Probing the Stereochemical Requirements for Receptor Recognition of δ Opioid Agonists through Topographic Modifications in Position 1

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Abstract: A series of side-chain constrained tyrosine derivatives, 2′,6′-dimethyl-β-methyltyrosines (TMT), has been designed and incorporated into position 1 of the highly selective δ opioid agonists DPDPE (Tyr-D-Pen[2]-Gly-Phe-D-Pen[5]-OH) and deltorphin I (DELT I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2). Molecular mechanics calculations on isolated TMT residues and nuclear magnetic resonance (NMR) studies of the TMT[1]-containing peptides in DMSO showed that each of the four stereoisomers of TMT favors one particular rotamer of the side-chain χ_1 torsional angle. Therefore, substitution of four TMT isomers for Tyr[1] allows us to perform a systematic conformational scan through three staggered rotamers of the aromatic side chain, *gauche* (−), *trans*, and *gauche* (+), and to explore specific binding requirements of the receptor in relation to the side chain conformation. The potency and selectivity of four isomers of [TMT[1]]DPDPE and four isomers of [TMT[1]]DELT I were evaluated by radioreceptor binding assays in the rat brain using μ- and δ-selective radiolabeled ligands and by bioassays with guinea pig ileum (GPI, μ receptor) and mouse vas deferens (MVD, δ receptor). In the DPDPE series only one isomer, [(2*S*,3*R*)-TMT[1]]-DPDPE showed high potency and selectivity for the δ opioid receptors. The favorable side-chain rotamers found for this analogue, i.e., the *trans* rotamer of TMT[1] and the *gauche* (−) rotamer of Phe[4], were proposed as the most probable δ receptor-binding conformations of DPDPE analogues. Two [TMT[1]]DELT I isomers possessed considerable δ receptor potencies. The (2*S*,3*R*)-TMT[1] isomer appeared to be a superpotent, but moderately δ-selective agonist, while the (2S,3S)-TMT[1] isomer showed the highest selectivity for the δ receptors in this series. Surprisingly, [(2*R*,3*R*)-TMT[1]]DELT I also was moderately potent at the δ receptor. These results suggest that the δ receptor requirements for the linear DELT I analogues may be satisfied with two different modes of binding of the (2S,3S)- and (2*S*,3*R*)-TMT[1] isomers. This study provides important guidance for the design of peptide and non-peptide ligands selective for the δ opioid receptor.

Introduction

Existence of multiple opioid receptors (μ, δ, κ, and possibly others) is now well documented[1] and demonstrated by direct cloning of the three major opioid receptor types.[2] The endogenous opioid peptides including enkephalins, dynorphins, and β-endorphin are not very selective for any of the opioid receptors.[3] Hence, systematic, rational approaches are needed to design potent and selective ligands for each type of opioid receptors.

Selective δ opioid agonists have several potential clinical advantages over μ or κ receptor agonists,[4] as the analgesia mediated through the δ receptors is likely not accompanied with respiratory depression, constipation, or other adverse effects. Enkephalins, the endogenous δ opioid ligands,[5] are not selective or stable enough to be considered as potential nonaddictive pain relievers. Therefore, considerable effort has been made to develop highly potent and selective ligands for δ opioid receptors. A successful approach in our laboratories involved applying global conformational constraints to the linear enkephalins. This led to the highly selective δ opioid ligand *cyclo*[D-Pen[2], D-Pen[5]]enkephalin (DPDPE, Figure 1a),[6] which also is highly resistant to enzymatic degradation.[7] Extensive NMR and molecular modeling studies[8] as well as the recently published X-ray crystal structure[9] have revealed a constrained conformation of the 14-membered disulfide ring of DPDPE.

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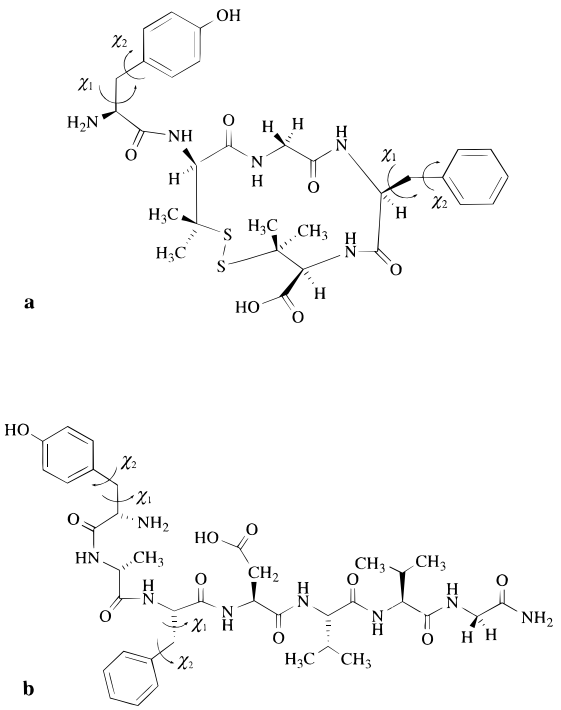

Figure 1. (a) Structure of c[D-Pen^2, D-Pen^5]enkephalin (**1**, DPDPE). (b) Structure of deltorphin I (**6**, DELT I).

However, determination of the three-dimensional δ-receptor pharmacophore of DPDPE has remained a challenging problem due to the conformation mobility of the Tyr^1 residue and the Phe^4 side chain. Even in the crystal structure of DPDPE,[9] three molecules found within the same crystal unit differ in conformations of the Tyr^1 residue. Therefore, it was important to explore topographical requirements of the δ receptor for the Tyr^1 and Phe^4 side chains of DPDPE using substitutions with conformationally constrained amino acids.

During the past few years, design of side-chain-constrained amino acids[10−13] such as β-methyl derivatives of aromatic amino acids have been of special interest, because they presented a unique possibility to constrain or bias side-chain conformational preferences systematically, using four isomers with different stereochemistry, but with similar physicochemical properties (electronegativity, hydrophobicity, etc.). Since methods of asymmetric synthesis of β-methyl amino acids have been developed,[12] β-methyl derivatives of phenylalanine (β-MePhe), tyrosine (β-MeTyr), and tryptophan (β-MeTrp) were incorporated into several biologically active peptides.[10−17] A second generation of DPDPE analogues was prepared by introduction of four stereoisomers of β-MeTyr and β-MePhe (or *p*-NO_2-β-MePhe) into positions 1 and 4, respectively.[14,15] The modifications in position 4 resulted in several potent and highly δ-selective analogues,[14] which allowed us to suggest a probable "bioactive" conformation of the Phe^4 side chain in DPDPE.[16] The modifications in position 1[15] gave only one moderately potent and selective (2*S*,3*R*)-stereoisomer of [β-MeTyr^1]DPDPE, which showed that an optimal topography for the Tyr^1 side chain has not been achieved in that study. On the other hand, our recent NMR studies of β-MePhe-containing analogues of cholecystokinin[17] and DPDPE [16] have shown that β-methyl substitution does not constrain conformational mobility of aromatic side chains dramatically. Two of three χ_1 rotamers of β-MePhe in both series of analogues were relatively highly populated. Therefore, further modification still was desirable, which would combine the advantage of β-methylated amino acids, as a tool for systematical exploration of receptor topography, with more substantial restrictions of side-chain mobility. In this study we present a new tyrosine derivative, 2′,6′-dimethyl-β-methyltyrosine (TMT, Figure 2), which was designed[18] to constrain rotation around both the χ_1 and χ_2 torsional angles of the aromatic side chain.

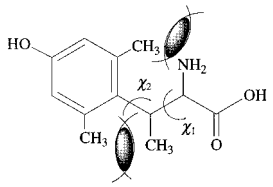

Figure 2. Structure of β-methyl-2′,6′-dimethyltyrosine (TMT).

Deltorphins, natural amphibian skin peptides with the amino acid sequence Tyr-D-Ala-Phe-Xxx-Val-Val-Gly-NH_2, where Xxx = Asp (DELT I, Figure 1b), or Glu (DELT II), have been found to possess high affinity and selectivity for the δ opioid receptor.[19] Unlike enkephalin analogues, the tyrosine and phenylalanine in deltorphins are separated by only one residue, a D-amino acid residue. It was interesting to compare the influence of similar side-chain constraining modifications on the biological activities of DPDPE and linear deltorphin analogues. In our preliminary study[20] incorporation of the (2S,-3S)-stereoisomer of TMT into position 1 of DPDPE and deltorphin I (DELT I) provided analogues with considerably different potencies and selectivities for the δ opioid receptor. In the present study, all four optically pure isomers of TMT were asymmetrically synthesized using recently reported methodologies[21] and incorporated into DPDPE and DELT I by solid phase peptide synthesis. Using a combination of extensive biological, pharmacological, and conformational studies we have determined optimal side-chain conformations of the residue in position 1 which are required for the specific recognition of the two series of opioid peptides selective for the δ opioid receptor.

