Development of Potent Bifunctional Endomorphin-2 Analogues with Mixed *μ*-/*δ*-Opioid Agonist and *δ*-Opioid Antagonist Properties

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The C terminus of endomorphin-2 (EM-2) analogues (Tyr-Pro-Phe-NH-X) was modified with aromatic, heteroaromatic, or aliphatic groups (X = phenethyl,benzyl, phenyl, naphthyl, pyridyl, quinolyl, isoquinolyl, *tert*-butyl, cyclohexyl, or adamantyl; **3**−**18**) to study their effect on opioid activity. Only **9** (1-naphthyl), **11** (5-quinolyl), **16** (cyclohexyl), and **18** (2-adamantyl) exhibited *μ*-opioid receptor affinity in the nanomolar range (K_i = 2.41−6.59 nM), which, however, was 3- to 10-fold less than the parent peptide. Replacement of Tyr[1] by Dmt (2′,6′-dimethyl-L-tyrosine) (**19**−**32**) exerted profound effects: (i) acquisition of high *μ*-opioid receptor affinity (K_i = 0.11−0.52 nM) except **23** (Ph); (ii) presence of potent functional *μ*-opioid receptor agonism (IC_{50} < 1 nM) for **19** ([Dmt[1]]EM-2), **27** (1-naphthyl), **29** (5-quinolyl), and **32** (5-isolquinolyl); (iii) association of weak *δ*-opioid antagonist activity (pA_2 = 5.41−7.18) except **19** ([Dmt[1]]EM-2), **20** (H), **27** (1-naphthyl), and in particular **29** (5-quinolyl) with its potent *δ*-agonism (IC_{50} = 0.62 nM, pA_2 = 5.88); (iv) production of antinociception after ic administration of **32** (5-isoquinolyl) in mice, a bioactivity absent in the corresponding Tyr[1] analogue (**14**); and (v) preferential cis orientation (cis/trans = 3:2 to 7:3) at the Dmt-Pro amide bond, in contrast to the Tyr−Pro amide trans orientation (cis/trans = 1:2 to 1:3). Thus, [Dmt[1]]EM-2 analogues with hydrophobic C-terminal extensions provide model compounds with potent *μ*-opioid receptor bioactivity and dual functional agonism.

Introduction[1]

Although the three major opioid receptor subtypes, *μ*, *δ*, and *κ*, are important targets for developing therapies to treat acute pain,[2,3] only the *μ*-opioid receptor appears to be involved in morphine tolerance and addiction.[4a] However, opiate tolerance and physical dependence could be blocked by *δ*-opioid receptor antagonists without compromising the antinociception produced by drug interaction at *μ*-opioid receptors.[4b] Therefore, it might be ideal to produce opioidmimetics with mixed *μ*-opioid agonist and *δ*-opioid antagonist properties. The plethora of endogenous ligands that interact with these receptors are structurally distinct but can be clearly divided into two unique classes of endogenous peptides: one contains the Tyr-Pro amide bond and the other has the Tyr-Gly-Gly-Phe sequence. In the former group, endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2) from bovine[5] and human brain[6] are highly *μ*-opioid receptor selective and share a common structural feature with morphiceptin (Tyr-Pro-Phe-Pro-NH_2),[7] hemorphin (Tyr-Pro-Phe-Thr),[8] Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2),[9,10] and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2).[11,12] The latter group of opioid peptides consists of the enkephalins,[13] endorphins,[14,15] and dynorphins,[16] which exhibit partial or weak selectivity for *δ*, *μ*, and *κ* opioid receptor subtypes, respectively. Modification or substitution at the second and fifth positions in enkephalin yielded DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol)[17] and DADLE ([D-Ala[2],D-Leu[5]]enkephalin),[18] which acquired selectivity and agonism for *μ*- and *δ*-opioid receptors, respectively. Similarly, the presence of a D-isomer at position 2 of the natural occurring amphibian skin opioid heptapeptides dermorphin[19] and deltorphin[20] suggests that the conformation around the Tyr[1]−Xaa[2] bond and residues C-terminal to Phe[3] might affect opioid activity. Although recent data revealed that the peptide bond of Tyr−Pro of endomorphins and morphiceptin occurs in a trans orientaion,[21−23] other results indicated that the cis orientation might be a characteristic of the bioactive form in EM-2[23] and morphiceptin.[23,24]

Rationale

The replacement of 2′,6′-dimethyl-L-tyrosine[25] (Dmt) for Tyr in opioid ligands dramatically enhanced their binding affinities and functional potency.[26−29] The structure−activity relationships in a series of [Dmt[1]]EM-2 analogues[30] indicated that Dmt enhanced the binding affinities to and bioactivity of *δ*- and *μ*-opioid receptors

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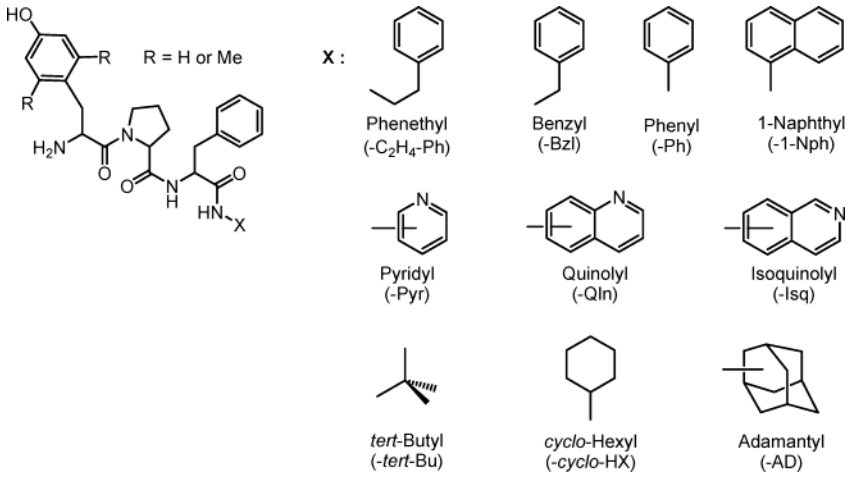
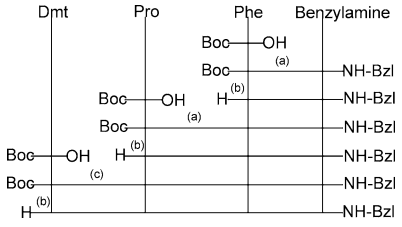


Figure 1. Design of EM-2 analogues and [Dmt[1]]EM-2 analogues.

