, respectively.[25,60,61] Agonist 3 or 4 was then injected and antinociception assessed at the time of peak drug effect (10 min). Data are presented as the means ± the standard errors of the mean for groups of 10 mice. Significant difference were determined by a researcher's *t*-test set at the $p < 0.05$ level.

Nuclear Magnetic Resonance Experiments. All NMR experiments were performed on peptide samples dissolved in DMSO-d_6 at 305 K with a concentration of 3.5 mg/0.5 mL for [(2S,3S)-TMT^1]-DPDPE and 10 mg/0.5 mL for [(2S,3S)-TMT^1]-DELT I, respectively. The proton and the carbon chemical shifts were referenced to the residual ^{1}H-DMSO solvent signal (at 2.49 ppm) and to the ^{13}C solvent signal (at 39.5 ppm), respectively. All NMR parameters used in the present study were obtained from 1D and 2D experiments performed on a BRUKER AM500 spectrometer (500 MHz proton frequency) using a 5-mm inverse probehead. Proton resonance assignments were obtained using 2D total correlation spectroscopy (*z*-filtered TOCSY)[21] and 2D ROESY experiments.[22] ROE cross-peaks were classified according to their intensities as strong (s), medium (m), and weak

(w) correlations corresponding to 1.8–2.5-, 1.8–3.5-, and 1.8–4.5-Å distance constraints. Resolution-enhanced 1D spectra, or in case of signal overlap, the highly digitized 1D traces of z-filtered TOCSY spectra were used to measure the conformationally important homonuclear vicinal coupling constants. Proton detected heteronuclear spectroscopy, namely the z-filtered carbon coupled HSQC-TOCSY experiment,[23] was used for the assignment of carbon resonances and for the evaluation of long range heteronuclear coupling constants ($^3J_{H_\alpha C_\gamma}$) in the peptide 4. The low sample concentration of peptide 3 allowed only the assignment of protonated carbons by means of HMQC experiment.[62]

The homonuclear coupling constants $^3J_{NH_\alpha}$ were used to estimated the ϕ angles[32a] for the corresponding amino acids residues and to assess the consistency with our computer-assisted modeling study. The $^3J_{H_\alpha H_\beta}$ coupling constants in combination with the observed intraresidue ROE patterns were used for stereospecific assignment of H_β-protons and determination of preferred side-chain conformations.[32,33] The side-chain conformation of the (2S,3S)-TMT[1]-residue (with one H_β-proton) in peptide 4 was deduced from the measured homonuclear ($^3J_{H_\alpha H_\beta}$ and heteronuclear ($^3J_{H_\alpha C_\gamma}$) vicinal coupling constants using the following equations:[32,33] $J_{H_\alpha H_\beta} = P^{ap}J_{H_\alpha H_\beta} + (1 - P)^{sc}J_{H_\alpha H_\beta}$ and $J_{H_\alpha C_\gamma} = P'^{ap}J_{H_\alpha C_\gamma} + (1 - P')^{sc}J_{H_\alpha C_\gamma}$ where P and P' are rotamer populations corresponding to the antiperiplanar (ap) arrangements of the relevant spins. The following values $^{ap}J_{H_\alpha H_\beta} = 13.9$ Hz, $^{sc}J_{H_\alpha H_\beta} = 3.55$ Hz, $^{ap}J_{H_\alpha C_\gamma} = 8.5$ Hz, and $^{sc}J_{H_\alpha C_\gamma} = 1.4$ Hz were used for antiperiplanar and synclinal (sc) arrangements. An error of ±5% for rotamer populations can be estimated from the inaccuracy of the coupling constants. In the [(2S,3S)-TMT[1]]-DPDPE case, however, the low sample concentration did not allow the measurement of the heteronuclear long-range coupling constant. The carbon chemical shift of β-Me carbon of (2S,3S)-TMT[1]-residue, however, can be put to good use as a sensitive measure of side-chain conformation, taking advantage of the well-known conformational dependent γ-effect.[34] The contributions to the β-Me carbon chemical shift from the NH_2 [of the (2S,3S)-TMT[1]-residue] and C=O functionality [of (2S,3S)-TMT[1]-residue] are given by the following equation:

$$\delta(\beta\text{-Me-}^{13}\text{C}) = \delta(\text{ref}) + P_{\text{I}}{}^{\text{gauche}}\delta_{\text{CO}} + P_{\text{II}}{}^{\text{guache}}\delta_{\text{NH}} + P_{\text{III}}{}^{\text{gauche}}\delta_{\text{CO,NH}}$$

where $P_{\text{I-III}}$ are the populations of the staggered rotamers and $^{\text{gauche}}\delta_{\text{CO}}$, $^{\text{gauche}}\delta_{\text{NH}}$ and $^{\text{gauche}}\delta_{\text{NH}}$ and $^{\text{gauche}}\delta_{\text{CO,NH}}$ are the shielding parameters of the relavent substituents which are −3.2, −4.6, and −7.8 ppm, respectively. δ (ref) is the β-Me carbon reference chemical shift, that was calculated from the rotamer populations and the β-Me carbon shift (ppm) of the [(2S,3R)-TMT[1]]DPDPE isomer which was available at sufficient concentration for evaluation of the heteronuclear long-range coupling constants.