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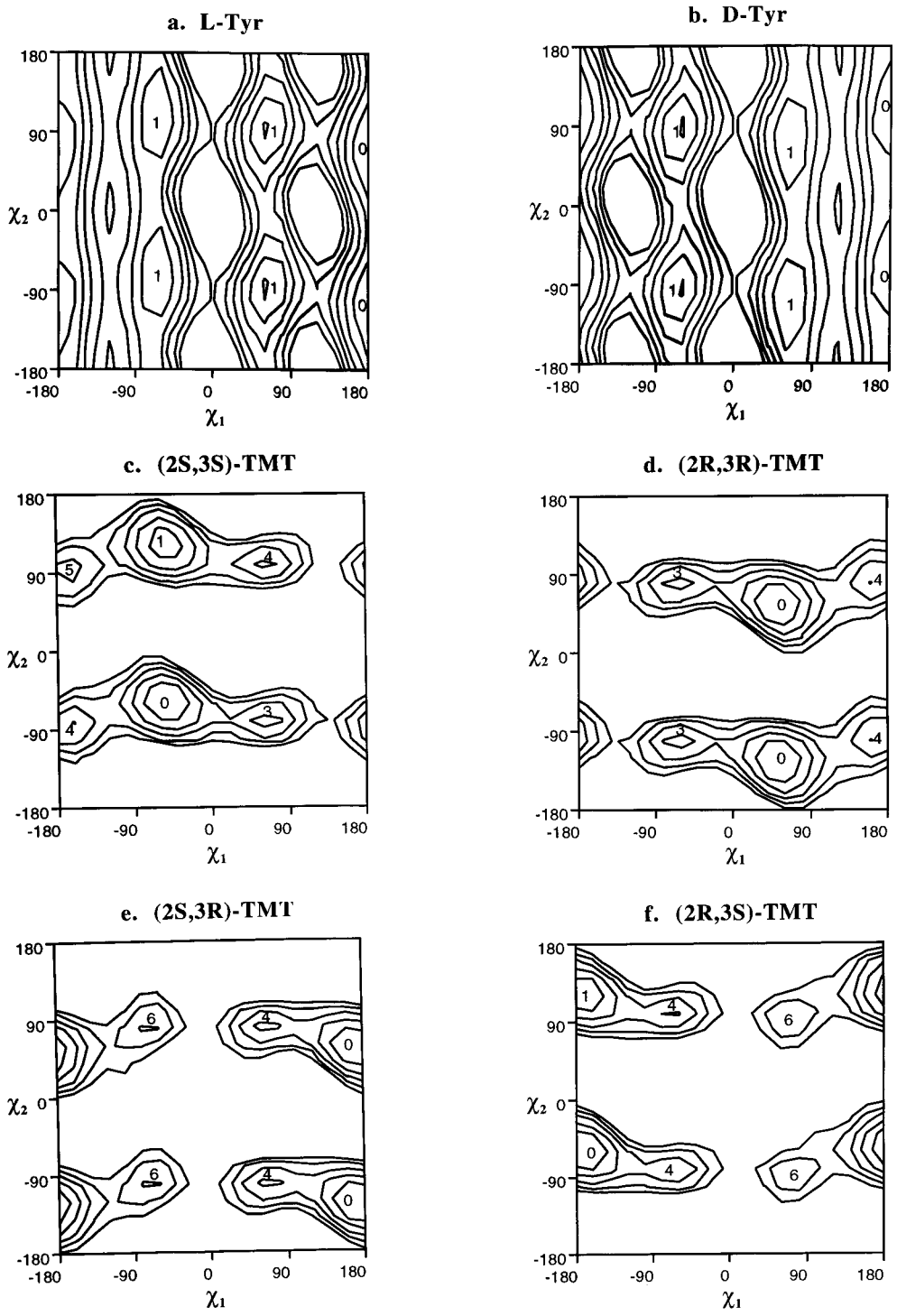

Figure 3. The (χ_1,χ_2) energy maps for N'-methylamides of tyrosine and β-methyl-2′,6′-dimethyltyrosine (TMT): (a) L-tyrosine; (b) D-tyrosine; (c) (2*S*,3*S*)-TMT; (d) (2*R*,3*R*)-TMT; (e) (2*S*,3*R*)-TMT; (f) (2*R*,3*S*)-TMT. Conformational energies were calculated using the united-atom AMBER force field with distance-dependent dielectrics $\epsilon = 4.0r_{ij}$. The χ_1,χ_2 torsional angles were varied with the 20° steps, and energy was minimized over all other degrees of freedom, starting with the extended backbone conformation $\phi,\psi = 180°$. Contours of equal energy $E - E_{min}$ are drawn with the 1.0 kcal/mol intervals from 1.0 to 7.0 kcal/mol for the Tyr plots **a** and **b** and with 2.0 kcal/mol intervals from 1.0 to 9.0 kcal/mol for the TMT plots **c**−**f**. Higher energy contours are not shown. Relative energies at local minima are shown to the closest integers (in kcal/mol).

Results

Molecular Modeling of 2′,6′-Dimethyl-β-Methyltyrosine Residues. The (χ_1,χ_2) energy maps of L- and D-tyrosine and of the four stereoisomers of 2′,6′-dimethyl-β-methyltyrosine (Figure 3) were calculated to examine how the combination of constraining modifications in the 2′,6′- and β-positions could affect the conformational space available to the tyrosine side chain. Since the TMT isomers are incorporated into the first position of the δ opioid agonists, the free amino group in its neutral NH_2 form was retained in the models, while the carboxylic groups of Tyr and TMT were converted to *N*-methylamides to mimic their connection to the rest of a peptide. Comparison of the Tyr and TMT (χ_1,χ_2) maps in Figure 3 shows that the three methyl substitutions restrict the side-chain conformational space dramatically. There are very small energy differences (less than 1 kcal/mol) between the three low energy χ_1 rotamers in the L- and D-tyrosine derivatives (Figure 3a,b). The energy differences between the lowest-energy conformer and two other χ_1 conformers of the TMT side chain range from about 3 to more than 6 kcal/mol. The most interesting result

obtained from this modeling is that each individual TMT isomer provides a single highly preferred rotamer of the χ_1 angle. Thus, (2*S*,3S)-TMT favors the *gauche* (−) rotamer, (2*R*,3*R*)-TMT favors the *gauche* (+) rotamer, and (2*S*,3*R*)-TMT and (2*R*,3*S*)-TMT favor the *trans* rotamer. Therefore, the clear advantage of TMT residues is that by incorporating all four isomers into δ opioid agonists, we can systematically probe the side chain topology of the tyrosine residue which is critical for the receptor recognition. In a previous NMR study[18] we have demonstrated that the energy barrier for rotation about the χ_2 angle in TMT derivatives ranged from 15 to 20 kcal/mol. The calculated (χ_1,χ_2) energy surfaces of TMT isomers have two saddle points at $\chi_2 \approx 0°$ with energies of about 13 and 19 kcal/mol above the global minima (the high-energy contours are not shown in Figure 3). These values, which may be accepted as theoretical estimates of energy barriers for the χ_2 rotation, are in good agreement with the previous experimental determinations.[18]

Nuclear Magnetic Resonance (NMR) Studies. 1D proton NMR spectroscopy, *z*-filtered total correlation spectroscopy (*zf*-TOCSY),[22] rotating frame Overhauser enhancement spectroscopy (ROESY),[23] proton detected heteronuclear single quantum correlation spectroscopy (HSQC),[24] and temperature dependent studies of NH proton chemical shifts were performed on all eight TMT[1]-analogues (**2**−**5** and **7**−**10**) of DPDPE (**1**) and DELT I (**6**). Details of the methods are described in the Experimental Section. The sequential assignment of proton resonances was carried out by using *z*-filtered total correlation spectroscopy (*zf*-TOCSY) and ROESY spectra, and the corresponding chemical shifts are listed in Tables 1 and 2. One-dimensional (1D) proton and two-dimensional (2D) *zf*-TOCSY spectra were used to measure the homonuclear vicinal coupling constants listed in Tables 1 and 2. *z*-Filtered carbon coupled HSQC-TOCSY experiments[25] were used for the assignment of carbon resonances and for the evaluation of long range heteronuclear coupling constants. The rotamer populations of side chain conformations were obtained from the measured homonuclear ($^3J_{H\alpha H\beta}$) and heteronuclear ($^3J_{H\alpha C\gamma}$) coupling constants. In the case of peptides **2**, **5**, **7**, **9**, and **10**, the conformational dependence of γ-substituent effects[26,27] also were used for determination of the side chain rotamer populations of TMT[1]. Details of the calculations are described in the Experimental Section, and the calculated rotamer populations for the χ_1 torsional angles of TMT isomers and the Phe[3]/Phe[4] residues are listed in Tables 3 and 4.

Binding Assays. The results obtained for compounds **1**−**10** in the *in vitro* binding assays using [^{3}H][*p*-Cl-Phe[4]]DPDPE (δ selective ligand) and [^{3}H]-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 ([^{3}H]CTOP, μ selective ligand) are shown in Table 5. Incorporation of (2*S*,3*S*)-TMT into DPDPE led to a 130-fold decrease in the binding affinity to the δ opioid receptor but

Table 1. ^{1}H Chemical Shifts (δ in ppm) and Coupling Constants (*J* in Hz) for [TMT[1]]DPDPE Analogues[a]