Scheme 1. Synthetic Scheme for Dmt-Pro-Phe-NH-Bzl (**22**)[a]



[a] Reagents: (a) IBCF, NMM; (b) HCl/dioxane; (c) PyBop, DIPEA.

and that the Dmt[1]-Pro[2] amide bond preferred a cis orientation. It is recognized that the presence of additional aromatic residues enhances the affinity and selectivity of various opioid analogues, such as observed with the TIPP family of δ-opioid receptor antagonists[31] and the Dmt-Tic-NH-CH(R)R′ analogues with mixed μ- and δ-opioid receptor agonist activity or with μ-agonist and δ-antagonist profiles.[32,33] In light of these observations and the effect that substitution with an ethylphenyl group at the C terminus of [Dmt[1]]EM-2 elicited δ-antagonism,[30] we sought to further investigate the effect of an increase of the hydrophobicity of EM-2 analogues. This study details the alteration of affinity and functional activity caused by replacement of the C-terminal Phe[4]$-NH_2$ with aromatic amines (benzyl, phenyl, naphthyl, pyridyl, quinolyl, isoquinolyl) or aliphatic amines (*tert*-butyl, cyclohexyl, adamantyl) (Figure 1). The alteration in receptor binding and bioactivity profiles suggests that substitution of Phe-NH_2 at the fourth position by more hydrophobic amides of [Dmt[1]]EM-2 appears to play a role also in the acquisition of both μ- and δ-opioid receptor agonism, comparable to that found in some Dmt-Tic-NH-CH(R)-1*H*-benzimidazol-2-yl analogues.[32,33]

Chemistry

Optically pure (98%) Dmt was prepared as described,[25] and the purity was ascertained by both HPLC using a chiral column [CROWNPAK CR(+)] and reaction with D- and L-amino acid oxidases[34] followed by amino acid analysis. Boc-Dmt-OH was prepared as published.[29] EM-2 and [Dmt[1]]EM-2 analogues were synthesized by a solution method (Scheme 1) and detailed in Figure 1. The Boc group and the Z group were used for α-amino protection. The peptide bond between Phe and 2-aminopyridine was formed by a phosphazo method[35] because of poor nucleophilicity of the amino group. Other peptide bonds were formed by a mixed-anhydride method using isobutyl chloroformate (for activation of amino acids without functional side chain),[36] an azide method (for activation of Tyr or Dmt),[37] or PyBop reagent (for activation of Dmt).[38] α-Amino protected peptide intermediates were characterized by using TLC, ^{1}H NMR, ^{13}C NMR, and elemental analysis. Deprotection of Boc was performed using HCl/dioxane or TFA, and for deprotection of the Z group, catalytic hydrogenation over Pd/C was employed. All final products were purified by semipreparative reversed-phase HPLC. The analytical data for compounds **4**−**18** and **22**−**32** are in Supporting Information (Tables 1 and 2). Each compound exhibited a single peak on analytical HPLC with a unique retention time. Analysis by MALDI-TOF mass spectrometry (MS) and by HPLC revealed that all compounds were the desired peptides with greater than 98% purity.

Results

Endomorphin-2 Analogues. The binding affinities for μ- and δ-opioid receptors for **1**−**18** are summarized in Table 1. All the analogues exhibited negligible δ-opioid receptor affinities (micromolar range). In the substitution of the C-terminal Phe-NH_2 by a monocyclic aromatic residue, the μ-opioid receptor binding affinities of **3** (C_2H_4−Ph), **4** (Bzl), and **5** (Ph) decreased as a function of the loss in the number of interposing methylene units (K_i = 13.3, 21.6, and 33.5 nM, respectively). Furthermore, the heteromonocyclic aromatic residue analogues **6** (4-Pyr), **7** (3-Pyr), and **8** (2-Pyr) exhibited similarly weak μ-opioid receptor affinities (26.5−48.5 nM). On the other hand, the bicyclic aromatic residue **9** (Nph) exhibited a 5.5-fold higher affinity (K_i = 2.41 nM) than **3** (phenethyl). In the heterocyclic group, only **11** (5-Qln) had moderate μ-opioid receptor affinity (K_i = 4.07 nM); analogues **10** (3-Qln), **12** (6-Qln), **13** (8-Qln), and **14** (5-Isq) had generally low μ-opioid receptor affinities. Analogues containing bulky aliphatic groups (**15**−**18**) also displayed comparable weak μ-opioid receptor affinities (Table 1).

[Dmt[1]]endomorphin-2 Analogues. Table 2 summarizes the opioid receptor affinities of the [Dmt[1]]EM-2 analogues (**19**−**32**). With the exception of Dmt-Pro-

Table 1. Receptor Binding Profile of EM-2 Analogues[a]

compd	peptide	μ-opioid receptor K_i (nM)		δ-opioid receptor K_i (nM)		binding selectivity $K_i(\delta)/K_i(\mu)$
1	EM-2	0.69 ± 0.16		9230 ± 200		13400
	Tyr-Pro-Phe-NH-X					
2	X = H	46.3 ± 3.8		15900 ± 2300		343
3	X = C_2H_4-Ph	13.3 ± 2.6	(3)	4310 ± 635	(3)	324
4	X = Bzl	21.6 ± 3.8	(4)	1730 ± 292	(3)	80
5	X = Ph	33.5 ± 6.1	(4)	23170 ± 11500	(3)	692
6	X = 4-Pyr	48.2 ± 2.1	(4)	25070 ± 2710	(3)	521
7	X = 3-Pyr	48.5 ± 5.0	(4)	19360 ± 3330	(3)	400
8	X = 2-Pyr	26.5 ± 3.9	(4)	19660 ± 633	(3)	742
9	X = 1-Nph	2.41 ± 0.5	(4)	3640 ± 1260	(4)	1510
10	X = 3-Qln	55.8 ± 5.03	(4)	18600 ± 1740	(3)	333
11	X = 5-Qln	4.07 ± 0.45	(4)	12700 ± 2470	(3)	3110
12	X = 6-Qln	72.9 ± 6.6	(4)	9880 ± 2640	(3)	136
13	X = 8-Qln	249 ± 20.6	(4)	17100 ± 3590	(3)	68
14	X = 5-Isq	61.1 ± 14.2	(5)	3480 ± 640	(3)	57
15	X = Bu^t	19.6 ± 4.5	(3)	13300 ± 1940	(3)	680
16	X = cyclo-HX	5.79 ± 1.7	(3)	8110 ± 1760	(3)	1400
17	X = 1-AD	23.6 ± 1.7	(3)	6260 ± 1310	(3)	265
18	X = 2-AD	6.59 ± 0.06	(3)	5560 ± 1770	(3)	844

[a] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DPDPE (δ-selective) from rat brain membrane synaptosomes. The K_i values are the mean ± SE.