The z-filtered TOCSY spectra were recorded using a repetition delay of 1 s between the subsequent transients, and the isotropic mixing periods (MLEV-17)[63] were set to 60 ms. A total of 256 experiments of 128 scans for the peptide 3 and 96 scans for the peptide 4 were accumulated; 4096 data points were recorded in the acquisition dimension (F_2) and the spectra width was 5430 Hz. The spectra were recorded in the phase-sensitive mode using the TPPI method.[64] z-Filter delay of 15 ms was randomly varied to obtain pure absorption phase data. Zero-filling in both F_1 and F_2 multiplication with a squared cosine function was performed prior to 2D Fourier transformation. For evaluation of coupling constants a final digital resolution of 0.3 Hz/point was achieved by inverse Fourier transformation, zero-filling, and back-transformation of selected traces. The duration of the ^{1}H 90° pulse was 26 μs.

ROESY experiments were carried out in reverse configuration using the decoupler for ^{1}H pulsing. Decoupler power was attenuated to give a 90° pulse of 75 μs (spin-lock field strength of 3333 Hz). The duration of CW spin-lock pulse was 200 and 160 ms for the peptide 3 and the peptide 4, respectively. A total of 256 experiments with 224 transients for the peptide 3 and 320 experiments with 192 transients for the peptide 4 were carried out. A relaxation delay of 1 s was allowed between the subsequent transients; 2018 data points were recorded in F_2. Zero-filling in both F_1 and F_2 multiplication with a squared cosine function was performed prior to 2D Fourier transformation. HMQC experiments extended with TANGO and spin-lock preparatory pulses were carried out on peptide 3; 160 transients were accumulated for each 256 experiments. A relaxation delay of 0.8 s was allowed between the subsequent transients. A total 2048 data points were recorded in F_2. The spectral widths were 5430 and 16 000 Hz in proton and carbon dimensions, respectively. The durations of the ^{1}H and ^{13}C 90° pulses were 13.2 and 15.5 μs. A spin-lock pulse of 4 ms was employed after the TANGO sandwich pulse to improve the suppression of the unwanted ^{1}H – ^{12}C magnetization.

The phase-sensitive z-filtered ^{13}C-coupled HSQC-TOCSY spectrum on the peptide 3 was obtained by recording 256 experiments of 320 transients each. A relaxation delay of 0.8 s was allowed after each acquisition. The spectral widths were 5430 and 16 000 Hz in proton and carbon dimensions, respectively; 4096 data points were recorded in the F_2 dimension. The durations of ^{1}H and ^{13}C 90° hard pulses were 13.7 and 15.5 μs, respectively. For the MLEV-17 mixing sequence the decoupler was attenuated to give a proton 90° pulse of 27 μs. Prior to acquisition a z-filtered consisting of two 90° pulses separated by a randomly varied delay of 15 ms was employed to provide pure absorption phase data. The heteronuclear long-range coupling constants were obtained by comparison of the corresponding multiplet width in the z-filtered homonuclear TOCSY and heteronuclear HSQC-TOCSY spectra.[23] The same experiments were carried out on DPDPE and DELT I and the same methods were used to measure the side-chain rotamer populations of DPDPE and DELT I.

Methods for Computer-Assisted Modeling. Molecular modeling for [(2S,3S)-TMT[1]]DPDPE was performed with SYBYL 6.01 program implemented on a Silicon Graphics workstation IRIS. The initial backbone conformation was taken directly from the crystal structure of DPDPE[31] because it was generally consistent with the NMR data. The two most populated conformers of χ_1 torsion angle of TMT[1] were considered together with two conformers of the Phe[4] side chain. Energy minimization was performed using the TRIPOS force field[43] with partial atomic charges determined by the Delre technique.[65] A dielectric constant $\epsilon = 45$ corresponding to the DMSO environment was assumed in these calculations.

Preliminary stages of molecular modeling for [(2S,3S)-TMT[1]]-Deltorphin I were performed with ECEPP/2 force field[41,42] and associated rigid-valence geometry. Initial conformations were taken from an earlier study of a Deltorphin I analogue.[40] Distance constraints derived from selected NOEs were applied during energy minimization as one-side harmonic penalty potentials $U_{pen} = U_o(d - d_0)^2$, where force constant U_o was set to zero when a current interatomic distance d was less then a predetermined upper limit d_o. The force constants $U_o = 5$ kcal/mol·Å^2 were used with upper limits of 5.0 Å for carbon–carbon distances and 3.5 Å for carbon–proton distances. Selected conformers obtained in the ECEPP/2 calculations were transferred into SYBYL 6.0 program and refined by constrained energy minimization using the TRIPOS force field with $\epsilon = 45$.[43,65] Two-side harmonic potentials with $U_o = 25$ kcal/mol·Å^2 and $d_0 = 3.5$ Å were applied to constrain selected interatomic distances.

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Supplementary Material Available: The synthetic methodology including analytical data for the asymmetric synthesis of the specialized amino acid (2S,3S)-β-methyl-2′,6′-dimethyltyrosine is available (8 pages). Ordering information is given on any current masthead page.

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