	NH					Hα					Hβ					Hγ				
residue	2	3	4	5		2	3	4	5		2	3	4	5		2	3	4	5	
TMT[1]						4.17	4.41	4.53	4.30		3.28	3.57	3.32	3.49		1.15	1.25	1.12	1.31	
						$J_{\alpha\beta}$ = 10.1	$J_{\alpha\beta}$ = 11.3	$J_{\alpha\beta}$ = 11.4	$J_{\alpha\beta}$ = 11.1							$J_{\beta\gamma}$ = 7.1	$J_{\beta\gamma}$ = 7.0	$J_{\beta\gamma}$ = 7.4	$J_{\beta\gamma}$ = 7.4	
D-Pen[2]	8.67	8.08	8.99	7.52		4.57	4.34	4.63	4.12							1.50	1.01	1.50	1.33 (γ)	
	$J_{NH\alpha}$ = 6.3	$J_{NH\alpha}$ = 9.0	$J_{NH\alpha}$ = 8.9	$J_{NH\alpha}$ = 8.9												1.26	0.46	1.33	1.14(γ′)	
Gly[3]	8.51	8.45	8.54	8.15		4.33 (α)	4.34 (α)	4.43 (α)	4.23 (α)											
	$J_{NH\alpha}$ = 9.3	$J_{NH\alpha}$ = 9.0	$J_{NH\alpha}$ = 9.1	$J_{NH\alpha}$ = 8.4		3.27(α′)	3.18(α′)	3.21(α′)	3.07(α′)											
	$J_{NH\alpha'}$ = 2.6	$J_{NH\alpha'}$ = 1.4	$J_{NH\alpha'}$ = 3.0	$J_{NH\alpha'}$ = 3.3		$J_{\alpha\alpha'}$ = 14.6	$J_{\alpha\alpha'}$ = 14.6	$J_{\alpha\alpha'}$ = 14.9	$J_{\alpha\alpha'}$ = 14.5											
Phe[4]	8.77	8.78	8.78	8.69		4.30	4.28	4.32	4.28		3.09 (β)	3.07 (β)	3.09 (β)	3.09 (β)						
	$J_{NH\alpha}$ = 7.6	$J_{NH\alpha}$ = 8.1	$J_{NH\alpha}$ = 7.8	$J_{NH\alpha}$ = 7.8		$J_{\alpha\beta}$ = 3.8	$J_{\alpha\beta}$ = 4.0	$J_{\alpha\beta}$ = 4.2	$J_{\alpha\beta}$ = 3.8		2.84(β′)	2.79(β′)	2.84(β′)	2.83(β′)						
						$J_{\alpha\beta'}$ = 11.3	$J_{\alpha\beta'}$ = 10.9	$J_{\alpha\beta'}$ = 10.7	$J_{\alpha\beta'}$ = 10.9		$J_{\beta\beta'}$ = 14.3	$J_{\beta\beta'}$ = 14.3	$J_{\beta\beta'}$ = 14.2	$J_{\beta\beta'}$ = 14.1						
D-Pen[5]	7.28	7.20	7.26	7.28		4.32	4.26	4.38	4.32							1.37	1.30	1.38	1.32 (γ)	
	$J_{NH\alpha}$ = 8.6	$J_{NH\alpha}$ = 8.0	$J_{NH\alpha}$ = 8.5	$J_{NH\alpha}$ = 8.5												1.31	1.24	1.33	1.27(γ′)	

[a] The chemical shifts and coupling constants are dependent on individual isomers of TMT: **2**, [(2*S*,3*S*)-TMT[1]]DPDPE; **3**, [(2*S*,3*R*)-TMT[1]]DPDPE; **4**, [(2*R*,3*R*)-TMT[1]]DPDPE; **5**, (2*R*,3*S*)-TMT[1]]DPDPE. Chemical shifts of the TMT[1] aromatic protons are between 6.2 and 6.5 ppm; chemical shifts of 2′,6′-dimethyl protons are at 2.22, 2.32 ppm in **2**, at 2.15, 2.37 ppm in **3**, at 2.33, 2.35 ppm in **4**, and at 2.09, 2.31 ppm in **5**. Chemical shifts of the Phe[4] aromatic protons are between 7.2 and 7.3 ppm in each analogue. *T* = 305 K, DMSO-d_6.

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Table 2. ^{1}H Chemical Shifts (δ in ppm) and Coupling Constants (J in Hz) for [TMT^1]Deltorphin I Analogues[a]

	NH				H$_\alpha$				H$_\beta$				H$_\gamma$			
residue	7	8	9	10	7	8	9	10	7	8	9	10	7	8	9	10
TMT^1					4.19	4.06	4.17	4.08	3.31	3.51	3.29	3.53	1.09	1.28	1.10	1.26
					$J_{\alpha\beta}$ = 11.3	$J_{\alpha\beta}$ = 11.1	$J_{\alpha\beta}$ = 11.1	$J_{\alpha\beta}$ = 11.1					$J_{\beta\gamma}$ = 7.3	$J_{\beta\gamma}$ = 7.4	$J_{\beta\gamma}$ = 7.3	$J_{\beta\gamma}$ = 7.3
D-Ala^2	8.95	7.66	8.86	7.82	4.53	4.11	4.41	4.06	0.92	0.22	0.96	0.72				
	$J_{NH\alpha}$ = 7.9	$J_{NH\alpha}$ = 8.0	$J_{NH\alpha}$ = 7.8	$J_{NH\alpha}$ = 7.6	$J_{\alpha\beta}$ = 7.0	$J_{\alpha\beta}$ = 6.8	$J_{\alpha\beta}$ = 7.2	$J_{\alpha\beta}$ = 6.8								
Phe^3	8.44	8.28	8.27	8.00	4.64	4.54	4.67	4.57	3.06 (β)	2.99 (β)	3.08 (β)	3.01 (β)				
	$J_{NH\alpha}$ = 8.9	$J_{NH\alpha}$ = 8.7	$J_{NH\alpha}$ = 8.8	$J_{NH\alpha}$ = 8.8	$J_{\alpha\beta}$ = 2.8	$J_{\alpha\beta}$ = 2.9	$J_{\alpha\beta}$ = 3.7	$J_{\alpha\beta}$ = 3.6	2.68(β')	2.60(β')	2.75(β')	2.62(β')				
					$J_{\alpha\beta'}$ = 11.4	$J_{\alpha\beta'}$ = 11.8	$J_{\alpha\beta'}$ = 11.4	$J_{\alpha\beta'}$ = 10.8	$J_{\beta\beta'}$ = 13.7	$J_{\beta\beta'}$ = 13.4	$J_{\beta\beta'}$ = 13.8	$J_{\beta\beta'}$ = 13.9				
Asp^4	8.59	8.58	8.51	8.53	4.61	4.59	4.65	4.67	2.69 (β)	2.69 (β)	2.73 (β)	2.75 (β)				
	$J_{NH\alpha}$ = 7.6	$J_{NH\alpha}$ = 7.7	$J_{NH\alpha}$ = 7.7	$J_{NH\alpha}$ = 7.6	$J_{\alpha\beta}$ = 5.4	$J_{\alpha\beta}$ = 5.3	$J_{\alpha\beta}$ = 6.1	$J_{\alpha\beta}$ = 6.0	2.51(β')	2.49(β')	2.53(β')	2.57(β')				
					$J_{\alpha\beta'}$ = 8.6	$J_{\alpha\beta'}$ = 8.7	$J_{\alpha\beta'}$ = 7.5	$J_{\alpha\beta'}$ = 7.1	$J_{\beta\beta'}$ = 16.9	$J_{\beta\beta'}$ = 16.8	$J_{\beta\beta'}$ = 16.6	$J_{\beta\beta'}$ = 16.7				
Val^5	7.66	7.64	7.63	7.62	4.24	4.24	4.26	4.27	1.99	1.98	1.98	1.99	0.81	0.80	0.84, 0.81	0.84, 0.81
	$J_{NH\alpha}$ = 8.6	$J_{NH\alpha}$ = 8.7	$J_{NH\alpha}$ = 8.9	$J_{NH\alpha}$ = 8.8	$J_{\alpha\beta}$ = 6.4	$J_{\alpha\beta}$ = 6.2	$J_{\alpha\beta}$ = 6.4	$J_{\alpha\beta}$ = 6.2					$J_{\beta\gamma}$ = 6.8	$J_{\beta\gamma}$ = 7.0	$J_{\beta\gamma}$ = 7.5, 6.7	$J_{\beta\gamma}$ = 7.0, 6.8
Val^6	7.86	7.85	7.85	7.87	4.10	4.09	4.09	4.10	1.97	1.96	1.97	1.99	0.85	0.84	0.85	0.86
	$J_{NH\alpha}$ = 8.5	$J_{NH\alpha}$ = 8.3	$J_{NH\alpha}$ = 8.1	$J_{NH\alpha}$ = 8.2	$J_{\alpha\beta}$ = 6.8	$J_{\alpha\beta}$ = 6.6	$J_{\alpha\beta}$ = 7.2	$J_{\alpha\beta}$ = 6.6					$J_{\beta\gamma}$ = 6.7	$J_{\beta\gamma}$ = 7.3	$J_{\beta\gamma}$ = 7.0	$J_{\beta\gamma}$ = 6.8
Gly^7	8.04	8.02	8.02	8.02	3.66 (α)	3.65 (α)	3.65 (α)	3.67 (α)								
					3.60 (α')	3.59(α')	3.61 (α')	3.59 (α')								
	$J_{NH\alpha}$ = 5.7	$J_{NH\alpha}$ = 5.7	$J_{NH\alpha}$ = 5.8	$J_{NH\alpha}$ = 5.8	$J_{\alpha\alpha'}$ = 16.5	$J_{\alpha\alpha'}$ = 16.4	$J_{\alpha\alpha'}$ = 16.7	$J_{\alpha\alpha'}$ = 16.7								

[a] The chemical shifts and coupling constants are dependent on individual isomers of TMT: **7**, [(2*S*,3*S*)-TMT^1]DELT I; **8**, [(2*S*,3*R*)-TMT^1]DELT I; **9**, [(2*R*,3R)-TMT^1]DELT I; **10**, (2*R*, 3*S*)-TMT^1]DELT I. Chemical shifts of the TMT^1 aromatic protons are between 6.2 and 6.5 ppm; chemical shifts of 2′,6′-dimethyl protons are at 2.23, 2.29 ppm in **7**, at 2.12, 2.28 ppm in **8**, 2.23, 2.29 ppm in **9**, and at 2.2 ppm in **10**. Chemical shifts of the Phe^4 aromatic protons are between 7.0 and 7.5 ppm in each analogue. T = 305 K, DMSO-d_6.