Table 2. Receptor Binding Profile of [Dmt^1]EM-2 Analogues[a]

compd	peptide	μ-opioid receptor K_i (nM)		δ-opioid receptor K_i (nM)		binding selectivity $K_i(\delta)/K_i(\mu)$
1	EM-2	0.69 ± 0.16		9230 ± 200		13400
19	[Dmt^1]EM-2	0.15 ± 0.035	(3)	28.2 ± 8.1	(3)	188
	Dmt-Pro-Phe-NH-X					
20	X = H	0.12 ± 0.09	(3)	53.2 ± 6.1	(3)	443
21	X = C_2H_4-Ph	0.51 ± 0.15	(3)	18.0 ± 2.5	(3)	35
22	X = Bzl	0.52 ± 0.055	(3)	13.8 ± 1.3	(3)	27
23	X = Ph	1.11 ± 0.18	(3)	20.6 ± 2.5	(3)	19
24	X = 4-Pyr	0.36 ± 0.063	(3)	52.7 ± 9.9	(3)	424
25	X = 3-Pyr	0.17 ± 0.02	(3)	287.5 ± 55	(4)	1732
26	X = 2-Pyr	0.13 ± 0.01	(3)	157.2 ± 25	(4)	1191
27	X = 1-Nph	0.29 ± 0.037	(4)	19.9 ± 3.0	(4)	68
28	X = 3-Qln	0.33 ± 0.017	(4)	190.4 ± 22.3	(3)	577
29	X = 5-Qln	0.11 ± 0.014	(3)	30.0 ± 3.8	(3)	283
30	X = 6-Qln	0.22 ± 0.038	(4)	46.6 ± 2.4	(3)	212
31	X = 8-Qln	0.49 ± 0.057	(5)	33.1 ± 1.71	(4)	68
32	X = 5-Isq	0.19 ± 0.018	(5)	98.3 ± 8.83	(4)	517

[a] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DPDPE (δ-selective) from rat brain membrane synaptosomes. The K_i values are the mean ± SE.

Phe-NH-Ph (**23**) (K_i = 1.11 nM), all of the [Dmt^1]EM-2 analogues demonstrated μ-opioid receptor affinity higher than EM-2 (K_i = 0.69 nM) with K_i ranging from 0.11 to 0.52 nM. Concomitantly, the δ-opioid receptor affinity increased, thereby decreasing μ-opioid receptor selectivity. However, **24** (4-Pyr), **25** (3-Pyr), **26** (2-Pyr), **28** (3-Qln), and **32** (5-Isq) indicated moderate μ-opioid receptor selectivities (424, 1732, 1191, 577, and 517, respectively).

Pharmacological Activity. All [Dmt^1]EM-2 analogues acted as μ-opioid agonists in a guinea pig ileum (GPI) bioassay (Table 3). In particular, [Dmt^1]EM-2 (**19**) and analogues **27** (1-Nph), **29** (5-Qln), and **32** (5-Isq) exhibited potent μ-opioid receptor agonism (IC_{50} < 1 nM). In the mouse vas deferens (MVD) bioassay, all analogues were weak δ-opioid receptor antagonists (pA_2 = 5.41−7.18) except **27** (1-Nph) and **29** (5-Qln), which were also δ-opioid receptor agonists (IC_{50} = 5.47 and 0.62 nM, respectively). Interestingly, **29** also exhibited weak δ-opioid antagonism (pA_2 = 5.88) but only at high concentrations.

In Vivo Antinociception. Dmt-Pro-Phe-NH-5-Isq (**32**) produced a dose-dependent antinociceptive effect after ic administration, which subsided within 60 min (Figure 2) and was antagonized completely by naltrexone, a μ-opiate antagonist (Figure 3). This indicates that the antinociceptive effect of **32** acts through μ-opioid receptors similar to that of morphine. This antinociceptive effect of **32** was weaker and had a duration shorter

Table 3. In Vitro Bioactivity Profile of [Dmt^1]EM-2 Analogues

compd	peptide	GPI assay[a] IC_{50} (nM)	MVD assay[b] IC_{50} (nM)	pA_2 value vs dertorphin II
1	EM-2	5.79 ± 0.4	344 ± 93	
19	[Dmt^1]EM-2	0.07 ± 0.016	1.87 ± 0.61	
	Dmt-Pro-Phe-NH-X			
20	X = H	2.33 ± 0.49	113 ± 35	
21	X = C_2H_4-Ph	5.03 ± 0.99	>10000	7.05
22	X = Bzl	22.0 ± 6.1	>10000	7.18
23	X = Ph	37.7 ± 7.0	>10000	6.94
24	X = 4-Pyr	11.8 ± 2.7	>10000	6.52
25	X = 3-Pyr	72.8 ± 13.0	>10000	6.33
26	X = 2-Pyr	15.0 ± 3.2	>10000	6.70
27	X = 1-Nph	0.49 ± 0.183	5.47 ± 1.01	
28	X = 3-Qln	9.14 ± 0.81	>10000	5.93
29	X = 5-Qln	0.26 ± 0.04	0.616 ± 0.17	5.88
30	X = 6-Qln	6.21 ± 3.1	>10000	5.41
31	X = 8-Qln	445 ± 11.0	2981 ± 685	6.14
32	X = 5-Isq	0.94 ± 0.112	>10000	6.12

[a] Values are the mean of seven experiments ± SE. [b] Values are the mean of six experiments ± SE.