Table 3. Populations of the Side Chain Rotamers in DPDPE and [TMT^1]DPDPE Analogues Calculated from NMR Data

		population (%)[a]		
peptide	amino acid residue	*gauche* (−)	trans	*gauche* (+)
DPDPE	Tyr^1	42	30	28
	Phe^4	72	7	21
[(2*S*,3*S*)-TMT^1]DPDPE	(2*S*,3*S*)-TMT^1	**63**	5	32
	Phe^4	75	3	22
[(2*S*,3*R*)-TMT^1]DPDPE	(2*S*,3*R*)-TMT^1	18	**75**	7
	Phe^4	71	4	25
[(2*R*,3*R*)-TMT^1]DPDPE	(2*R*,3*R*)-TMT^1	0	24	**76**
	Phe^4	69	6	25
[(2*R*,3*S*)-TMT^1]DPDPE	(2*R*,3*S*)-TMT^1	5	**73**	22
	Phe^4	71	2	27

[a] The most populated rotamers of TMT^1 side chains are shown in bold.

Table 4. Populations of the Side Chain Rotamers in Deltorphin I and [TMT^1]DELT I Analogues Calculated from NMR Data

		population (%)[a]		
peptide	amino acid residue	*gauche* (−)	*trans*	*gauche* (+)
Deltorphin I	Tyr^1	31	20	49
	Phe^3	86	4	10
[(2*S*,3*S*)-TMT^1]DELT I	(2*S*,3*S*)-TMT^1	**75**	3	22
	Phe^3	80	2	18
[(2*S*,3*R*)-TMT^1]DELT I	(2*S*,3*R*)-TMT^1	13	**73**	14
	Phe^3	84	3	13
[(2*R*,3*R*)-TMT^1]DELT I	(2*R*,3*R*)-TMT^1	15	15	**70**
	Phe^3	76	1	23
[(2*R*,3*S*)-TMT^1]DELT I	(2*R*,3*S*)-TMT^1	12	**73**	15
	Phe^3	70	1	29

[a] The most populated rotamers of TMT^1 side chains are shown in bold.

hardly affected the binding affinity to the μ receptor (**2**, Table 5), thus providing more than a 100-fold decrease in the δ vs μ receptor selectivity of analogue **2** compared to DPDPE. Incorporation of (2*S*,3*R*)-TMT into DPDPE led to a 3- and 7-fold decrease in binding affinity to the δ and μ opioid receptors, respectively. Analogue **3** is the most potent and selective δ opioid ligand in the [TMT^1]DPDPE series with a nanomolar binding affinity and higher selectivity than DPDPE. Incorporation of the D-TMT isomers did not provide either high-affinity or highly selective DPDPE analogues (**4** and **5**, Table 5). The binding affinity of [(2*R*,3*R*)-TMT^1]DPDPE (**4**) decreased more than 2000-fold at the δ receptor and more than 100-fold at the μ receptor relative to DPDPE. Incorporation of (2*R*,3*S*)-TMT into DPDPE provided the inactive analogue **5**. In the series of DELT I analogues, incorporation of (2*S*,3*S*)-TMT (**7**, Table 5) resulted in a 5- and 8-fold decrease in binding affinity at the δ and μ receptors, respectively, making analogue **7** slightly more δ receptor selective but less potent than DELT I. Incorporation of (2*S*,3*R*)-TMT into DELT I (analogue **8**) led to a 1.7-fold decrease in binding affinity at δ receptors and a 3.4-fold increase at μ receptors, thus providing a slightly less potent and selective δ opioid ligand than DELT I. Incorporation of the D-TMT isomers into DELT I provided analogues **9** and **10** with lower affinities and selectivities for the δ opioid receptor. However, the (2*R*,3*R*)-TMT isomer (**9**) retained surprisingly high binding potency at the δ opioid receptor, (IC_{50} = 39 nM), with a moderate selectivity for the δ opioid receptor, while [(2*R*,3*S*)-TMT^1]DELT I (**10**) did not bind well either to δ or to μ receptors.

MVD and GPI Bioassays. The results obtained for compounds **1**−**10** in the *in vitro* guinea pig ileum (GPI, μ) and mouse vas deferens (MVD, δ) bioassays are shown in Table 6. [(2*S*,3*S*)-TMT^1]DPDPE (analogue **2**) showed a 40-fold decrease

Table 5. Binding Affinities of [TMT[1]]DPDPE and [TMT[1]]DELT I Analogues

peptide[a]	binding data IC_{50} (nM) ± SEM [^{3}H]CTOP	[^{3}H][*p*-ClPhe^4]DPDPE	selectivity (μ/δ)	peptide	binding data IC_{50} (nM) ± SEM [^{3}H]CTOP	[^{3}H][*p*-ClPhe^4]DPDPE	selectivity (μ/δ)
DPDPE (**1**)	609 ± 70	1.6 ± 0.2	380	DELT I (**6**)	2100 ± 690	0.6 ± 0.3	3500
2	722 ± 126	211 ± 33	3	**7**	17100 ± 3900	3.0 ± 0.2	5740
3	4270 ± 820	5.0 ± 0.1	850	**8**	613 ± 47	1.0 ± 0.2	610
4	77100 ± 5900	3500 ± 228	22	**9**	7000 ± 3000	39 ± 1.0	180
5	0% at 10 μM[b]	9% at 10 μM[b]	N/A	**10**	31200 ± 4100	655 ± 123	48

[a] **2**-[(2*S*,3*S*)-TMT[1]]DPDPE; **3**-[(2*S*,3*R*)-TMT[1]]DPDPE; **4**-[(2*R*,3*R*)-TMT[1]]DPDPE; **5**-[(2*R*,3*S*)-TMT[1]]DPDPE; **7**-[(2*S*,3*S*)-TMT[1]]DELT I; **8**-[(2*S*,3*R*)-TMT[1]]DELT I; **9**-[(2*R*,3*R*)-TMT[1]]DELT I; **10**-[(2*R*,3*S*)-TMT[1]]DELT I. [b] Percent of maximum binding achieved at this concentration of the peptide.

Table 6. Biological Potencies of [TMT[1]]DPDPE and [TMT[1]]DELT I Analogues

peptide[a]	bioassay data EC_{50} (nM) ± SEM GPI (μ)	MVD (δ)	selectivity (μ/δ)	peptide	bioassay data EC_{50} (nM) ± SEM GPI (μ)	MVD (δ)	selectivity (μ/δ)
DPDPE (**1**)	7300 ± 1700	4.1 ± 4.6	1780	DELT I (**6**)	2900 ± 250	0.36 ± 0.04	8100
2	293 ± 1	168 ± 37	2	**7**	3840 ± 850	0.66 ± 0.06	5800
3	0% at 60 μM[b] (antagonist, $IC^{50} = 5$ μM)	1.8 ± 0.3	>33000	**8**	147 ± 3	0.07 ± 0.01	2100
4	49900 ± 33000	2200 ± 780	23	**9**	5330 ± 3640	11.0 ± 3.6	485
5	75% at 82 μM[b]	28% at 10 μM[b]	N/A	**10**	23100 ± 6400	2090 ± 260	11

[a] **2**-[(2*S*,3*S*)-TMT[1]]DPDPE; **3**-[(2*S*,3*R*)-TMT[1]]DPDPE; **4**-[(2*R*,3*R*)-TMT[1]]DPDPE; **5**-[(2*R*,3*S*)-TMT[1]]DPDPE; **7**-[(2*S*,3*S*)-TMT[1]]DELT I; **8**-[(2*S*,3*R*)-TMT[1]]DELT I; **9**-[(2*R*,3*R*)-TMT[1]]DELT I; **10**-[(2*R*,3*S*)-TMT[1]]DELT I. [b] Percent of maximum effect achieved with this concentration of peptide.

Table 7. Analytical Characterization of DPDPE, DELT I, and Their [TMT[1]]-Analogues

peptide	TLC[a] R_f values I	II	III	IV	HPLC k' (T_R, min)[b] V	VI	FAB-MS $(M + 1)^+$ calc	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	0.44	0.66	0.43	0.72	6.90 (19.6)	5.38 (15.8)	688	688
5	0.45	0.69	0.44	0.68	7.23 (20.4)	5.65 (16.5)	688	688
6	0.41	0.62	0.34	0.67	6.77 (19.3)	5.14 (15.2)	688	688
7	0.32	0.71	0.44	0.74	7.38 (20.8)	5.93 (17.2)	811	811
8	0.24	0.69	0.24	0.73	7.00 (19.8)	5.51 (16.2)	811	811
9	0.22	0.65	0.19	0.69	7.07 (20.0)	5.68 (16.6)	811	811
10	0.18	0.63	0.22	0.68	6.74 (19.2)	5.14 (15.2)	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 factor (k') and retention time (T_R, min) was 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. **1**-DPDPE, **2**-DELT I, **3**-[(2*S*,3*S*)-TMT[1]]DPDPE, **4**-[(2*S*,3*R*)-TMT[1]]DPDPE, **5**-[(2*R*,3*R*)-TMT[1]]DPDPE, **6**-[(2*R*,3*S*)-TMT[1]]DPDPE, **7**-[(2*S*,3*S*)-TMT[1]]DELT I, **8**-[(2*S*,3*R*)-TMT[1]]DELT I, **9**-[(2*R*,3*R*)-TMT[1]]DELT I, **10**-[(2*R*,3*S*)-TMT[1]]DELT I.

of the potency in the MVD (δ) assay and a 25-fold increase of the potency in the GPI (μ) assay, which lead to a weakly potent and nonselective opioid ligand. In contrast, incorporation of (2*S*,3*R*)-TMT isomer into DPDPE led to a highly potent and exceptionally selective δ opioid agonist **3** (Table 6). The potency of analogue **3** in the MVD assay increased 2.3-fold with respect to DPDPE, and it showed no agonist activity in the GPI (μ receptor) assay. In fact this analogue was found to be a weak antagonist in the GPI assay. To our knowledge, analogue **3** is the first example of an enkephalin analogue that is a δ-agonist/μ-antagonist opioid ligand. Incorporation of the two D-TMT isomers provided DPDPE analogues (**4** and **5**) which have little or no activity at either δ or μ receptors.

The potencies of [(2*S*,3*S*)-TMT[1]]DELT I (**7**) both in the GPI and in the MVD assays were very similar to those for DELT I (**6**, Table 6). Incorporation of (2*S*,3*R*)-TMT isomer into DELT I (analogue **8**) led to a 5-fold increase in potency in the MVD assay and a 20-fold increase in potency in the GPI assay, thus providing a super-potent, but slightly less selective δ opioid agonist than DELT I. Incorporation of (2*R*,3*R*)-TMT isomer into DELT I led to a 30-fold decrease of potency in the MVD assay but did not affect potency in the GPI assay, thus providing a moderately potent ($EC_{50} = 11$ nM) and quite selective δ opioid agonist **9**. The other D-TMT[1] containing isomer analogue **10** was a poorly potent and nonselective δ opioid ligand. The (2*S*,3*S*)- and (2*S*,3*R*)-TMT[1] isomers of DPDPE and all four TMT[1] isomers of DELT I showed considerable analgesic activities in mice (to be published elsewhere).

Discussion

NMR Data and Side-Chain Rotamer Populations. The essential parameters of the NMR spectra of [TMT[1]]DPDPE and [TMT[1]]DELT I analogues including proton chemical shifts and coupling constants are summarized in Tables 1 and 2. Preliminary models for the solution conformations for the (2*S*,3*S*)-stereoisomers in [TMT[1]]DPDPE and [TMT[1]]DELT I (analogues **2** and **7**) have been reported earlier.[20] In this section we will note the general similarities and differences in the solution structures of the two series of peptides, as they appear from the basic NMR characteristics, and discuss in detail the rotamer populations obtained for the biologically important TMT and phenylalanine side chains.

Very similar chemical shifts and $^3J_{NH\alpha}$ coupling constants were observed for NH and $C^{\alpha}H$ protons in positions 3, 4, and 5 of all four [TMT[1]]DPDPE analogues (Table 1). The most striking feature revealed by these NMR parameters is a considerable nonequivalence of the two diastereotropic $C^{\alpha}H$

protons of the Gly[3] residues, which is manifested by the extreme difference both between their chemical shifts ($\Delta\delta$ = 1.0−1.2 ppm) and between their $^3J_{NH\alpha}$ coupling constants (about 9.0 Hz and less than or about 3.0 Hz). These common features indicate that the disulfide rings in the four [TMT[1]]DPDPE isomers have similar well-defined conformations. In contrast, chemical shifts of the NH and C$^\alpha$H protons in residues 1 and 2 and the $^3J_{NH\alpha}$ coupling constants of D-Pen[2] differ considerably in the four analogues, which suggests that the isomers of [TMT[1]]-DPDPE may have different favorable conformations for the exocyclic N-terminal TMT[1] moiety. Similar sets of NH and C$^\alpha$H proton chemical shifts and vicinal coupling constants observed for the four isomers of [TMT[1]]DELT I (Table 2) indicate similar conformational properties for analogues **7**−**10** in DMSO. The only noticeable difference is in the NH and C$^\alpha$H chemical shifts of the D-Ala[2] residues, which again may reflect different conformational preferences in the N-terminal moieties of the four isomers. However, in contrast to the DPDPE analogues, these NMR characteristics do not indicate the presence of a unique backbone conformation of the DELT I analogues in DMSO. A preliminary computer modeling based on nuclear Overhauser effects (NOE) observed for [(2*S*,3*S*)-TMT[1]]DELT I[20] resulted in two alternative conformational models, none of which satisfied all of the NMR data. Thus, the [TMT[1]]DPDPE analogues all seem to possess well-defined solution conformations which are similar to that previously found for DPDPE[8] and differ only in the acyclic N-terminal moieties of the four analogues, while the [TMT[1]]DELT I analogues appear to be flexible in DMSO.

Despite these differences in overall conformational features, a striking similarity has been found between the side-chain conformational preferences of TMT isomers in the two series of analogues in DMSO (Tables 3 and 4). Each stereoisomer of TMT both in DPDPE and in DELT I favors one particular χ_1 rotamer, the *gauche* (−) rotamer ($\chi_1 \approx -60°$) for (2*S*,3*S*)-TMT, the *gauche* (+) rotamer ($\chi_1 \approx +60°$) for (2*R*,3*R*)-TMT, and the *trans* rotamer ($\chi_1 \approx 180°$) for (2*S*,3*R*)- and (2*R*,3*S*)-TMT. The most favorable χ_1 rotamers are populated at about 70%, and the rotamer preferences are similar for the same TMT[1] isomers in DPDPE and DELT I analogues. Moreover, the most populated rotamers of the TMT[1] side chains in DMSO exactly correspond to the lowest-energy χ_1 conformers found in the computational studies on isolated N-terminal TMT residues (see Figure 3). This close correlation between the most favorable TMT side-chain rotamers found for two structurally different series of peptides in DMSO and calculated for isolated TMT residues shows that the χ_1 rotamer preferences are determined by local steric interactions within the TMT isomers and are not influenced substantially either by an overall molecular structure or by environment.

The above features make TMT isomers an important and effective tool for investigating the topographical and conformational requirements for peptide−receptor interactions. Since the dominant χ_1 conformations of different TMT isomers cover all three staggered χ_1 rotamers available for natural aromatic amino acids, systematic substitution with four TMT isomers allows one to perform a "rotamer scan" over all χ_1 rotamers of Tyr[1] and to explore specific binding requirements of the receptor for a side-chain conformation. Figure 4 shows pairwise overlaps of the four stereoisomers of the N-terminal TMT residues in their most favorable conformations. The overlaps were performed by a best-fit matching of N, C$^\epsilon$, C$^\zeta$, and O$^\zeta$ atoms, assuming that the α-amino and *p*-hydroxyl groups as well as the *para* and *ortho* carbons of the aromatic ring of TMT[1] are directly involved in receptor binding. Despite the opposite configuration of substituents both in the α and in the β positions, the *erythro*-isomers (2*S*,3*S*) and (2*R*,3*R*) display quite good overlap of the above atoms (Figure 4a; RMS = 0.1 Å) in their preferred side chain conformations, *gauche* (−) and *gauche* (+), respectively. The same is true for the pair of *threo*-isomers (2*S*,3*R*) and (2*R*,3*S*) with the *trans* χ_1 rotamers (Figure 4b; RMS = 0.1 Å). These similarities mean that the respective pairs of stereoisomers might have comparable receptor binding affinities, if the receptor does not require a strict stereospecific fit of substituents at the α and/or β positions. On the contrary, the pair of (2*S*,3*S*)- and (2*S*,3*R*)-stereoisomers with their favorable χ_1 rotamers do not display a close overlap of the amino and aromatic moieties (Figure 4c; RMS = 0.64 Å), and, at the best overlap, the amide bonds connecting the two isomers of TMT[1] with the rest of a peptide are directed almost oppositely. Therefore, one cannot expect similar binding affinities of the (2*S*,3*S*)- and (2*S*,3*R*)-TMT[1]-containing peptides when they interact with the same receptor binding site. The same expectation is true for the pair of (2*R*,3*R*)- and (2*R*,3*S*)-TMT[1] isomers.

From the NMR study the Phe[4] side chain in DPDPE analogues and the Phe[3] side chains in DELT I analogues showed a clear preference for a *gauche* (−) χ_1 rotamer. A similar, but less pronounced preference for the *gauche* (−) rotamer have been reported[8b] for the Phe[4] residue of DPDPE in DMSO.

Structure−Activity Relations of [TMT[1]]DPDPE Analogues. Introduction of the three additional methyl groups increases the volume and lipophilicity of the tyrosine side chain and considerably restricts its conformational mobility. All these effects can affect the receptor binding affinities and biological potencies of the TMT[1]-containing peptides. It was shown previously that increase in the lipophilicity of the Tyr[1] residue, such as incorporation of 2′-methyltyrosine[15] and 2′,6′-dimethyltyrosine[28] into DPDPE, leads to more potent, although less selective δ ligands. Incorporation of the χ_1-constrained β-MeTyr[1] derivatives did not provide highly potent and selective DPDPE analogues.[15] The most potent isomer of [β-MeTyr[1]]DPDPE, (2*S*,3*R*), had 50 times lower binding affinity than DPDPE and five times lower activity in the MVD bioassay. In the present study the (2*S*,3*R*)-isomer of [TMT[1]]DPDPE showed only slightly lower δ receptor affinity than DPDPE, but its potency in the MVD assay was two times higher than that of DPDPE (Tables 5 and 6). Comparison of these analogues suggests that the highly constrained (2*S*,3*R*)-TMT[1] side chain exactly fits the topographical requirements of the δ receptor, and its three additional methyl groups do not cause steric hindrance with the receptor. Increased lipophilicity of the TMT[1] residue may enhance interactions of analogue **3** with the receptor. In addition, analogue **3** showed exceptionally high selectivity for the δ opioid receptor, which is due in part to its poor affinity and potency at the μ receptors. The latter effect can be attributed either to the constrained mobility of the (2*S*,3*R*)-TMT[1] side chain which completely loses complimentarity for the μ receptor or to unfavorable interaction of the 3*R*-methyl group with the μ receptor. In contrast, the (2*S*,3*S*)-TMT[1] isomer showed considerably decreased potency at the δ receptor but increased potency at the μ receptor, which suggests that the conformational preferences of the (2*S*,3*S*)-TMT[1] side chain are inconsistent with the topographical requirements of the δ receptor but may enhance its interactions with the μ receptor. The very low potencies of (2*R*,3*R*)- and (2*R*,3*S*)-TMT[1] isomers **3** and **4** were not surprising, as an L-configuration at the α-position of Tyr[1] is known to be important both for δ and for μ receptor binding and D-Tyr[1] derivatives have never led to potent analogues of DPDPE.