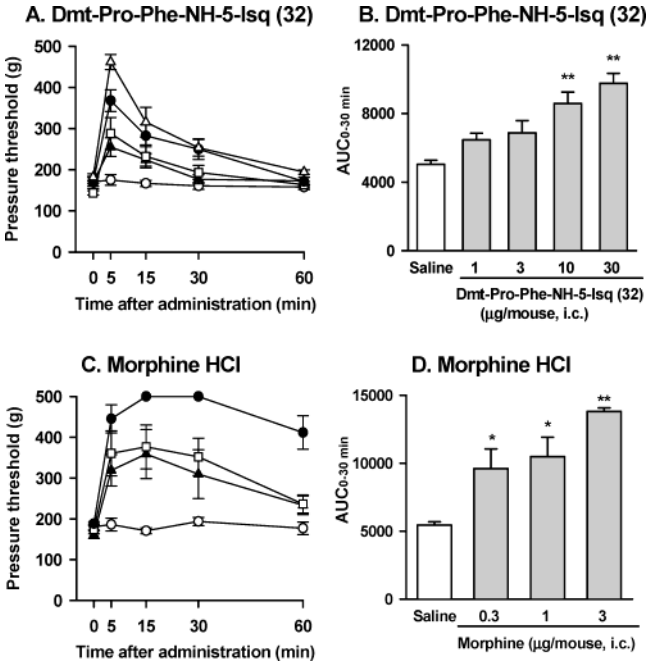


Figure 2. Antinociceptive effect of Dmt-Pro-Phe-NH-5-Isq (**32**) after intracisternal (ic) administration in the tail pressure test in mice. (A) Time course of the antinociceptive effect induced by Dmt-Pro-Phe-NH-5-Isq (**32**). Each symbol represents the mean ± SE of five mice: (○) saline; (▲) Dmt-Pro-Phe-NH-5-Isq **32** (1 μg/mouse); (□) Dmt-Pro-Phe-NH-5-Isq **32** (3 μg/mouse); (●) Dmt-Pro-Phe-NH-5-Isq **32** (10 μg/mouse); (△) Dmt-Pro-Phe-NH-5-Isq **32** (30 μg/mouse). (B) The area under the curve (AUC) was calculated from the time course from 0 to 30 min after administration. Each column represents the mean ± SE of 5 mice: (**) $P < 0.01$, significantly different from the saline-treated group (Dunnett's munltiple comparison test). (C) Time course of the antinociceptive effect induced by morphine HCl. Each symbol represents the mean ± SE of four or five mice: (○) saline; (▲) morphine HCl (0.3 μg/mouse); (□) morphine HCl (1 μg/mouse); (●) morphine HCl (3 μg/mouse). (D) The area under the curve (AUC) was calculated from the time course from 0 to 30 min after administration. Each column represents the mean ± SE of four or five mice: (*) $P < 0.05$, (**) $P < 0.01$, significantly different from the saline-treated group (Dunnett's multiple comparison test).

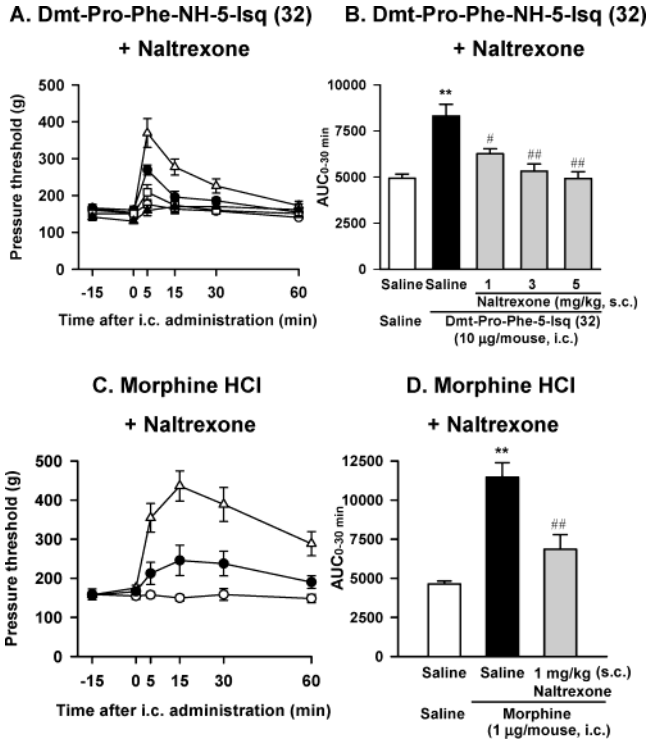


Figure 3. Antagonism by naltrexone of the antinociceptive effect induced by Dmt-Pro-Phe-NH-5-Isq (**32**) and morphine HCl in the tail pressure test in mice. (A) Time course of the antinociceptive effect induced by Dmt-Pro-Phe-NH-5-Isq (**32**), showing the dose−response relation in the antagonism by naltrexone. Each symbol represents the mean ± SE of five mice. Naltrexone was administration sc 15 min before intracisternal (ic) administration of Dmt-Pro-Phe-NH-5-Isq **32**: (○) saline (sc) + saline (sc); (△) saline (sc) + Dmt-Pro-Phe-NH-5-Isq **32** (10 μg/mouse, ic); (●) naltrexone HCl (1 mg/kg, sc) + Dmt-Pro-Phe-NH-5-Isq **32** (10 μg/mouse, ic); (□) naltrexone HCl (3 mg/kg, sc) + Dmt-Pro-Phe-NH-5-Isq **32** (10 μg/mouse, ic); (▲) naltrexone HCl (5 mg/kg, sc) + Dmt-Pro-Phe-NH-5-Isq **32** (10 μg/mouse, ic). (B) The area under the curve (AUC) was calculated from the time course from 0 to 30 min after administration. Each column represents the mean ± SE of five mice: (**) $P < 0.01$, significantly different from the saline-treated group (Student's *t*-test); (#) $P < 0.05$, (##) $P < 0.01$, significantly different from the Dmt-Pro-Phe-NH-5-Isq (**32**) alone-treated goup (Dunnett's multiple comparison test). (C) Time course of the antinociceptive effect induced by morphine HCl, showing the dose−response relation in the antagonism by naltrexone. Each symbol represents the mean ± SEM of five mice. Naltrexone was administered sc 15 min before intracisternal (ic) administration of morphihe HCl: (○) saline (sc) + saline (sc); (△) saline (sc) + morphine HCl 1 μg/mouse (ic); (●) naltrexone HCl 1 mg/kg (sc) + morphine HCl 1 μg/mouse (ic). (D) The area under the curve (AUC) was calculated from the time course from 0 to 30 min after administration of morphine HCl. Each column represent the mean ± SEM of five mice: (**) $P < 0.01$, significantly different from the saline-treated group (Student's *t*-test); (#) $P < 0.05$, (##) $P < 0.01$, significantly different from the morphine alone-treated group (Student's *t*-test).

than that of morphine (Figure 2). The maximum effect of analogue **32** (30 μg/mouse) was about one tenth of that with morphine.

Solution Conformational Study. NMR studies of [Dmt[1]]EM-2 analogues were conducted in DMSO-d_6. Despite the molecular differences in the compounds, the chemical shift of the Pro β and γ positions of the cis and trans conformations in ^{13}C NMR remained constant; chemical shifts of cis γ, trans γ, trans β, and cis β were observed at 21, 24, 29, and 31 ppm, respectively. The cis and trans isomers were identified by ^{1}H NMR, ^{13}C NMR, H−H COSY, C−H COSY, and NOESY. Figure 4 shows the characteristic NOE pairs of the cis and trans conformations at the Dmt−Pro peptide bonds. In the cis isomer, the methyl groups and the Cα proton of Dmt are spatially close to the Cα proton of Pro. As a result, the chemical shift of Pro Cα H of the cis isomer was observed at a higher field than that of trans isomer. While Dmt exists as a trans isomer, the methyl groups and the Cα proton of Dmt are spatially close to the Cδ proton of Pro; consequently, the chemical shift of Pro Cδ H of the trans isomer was observed at higher field than that of the cis isomer. The cis/trans ratios of the [Dmt[1]]EM-2 analogues were determined from relative peak intensities obtained by ^{1}H NMR spectroscopy (Table 4).