Results of the present biological, NMR, and molecular modeling studies enable us to formulate the conformational

requirements to the Tyr[1]/TMT[1] side chains of DPDPE analogues for an efficient interaction with the δ opioid receptor. The conformationally flexible Tyr[1] side chain of DPDPE can fit the δ receptor topography without a noticeable increase in potential energy of intramolecular interactions. In contrast, the TMT[1] side chains have a limited freedom for an induced fit to the tyrosine-binding site of the δ receptor. The TMT[1] containing analogues whose favorable side-chain conformation and/or configuration of substituents at α or β carbons do not fit steric and topographical requirements of the receptor will have considerably lower receptor affinity than the parent peptide. The high affinity and biological activity of [(2*S*,3*R*)-TMT[1]]DPDPE strongly suggest that this isomer binds to the δ receptor with the sterically most favorable *trans* conformation of the TMT[1] side chain. In this case, a properly constrained χ_2 torsional angle also may be crucial for its high affinity and selectivity for the δ opioid receptors. The high affinity of this analogue suggests also that the additional 3*R*-methyl group does not cause a noticeable steric hindrance at the δ receptor. The (2*S*,3*S*)-isomer of TMT[1] which favors the *gauche* (−) χ_1 rotamer and strongly disfavors the *trans* rotamer (relative energy above 4 kcal/mol; see Figure 3c) leads to a considerably less potent and selective analogue. It may be suggested that in this case part of the energy of ligand−receptor interaction is spent to move the (2*S*,3*S*)-TMT[1] side chain in its sterically unfavorable *trans* conformation, which causes an overall loss in the free energy of binding to the δ receptor. A steric hindrance of the 3*S*-methyl with the receptor also may be responsible for the decreased affinity of this analogue. Thus, this study supplies strong evidence that the δ opioid receptor recognizes the *trans* conformation ($\chi_1 \approx 180°$) of the Tyr[1]/TMT[1] aromatic side chain in DPDPE analogues.

NMR data show also that the Phe[4] side chains of the DPDPE analogues **2**−**4** favors a *gauche* (−) χ_1 rotamer in DMSO. The previous study on the β-MePhe[4] derivatives of DPDPE[14] did not reveal a strong discrimination between (2*S*,3*S*)- and (2*S*,3*R*)-β-MePhe[4] isomers in δ receptor binding affinities and biological potencies. Our recent NMR study[16] has shown that the *gauche* (−) rotamer was the only common, highly populated rotamer of the phenylalanine side chains in the two most potent (2*S*,3*S*)- and (2*S*,3*R*)-stereoisomers of [β-Me-*p*-NO_2Phe[4]]DPDPE. The *gauche* (−) rotamer of Phe[4] was indicated also in a model for the biologically active conformation of DPDPE proposed in an earlier conformation-activity study.[29] Although the preferred conformation of the nonconstrained phenylalanine side chain may, in principle, change upon interaction with the receptor, the consistency of all the above data allows us to suggest the *gauche* (−) χ_1 rotamer as a putative "bioactive" conformation of the Phe[4] side chain in DPDPE analogues.

Structure−Activity Relations of [TMT[1]]Deltorphin I Analogues. The four stereoisomers of [TMT[1]]DELT I revealed more complicated structure−activity relations than the [TMT[1]]-DPDPE analogues. Three of the four isomers showed considerable binding affinities for the δ receptor and biological activities in the MVD bioassay (analogues **7**, **8**, and **9** in Tables 5 and 6). The (2*S*,3*R*)-stereoisomer (analogue **8**) was a superagonist in the δ receptor MVD assay with a potency about five and 60 times higher than that of DELT I and DPDPE, respectively. This allows us to suggest that [(2*S*,3*R*)-TMT[1]]DELT I with the TMT[1] side chain in its most favorable *trans* conformation ideally fits binding requirements for the δ opioid receptor. The increased lipophilicity of the TMT side chain also may aid to the increased potency of this analogue. Models of the δ receptor-bound conformations proposed recently[30] for the highly constrained deltorphin-related tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13) and its analogues also suggest a *trans* χ_1 rotamer for the side chains in position 1 of this ligand.

On the other hand, the (2*S*,3*S*)-stereoisomer (analogue **7**) was almost equipotent to the parent DELT I and had only nine times lower MVD activity than the super-potent (2*S*,3*R*)-stereoisomer. The (2*S*,3*S*)-stereoisomer prefers the *gauche* (−) conformation of the TMT[1] side chain and definitely disfavors the *trans* conformation (Figure 3c). If the two stereoisomers of [TMT[1]]-DELT I interacting with a receptor binding site assume the same conformation, which is favorable for one of them and sterically unfavorable for the other, one would expect a considerable difference in binding affinities of analogues **7** and **8**, as was observed for the respective isomers of [TMT[1]]DPDPE (analogues **2** and **3**). Therefore, the high potencies shown by both (2*S*,3*R*)- and (2*S*,3*S*)-stereoisomers of [TMT[1]]DELT I suggest that each of them may interact with δ opioid receptors in its sterically favorable conformation, i.e., that the deltorphin-related ligands may have two different modes of binding to δ opioid receptors. If the latter is the case, the relatively high potency of the (2*R*,3*R*)-stereoisomer (analogue **9**) can be explained taking into account a good overlap between the tyramine moieties of the (2*S*,3*S*)- and (2*R*,3*R*)-TMT residues as shown in Figure 4a. In this case, it seems that stabilization of a preferred side chain conformation may overcome a negative effect of the D-configuration at α-position. The (2*S*,3*S*)- and (2*R*,3*R*)-stereoisomers with the *gauche* (−) and *gauche* (+) rotamers of the TMT[1] side chain, respectively, may represent the same mode of δ receptor binding, while the (2*S*,3*R*)-stereoisomer represents another, more selective mode of the receptor interaction, which does not allow a tight binding of its (2*R*,3*S*)-TMT[1] counterpart (analogue **10**). The suggested diversity may be possible due to a relative conformational mobility of the message domain of deltorphin analogues, which may allow a proper orientation of the tyramine and phenylalanine pharmacophores in more than one binding conformation. On the contrary, the conformationally constrained backbone of DPDPE allows only one "bioactive" arrangement of pharmacophores, which may be achieved effectively only by the parent peptide and one of its TMT[1]-containing isomers. Furthermore, the conformationally constrained deltorphin-like tetrapeptide JOM-13 also appeared to possess a unique δ receptor binding conformation.[30] A combination of TMT[1] substitutions with backbone-constraining modifications seems to be a promising approach for further conformation-activity studies of deltorphins.

It is interesting to note that among the [TMT[1]]DELT I analogues only the (2*S*,3*R*)-stereoisomer possesses a noticeable binding affinity and biological potency in μ receptor assays. This, however, makes the super-potent agonist **8** a less selective δ opioid ligand than the parent deltorphin I and its (2*S*,3*S*)-TMT[1]- analogue **7**. The fact that two different TMT[1]-stereoisomers of DELT I and DPDPE show the highest μ-receptor affinities may reflect different topographical requirements for the μ receptor binding of peptide ligands with phenylalanine in positions 3 and 4. However, the generally low level of μ-receptor affinities for analogues under this study does not allow us to determine these requirements explicitly.

Thus, the TMT[1] stereoisomers appears to be a powerful tool to enhance the δ vs μ opioid receptor selectivity of DPDPE

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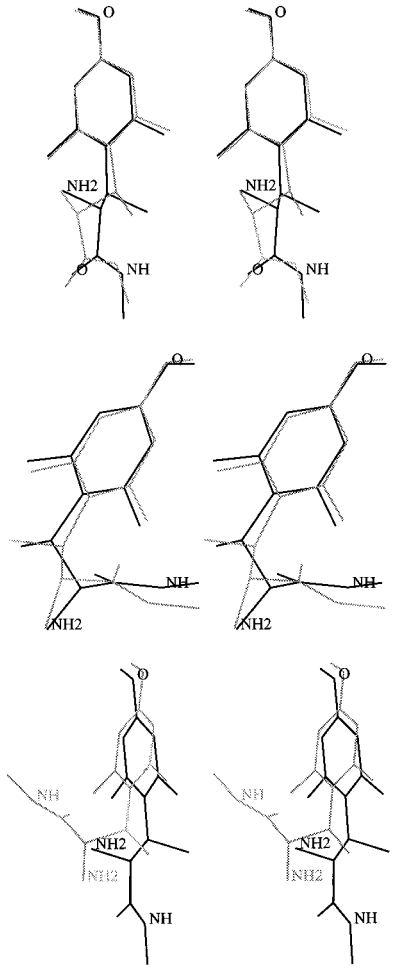

Figure 4. Superposition of the lowest-energy conformers of four stereoisomers of TMT by the best-fit matching of N^{α}, C^{ϵ}, C^{ζ} and O^{ζ} atoms: (a) (2*S*,3*S*)-TMT (bold) vs (2*R*,3*R*)-TMT (shadow); (b) (2*S*,3*R*)-TMT (bold) vs (2*R*,3*S*)-TMT (shadow); (c) (2*S*,3*S*)-TMT (bold) vs (2*S*,3*R*)-TMT (shadow).

and DELT I analogues. Furthermore, antinotiception studies *in vivo* indicated that the two series of analogues tend to interact with two different subtypes of the δ opioid receptor (results of this pharmacological study will be published elsewhere).