Discussion

Influence of Dmt and the Substitution at the Fourth Position on Peptide Activity. The receptor binding profiles of EM-2 analogues and [Dmt[1]]EM-2 analogues summarized in Tables 1 and 2 demonstrated

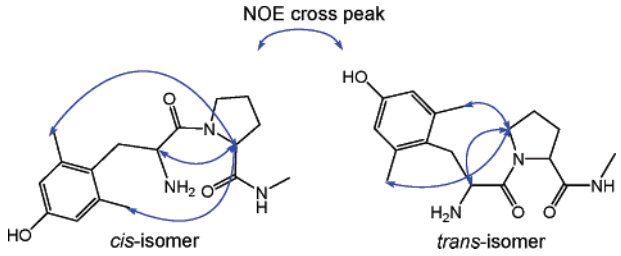


Figure 4. Characteristic NOE pairs of cis and trans conformations at the Dmt-Pro peptide bond.

Table 4. Cis/Trans Ratio of [Dmt[1]]EM-2 Analogues in DMSO[a]

compd	peptide	cis/trans
	Tyr-Pro-Trp-Phe-NH_2 (EM-1)	1/3
1	Tyr-Pro-Phe-Phe-NH_2 (EM-2)	1/2
2	Tyr-Pro-Phe-NH_2	1/2
19	[Dmt[1]]EM-2	70/30
	Dmt-Pro-Phe-NH-X	
20	X = H	65/35
21	X = C_2H_4-Ph	65/35
22	X = Bzl	65/35
23	X = Ph	60/40
24	X = 4-Pyr	60/40
25	X = 3-Pyr	65/35
26	X = 2-Pyr	65/35
27	X = 1-Nph	60/40
28	X = 3-Qln	65/35
29	X = 5-Qln	60/40
30	X = 6-Qln	60/40
32	X = 5-Isq	60/40

[a] Determined from relative peak intensities obtained by ^{1}H NMR.

that Dmt enhanced interaction with the receptors and altered their bioactivity profiles. Similarly, the replacement of Tyr with Dmt in the Tyr-Tic pharmacophore[39] resulted in a potent and highly selective δ-opioid receptor antagonist,[40−42] and our previous studies indicated that the replacement of Tyr with Dmt in EM-2 enhanced both δ- and μ-opioid receptor affinities.[30] These data and other observations substantiate the fact that Dmt plays a dominant role in determining the efficacy of the interaction of an opioid ligand with receptors.

In addition to a hypothesis that the dimethyl groups on the tyramine ring interact with hydrophobic binding sites within the receptor pocket,[43] experimental evidence provided herein demonstrates that Dmt also shifts the conformation at the Dmt-Pro amide bond in endomorphin-2 from trans to cis. A common structural feature of Dmt-Pro and Dmt-Tic is the cyclic imino acid at the second position; however, the size of these rings (five-membered ring and six-membered ring, respectively) and the existence of a second aromatic residue in the latter compound represent major differences between Pro and Tic. The cis/trans equilibrium at the Dmt-Pro peptide bond in DMSO favored the cis orientation in contrast to the Tyr-Pro amide bond, which indicated a higher ratio for the trans orientation.[30] The preferred cis orientation of the Dmt-Pro amide bond may be explained by the presence of methyl groups at the 2′ and 6′ positions of Dmt that could make the trans orientation less favorable (Table 4). Molecular modeling and energy minimization of 1-Nph (**27**) (μ/δ-agonist), 5-Qln (**29**) (μ/δ-agonist and δ-antagonist), and 6-Qln (**30**) (μ-agonist, weak δ-antagonist) revealed a different orientation of the heteroaromatic ring (Figure 5), supporting a hypothesis that the orientation of the heterocyclic ring may be important for the appearance of δ-agonism.

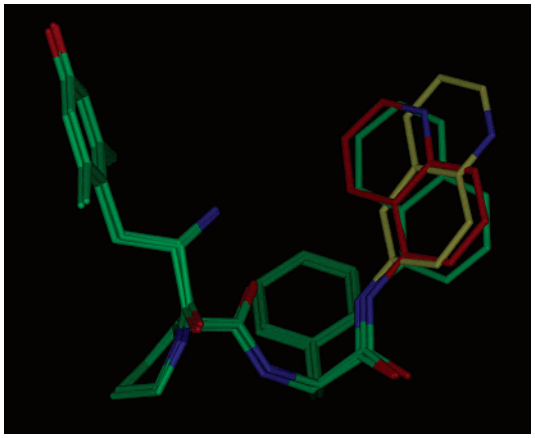


Figure 5. Superimposition of the endomorphin-2 analogues, Dmt-Pro-Phe-NH-1-Nph (**27**) (green), Dmt-Pro-Phe-NH-5-Qln (**29**) (red), and Dmt-Pro-Phe-NH-6-Qln (**30**) (yellow). The models were developed on the basis of data from ^{1}H NMR and molecular modeling. Carbon atoms are displayed in green, oxygen atoms in red, and nitrogen atoms in blue with the exception the C-terminal heteroaromatics, which are colored according to the substitution. Hydrogens are not displayed.

The μ-opioid receptor affinities of EM-2 analogues containing a third aromatic residue, such as **3** (C_2H_4−Ph, 13.3 nM), **4** (Bzl, 21.6 nM), and **5** (Ph, 33.5 nM), decreased as a function of the loss in the number of methylene units. Therefore, the distance between the phenol moiety of the Tyr residue and the other aromatic residue might be an important factor in a proper fit within an opioid receptor active site.[43] On this point, Dmt-Tic-NH-CH(R)-R′ analogues containing Bid (1*H*-benzimidazol-2-yl) as the R′ functional group acquired high affinity and moderate agonist activity for μ-opioid receptors, whereas the change in spacer (R) influenced the acquisition of agonist or antagonist activity.[32,33]

While all the analogues studied were μ-opioid receptor agonists, the functional bioactivity of [Dmt[1]]EM-2 analogues for the δ-opioid receptor indicated considerable differences, the analogues behaving as agonists (**19**, **20**, **27**) or antagonists (**21**−**26**, **28**, **30**, **32**) or having mixed agonist/antagonist properties (**29**, **31**). Interestingly, with respect to the δ-receptor, those compounds containing monocyclic aromatic residues (**21**−**26**) exhibited antagonist activity but in the presence of a bicyclic aromatic residue attached through the α-position (**27**, **29**, **31**) tended to act as agonists, while those covalently bound through the β position (**28**, **30**) exhibited antagonist activity.

In Vivo Antinociceptive Activity of a [Dmt[1]]EM-2 Analogue. Peptides are easily hydrolyzed by proteolytic enzymes such as aminopeptidase, carboxypeptidase, and enkephalinase.[44] While the Pro[2]-Phe[3] amide bond of endomorphin-2 was cleaved by dipeptidyl peptidase IV,[45a] [D-Pro[2]]EM-2 is totally resistant.[45b] Cardillo et al.[46] reported that endomorphin-1 analogues containing homo-Pro acquired resistance to enzymatic degradation and the replacement of Pro by homo-Pro effected a significant change in peptide−enzyme recognition. Enzymatic stability of opioid peptides containing Dmt at position 1 was examined by use of aminopeptidase and rat brain homogenates, which revealed that they were

more stable than the original peptides.[29] Therefore, the [Dmt[1]]EM-2 analogue Dmt-Pro-Phe-NH-5-Isq (**32**) might be similarly resistant to enzymatic degradation by aminopeptidase or carboxypeptidase, although the Pro^2−Phe^3 bond is metabolized.[45a] Therefore, modification of the Pro^2−Phe^3 peptide bond might be necessary for future research in order to develop a more potent and long lasting opioid ligand.

Conclusions

The data indicated that Dmt markedly enhanced the biological properties of EM-2 analogues in both the competitive binding and in vitro biological assays. Furthermore, the binding affinity of **19**−**32**, except for **23** (Ph), is also higher than that of EM-2 and their in vitro bioactivity profiles are also different from that of EM-2. Although [Dmt[1]]EM-2 and its analogues are μ-opioid agonists, Dmt-Pro-Phe-NH-1-Nph (**27**) and -5-Qln (**29**) exhibited μ- and δ-opioid agonism. These remarkable changes are comparable to those observed in other, unrelated opioid peptides such as enkephalin[26] and analogues of deltorphin,[19] dynorphin A (1−11),[20] as well as the Dmt-Tic-R[32] and Dmt-Tic-NH−(R)-R′ family of peptides.[33] The ic administration of Dmt-Pro-Phe-NH-5-Isq (**32**) produced a significant dose-dependent antinociceptive effect that was antagonized completely by naltrexone, proving that the effect was mediated by the μ-opioid receptors in a manner similar to that of morphine. Thus, these data clearly indicate that N-terminal Dmt has the potential to act in the development of novel bioactive opioidomimetics for potential therapeutic and clinical applications. The difference in the conformation about the Xaa-Pro amide bond in solution in peptides containing Tyr (trans-rich) and Dmt (cis-rich) (Table 4) suggests that conformational features of the peptide affect receptor recognition and biological function. Finally, the methyl groups on the tyramine ring of Dmt undoubtedly play a dominant role in the interaction within the opioid binding domains by direct interaction with hydrophobic side chains of receptor residues or by stabilization of a favored cis conformer in solution prior to and during binding.

Experimental Section

Peptide Synthesis. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-1000 (Japan Spectroscopic Co.). MALDI-TOF mass spectrometry (MS) was carried out on a KOMPACT MALDI IV instrument (Kratos Analytical Co.) using sinapinic acid as the matrix. ^{1}H (400.13 MHz) and ^{13}C (100.62 MHz) NMR spectra were recorded on a Bruker DPX-400 spectrometer. Final compounds (30 mg) were dissolved in 1.0 mL of DMSO-d_6 (99.9% isotopic purity). Chemical shift values were expressed as ppm downfield from tetramethylsilane, used as an internal standard (δ value). The J values are given in Hz. The ^{13}C signals were assigned with the aid of distortionless enhancement by polarization transfer (DEPT) and two-dimensional experiments (H−H COSY, C−H COSY), and classification of carbon atoms are indicated by CH_3 (primary), CH_2 (secondary), CH (tertiary), or Cq (quaternary). Dmt was prepared according to the method of Dygos et al.,[25] and its chirality was assessed by both HPLC [column, CROWNPAK CR(+) (Daisel Chemical, Ltd., 15 cm × 4.6 mm); flow rate, 0.4 mL/min; mobile phase, perchloric acid (pH 2); UV detection, 220 nm] and amino acid analysis. Peptide bonds were formed by the mixed-anhydride method using isobutyl chloroformate (IBCF),[36] the phosphazo method,[35] the azide method[37] or using the PyBop reagent.[38] The Boc and Z groups were used for N-terminal protection. Deprotection of Boc was performed using hydrochloric acid in dioxane (HCl/dioxane) or TFA. Deprotection of Z was performed by catalytic hydrogenolysis using Pd/C. On TLC (Kieselgel G/ Merck), R_f values refer to the systems $CHCl_3$/MeOH/AcOH (90:8:2), hexane/AcOEt (1:1), and hexane/AcOEt (1:3). These peptides exhibited greater than 98% purity by analytical HPLC (Waters model 600 E), using a Cosmosil 5C18-AR column (NAKARAI TESQUE, Inc., 25 cm × 4.6 mm) with the absorbance monitored at 220 nm, and run (1 mL/min) in the following solvents: A, 0.05% TFA in H_2O; B, 0.05% TFA in CH_3CN.

General Procedure for Synthesis of Boc-Phe-NH-X (X = Bzl, Ph, 4-Pyr, 3-Pyr, Nph, Qln, Isq, Bu^t, Cyclo-HX, AD). A mixed anhydride [prepared from Boc-Phe-OH (3.8 mmol), IBCF (3.8 mmol), and NMM (4.2 mmol)] in THF (30 mL) was added to a solution of the corresponding amine component (4.2 mmol) in DMF (20 mL) at −15 °C. The reaction mixture was stirred at 0 °C for 15 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3, and saturated NaCl, dried over Na_2SO_4, and evaporated. Petroleum ether was added to the residue to form crystals, which were collected by filtration. The yield, melting point, $[\alpha]_D^{20}$, R_f, and elemental analysis data are summarized in Table 3 in Supporting Information along with the ^{1}H NMR values for the intermediates in the peptide synthesis.