Conclusions

A series of novel, highly side-chain constrained tyrosine derivatives (TMT) have been designed which can selectively bias rotamer populations of the χ_1 torsional angle and constrain rotational mobility around the χ_2 torsional angle. The TMT[1] substitution allowed us to evaluate in a systematic way the side-chain conformation-biological activity relationships of two series of opioid peptides. Modifications with the same TMT[1] isomers resulted in different profiles of biological activities for DPDPE and DELT I analogues. Incorporation of TMT[1] isomers into the backbone-constrained DPDPE led to a potent and extremely selective δ opioid ligand [(2*S*,3*R*)-TMT[1]]DPDPE. We suggest that the most favorable side chain conformations of this analogue, i.e., the *trans* χ_1 rotamer of TMT[1] and the *gauche* (−) rotamer of Phe[4] represent the topography of DPDPE analogues which is selectively recognized by the δ opioid receptor. Incorporation of TMT[1] isomers into the flexible deltorphin I resulted in analogues with a broad spectrum of potencies and opioid receptor selectivities, including a super-potent analogue, [(2*S*,3*R*)-TMT[1]]DELT I, and a highly selective analogue for the δ opioid receptor, [(2*S*,3*S*)-TMT[1]]DELT I. Based on these structure−activity relations, two possible modes of binding of [TMT[1]]DELT I analogues to δ receptors is suggested.

Since many peptide hormones and neurotransmitters have aromatic pharmacophores important for their receptor binding and bioactivities, the approach used here to probe the stereochemical requirements for selective recognition of the δ opioid receptors also should be very useful in the design of potent and selective constrained peptide ligands of other opioids and such bioactive peptides as oxytocin, vasopressin, cholecystokinin, melanocyte-stimulating hormone, etc. The systematic "rotamer scan" with topographically constrained analogues of aromatic amino acids may provide an important supplement to such traditional methods of structure−activity studies for peptides, as "alanine scan" and "D-amino acid scan". The "rotamer scan" will supply valuable information about receptor-ligand interactions and can help to obtain new peptide leads for the design of non-peptide mimetics.

Experimental Section

General Methods for Peptide Synthesis and Purification. All analogues were synthesized by solid phase peptide methods using procedures similiar to those previously used for DPDPE, deltorphin I, and their analogues.[6,20] 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 analogues. 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 analogues.[31] All syntheses were carried out on a Vega 1000 semiautomatic peptide synthesizer. N^{α}-*t*-Butyloxycarbonyl (Boc) protected amino acids were used throughout. The unprotected amino acids (Gly, Val, Phe) (obtained from Aldrich, Milwaukee, WI) and the D-Pen-*S*-*p*-MeBzl (obtained from Peptides International, Louisville, KY) were converted to their N^{α}-*tert*-butyloxycarbonyl derivatives with di-*tert*-butyldicarbonate (Bachem California, Torrance, CA). The N^{α}-Boc-D-Ala and the N^{α}-Boc-L-Asp-β-benzyl ester were obtained from Bachem California (Torrance, CA). The four isomers of 2′,6′-dimethyl-β-methyltyrosine were prepared as described previously[21] and were converted to their N^{α}-*tert*-butyloxycarbonyl derivatives with di-*tert*-butyldicarbonate (Bachem California, Torrance, CA) following standard procedures. The N^{α}-Boc-D-Pen-*S*-*p*-MeBzl was attached to the chloromethylated polystyrene resin by Gysin's method.[32] Diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBT) (Aldrich, Milwaukee, WI) were prepared as 1 M solutions in DMF prior to use in the coupling reactions which were monitored by the ninhydrin test.[33] Benzotriazol-1-yloxytris(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 the N^{α}-Boc-TMT isomers. Following completion of the syntheses, 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 peptide resin was washed with anhydrous ether (3 × 60 mL), and the peptide was then extracted into acetic acid (5 × 60 mL). The acetic acid solutions were combined, frozen, and lyophilized to afford the crude peptide. The linear DPDPE analogue was then cyclized using a 0.1 M solution of $K_3[Fe(CN)_6]$ using previously published methods.[34] 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 on silica gel in four solvent systems and by analytical HPLC (Table 7). Amino acids analyses were performed on a 420A ABI amino acid analyzer. The $(M + 1)^+$ molecular ions and fragmentation patterns were obtained

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by FAB-MS and were consistent with the amino acid sequence and structure of the peptides. The analytical results are listed in Table 7.

c-[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.[6,20]

Deltorphin I (6). The title compound was prepared by the methods described above (without the cyclization procedure) and was found to be identical to the compound previously synthesized.[31]

General procedure for synthesizing [TMT^1]DPDPE analogues is illustrated by the preparation of [(2*S*,3*R*)-TMT^1]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*R*)-TMT. The analytical data of N^{α}-Boc-(2*S*,3*R*)-TMT are mp 67.5−68.3 °C. ^{1}H-NMR ($CDCl_3$, TMS) δ 6.42 (s, br, 2H, 3′, 5′ aromatic-Hs), 4.72−4.79 (m, 1H, C_{α}-H), 3.34 (m, 1H, C_{β}-H), 2.40 (s, Ar-CH_3), 2.16 (s, Ar-CH_3′), 1.46 (s, 9H, *t*-Bu), 1.36 (d, J = 7.3 Hz, 3H, C_{β}-CH_3). ^{13}C-NMR ($CDCl_3$) δ 176.2, 155.7, 153.7, 138.7, 129.4, 117.0, 115.2, 80.5, 56.6, 38.5, 28.3, 21.5, 17.5, 15.6. IR (KBr, cm^{-1}): 3375, 2975, 1712, 1689, 1609, 1161, 856. CIMS *m*/*e* (relative intensity) 324.20 ($M^+ + 1$, 0.5), 73.15 (100). HR-CIMS calcd for $C_{17}H_{25}NO_5$ 323.1733; found ($M^+ + 1$) 323.1747. $[\alpha]_D^{23} = +1.55°$ (*c* 0.38, $CHCl_3$). All the N^{α}-*tert*-butyloxycarbonyl (Boc) protected amino acids (2 equiv) except for N^{α}-Boc-(2*S*,3*R*)-TMT were coupled to the growing peptide chain using diisopropylcarbodiimide (DIC 2.5 equiv) and *N*-hydroxybenzotriazole (HOBT, 2.5 equiv) as coupling reagents. N^{α}-Boc-(2*S*, 3*R*)-TMT (1.2 equiv)[21] 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 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, as outlined above followed by cyclization.[34] 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 15−75% CH_3-CN in 0.1% trifluoroacetic acid (aqueous solution) 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 lyophilized to afford a white powder. Yield 55 mg (16%). Amino acid analysis result for [(2*S*,3*R*)-TMT^1]DPDPE (**3**): (2*S*, 3*R*)-TMT 0.95 (1.00), Gly 1.04 (1.00), Phe 1.00 (1.00). The analytical data are presented in Table 7.

The analytical data of N^{α}-Boc-(2*R*,3*R*)-TMT are mp 139.0−140.0 °C dec. ^{1}H-NMR ($CDCl_3$, TMS) δ 6.49 (s, br, 2H, 3′, 5′ aromatic-Hs), 4.52−4.67 (m, 1H, C_{α}-H), 3.54 (m, 1H, C_{β}-H), 2.36 (s, Ar-CH_3), 2.30 (s, Ar-CH_3′), 1.32 (s, 9H, *t*-Bu), 1.38 (d, J = 7.4 Hz, 3H, C_{β}-CH_3). ^{13}C-NMR ($CDCl_3$) δ 186.6, 117.2, 115.5, 80.7, 57.6, 36.3, 28.0, 21.8, 16.6, 14.8. IR (KBr, cm^{-1}) 3353, 2977, 2936, 1712, 1610, 1592, 1486, 1454, 1393, 1367, 1302, 1161, 1030, 860. HR-EIMS calcd for $C_{17}H_{25}NO_5$ 323.1733; found ($M^+ + 1$) 323.1730. $[\alpha]_D^{23} = +23.3°$ (*c* 1.05, $CHCl_3$).