Z-Phe-NH-2-Pyr. PCl_3 was added to distilled pyridine (50 mL) containing 2-aminopyridine (1.0 g, 10.6 mmol) at 0 °C. After 1 h, Z-Phe-OH (3.17 g, 10.6 mmol) was added to the mixture. The mixture was stirred at 80 °C for 5 h and at room temperature for 2 days. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% Na_2CO_3, and water, dried over Na_2SO_4, and evaporated to dryness. Petroleum ether was added to the residue to form crystals, which were collected by filtration: yield 2.93 g (73.6%), mp 137−139 °C, $[\alpha]_D^{20}$ +8.9° (*c* 1.0, MeOH), R_f = 0.77 ($CHCl_3$/MeOH/AcOH = 90:8:2). Anal. Calcd for $C_{22}H_{21}N_3O_3 \cdot {}^1/_4H_2O$: C, 69.6; H, 5.66; N, 11.00. Found: C, 69.4; H, 5.64; N, 10.6.

General Procedure for Synthesis of Boc-Pro-Phe-NH-X (X = Bzl, Ph, 4-Pyr, 3-Pyr, Nph, Qln, Isq, Bu^t, Cyclo-HX, AD). A mixed anhydride [prepared from Boc-Pro-OH (11 mmol), IBCF (11 mmol), and NMM (11 mmol)] in THF (30 mL) was added to a solution of the corresponding amine component [prepared from Boc-Phe-NH-X (10.1 mmol) and 7.4 N HCl/dioxane (30 mmol)] in DMF (30 mL) containing NMM (21 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 15 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was worked up in a manner similar to that for Z-Phe-NH-2-Pyr to give crystals. The yield, melting point, $[\alpha]_D^{20}$, R_f, and elemental analysis data are summarized in Supporting Information (Table 4) along with the ^{1}H NMR and ^{13}C NMR values for the intermediates in peptide synthesis.

Boc-Pro-Phe-NH-2-Pyr. The title compound was prepared as for the synthesis of Boc-Pro-Phe-NH-X starting from Boc-Pro-OH (0.50 g, 2.32 mmol), IBCF (0.30 mL, 2.32 mmol), NMM (0.25 mL, 2.32 mmol), and H-Phe-NH-2-pyridine·2AcOH [prepared from hydrogenolysis of Z-Phe-NH-2-pyridine (1.0 g, 2.66 mmol) in MeOH (30 mL) in the presence of Pd/C and acetic acid].

General Procedure for Synthesis of Boc-Tyr-Pro-Phe-NH-X (X = Ph, Pyr, Nph, Qln, Isq, Bu^t, Cyclo-HX, AD). Boc-Tyr-$NHNH_2$ (2.28 mmol) was dissolved in DMF (10 mL). Under cooling with ice/NaCl, 7.2 N HCl/dioxane (4.56 mmol) and isoamyl nitrite (2.5 mmol) were added. Stirring was continued for 10 mim, when the hydrazine test became negative. The solution, after neutralization with NMM (4.56 mmol), was combined with a solution of the corresponding amine component [prepared from Boc-Pro-Phe-NH-X (2.28 mmol) and 7.2 N HCl/dioxane (22.8 mmol)] and NMM (6.84 mmol) in DMF (20 mL). After the reaction mixture was stirred

at 4 °C overnight, the solvent was removed. The residue was extracted with AcOEt. The extract was worked up in a manner similar to that for Z-Phe-NH-2-Pyr to give crystals. The yield, melting point, $[\alpha]_D^{20}$, R_f, and elemental analysis data are summarized in Table 5 in Supporting Information along with the ^{1}H NMR and ^{13}C NMR values for the intermediates in the peptide synthesis.

Boc-Tyr(Bu^t)-Pro-Phe-NH−Bzl. A mixed anhydride [prepared from Boc-Tyr(Bu^t)-OH (2.22 mmol), IBCF (2.22 mmol), and NMM (2.22 mmol)] in THF (30 mL) was added to a solution of H-Pro-Phe-NH-Bzl·HCl [prepared from Boc-Pro-Phe-NH-Bzl (2.22 mmol) and 7.4 N HCl/dioxane (7.0 mmol)] in DMF (30 mL) containing NMM (2.44 mmol) at −15 °C. The reaction mixture was stirred at 0 °C for 30 min and at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was worked up in a manner similar to that for Z-Phe-NH-2-Pyr to give crystals. Yield 1.17 g (71.8%), mp 151−160 °C, $[\alpha]_D^{20}$ −45.9° (*c* 1.0, MeOH), R_f = 0.78 ($CHCl_3$/MeOH/AcOH = 90:8:2). Anal. Calcd for $C_{39}H_{50}N_4O_6$: C, 69.8; H, 7.51; N, 8.35. Found: C, 69.6; H, 7.53; N, 8.31.

General Procedure for Synthesis of HCl·H-Tyr-Pro-Phe-NH-X (4−18) (X = Bzl, Ph, Pyr, Nph, Qln, Isq, Bu^t, Cyclo-HX, AD). Boc-Tyr-Pro-Phe-NH-X (0.068 mmol) was treated with 6.9 N HCl/dioxane (0.68 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was collected by centrifugation, dried over KOH pellets, and purified by semipreparative reversed-phase HPLC. The purified peptide was lyophilized from 1 N HCl to give an amorphous powder. The yield, HPLC retention time, and MS and elemental analysis data are summarized in Table 1 of Supporting Information.

General Procedure for Synthesis of Boc-Dmt-Pro-Phe-NH-X (X = Bzl, Ph, Pyr, 5-Qln, 6-Qln). To a solution of the corresponding amine component [prepared from Boc-Pro-Phe-NH-X (1.1 mmol) and 6.9 N HCl/dioxane (4.3 mmol)] in DMF (30 mL) containing DIPEA (3.3 mmol), Boc-Dmt-OH (1.1 mmol) and PyBop (1.1 mmol) were added. The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was worked up in a manner similar to that for Z-Phe-NH-2-Pyr to give crystals. The yield, melting point, $[\alpha]_D^{20}$, R_f, and elemental analysis data are summarized in Table 6 in Supporting Information along with the ^{1}H NMR and ^{13}C NMR values for the intermediates in the peptide synthesis.