The analytical data of N^{α}-Boc-(2*R*,3*S*)-TMT are mp 65.5−66.0 °C. ^{1}H-NMR ($CDCl_3$, TMS) δ 6.42 (s, br, 2H, 3′, 5′ aromatic-Hs), 4.70−5.10 (m, 1H, C_{α}-H), 3.33 (m, 1H, C_{β}-H), 2.41 (s, Ar-CH_3), 2.16 (s, Ar-CH_3′), 1.54 (s, 9H, *t*-Bu), 1.36 (d, J = 6.84 Hz, 3H, C_{β}-CH_3). ^{13}C-NMR ($CDCl_3$) δ 175.2, 155.6, 153.7, 138.2, 129.4, 117.0, 80.4, 56.6, 38.6, 28.3, 27.7, 21.5, 15.6, 14.2. CIMS *m*/*e* (relative intensity) 324.20 ($M^+ + 1$, 8), 268.20 (100). IR (KBr, cm^{-1}): 3329, 2967, 1683, 1592, 1515, 1450, 1369, 1260, 1142, 1048, 883, 855, 800, 669. HR-CIMS: calcd for $C_{17}H_{25}NO_5$ 323.1733; found ($M^+ + 1$) 323.1757. $[\alpha]_D^{23} = -1.38°$ (*c* 0.44, $CHCl_3$).

Amino acid analysis result for [(2*R*,3*R*)-TMT^1]DPDPE (**4**): (2*R*, 3*R*)-TMT 0.90 (1.00), Gly 1.12 (1.00), Phe 1.00 (1.00). Amino acid analysis result for [(2*R*,3*S*)-TMT^1]DPDPE (**5**): (2*R*,3*S*)-TMT 0.93 (1.00), Gly 1.15 (1.00), Phe 1.00 (1.00). The analytical data for (2*S*,3*S*)-TMT^1 and [(2*S*,3*S*)-TMT^1]DPDPE were reported in our previous study.[20]

General procedure for synthesizing [TMT^1]deltorphin I analogues is illustrated by the preparation of [(2*S*,3*R*)-TMT^1]DELT I **(8)**. 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 **8**, 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-L-Asp-β-benzyl ester, N^{α}-Boc-Phe, N^{α}-Boc-D-Ala, and optically pure N^{α}-Boc-(2*S*, 3*R*)-TMT. An excess (2 equiv) of protected amino acids [except for N^{α}-Boc-(2*S*, 3*R*)-TMT], HOBT, and DIC was used for the coupling reactions, which were monitored by ninhydrin tests. N^{α}-Boc-(2*S*,3*R*)-TMT (1.2 equiv)[20] 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 analogues (see above). 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_3-CN in 0.1 trifluoroacetic acid (aqueous solution) 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 lyophilized to afford a white powder; yield 45%. Amino acid analysis result for [(2*S*,3*R*)-TMT^1]-DELT I (**8**): (2*S*,3*R*)-TMT 0.95 (1.00), D-Ala 1.03 (1.00), Phe 1.00 (1.00), Asp 1.16 (1.00), Val 1.80 (2.00), Gly 1.10 (1.00). The analytical data are presented in Table 7.

Amino acid analysis result for [(2*R*,3*R*)-TMT^1]DELT I (**9**): (2*R*,3*R*)-TMT 0.98 (1.00), D-Ala 1.10 (1.00), Phe 1.00 (1.00), Asp 1.10 (1.00), Val 1.92 (2.00), Gly 1.09 (1.00). Amino acid analysis result for [(2*R*,3*S*)-TMT^1]DELT I (**10**): (2*R*, 3*S*)-TMT 0.86 (1.00), D-Ala 1.10 (1.00), Phe 0.96 (1.00), Asp 1.04 (1.00), Val 1.80 (2.00), Gly 1.09 (1.00). The analytical data of [(2*S*,3*S*)-TMT^1]DELT I was reported previously.[20]

Radioligand Binding Assays. 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 memberane fraction obtained by centrifugation at 48 000*g* 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 was resuspended in 20 volumes of fresh Tris-HCl buffer.

Radioligand binding inhibition assay samples were done as previously published[14] using cyclo-[^{3}H][D-Pen^2, *p*-Cl-Phe^4, D-Pen^5]enkephalin[35] ([^{3}H][*p*-ClPhe^4]]DPDPE, δ) at a concentration of 0.75 nM and [^{3}H]-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, [^{3}H]CTOP, μ)[36] (New England Nuclear, Boston, MA) at a concentration of 0.5 nM as the radioligands.

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.[37] Data best fitted by a one site model were reanalyzed using the logistic equation.[38] Data obtained from independent measurements are presented as the arithmetic mean ± SEM.

***In Vitro* Bioassay Methods.** Electrically induced smooth muscle contractions from mouse vas deferens (MVD) and guinea pig ileum (GPI) longitudinal muscle-myenteric plexus were used for bioassays.[39] 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

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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 tissue baths for 3 min and 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. EC_{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.[40] In some cases, the weak μ agonist action of these analogues did not permit completion of dose-response curves in the GPI.

Nuclear Magnetic Resonance Experiments. All NMR parameters used in the present study were obtained from 1D and 2D experiments performed at 305 K with Bruker AM500 or Bruker AC400 spectrometer (500 or 400 MHz proton frequency) equipped with ASPECT 3000 computer and 5 mm inverse probehead. Peptide samples were dissolved in DMSO-d_6 at a concentration of 3.5 mg/0.4 mL for [(2*S*,3*S*)-TMT[1]]-DPDPE (**2**) and 10−14 mg/0.4 mL for the other analogues. The proton and the carbon chemical shifts were referenced to the solvent (2.49 ppm for residual ^{1}H signal of DMSO-d_6 and 39.5 ppm for ^{13}C signal). Sequential assignments[41] of proton resonances have been achieved by the combined use of 2D total correlation spectroscopy (*z*-filtered TOCSY)[22,42] and ROESY experiments.[23,43] 2D TOCSY spectra were recorded in phase sensitive mode using TPPI method,[44] and the isotropic mixing was achieved by the MLEV-17 sequence[45] or the recently introduced TOWNY sequence[42] which suppresses unwanted cross-relaxation peaks in TOCSY experiments. In ROESY experiments, continuous wave spin-lock field or a recently proposed multiple pulse sequence, $[180°(x)\ 180°(-x)]_n$, was employed for spin-locking.[43] The advantage of the latter is to avoid undesired TOCSY transfer in ROESY measurements. The ^{1}H chemical shifts and the conformationally important homonuclear vicinal coupling constants[46] were extracted from the resolution enhanced 1D spectra, or in case of signal overlap, from the highly digitized 1D traces of 2D *z*-filtered TOCSY spectra. The $^3J_{H\alpha H\beta}$ coupling constants in combination with the observed intra-residue NOE patterns were used for the stereospecific assignment of H_β protons and for the determination of preferred side chain conformations.[47] Proton detected heteronuclear spectroscopy including heteronuclear multiple quantum correlation (HMQC)[24a,b] experiments, multiplet edited HSQC,[24c] and *z*-filtered carbon coupled HSQC-TOCSY[25] experiments were used for the assignment of carbon resonances and for the evaluation of long-range heteronuclear coupling constants $^3J_{H\alpha C\gamma}$. Side-chain conformations of the TMT[1] residues (with one H_β proton) in peptide analogues **3**, **7**, and **8** were deduced from the measured homonuclear ($^3J_{H\alpha H\beta}$) and heteronuclear ($^3J_{H\alpha C\gamma}$) vicinal coupling constants using the following equations[47,48]

$$J_{H\alpha H\beta} = P\ {}^{ap}J_{H\alpha H\beta} + (1-P)\ {}^{sc}J_{H\alpha H\beta}$$

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.[46−48] An error of ±5% for rotamer populations can be estimated from the inaccuracy of the coupling constants. For the other peptide analogues, the β-methyl carbon chemical shifts of the TMT[1] residue were used to probe the side chain conformations by taking advantage of the well-known conformational dependent γ-effect.[26,27] The contributions to the β-Me carbon chemical shift from the NH_2 and C═O functionalities of the TMT[1] residue are given by the following equation

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

where P_{I-III} are the populations of the staggered rotamers, and ${}^{gauche}\delta_{CO}$, ${}^{gauche}\delta_{NH}$, and ${}^{gauche}\delta_{CO,NH_2}$ are the shielding parameters of the relevant substituents equal to −3.2, −4.6, and −7.8 ppm, respectively.[27] δ_{ref} is the β-Me carbon reference chemical shift which was calculated from the known rotamer populations and the β-Me carbon shifts of analogues **3**, **7**, and **8** using the above equation.

Molecular Modeling. The (χ_1,χ_2) energy maps for H_2N-(D,L)-Tyr-CO-NHMe and H_2N-TMT-CONMe isomers were calculated using the *Drive* module of the MacroModel[49] (version 4.5)[50] program. The model Tyr and TMT residues with free neutral amino group and blocked carboxyl group were chosen in order to mimic incorporation of these residues into N-terminal position of opioid peptides. The (χ_1,χ_2) energy maps were calculated with the 20° steps in both directions within the intervals of −180° to 180°. Energy was minimized over all other degrees of freedom using the united-atom AMBER* force field[51] implemented in the MacroModel 4.5, with a distance-dependent dielectrics $\epsilon = 4.0r_{ij}$. The dielectric constant of 4.0 was recommended[52] with the AMBER force field instead of the original value of 1.0[51] in order to reproduce better experimental characteristics of peptides and proteins. Extended initial backbone conformers with ϕ, ψ = 180° were considered at each (χ_1,χ_2) grid point. Contours of equal relative energies $E - E_{min}$ of the (χ_1,χ_2) maps were drawn with a step of 1.0 kcal/mol (Tyr) or 2 kcal/mol (TMT) using the *Plt2D* module of MacroModel 4.5.

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Supporting Information Available: Tables providing the ^{13}C chemical shifts for the four [TMT[1]]DPDPE analogues **2**, **3**, **4**, and **5** and the four [TMT[1]]Deltorphin I analogues **7**, **8**, **9**, and **10** (3 pages). See any current masthead page for ordering and Internet access instructions.

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