General Procedure for Synthesis of Boc-Dmt-Pro-Phe-NH-X (X = Nph, 3-Qln, 8-Qln, 5-Isq). Boc-Dmt-$NHNH_2$ (2.35 mmol) was dissolved in DMF (10 mL). Under cooling with ice/NaCl, 5.0 N HCl/dioxane (2.82 mmol) and isoamyl nitrite (2.82 mmol) were added. Stirring was continued for 10 mim, when the hydrazine test became negative. The solution, after neutralization with NMM (5.88 mmol), was combined with a solution of the corresponding amine component [prepared from Boc-Pro-Phe-NH-X (2.17 mmol) and 5.0 N HCl/dioxane (7.0 mmol)] and NMM (4.7 mmol) in DMF (20 mL). After the reaction mixture was stirred at 4 °C overnight, the solvent was removed and the residue was extracted with AcOEt. The extract was worked up in a manner similar to that for Z-Phe-NH-2-Pyr to give crystals. The yield, melting point, $[\alpha]_D^{20}$, R_f, and elemental analysis data are summarized in Table 6 in Supporting Information along with the ^{1}H NMR and ^{13}C NMR values for the intermediates in the peptide synthesis.

General Procedure for Synthesis of HCl·H-Dmt-Pro-Phe-NH-X (22−32) (X = Bzl, Ph, Pyr, Nph, Qln, Isq). Boc-Dmt-Pro-Phe-NH-X (0.068 mmol) was treated with 6.9 N HCl/dioxane (0.68 mmol) for 1 h at room temperature. Et_2O was added to the solution until the product precipitated. The precipitate was collected by centrifugation, dried over KOH pellets, and purified by semipreparative reversed-phase HPLC. The purified peptide was lyophilized from 1 N HCl to give an amorphous powder. The yield, HPLC retention time, and MS and elemental analysis data are summarized in Table 2 in Supporting Information along with the ^{1}H NMR and ^{13}C NMR values.

Radioligand Binding Assays. Synaptosomal brain membrane P_2 preparations from Sprague-Dawley rats were prepared and used as the source for μ- and δ-opioid receptors after the removal of endogenous opioids.[47,48] The competitive displacement assay used 5.57 nM [^{3}H]DPDPE (NEN-DuPont) and 3.5 nM [^{3}H]DAMGO (Amersham) for the δ and μ sites, respectively, as published.[47,48] Affinity constants (K_i) were determined as given earlier.[48]

In Vitro Bioassay. For the GPI assay, the myenteric plexus-longitudinal muscle was obtained from male Hartley strain guinea pig (250−300 g) ileum as described by Rang,[49] and the tissue was mounted in a 10 mL bath that contained aerated (95% O_2, 5% CO_2) Krebs−Henseleit solution at 35 °C. The tissue was stimulated transmurally between the platinum wire electrodes using pulses of 0.5 ms duration with a frequency of 0.1 Hz at the supramaximal voltage. Longitudinal contractions were recorded via an isometric transducer. For the MVD assay, the vas deferens of male ddY strain mouse (25−35 g) were prepared as described by Hughes et al.[50] A pair of vasa was mounted in a 10 mL bath that contained aerated (95% O_2, 5% CO_2), modified Mg^{2+}-free Krebs solution containing ascorbic acid (0.1 mM) and EDTA·4Na (0.027 mM) at 35 °C. The tissue was stimulated transmurally with trains of rectilinear pulses of 1 ms. Stimulation trains were given at intervals of 20 s and consisted of seven stimuli of 1 ms duration with intervals of 10 ms.

In both assays, three to four washings were done with intervals of 15 min between each dose. Dose−response curves were constructed, and IC_{50} values (concentration causing a 50% reduction of the electrically induced twitches) were calculated graphically. For antagonism experiments, the test sample was added to the bath 15 min prior to addition of deltorphin II as agonist. The concentration−response curves of the agonist were performed in the absence and in the presence of increasing concentrations of the test compound, and the pA_2 values were calculated according to the method of Arunlakshana and Schild.[51]

Determination of Antinociception. Antinociception was evaluated by the tail-pressure test in male ddY mice. Incremental pressure was applied onto the tail of mice at a constant rate with an analgeseometer (Ugo Basile), and the pressure required to elicit a struggle behavior was measured as the pressure threshold before and at various times after administration of test compound. A cutoff value of 500 g was used. The test compound was dissolved in sterile saline or water, and intracisternal administration (ic) was done. Intracisternal administration was performed according to the method of Ueda et al.[52] at a volume of 10 μL/mouse.

Molecular Modeling. Molecular models of Dmt-Pro-Phe-NH-1-Nph (**27**), Dmt-Pro-Phe-NH-5-Qln (**29**), and Dmt-Pro-Phe-NH-6-Qln (**30**) were developed on the basis of 2D (NOESY) ^{1}H NMR analyses and computational modeling strategies. Molecular modeling was performed on a SGI Octane2 computer system using Insight98 software (Accelrys). The starting structures were derived on the basis of torsion angles and approximate H−H distances from *J* coupling constants and NOEs from 2D ^{1}H NMR analyses. Constrained energy minimizations were performed on each molecule using the CFF91 force field and the distant-dependent dielectric constant. The steepest descent algorithm was used for 100 cycles followed by another 1000 cycles of minimization using the conjugate gradient algorithm in the same force field, stopping with a root-mean-square gradient of 0.001 kcal mol^{-1} Å^{-1}. Internal rotation coordinates and energies for the three compounds, Dmt-Pro-Phe-NH-1-Nph (**27**), Dmt-Pro-Phe-NH-5-Qln (**29**), and Dmt-Pro-Phe-NH-6-Qln (**30**), are listed in Table 7 in Supporting Information.

Supporting Information Available: The yield, analytical data, ^{1}H NMR and ^{13}C NMR values of intermediates; the yield and analytical data of EM-2 analogues; a list of internal rotation coordinates and energies for the three compounds **27**, **29**, and **30**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(1) Abbreviations used in this report for amino acids, peptide, and their derivatives are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry* **1966**, *5*, 2485−2489; **1966**, *6*, 362−364; **1972**, *11*, 1726−1732. The customary L-configuration for amino acid residues is omitted. The following additional abbreviations are used: AcOEt, ethyl acetate; AcOH, acetic acid; AD, adamantine; Boc, *tert*-butyloxycarbonyl; Bu, butyl; Bzl, benzyl; DADLE, [D-Ala^2,D-Leu^5]-enkephain; DAMGO, Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; Dmt, 2′,6′-dimethyl-L-tyrosine; DPDPE, Tyr-*c*(D-Pen-Gly-Phe-D-Pen); EDTA, ethylenediaminetetraacetic acid; EM-2, endomorphin-2; Et_3N, triethylamine; Et_2O, diethyl ether; GPI, guinea pig ileum; HX, hexane; IBCF, isobutyl chloroformate; Isq, isoquinoline; MeOH, methanol; MVD, mouse vas deference; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser and exchange spectroscopy; Nph, naphthalene; Ph, phenyl; PyBop, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; Pyr, pyridine; Qln, quinoline; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Z, benzyloxycarbonyl.

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